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SPECTROSCOPIC STUDIES OF MITOCHONDRIAL IRON SULFUR PROTEINS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Chemistry

by Joyce Elaine Morningstar
B.A., New College of the University of South Florida, 1978
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May 1986
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ABSTRACT

Aerobic respiration is linked to oxidative phosphorylation and involves a redox-linked proton translocation across the mitochondrial inner membrane. Deeper understanding of the mechanism of mitochondrial electron transport requires detailed characterization of the redox centers present in respiratory enzyme complexes.

A combination of low temperature MCD spectra and magnetization data, with parallel EPR, CD, and UV-visible experiments, has been used to characterize the structural, electronic, and magnetic properties of the iron sulfur centers in respiratory enzymes. The enzymes studied were those associated with electron transport in mammalian mitochondria, with comparative studies of similar enzymes and proteins in heterotrophic bacteria.

The results from studies of soluble high molecular weight NADH dehydrogenase are consistent with the presence of one [2Fe-2S] and three [4Fe-4S] centers, each approximately stoichiometric with FMN. Comparison of EPR spectra of Complex I and soluble enzyme indicates that the same clusters are present in the more intact particulate preparation.

Spectroscopic experiments demonstrate that both yeast and mammalian succinate dehydrogenase, and the analogous fumarate reductase from E. coli, contain a [2Fe-2S], a [3Fe-xS], and a [4Fe-4S] cluster in amounts stoichiometric with FAD. The trinuclear cluster was shown to be a necessary requirement for reconstitutive activity. In addition, evidence is presented to show that the [3Fe-xS] and [4Fe-4S] centers are distinct entities rather than interconversion products.
of the same cluster.

Spectroscopic studies of the iron sulfur center in mammalian electron transfer flavoprotein (ETF) dehydrogenase were consistent with the presence of one [4Fe-4S] cluster. In addition, dithionite reduction of ETF dehydrogenase was shown to involve addition of two electrons to FAD and one electron to the iron sulfur cluster, whereas the enzymatic process involves one electron reduction of both redox centers.

Comparison of spectra from *T. thermophilus* Rieske protein with that from spinach ferredoxin indicated substantial differences in the electronic structure of their [2Fe-2S] clusters. The differences most likely relate to variations in the ligand amino acid groups.
I. INTRODUCTION

THE MITOCHONDRIAL ELECTRON TRANSPORT CHAIN

Mitochondrial electron transport releases large amounts of free energy, much of which is conserved in the form of the phosphate bond energy of adenosine triphosphate in the process known as oxidative phosphorylation (1). The process is believed to involve redox-driven proton translocation across the mitochondrial inner membrane (2). Oxidative phosphorylation is fundamental to all aspects of cellular life in aerobic organisms, since it provides the main source of energy for life processes.

Although it was observed in 1886 that respiratory electron transport was associated with a chain of heme proteins terminating in cytochrome oxidase, forty years passed before the discovery of oxidative phosphorylation. Ten years later Krebs fashioned the tricarboxylic acid (TCA) cycle. Respiratory metabolism is now known to entail glycolic conversion of a primary substrate to pyruvate in the cytosol, followed by oxidation of pyruvate in the mitochondria by way of the TCA cycle. The electrons pass to molecular oxygen through a series of carriers to cytochrome oxidase (3, 4). Electron transfer is linked to, and controlled by oxidative phosphorylation.

Mitochondrial electron transport chains consist of a number of large multiprotein complexes which generally span a lipid bilayer membrane in which they are situated. These complexes are often functionally connected by smaller components, eg. ubiquinone (UQ), which may be located in the membrane or in the cytoplasm.

Historically, the mitochondrial electron transport chain has been
divided into segments as illustrated in Figure 1-1. The region of the respiratory chain mediating transfer of reducing equivalents from NADH to UQ, NADH-UQ oxidoreductase, is known as Complex I. Other respiratory substrates are dehydrogenated by two flavin-linked dehydrogenases: succinate-UQ oxidoreductase (Complex II) and electron transfer flavoprotein (ETF) dehydrogenase. These enzymes funnel electrons into the electron transport chain via UQ. Oxidation of ubiquinol and reduction of cytochrome c is catalyzed by ubiquinol-cytochrome c oxidoreductase, Complex III. Electrons are passed from cytochrome c, through Complex IV, to ultimately reduce molecular oxygen to water. The energy sites of oxidative phosphorylation associated with respiration are Complex I, Complex III, and Complex IV.

One of the goals of mitochondrial research has been to establish the electron carrier sequence in the respiratory chain. An approach that has been very successful is a combination of inhibition of electron exchange by site-specific reagents and determination of the time course of redox state changes of the carriers induced by adding a pulse of oxidant or reductant.

Deeper understanding of the mechanism of mitochondrial electron transport requires a more detailed characterization of the individual redox components and their interactions. Recent advances in spectroscopy have allowed discovery and characterization of multiple iron-sulfur centers in the mitochondria. It has become evident that iron-sulfur clusters greatly outnumber cytochromes in the respiratory chain. Since iron-sulfur proteins are not amenable to kinetic or potentiometric measurements by spectroscopic methods at ambient
temperature, their role in the catalytic mechanism is not well understood. In addition, in many of the enzyme complexes, the number and identities of these iron-sulfur centers is a matter of vigorous dispute.

The scope of this work is limited to the study of those enzymes associated with electron transport in mammalian mitochondria, with comparative studies of similar enzymes and proteins in heterotrophic bacteria. The objective of the research is to provide new insight into the role and diversity of the iron-sulfur centers present in the mitochondrial electron transport chain. To achieve this goal the techniques of magnetic circular dichroism (MCD) and electron paramagnetic resonance (EPR) spectroscopies have been used to characterize the structural, electronic, and magnetic properties of the iron-sulfur centers in respiratory enzyme complexes. The enzymes investigated include NADH-UQ oxidoreductase, succinate-UQ oxidoreductase, electron transfer flavoprotein dehydrogenase, fumarate reductase from Escherichia coli, and the iron-sulfur protein (Rieske center) in ubiquinol-cytochrome c oxidoreductase from Thermus thermophilus.

IRON-SULFUR PROTEINS

Iron-sulfur proteins contain iron and inorganic sulfur in the form of an iron-sulfur cluster. They are generally involved in biological electron transfer and have been the subject of numerous books and reviews (5-7). Iron-sulfur proteins are involved in the principal life-sustaining process: photosynthesis, nitrogen fixation, and respiration. Although iron-sulfur clusters are now known to be
the most common electron transfer prosthetic groups in nature, their existence was only discovered about 30 years ago.

Initially, iron-sulfur centers were empirically identified in numerous biological samples by use of low temperature EPR. Exploration of the structure of iron-sulfur proteins has been greatly enhanced by study of inorganic model complexes (8-10). Further study of the structure of the centers had to await development of other suitably non-destructive methods of analysis: X-ray crystallography, extended X-ray absorption fine structure (EXAFS), electron nuclear double resonance (ENDOR), Mossbauer, resonance Raman, and MCD spectroscopies. Analytical techniques, such as atomic absorption and colorimetric techniques, yield information about the number of non-heme iron and acid-labile sulfide atoms in an iron-sulfur protein.

**Nomenclature**

Figure 1-2 shows a classification scheme for iron-sulfur proteins which emphasizes their variety and diversity. Rules for their official nomenclature can be found in reference 11.

The term "simple" iron-sulfur protein refers to a protein in which no prosthetic group other than an iron-sulfur center is present. Studies of this type of protein and the corresponding synthetic inorganic analog led to the conclusion that there are three types of iron-sulfur centers in simple iron-sulfur proteins. The existence of a fourth type, a trinuclear cluster, has been accepted only in the last few years (12).

A shorthand designation for simple iron-sulfur proteins has been devised. The term rubredoxin, abbreviated Rd, refers to a one-iron center (Figure 1-3a). Centers with two or more iron atoms are termed
ferredoxins, abbreviated Fd. The notations [2Fe-2S], [3Fe-xS], and [4Fe-4S] indicate the presence of two-, three- and four-iron centers respectively. The number of inorganic sulfur atoms in trinuclear clusters is presently unclear, therefore the notation for the center is [3Fe-xS]. Superscripts are used to indicate the net charge, or range of net charges for the cluster. A numerical prefix to the bracket indicates the number of centers present in the protein. A similar notation is used for the synthetic analogues.

The term "complex" iron-sulfur protein was originally coined to distinguish enzymes such as succinate dehydrogenase from the electron transfer proteins such as ferredoxins. Complex iron-sulfur proteins contain additional prosthetic groups, such as molydebenum, nickel, flavin, or cytochrome and generally have molecular weights in excess of 50 kilodaltons (KDa). The enzymes involved in the mitochondrial electron transport chain are complex iron-sulfur proteins.

**Binuclear iron-sulfur clusters**

The planar [2Fe-2S] center, consisting of two iron atoms linked by two bidentate sulfur atoms, is bound to the protein by four cysteinyl sulfurs (Figure 1-3b). This iron-sulfur center is the simplest cluster that contains a bridging acid-labile sulfur. Detailed characterizations of [2Fe-2S] centers have been made with simple systems.

An X-ray crystal structure of the chloroplast-type ferredoxin from *Spirulina platensis* shows each iron atom to be in a tetrahedral environment (13). The dimensions agree with those of a synthetic analog (14). The iron-iron distance is 2.72Å, giving a possibility of weak metal-metal interaction.
Oxidized [2Fe-2S] clusters consist of two antiferromagnetically coupled, high spin ferric ions giving an EPR-silent ground state of S=0. Although Mossbauer experiments show strong delocalization, the spectrum exhibits some broadening which was attributed to a slight inequivalence of the iron atoms (15). Upon reduction, discrete Fe(III) and Fe(II) sites are observed by Mossbauer spectroscopy, indicating localization of the unpaired electron on one of the iron atoms (15). The high spin ferrous (S=2) and high spin ferric (S=5/2) ions are antiferromagnetically coupled to give a ground state of S=1/2. This overall picture is supported by ENDOR (16), low temperature MCD (17), and magnetic susceptibility experiments (18). Reduced [2Fe-2S] centers display an EPR spectrum, generally observed at temperatures up to 120K, with g_\text{av} greater than 2 (19). Homology comparisons between [2Fe-2S] ferredoxins from diverse organisms indicate that the sequence of cysteines associated with the binding core is highly conserved (20, 21).

**Tetranuclear iron-sulfur clusters**

The [4Fe-4S] proteins are known to have at least three possible oxidation states (22). In vivo, the [4Fe-4S] center operates between two of the three oxidation levels, +2/+1 or +3/+2. The first redox couple is more common and has an E_m of around -400mV. The latter couple, known as a high potential iron-sulfur protein (HiPIP), has an E_m of around +350mV. The factors determining differences in the redox properties of these two types of centers are not yet understood. Both types of [4Fe-4S] center have similar cubane-type structures.

A [4Fe-4S] center can be visualized as two interpenetrating tertahedra of four iron and four sulfur atoms (Figure 1-3d).
Structures of two [4Fe-4S] proteins have been determined by X-ray diffraction methods, oxidized and reduced *Chromatium vinosum* HiPIP (23) and oxidized *Peptococcus aerogenes* Fd (24). The average iron-iron distance in the 2+ oxidation state is 2.75 Å and the sulfur-sulfur distance is 3.5 Å. The tetranuclear clusters are bound to the polypeptide by four cysteine ligands. The *C. vinosum* HiPIP iron-iron distances determined by EXAFS studies are in agreement with X-ray diffraction data (25).

Oxidized *C. vinosum* HiPIP formally consists of three ferric and one ferrous ions, antiferromagnetically coupled to give a ground state of S=1/2 (26, 27). Mossbauer experiments show strong electron delocalization although there is some broadening and asymmetry in the spectrum, indicating that the four iron atoms are not exactly equivalent (26). Oxidized HiPIP exhibits an essentially axial EPR spectrum with $g_{av}$ greater than 2 (28). One electron reduction of oxidized *C. vinosum* HiPIP results in a diamagnetic species, as shown by EPR (28), magnetic susceptibility (27), and MCD (29) experiments. Formally, the reduced center consists of two ferric and two ferrous atoms. Mossbauer data show the four iron atoms to be equivalent through antiferromagnetic coupling (26).

As isolated, bacterial [4Fe-4S] ferredoxins are diamagnetic. The diamagnetic form of bacterial [4Fe-4S] ferredoxins such as *Bacillus polymyxa* is structurally similar to oxidized *C. vinosum* HiPIP (22). Comparisons of the two types of diamagnetic [4Fe-4S] proteins with synthetic analogues demonstrate an isoelectronic relationship (30).

The reduced form of *B. polymyxa* Fd exhibits a rhombic EPR spectrum with principal $g$ values of 2.06, 1.92, 1.88 (31). More
complex spectra, centered about a $g_{av}$ of 1.94, are observed for reduced eight-iron ferredoxins such as that from *Clostridium pasteurianum* (32). The complex EPR spectrum is thought to arise from weak magnetic coupling between the two [4Fe-4S] centers (33). Mossbauer studies of reduced ferredoxins from *Bacillus* and *Clostridium* show strong electron delocalization (34). As with other iron-sulfur clusters, the Mossbauer spectra of these tetranuclear clusters exhibited broadening, attributed to slight inequivalence of the iron atoms. Formally, the one ferric and three ferrous ions in the [4Fe-4S] core are antiferromagnetically coupled to give a ground state of $S=1/2$.

**Trinuclear iron-sulfur clusters**

Only recently has there been direct evidence for [3Fe-xS] centers (35, 36). A recent review of trinuclear iron-sulfur clusters can be found in reference 12. The structure of the cluster and number of cysteine ligands is still unclear. An X-ray crystal structure of *Azotobacter vinelandii* ferredoxin I suggests an apparently planar Fe$_3$S$_3$ ring with cysteinyl sulfurs completing tetrahedral coordination about two of the iron atoms (Figure 1-3c). The third iron atom appeared to be coordinated by both a cysteine and an oxygen atom supplied by an exogenous ligand (37). The iron-iron distance in this essentially planar structure is 4.1Å. However, resonance Raman studies of the three-iron centers in *A. vinelandii* Fd I, aconitase, and *Desulfbirio gigas* Fd II have been interpreted in terms of a modified cubane-type structure, with iron-iron distances of about 2.7Å, in which one of the iron atoms is missing (38). Moreover, EXAFS data on *D. gigas* Fd II and aconitase (39, 40), and a recent X-ray
structure of aconitase (41) support a model in which iron-iron bond distances are no greater than 2.7Å. Analytical data for *D. gigas* Fd II and aconitase are consistent with the species [3Fe-4S]. It is not known if all trinuclear clusters have this stoichiometry.

Oxidized [3Fe-xS] centers are paramagnetic with a ground state of S=1/2, exhibiting an almost isotropic EPR signal centered at g=2.01 (42). Mossbauer data for oxidized *D. gigas* Fd II suggests that there are three equivalent, high spin Fe(III) atoms in a tetrahedral environment of sulfur atoms (43). Similar results were observed for the [3Fe-xS] center in aconitase, *A. vinelandii* Fd I, and *T. thermophilus* Fd. The reduced [3Fe-xS] center is paramagnetic and EPR-silent for reasons of zero field splitting. Mossbauer data from reduced *D. gigas* Fd II show two types of iron atoms, one high spin Fe(III) and two iron sites which equally share the electron which has entered the cluster upon reduction (43). Low temperature MCD (44) shows that the three iron atoms spin couple to give a S=2 ground state for the system.

There have been questions raised as to the biological significance of [3Fe-xS] centers, because, in some cases, there is interconversion between [3Fe-xS] and [4Fe-4S] centers (29). Trinuclear clusters are known to be formed by oxidative degradation of [4Fe-4S] centers during the isolation of enzymes such as aconitase (33). Since many enzymes and proteins are now known to contain [3Fe-xS] clusters, experiments need to be performed to determine whether or not [3Fe-xS] centers are always artifacts induced by oxidative degradation of [4Fe-4S] clusters during isolation.
REFERENCES


Figure 1-1. Respiratory chain redox components present in the inner mitochondrial membrane. Fe-S clusters associated with NADH-UQ and succinate-UQ oxidoreductase segments are designated with suffixes N-x and S-x respectively. The Q₈, Q₉, and Qₐ are protein-associated pools of ubiquinone in succinate-UQ, NADH-UQ, and ubiquinol-cytochrome c oxidoreductase segments respectively. (Taken from reference 7.)
Figure 1-2. Classification of iron proteins emphasizing the Fe-S proteins.
Figure 1-3. Approximate geometries of the four types of iron-sulfur clusters. (Taken from reference 7.)
II. THEORY

ELECTRON PARAMAGNETIC RESONANCE

EPR spectroscopy involves the absorption of microwave radiation by a system with net electronic angular momentum placed in an external magnetic field. Magnetic dipoles, arising from the angular momenta, interact with the magnetic component of the microwave radiation, resulting in magnetic dipole transitions. There are several factors which affect an EPR spectrum. The EPR interactions of the most importance to the study of iron-sulfur clusters are: the electron-spin interaction with the magnetic field, spin-orbit coupling, and intercluster spin coupling. EPR can be used to characterize the local environment of a paramagnetic center by defining the size and shape of the magnetic moment. General reviews of the application of EPR to the study of iron-sulfur proteins can be found in references 1-3.

EPR is a very powerful tool in the study of iron-sulfur clusters in biological systems. Detection of a signal requires paramagnet concentrations in the 10 to 100µM range and is unaffected by the presence of diamagnetic impurities. Since paramagnets can often be distinguished by their different spectroscopic properties, EPR can be used to study systems containing more than one type of paramagnetic center, e.g. the mitochondrial respiratory enzymes. In addition to the study of isolated enzymes, EPR has been used to study iron-sulfur clusters in membranes, intact organelles, and bacterial cells.

Due to zero-field splitting, EPR generally only detects paramagnetic transition metal centers with an odd number of unpaired electrons. Therefore it can detect either the oxidized or one-
electron reduced state of an iron-sulfur cluster, but not both states. Since EPR signal intensity is related to the spin concentration of a paramagnetic center, EPR is often used to monitor redox titrations of iron-sulfur clusters in biological systems.

**Basic EPR theory**

A brief description of basic EPR theory can be found in physical chemistry texts such as reference 4. The following description discusses some of the aspects of EPR applicable to the study of iron-sulfur centers.

In EPR spectroscopy, different energy states arise from the interaction of an unpaired electron's magnetic moment with an external magnetic field. The Zeeman Hamiltonian describing this interaction is:

\[ \hat{H} = g \beta \hat{S} \cdot B \]  

(1)

where \( g \) is the gyromagnetic ratio (g-factor or g value), \( \beta \) is the Bohr magneton, \( \hat{S} \) is the spin angular momentum operator, and \( B \) is the applied field strength.

For a \( S=1/2 \) system, the quantum number \( M_S \) is \( \pm 1/2 \), and the two possible Zeeman energies are \( \pm 1/2 (g \beta B) \). The separation between the Zeeman levels therefore increases linearly with increasing magnetic field, as shown in Figure 2-1. Transitions between the two Zeeman levels can be induced by electromagnetic radiation of the appropriate frequency \( \nu \), where \( \Delta E = h\nu \). The selection rule for absorption of microwave radiation is \( \Delta M_S = \pm 1 \).

For systems with very little effective orbital angular momentum, \( g=2.0023 \), the free electron value. However, if the electron has
orbital angular momentum, the g value can deviate markedly from the free electron value. This deviation is commonly observed in transition metal compounds and arises from coupling of orbital and spin angular momenta. For iron-sulfur clusters the g factor will differ from the free electron value in a way characteristic of the electronic structure of the cluster.

Local electric fields, originating from the ligand electrons, produce orbital coupling to the electron's magnetic moment. The spin-orbit Hamiltonian is:

\[ \hat{H} = \lambda (\hat{S} \cdot \hat{L}) \]

where \( \hat{L} \) is the orbital angular momentum operator and \( \lambda \) is the spin-orbit coupling constant. When the effects of the orbital moment are small, they are incorporated into the g value. The local electric fields can be asymmetric in shape, resulting in anisotropy of the g factor. The g-factor is therefore a tensor quantity. In an anisotropic system, the magnetic field required for resonance is related to the orientation of the molecular axes with respect to the applied field.

Description of systems with anisotropic behavior usually requires six independent parameters to be specified since the g-factor is a tensor. Except for certain cases, principal molecular axes may be chosen such that off-diagonal terms of the g tensor vanish, and only the principal g values: \( g_{xx} \), \( g_{yy} \), and \( g_{zz} \) are considered. For an arbitrary orientation of a single crystal the effective g value is:

\[ g_{\text{eff}}^2 = g_x^2 \cos^2 \theta_x + g_y^2 \cos^2 \theta_y + g_z^2 \cos^2 \theta_z \]

where \( \theta_x \), \( \theta_y \), and \( \theta_z \) are the angles between the magnetic field \( B \) and
the molecular axes $x$, $y$, and $z$.

The anisotropy of the $g$ factor has a significant effect on the lineshape of the EPR spectrum. There are three basic types of symmetry: cubic or isotropic, axial, and rhombic. In perfect cubic symmetry, ignoring Jahn-Teller distortion, $g_z = g_x = g_y$ and there is only one EPR resonance frequency. For the case of axial symmetry with $g_z \neq g_x = g_y$, there are two principal resonance frequencies. The $g_z$ is denoted as $g_{||}$ while the $g_x$ and $g_y$ are called $g_{\perp}$. In a system with rhombic symmetry, $g_z \neq g_x \neq g_y$ there are three principal resonance frequencies.

Biological samples are usually in the form of a glass, i.e., the molecules are randomly ordered in a powder. The observed spectrum is an average of spectra from all possible orientations of the molecules. Information about the anisotropy in these systems can be obtained because the resulting spectra are not those from a motionally averaged system.

For example, in an axial system, the $g$ value for any orientation is given by:

$$g^2 = g_{||}^2 \sin^2 \Theta + g_{\perp}^2 \cos^2 \Theta$$

(4)

where $\Theta$ is the angle between the principal axis and the applied field, and $g_{||}$ and $g_{\perp}$ refer to the molecular principal axes. The spectrum will be spread over a field range from $g_{||}$ to $g_{\perp}$, with the most intense absorption corresponding to $g_{\perp}$. A computer simulation, taking into account the probabilities of the various orientations and their associated transition probability, gives rise to the absorption spectrum, and its corresponding derivative, shown in Figures 2-2b and
Similar arguments apply to the rhombic powder spectra shown in Figure 2-2d. For net $S=1/2$ systems, the principal $g$ values refer both to the principal components of the $g$ tensor, and therefore to the positions of maxima, cross-over, and minima of the EPR spectrum as illustrated in Figure 2-2.

**Zero field splitting for systems with $S>1/2$**

Kramer's theorem states that for all systems with an odd number of electrons, there is at least a two-fold degeneracy of the spin state, which can be removed only by application of an external magnetic field. Thus, for isolated, non-integer systems ($S=1/2, 3/2, 5/2$), conditions can usually be found for obtaining an EPR resonance, $\Delta M_s = \pm 1$. For systems with even numbers of unpaired electrons, the degeneracy of spin states may be removed even in the absence of a magnetic field. The phenomena of removal of spin degeneracy in the absence of a magnetic field is known as zero-field splitting. A Hamiltonian for zero-field splitting can be written in terms of two independent parameters, $D$ and $E$, as shown in equation 6. The $D$ and $E$ parameters indicate the magnitude of axial and rhombic zero-field splitting, respectively.

$$\hat{H} = D\left(S_z^2 - \frac{1}{3}S(S + 1)\right) + E(S_x^2 - S_y^2)$$

In systems of even spin greater than zero, the EPR spectrum can generally be observed only if zero-field splitting is very small. If the separation between energy states is larger than the microwave quantum, it may be impossible to observe the EPR spectrum. This is frequently true for transition metals and invariably true for iron sulfur clusters.

For example, in a reduced $[3Fe-xS]$ cluster ($S=2$), the individual
electron spins correlate to produce the zero-field splitting illustrated in Figure 2-3. In the absence of a magnetic field and \( E=0 \), there are two doubly degenerate levels, \( M_s=\pm 1 \) and \( M_s=\pm 2 \), and a nondegenerate level, \( M_s=0 \). Rhombic distortion \((E\neq 0)\) or application of a magnetic field splits the system into five spin states. Due to the selection rule, \( \Delta M_s=\pm 1 \), transitions within doublets are forbidden. While a transition between doublets is allowed, the zero field splitting is usually large enough that the microwave energy is not sufficient to result in resonant absorption for the attainable magnetic fields.

**Spin relaxation**

In the case of an \( S=1/2 \) system, the presence of an applied magnetic field divides the set of spins into two populations, those with their spins aligned with the field, and those with their spins antiparallel to the field. The two sets of spins are related by the Boltzmann distribution:

\[
\frac{N_a}{N_b} = \exp\left(-\frac{g\beta B}{kT}\right)
\]  

(6)

where \( N_a \) and \( N_b \) are populations of the upper and lower states. When a sample absorbs energy of \( g\beta B \), transitions between the two states are induced. Since upward and downward transitions are of equal probability, net absorption depends on the fact there is a greater population in the lower state than in the upper state. The processes which tend to maintain this population difference are called relaxation processes \((4-7)\). Due to spin-orbit coupling and the presence of low lying excited states, iron sulfur clusters undergo rapid spin relaxation.
Relaxation processes can broaden the EPR spectrum of the paramagnet for reasons due to the Heisenberg Uncertainty Principle:

$$\Delta E \Delta \tau \geq \frac{\hbar}{2\pi}$$ (7)

Using the spin-lattice relaxation time, $T_1$, as an estimate of $\Delta \tau$, a small $T_1$ value will lead to a large $E$. The result is a smearing of energy levels and a broadened EPR spectrum.

A phenomenon known as microwave power saturation occurs when the incident radiation becomes sufficiently intense that the rate of transitions to the upper state exceeds the rate at which spins can relax to the lower state. Spin-lattice relaxation results from interaction of the paramagnet with the thermal vibrations of the lattice. The mechanisms of spin-lattice relaxation depend upon the presence of extended lattice motions which diminish upon going to lower temperature. Thus, the intensity of the microwave power which can be used, and therefore the EPR signal intensity, diminishes as the temperature is lowered. The temperature used in EPR spectroscopy is usually a compromise between the saturation effect, the linewidth effect, and the Curie law. The Curie law states that magnetic susceptibility is proportional to the reciprocal of the temperature.

Broadening of the EPR spectrum can also result from dipolar coupling between two paramagnets. Interaction between spins will lead to splitting of the EPR spectrum, which may become very complex depending on the anisotropy of the g tensors. The magnitude of the interaction depends strongly on the distance between the paramagnets. As the distance between the paramagnets increases, the splittings are not resolved and are detected as line broadening. Dipolar spin-spin interactions sometimes result in enhancement of the spin relaxation.
rates for individual paramagnetic centers. In principle, it is possible to calculate the spin-lattice and spin-spin relaxation times, $T_1$ and $T_2$, for Lorentzian lineshapes. However, the methods require homogeneous broadening, i.e. the sample contains identical paramagnets which all resonate at the same magnetic field. Because of the complicated relaxation behavior of iron-sulfur proteins, usually only the power saturation behavior of the EPR signal is studied, without attempting to determine relaxation times.

In order to characterize power saturation behavior, and hence relate spin relaxation rates, EPR spectra of iron-sulfur clusters are recorded at microwave powers ranging from negligible saturation to pronounced saturation. Microwave saturation can be used to distinguish overlapping EPR signals since features arising from different species often have different saturation behavior. Saturation behavior can be compared by a plot of $\log(I/\sqrt{P})$ versus $\log P$, where $I$ is the signal intensity and $P$ is the microwave power. As power saturation is reached, the plot of $\log(I/\sqrt{P})$ versus $\log P$ slopes downward with increasing microwave power. Comparison of power saturation curves for similar iron-sulfur clusters can be useful in detecting spin-spin interactions between centers in a protein.

Spin Quantitation

EPR signals can be quantified in terms of the concentration of the paramagnet which gives rise to the signal (8). EPR signal intensity, under non-saturating conditions, depends on the sample concentration, sample temperature, and spectrometer settings. Comparison of the EPR signal intensity for an unknown sample with the signal intensity of a sample of known concentration, under identical
conditions, yields a spin concentration for the unknown sample. Differences in spectrometer settings such as spectrometer gain, magnetic field modulation amplitude, and microwave power can be corrected for.

The EPR spectrum is obtained as the first derivative of the absorption spectrum. Calculation of signal intensity involves calculation of the area under the absorption, $A$, by double integration of the derivative signal according to the equation given in reference 9:

$$A = h^2 \sum_{r=1}^{n} r y_r$$

where $h$ is the magnetic field interval, $y_r$ is the magnitude of the EPR signal for the $r$th interval, and $n$ is the number of intervals.

For temperatures between 100K and 20K, and microwave powers of 1mW, a 1mM aqueous solution of copper EDTA was used as the standard. The equation used is:

$$\frac{N_u}{N_s} = \left( \frac{A_u}{A_s} \right) \left( \frac{H_u}{H_s} \right) \left( \frac{P_u}{P_s} \right) \left( \frac{G_s}{G_u} \right) \left( \frac{g_s}{g_u} \right)$$

where subscripts $s$ and $u$ refer to standard and unknown, $H$ refers to field modulation amplitude, $P$ is microwave power, $G$ is the spectrometer gain, $N$ refers to the number of spins in a sample, and $g$ is the average $g$ value. The average $g$ value for a compound is calculated as:

$$g = \sqrt{\left( \frac{g_x^2 + g_y^2 + g_z^2}{3} \right)}$$

Due to power saturation of copper EDTA at temperatures below 20K for the available microwave powers, another standard, 1mM metmyoglobin cyanide, was used for temperatures below 20K. The $g_x$ and $g_y$
absorbances of metmyoglobin are too broad to enable accurate spin quantitation using the entire spectrum. However, Aasa and Vangard (8) have developed a procedure to determine the spin quantitation of a rhombic or axial EPR resonance, using only the isolated "absorption-shaped" component of the signal. In the case of metmyoglobin cyanide, the $g_z$ peak was used. The equation used for EPR quantitation using metmyoglobin as the standard is given in equation 12. Calculation of signal intensity for the entire EPR spectrum of the unknown sample follows equation 9.

In this instance, $A_g$ corresponds to the area under the $g_z$ resonance, i.e.:

$$A_g = \sum_{r=1}^{n} y_r$$

and

$$T_S = \frac{\beta}{h \nu} \left( \frac{g_x + g_y}{2\left(1-\rho_x\right)^2\left(1-\rho_y\right)^2} \right) \rho_i = \frac{g_i}{g_z}$$

MAGNETIC CIRCULAR DICHROISM

The Faraday effect was discovered in 1845, but has only recently been applied to the study of metalloproteins. For reviews of application of low temperature MCD to study of metalloproteins, see references 10-12. Magnetic optical activity is induced in all matter by application of a longitudinal magnetic field. The Faraday effect arises from the interaction of the magnetic field with the electronic states of chromophores, resulting in differential absorption of right circularly polarized (RCP) and left circularly polarized (LCP) light, $A=A_{LCP}-A_{RCP}$. Since MCD can be positive or negative, it shows more detail and greater resolution than the corresponding absorption
spectrum. Thus, MCD is particularly useful in characterizing the
electronic transitions of iron-sulfur proteins. Iron-sulfur proteins
exhibit broad, featureless absorption spectra due to numerous
overlapping sulfur to iron charge transfer transitions.

All of the known iron-sulfur clusters are paramagnetic in at
least one accessible oxidation state. In paramagnetic species, MCD
signal intensity can be increased by a factor of up to 70 upon going
from ambient temperature to liquid helium temperatures. Therefore,
low temperature MCD is a sensitive optical probe for paramagnets, and
can be used to study paramagnets in the presence of overlapping bands
arising from other chromophores.

In addition to providing information on electronic transitions,
MCD can probe the magnetic properties of electronic ground states by
means of magnetization curves at discrete wavelengths. While similar
information is available from EPR, Mossbauer, and magnetic
susceptibility experiments, MCD offers certain advantages over each of
the techniques when applied to the study of metalloproteins. MCD is
less limited than EPR, in that transition metal centers with an even
number of unpaired electrons can be studied. Therefore MCD can be
used to obtain electronic ground state information from EPR-silent
paramagnetic clusters. Mossbauer studies of metalloproteins generally
require $^{57}$Fe enrichment, which is not always feasible. Both Mossbauer
and magnetic susceptibility experiments can be very difficult to
interpret for multicomponent proteins, such as mitochondrial enzymes.
In MCD spectroscopy, the magnetic properties of the individual centers
can be studied by use of magnetization curves at discrete wavelengths.
However, it should be emphasized that each technique is the most
powerful when used in conjunction with one or more of the others. The information content of the various techniques used to study metalloproteins is complimentary. Since EPR studies have been reported in the literature for many of the enzymes studies in this work, parallel MCD and EPR experiments were performed on the enzyme samples. This permits comparison and reinterpretation of published work.

**Basic MCD theory**

The theoretical basis for MCD can be found in references 13-15. A brief description follows. The angular momentum of a spherically symmetric system is specified by the quantum numbers $J$ and $M_J$, which represent the eigenvalues of the total angular momentum and the $z$-component of the angular momentum, respectively. In the absence of a magnetic field, a state specified by $J$ has $(2J+1)$-fold degeneracy. In the presence of a magnetic field, this degeneracy is removed. The relative energies of the states are determined by the gyromagnetic ratio or $g$ factor.

The selection rule governing an electric dipole transition between electronic states with $J=0$ and $J=1$ is $\Delta M_J = \pm 1$. The $\Delta M_J = 0$ transition is polarized parallel to the magnetic field, while the $\Delta M_J = \pm 1$ transitions are polarized in the plane perpendicular to the field. CP light can be thought of as being composed of photons having total angular momenta of $J=1$ with $M_J = +1$ or $-1$. Therefore, absorption of a photon of CP light causes the system to change in total angular momentum by 1, while the $z$-component of the angular momentum changes by $+1$ or $-1$ for RCP and LCP, respectively.

MCD intensity for a particular electronic transition $a \rightarrow j$, $\Delta \Lambda$, \ldots
can be described as the sum of three terms: A, B, and C terms.

$$\Delta A(a \rightarrow j) = \gamma \left\{ -A_1 \left( \frac{\partial f}{\partial E} \right) + \left( B_0 + \frac{C_0}{kT} \right) f \right\} \beta b b l$$

(13)

where B is the magnetic field strength, k is the Boltzmann constant, b is the sample concentration, and l is the pathlength. The f refers to a normalized bandshape function which describes the distribution of transition energies E, about the absorption maximum. The γ is a constant and is expressed as:

$$\gamma = \frac{N \pi \alpha^2 \log e}{250 n \pi n}$$

where α is the absorption coefficient and n is the refractive index of the material.

The form of the A and C terms can be readily understood by considering a simple atomic transition, e.g., $^1S \rightarrow ^1P$. The A term results from a transition from a degenerate, or non-degenerate ground state to a degenerate excited state (Figure 2-4a). Absorption of RCP and LCP light each give a transition, separated in energy by the Zeeman splitting of the excited state (Figure 2-4b). MCD corresponds to the difference between these two bands and is expressed as $\Delta A = A_L - A_R$. It has the form of a symmetrical, temperature-independent, biphasic signal crossing the abscissa at $\nu_0$, the frequency at the maximum of the absorption. Hence, the A term has a derivative dispersion and is independent of temperature (Figure 2-4c).

If the ground state is degenerate and split by a magnetic field, a C term results. The splitting pattern and selection rules are the same, but the electronic population of the two ground states, $M_j=-1$ and $M_j=+1$, is dependent on the temperature according to the Boltzmann
distribution (Figure 2-5a). Transitions from the lower state will have a higher intensity, and $A_L$ will be greater than $A_R$ (Figure 2-5b). The resultant MCD band will therefore have absorption-shaped dispersion and will be inversely dependent on temperature (Figure 2-5c). For paramagnetic chromophores, this term dominates the low temperature MCD spectrum due to its temperature dependence, and enables low temperature MCD to become a sensitive optical probe for paramagnetic metal centers in biological systems.

A third term is the B term, arising from magnetically-induced mixing of states that are not necessarily degenerate. The magnetic field causes levels to be mixed due to the presence of magnetic dipole transition moments between the states. The amount of mixing is inversely proportional to the energy separation. The B term has a lineshape similar to that of the C term, but it has no temperature dependence.

By various techniques, MCD spectra of highly symmetrical mononuclear transition metal complexes can be analyzed, and the terms assigned to individual electronic transitions. Due to the relatively low symmetry, lack of detailed structural information, and complexity of the electronic structure, similar analysis of the MCD spectrum is generally not possible for biological metal centers such as iron-sulfur proteins. However, different types of iron-sulfur clusters have been found to exhibit characteristic MCD spectra, enabling cluster type to be determined by the pattern of bands and magnetization data.

Magnetization curves

The exact form of the field and temperature dependence of the MCD
spectrum of a paramagnet can be used to yield information about the nature of the ground state and an estimation of the ground state \( g \) factors (17, 18). The MCD signal intensity is directly proportional to \( B/T \) when the Zeeman splitting, \( g\beta B \), is much less than \( kT \). When \( g\beta B/kT \) approaches 1, \( \Delta A \) becomes non-linear as a function of \( B/T \). At very low temperatures and high fields, only the lowest Zeeman sublevel is populated and \( \Delta A \) is independent of \( B/T \). At this point, the MCD signal is said to be magnetically saturated. Figure 2-6 illustrates theoretical MCD magnetization curves for systems with \( S=2 \) and \( S=1/2 \).

Theoretical expressions have been developed to analyze the complete temperature and field dependence of an MCD spectrum of a randomly oriented system for the case of an axial, "isolated" Kramer's doublet ground state \( (S=1/2) \) (18). The expressions relate the intensity of the MCD signal at a given magnetic field and temperature to the ground state \( g \) factors of an axial system, \( g_\parallel \) and \( g_\perp \), and to the polarization ratio of the electronic transition, \( \frac{m_z}{m_+} \), where \( m_+^2 \) and \( m_z^2 \) are the electric transition dipole moments in the molecular xy and z directions, respectively. The expression is a function of the angles between the molecular principal axes and the direction of the applied field. The expression must be averaged over all angles since samples are in the form of a frozen glass which contains all possible orientations of an assembly of molecules.

The MCD equation for the case of an axial, \( S=1/2 \) ground state is:

\[
\langle A_0 \rangle = K \left( \sqrt{2} \right) \mathcal{F}_1(n) \tanh(GV)dn + \mathcal{F}_2(n) \tanh(GV)dn
\]

where:

\[
G = g_\perp \beta B/2kT, \quad V = \sqrt{n^2(S^2-1)+1}, \quad S = g_\parallel/g_\perp
\]
and $V$ is the Zeeman splitting at angle $\theta$. The MCD magnetization behavior of reduced [2Fe-2S] and [4Fe-4S] clusters are described by this equation.

Oxidized [3Fe-xS] clusters are isotropic, enabling simplification of the above expression. Their MCD magnetization behavior is described by the equation:

$$\langle A_0 \rangle = k \left( 2 \sqrt{2} m_x m_+ m^2 \right) \tanh(G)$$

(15)

For reduced [3Fe-xS] clusters with axial zero-field splitting, the lowest doublet would be expected to have $g=8, 0, 0$. MCD magnetization data for this special case can be described by the equation:

$$\langle A_0 \rangle = k m_+^2 \int_0^1 \frac{1}{n} \tanh \left( \frac{ng \beta B}{2kT} \right) dn$$

(16)

If the anisotropy of the $g$ factor is determined through EPR experiments, comparison of theoretical and experimental MCD magnetization curves can show that the EPR and MCD transitions arise from the same ground state. In addition, ground state $g$ factors can be estimated from magnetization curves in certain limiting cases. When the ground state is isotropic, $g_\parallel = g_\perp$, then the ratio of the asymptotic limit of the curve to the initial slope equals $1/g$. For a completely axial doublet such that $g_\parallel \neq 0$ and $g_\perp = 0$, this ratio equals $3/(2g_\parallel)$.

For systems such as a reduced [3Fe-xS], $S=2$, the nature of the
lowest energy state can be studied by analyzing magnetization data collected at temperatures below 2K. At very low temperatures where there is a significant population of only the lowest doublet, theoretical expressions for an isolated, doublet ground state can then be applied to yield estimations of the ground state effective g factors. Both field-induced mixing of zero-field components and rhombic distortion are assumed to be negligible in such an analysis.

It is possible to recognize spin states of greater than 1/2 from magnetization curves in three ways. (1) Experimental magnetization data cannot be adequately simulated by theoretical data based on g values around 2.0. (2) Plots of $\Delta A$ versus $1/T$ will only become linear when $kT$ is much larger than the energy spread of the zero field components. (3) A plot of $\Delta A$ versus $B/2kT$ generally results in a series of "nested" curves, rather than the smooth curve obtained for the $S=1/2$ system, due to population of low lying zero field components.

REFERENCES


Figure 2-1. Energy level diagram for $S=1/2$ in presence and absence of a magnetic field $B$. $\Delta E$ is the transition energy for the EPR experiment.
Figure 2-2. Idealized absorption and derivative EPR spectra for unoriented S=1/2 systems.
Figure 2-3. Energy level diagram for a $S=2$ system with axial ($E=0$) and rhombic ($E \neq 0$) zero-field splitting.
Figure 2-4. The transitions and expected MCD spectra for a $^1S \rightarrow ^1P$ transition, A term. (a) energy level diagram; (b) absorption spectrum; (c) MCD spectrum.
Figure 2-5. The transitions and expected MCD spectra for a $^1P \rightarrow ^1S$ transition, C term. (a) energy level diagram; (b) absorption spectrum; (c) MCD spectrum.
Figure 2-6. MCD magnetization curves. Solid line is theoretical curve for the lowest $M_s = \pm 2$ doublet of a $S=2$ ground state, $g_\parallel = 8.0$, $g_\perp = 0.0$. Broken line is theoretical curve for $S=1/2$, $g_\parallel = 2.026$, $g_\perp = 1.927$, $m_2/m_+ = -1$. 
III. MATERIALS AND METHODS

INSTRUMENTATION

MCD spectrometer

MCD spectra were obtained using an Oxford Instruments SM3, split coil, superconducting magnet mated to a Jasco J500C spectropolarimeter (Figure 3-1). This system permits measurement of a MCD spectrum in the wavelength range of 200 to 1000nm, at temperature ranges between 1.5 and 300K, and at magnetic fields between 0 and 4.5 Tesla.

A variable, longitudinal magnetic field is applied to the sample mounted in the optical chamber of the split-coil superconducting solenoid (Figure 3-2a). The sample chamber of the magnet is centered between the split coil and can be filled with liquid helium from the main helium reservoir by opening a needle valve. Magnetic fields were calibrated using a transverse Hall probe (Lake Shore Cryogenics) in place of the sample on the sample probe. The magnet calibration was linear over the range of 0 to 5 Tesla. The accuracy of the magnetic field measurement was ± 0.03 Tesla.

A strong magnetic field can affect the operation of some of the components of the dichrograph, e.g. the photomultiplier tube (PMT) and the photoelastic modulator (PEM). To avoid this problem, the magnet was spatially separated from both the optical system and the PMT by one meter. Tests involving measurement of the CD baseline in the absence of a sample, and the CD spectrum of a standard, D-tris(ethylenediamine)cobalt(III), (D-Co(en)_3), placed after the magnet, in the presence and absence of magnetic fields, gave no indication of
magnetic field effects on dichrograph components, or strain birefringence in magnet windows. In this configuration, the presence of a 4.5T magnetic field had no effect on the CD spectrum of D-tris(ethylenediamine) cobalt(III) trichloride (Co(en)$_3$).

The dichrograph exit slit, the optical chamber of the magnet, and the FMT grid were aligned using an optical rail. Stray light was eliminated by means of an optical tube connecting the windows of the magnet's optical chamber to the dichrograph. The slight decrease in the dichrograph's signal to noise ratio due to the slightly divergent light beam had no noticeable effect on the quality of the MCD or CD spectra.

Linearly polarized light is converted alternately, at a frequency of 50KHz, to LCP and RCP light by the PEM. The FMT is phase-locked to the PEM frequency for measuring the differential absorption of RCP and LCP light. Phase-sensitive detection greatly increases the instrument's signal-to-noise ratio. The difference in absorption between LCP and RCP was recorded as a function of wavelength.

Typically, MCD data were collected at temperatures of approximately 1.6K, 4.218K, 10K, and occasionally at a higher temperature, e.g. 80K. Temperatures below 4.218K, the normal boiling point of liquid helium, are obtained by pumping on a bath of liquid helium with a two stage rotary pump. Sample temperatures above 4.218K are achieved by pulling cold helium gas into the sample chamber and were controlled by a Rh/Fe resistor and heater connected to an Oxford Instruments DTC2 temperature controller. Due to the increased signal noise resulting from the bubbling helium, MCD spectra were not collected between 4.218 and 2.2K. Below the lambda point of helium,
2.2K, the helium bath becomes optically clear, and moreover, helium becomes a good heat conductor at temperatures below 2K, eliminating problems with thermal gradients.

**MCD temperature measurements**

Sample temperatures were measured with calibrated carbon glass resistors (Lake Shore Cryogenics) placed directly above and below the sample (Figure 3-2b). Placement of the thermistors allows temperature gradients in the sample to be detected. The resistors were calibrated by Lake Shore Cryogenics at 42 points over the temperature range of 1.5 to 300K. Temperature measurement accuracy was ±0.5%.

**MCD sample probe**

The sample cell is mounted on the end of a probe as shown in Figure 3-2b. The probe also houses the temperature sensors and heater. Samples were frozen by lowering the sample probe directly into the sample chamber filled with liquid helium.

**MCD sample cells**

MCD sample cells consist of a rubber spacer (ca. 1.5mm thick) sandwiched between two polished quartz plates (1.5 X 1.5cm, 1mm thick), cemented together with an epoxy resin. After curing, the cell is sealed by application of the epoxy resin over the external surface of the rubber spacer. Two small gaps are left for needle entry and injection of the sample into the cell. The difference between cell width and the sum of the widths of the two quartz plates yields the cell pathlength.

The MCD cell is pumped and flushed with argon prior to introduction of the enzyme sample. The rubber spacer of the cell is pierced with a gas-tight syringe and the sample is injected into the
Glassing agents

Frozen MCD samples are in the form of a glass to allow transmittance of light. For the formation of the optical quality glass, protein solutions were made up to be 50% in ethylene glycol, or 40% in glycerol, and rapidly frozen by immersion into the magnet's bath of liquid helium.

The presence of the glassing agent had no effect on the activity, UV-visible absorption, CD, or EPR characteristics of the various enzymes. Strain in the frozen glass may cause some depolarization of the light beam, leading to decreased signal intensity. Strain birefringence in the glass is assessed, and corrected for, by measuring the natural CD of D-Co(en)$_3$ placed after the sample, in the absence of an applied field. The depolarization correction is the percentage difference between the CD signal height of D-Co(en)$_3$ with the MCD sample and the CD signal height of D-Co(en)$_3$. The depolarizations in the enzyme samples studied were less than 10%.

MCD measurements

MCD spectra are measured at positive, negative, and zero magnetic field. Positive magnetic field is defined as magnetic field aligned with the direction of light propagation. Similarly, negative field is when the magnetic field is aligned against the direction of light propagation.

Acceptable MCD data at positive and negative field must form mirror images of one another reflected in the zero field, natural CD baseline. MCD spectra were corrected for contributions from natural CD by subtracting negative field from positive field and dividing by
two. The MCD intensity is expressed as the difference in the molar extinction coefficients for LCP and RCP light, \( \Delta \epsilon = \epsilon_{\text{LCP}} - \epsilon_{\text{RCP}} \) in units of \( \text{M}^{-1}\text{cm}^{-1} \).

Spectra were recorded digitally with an OKI IF800 model 30 microcomputer interfaced via a Jasco IF500 interface. Data manipulation and storage were performed using the commercially available Jasco computer program. Subtraction of natural CD and the differencing of MCD spectra were accomplished by use of the Jasco computer program which digitally subtracted the two appropriate spectra. The Jasco computer program was also used to correct MCD spectra for differences in concentration, depolarization, pathlength, and sensitivity.

In a typical MCD spectrum, data were sampled every 0.4nm at a scan speed of 50nm per minute and a time constant of 2 seconds. Between 800nm and 510nm the slit openings were fixed at 100\( \mu \text{m} \). Slits programmed for constant resolution were used in the region between 510 and 250nm.

MCD magnetization plots were measured by monitoring MCD intensity at several fixed temperatures as a function of the magnetic field strength. The data are presented as plots of \% magnetization versus \( \beta B/2kT \) where \% magnetization refers to the MCD intensity as a percentage of the intensity at magnetic saturation. Theoretical magnetization curves, generated from the appropriate equation given chapter 1 were fit to the experimental data. The BASIC computer program used for calculating the theoretical data can be found in reference 1.
**UV-visible and CD spectroscopy**

Room temperature absorption spectra were recorded under anaerobic conditions in serum-capped 1mm quartz cuvettes with a Cary 219 UV-visible absorption spectrometer. Room temperature CD spectra were recorded in the same quartz absorption cell using the circular dichrograph described previously.

**EPR spectroscopy**

EPR spectra were recorded on a Varian E-line X-band spectrometer fitted with an Air Products Helitrans low temperature cryostat. This system permits recording of spectra at microwave powers between 0.5 and 100mWatt and temperatures between 8.5 and 300K. Sample temperatures were varied by balancing helium flow against a heater connected to the temperature controller. The magnetic field calibration was checked using 2,2-diphenyl-1-picrylhydrazine (DPPH).

Sample temperatures were accurately measured for each sample temperature using a calibrated silicon diode (Lake Shore Cryogenics) in a water filled EPR tube, positioned in place of the sample in the cryostat. The diode was calibrated at 95 points over the range of 4 to 300K. Temperature reading accuracy was ±2%.

The EPR spectra were quantified by double integration according to published procedures (2, 3). Spin quantitations of each EPR sample were performed at least three times. Care was taken to ensure that the quantitation was made under non-saturating conditions. When appropriate, e.g. high gain and low temperature, the baseline spectrum was obtained and manually subtracted off from the sample EPR spectrum.

EPR samples were prepared simultaneously with MCD samples.
enzyme solution was injected into an argon-flushed EPR tube, rapidly frozen by immersion in liquid nitrogen, and stored in liquid nitrogen until use.

SAMPLE HANDLING

Samples were handled anaerobically using either a vacuum/argon line equipped with a BASF copper catalyst column, or an inert atmosphere box (Vacuum Atmospheres model HE-43-2) equipped with a dri-train (Vacuum Atmospheres model HE-493) and an oxygen analyzer (Vacuum Atmospheres model AO-316-C). Oxygen levels in the inert atmosphere box and the gas handling line were below 1ppm. All solutions used were rigorously degassed by three freeze-pump-thaw cycles. Solutions were contained in Schlenk flasks or serum-capped vials, and transferred by argon-flushed gas-tight syringes. Solids were degassed by three evacuation-flush cycles while in Schlenk flasks. Transfer of solids and manipulation of intact bacterial cells was performed in the inert atmosphere box. Frozen enzyme pellets were taken up in the appropriate degassed buffer using either the gas handling line or the inert atmosphere box.

Protein reductions were performed under anaerobic conditions in a serum-capped 1mm quartz cuvette. Extent of reduction was monitored by UV-visible spectroscopy. The iron-sulfur proteins usually were reduced by addition of approximately 10μL of a freshly prepared, degassed sodium dithionite solution. Preparation of the dithionite solution was as follows. A weighed amount of sodium dithionite was placed in a Schlenk flask, degassed, and dissolved in 10ml of degassed water. The solution was then degassed once and used immediately.
Unless otherwise indicated, a ten-fold excess of dithionite, based on the protein concentration, was added. Solutions of other oxygen-sensitive compounds, e.g., ammonium iron(II) sulfate and NADH, were prepared, in a similar manner.

PROTEIN ISOLATION, MANIPULATION, AND EXPERIMENTS

NADH-UQ OXIDOREDUCTASE

Preparation of high molecular weight NADH dehydrogenase and Complex I

Enzyme purification and assays were performed by Drs. Rona Ramsey and Thomas Singer at the Veterans Administration Medical Center in San Francisco, California. Complex I and Type I enzyme were prepared from beef heart mitochondria according to published procedures (4, 5). Enzyme samples were analyzed for non-heme iron (6), acid-labile sulfide (6), FMN (6), protein concentration (7), and activity (8) prior to shipment to Baton Rouge. Enzyme activity was assayed by the ferricyanide method.

Since freezing of the enzyme causes a loss of activity in NADH dehydrogenase, the enzyme samples were shipped overnight to Louisiana State University, Baton Rouge on wet ice. Upon arrival, the enzyme solutions were concentrated, made to be 50% in ethylene glycol, separated into portions, and then treated with the appropriate reagents prior to spectroscopic study. As received, Type I enzyme was in 30mM potassium phosphate, pH 7.8. Complex I was suspended in a buffer of 50mM Tris-HCl, pH 8.0 containing 0.66M sucrose and 1mM histidine. Parallel MCD and EPR experiments were performed on Type I enzyme. Complex I was studied by EPR spectroscopy.

Enzyme samples were treated with: a 20-fold excess of dithionite;
a 5-fold excess of freshly prepared NADH; or a 20-fold excess of
dithionite followed by addition of a 5-fold excess of NADH. (NADH is
photolabile and a solution of it must be prepared immediately before
use.) Two EPR samples of NADH-reduced soluble and complex enzyme were
prepared. One EPR sample was frozen immediately after addition of
NADH. The other sample was frozen at the same time the MCD sample was
placed in the magnet's liquid helium bath, approximately 2 minutes
after addition of the reductant.

Photochemical reduction of NADH-UQ oxidoreductase

In an adaptation of the procedure described for photochemical
reduction of flavoproteins (9, 10), a sample of enzyme was made to be
10mM in EDTA and 1.1mM in deazaflavin, placed in an EPR tube under
argon, and immersed in an ice-water bath. The sample was irradiated
with a 200Watt xenon-mercury arc lamp for 30 minutes and frozen by
immersion in liquid nitrogen. Additional samples of photoreduced Type
I enzyme and Complex I were prepared, treated with a 5-fold excess of
NADH, and frozen in EPR tubes.

SUCCINATE-UQ OXIDOREDUCTASE

Preparation of succinate dehydrogenase and Complex II

The purification and assays of SDH were performed by Drs. Brian
A.C. Ackrell and Edna Kearney at the Veterans Administration Medical
Center in San Francisco, California. The enzyme preparations, in the
form of ammonium sulfate pellets, were shipped in liquid nitrogen to
Louisiana State University, Baton Rouge. After spectroscopic study,
the frozen protein solutions were shipped, on dry ice, to San
Francisco for determinations of protein (7), flavin (11), and non-heme
iron (11) content; acid-labile sulfide (11), and for assay of FMS-
reductase and "low $K_m$"-ferricyanide reductase activities (12). A
brief description of the purification methods emphasizing the
differences from published procedures follows.

Complex II was prepared from beef heart mitochondria according to
published procedures (13). Reconstitutively active SDH (BS-SDH) was
extracted from butanol-treated Complex II at pH 9.2 under strictly
anaerobic conditions, in the presence of 20mM succinate and 1mM
dithiothreitol (DTT) and purified as previously described (12). A
second enzyme preparation (P-SDH) was purified according to the method
of Davis and Hatefi, except that succinate was absent (14). The
procedure involved sequential treatment of Complex II with 0.4M and
0.8M sodium perchlorate, under argon, in the presence of 1mM DTT.
This enzyme preparation was expected to have little reconstitutive
activity since succinate was absent and strict anaerobiosis is
difficult to maintain during homogenation steps in the procedure. The
Complex II used for EPR studies was incubated with 20mM succinate for
10 minutes at 30°C to dislodge tightly bound oxaloacetate. It was
then washed three times by centrifugation to remove free oxaloacetate
and succinate (15).

For use in spectroscopic experiments, pellets of BS-SDH or P-SDH
were taken up in a pH 7.8 buffer containing 100mM potassium phosphate,
100mM succinate, 1mM DTT, and 50% ethylene glycol. Samples designated
as-isolated P-SDH were taken up in the phosphate buffer without
succinate. Parallel MCD and EPR experiments were performed on soluble
SDH. Complex II, which was not studied with MCD, was suspended in a
buffer of 100mM potassium phosphate, pH 7.8, 100mM succinate, 1mM DTT,
and 3% Triton X-100. The presence of Triton X-100 was necessary for the suspension of the particulate preparation.

BS-SDH core conversion experiments

In an adaptation of the procedure described for the activation of aconitase (16), a 15-fold excess of aqueous ammonium iron(II) sulfate was added to a solution of BS-SDH in 100mM potassium phosphate, pH 7.8, 100mM succinate, 10mM DTT, and 50% ethylene glycol. The samples were incubated at 35°C or at room temperature, and then reduced with a 20-fold excess of dithionite for use in EPR and MCD experiments. Incubation times were 30 and 15 minutes.

Attempted reconstitution of [3Fe-xS] core in P-SDH

In a slight modification of the procedure described by Baginsky and Hatefi (17), P-SDH was taken up in 20mM piperazine-N,N'-bis[2-ethansulfonic acid] (PIPES), pH 7.0, and 100mM succinate to a final concentration of 10mg/ml of protein. The enzyme solution was made to be 4mM in ammonium iron(II) sulfate, 65mM in mercaptoethanol, and 4mM in sodium sulfide, in the order indicated. All manipulations were performed in an inert atmosphere box at room temperature. After standing for 20 minutes, the solution was applied to a Sephadex G25 column and the sample was reduced with excess dithionite and frozen for use in EPR.

Oxygen damage experiments with BS-SDH

BS-SDH pellets were taken up in an aerobic buffer of 0.15 Tris-HCl, pH 7.4, and 50% ethylene glycol. The enzyme was divided into four portions. One portion was placed in an EPR tube and frozen immediately. The second sample was exposed to air for one hour before being frozen in an EPR tube. The third and fourth portions were
exposed to air for one hour, reduced with succinate, and succinate and dithionite, and then were frozen in EPR tubes.

In a subsequent experiment, pellets of BS-SDH were taken up in an anaerobic buffer of 100mM potassium phosphate, 100mM succinate, pH 7.8 containing 50% ethylene glycol. Parallel MCD and EPR experiments were performed on the enzyme samples. The enzyme was divided into three portions which were exposed to air for 0, 1 and 4 hours, respectively, before being reduced with dithionite and used for spectroscopic study.

**Oxygen damage experiments with P-SDH**

Pellets of aerobically isolated P-SDH were taken up in an anaerobic buffer of 100mM potassium phosphate and 100mM succinate, pH 7.8 containing 50% ethylene glycol. This sample of SDH was almost completely deficient in center S3. Parallel MCD and EPR experiments were performed on all samples. The enzyme solution was divided into three portions which were exposed to air for 0, 1, and 4 hours, respectively, before being reduced with dithionite and used for spectroscopic study.

**Preparation of yeast Complex II**

The purification and assays of yeast Complex II were performed by Drs. Roger Schilling and Graham Palmer at Rice University, Houston, Texas. Many of the established procedures generally applied to purification of beef heart mitochondrial enzymes are unsuccessful with yeast enzymes. The detailed procedure for purification of yeast Complex II can be found in reference 18.

The enzyme preparations were transported on ice to Louisiana State University, Baton Rouge, Louisiana. For spectroscopic experiments, yeast Complex II was in a buffer of 10mM N-2-
hydroxyethylpiperazine-N-ethanesulfonic acid (HEPES), pH 7.5, 0.5mM EDTA, 0.05% octyl glucoside, and 50% ethylene glycol. After concentration, the enzyme was divided into two portions, one of which was reduced with a 10-fold excess of sodium dithionite for use in MCD and EPR experiments.

**E. coli Fumarate Reductase**

**Preparation of fumarate reductase from E. coli**

Amplification, enzyme isolation, and assays were performed by Drs. Gary Cecchini and Brian Ackrell at the Veterans Administration Medical Center, San Francisco, California. Enzyme samples and whole *E. coli* cells were shipped to Louisiana State University, Baton Rouge packed in dry ice. After spectroscopic study, the frozen enzyme solutions were shipped, packed in dry ice, to San Francisco for determinations of protein (7), flavin (11), non-heme iron (11), acid-labile sulfide (11), and specific activity (19). A brief description of the cell growth and enzyme preparation follows.

*E. coli* cell 1100 (bglR thi lack Nfr Pol) was transformed with plasmid pGC 1002 which codes for the entire fumarate reductase operon and amplifies levels of this enzyme in the cell (19). To induce maximal levels of fumarate reductase, the bacterium was grown anaerobically on a glycerol-fumarate medium (20). Details of the isolation of the fumarate reductase complex can be found in reference 21. The procedure entails extracting the cytoplasmic fraction with a pH 6.8, 100mM phosphate buffer containing 30mM octyl-β-D-glycopyranoside, 0.1mM phenylmethylsulfonylfluoride, and 1mM DTT. The extract was centrifuged, dialyzed against a pH 6.8 phosphate buffer
containing 1mM DTT, and centrifuged again. The resulting pellet of fumarate reductase complex was stored in liquid nitrogen until use.

Soluble fumarate reductase was obtained from the complex enzyme essentially as described by Davis and Hatefi (14). Details of the procedure can be found in reference 21. Two forms of the soluble enzyme were obtained, that treated with 0.4M sodium perchlorate and that treated with 0.8M sodium perchlorate. It was anticipated that the enzyme purity and the relative amounts of iron-sulfur centers in these preparations would be different. Perchlorate treatment has been shown to damage the [3Fe–xS] center in mammalian SDH (22).

For use in spectroscopic experiments, the soluble fumarate reductase pellets were taken up in a pH 7.8, 100mM potassium phosphate buffer containing 1mM DTT, 100mM succinate, and 50% ethylene glycol. The complex enzyme was taken up in the same buffer containing 3% Triton X-100. The samples designated as-isolated were taken up in the above buffers, except that the buffer did not contain succinate.

Amplification of discrete fumarate reductase subunits in E. coli cells

E. coli cell 1100 (bglR thl rell Nfr Pol) was transformed with plasmids which amplified levels of specific fumarate reductase subunits in the cell. The plasmids used in this work were derived from pGC1002 (19) and pFRD5-10 (see below). Information concerning plasmid size, location of pertinent restriction endonuclease sites, and the fumarate reductase subunits for which the plasmid codes can be found in reference 23. Reference 23 also gives the details of construction of pFRD23, which codes for intact A and B subunits, and pFRD105, which codes for an intact A subunit and a truncated B subunit. Amplification of cells for subunits ABCD is described above.
For construction of pFRD39, the 1.4-kb **SalI/BglII** fragment of pGC1002 was ligated into the **BamHl/SalI** sites of pRK9. Plasmid pRK9 is derived from pBR322, which contains the **trp** promoter/operator region in front of the **BamHl** site. Therefore, plasmid pFDR39 codes for synthesis of the B subunit and expression of the gene product can be regulated by conditions which turn on tryptophan biosynthesis. For construction of pFRD40, which codes for the synthesis of the BCD subunit, the **BglII/BamHl** fragment from plasmid pFRD5-10 was ligated into the **BamHl** site of pRK9. Therefore, synthesis of subunits BCD is also under **trp** promoter control. Plasmid pFRD5-10 is very similar to pGC1002 except that the fumarate reductase operon has been ligated into a different plasmid vector, pACYC184, rather than pBR322, and pFRD5-10 has the genes in reverse orientation to pGC1002.

Samples for EPR spectroscopy were harvested anaerobically and suspended in 50mM potassium phosphate buffer, pH 7.8, followed by addition of 10µL of the appropriate reagent. Cells were incubated in (1) 50mM fumarate, (2) 40mM glycerol and 20mM dithionite, or (3) 1mM ferricyanide.

**Fumarate reductase complex core conversion experiments**

Fumarate reductase complex was taken up in a 50mM potassium phosphate buffer, pH 7.8 with 2% Triton X-100. The enzyme solution was divided into five portions. EPR samples were prepared for the following samples: as-isolated; dithionite-reduced; dithionite-reduced followed by incubation at room temperature for 30 minutes; dithionite-reduced followed by incubation for 30 minutes with a 5-fold excess of aqueous ammonium iron(II) sulfate; and incubation for 30 minutes at room temperature in the presence of EDTA (100µM).
Soluble fumarate reductase core conversion experiments

Pellets of 0.8M perchlorate treated fumarate reductase were taken up in a 20mM PIPES buffer, pH 7.8, containing 100mM succinate and 50% ethylene glycol. This enzyme sample was completely deficient in center FR3. Parallel EPR and MCD experiments were performed on all samples. The enzyme was divided into two portions. One portion was reduced with dithionite. The remainder was incubated 15 minutes at room temperature with a ten-fold excess of ammonium iron(II) sulfate before reduction with dithionite.

Oxidative damage experiments with fumarate reductase

Fumarate reductase complex was taken up in an anaerobic buffer of 50mM potassium phosphate, pH 7.8, 2% Triton X-100. The protein was stirred in air for 10 minutes, made to be 50% in ethylene glycol, and divided into two portions. One portion was studied by MCD and EPR. The remainder was treated with excess of potassium ferricyanide, desalted, concentrated, and made to be 50% in ethylene glycol for use in MCD and EPR experiments. After spectroscopic study, the oxidized samples were thawed anaerobically, reduced with dithionite, and examined by EPR. In a similar experiment, soluble fumarate reductase was taken up in 50mM potassium phosphate, pH 7.8, exposed to air, and studied by MCD and EPR.

ETF-UQ OXIDOREDUCTASE

Preparation of electron transfer flavoprotein-UQ oxidoreductase

Enzyme isolation and assays were performed by Dr. Frank E. Frereman at the Medical College of Wisconsin, Milwaukee, Wisconsin. Enzyme samples were shipped in dry ice to Louisiana State University,
Baton Rouge for spectroscopic study.

Details of enzyme purification can be found in reference 24. Briefly, pig liver submitochondrial particles were extracted with cholate. The extract was centrifuged, desalted, and fractionated by chromatography on DEAE cellulose, followed by ion-exchange chromatography on hydroxylapatite. Samples were then desalted, concentrated to 1mg/ml, made to be 20% in glycerol, and stored in liquid nitrogen until use. The glycerol is necessary to prevent an approximate 50% inactivation by a single freeze-thaw cycle.

ETF dehydrogenase, as received, was in a buffer of 10mM Tris-HCl, pH 7.4 containing 10mM potassium phosphate, 20% glycerol, and 8mM CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate). The presence of CHAPS is necessary to prevent precipitation of the protein. The enzyme was concentrated and made to be 40% in glycerol for a final concentration of 55μM. The enzyme was reduced with a 20-fold excess of dithionite. Use of ethylene glycol as the glassing agent resulted in degradation and precipitation of the protein. Glycerol, although more viscous and therefore more difficult to handle, was therefore used as a glassing agent for preparation of MCD samples.

Enzymatic reduction of ETF dehydrogenase

According to the procedure described for enzymatic reduction of ETF dehydrogenase (24), the enzyme in 50mM TAPS (tris[hydroxymethyl]methylaminopropanesulfonic acid), pH 8.5, and 40% glycerol, was made to be 0.1μM in General acylCoA dehydrogenase and 0.1μM in ETF. The substrate, octylCoA, was added in portions while monitoring changes in the UV-visible absorption spectrum of the enzyme mixture. After no
further decreases in absorption intensity could be detected, the sample was frozen for use in EPR spectroscopy.

**T. THERMOPHILUS RIESKE PROTEIN**

**Preparation of Thermus thermophilus Rieske protein**

Protein isolation was performed by Dr. James A. Fee at the University of Michigan, Ann Arbor, Michigan. Protein samples were shipped on dry ice to Louisiana State University, Baton Rouge for spectroscopic study. A detailed description of the purification of *T. thermophilus* Rieske protein can be found in reference 25. The procedure involved extraction of *T. thermophilus* membranes with a nonionic detergent followed by ion exchange chromatography. The protein, as received, was in a pH 7.8 buffer of 75mM Tris-HCl and 0.1mM EDTA. For use in spectroscopic study, the protein was made to be 50% in ethylene glycol for a final protein concentration of 218μM (ε₄₀₀=28.14M⁻¹cm⁻¹), and was reduced with a 10-fold excess of dithionite.

**SPINACH FERREDOXIN**

**Preparation of spinach ferredoxin**

Crude spinach ferredoxin was obtained from Sigma Chemical Company and then purified according to an adaptation of the procedure described by Petering and Palmer (26). The protein was made to be 85% saturated in ammonium sulfate, centrifuged twice at gX22K for 20 minutes, and desalted by ultrafiltration. The ratio of the UV-visible absorbance at 420nm to the absorbance at 275nm was 0.37, an acceptable level of purity. (The ratio reported in reference 26 was 0.4.)
After concentration, the protein was made to be 50% in ethylene glycol and reduced with dithionite for use in MCD and EPR experiments.

REFERENCES


Figure 3-1. Arrangement of Jasco J-500 spectropolarimeter and Oxford Instruments Superconducting magnet.
Figure 3-2. (a) Schematic representation of Oxford's Instruments, split coil superconducting magnet, type SM3: (S) sample holder, lowered into sample space; (W) optical windows; (M) coils of superconducting magnet; (T) tube for transferring liquid helium from main reservoir to sample chamber.

(b) Schematic of MCD sample probe.
IV. NADH-UBIQUINONE OXIDOREDUCTASE

BACKGROUND

NADH-Ubiquinone (NADH-UQ) oxidoreductase is an enzyme complex present in the mitochondrial inner membrane. It is one of several enzymes involved in the mitochondrial electron transport chain (1-4). The enzyme catalyzes proton translocation coupled to the oxidation of NADH by UQ (5, 6). Thus, the oxidations initiated by NADH-UQ oxidoreductase are responsible for the majority of the ATP synthesized by oxidative phosphorylation. NADH-UQ oxidoreductase is thought to be comprised of twenty six polypeptides and five or six redox centers.

NADH-UQ oxidoreductase is the most complex flavoprotein in the respiratory chain, and the most difficult to isolate due to its high affinity for membrane lipids. In addition detergents can entrap the enzyme, causing aggregation problems. Therefore, very few enzyme preparations are homogeneous. Reported molecular weights of the enzyme complex and of its constituent polypeptides vary widely depending on the isolation procedure and the method of molecular weight determination.

Enzyme preparations

NADH-UQ oxidoreductase has been isolated and studied in three basic forms: Complex I, Type I, and Type II enzyme. Phosphorylating and non-phosphorylating submitochondrial particles are also used to study the enzyme. Submitochondrial particles (SMP) are obtained by fragmentation of isolated mitochondrial preparations through mechanical disruption (7). These fragments are pieces of the inner mitochondrial membrane which contain all the enzymes of the
respiratory chain. The fragments have an inverted membrane orientation relative to intact mitochondria. Therefore, membrane-bound enzymes are freely accessible to the chemical species present in the suspending medium.

Study of processes occurring in SMP is more complicated than working with isolated enzymes, particularly in spectroscopic experiments. However, SMP are less complex than the intact mitochondria. Since it is not certain how closely these isolated extracts of NADH-UQ oxidoreductase model the enzyme complex present in the intact mitochondria, comparative experiments with SMP are often performed.

Complex I (8-10), in addition to the NADH dehydrogenase portion, contains components which are required for energy coupling at site Ia. This preparation is in a highly aggregated state and is often contaminated with proteins from other components of the respiratory chain. It is highly useful for comparison with simpler NADH-UQ oxidoreductase extracts since it retains all known properties of the intact enzyme. The EPR spectrum shows the presence of at least four iron-sulfur centers (1-4).

Complex I has been resolved into three components by a combination of chaotropic agents and ammonium sulfate fractionation (10). Such treatment separates the enzyme complex into a hydrophilic flavo-iron-sulfur protein (FP), a soluble iron-sulfur protein (IP), and an insoluble protein fraction (Figure 4-1).

The FP fraction corresponds to Type II enzyme. It contains three a The site where a proton gradient is generated by the flow of electrons from NADH.
polypeptides (FP-I, FP-II, FP-III) of molecular weights 53, 27, and 11 Kda (11-13). The FP fraction has been further resolved into two subfractions, FP-I and FP-II+FP-III. FP-I contains four non-heme iron atoms and four acid-labile sulfides (14). FP-II+FP-III contains two non-heme iron atoms and two acid-labile sulfides. The IP fraction has also been resolved into two subfractions, IP-I and IP-II (11-13). IP-I is a 75 Kda polypeptide. IP-II contains 40, 33, and 56 Kda polypeptides. Both of these subfractions contain non-heme iron and equivalent acid-labile sulfides (14).

Type I enzyme contains most of the polypeptides present in Complex I, plus a 30 Kda polypeptide (15, 16). Its catalytic properties, non-heme iron and sulfide contents, and EPR signals are similar to those of Complex I (17-19). The difference between the preparations is the absence of rotenone-sensitive NADH-ubiquinone reductase activity in Type I enzyme.

Type II NADH enzyme is very different from Type I enzyme. It is composed of three polypeptides of molecular weights 53, 27, and 11 Kda (20-23), and contains five to six non-heme iron atoms and equivalent acid-labile sulfides. The turnover number, substrate specificity, and EPR signals exhibited by Type II enzyme are quite different from those observed in Complex I and Type I enzyme. NADH-reduced Type II enzyme exhibits two types of EPR signals corresponding to iron-sulfur clusters (24). The slower relaxing resonance, observed at about 40K, has g values of 2.03, 1.94, and 1.92 and is believed to correspond to center N1b in Complex I (see below). The other EPR signal, observed at temperatures below 30K, has g values of 2.045, 1.95, and 1.87 with a spin concentration of 0.5 spins per FMN.
It is believed that Type I enzyme contains the FP and IP fractions isolated from Complex I. Type II enzyme is thought to contain the FP fraction. Type I enzyme can be converted to Type II by heat treatment, chaotropic agents, or proteolysis (21, 25), although the reverse conversion has not been accomplished. Reconstitution of either Complex I or Type I enzyme has not been accomplished.

Iron-sulfur clusters

In addition to FMN, NADH-UQ oxidoreductase contains multiple iron-sulfur centers which are involved in electron transfer. The number and type of iron-sulfur centers present in the enzyme complex is still a controversial subject. Complex I and Type I preparations each contain 16 to 24 iron atoms per molecule of FMN (8, 14, 15). In Complex I, acid-labile sulfide and iron are present in equal amounts (18, 26). In most Type I preparations the molar ratio of sulfide to iron is 1.5 to 1.6. The difference in the iron to sulfide ratio has not been explained.

Upon reduction with NADH, four iron-sulfur centers become detectable by EPR at 12K in SMP, in Complex I, and in Type I enzyme. These centers are designated N1, N2, N3, and N4. While there is good agreement that N2, N3, and N4 are present in concentrations approximately stoichiometric with FMN, N1 is reported to be present in concentrations ranging from 0.4 to 0.8 spins per FMN (1). An additional center N5, accounting for up to 0.25 spins per FMN, has been proposed by Ohnishi. The EPR lineshapes of the individual iron-

b Reconstitution refers to the ability of an enzyme preparation to bind NADH dehydrogenase-depleted SMP and restore electron transfer activity from NADH to oxygen.
sulfur centers are obtained by computer simulation of Complex I spectra (27). Since the EPR signals of the iron-sulfur centers are very sensitive to minor changes in the core environment (14), EPR spectra of the individual subunits isolated from Complex I do not provide reliable information on native NADH-UQ oxidoreductase. Therefore, information is obtained from more complex systems, i.e., SMP and Complex I, by indirect methods. The redox behavior of iron-sulfur centers giving rise to EPR signals is known to change depending on the degree of purification of the enzyme.

The EPR lineshape obtained for NADH-reducible N1 at 40K was simulated by Albracht arising from two axial species (N1a and N1b) present in equal concentrations (28). Ohnishi reported that one rhombic signal (N1b) provided the best fit to the data (24). This species has g values of 2.02, 1.94, 1.92. In later work, the two research groups agreed that the N1 spectrum could be satisfactorily fit by a set of parameters describing one rhombic center, N1b (29). The rhombic EPR signal for N1b is observed in beef heart SMP (28, 30). However, EPR signals for N1 in bacteria (31) or yeast (28, 30) SMP are simulated as an axial species.

Ohnishi believes that there are two components to N1 (32-34). This second center N1a, has a midpoint redox potential that is too low to allow reduction by NADH (24, 32-34). The N1a EPR signal in pigeon heart mitochondria is observed upon reduction with dithionite in the presence of mediator dyes. The signal has axial symmetry with g values of 1.94 and 2.03. Experiments with beef heart preparations were interpreted in terms of two centers, N1a and N1b, with slightly different redox potentials and EPR lineshapes (24). In Complex I,
Ohnishi measured a midpoint potential of lower than \(-500\) mV at pH 8.0 for Nla. The value is higher, ca. \(-380\) mV, for intact mitochondria. Albracht does not believe in the existence of Nla. The reader should note that in the early literature Albracht's center Nla and Nlb correspond to Ohnishi's center Nlb. Both Ohnishi's and Albracht's groups do agree that each of their two components of N1 is present in lower concentrations than that of FMN. According to Ohnishi, the concentration of N1 in Complex I is at most 0.8 per FMN (35). Albracht reports a value of 0.5 per FMN in Complex I (28).

N2 has a well established EPR spectrum at 15K (37). The center has a relatively high midpoint redox potential (greater than \(-140\) mV)\(^c\) and can be reduced by NADH alone (32-34, 38). The signal has axial symmetry with g values of 2.054 and 1.928. The concentration of N2 in Complex I is reported to be between 0.77 and 0.89 per FMN (28, 36).

EPR signals for N3 and N4 have been resolved by a combination of potentiometric titrations and computer simulations of spectral lineshapes (28). Albracht designated N3 as the signal g= 2.103, 1.935, 1.885, and N4 as the signal g=2.032, 1.925, 1.862 (28). Ohnishi uses the opposite designation (39). Albracht's designation for N3 and N4 will be used in this work.

The intensity of the combined signals of N3 and N4 give a spin concentration of 1.8 per FMN in Complex I (35, 36). The N3 concentration is estimated to be 0.56 to 0.66 per FMN (28). The concentration of N4 is estimated to be 0.86 to 1.08 per FMN. The latter concentration is not reliable since it is obtained from the

\(^c\) The midpoint potential is preparation dependent. Some show \(-20\) mV, while others show \(-140\) mV.
high-field half of the $g_x$ line at 1.86 which is partly obscured by the $g=1.88$ line. Albracht reported that a concentration ratio of 1:1 of N4 to N2 gave the best computer simulation. A ratio of N3 to N2 which was slightly less than one gave the best fit.

Signals originally ascribed to N5 are no longer thought to be due to a component of NADH–UQ oxidoreductase (28, 39). The center N6 is now called N5. The EPR signal is observed in beef heart in Complex I and SMP, and in pigeon heart SMP at $g=1.89, 1.92$, N5 is present in variable amounts, from less than 6% to less than 25% of FMN in Complex I (39). It is rapidly reduced by NADH, and its spin relaxation is more rapid than that of the other iron-sulfur centers.

EPR computer simulations of the hyperfine interaction in $^{57}$Fe-enriched SMP from yeast concludes that N1 is a [2Fe-2S] center (30). The conclusion that N1 is a binuclear center is in agreement with the size of the coupling constant between iron atoms in Type I enzyme and the temperatures at which N1 EPR signals can be observed (40). The g values, hyperfine interaction, and temperature dependence of N2 suggest that it is a [4Fe-4S] center. Broadening due to hyperfine interactions was not detected for the $g_x$ or $g_z$ tensors of N3, nor on $g_x$ of N4. However, hyperfine interactions observed with ENDOR in $^{57}$Fe-substituted [4Fe-4S] proteins are smaller than for [2Fe-2S] proteins (41). It was concluded that N3 and N4 are tetranuclear clusters, in agreement with their spin relaxation behavior.

These EPR results disagree with iron-sulfur core extrusion studies on Type I enzyme (42). Using $^{19}$F NMR spectroscopy, binuclear and tetranuclear clusters were detected in a ratio of 2:1. The concentration of non-heme iron and acid-labile sulfide after extrusion
was equal to the concentration in the original enzyme extract. However, it is possible that [4Fe-4S] clusters present in Complex I are converted to [2Fe-2S] clusters during cluster extrusion or preparation of Type I enzyme. It also has been suggested that some binuclear cores in Complex I could either be inaccessible to reductants, and therefore diamagnetic in the usual redox potential range, or that the cores are EPR-undetectable due to spin-spin interaction with neighboring redox components (43).

**Spatial organization of NADH dehydrogenase components**

Various biophysical and biochemical approaches have been useful in the determination of the spatial arrangement of the components of the respiratory chain. Spatial relationships of various redox centers are important in forming a coherent picture of electron and proton transfer mechanisms. Up to twenty six polypeptides can be detected in Complex I by SDS-polyacrylamide gel electrophoresis (13). Distribution of the constituent polypeptides has been studied using surface labeling of Complex I, of SMP, and of intact mitochondria with impermeable probes, with diazobenzene \(^{35}S\) sulfonate, or with lactoperoxidase-catalyzed \(^{125}I\) iodination, followed by SDS-polyacrylamide gel electrophoresis (44-45). Results show that none of the three FP subunits are exposed to the surface, even in Complex I. This is interesting since the NADH binding site is thought to be on the 53 Kda subunit (46). Some part of this subunit must be close to the matrix side of the membrane for NADH oxidation to occur.

The IP fraction is transmembranous, but is excluded from direct interaction with the lipid phase of the membrane by the hydrophobic shell. The 40 Kda polypeptide, thought to be responsible for NADH-NAD
transhydrogenation (47), is located in the IP segment. The location of the 130 Kda subunit, involved in NADPH-NAD transhydrogenation (48), is unclear but is probably transmembrane. Several hydrophobic subunits are not exposed to either side of the mitochondrial inner membrane surface, but are in contact with the hydrophobic interior of the membrane. Figure 4-2 shows how some of the subunits are thought to be located in the membrane.

Image reconstruction electron microscopy shows NADH-UQ oxidoreductase to be a tetramer (49). Using an estimated molecular weight of 750 Kda, it was concluded that each tetramer cannot contain more than one FMN. This implies that the tetramer is only part of Complex I, since there is evidence that one functional enzyme unit of Complex I contains two FMN (50-51). Structural and functional analysis of NADH-UQ oxidoreductase could be aided by fragmentation of the enzyme complex and study of the fragments. Questions remain as to whether the iron-sulfur centers in these subfractions are different from those observed in Complex I.

Based on visible and EPR spectroscopies, and on iron and acid-labile sulfide contents of Type II enzyme, it was concluded that FP-II+FP-III contains a binuclear center (14, 46). Determination of the primary structure of FP-II showed that it has the four cysteine residues necessary for a binuclear center (40). Presumably, this cluster is Ni. FP-I also appears to contain an iron-sulfur center, but it is unclear which center it contains. The EPR behavior is indicative of a [2Fe-2S]. Amino acid analysis shows six cysteine residues, enough for one [4Fe-4S], but not enough for the two binuclear centers indicated by the iron content (20). N2 is believed
to be at or near the active site (46). Since Type II enzyme is capable of catalyzing the oxidation of NADH by artificial electron acceptors, it is reasonable to assume that the iron-sulfur center on FP-I is N2. Therefore, centers N3 and N4 must be in the IP fragment of NADH-UQ oxidoreductase.

Rationale

Clearly the number and type of iron-sulfur clusters in NADH-UQ oxidoreductase remains a matter of dispute. Conclusions about the cluster composition are primarily based on EPR spectroscopy, which may be an inadequate tool since paramagnetic iron-sulfur clusters can be EPR-silent for reasons of zero-field splitting or weak magnetic interactions between paramagnetic centers. Low temperature MCD spectroscopy can resolve some of the uncertainty about the cluster composition of NADH dehydrogenase since the temperature dependences of the spectra are unaffected by small zero-field splittings or weak spin interactions.

Complex I cannot be studied by MCD due to the amount of cytochrome impurities which totally dominate the low temperature MCD spectrum. Therefore, MCD spectra and magnetization data, with parallel EPR experiments were performed on Type I enzyme. Comparative EPR studies of Complex I and soluble enzyme were necessary because of the paucity of reported EPR data on Type I enzyme.

RESULTS AND DISCUSSION

Identical MCD and EPR experiments were performed on two preparations of Type I NADH dehydrogenase. Complex I was studied with
similar EPR experiments.

**Analytical data**

Table 4-1 summarizes the analytical and assay data for the Complex I and Type I enzyme used in this work. Assuming a molecular weight of 683 kDa, the FMN content and specific activity assay indicated that Complex I was approximately 50% pure. The non-heme iron content, 16 g-atoms Fe/mole FMN, is at the lower end of the range reported for Complex I preparations, indicating that the contaminating protein does not contain non-heme iron. The absence of FAD suggests that Complex II and ETF dehydrogenase are not the contaminates. Analytical and assay data for soluble enzyme are in good agreement with previously reported values (52).

**EPR data for Complex I**

Table 4-2 summarizes the EPR data for Complex I and Type I enzyme preparations used in these experiments. Spin quantitations are based on flavin determinations for the samples.

EPR spectra at temperatures between 8 and 70K for Complex I after reduction with dithionite in the presence and absence of NADH are illustrated in Figures 4-3 and 4-4. The spectra can be interpreted in terms of four distinct S=1/2 iron-sulfur clusters, corresponding to N1, N2, N3, and N4. EPR signals originating from other mitochondrial iron-sulfur proteins were not observed. Spectra similar to those shown in Figure 4-4 were observed for enzyme samples reduced with NADH or via photochemical reduction in the presence of EDTA and deazaflavin (Figure 4-5).

As previously observed, Complex I contains an iron-sulfur center which is reduced by NADH but not by dithionite. This signal, observed
at temperatures above 30K, corresponds to N1, \( g=2.021, 1.940, 1.925 \).
N1 is present in amounts approximately stoichiometric with FMN, in agreement with values reported by Ohnishi (24). However, no increase in EPR signal intensity was observed for the photochemically reduced enzyme, either in the presence or absence of NADH. Therefore, either the controversial, low potential center, N1a, is not present in Complex I, or the center cannot be reduced under these conditions.

Below 30K, EPR signals arising from three additional iron-sulfur centers can be observed. These signals correspond to N2, N3, and N4; and can be resolved into their individual rhombic components by differences in microwave power saturation behavior and reducibility with dithionite. In the presence of NADH, these clusters are fully reduced and occur in amounts approximately stoichiometric with FMN. The EPR spectrum of N2 exhibits an axial resonance with \( g=2.05, 1.928 \). N2 is the only center which is completely reduced by dithionite in the absence of NADH. The signal for N2 can be most clearly observed in the 20K EPR spectrum of dithionite-reduced Complex I. The more rapidly relaxing signals for N3 and N4 are only partially reduced in the presence of dithionite, and thus their contribution to the 20K EPR spectrum is small.

At 8K and 1mW microwave power, N2 is strongly power saturated and the EPR resonances for N3 and N4 can be observed clearly in dithionite reduced Complex I. Since the \( g \) resonances of both centers are obscured by the N1 and N2 signals, the \( g \) values quoted for the middle resonance frequency are those derived from spectral simulations (28). Unfortunately, accurate spin quantitation was not possible for N4 since none of the resonances attributed to N4 can be completely
separated from the other components of the composite EPR spectrum. Estimation of the lineshape for the high field band of N4 results in a value which is probably slightly high, but it suggests that N4 is present in amounts approximately stoichiometric with FMN.

**EPR data for soluble Type I enzyme**

As-isolated soluble NADH dehydrogenase exhibits a EPR spectrum centered about \( g = 2.00 \) attributed to the flavin semiquinone. No other signals were observed in the 8.5 to 100K temperature range.

For reasons which are presently unclear, some samples of reduced, soluble NADH dehydrogenase exhibited EPR signals similar to those observed in Complex I (Figures 4-6 and 4-7), while other samples exhibited the very broad, rhombic EPR signals illustrated in Figure 4-8. The spin quantitations of the entire EPR spectrum for these two types of spectra are similar (Table 4-2). It was not possible to resolve contributions from the individual centers in the broadened spectra.

The four iron-sulfur cluster EPR signals observed in Complex I are apparent in both types of soluble enzyme, although they are significantly broader. As in Complex I, there was no increase in the EPR signal intensity, corresponding to reduction of a very low potential center, during photochemical reduction in the presence or absence of NADH. Based on similarities in EPR spin quantitations, it appears that the iron-sulfur cluster compositions of Complex I and soluble NADH dehydrogenase are the same. One difference between Complex I and Type I enzyme is that dithionite reduction of soluble enzyme resulted in partial reduction of N1 (Figure 4-6), whereas in Complex I, N1 remained oxidized in the presence of dithionite.
Unfortunately, the soluble enzyme samples used in the MCD experiments exhibited the broadened EPR signals shown in Figure 4-8. Due to the unavailability of additional enzyme, it was not possible to determine if there are differences in the MCD spectra corresponding to the two types of EPR spectra. Based on the similarities of the EPR lineshapes, spin quantitations, and power saturation behavior, it is not unreasonable to believe that the two types of soluble samples exhibit similar MCD spectra. The broadening of the EPR signals could result from changes in the magnetic interactions between paramagnetic centers, due to slight changes in the conformation of the enzyme. However, changes in the EPR spectrum could also indicate structural changes in the clusters, or changes in cluster composition. The form of the spectra illustrated in Figure 4-8 does resemble that of a superimposition of EPR signals from the various subfractions resolved from Complex I by chaotrophic agents (53). Experiments in which conditions of sample handling were varied suggested that the type of EPR signal elicited does not depend on the glassing agent, the buffer, or length of reductant incubation time. Slight differences in enzyme isolation and/or storage may be responsible for the observed differences in the EPR spectra.

**MCD data for soluble Type I enzyme**

The low temperature MCD spectrum of NADH-reduced soluble NADH dehydrogenase was dominated by electronic transitions arising from low spin \((S=1/2)\) ferric cytochrome (not shown). Attempts to obtain the form of the MCD spectrum for the paramagnetic iron-sulfur centers by subtracting the 90K MCD spectrum from the lowest temperature spectrum (1.5K) were not successful.
Figure 4–9 shows the room temperature absorption and low temperature MCD spectra at 1.53, 4.22, 7.5, and 90K at a magnetic field of 4.5 tesla for dithionite-reduced soluble NADH dehydrogenase. The absorption spectrum indicates the presence of low spin (S=0) ferrous cytochrome in the bands centered around 558 and 426 nm. The MCD spectra exhibit sharp temperature-independent bands between 500 and 560 nm, and a very weak temperature-independent, derivative-shaped Soret band centered around 416 nm which are attributed to low spin (S=0) cytochrome. The temperature-dependent derivative-shaped band between 400 and 455 nm is indicative of a trace of high spin (S=2) ferrous cytochrome. All other MCD transitions originate from paramagnetic iron-sulfur centers. The paramagnetic contributions in the low temperature MCD spectrum were obtained by subtracting the 90K MCD spectrum from the 1.53K MCD spectrum (Figure 4–9c).

MCD spectra have been recorded for a wide range of proteins which contain well characterized binuclear or tetranuclear clusters (54). Reduced [4Fe-4S] clusters characteristically exhibit broad positive MCD bands centered around 750 nm, multiple bands between 350 and 500 nm, and negative features around 320 nm. In addition, the MCD intensities for the most intense transitions in a reduced [4Fe-4S] center are in the range of 50 to 75 M^−1 cm^−1. In contrast, the dominant feature in all reduced [2Fe-2S] clusters reported thus far is an intense (∆ε=200 to 300 M^−1 cm^−1 at 4.2K and magnetic 4.5 tesla) negative band centered around 325 nm. Therefore, the intensity of the low temperature MCD spectrum of dithionite-reduced soluble NADH dehydrogenase is consistent with the presence of 2 to 3 reduced tetranuclear centers, and maximally 0.3 of a reduced [2Fe-2S] center, in agreement with EPR
spin quantitations.

Magnetization data were collected at 750 nm (Figure 4-10) and 320 nm (not shown). The data were well fit by a theoretical curve for a system with $S=1/2$ and $g$ values centered around 2, indicating that the MCD transitions arise from reduced [4Fe-4S] and/or [2Fe-2S] clusters (54). Magnetization data cannot distinguish between the presence of reduced binuclear and tetranuclear clusters. However, it does exclude the presence of EPR-silent paramagnetic iron-sulfur centers with $S > 1/2$.

Figure 4-11 shows the room temperature absorption and low temperature MCD spectra at 1.53, 4.22, 8.0, and 90 K at magnetic field 4.5 tesla for soluble NADH dehydrogenase reduced with dithionite in the presence of NADH. The form of the spectrum is similar to that obtained for the dithionite-reduced enzyme, including the diamagnetic contributions from cytochromes. Differencing the 1.53 K and the 90 K MCD spectra reveals the form of the MCD spectrum for the paramagnetic iron-sulfur clusters (Figure 4-11c). Comparison of the MCD spectrum of dithionite-reduced enzyme with that of enzyme reduced with dithionite in the presence of NADH is shown in Figure 4-12. Increases in MCD intensities for the dithionite plus NADH reduced soluble NADH dehydrogenase can be attributed to further reduction of iron-sulfur clusters, in accord with EPR results. The form of the low temperature MCD spectrum corresponding to these additional contributions to the spectrum (Figure 4-12c) was obtained by differencing Figures 4-8c and 4-12c. The form and intensity of the resulting spectrum is best rationalized in terms of the presence of a mixture of between 0.4 and 0.7 reduced [2Fe-2S] centers and between 0.5 and 1.0 reduced [4Fe-4S]
centers, in agreement with EPR results. Magnetization data collected at 750 and 320nm (not shown) were well fit by theoretical curves for a system with $S=1/2$ and $g$ values centered about 2, indicating that there are no other EPR-silent paramagnetic centers with $S>1/2$.

**SUMMARY AND CONCLUSIONS**

The MCD studies of soluble Type I NADH dehydrogenase agree with the iron-sulfur cluster assignment based on quantitative EPR experiments and give no indication of the presence of any paramagnetic, EPR-silent iron-sulfur clusters. The results are best rationalized in terms of four NADH-reducible iron-sulfur clusters, each approximately stoichiometric with FMN. Based on the EPR properties and MCD intensities of these species, NADH dehydrogenase contains one [2Fe-2S] cluster (N1), and three [4Fe-4S] clusters (N2, N3, and N4). The cluster composition accounts for 14 non-heme iron atoms, and therefore is in reasonable agreement with the iron to FMN ratio of 16:1 observed in Complex I. The variation in the literature values for the iron to FMN ratio (8, 14, 15, 36, 55-58) and the higher value (Fe/FMN of 22:1) for the soluble enzyme, can be attributed to impurities, or to partial loss of FMN during enzyme isolation.

Ohnishi et al. has reported evidence for the presence of a non-NADH reducible iron-sulfur cluster, Nla, in Complex I (32-34). However, samples of Complex I and Type I NADH dehydrogenase which were photochemically reduced with EDTA and deazaflavin did not exhibit any increase in EPR signal intensity, relative to the dithionite plus NADH-reduced enzyme. Since the deazaflavin radical is a powerful one electron reductant ($E_o=-650\text{mV}$ at pH 7.9 (59)), center Nla is either
inaccessible to the redox mediator, or is not present in the enzyme samples.

The EPR and MCD analysis of intact NADH dehydrogenase disagree with results from EPR studies of resolved subfractions of Complex I which were interpreted in terms of three [4Fe-4S] clusters and five or six [2Fe-2S] clusters (54). Since the MCD results eliminate the possibility that some of the [2Fe-2S] clusters are EPR-silent due to weak spin interactions, the only other rationalization for such a cluster composition is that most of the [2Fe-2S] clusters in intact NADH dehydrogenase are inaccessible to reductant or have redox potentials below -650mV. Such a situation seems unlikely. In addition, there are questions as to whether the iron-sulfur clusters observed in the resolved subfractions are different from those observed in Complex I. Only a small amount of the iron in any subfraction can be accounted for by the EPR signals due to iron-sulfur clusters, suggesting cluster degradation. Therefore, the observed EPR signals may represent artifacts of the fractionation procedure rather than intrinsic components of NADH dehydrogenase.

The results of cluster extrusion experiments with Type I enzyme (42) appear to disagree with the cluster composition assigned by MCD spectroscopy. However, breakdown of one of the [4Fe-4S] clusters into two [2Fe-2S] clusters during extrusion could account for the apparent discrepancy. The tetranuclear cluster in the nitrogenase iron protein from *Azotobacter vinelandii* is known to fragment to give a binuclear cluster upon treatment with bipyridyl compounds (60). It would be of interest to determine if such a cluster conversion could occur in NADH dehydrogenase during the extrusion experiment.
There is some concern as to the relationship between Complex I and the soluble NADH dehydrogenase used in the MCD experiments. As previously discussed, reduced soluble Type I NADH dehydrogenase can exhibit two types of EPR signals, signals similar to those observed in Complex I, or the broader spectra shown in Figure 4-8. The source of the line broadening is unknown. However, activity assays, iron/sulfide analysis, and similarities in EPR characteristics of these samples suggest that the differences in the EPR spectra do not indicate a different cluster composition.

Broadening of EPR resonances can result from dipolar coupling between paramagnets (see chapter 2). Slight changes in the conformation of the enzyme, and hence the orientation and/or distance between iron-sulfur centers, could account for differences in EPR spectra. It is important to determine the conditions required to elicit EPR line broadening, and to verify that there is no corresponding change in the MCD spectrum of the enzyme. Unfortunately, there was insufficient enzyme for such studies.

REFERENCES


York.


Table 4-1

Analytical and assay data for samples of Complex I and Type I NADH dehydrogenase used in spectroscopic studies

<table>
<thead>
<tr>
<th></th>
<th>Complex I</th>
<th>Type I</th>
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<tbody>
<tr>
<td>FMN content (nmol/mg)</td>
<td>0.73</td>
<td>0.78</td>
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<tr>
<td>Non-heme iron/FMN (gatoms Fe/mol FMN)(a)</td>
<td>16</td>
<td>22</td>
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<tr>
<td>Specific activity (mol of NADH/min/mg)(b)</td>
<td>181</td>
<td>453</td>
</tr>
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\(a\) Average values based on at least three determinations of non-heme iron, FMN, and protein.

\(b\) \(V_{\text{max}}\) for ferricyanide
Table 4-2

EPR spin quantitations for iron-sulfur centers in Complex I and soluble NADH dehydrogenase

<table>
<thead>
<tr>
<th>Complex I</th>
<th>N_1^b</th>
<th>N_2^c</th>
<th>N_3^d</th>
<th>N_4^e</th>
<th>Entire spectrum^f</th>
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<tbody>
<tr>
<td>Dithionite-reduced</td>
<td>&gt;0.1</td>
<td>1.02</td>
<td>0.30</td>
<td>0.42</td>
<td>1.8</td>
</tr>
<tr>
<td>Dithionite-reduced plus NADH</td>
<td>0.90</td>
<td>1.03</td>
<td>1.05</td>
<td>1.22</td>
<td>4.4</td>
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<tr>
<td>NADH-reduced</td>
<td>1.02</td>
<td>0.96</td>
<td>1.42</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>Photochemical reduction</td>
<td>1.03</td>
<td>1.16</td>
<td>1.27</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>Photochemical plus NADH</td>
<td>1.02</td>
<td>1.29</td>
<td>1.37</td>
<td>4.2</td>
<td></td>
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</tbody>
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<table>
<thead>
<tr>
<th>Soluble NADH dehydrogenase</th>
<th>Complex I-type signals</th>
<th>Rhombic-type signals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dithionite-reduced</td>
<td>N_1^b</td>
<td>Entire spectrum^f</td>
</tr>
<tr>
<td>Dithionite-reduced plus NADH</td>
<td>0.22</td>
<td>2.7</td>
</tr>
<tr>
<td>plus NADH</td>
<td>0.75</td>
<td>4.2</td>
</tr>
<tr>
<td>Photochemical plus NADH</td>
<td>0.65</td>
<td>3.54</td>
</tr>
</tbody>
</table>

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a Average values for several independent samples. Values are to ±15% for Complex I and ±20% for soluble NADH dehydrogenase.
b Spin quantitation at 70K and 1 mW microwave power.
c Spin quantitation using g=2.054 feature. Conditions: temperature 18K and lmW microwave power.
d Spin quantitation using g=2.103 feature. Conditions: temperature 12K and lmW microwave power.
e Spin concentration using g=1.862 feature. Conditions: temperature 12K and lmW microwave power.
f Maximum spin quantitation obtained for entire spectrum. Conditions: temperatures between 14 and 17K and lmW microwave power.
Figure 4-1. Schematic for chaotropic resolution of Complex I.
Figure 4-2. Schematic for the organization of NADH dehydrogenase in the mitochondrial membrane. Arrows indicate direction of electron and proton flow through enzyme complex.
Figure 4-3. EPR spectra for dithionite-reduced Complex I. Complex I was suspended in anaerobic pH 8.0 Tris-sucrose-histidine buffer containing 0.66M sucrose, 50mM Tris-chloride, 1mM histidine, and 1mM sodium dithionite. Sample is 13.9μM in FMN. Conditions: 1mW microwave power, 0.63 mT modulation amplitude, frequency 8.985 GHz, temperatures as indicated. Multiplication factors indicate the relative gains for each spectrum.
Figure 4-4. EPR spectra for dithionite-reduced Complex I after addition of NADH. Buffer and conditions as in Figure 4-3, except that the sample was made to be 0.6mM in NADH. Sample is 12.2μM in FMN.
Figure 4-5. Comparison of EPR spectra for Complex I at 13K. Buffer and conditions as in Figure 4-3. except for the reducing agent: (a) 1mM sodium dithionite and 0.6mM NADH; (b) 0.6mM NADH; (c) photolysis in the presence of 0.6mM EDTA and 1mM deazaflavin; (d) photolysis in the presence of 0.6mM EDTA and 1mM deazaflavin followed by addition of NADH (0.6mM in NADH). Sample concentration is 12.2μM in FMN. Multiplication factors indicate relative gains for each spectrum.
Figure 4-6. EPR spectra for dithionite-reduced soluble NADH dehydrogenase. Enzyme was in pH 7.8, 30mM potassium phosphate buffer containing 50% ethylene glycol and 1mM sodium dithionite. Sample concentration is 18.1μM in FMN. Conditions as in Figure 4-3.
Figure 4-7. EPR spectra for dithionite-reduced soluble NADH dehydrogenase after addition of NADH. Buffer and conditions as in Figure 4-6, except that sample was made to be 0.6mM in NADH. Sample concentration is 16.0μM.

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Figure 4-8. Comparison of rhombic-type EPR spectra for soluble NADH dehydrogenase at 8.5K. Buffer and conditions as in Figure 4-6. except for the reducing agent: (a) 1mM sodium dithionite; (b) 0.6mM NADH; (c) 0.6mM NADH and 1 mM sodium dithionite. Sample concentration is 40µM. Multiplication factors indicate the relative gains for each spectrum.
Figure 4-9. MCD and room temperature absorption spectra for dithionite-reduced soluble NADH dehydrogenase. Sample as in Figure 4-8a. (a) Room temperature absorption spectrum. (b) MCD recorded at 1.5, 4.2, 7.5, and 90K, at a magnetic field of 4.5 tesla, pathlength 0.17 cm. All transitions increasing in intensity with decreasing temperature except for the temperature independent region around 550 nm. (c) MCD difference spectrum; 1.5K spectrum minus 90K spectrum.
Figure 4-10. MCD magnetization plot for dithionite-reduced soluble NADH dehydrogenase. Data collected at wavelength 750nm at temperatures 1.55K (●), 4.22K (X), 12.5K (0) and magnetic fields varying between 0 and 4.5 tesla. Solid line represents theoretical magnetization for an isotropic system with $g=1.96$. 

$\beta B/2kT$
Figure 4-11. MCD and room temperature absorption spectra for dithionite-reduced soluble NADH dehydrogenase after addition of dithionite. Sample as in Figure 4-8c. (a) Room temperature absorption spectrum. (b) MCD spectra recorded at 1.53, 4.22, 8.0, and 90K at magnetic field of 4.5 tesla and pathlength of 0.16 cm. All transitions increasing with decreasing temperature except for the temperature-independent region around 550 nm. (c) MCD difference spectrum; 1.53K spectrum minus 90K spectrum.
Figure 4-12. Comparison of low temperature MCD spectra for dithionite-reduced soluble NADH dehydrogenase in the presence and absence of NADH. Enzyme samples as used in Figures 4-9 and 4-11. Magnetic field is 4.5 tesla. (a) Solid line is dithionite-reduced, NADH-treated enzyme; dashed line is dithionite-reduced enzyme in absence of NADH. Spectra are derived from the difference between the 1.5K and 90K MCD spectra. (b) difference between the MCD spectra of dithionite-reduced enzyme in presence and absence of NADH.
V. SUCCINATE-UBIQUINOL OXIDOREDUCTASE

BACKGROUND

Succinate-UQ oxidoreductase (Complex II) is an enzyme complex present in the mitochondrial respiratory chain (1-4). It catalyzes electron transfer from succinate to UQ, and has been isolated from a variety of eukaryotic and prokaryotic sources. Although these enzymes appear to be similar based on their subunit composition and amounts of covalently bound flavin, non-heme iron, and acid-labile sulfide, the best characterized enzyme is that isolated from beef heart.

The properties of succinate dehydrogenase (SDH) in inner membrane preparations are believed to be those of the pristine enzyme. Unfortunately, such preparations are difficult to study by EPR and MCD. The presence of other components of the respiratory chain complicates interpretation of EPR data. Moreover, relatively small amounts of cytochrome can completely dominate a low temperature MCD spectrum, obscuring the MCD signals arising from iron-sulfur clusters. Therefore, samples of soluble SDH or Complex II, a particulate preparation, are used in spectroscopic experiments.

Complex II has been extensively studied by EPR spectroscopy. Unfortunately, Complex II, as prepared, is almost invariably contaminated with small amounts of cytochrome and is not suitable for study by MCD. Complex II can be resolved into two components: succinate dehydrogenase (SDH), and the quinol binding proteins. Elimination of the quinol binding proteins yields soluble SDH preparations. These soluble SDH extracts are almost completely free of cytochromes and can be studied by MCD.
Soluble SDH

The first soluble SDH, isolated in 1955, could only catalyze electron transfer from succinate to non-physiological electron acceptors (6). Over the years, improvements in the isolation of SDH have resulted in preparations which appear to be identical to SDH in intact mitochondria. Pure and fully reconstitutively active mammalian SDH is isolated by extraction of the enzyme from purified particulate Complex II with butanol at pH 9 in the presence of succinate (8). A similar high purity, reconstitutively active, SDH preparation was subsequently isolated from Complex II using chaotropic agents under strictly anaerobic conditions and in the presence of succinate (9). Incomplete anaerobiosis resulted in loss of reconstitutive activity.

Iron-sulfur clusters

SDH consists of two non-identical subunits: a flavoprotein (FP) of molecular weight 70 KDa, and an iron-sulfur protein (IP) of molecular weight 27 KDa (10, 11). The FP subunit contains one mole of covalently bound FAD. Purified preparations of soluble SDH contain an average of eight non-heme iron atoms and eight acid-labile sulfides per molecule of enzyme. Initial EPR experiments on partially reconstitutively active SDH showed three distinct EPR signals, corresponding to three iron-sulfur clusters (12-15). The number, type, and subunit location of the iron-sulfur clusters in SDH have been subjects of vigorous controversy (1-5).

A preparation which can reconstitute the respiratory chain, i.e., it can bind SDH-depleted SMP and restore electron activity from succinate to oxygen (7).
Evidence for the two conflicting points of view are summarized in reference 3. Ohnishi and co-workers believe that the FP subunit contains two spin-coupled binuclear centers, and that the IP subunit contains a HiPIP-type [4Fe-4S] center (2). In contrast, Albracht found evidence for only two iron-sulfur clusters, one [2Fe-2S] cluster bound to the IP subunit and a HiPIP-type [4Fe-4S] in the FP subunit (17).

There has been agreement on the identity of one of the binuclear clusters, S1. At 70K, S1 has an EPR spectrum with rhombic symmetry with g values of 2.025, 1.930, and 1.910 in the reduced state (18). The midpoint redox potential is about 0mV in soluble SDH, the center being completely reduced in a high concentration of succinate. The spin concentration of S1 is approximately equal to that of the flavin (13). The spin relaxation behavior of the center is slow enough that its EPR signals are readily saturated at temperatures below 30K (13, 14).

A second FP iron-sulfur cluster S2, was proposed to explain differences in the EPR spectra of succinate- and dithionite-reduced samples of partially reconstitutively active SDH (18). Succinate-reduced and dithionite-reduced SDH show a similar spin intensity above 40K. At temperatures below 20K, the dithionite-reduced SDH gave 1.5 to 1.75 spins per flavin compared to a smaller value (0.8 to 1.0) for the succinate-reduced enzyme. Other workers also reported differences in spin concentration for succinate- and dithionite-reduced SDH, but the apparent S2 spin concentration varied considerably depending on the preparation used (19). Potentiometric EPR experiments in which the spin relaxation of S1 is monitored as a function of redox
potential indicate that the redox midpoint potential of S2 is very low, approximately −400mV in soluble SDH and −260mV in particulate preparations (20). Circumstantial evidence for the presence of a cluster S2 came from the observed enhancement of S1 spin relaxation upon reduction of SDH with dithionite. This phenomenon could then be attributed to spin coupling between S1 and a rapidly relaxing species, S2, that becomes paramagnetic only upon dithionite reduction.

The lineshape for S2 was estimated from the difference between spectra of dithionite-reduced and succinate-reduced reconstitutively inactive enzyme (18). The lineshapes and principal g values of S1 and S2 are identical. However, subsequent EPR experiments have reported that the EPR signal for S2 is not detectable in fully reconstitutively active SDH, or in intact mitochondria (1, 17). However, in both types of preparations, enhancement of S1 spin relaxation upon dithionite reduction was observed.

One explanation of this data is that S2 is an artifact of the earlier isolation procedure, and that the spin relaxation enhancement observed is caused by a protein conformational change induced by reduction of the flavin (1, 17). Questions have also been raised about the reliability of core extrusion and core transfer data (1). Arguing against this idea is the observation that reduction of the SDH flavin free radical is an n=2 redox process with a midpoint potential of −81mV at pH 7 (22). The spin relaxation enhancement corresponds to an n=1 redox process, and occurs at much lower redox potentials.

Another proposal is that the apparent non-detectability of S2 in intact mitochondria, or in fully reconstitutively active SDH, is caused by spin coupling between S1 and S2, which reduces the total
EPR-detectable spin concentration of S1 and S2 to that of the flavin concentration of the system (1). That such spin coupling would occur without some change in the lineshape of the EPR spectrum is difficult to explain.

The third EPR signal, S3, is observed in fully oxidized intact mitochondria, in Complex II, and in reconstitutively active SDH. This signal is not observed in reconstitutively inactive SDH preparations. The EPR signal at 20K is relatively isotropic, with principal g values at 2.015, 2.014, and 1.990 (23-25). The redox midpoint potential of S3 is +65mV in Complex II and +120mV in intact mitochondria. In the membrane-bound enzyme, and in Complex II, the spin concentration is approximately equal to that of FAD. In soluble SDH, S3 is extremely labile towards oxygen (19). The amount of S3 in soluble preparations correlates with the preparation's reconstitutive activity and with its "low K_m"-ferricyanide reductase ability (26).

Center S3 was originally thought to be a HiPIP based on core extrusion data and on its EPR characteristics. In recent years trinuclear centers have been identified in various iron-sulfur proteins (27). The EPR characteristics of these [3Fe-xS] centers are similar to those of S3. Very recently, the linear electric field effect (LEFE) method has been applied to the study of S3 in oxidized Complex II (28). Based on the similarity of the LEFE data for the EPR signal in oxidized Complex II and that obtained with other [3Fe-xS] centers, the authors tentatively concluded that S3 is a [3Fe-xS] cluster (29).

Quinol binding proteins

In Complex II there are two low molecular weight polypeptides,
The molecular weights of these subunits are 13.5 and 7 Kda, respectively. Recombination of soluble SDH, C_{II-3}, and C_{II-4} gave UQ reductase activity (26), with a ratio of 6 or 7 moles of polypeptide per SDH giving maximum activity (31). The quinol binding polypeptides confer oxygen stability on SDH. There is direct evidence for physical association of SDH and the polypeptides during reconstitution (31). The subunit C_{II-3} is thought to be involved in quinone binding to SDH. C_{II-4} is believed to bind SDH to the membrane, or to bind and orient C_{II-3} for UQ reduction.

Cytochrome b is thought to be present in Complex II in amounts equimolar to FAD (32). The heme center is believed to be associated with C_{II-3}. It cannot be distinguished by its absorption spectrum from the cytochrome b present in Complex III. The cytochrome from Complex III differs from the resolved cytochrome b of Complex II in that it does not react with carbon monoxide, and by its unusually low reduction potential of -200mV.

**Spatial organization of the components of Complex II**

Surface labeling of mitochondria combined with immunoprecipitation experiments showed Complex II to span the inner mitochondrial membrane (33) (Figure 5-1). Both the FP and IP subunits of SDH are on the matrix side of the membrane, but IP is partially shielded from water. The FP subunit was shown to be held above the lipid bilayer (34), while the IP, C_{II-3}, and C_{II-4} subunits were within the bilayer (33). The 13.5 Kda subunit is exposed to the cytosolic surface, but not to the matrix surface of the inner membrane.

The location of the iron-sulfur clusters shown in Figure 5-1
should be viewed as tentative and, moreover, it disagrees with conclusions reached from studies with *Escherichia coli* fumarate reductase (see chapter 6). The amino acid sequences of flavoprotein and iron protein of *E. coli* SDH and fumarate reductase have been determined recently (35). The iron protein subunits of these enzymes exhibit striking homology. The arrangement of the cysteine residues in the subunits, coupled with the iron sulfur cluster composition indicated by MCD experiments, suggests that no iron-sulfur centers are bound to the flavoprotein and that the iron protein contains the three iron-sulfur centers.

**Objective**

The iron-sulfur cluster composition of SDH is a complex problem. The controversies in the number and identity of the clusters arise from the possibility that iron-sulfur centers may be paramagnetic but EPR-silent for reasons of zero-field splitting (e.g. reduced [3Fe-xS]) or weak spin interaction between paramagnetic centers (e.g. S1 and S2). Low temperature MCD is well suited to resolve these uncertainties since small zero-field splittings or weak magnetic interactions between paramagnetic centers will not generally alter the magnitude of the MCD C term for a paramagnetic chromophore. Furthermore, ground state electronic parameters, e.g. spin state and g values, can be estimated by analyzing MCD intensity as a function of magnetic field strength and temperature. Such information is particularly useful for study of paramagnetic EPR-silent centers.

This work presents the low temperature MCD spectra and magnetization data, together with parallel EPR studies, for both reconstitutively active and reconstitutively inactive SDH.
results resolve many of the controversies concerning the EPR and core extrusion data, provide the first definitive assessment of the nature of all of the iron-sulfur clusters in SDH, and address the question of whether S3 is able to sustain Q reductase activity as a [3Fe-xS] center.

RESULTS AND DISCUSSION

BEEF HEART SDH

Identical MCD and EPR experiments were performed on two preparations of butanol-extracted beef heart SDH (BS-SDH) and four preparations of perchlorate-treated beef heart SDH (P-SDH) in the comparisons of succinate- and dithionite-reduced enzymes. The P-SDH was prepared in the absence of succinate, and thus was expected to be deficient in S3 and largely reconstitutively inactive. The enzyme preparations were studied in succinate- and dithionite-reduced forms in order to demonstrate whether S3 was present as a [3Fe-xS] center in both forms and to allow deconvolution of the multicomponent MCD spectrum. EPR experiments were performed on two batches of succinate- and dithionite-reduced Complex II.

Analytical data for bovine SDH

Table 5-1 shows the relevant analytical and assay data for soluble SDH used in the MCD experiments. Enzyme concentrations were equated with the flavin content of the preparations. Solubilization of SDH results in the appearance of a new catalytic activity, a succinate-ferricyanide reductase which is functional at low concentrations of ferricyanide (referred to as "low $K_m$-ferricyanide reductase). This activity has been shown to be directly related to
the ability of the sample to restore electron transfer from succinate to oxygen in submitochondrial particles depleted in SDH. Therefore, the assay of this activity is used as a measure of reconstitutive capacity.

Both SDH preparations contained 8 to 9 moles of non-heme iron per mole of FAD. Based on the FAD content, subunit molecular weights, and the assumption of 1 FAD per mole enzyme, the preparations of BS-SDH and P-SDH were approximately 79 and 100% pure, respectively. As expected, P-SDH was essentially reconstitutively inactive, exhibiting a low turnover number in the "low K_m"-ferricyanide reductase assay. The activities for both preparations were similar to those reported previously (36).

**EPR data for BS-SDH Center S1**

EPR data for the MCD samples of BS-SDH and P-SDH are summarized in Table 5-2.

As reported previously (18), BS-SDH exhibited a slowly relaxing rhombic EPR signal at 70K, corresponding to slightly more than 1 spin per FAD (Figure 5-2). This signal has been designated S1. Other than the flavin free radical signal at g=2.004, no additional EPR signals were observed in the temperature range of 15 to 100K.

Addition of a 20-fold excess of sodium dithionite to BS-SDH resulted in loss of the flavin free radical EPR signal, with a small increase in the 70K spin quantitation of S1 (Figure 5-2). The form and g values of S1 are essentially unchanged in these two samples.

In agreement with previous work (13, 17, 19), there is a dramatic enhancement of the spin relaxation rate of S1 upon addition of excess dithionite (Figure 5-3). The power dependences of S1 at 15K for BS-
SDH and P-SDH were monitored over the range of 0.5 to 100mW. Plots of log (I/\sqrt{P}) versus log P (where I is EPR signal intensity) were essentially the same to those shown in previous work (2).

**EPR data for P-SDH Center S1**

The 70K EPR spectra for succinate- and dithionite-reduced P-SDH are shown in Figure 5-4. The spectra closely resemble those of BS-SDH in terms of g values and spin relaxation properties (Figure 5-5). There was no apparent increase in spin intensity upon dithionite reduction. In the absence of succinate, P-SDH exhibited a signal corresponding to S1 of about 15% of that observed for the succinate-treated P-SDH. In addition, a very weak (accounting for less than 0.03 spins per molecule) almost isotropic signal, centered about g=2.02, was observed at temperatures below 20K. This signal corresponds to a trace of S3. Subsequent results show the low spin quantitation for S3 is primarily due to absence of the center, as a result of oxidative damage of S3 during purification.

**CD spectra for SDH**

The natural CD spectra for the oxidized and reduced [2Fe-2S] centers are known to be more intense than for other types of iron-sulfur centers (37). Transitions from this type of center would be expected to dominate a CD spectrum which has contributions from other types of iron-sulfur centers. CD spectra for [2Fe-2S] proteins are known to be largely invariant for plant and hydroxylase type ferredoxins (37). The CD spectrum of the as-isolated P-SDH is consistent with an oxidized [2Fe-2S] center (Figure 5-6). The CD spectra of the reduced SDH enzymes (Figure 5-6) closely resemble those of other reduced [2Fe-2S] proteins, both in form and intensity.
MCD data for BS-SDH

Figure 5-7 shows the room temperature absorption and low temperature MCD spectra of BS-SDH at 1.54, 4.22, and 14.9K. The low temperature MCD spectrum revealed a number of electronic transitions. The biphasic band with a positive peak at 404nm and a negative trough at 424nm is indicative of a trace (less than 1%) of oxidized, low spin cytochrome b, and magnetization data (not shown) confirm this assignment. All other electronic transitions were attributed to paramagnetic iron-sulfur centers, based on their intensity and temperature dependence.

In an attempt to determine the magnetic properties of the contributing paramagnetic iron-sulfur clusters, magnetization data were collected at three wavelengths: 320, 690, and 720nm (Figure 5-8). Plots of this data cannot be fit to the theoretical curve for the S=1/2 ground state of reduced SI. For a S=1/2 ground state with \( g \) values close to 2, the form of the magnetization curve will not depend significantly upon the wavelength of measurement, or on the polarization of the electronic transition (38, 39). Therefore, the presence of an additional paramagnetic, EPR-silent center is indicated.

MCD magnetization characteristics observed at 720nm are uniquely indicative of a reduced [3Fe-xS] center (38). MCD spectra from a variety of [3Fe-xS] proteins show broad, intense bands centered around 720nm, with magnetization curves that are fit, at temperatures below 2K, to theoretical data for \( g=8, 0, 0 \). These are the effective \( g \) values expected for a \( M_s=\pm 2 \) doublet resulting from axial zero field splitting of an \( S=2 \) ground state. Magnetization data at temperatures
higher than 2K are not expected to lie on the same curve since population of higher $M_s=\pm 1$ and $M_s=0$ zero field components can occur, depending on the magnitude of the zero field splitting parameter $D$. This result is the first evidence for the presence of a reduced [3Fe-xS] cluster in soluble, reconstitutively active SDH. Together with the conclusions of the LEFE measurement of oxidized Complex II (28), this result leaves little doubt that S3 is a [3Fe-xS] center.

Magnetization data at temperatures below 2K were well fit to theoretical curves constructed from combinations of theoretical magnetization curves for S1 and S3. The experimental data can be simulated with S1 contributing 60% and reduced [3Fe-xS] contributing 40% of the MCD intensity at 320nm. At 690nm, S1 contributed 30% and S3 contributed 70% of the MCD intensity.

Figure 5-9 shows room temperature absorption and low temperature MCD spectra at 1.56, 4.22, and 14.0K for dithionite reduced BS-SDH. In comparison with the succinate-reduced enzyme, there is a substantial decrease of absorption intensity in the visible region. The brown color of the enzyme was noticeably bleached upon addition of dithionite. The shoulders at 310 and 430nm in the room temperature absorption spectrum were attributed to excess dithionite and a trace of reduced cytochrome, respectively. In the MCD spectrum, the sharp, biphasic band centered at 552nm indicates the presence of a trace of low spin ($S=0$) ferrous cytochrome $b$ (40). The positive band at 438nm is typical of that observed for a trace of high spin ($S=2$) ferrous cytochrome (41, 42). The remaining temperature dependent features in the MCD spectra were assigned to paramagnetic iron-sulfur centers.

If another paramagnetic iron-sulfur center, i.e. S2, is not
generated upon addition of excess dithionite, there should be no change in the regions of the MCD spectrum associated with iron-sulfur centers. However, significant changes in the MCD intensities were observed (Figure 5-10). There were increases in the bands around 320, 350, 530, and 750nm; and a decrease in intensity between 580 and 680nm. This result strongly implies the existence of an additional iron-sulfur cluster, S2, in dithionite-reduced BS-SDH.

Magnetization data were collected at 320, 695, and 725nm (Figure 5-11). At these wavelengths, at least 50%, and probably the majority, of the observed increase in MCD intensity arises from a paramagnet with a \( S=1/2 \) ground state with \( g \) values close to 2. For example, at 320nm contributions of 70% for S1 and 30% for S3 are required to fit the lowest temperature experimental data for dithionite-reduced BS-SDH. The succinate-reduced enzyme requires contributions of 60% and 40% for S1 and S3, respectively.

Assuming that increases in the MCD intensity result solely from the reduction of an additional iron-sulfur center, the magnetization data are consistent with a S2 being a \( S=1/2 \) ground state with \( g \) values close to 2. Center S2 could be either a \([2Fe-2S]\) or a \([4Fe-4S]\) center. Experimental magnetization curves cannot distinguish between these two types of centers. Magnetization plots of dithionite-reduced BS-SDH at 438nm are consistent with a trace amount of high spin ferrous cytochrome.

**MCD data for P-SDH**

Figure 5-12 shows room temperature absorption and low temperature MCD spectra at 1.6, 4.22, and 12.0K for succinate-reduced P-SDH. The form of the MCD spectrum is significantly different from that of the
succinate reduced BS-SDH sample in regions associated with iron-sulfur centers. Both spectra exhibit a trace of low spin ferric heme, but in P-SDH the impurity is attributed to cytochrome c, rather than cytochrome b.

Magnetization data were collected at 320nm (Figure 5-13). A plot of the data gave a good fit to the theoretical curve for $S=1/2$ and $g$ values for $S_1$. Contributions of 10% for $S_3$ are required for the best fit to the lowest temperature data. Comparing this result with the magnetization curve obtained for BS-SDH leads to the conclusion that BS-SDH contains at least 4 to 5 times the amount of $[3\text{Fe}-x\text{S}]$ centers as does P-SDH. Differences in the MCD spectra of succinate- and dithionite-reduced P-SDH and BS-SDH, particularly in the region of 450 to 800nm, are consistent with this interpretation. Clearly, the absence of succinate and lack of complete anaerobidity during the isolation of P-SDH results in significant loss of center $S_3$.

Figure 5-14 shows the room temperature absorption and low temperature MCD spectra at 1.63, 4.22, and 8.5K for dithionite-reduced P-SDH. The absorption spectrum is featureless, increasing monotonically in the visible region, with a shoulder around 310nm due to excess dithionite. As with BS-SDH, there is a substantial decrease in intensity in the visible region compared to the succinate-reduced enzyme. The brown color of the enzyme was noticably bleached upon addition of dithionite. The low temperature MCD spectrum shows no evidence of high spin ferrous heme. The entire spectrum arises from transitions originating from paramagnetic iron-sulfur centers. Comparison of low temperature MCD spectra of succinate- and dithionite-reduced P-SDH reveals similar changes to those observed for
Therefore, S2 is also present in P-SDH.

Magnetization data at 320 and 548 nm are in good agreement with the theoretical curve for a S=1/2 ground state with g values around 2 (Figure 5-15). Only minor contributions from a reduced [3Fe-xS] center are required for a best fit to the lowest temperature data. Magnetization data at 690 nm also indicates the presence of small amounts of a reduced [3Fe-xS] center (Figure 5-15). In this region of the spectrum, reduced [3Fe-xS] centers have a greater MCD intensity at low temperatures relative to that of other iron-sulfur centers. Since magnetization curves for succinate-reduced P-SDH at 690 nm (not shown) exhibit a similar contribution from a reduced [3Fe-xS] center, this MCD contribution was attributed to S3 rather than to S2.

The MCD spectrum at 4.22 K for P-SDH in the absence of succinate, along with spectra of the succinate- and dithionite-reduced forms, is shown in Figure 5-16. The dominant feature of the MCD spectrum of as-isolated P-SDH is cytochrome c1. Magnetization curves in other regions of the spectrum suggest that the MCD bands originate from small amounts of reduced [3Fe-xS] and S1. The EPR data show that only 15% of S1 is reduced in the as-isolated sample. The MCD spectrum shows that the majority of the [3Fe-xS] present in P-SDH is in the reduced state, even in the absence of succinate.

Form of the MCD components of SDH

In order to deconvolute the complex MCD spectra from SDH, the appropriate spectra, under identical conditions, were differenced. Difference MCD spectra corresponding to S1, S2 and S3 are shown in Figure 5-17.

The form of the MCD spectrum for S1 was obtained from the
difference between succinate-reduced and as-isolated P-SDH. The amount of oxidized cytochrome $c_1$ and reduced [3Fe-xS] is essentially the same in both samples, as previously discussed. Therefore, the difference between the two spectra corresponds entirely to reduced S1 and is essentially free from cytochrome.

Low temperature MCD spectra have been reported for plant-type ferredoxins, typified by spinach Fd, and for hydroxylase-type ferredoxins, typified by adrenodoxin (43). The form of the spectra for these two types of reduced [2Fe-2S] centers are markedly different. (See chapter 8 for a comparison of the spectroscopic and magnetic properties of spinach Fd and adrenodoxin.) The MCD spectrum for reduced S1 most closely resembles that of adrenodoxin, although it is somewhat intermediate between that from a plant-type ferredoxin and that from a hydroxylase-type ferredoxin. Interestingly, the EPR spectrum of reduced S1 also is intermediate between that of the two types of [2Fe-2S] centers, most closely resembling that of adrenodoxin. The factors that are responsible for changes in the electronic structure of [2Fe-2S] proteins revealed by MCD and EPR are not well established. It is believed that differences in the orientation of the ligating cysteine ligands may play a major role.

Obtaining the form of the low temperature MCD spectrum for S2 was difficult because of the cytochrome impurities. The difference between spectra of dithionite- and succinate-reduced BS-SDH is shown in Figure 5-17b. The cytochrome Soret region has been excluded in this spectrum. The biphasic band centered around 552nm is due to a trace of reduced cytochrome b. The analogous difference spectrum for P-SDH shows similar features. The broad, positive bands around 730nm,
and between 560 and 510nm, and the negative band centered at 630nm are characteristic of a reduced [4Fe-4S] center of the type seen in bacterial ferredoxins (39, 44). The intensity of this component is consistent with approximately one [4Fe-4S] per flavin in both BS-SDH and P-SDH.

The form of the reduced S3 was obtained by subtracting the spectrum of S1 (obtained as previously described) from that of the succinate-reduced BS-SDH. The region between 390 and 470nm was excluded due to the presence of cytochrome impurities. In agreement with magnetization data, the form of the difference spectrum is typical for that of reduced [3Fe-xS] centers of the type observed in bacterial ferredoxins (45-47). The MCD spectrum of a reduced [3Fe-xS] center is much more intense than other known iron-sulfur centers. The intensity of the S3 component of reduced BS-SDH, compared to other well-characterized, reduced [3Fe-xS] centers, suggests that the enzyme does not have a full complement of reduced [3Fe-xS] clusters.

EPR data for beef heart Complex II

The EPR spectra at 13K for bovine Complex II reduced with succinate or dithionite are shown in Figure 5-18. Both samples exhibit the rhombic EPR signal of S1. EPR data for Complex II are summarized in Table 5-2.

Both succinate- and dithionite-reduced forms of Complex II exhibit a EPR signal corresponding to reduced S1. The spin relaxation properties of S1 in Complex II are similar to those observed in BS-SDH and P-SDH. In succinate-reduced Complex II, S1 is strongly microwave power saturated at temperatures below 10K, relative to dithionite-reduced Complex II. As a result of slower spin relaxation, the signal
intensity of S1 is lower in the 13K EPR spectrum of succinate-reduced enzyme compared to dithionite-reduced Complex II, as illustrated in Figure 5-18.

At temperatures below 10K, additional weak features, positive at \(g=2.06\) and 1.992, and negative at \(g=1.84\) were observed at high microwave powers and modulation amplitudes in the dithionite-reduced enzyme (Figure 5-18). This signal, not observed in the succinate-reduced enzyme, results from a center with rapid spin relaxation. The signal was only clearly discernable at very low temperatures and, at these temperatures, shows no sign of power saturation up to 100mW. EPR signals with similar properties, although broader and less well resolved, were observed in dithionite-reduced BS-SDH (Figure 5-20) and P-SDH. The differences in the observed form of the EPR spectra probably arises from variations in spin interaction in the different types of enzyme preparations.

The \(g\) values and spin relaxation properties of this signal are typical of those observed for bacterial [4Fe-4S] ferredoxins (48). The \(g\) values of the signal are different from those of iron-sulfur centers in Complex I, ETF dehydrogenase, or Complex III. And, since this signal is observed in soluble SDH, it is reasonable to conclude that a [4Fe-4S] center, S2, is an intrinsic component of succinate-UQ oxidoreductase. This is in agreement with the interpretation of the MCD data from soluble SDH described previously.

Based on the intensity of the low temperature MCD spectrum of reduced S2 and the results from extensive analytical and core extrusion experiments (16), SDH contains one [4Fe-4S] cluster. However, initial attempts to estimate the spin concentration of S2 in
dithionite-reduced Complex II, by assuming a rhombic EPR signal and integrating the $g=2.06$ or $g=1.84$ absorption-shaped bands (49), gave spin concentrations of only 0.20 spins per FAD. EPR experiments over a wider magnetic field range revealed additional broad features around $g=2.29$ and $g=1.63$ (Figure 5-19). These features have identical spin relaxation behavior to that observed for the $g=2.05$ and $g=1.86$ bands.

Therefore, it was concluded that reduced S2 does not exhibit the simple rhombic EPR spectrum associated with a magnetically isolated $S=1/2$ center. Rather, it is a complex signal, spanning almost 150 mTesla at X-band. Similar complex spectra have been observed in reduced, spin-coupled eight-iron ferredoxins (50). In these systems, EPR experiments at different frequencies show that these complex signals result from weak interactions between the two [4Fe-4S] centers.

Accurate spin quantitation of S2 is not possible since the EPR signal is partially obscured by that of S1. However, an estimation of the form of the spectrum yielded a value of approximately 1 spin per FAD. In addition to the features at $g=2.29$, 2.06, 1.84, and 1.63, this spin quantitation assumes the presence of a symmetrical, derivative-shaped band centered at about $g=1.93$, having a band width of about 20 mT. The maximum and minimum of the peaks are slightly larger than the features at $g=2.06$ and $g=1.84$ (Figure 5-19). Broad complex signals, with slightly different bandshapes, but similar spin quantitations were subsequently observed in samples of dithionite-reduced fumarate reductase from E. coli, which is believed to be very similar in terms of iron-sulfur centers to SDH.
Spin interactions involving S2

The complex spectrum observed for S2 is probably due to spin interaction involving the S=1/2 [4Fe-4S] center and one, or both, of the other paramagnetic centers present in SDH. Direct evidence for spin interaction between S1 and S2 came from redox titration experiments with parallel EPR analysis performed in collaboration with Dr. John Maguire, University of California, Berkeley (51). Complex II and BS-SDH were titrated in the presence of mediator dyes, using dithionite as the reductant and ferricyanide as the oxidant. EPR signals at \( g=1.935 \) and \( g=1.847 \) were monitored under conditions where S1 was power saturated. These conditions resulted in a substantial increase in signal intensity as the spin relaxation was enhanced.

Figure 5-21a shows the redox titration of Complex II in which the intensity of the \( g=1.935 \) feature of S1 and the \( g=1.847 \) feature of S2 were plotted as a function of redox potential. Two redox processes are apparent; reduction of S1 at about 0mV, and a dramatic increase in the spin relaxation rate of S1 at around -260mV. The enhancement of spin relaxation coincided with the appearance of the EPR signal for S2, demonstrating that the reduction occurs concomitantly with the change in the relaxation properties of S1. Similar results were obtained for soluble SDH (Figure 5-21b), except that the midpoint potential for S2 was -360mV, and the EPR signal for S2 was less well resolved. In order to avoid oxidative damage to BS-SDH, redox titrations on BS-SDH were performed in the presence of 5mM succinate, and the potential was not allowed to go higher than -100mV.

The spin interaction between reduced S2 and S1 does not significantly alter the form of the EPR signal of S1. Therefore, it
seems unlikely that spin interaction between S1 and S2 is the only interaction occurring. Additional weak spin interaction between reduced S2 and reduced S3 is one possibility. Weak spin-spin interactions between reduced [4Fe-4S] and [3Fe-xS] centers have been observed in fully reduced T. thermophilus ferredoxin (52). The observation that the EPR signal for S2 is more resolved in P-SDH, compared to BS-SDH, suggests that there may be spin interaction between reduced S2 and S3. However, there is no direct evidence for such an interaction.

Microwave power saturation studies provide evidence against spin interaction between S1 and S3. The power saturation behavior of S1 in succinate-reduced Complex II and S-3 deficient P-SDH at 15K was the same. In addition, a comparison of the power saturation behavior of dithionite-reduced Complex II, BS-SDH, and P-SDH at 10K, 16K, and 20K, showed no difference in the spin relaxation properties of S1. EPR experiments at different microwave frequencies are necessary to explore the nature of the intercluster spin interactions in SDH. These experiments will be the subject of future investigation.

YEAST COMPLEX II

Although the soluble form of SDH is the easiest to study spectroscopically, there is the concern that the isolation procedure has altered the enzyme relative to its native state. Low temperature MCD measurements on mammalian Complex II are not possible due to the amount of heme present in the preparation. Relatively small amounts of heme can completely dominate a low temperature MCD spectrum. However, samples yeast Complex II are reported to be essentially free of cytochrome b (53) and thus offer the possibility of studying a more
intact form of SDH by MCD. In addition, it is of interest to compare enzyme systems of organisms which have evolved through different evolutionary pathways. MCD and parallel EPR experiments were performed on two preparations of yeast Complex II.

**EPR data for yeast Complex II**

Two derivatives of yeast Complex II were studied: the as-isolated enzyme and the dithionite-reduced enzyme. EPR data is summarized in Table 5-2.

At temperatures below 20K, the as-isolated enzyme exhibited a broad, isotropic EPR signal centered about $g = 2.01$ (Figure 5-22). The EPR characteristics of this resonance were similar to those observed for S3 in oxidized beef heart Complex II. The isotropic EPR signal was not observed at temperatures above 30K, and became rapidly power saturated at temperatures below 10K. The spin quantitation of this signal was low, approximately 0.25 spins per FAD. The low spin quantitation could be due to oxidative damage of the high potential center during manipulation, or to partial reduction yielding an EPR-silent, reduced [3Fe-xS] core. As subsequent oxygen-exposure experiments on BS-SDH demonstrate, center S3 in soluble mammalian SDH is extremely sensitive to oxygen. No additional EPR signals were detected in as-isolated yeast Complex II at higher temperatures.

Upon dithionite reduction, the $g = 2.01$ signal disappeared, to be replaced by a slowly relaxing, rhombic signal with $g = 2.03$, 1.93, and 1.91 (Figure 5-23). The rhombic EPR signal was observed at temperatures above 70K, and accounted for approximately 0.7 spins per FAD. The EPR characteristics of this signal are identical to those observed for dithionite-reduced S1 in beef heart SDH. At temperatures
below 20K, the power saturation behavior of the rhombic EPR signal in the dithionite-reduced yeast Complex II was identical to that observed for SI in dithionite-reduced mammalian Complex II.

At very low temperatures and high microwave powers, weak features, positive at \( g = 2.07 \) and negative at \( g = 1.85 \), were observed in the dithionite-reduced enzyme. This signal was only clearly discernable at very low temperatures, and showed no sign of power saturation at 8.5K and 100mW. The characteristics of this broad resonance are similar to those observed for S2 in dithionite-reduced mammalian SDH and for the [4Fe-4S] center in dithionite-reduced fumarate reductase. Accurate spin quantitation of this rapidly relaxing EPR signal was not possible since it is partially obscured by the more slowly relaxing [2Fe-2S] resonance. Based on MCD intensities and approximate estimates of EPR spin concentrations, the [4Fe-4S] center present in mammalian SDH and in \( \text{E. coli} \) fumarate reductase was found to be equimolar with the [2Fe-2S] center. It is not unreasonable to assume that the relative amounts of iron-sulfur centers are the same in mammalian and yeast SDH.

**CD data for yeast Complex II**

The CD spectra of oxidized and dithionite-reduced yeast Complex II were similar to the corresponding CD spectra for bovine SDH (Figure 5-24).

**MCD data for yeast Complex II**

Unfortunately, MCD measurements of the oxidized [3Fe-xS] center in as-isolated yeast Complex II were not possible due to the amount of cytochrome contamination. A low spin, ferric cytochrome signal dominates the low temperature MCD spectrum (Figure 5-25).
Figure 5-26 shows the room temperature absorption and low temperature MCD spectrum at 1.53, 4.22, and 10.9K for dithionite-reduced yeast Complex II. The MCD spectrum revealed a number of electronic transitions. The biphasic band centered at 522nm and the positive band at 438nm were assigned to low spin (S=0) and high spin (S=2) ferrous heme, respectively. All other transitions were attributed to paramagnetic iron-sulfur centers, based on their temperature dependences and intensities. Comparison of the 4.22K MCD spectrum of the dithionite-reduced yeast Complex II with the corresponding MCD spectrum for BS-SDH demonstrates their striking similarity (Figure 5-27). Although a composite spectrum of three iron-sulfur cores, the similarity in the form of the MCD spectra of the two SDH enzymes suggests that the structures and the relative amounts of the iron-sulfur centers are very similar.

The MCD spectrum of a reduced \([3\text{Fe-xS}]\) center is much more intense than other known iron-sulfur centers (38). Transitions from this center would be expected to dominate a MCD spectrum that has contributions from several different iron-sulfur centers. Based on the MCD intensity at 715nm, a region characteristic of reduced \([3\text{Fe-xS}]\) centers, yeast Complex II probably does not have a full complement of \([3\text{Fe-xS}]\).

Magnetization data collected at 740, 690, and 320nm, are shown in Figure 5-28. As with beef heart BS-SDH, at no wavelength are the experimental data fit adequately by the theoretical data based solely on the EPR-determined, S=1/2 ground state of a reduced \([2\text{Fe-2S}]\) center. A theoretical curve, constructed from a combination of 90% of S=2 with g=8, 0, 0 and 10% of S=1/2 with g values around 2, gave a
good fit to the lowest temperature data at 740nm. Lowest temperature data at 690 and 320nm were fit to a curve with contributions of 70% of $S=2$ and 30% of $S=1/2$, and 45% of $S=2$ and 55% of $S=1/2$. Although the theoretical $S=1/2$ contribution was based on EPR-determined $g$ values for the [2Fe-2S] center, any other $S=1/2$ center with $g$ values about 2, i.e., a [4Fe-4S] core, would give a similar curve.

EXPERIMENTS WITH BEEF HEART SDH

Until very recently, no unambiguous cases had been reported in which a [3Fe-xS] center could be considered to be an intrinsic component of an in vivo protein. Hence the possibility exists that all [3Fe-xS] centers arise from [4Fe-4S] cores by oxidative degradation during isolation (46, 54, 55). In aconitase, the [3Fe-xS] center has been shown to be rapidly converted to a [4Fe-4S] center under reducing conditions to yield the catalytically active form (54, 55). However, in nitrate reductase (47), and in fumarate reductase from E. coli (see chapter 6), a stoichiometric amount of a [3Fe-xS] center was shown to be present in the catalytically active form of the enzyme. The question arises as to whether S2 and S3 are distinct clusters or interconversion products of the same cluster.

The MCD and EPR results, discussed above, argue against conversion between S2 and S3. MCD experiments show S3 to be present in reconstitutively active SDH and essentially absent in nonreconstitutively active enzyme, strongly implying that the [3Fe-xS] center in SDH is a requirement for reconstitutive activity. Conversion of this center to a paramagnetic [4Fe-4S] does not occur upon addition of substrate, since the increase in the MCD intensity ascribed to S2 in the S3-deficient P-SDH was approximately the same as
in BS-SDH. In addition, the BS-SDH preparations used in MCD experiments gave no indication of oxygen-damage in the "low-K_m" ferricyanide reductase assay.

The MCD spectra and magnetization data for succinate- and dithionite-reduced BS-SDH give no indication that the spectral changes induced by the addition of dithionite result from loss of reduced S3. However, partial core conversion of S3 to yield a diamagnetic [4Fe-4S] center in the presence of substrate still remains a possibility. This cannot be ruled out by the data since the magnitude of the MCD spectrum of S3 is less than that generally observed for [3Fe-xS] centers in bacterial ferredoxins (56). The less than stoichiometric amounts of reduced S3 in BS-SDH could also be due to the extreme oxygen lability of this center in soluble SDH.

The extensive analytical and extrusion data for SDH are also consistent with distinct S2 and S3 clusters. Purified preparations of soluble SDH contain an average of eight non-heme iron and eight acid-labile sulfides per enzyme molecule. A 1:1:1 ratio of S1:S2:S3 in SDH accounts for nine non-heme irons per FAD. The less than stoichiometric amount of S3 rationalizes the slightly lower experimental iron content. Core extrusion and interprotein core transfer experiments with BS-SDH were interpreted in terms of two [2Fe-2S] and one [4Fe-4S] centers (16). However, the [3Fe-xS] center in aconitase is known to extrude as a [2Fe-2S] cluster (54). A trinuclear center has not been extruded intact from a metalloprotein.

In order to address the question of possible core interconversion, three types of experiments were performed on SDH, using MCD and parallel EPR studies to monitor cluster conversion.
The three approaches included attempts to convert S3 into a [4Fe-4S] cluster by incubation with ferrous iron, attempts to reconstitute S3 in P-SDH, and attempts to breakdown clusters in BS-SDH and P-SDH by exposure of the enzymes to air.

**Attempted [3Fe-xS] to [4Fe-4S] conversion in SDH**

In an adaptation of the aconitase activation procedure, BS-SDH was treated with aqueous ammonium iron(II) sulfate. The enzyme samples used in these experiments were from a third BS-SDH preparation, i.e., a preparation different from that described in Table 5-1 and the above MCD and EPR experiments. Assays for "low K_m" ferricyanide activity were not performed on these samples. However, MCD data indicated that these samples contained a lower amount of [3Fe-xS] center relative to the BS-SDH preparations used in the analysis of SDH's cluster composition.

MCD and EPR spectra were obtained for the following BSDH derivatives: iron-treated, succinate-reduced; iron-treated, dithionite-reduced; and dithionite-reduced. Samples treated with iron were centrifuged in order to remove a precipitate which formed upon addition of the ferrous sulfate. The room temperature absorption spectra showed no change in the sample before or after addition of the ferrous sulfate. Increased light scattering resulting from suspended particles in the some of the enzyme samples resulted in increased depolarization of the CD light beam. These particles could not be removed by centrifugation. Although corrected for, the variable depolarization makes it difficult to accurately compare MCD intensities between the iron-treated samples and the control samples.

In two experiments, one with incubation for 30 minutes at 35°C
and one with incubation at room temperature, it was difficult to see any difference between the 4.22K MCD spectra of the iron-treated, dithionite-reduced enzyme and that of the control. In contrast to previous samples of BS-SDH, the MCD spectra and magnetization data indicated that there was considerably less [3Fe-xS] cluster present in the enzyme preparation used for these experiments. Magnetization data (not shown) collected at 320nm were well fit by a theoretical curve corresponding to reduced S1. Scatter in the data would account for no more than a 10% contribution of reduced S3, in comparison to the 30% contribution of S3 previously observed in dithionite-reduced BS-SDH. The scatter in the data at 715 and 680nm was too bad to allow a fit.

Based on the MCD data (above), the room temperature absorption spectra and EPR spin quantitations for the two samples, it appeared that there was no difference in the iron-sulfur cluster composition of iron-treated SDH and the control. The absorption spectra for the two samples were the same, indicating that there was no change in the total iron-sulfur cluster concentration resulting from incubation with iron. EPR indicated that there was no change in the amount of S1 present in either sample. Unfortunately, the features of S2 in BS-SDH are too indistinct to allow comparison of signal intensities except at very high enzyme concentrations. However, indirect evidence for the presence of reduced S2 was provided by a comparison of the spin relaxation properties of S1 at 15K, which were the same in the iron-treated SDH and the control sample.

In a subsequent experiment with a 15 minute incubation at room temperature, results again suggested that S2 and S3 do not interconvert. There were no noticeable differences in the 800 to
600nm region of the MCD spectra for succinate-reduced, iron-treated; iron-treated, dithionite-reduced; and dithionite-reduced samples (Figure 5-29). However, in these samples the presence of reduced S3 was indicated. Due to the amount of cytochrome, it was not possible to compare differences between the MCD spectra for succinate- and dithionite-reduced enzyme in order to estimate the amount of S2. Magnetization data (Figure 5-30) at 715nm for iron-treated samples were fit to a curve corresponding to reduced S3. Magnetization data (Figure 5-30) collected at 320nm were fit to a curve with contributions of 90% of S=1/2 with g values for S1 and 10% of reduced S3. The similarity in the room temperature absorption spectra indicated that the total iron-sulfur cluster concentration remained the same in the iron-treated and control samples. EPR showed no changes in S1 resulting from incubation of the enzyme with iron.

The extreme lability of S3 in soluble SDH makes it difficult to draw conclusions from these experiments. None of the BS-SDH preparations studied in this work had a stoichiometric amount of S3, and unfortunately the preparations used in the conversion experiments were deficient in S3. The amount of [3Fe-xS] cluster present was not converted to [4Fe-4S] under the reaction conditions. Analogous EPR experiments with fumarate reductase (see chapter 6) provide more definitive evidence that the [3Fe-xS] clusters are not always converted to [4Fe-4S] clusters.

**Attempted reconstitution of S3 in P-SDH**

An alternate strategy in addressing the question as to whether S2 and S3 are distinct clusters or interconversion products of the same cluster is to reconstitute S3 in air-treated SDH. The presence of a
stoichiometric amount of reduced S3 in reconstitutively active SDH would provide definitive proof that S3 is not a degradation product of S2.

There has been one report that treatment of non-reconstitutively active SDH with ferrous ammonium sulfate and sodium sulfide in the presence of mercaptoethanol restores reconstitutive activity to SDH (57). While uptake of iron was not demonstrated, related experiments did show that SDH incorporates the sulfane sulfur of \[^35S\]rhodanese (58, 59). In the presence of unlabeled rhodanese, SDH incorporated the labeled sulfur from \[^35S\]thiosulfate, and reconstitutive activity increased in parallel with sulfur incorporation. Presumably, the increase in SDH reconstitutive activity was due to reassembly of S3, since its presence has been correlated with such activity.

As reported, anaerobic addition of aqueous ammonium iron(II) sulfate and sodium sulfide to P-SDH resulted in the formation of a greenish black suspension, presumably iron sulfide. Attempts to completely remove what appeared to be a finely divided, black precipitate were unsuccessful. Centrifugation, exclusion chromatography, and ultrafiltration removed some, but not all of the color from the enzyme sample. Unfortunately the black color made the study of the enzyme sample by an optical technique impossible. EPR showed that center S1 had not been significantly damaged during this experiment. A similar experiment using rhodanese as the sulfide source should avoid the problem of iron sulfide formation, however this enzyme was not available.

Oxygen damage experiments with BS-SDH
Assuming that the trinuclear iron-sulfur cluster, S3, in SDH arises by oxidative degradation of a [4Fe-4S] center, intentional exposure of BS-SDH to air should, at least initially, result in an increased amount of S3. Conversely, if S3 is an intrinsic, oxygen-labile component of SDH, exposure of BS-SDH will result in loss of S3. In order to demonstrate whether or not S3 is formed by oxygen damage to S2, as-isolated BS-SDH was taken up in aerobic buffer, allowed to stand for various periods of time, reduced with succinate or dithionite, and then frozen in EPR tubes. The samples of BS-SDH enzyme used in this experiment were of the preparation described in Table 5-2, i.e. the samples contain S3.

EPR spectra of iron sulfur proteins often exhibit a signal at g=4.3 corresponding to adventitiously bound iron, i.e. non-heme iron which is not organized into an iron-sulfur center. This signal is often referred to as the "rhombic iron" signal. The air-treated samples showed an increase in the g=4.3 rhombic iron signal, relative to samples of dithionite-reduced BS-SDH which were taken up in an anaerobic buffer. In addition, EPR signal intensity for the rhombic iron resonance was greater in samples which were allowed to stand for longer periods of time, suggesting that iron sulfur cluster breakdown is occurring upon exposure to air. No EPR resonance corresponding to a oxidized [3Fe-xS] cluster was observed in any of the enzyme samples. However, MCD results indicate considerable breakdown of S3, since the form of the 4K MCD spectrum of dithionite-reduced air-treated BS-SDH was similar to that of dithionite-reduced P-SDH.

The lineshape of the EPR signal corresponding to the [2Fe-2S] center was unaltered by exposure to air. The spin concentration of
reduced [2Fe-2S] is different in the samples of air-treated, air-treated/dithionite-reduced, and air-treated/succinate-reduced enzymes. However, the differences in spin concentration is probably due to variable amounts of the [2Fe-2S] cluster being in the oxidized, diamagnetic form. Unfortunately, since a control sample, i.e. dithionite-reduced BS-SDH, was not prepared, the amount of [2Fe-2S] cluster degradation could not be assessed.

In a subsequent experiment, succinate-reduced BS-SDH was exposed to air for either one hour or four hours and then reduced with dithionite. The samples of BS-SDH enzyme used in this experiment were from a preparation different from those previously described. Assays of "low K_m"-ferricyanide activity were not performed on this preparation, however MCD data indicates that S3 was present in the samples.

MCD experiments showed that intentional oxygen damage to succinate-reduced BS-SDH results in loss of the [3Fe-xS] and the [2Fe-2S] center (Figure 5-31). The lineshape of the 70K EPR spectrum of S1 was unchanged. Spin quantitation of this signal showed a 20% decrease in the amount of [2Fe-2S] after one hour exposure to air, in agreement with MCD results. EPR spin quantitations showed that the the amount of S1 was reduced about 50% by exposure to air for four hours, in agreement with losses in MCD intensity.

Estimates of the amount of S2 in these samples were not possible. However, the presence of S2 can be detected by studying the spin relaxation properties of S1 at temperatures near 15K. Although relief from microwave power saturation was not as great as in the dithionite-reduced control sample, the spin relaxation properties of S1 in
dithionite-reduced, air-treated BS-SDH were quite different from those of succinate-reduced BS-SDH, suggesting the presence of the reduced [4Fe-4S] cluster. The somewhat slower spin relaxation of S1 in the air-treated BS-SDH can be rationalized in terms of damage to some of the S2 centers. Since excess dithionite was present, as observed by the UV-visible spectrum, all of S2 present should be reduced.

**Oxygen damage experiments with P-SDH**

In order to further demonstrate that S3 is not formed by oxidative damage to SDH, succinate-reduced P-SDH was exposed to air for either one or four hours and then reduced with dithionite. This enzyme preparation was completely deficient in S3. MCD and EPR experiments showed that intentional exposure to oxygen had no effect on the iron-sulfur clusters in P-SDH (Figure 5-32). Although unexpected, the oxygen-stability of P-SDH is not unreasonable since this batch of P-SDH was isolated in the absence of argon. If the iron-sulfur clusters in P-SDH were as oxygen-labile as those in BS-SDH, S1 and S2 would not survive the aerobic purification procedure.

It is interesting to speculate as to the origin of the difference in oxygen sensitivity of BS-SDH and P-SDH. Possibly SDH undergoes some type of structural modification, resulting in resistance to oxygen damage, when it is exposed to air during the aerobic isolation procedure. Or perhaps the butanol extraction procedure used for the preparation of BS-SDH somehow imparts extreme oxygen lability to SDH. Since the form of the MCD and EPR spectra for S1 are the same in P-SDH, BS-SDH, and air-treated BS-SDH, it would appear that these differences in oxygen stability do not result from changes in the structure of the binuclear cluster.
SUMMARY AND CONCLUSIONS

The combination of low temperature MCD spectroscopy and magnetization data with parallel EPR experiments demonstrates that SDH contains a [2Fe-2S], a [4Fe-4S], and a [3Fe-xS] center. This is the first metalloenzyme reported to contain all three types of iron-sulfur clusters. The results rationalize previous analytical and core extrusion data, as well as the differences in catalytic properties of enzyme preparations which contain center S3 and those which do not.

Previous EPR studies of SDH had been interpreted in terms of a HiPIP-type [4Fe-4S] center, and one or two [2Fe-2S] centers. The principal evidence for the presence of two binuclear centers comes from the marked enhancement of spin relaxation of an axial EPR resonance, centered about g=2, upon dithionite reduction of the succinate-reduced enzyme. In addition, increases in the spin intensity of the EPR signal for S1 were observed for the dithionite-reduced versus succinate-reduced enzyme. Increases in spin intensity varied depending upon the enzyme preparation. Results from core extrusion experiments and the non-heme iron/acid-labile sulfide content of SDH appeared to support this assignment of cluster composition.

The EPR characterization of S1 as a succinate-reducible [2Fe-2S] cluster was confirmed by MCD and CD spectroscopies. Comparison of the EPR and MCD spectra for S1 with corresponding spectra from adrenodoxin and spinach ferredoxin shows S1 to be most similar to the hydroxylase-type ferredoxin. In addition, the MCD intensity and EPR quantitations of reduced SDH are consistent with one binuclear center per enzyme molecule. Based on the EPR lineshape, S1 is the same in intact
mitochondria, soluble and complex beef SDH, and complex yeast SDH.

Proponents of the existence of center S2 in SDH have generally assumed it to be a binuclear cluster. The results presented here provide evidence that S2 is a spin-coupled tetranuclear iron-sulfur cluster which becomes paramagnetic upon dithionite reduction. At temperatures below 20K, weak features were observed to high and low field of S1. The signal, S2, is very broad with a spin intensity of approximately one spin per FAD. Redox titrations, monitored by EPR spectroscopy, demonstrate that the appearance of S2 coincides with a change in the spin relaxation properties of S1. The form and intensity of the MCD spectrum of S2 are consistent with the presence of one [4Fe-4S] center in SDH, in agreement with the estimated EPR spin quantitation.

In addition to centers S1 and S2, MCD data demonstrate the presence of a succinate-reducible trinuclear center, S3, in SDH. Based on the results from the succinate-FMS oxidoreductase assay, this center is necessary for reconstitutive activity. The intensity of the 4.22K MCD spectrum of S3 in reconstitutively active SDH is less than that generally observed for reduced [3Fe-xS] centers from bacterial ferredoxins. However, the less than stoichiometric amount of S3 is probably due to the extreme lability of the center in solubilized preparations.

Results of MCD and EPR experiments strongly suggest that center S3 is an intrinsic component of SDH and not an isolation artifact. MCD results indicate that conversion of S3 to a [4Fe-4S] cluster does not occur upon addition of substrate. In addition MCD and EPR experiments show that intentional exposure of reconstitutively active
SDH to air results in loss, not formation, of the [3Fe-\(x\)S] center. Attempts to convert S3 into a [4Fe-4S] center were unsuccessful, further supporting the idea that the trinuclear cluster is an intrinsic component of this enzyme. Furthermore, analytical data and results of core extrusion experiments are consistent with the [3Fe-\(x\)S] and [4Fe-4S] clusters being distinct entities in SDH.

Understanding of SDH is not yet complete. There remain several unresolved problems. For example, it is unclear as to why the tetranuclear cluster in SDH exhibits different EPR spectra for complex and soluble preparations. Preparation-dependent differences in the magnitude and/or orientation of the spin coupling between the [4Fe-4S] center and the other two clusters could account for the different EPR lineshapes. There is circumstantial evidence to support this view. Turnover numbers for SDH in the inner membrane, Complex II, and soluble preparations are different, and it is believed that the differences result from changes in conformation of the enzyme in the various preparations. In addition, the intercluster magnetic interactions in SDH have not been fully characterized. A more detailed study of these interactions will be useful in determining intercluster distances and relative orientations. Such information is crucial for an understanding of electron transfer in SDH. EPR studies of the clusters in the various preparations at frequencies other than X-band are necessary for this type of analysis.

SDH is the first example of a metalloprotein which contains three types of iron-sulfur clusters. It is unclear as to why this enzyme requires three different types of iron-sulfur centers and covalently bound FAD. There also the question about the function of the low
potential [4Fe-4S] center, which has a midpoint redox potential far below that of the succinate/fumarate couple.

Very little is known about the mechanistic details of electron transfer in the mitochondrial electron transport system. The sequence of electron flow through the redox centers has yet to be established. It is clear that the trinuclear cluster in SDH is necessary for transfer of electron from SDH to ubiquinone. Presumably, S3 is the final electron acceptor in enzymatic turnover of SDH. The function of the other redox centers in SDH is less certain.

Ohnishi believes that the FAD in SDH may function to convert the n=2 redox process of the TCA cycle into two n=1 redox steps at SDH, i.e. the flavin accepts two electrons from succinate and then reduces the iron sulfur clusters in sequential one-electron steps (22). This proposal was based on the observation that the flavin semiquinone formation constant (k=2.5X10^-2) indicates a more stable intermediate redox state than the typical n=2 redox components, e.g. the free ubiquinone/ubiquinol couple of k=10^-10 (60). The hypothesis fits in with the currently accepted model of electron transfer in Complex III, which involves a one electron reduction by a dehydrogenase (61). However, there is no definitive evidence for the splitting of the electron pair at FAD.

Another interesting question concerns the location of the iron-sulfur clusters in SDH. Studies of a similar enzyme, fumarate reductase from E. coli (see chapter 6), coupled with the published amino acid sequence (Figure 6-22) strongly suggest that all three clusters in both enzymes are located in the iron protein subunit. Selective deletion of the individual iron-sulfur clusters through
replacement of specific cysteine residues in bacterial SDH, coupled with EPR and MCD analysis of the enzyme, could be used to resolve questions of subunit location and function of the redox components of SDH. In addition, this approach has the potential of giving a very detailed mechanism for electron transfer in SDH.

REFERENCES


Biol. Chem. 255, 2761-2769.


48 Orme-Johnson, W.H. and Sands, R.H. (1973) in "Iron Sulfur


<table>
<thead>
<tr>
<th></th>
<th>BS-SDH</th>
<th>P-SDH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Analytical data</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAD content (nmol/mg)</td>
<td>7.9 ± 0.3</td>
<td>10.0 ± 0.3</td>
</tr>
<tr>
<td>Nonheme iron/ FAD&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.6 ± 0.3</td>
<td>7.8 ± 0.5</td>
</tr>
<tr>
<td><strong>Assays of activity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;Low K&lt;sub&gt;m&lt;/sub&gt;&quot;-ferricyanide reductase&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10,000-11,000</td>
<td>1,700-1,900</td>
</tr>
<tr>
<td>% Reconstitutive capacity&lt;sup&gt;c&lt;/sup&gt;</td>
<td>100</td>
<td>15-19</td>
</tr>
</tbody>
</table>

<sup>a</sup> Based on at least 3 determinations of nonheme iron, FAD, and protein.

<sup>b</sup> Moles of succinate oxidized/min/mol of FAD at 38°C.

<sup>c</sup> Calculated from the respective "low K<sub>m</sub>"-ferricyanide reductase activities (8).
### Table 5-2.

EPR. data for S1 and S3 in BS-SDH, P-SDH, beef heart Complex II, and yeast Complex II

<table>
<thead>
<tr>
<th></th>
<th>g values</th>
<th>spins/molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BS-SDH</strong>a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>succinate reducedb</td>
<td>2.026, 1.935, 1.912</td>
<td>1.13 ± 0.05</td>
</tr>
<tr>
<td>dithionite reducedb</td>
<td>2.026, 1.935, 1.912</td>
<td>1.28 ± 0.05</td>
</tr>
<tr>
<td><strong>P-SDH</strong>c,d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>as isolated</td>
<td>isotropic, 2.02</td>
<td>&lt; 0.03</td>
</tr>
<tr>
<td>succinate reduceda,b</td>
<td>2.026, 1.935, 1.913</td>
<td>0.15 ± 0.05</td>
</tr>
<tr>
<td>dithionite reduceda,b</td>
<td>2.026, 1.935, 1.913</td>
<td>1.08 ± 0.05</td>
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<tr>
<td><strong>Beef heart Complex II</strong>e</td>
<td></td>
<td></td>
</tr>
<tr>
<td>succinate reducedb</td>
<td>2.026, 1.935, 1.912</td>
<td>1.05 ± 0.05</td>
</tr>
<tr>
<td>dithionite reducedb</td>
<td>2.026, 1.935, 1.912</td>
<td></td>
</tr>
<tr>
<td><strong>Yeast Complex II</strong>f</td>
<td></td>
<td></td>
</tr>
<tr>
<td>as isolatedd</td>
<td>isotropic, 2.01</td>
<td>0.25 ± 0.05</td>
</tr>
<tr>
<td>dithionite reducedb</td>
<td>2.03, 1.93, 1.91</td>
<td>0.7 ± 0.05</td>
</tr>
</tbody>
</table>

---

*a* Enzyme taken up in 100mM potassium phosphate, pH 7.8, 100mM succinate, 1mM DTT, 50% (v/v) ethylene glycol; when indicated enzyme was reduced under anaerobic conditions with a 10-fold excess of sodium dithionite.

*b* Signal observed up to 120K; signal quantitation at 70K and 1mW microwave power versus 1mM CuEDTA standard; g values taken at peak maxima, crossover, and minima, accurate to ±0.001.

*c* Enzyme taken up in 100mM potassium phosphate, pH 7.8, 1mM DTT, 50% (v/v) ethylene glycol.

*d* Signal observed only below 30K; signal quantitation about 15K and 1mW microwave power versus 1mM metmyoglobin cyanide standard.

*e* Complex solubilized in 100mM potassium phosphate, pH 7.8, 100mM succinate, 1mM DTT, 3% (v/v) Triton X-100, 50% (v/v) ethylene glycol; when indicated complex was reduced under anaerobic conditions with a 10-fold excess of sodium dithionite.

*f* Complex was in 10mM HEPES, pH 7.5, 0.5mM EDTA, 0.05% (v/v) octyl glucoside, 50% (v/v) ethylene glycol; when indicated complex was reduced with a 10-fold excess of sodium dithionite.
Figure 5-1. Schematic representation of the organization of components of succinate dehydrogenase in the mitochondrial inner membrane.
Figure 5-2. EPR spectra for succinate- and dithionite-reduced BS-SDH. Solid line: succinate-reduced, enzyme concentration 135 μM. Broken line: dithionite-reduced, enzyme concentration 133 μM. Buffer and dithionite reduction as described in Table 5-2, footnote a. Conditions: 70K, 1 mW microwave power, 0.63 mtesla, modulation amplitude, frequency 9.02 GHz, gain the same for both spectra.
Figure 5-3. Power saturation behavior of the g=1.935 EPR signal of BS-SDH. (□) succinate-reduced, (X) dithionite-reduced. Samples as used in MCD and EPR studies. Signal amplitudes correspond to peak to trough of the g=1.935 feature. Conditions: 15K, 0.63 mtesla modulation amplitude, frequency 9.02 GHz.
Figure 5-4. EPR spectra for succinate- and dithionite-reduced P-SDH. Solid line: succinate-reduced, enzyme concentration 74 μM. Broken line: dithionite-reduced, enzyme concentration 72 μM. Buffer and dithionite reduction as described in Table 5-2, footnote a. Conditions: 70K, 1 mW microwave power, 0.63 mtesla modulation amplitude, frequency 9.02 GHz, gain the same for both spectra.
Figure 5-5. Power saturation behavior of the $g=1.935$ EPR signal for P-SDH. (□) succinate-reduced, (▲) dithionite-reduced. Samples as used in MCD and EPR studies. Signal amplitudes correspond to peak to trough of $g=1.935$ feature of EPR feature. Conditions: 15K, 0.63 mtesla modulation amplitude, frequency 9.02GHz.
Figure 5-6. Room temperature CD spectra of P-SDH. As isolated (---), succinate-reduced (---), dithionite-reduced (---). Enzyme samples identical to those used in MCD and EPR studies.
Figure 5-7. MCD and room temperature absorption spectra for succinate-reduced BS-SDH. Enzyme concentration 135 μM, buffer as in Table 5-2, footnote a. Upper spectrum: room temperature absorption spectrum. Lower spectra: MCD spectra at 1.54K (--), 4.22K (---), and 14.9K (••••) at magnetic field of 4.5 tesla, pathlength 0.165 cm.
Figure 5-8. MCD magnetization curves for succinate-reduced BS-SDH. Upper: wavelength 320nm; 1.60K (X), 4.22K (△), and 15.1K (●); magnetic fields between 0 and 4.5 tesla. --- represents theoretical magnetization curve for \( g_\parallel = 2.025 \), \( g_z = 1.925 \), and \( m_z/m_\perp = -1 \) (type A). -- represents theoretical magnetization curve for \( g_\parallel = 8.0 \) and \( g_z = 0.0 \) (type B). — represents 60% type A and 40% type B theoretical magnetization curves. Middle: wavelength 690 nm; 1.62K (X), 4.22K (△), magnetic fields between 0 and 4.5 tesla, various temperatures between 10 and 106K at magnetic field 4.2 tesla (●). — represents 30% type A and 70% type B theoretical magnetization curves. Bottom: wavelength 720 nm; 1.62K (X), 4.22K (△), magnetic fields between 0 and 4.5 tesla, various temperatures between 10 and 106K at magnetic field 4.2 tesla (●). — represents type B theoretical magnetization curve.
Figure 5-9. MCD and room temperature absorption spectra for dithionite-reduced BS-SDH. Enzyme concentration 133 μM, buffer mixture as in Table 5-2, footnote a. Upper spectrum: room temperature absorption spectrum. Lower spectra: MCD spectra at 1.56K (----), 4.22K (-----), and 14.0K (*****)) at magnetic field 4.5 tesla, pathlength 0.170 cm.
Figure 5-10. Comparison of low temperature MCD spectra for succinate and dithionite-reduced BS-SDH. Conditions: 4.2K, magnetic field 4.5 tesla. Succinate-reduced (-----), dithionite-reduced (-----).
Figure 5-11. MCD magnetization curves for dithionite-reduced BS-SDH. Upper: wavelength 320 nm; 1.54K (X), 4.22K (Δ), 12.6K (○), magnetic fields between 0 and 4.5 tesla. —— represents theoretical magnetization curve for $g_H=2.025$, $g_L=1.925$, and $m_σ/m_π=-1$ (type A). ——— represents theoretical magnetization curve for $g_H=8.0$ and $g_L=0.0$ (type B). —— represents 70% type A and 30% type B theoretical magnetization curves. Middle: wavelength 695 nm; 1.61K (X), 4.22K (Δ), magnetic fields between 0 and 4.5 tesla. —— represents 35% type A and 65% type B theoretical magnetization curves. Lower: wavelength 725 nm; 1.61K (X), magnetic fields between 0 and 4.5 tesla. —— represents 15% type A and 85% type B theoretical magnetization curves.
Figure 5-12. MCD and room temperature absorption spectra for succinate-reduced P-SDH. Enzyme concentration 74 \mu M, buffer mixture as in Table 5-2, footnote c. Upper spectrum: room temperature absorption spectrum. Lower spectra: MCD spectra at 1.60K (---), 4.22K (--), and 12.0K (●●●) at magnetic field 4.5 tesla, pathlength 0.156 cm.
Figure 5-13. MCD magnetization curve for succinate-reduced P-SDH. Wavelength 320 nm; 1.55K (X), 4.22K (Δ), 8.5K (●), magnetic fields between 0 and 4.5 tesla. —— represents theoretical magnetization curve for $g_\parallel=2.025$, $g_\perp=1.925$, $m_z/m_+=-1$. 

\[ \lambda = 320 \text{ nm} \]
Figure 5-14. MCD and room temperature absorption spectra for dithionite-reduced P-SDH. Enzyme concentration 72 uM, buffer mixture as in Table 5-2, footnote c. Upper spectrum: room temperature absorption spectrum. Lower spectra: MCD spectra at 1.63K (----), 4.22K (----), 8.5K (●●●) at magnetic field 4.5 tesla, pathlength 0.170 cm.
Figure 5-15. MCD magnetization curves for dithionite-reduced P-SDH. Temperatures of 1.54K (X), 4.22K (A), 10.0K (●); magnetic fields between 0 and 4.5 tesla. Upper: wavelength 320 nm; —— represents theoretical magnetization curve for $g_\|_1=2.025$, $g_\perp_1=1.925$, $m^2/m^+=-1$ (type A). Middle: wavelength 548 nm; —— represents type A theoretical magnetization curve. Lower: wavelength 690 nm; —— represents type A theoretical magnetization curve; —— represents theoretical magnetization curve for $g_\|_1=8.0$ and $g_\perp=0.0$ (type B); —— represents 70% type A and 30% type B theoretical magnetization curves.
Figure 5-16. Comparison of the low temperature MCD spectra of P-SDH: as-isolated (••••), succinate-reduced (-----), and dithionite-reduced (- - - -). Conditions: 4.22K, magnetic field of 4.5 tesla.
Figure 5-17. MCD spectra of reduced S1, S2, and S3 in succinate dehydrogenase. Conditions: 4.22K, magnetic field 4.5 tesla. (a) succinate-reduced minus as-isolated P-SDH (corresponds to reduced S1). (b) dithionite-reduced minus succinate-reduced BS-SDH (corresponds to reduced S2). Arrows indicate sharp derivative due to a trace of cytochrome b impurity. (c) succinate-reduced BS-SDH minus reduced S1 (corresponds to reduced S3). ——— represent regions where Soret MCD from heme impurities overlap.
Figure 5-18. EPR spectra of succinate- and dithionite-reduced beef heart Complex II. Protein concentration 18.2 mg/ml, flavin concentration 86 μM. Buffer as in Table 5-2, footnote e. Upper spectrum: dithionite-reduced enzyme. Lower spectrum: as prepared enzyme. Conditions: 13K, 1 mW microwave power, 10 mtesla modulation amplitude, frequency 9.012 GHz. Multiplication factors indicate the relative gain for the spectra.
Figure 5-19. EPR spectrum of dithionite-reduced beef heart Complex II. Protein concentration 32 mg/ml, flavin concentration 151 μM. Buffer as in Table 5-2, footnote e. Conditions: 9.5K, 5 mW microwave power, 0.63 mT modulation amplitude, frequency 8.983 GHz.
Figure 5-20. EPR spectra of succinate- and dithionite-reduced BS-SDH. Protein concentration 7.3 mg/ml, flavin concentration 58 μM. Buffer as in Table 5-2, footnote a. Upper panel: dithionite-reduced enzyme. Lower panel: as prepared enzyme. Conditions: 13K, 1 mW microwave power, 1 mtesla modulation amplitude, frequency 9.012 GHz.
Figure 5-21. EPR reduction-oxidation titration of beef heart Complex II and BS-SDH. The solid lines represent n=1 Nernst plots which have been fit to the data points. Upper panel: Complex II; points obtained by measuring the height of the g=1.935 EPR signal (●) and the g=1.847 feature (■). Conditions: 12K, 20 mW microwave power, 1 mtesla modulation amplitude, frequency 9.012 GHz. Lower panel: BS-SDH; points obtained by measuring the height of the g=1.935 EPR signal (●) and the difference in height between the g=1.830 and 1.795 features (■). Conditions: 12K, 50 mW microwave power, 1 mtesla modulation amplitude, frequency 9.012 GHz.
Figure 5-22. EPR spectrum of as isolated yeast Complex II. Enzyme concentration 51.5 μM, buffer mixture as in Table 5-2, footnote f, except no dithionite. Conditions: 15K, 1 mW microwave power, 0.63 mtesla modulation amplitude, frequency 9.02 GHz.
Figure 5-23. EPR spectrum of dithionite-reduced yeast Complex II. Enzyme concentration 51.5 μM, buffer mixture as in Table 5-2, footnote f. Conditions: 70K, 1 mW microwave power, 0.63 mtesla modulation amplitude, frequency 9.02 GHz.
Figure 5-24. Room temperature CD spectra for yeast Complex II. Upper spectrum: as isolated enzyme. Lower spectrum: dithionite-reduced enzyme. Samples as used in MCD and EPR studies.
Figure 5-25. MCD and room temperature absorption spectra for as isolated yeast Complex II. Enzyme concentration 51.5 μM, buffer mixture as in Table 5-2, footnote f, except no dithionite. Upper spectrum: room temperature absorption spectrum. Lower spectrum: 4.22K MCD spectrum, magnetic field 4.5 mtesla, pathlength 0.160 cm.
Figure 5-26. MCD and room temperature absorption spectra of dithionite-reduced yeast Complex II. Enzyme concentration 51.5 µM, buffer mixture as in Table 5-2, footnote f. Upper spectrum: room temperature absorption spectrum. Lower spectra: MCD spectra at 1.53, 4.22, and 10.9 K (intensity of all transitions increases with decreasing temperature) at magnetic field 4.5 tesla, pathlength 0.1686 cm.
Figure 5-27. Comparison of low temperature MCD spectra for dithionite-reduced BS-SDH (upper spectrum) and dithionite-reduced yeast Complex II. Conditions: temperature 4.22K, magnetic field 4.5 tesla.
Figure 5-28. MCD magnetization curves for dithionite-reduced yeast Complex II. Wavelengths: 740 nm (+), 690 nm (□), and 320 nm(Δ). Temperature 1.57K, magnetic fields between 0 and 4.5 tesla. —— is theoretical magnetization curve for $g_{||}=8.0$, $g_{\perp}=0.0$. ----- is theoretical magnetization curve for $g_{||}=2.025$, $g_{\perp}=1.925$, $m_{z}/m_{+}=-1$. 
Figure 5-29. Comparison of low temperature MCD spectra for dithionite-reduced BS-SDH (upper spectrum) and iron-treated, dithionite-reduced BS-SDH (lower spectrum). Conditions: temperature 4.22K, magnetic field 4.5 tesla.
Figure 5-30. MCD magnetization curves for iron-treated, dithionite-reduced BS-SDH. Enzyme sample as in Figure 5-29. Wavelength: 715 nm (+) and 320 NM (△). Temperature 1.78K, magnetic fields between 0 and 4.5 tesla. —— represents theoretical magnetization curve for $g_\parallel=8.0$, $g_\perp=0.0$. ---- represents theoretical magnetization curve for $g_\parallel=2.025$, $g_\perp=1.925$, $m_z/m_+=-1$. 
Figure 5-31. Low temperature MCD spectra of air-treated, dithionite-reduced BS-SDH. —— corresponds to dithionite-reduced BS-SDH, ---- corresponds to BS-SDH exposed to air for 1 hour followed by dithionite reduction, ····· corresponds to BS-SDH exposed to air for 4 hours followed by dithionite reduction. Conditions: temperature 4.22K, magnetic field 4.5 tesla.
Figure 5-32. Comparison of low temperature MCD spectra for dithionite-reduced P-SDH (upper spectrum) and air-treated, dithionite-reduced P-SDH (lower spectrum). Conditions: temperature 4.22K, magnetic field 4.5 tesla.
VI. FUMARATE REDUCTASE

BACKGROUND

Electron transport phosphorylation with fumarate rather than oxygen as the terminal acceptor is a process which appears to be essentially similar to oxidative phosphorylation in mitochondria. It differs from mitochondrial respiration by being a simpler system and by the more negative redox potential of the fumarate acceptor. Therefore, only one molecule of ATP is formed for every two electrons transported. Reviews of anaerobic energy transduction can be found in references 1-4.

The formate-fumarate reductase in anaerobic bacteria is composed of two membrane-bound enzyme complexes as illustrated in Figure 6-1 (3, 4). The donor complex is thought to consist of two proteins, formate dehydrogenase and a cytochrome \( b \) containing peptide. The acceptor is composed of four subunits: a flavoprotein, an iron-sulfur protein, and two membrane anchors which contain cytochrome \( b \). The electron transport chain of formate-fumarate reductase (Figure 6-2) is similar to that proposed for formate-nitrate reductase in Escherchia coli (5, 6). Various models of the proton pump involved in electron transport have been proposed (1-4).

Until very recently, the enzyme from Vibrio succinogenes was the best characterized of the membrane-bound fumarate reductases. This enzyme is an iron-sulfur flavoprotein with subunits of molecular weights of 79, 31, and 25 Kda (7, 8). The 79 Kda subunit contains one covalently-bound molecule of FAD. EPR studies have concluded that fumarate reductase from V. succinogenes contains one \([2Fe-2S]\) center
and one HiPIP [4Fe-4S] per enzyme molecule (9). The [2Fe-2S] center has a midpoint redox potential of -59mV while the HiPIP has a midpoint potential of -24mV at pH 7.0 (10). Experiments with partially resolved subunits were interpreted to show that the [2Fe-2S] center is localized on the 31 Kda subunit and the HiPIP is on the 79 Kda peptide (9). Other experiments involving cleavage of the subunits, followed by reconstitution, showed that fumarate reductase has two reactive sites which are essential for phosphorylative electron transport (11). The site where substrate binds is situated on the 79 Kda subunit. Menaquinone interacts with cytochrome b, while the 31 Kda peptide mediates electron transport between cytochrome b and the 79 Kda peptide.

*E. coli* can also utilize fumarate as a respiratory oxidant. Fumarate reductase from *E. coli* has recently been cloned, amplified, sequenced, and isolated in soluble form (12-18). The enzyme has a composition similar to that in *V. succinogenes*. The soluble, two-subunit enzyme consists of a 66 Kda flavoprotein and a 27 Kda iron-sulfur protein. The two smaller peptides (15 and 13 Kda) present in the complex are required for attachment of the enzyme to the membrane. They also have a role in quinone reduction (19), similar to that of the peptides C\(_{II-3}\) and C\(_{II-4}\) of bovine mitochondrial SDH (20).

Results of recent EPR studies of *E. coli* fumarate reductase in electron transport particles were interpreted in terms of two [2Fe-2S] clusters, FR1 and FR2, and one HiPIP [4Fe-4S] cluster, FR3 (21, 22), a Reconstitution refers to restoration of fumarate reductase activity by recombination of the individual subunits of fumarate reductase.
In analogy with an older characterization of mammalian SDH (23). The [2Fe-2S] centers were shown to be on the cytoplasmic side of the membrane by use of the dysprosium-EDTA complex as an exogenous paramagnetic probe. One of the [2Fe-2S] centers (FR1) had a midpoint redox potential of -50mV at pH 7.0. Dithionite reduction resulted in a more rapidly relaxing EPR signal (FR2) with less rhombicity but similar g values compared to the succinate-reducible species, FR1. FR2 exhibited an apparent midpoint potential of -285mV at pH 7.0. Potentiometric titrations of the HiPIP (FR3) gave an E_m of -30mV at pH 7.0. A recent EPR study of purified E. coli fumarate reductase gave similar results, except for differences in the midpoint potentials of the redox centers (23). In the two-subunit form of the enzyme, the midpoint potentials were -20mV for FR1, -330mV for FR2, and -70mV for FR3. Preparation-dependent differences in redox potentials of iron-sulfur clusters in membrane-bound enzymes are not uncommon.

The nucleotide sequences of the genes encoding SDH in E. coli have recently been determined, revealing a remarkable structural homology between subunits of SDH and fumarate reductase from E. coli (24, 25). Similarities between primary structures of the 66K da and 27 Kda subunits are striking. Analysis with the DIAGON computer program showed 66% and 58% homology for the 66 and 27 Kda peptides, respectively (25). Similarities in the enzymology and amino acid sequence of SDH and fumarate reductase suggest that the two enzymes may have similar redox centers.

MCD and parallel EPR experiments with SDH have demonstrated that the enzyme contains a [2Fe-2S], a [3Fe-xS], and a [4Fe-4S] center (see chapter 5). As in SDH, the iron-sulfur cluster composition of
fumarate reductase is a complex problem. EPR spectroscopy alone is not adequate for assessing the nature of the clusters in this enzyme because of the possibility that iron-sulfur clusters may be paramagnetic but EPR-silent due to zero-field splitting (e.g., a reduced \([3\text{Fe}-x\text{S}]\) cluster) or weak spin interactions between paramagnetic centers. Low temperature MCD is well suited to resolving these uncertainties since small zero-field splittings or weak magnetic interactions do not result in the absence of a temperature-dependent MCD spectrum. The present work utilizes MCD and EPR spectroscopy to characterize the iron-sulfur cluster composition of fumarate reductase. In addition, preliminary studies addressing the question of location of clusters in the subunits are reported.

RESULTS AND DISCUSSION

Identical MCD and EPR experiments were performed on two batches of fumarate reductase complex, and on two batches, each, of 0.4M perchlorate-treated and 0.8M perchlorate-treated soluble fumarate reductase preparations.

Analytical data

Table 6-1 shows the relevant analytical and assay data for the complex and 0.4M perchlorate enzyme used in the MCD experiments. Comparing FAD and protein determinations, preparations of the complex and soluble enzymes were 53% and 83% pure, respectively. Since catalytic turnover of fumarate reductase is faster than the rate-limiting step of the respiratory chain, activity assays with artificial electron acceptors, e.g., phenazine methosulfate (PMS), are used. This ensures that full activities are measured. The soluble enzyme was
approximately 43% less competent in the succinate-PMS oxidoreductase assay compared to the complex, indicating that solubilization damages the enzyme. The ability of fumarate reductase to reduce the ubiquinone homologue, Q, is related to the presence of the two small subunits in the complex enzyme. The low level of Q-reductase activity exhibited by the soluble enzyme can be attributed to minor contamination by the complex enzyme.

EPR data for fumarate reductase complex

Table 6-2 summarizes the EPR data for the samples used in the MCD experiments. Spin concentrations are based on flavin determinations. At temperatures below 20K, as-isolated fumarate reductase complex exhibits an EPR signal with a sharp peak at g=2.02 and a broad trough around g=1.97, with an extended tail towards high field (Figure 6-3). There is a distinct shoulder in the EPR signal about g=1.99. This signal is designated as FR3. At 13K, FR3 is not power saturated up to 50mW and corresponds to approximately one spin per FAD.

Figure 6-3 illustrates the striking similarity between EPR spectra of as-isolated fumarate reductase complex and of as-isolated Thermus thermophilus ferredoxin. The centers show similar temperature and microwave dependences in EPR spectroscopy. Mossbauer and MCD studies have unambiguously shown the g=2.015, 1.98, 1.93 EPR signal in T. thermophilus ferredoxin to arise from an oxidized [3Fe-xS] center (34, 35). Other well characterized oxidized [3Fe-xS] centers in aconitase, Desulfibrio gigas Fd II, and Azotobacter vinelandii Fd I exhibit similar, but more isotropic, EPR resonances (36). The EPR data suggests the presence of one [3Fe-xS] center in as-isolated fumarate reductase complex. However, since oxidized HiPIP [4Fe-4S]
centers also exhibit an isotropic EPR signal centered about \( g = 2.01 \), a technique which can discriminate between the two possibilities is necessary.

Upon reduction of fumarate reductase complex with dithionite, the EPR signal corresponding to the oxidized high potential center disappears, to be replaced by a slower relaxing, axial resonance at \( g = 2.026, 1.934, 1.920 \) (Figure 6-4). This EPR signal is designated as FR1. FR1 is observed up to 120K and accounts for approximately one spin per FAD. The \( g \) values and relaxation behavior of FR1 are similar to those of center S1 in beef heart SDH. Center S1 has been characterized as a \([2Fe-2S]\) core (32, 37-39).

Succinate-reduced fumarate reductase complex also exhibits the axial EPR signal described above (Figure 6-5), but the spin quantitation was significantly lower (see Table 6-2). The less than stoichiometric spin concentration is attributed to partial reduction of the \([2Fe-2S]\) center. This rationale is consistent with the observation of a weak FR3 EPR signal in the succinate-reduced enzyme (Figure 6-6). FR3 is reported to have a midpoint redox potential between 20 and 50mV above that of the \([2Fe-2S]\) center (21-23). An EPR signal centered about 2.0, attributed to the flavin free radical is also observed.

The concentration of oxidized FR3 in the succinate-reduced enzyme was estimated by comparing an EPR spectrum of the as-isolated enzyme to the EPR spectrum of the succinate-reduced enzyme under identical conditions at 15K. This procedure gave a value of approximately 0.4 of a spin per FAD.

Dithionite reduction of fumarate reductase complex often resulted
in an increase in EPR signal rhombicity, relative to the succinate-reduced enzyme. For reasons that are unclear, some samples of both complex and soluble enzyme did not exhibit a significant increase in signal rhombicity upon dithionite reduction.

A marked enhancement of the [2Fe-2S] spin relaxation is observed upon dithionite reduction (Figure 6-7). This behavior is analogous to that reported for center S1 in mammalian SDH, and is attributed to a species designated as FR2 (23). Recent studies have shown that this behavior in SDH is due to intercluster spin interaction between the S=1/2 [2Fe-2S] and [4Fe-4S] centers (41).

At temperatures below 15K, dithionite-reduced fumarate reductase complex exhibits weak features to high and low field of the [2Fe-2S] resonance (Figure 6-8). This new signal has not been reported by other workers. The signal was attributed to FR2 since it is not observed in the succinate-reduced enzyme. FR2 spans almost 150mTesla at X-band and shows no sign of power saturation at 8.5K and 50mW. These properties are characteristic of a reduced [4Fe-4S] center, spin-coupled to another paramagnetic center. The form of the signal is similar to that observed for S2 in mammalian Complex II (see Figure 5-19).

Although partially obscured by the EPR signal arising from FR1, the broad resonance centered about g=2 is similar in form to that of the complex EPR spectra arising from spin-interacting [4Fe-4S] centers in fully reduced eight-iron ferredoxins (42). In addition to spin interaction with the [2Fe-2S] center, the possibility exists for interaction between the reduced [4Fe-4S] core and the EPR-silent reduced [3Fe-xS] center. EPR experiments at different frequencies are
necessary to determine the nature of the spin interaction.

By estimating several appropriate bandshapes for the region obscured by the FR1 resonance, an approximate spin concentration of one spin per FAD was obtained for FR2. Although relatively inaccurate, the spin quantitation does demonstrate that this signal arises from an intrinsic component of fumarate reductase, not from an impurity.

**EPR data for soluble fumarate reductase**

Results obtained for 0.8M perchlorate-treated and 0.4M perchlorate treated fumarate reductase were identical, except for the amount of cytochrome contamination. The discussion that follows is applicable to both preparations. EPR data is summarized in Table 6-2.

The oxidized high potential center in soluble fumarate reductase is extremely oxygen sensitive. Similar behavior has been observed for the equivalent iron-sulfur core in mammalian SDH. As in SDH, the soluble fumarate reductase was isolated in the presence of succinate in order to protect FR3 from oxidative damage.

The dithionite-reduced soluble enzyme exhibits an axial EPR signal at 70K identical to that observed for the enzyme complex (Figure 6-4). The spin intensity of FR1 corresponds to approximately one spin per FAD. As in the complex, succinate reduction of the soluble enzyme results in a less than stoichiometric amount of reduced [2Fe-2S]. Succinate-reduced soluble fumarate reductase exhibits EPR signals corresponding to reduced [2Fe-2S] and oxidized FR3 centers (not shown). Spin quantitations of those centers were similar to quantitations obtained for the complex enzyme.

As in fumarate reductase complex, dithionite reduction results in
a marked enhancement of FR1 spin relaxation. At temperatures below 15K, weak features to high and low field of the reduced [2Fe-2S] resonance are observed. The spin concentration of FR2 was estimated to be approximately one spin per FAD. The observation of the FR2 EPR signal in the more purified soluble enzyme reinforces the assertion that it is an intrinsic component of fumarate reductase, rather than an impurity.

**CD data for fumarate reductase**

The CD spectra for soluble and complex fumarate reductase were almost identical. The discussion that follows is applicable to both types of enzyme preparations.

Oxidized and reduced [2Fe-2S] proteins are known to have CD spectra that are more intense than those observed for other types of iron-sulfur proteins (43). Transitions from this type of center would be expected to dominate a CD spectrum which has contributions from other types of iron-sulfur centers. The room temperature CD spectrum of as-isolated fumarate reductase is consistent with the presence of an oxidized [2Fe-2S] core (Figure 6-9). The form and intensity of the spectrum shown in Figure 6-8 is very similar to the CD spectrum of as-isolated SDH (40). As-isolated fumarate reductase has a weak positive feature centered about 600nm; positive bands at 480, 400, and 350nm; and a negative band at 540nm. The dithionite-reduced protein has broad positive bands centered at 580 and 400nm, and negative bands between 520 and 430nm and between 380 and 300nm. The CD spectrum of reduced fumarate reductase is similar to those observed for other reduced [2Fe-2S]-containing proteins, including SDH. CD spectra of [2Fe-2S] proteins is known to be largely invariant for plant- and
hydroxylase-type ferredoxins (43°).

MCD data for fumarate reductase complex

Figure 6-10 shows the room temperature absorption and low temperature MCD spectra at 1.65, 4.22, and 7.9K of as-isolated fumarate reductase complex. At low temperatures the MCD spectra are dominated by intense Soret transitions arising from an oxidized, low spin ferric (S=1/2) cytochrome. The MCD intensity indicates that the cytochrome to FAD ratio is approximately 1 to 10. Although the quantity of cytochrome in the complex enzyme varied, it never exceeded 10% of the FAD concentration. Therefore, the cytochrome is probably not part of the plasmid encoded fumarate reductase. Other transitions in the spectrum were attributed to iron-sulfur centers based on their intensities and temperature dependences.

To obtain the MCD spectrum of the oxidized FR3 in the absence of cytochrome, use was made of the observation that different preparations of complex enzyme had different amounts of cytochrome. The form of the 4K MCD spectrum of cytochrome was obtained by differencing the MCD spectra of the two different preparations of the complex, after appropriate normalization (Figure 6-11). Subtraction of the cytochrome 4K MCD spectrum from the 4K MCD spectrum of the as-isolated enzyme yielded the 4K MCD spectrum of the oxidized high potential center. A comparison of this spectrum with the 4K MCD spectrum of the oxidized T. thermophilus ferredoxin is shown in Figure 6-11a. The striking similarity between these spectra demonstrates that as-isolated fumarate reductase complex contains an oxidized [3Fe-4S] center. The intensity of the spectrum is consistent with one trinuclear species per enzyme molecule. The MCD spectrum of a HiPIP
\([4\text{Fe}-4\text{S}]\) is quite different from the spectrum shown in figure 6-11 (36, 44, 45). MCD magnetization data collected at 465nm were fit to a theoretical curve for \(S=1/2\) and \(g=2.01\) (Figure 6-12).

Figure 6-13 shows the room temperature absorption and low temperature MCD spectra at 1.53, 4.22, and 12.4K for the dithionite-reduced complex enzyme. There is a substantial decrease of absorption intensity in the visible region relative to the as-isolated enzyme. The brown color of the enzyme was noticeably bleached upon addition of dithionite. The spectra reveal numerous temperature-dependent MCD transitions. The biphasic band centered at 556nm (indicated by arrows), superimposed on a temperature-dependent positive band, was attributed to low spin (\(S=0\)) ferrous cytochrome. The positive band at 440nm is typical of a trace of high spin (\(S=2\)) cytochrome. All other temperature-dependent MCD transitions originate from paramagnetic iron-sulfur centers.

The intense, positive MCD band centered about 715nm is characteristic of a reduced [3Fe-xS] center (35, 45). These type of clusters exhibit distinctive magnetization plots which are fit by \(g=8, 0, 0\) at temperatures below 2K (35, 36, 45). MCD magnetization data, collected at 715nm were fit to a theoretical curve for \(g=8, 0, 0\) (Figure 6-14), confirming the assignment and indicating an \(S=2\) ground state. The low temperature MCD spectra of reduced [3Fe-xS] cores are more intense than any other type of known iron-sulfur center. Transitions from this type of center would be expected to dominate a MCD spectrum which has contributions from several different paramagnetic centers. The intensity of the band at 715nm is consistent with one reduced [3Fe-xS] center in fumarate reductase
Paramagnetic [2Fe-2S] centers generally exhibit positive MCD bands centered about 680 and 550nm, and a negative band at about 320nm (45). Plots of magnetization data at these wavelengths are shown in Figure 6-14. Magnetization data collected at 680nm were fit to a theoretical curve constructed for 75% of S=2 and g=8, 0, 0 and 25% of S=1/2 with EPR-determined g values for FR1. Theoretical data constructed for 60% of S=2 and 40% of S=1/2 systems gave a good fit to the magnetization data collected at 320nm. Although the theoretical curve for the S=1/2 system is based on the EPR-determined g values for the [2Fe-2S] center, any S=1/2 paramagnetic center with g values close to 2 would give a similar curve, and the MCD magnetization data cannot distinguish whether FR2 is a [2Fe-2S] cluster of [4Fe-4S] cluster.

The room temperature absorption and low temperature MCD spectra at 1.57, 4.22, and 8.5K for succinate-reduced enzyme complex are shown in Figure 6-15. The decrease in absorption intensity in the visible region is not as great as was observed with dithionite reduction. As in the dithionite-reduced fumarate reductase, the sharp biphasic band at 556nm is indicative of low spin (S=0) reduced cytochrome. The biphasic band centered around 440nm is indicative of a trace of low spin ferric (S=1/2) cytochrome. All other MCD transitions are ascribed to paramagnetic iron-sulfur centers. The redox potentials of the [2Fe-2S] and [3Fe-xS] centers in E. coli fumarate reductase are too low for these centers to be completely reduced in the succinate-reduced enzyme. Contributions from reduced [3Fe-xS] and reduced [2Fe-2S] cores can be observed in the low temperature MCD spectra shown in Figure 6-15. The negative band at
about 340nm suggests the presence of oxidized \([3\text{Fe}-\text{xS}]\) clusters.

Lowest temperature magnetization data are shown in Figure 6-16. Data collected at 715nm were fit to a theoretical curve for 90% of a \(S=2\) system with \(g=8, 0, 0\), and 10% of a \(S=1/2\) system with \(g_\parallel=1.990, g_\perp=1.903\). Magnetization data at 680nm were fit to a curve constructed for 80% \(S=1/2, g=8, 0, 0\) and 20% of \(S=1/2\) with EPR-determined \(g\) values for FR1. At 320nm, the lowest temperature data were fit to 60% of \(S=2, g=8, 0, 0\) and 40% of \(S=1/2\) and \(g\) values about 2.

In mammalian SDH it was possible to extract the form of the MCD spectra for the individual iron-sulfur centers: \(S1, S2,\) and \(S3\) \((40)\). Deconvolution of the SDH's complex MCD spectrum was possible because of the ability to prepare the enzyme derivatives in which only certain iron-sulfur centers were reduced; i.e., \(S1, S1+S2, S1+S3,\) and \(S1+S2+S3\). In SDH, the substrate succinate effected complete reduction of both the \([2\text{Fe-2S}]\) and the \([3\text{Fe-xS}]\) centers. Since only partial reduction of FR1 and FR3 was accomplished by addition of succinate, it was not possible to obtain the form of the MCD spectrum of the individual iron-sulfur centers in fumarate reductase. A potentiometric titration of fumarate reductase, monitored by MCD would be most useful. However, the necessity to add chromophoric redox mediators would impair collection of MCD data. Although it is of interest to compare the form of the MCD spectra of the individual redox components in SDH and fumarate reductase, obtaining their spectra is not crucial to identification of cluster type.

**MCD data for soluble fumarate reductase**

Results obtained for 0.8M perchlorate-treated and 0.4M perchlorate-treated soluble enzyme were the same. The discussion that
follows is applicable to both preparations.

Room temperature absorption and low temperature MCD spectra at 1.53, 4.22, and 7.7K for the dithionite-reduced enzyme are shown in Figure 6-17. As with the complex, there is a substantial decrease in absorption intensity in the visible region compared to the as-isolated enzyme. The brown color of the enzyme was noticeably bleached upon addition of dithionite. There is no evidence of cytochrome contamination in this spectrum. All temperature-dependent MCD transitions arise from paramagnetic iron-sulfur centers. The form of the MCD spectrum for the soluble fumarate reductase is similar to that obtained for the complex, except that there is a decrease in the intensity at about 715nm. This decrease in MCD intensity corresponds to approximately a 50% decrease in the amount of reduced [3Fe-xS] center.

Magnetization data at 1.53K recorded at 715, 680, 550, 406, and 320nm are shown in Figure 6-18. The reduced [3Fe-xS] center is the only contributor to the MCD band at 715nm. At 550nm, the transitions originate from a S=1/2 ground state with g values about 2. Data at 680, 406, and 320nm were fit to theoretical plots constructed for 65% S=2 and 35% S=1/2, 70% S=2 and 30% S=1/2, and 45% S=2 and 55% S=1/2, respectively. Comparison of these plots with data obtained for the dithionite-reduced complex is consistent with a decrease in the amount of the reduced [3Fe-xS] core.

Core conversion experiments

Although [3Fe-xS] centers have been identified in several iron-sulfur proteins, many researchers question their biological significance (36). The observation that [3Fe-xS] cores can arise by
oxidative degradation of a [4Fe-4S] center has led many workers to believe that all trinuclear iron-sulfur centers are isolation artifacts (46, 47). However, in _E. coli_ nitrate reductase (48), mammalian SDH (40), and _E. coli_ fumarate reductase, [3Fe-xS] centers have been found to be present in the catalytically active form of the enzyme, arguing against this hypothesis. Experiments with mammalian SDH strongly imply that conversion between the [3Fe-xS] and [4Fe-4S] centers does not occur. Moreover, the observation that both centers FR2 and FR3 are present in amount approximately stoichiometric with FAD, and that no conversion of FR3 to a [4Fe-4S] cluster occurs on reduction, indicates that these centers are not interconversion products of the same cluster. To further address the question of possible core interconversion in fumarate reductase, attempts were made to induce conversion of FR3 to a [4Fe-4S] core while monitoring EPR and MCD spectra.

Inaconitase, treatment of the reduced enzyme with ferrous iron results in conversion a [3Fe-xS] center into a [4Fe-4S] center, yielding the catalytically active form of the enzyme (46). In an adaptation of this procedure, fumarate reductase complex was incubated 30 minutes with either aqueous ammonium iron(II) sulfate or EDTA, and then reduced with dithionite. The following control samples were prepared: dithionite-reduced with 30 minute incubation, dithionite-reduced with immediate freezing, and as-isolated fumarate reductase complex. Comparison of the broad, rapidly relaxing EPR signal (FR2) in the various enzyme derivatives showed no change in the concentration of this cluster on incubation with Fe(II) or EDTA. Moreover, the concentration of FR1 was the same in all reduced
samples, and spin quantitation of as-isolated fumarate reductase complex showed that the enzyme sample used in this experiment contained a full complement of trinuclear cluster. Hence, EPR experiments gave no indication of conversion of $[3\text{Fe-}x\text{S}]_1^\text{-ster}$ to $[4\text{Fe-}4\text{S}]$ cluster conversion in the fumarate reductase complex on incubation with Fe(II) under reducing conditions.

In a subsequent MCD experiment, 0.8M perchlorate-treated fumarate reductase was incubated 15 minutes with ammonium iron(II) sulfate and then reduced with dithionite. The MCD spectrum of this sample of soluble enzyme indicated that the sample almost completely deficient in $[3\text{Fe-}x\text{S}]$ clusters. Comparisons of spin relaxation behavior for FR1 in the dithionite-reduced enzyme, relative to that exhibited by succinate-reduced enzyme, indicated that FR2 was present in both the iron-treated and control samples. The 4K MCD spectrum of the iron-treated enzyme was identical to that of the control sample (Figure 6-19). Spin quantitations of the 70K EPR signal for FR1 are the same in both samples. Comparison of the UV-visible spectra of both samples shows no increase in absorption corresponding to formation of another iron-sulfur center. Therefore, incubation of fumarate reductase with ferrous iron under reducing conditions appears to have no effect on the iron-sulfur cluster composition of the enzyme.

**Oxidative damage experiments**

Treatment of as-isolated fumarate reductase complex with air or ferricyanide resulted in a decrease of the the rapidly relaxing, isotropic EPR signal centered about $g=2.01$ (FR3). In addition, decreases in the MCD intensity of the oxidized $[3\text{Fe-}x\text{S}]$ cluster indicated loss of FR3, and that there was not formation of reduced
[3Fe-xS] centers. The form of the EPR and MCD spectra of the oxidized samples were the same as for the as-isolated enzyme. Comparison of the UV-visible absorption at 444nm for the as-isolated, air-treated, and ferricyanide-treated enzyme, along with their EPR spin quantitations (Table 6-2), indicated that the majority of iron-sulfur core degradation involved the high potential center. If conversion of the oxidized [4Fe-4S] clusters to [3Fe-xS] clusters had occurred, the intensity of the UV-visible absorption spectrum should have remained the same, not decreased as observed. Dithionite reduction of both of the oxidized enzyme samples showed that the [2Fe-2S] center had not been damaged. Comparison of the spin relaxation behavior of FR1 indicated that FR2 was present in the oxidized enzyme. Experiments with soluble fumarate reductase gave similar results. Based on these experiments, and the results discussed above, the [3Fe-xS] and [4Fe-4S] clusters of fumarate reductase are considered to be distinct entities, not interconversion products of the same cluster. This conclusion is in agreement with results of similar experiments on SDH (see chapter 5).

EPR studies of E. coli cells with amplification of fumarate reductase

The core conversion experiments described above strongly argue against the premise that all trinuclear iron-sulfur centers are isolation artifacts. While the presence of [3Fe-xS] cores in catalytically active metalloenzymes has been unambiguously demonstrated, there has been no evidence reported for the existence of [3Fe-xS] centers in vivo.

EPR studies on E. coli fumarate reductase in the form of electron transport particles or intact membranes have been reported (21, 22).
Plasmid amplification of fumarate reductase in *E. coli* HB101 results in a bacterium in which 80% of the cell membrane mass is fumarate reductase. The high level of enzyme in the membrane permits useful EPR studies of pristine fumarate reductase in vivo.

Anaerobically harvested *E. coli* cells exhibit a rhombic EPR signal at 70K with $g=2.026, 1.934, 1.920$ (Figure 6-20). The $g$ values and spin relaxation behavior of this signal are identical to that of center FR1 in the fumarate reductase complex and soluble. The signal at $g=2.004$ is attributed to the flavin free radical. No additional EPR resonances were observed at temperatures below 30K. Failure to observe the oxidized [3Fe-xS] core is consistent with redox titrations of the cytoplasmic membrane fraction, which show that FR3 has a redox midpoint potential close to that of FR1 (21-23). An alternative explanation for the absence of EPR signals corresponding to the oxidized [3Fe-xS] is that FR3 is present, not as a EPR-silent reduced trinuclear cluster, but as a diamagnetic [4Fe-4S] cluster in vivo.

In order to distinguish between these two possibilities, it was necessary to oxidize the enzyme by anaerobic incubation of the cells with the natural substrate fumarate. Cells incubated at room temperature in a buffer containing 50mM fumarate for one hour showed a decrease in the spin intensity of FR1, observed at 70K, and a marked enhancement in intensity for the flavin resonance (Figure 6-21). Spin quantitation showed a decrease from 35μM to less than 5μM for the reduced [2Fe-2S] center in fumarate-treated cells. At temperatures below 30K, an EPR signal corresponding to the oxidized [3Fe-xS] center was observed (Figure 6-21). Assuming that the [3Fe-xS] center is present in a 1:1 ratio with the [2Fe-2S] core, and that the form of
FR3 is the same as in the isolated enzyme, it was estimated that between 60 and 80% of the [3Fe-xS] centers are in the oxidized state in the fumarate-treated E. coli cells. Based on these EPR experiments, the [3Fe-xS] cluster FR3 is an intrinsic component of pristine fumarate reductase. Although some trinuclear clusters are formed by degradation of tetranuclear clusters, others are clearly functional components of metalloenzymes under physiological conditions.

EPR experiments on E. coli cells plasmid-amplified for discrete fumarate reductase peptides

Complete amino acid sequences have been determined for both major polypeptides in SDH and fumarate reductase from E. coli (24, 25). The subunits of the two enzymes exhibit striking homology. In addition, the iron-sulfur subunit of both enzymes contain eleven cysteine residues, ten of which are conserved in clusters that closely resemble those found in bacterial ferredoxins (Figure 6-22). The cysteine residues of the flavoprotein are more randomly dispersed and are not organized in ferredoxin-like clusters. Following the convention of Darlison and Guest (25), the flavoprotein and iron-sulfur subunit will be referred to as A and B, respectively. The membrane anchor peptides will be referred to as C and D.

The clusters of cysteines are numbered I, II, and III, with I being the closest to the amino-terminal end of the polypeptide. Cysteine cluster I has an arrangement of cysteine residues similar to those bound to [2Fe-2S] centers in plant and cyanobacterial ferredoxins. The arrangement of the cysteines in the remaining two clusters, II and III, resemble those bound to [4Fe-4S] centers in
bacterial ferredoxins. The number of cysteine residues required to accommodate a [3Fe-xS] center is unknown. Recent spectroscopic studies suggest a common stoichiometry for all [3Fe-xS] centers and two structures have been proposed (see chapter 1). These structures require three or four cysteine ligands to coordinate to the iron atoms of the trinuclear core. The facile interconversion of [3Fe-xS] and [4Fe-4S] centers in some proteins supports the idea that these centers require an similar arrangement of cysteine residues.

Although the amino acid sequences of fumarate reductase and SDH strongly imply that all three iron-sulfur cores are bound to the subunit which does not contain FAD (25), there is no direct evidence demonstrating the location of the iron-sulfur cores. Results from EPR experiments on mutants of Bacillus subtilis, attempting to determine core location in the SDH subunits, were ambiguous (49). Attempts to cleanly cleave the two major polypeptides in V. succinogenes fumarate reductase have been unsuccessful (9). An alternate strategy is to amplify a bacterium selectively for one or both of the major peptides. Since the ubiquinone binding polypeptides are believed to confer stability to fumarate reductase, cells amplified for these subunits were also studied.

Anaerobically harvested E. coli cells, HB101 and W3100, amplified for the fumarate reductase peptides ABCD, AB, and A plus truncated B, exhibit the rhombic EPR signal attributed to FR1 70K (Figure 6-23). The intensity of the FR1 resonance in the plasmid amplified cells is similar, and an order of magnitude stronger than the FR1 EPR signal observed in wild type E. coli. The EPR signal at g=2.004 is due to the flavin semiquinone. In contrast, the amount of reduced FR1
evident in the EPR spectra of E. coli cells amplified for B, BCD, or two-thirds of A subunits is equivalent to that present for chromosomally encoded fumarate reductase. There was no evidence for oxidized [3Fe-xS] or reduced [4Fe-4S] cores in these samples.

Incubation of E. coli cells amplified for ABCD, AB, and a plus part of B, with 50mM fumarate resulted in a attenuation of the FR1 EPR resonance due to oxidation of the center to the diamagnetic [2Fe-2S]^{2+} state. An increase in the signal intensity of the flavin semiquinone was also observed. The changes in the EPR signal intensities are similar for the all transformed cells. At temperatures below 30K, fumarate-treated cells amplified for ABCD and AB exhibited EPR signals corresponding to the oxidized FR3 (Figure 6-24). The 16K EPR spectrum of fumarate-treated E. coli cells amplified for A and part of B shows no evidence of the oxidized trinuclear center (Figure 6-24c). EPR spectra of fumarate-treated E. coli cells, amplified for B, BCD, and part of A, were similar to that of wild type E. coli.

In an attempt to detect the presence of the reduced [4Fe-4S] cluster in E. coli cells, EPR experiments were performed with E. coli cells incubated with 40mM glycerol and 20mM dithionite. Dithionite-treated cells amplified for subunits ABCD, AB, and A plus part of B showed relief from microwave power saturation at 9K, analogous to that observed in the dithionite-reduced fumarate reductase complex. In contrast, the corresponding as-isolated cells exhibited spin relaxation properties similar to those observed in the succinate-reduced complex enzyme.

There was no increase in the spin intensity of FR1 in the transformed E. coli cells upon incubation with glycerol and
dithionite, however at temperatures near 9K, dithionite-treated *E. coli* cells amplified for ABCD or AB did exhibit weak features to high and low magnetic field of the FR1 resonance (Figure 6-25). The additional signal was attributed to a reduced [4Fe-4S] cluster since it has properties similar to the FR2 EPR resonance observed in dithionite-reduced, purified enzyme. This signal is not observed in samples of the as-isolated cells. Dithionite-reduced cells amplified for subunits B and BCD exhibited EPR spectra similar to that of wild type *E. coli*.

EPR experiments on anaerobically harvested *E. coli* W3110 cells amplified for subunits ABCD, AB, B, or BCD gave results similar to those described previously (Figure 6-23), except that the levels of chromosomally encoded fumarate reductase were higher in wild type cells. In an attempt to partially oxidize the [2Fe-2S] cluster and [3Fe-xS] cluster, the cells were incubated in 1mM ferricyanide for 45 minutes at room temperature. In contrast to the results previously described for incubation in 50mM fumarate, there was no decrease in the size of the 70K EPR signal for FR1, indicating that oxidation to the diamagnetic state had not occurred. In addition, the EPR resonance corresponding to the oxidized [3Fe-xS] was not observed at temperatures near 10K. Cell membranes are known to be impermeable to chemical reagents such as ferricyanide and dithionite.

The finding that EPR signals corresponding to iron sulfur centers are exhibited only by cells amplified for both the A and B subunits is consistent with EPR studies of SDH in *B. subtilis* mutants (49). In these studies, center S1 was found to be present only in mutants which contain a full-length FP polypeptide and a 15K, or larger, fragment of
the IP subunit. Lysates of mutants with defective FP and non-mutated IP subunits did not exhibit EPR signals for SDH iron-sulfur centers. Therefore a requirement of both the FP and IP subunits for assembly and/or stability of the iron-sulfur cores is strongly implied. Very little is known about the in vivo assembly of iron-sulfur cores.

SUMMARY AND CONCLUSIONS

The combination of low temperature MCD spectroscopy and magnetization data with parallel EPR experiments demonstrates that E. coli fumarate reductase contains a [2Fe-2S], a [4Fe-4S], and a [3Fe-xS] center. SDH has a similar cluster composition (40), therefore fumarate reductase is the second metalloenzyme reported to contain all three types of iron-sulfur clusters. These two enzymes are very similar based on their subunit composition, primary structure, and enzymology. SDH oxidizes succinate to fumarate during aerobic respiration, while fumarate reductase catalyzes the reduction of fumarate to succinate in anaerobic respiration. Both enzymes are involved in an electron transport chain which is linked to oxidative phosphorylation.

Previous EPR studies of fumarate reductase had been interpreted in terms of a HiPIP [4Fe-4S] and two [2Fe-2S] centers (9, 10, 21-23). The principal evidence for two binuclear centers comes from the marked enhancement of spin relaxation of an axial EPR resonance (FRI) upon dithionite reduction of the succinate-reduced enzyme. In addition, the EPR spin intensity of FRI in dithionite-reduced fumarate reductase was twice that in the succinate-reduced enzyme.

MCD and CD spectroscopies confirmed the EPR characterization of
FR1 as a reduced [2Fe-2S] center in *E. coli* fumarate reductase. However, the spin quantitation of dithionite-reduced fumarate reductase argues against the presence of two binuclear clusters. The observed doubling of the EPR spin intensity upon dithionite reduction of succinate-reduced enzyme is due to the [2Fe-2S] center only being partially reduced by succinate in fully activated preparations. MCD data agree with the presence of one binuclear cluster in fumarate reductase. Based on the EPR lineshape, FR1 is the same in the soluble and complex enzymes as in the intact *E. coli* cells.

Previously, center FR2 had been assigned as a spin-coupled binuclear cluster (9, 10, 21-23). FR1 is observed to undergo enhancement of spin relaxation upon dithionite reduction, analogous to the behavior reported for the equivalent center in mammalian SDH (40). Redox titrations of SDH, monitored by EPR spectroscopy, have demonstrated that this behavior is due to intercluster spin coupling between the $S=1/2$ [2Fe-2S]$^{+1}$ and [4Fe-4S]$^{+1}$ centers (41). The results presented here provide evidence that FR2 is a spin-coupled tetranuclear iron-sulfur cluster which becomes paramagnetic upon dithionite reduction. At temperatures below 20K, weak features are observed to high and low magnetic field of FR1. The spin relaxation behavior and redox properties of this signal, as well as the average g value, are characteristic of a [4Fe-4S]$^{+1}$ cluster (42). However, the signal is broader than that generally observed in bacterial ferredoxins, indicating spin interaction between this cluster and the [2Fe-2S]$^{+1}$ cluster. The form and intensity of the MCD spectrum of FR2 are consistent with the presence of one [4Fe-4S] center in fumarate reductase, in agreement with the estimated EPR spin quantitation of
one [4Fe-4S] center per enzyme molecule.

In addition to centers FR1 and FR2, MCD data demonstrate the presence of a trinuclear center, FR3, in fumarate reductase. Based on Q reductase activity, this center is necessary for reconstitutive activity. MCD results indicate that bulk conversion of FR3 to a tetranuclear center does not occur upon addition of substrate. The intensity of the 4K MCD spectrum of FR3 in fumarate reductase complex is similar to that generally observed for reduced [3Fe-xS] centers from bacterial ferredoxins (45). The lower amount of FR3 in the soluble enzyme is probably due to the lability of this center in solubilized preparations.

EPR experiments with intact E. coli cells amplified for fumarate reductase, provide definitive evidence for the presence of the trinuclear iron-sulfur cluster in the native enzyme. Due to the reducing environment of the cell matrix, the EPR signal corresponding to oxidized FR3 is only observed upon incubation of the cells with the natural substrate fumarate.

MCD and EPR experiment show that intentional oxygen damage to fumarate reductase results in loss of the [3Fe-xS] center. Attempts to convert the trinuclear cluster into a [4Fe-4S] center were unsuccessful, further supporting the idea that the trinuclear cluster is an intrinsic component of the enzyme, not an isolation artifact. Therefore, all evidence is consistent with the [3Fe-xS] and [4Fe-4S] clusters being distinct entities in fumarate reductase.

There remain several unresolved problems. For example it is unclear as to why the tetranuclear cluster in these enzymes gives different EPR spectra in complex and soluble preparations. It is
interesting to note that a similar phenomenon occurs in SDH (see chapter 5). Preparation-dependent differences in the magnitude and/or orientations of the spin coupling between the [4Fe-4S] center and the other two clusters could account for the different EPR lineshapes. The intercluster magnetic interactions in fumarate reductase have not been fully characterized. A more detailed study of these interactions will be useful in determining intercluster distances and relative orientations. Such information is crucial for an understanding of electron transfer in the enzyme. Therefore, EPR studies of various enzyme preparations at frequencies other than X-band are necessary.

As of yet, there is no definitive evidence concerning cluster location in fumarate reductase. Comparison of amino acid sequences of the flavoprotein and iron-sulfur protein subunits in SDH and fumarate reductase from _E. coli_ suggest that all three clusters are located in the iron-sulfur protein subunit (25). However, preliminary EPR studies of _E. coli_ cells amplified for discrete fumarate reductase subunits, or a portion of a subunit found that cells which were not amplified for the flavoprotein did not exhibit EPR signals corresponding to iron-sulfur clusters. In addition, iron-sulfur clusters appeared not to be present in cells amplified for production of the fumarate reductase flavoprotein alone. In agreement with analogous work with _E. coli_ SDH mutants, it appears that the flavoprotein is somehow necessary for assembly of the iron-sulfur clusters in the enzyme (48). Very little is known about the biosynthetic process for insertion of iron-sulfur clusters into proteins.

EPR experiments with _E. coli_ cells amplified for the flavoprotein
and a truncated iron protein indicate that the first cluster of cysteine residues in the iron protein binds the [2Fe-2S] cluster. This is in agreement with comparisons of the arrangement of cysteines in cluster I and those in plant [2Fe-2S] ferredoxins (25). Selective deletion of the individual iron-sulfur clusters in *E. coli* fumarate reductase, coupled with EPR and MCD analysis of the enzyme, should be able to resolve questions of subunit location.

Another question concerns why fumarate reductase requires three different types of iron-sulfur centers and covalently bound FAD. In addition, the sequence of electron flow through these redox centers has yet to be established. It is also unclear as to why the potential of the [4Fe-4S] center is so much lower than the succinate/fumarate couple. These and other questions remain to be explored.

REFERENCES


Table 6-1

Analytical and assay data for samples of fumarate reductase complex and soluble enzyme.

<table>
<thead>
<tr>
<th></th>
<th>Complex</th>
<th>Soluble</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Analytical data</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAD content (nmole/mg)</td>
<td>4.3±0.3</td>
<td>8.9±0.3</td>
</tr>
<tr>
<td>Nonheme iron/FAD</td>
<td>9.2±0.3</td>
<td>7.4±0.3</td>
</tr>
<tr>
<td><strong>Assay data</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q reductase activity</td>
<td>1539±151</td>
<td>235±11</td>
</tr>
<tr>
<td>PMS reductase activity</td>
<td>4052±399</td>
<td>2302±109</td>
</tr>
</tbody>
</table>

a Based on at least 2 determinations of nonheme iron, FAD, and protein.
b Moles of succinate oxidized/min/mol of FAD at 38°C.
c Q is ubiquinone.
d PMS is phenazine methosulfate.
Table 6-2

EPR data for FR1 and FR3 in fumarate reductase complex and soluble enzyme

<table>
<thead>
<tr>
<th></th>
<th>g values</th>
<th>spins/FAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>as isolated&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>2.015, 1.98, 1.93</td>
<td>0.94±0.05</td>
</tr>
<tr>
<td>air treated&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>2.015, 1.98, 1.93</td>
<td>0.77±0.05</td>
</tr>
<tr>
<td>ferricyanide treated&lt;sup&gt;a,d&lt;/sup&gt;</td>
<td>2.015, 1.98, 1.93</td>
<td>0.59±0.05</td>
</tr>
<tr>
<td>succinate reduced&lt;sup&gt;a,e&lt;/sup&gt;</td>
<td>2.015, 1.98, 1.93</td>
<td>~0.4</td>
</tr>
<tr>
<td>succinate reduced&lt;sup&gt;e,f&lt;/sup&gt;</td>
<td>2.026, 1.934, 1.920</td>
<td>1.20±0.05</td>
</tr>
<tr>
<td>dithionite reduced&lt;sup&gt;e,f&lt;/sup&gt;</td>
<td>2.026, 1.934, 1.920</td>
<td></td>
</tr>
<tr>
<td>Soluble enzyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td>as isolated&lt;sup&gt;a,h&lt;/sup&gt;</td>
<td>2.015, 1.98, 1.93</td>
<td>~0.4</td>
</tr>
<tr>
<td>succinate reduced&lt;sup&gt;a,g&lt;/sup&gt;</td>
<td>2.015, 1.98, 1.93</td>
<td></td>
</tr>
<tr>
<td>succinate reduced&lt;sup&gt;e,g&lt;/sup&gt;</td>
<td>2.026, 1.934, 1.920</td>
<td></td>
</tr>
<tr>
<td>dithionite reduced&lt;sup&gt;e,g&lt;/sup&gt;</td>
<td>2.026, 1.934, 1.920</td>
<td>0.92±0.05</td>
</tr>
</tbody>
</table>

<sup>a</sup> Signal observed only below 30K. Spin quantitation at 13K and 1mW microwave power versus 1mM metmyoglobin cyanide standard. g values based on computer simulation of a similar signal observed in *T. thermophilus* Fd, as isolated.

<sup>b</sup> Complex solubilized in 100mM potassium phosphate, pH 7.8, 3% (v/v) Triton X-100, 50% (v/v) ethylene glycol.

<sup>c</sup> As prepared complex enzyme exposed to air for 2 hours at room temperature.

<sup>d</sup> As prepared complex enzyme treated with a 10-fold excess of ferricyanide for 30 minutes at room temperature; ferricyanide removed by gel filtration using Sephadex G-25.

<sup>e</sup> Signal observed up to 120K; spin quantitation at 70K and 1mW microwave power versus 1mM CuEDTA standard; g values taken at peak maxima, crossover, and peak minima.

<sup>f</sup> Complex solubilized in 100mM potassium phosphate, pH 7.8, 100mM succinate, 1mM DTT, 3% (v/v) Triton X-100, 50% (v/v) ethylene glycol. When indicated, enzyme was reduced with a 10-fold excess of sodium dithionite.

<sup>g</sup> Soluble enzyme taken up in 100mM potassium phosphate, pH 7.8, 100mM succinate, 1mM DTT, 50% (v/v) ethylene glycol. When indicated, enzyme was reduced with a 10-fold excess of sodium dithionite.

<sup>h</sup> Soluble enzyme taken up in 100mM potassium phosphate, pH 7.8, 1mM DTT, 50% (v/v) ethylene glycol.
Figure 6-1. Schematic of formate-fumarate reductase complex. Arrows depict a possible mechanism for generating an electrochemical proton potential across the bacterial membrane during electron transfer from formate to fumarate. FDH = formate dehydrogenase; FR = fumarate reductase.
Figure 6-2. Sequence of electron flow through redox components of the formate-fumarate reductase complex. FDH = formate dehydrogenase, FR = fumarate reductase, MK = menaquinone, cyt b = cytochrome b.
Figure 6-3. EPR spectra of *E. coli* fumarate reductase complex, as prepared, and *T. thermophilus* Fd, as isolated. Upper spectrum: fumarate reductase complex, as prepared. Enzyme concentration 47 μM; buffer mixture as in Table 6-2, footnote b. Lower spectrum: *T. thermophilus*, as isolated. Conditions: 13K, 1 mW microwave power, 0.63 mtesla modulation amplitude, frequency 8.99 GHz.
Figure 6-4. EPR spectra of *E. coli* fumarate reductase complex and soluble enzyme after dithionite reduction. Upper spectrum: dithionite-reduced fumarate reductase complex; concentration 47 μM. Lower spectrum: dithionite-reduced soluble fumarate reductase; concentration 53 μM. Buffer mixture and conditions of reduction as described in Table 6-2, footnotes f and g. Conditions: 70K, 1 mW microwave power, 0.63 mtesla modulation amplitude, frequency 8.99 GHz.
Figure 6-5. Comparison of EPR spectra for succinate- and dithionite-reduced *E. coli* fumarate reductase. (a) Fumarate reductase complex, succinate-reduced. (b) Fumarate reductase, dithionite-reduced. Enzyme concentration for both samples 47 μM; buffer mixture as in Table 6-2, footnote f. Conditions: 70K, 1mW microwave power, 0.63 mtesla modulation amplitude, frequency 8.99 GHz, gain is the same for both samples.
Figure 6-6. EPR spectrum of succinate-reduced *E. coli* fumarate reductase complex. Enzyme concentration 47 \( \mu \text{M} \), buffer mixture as in Table 6-2, footnote f, except no dithionite. Conditions: 15K, 1 mW microwave power, 0.63 mtesla modulation amplitude, frequency 8.99 GHz.
Figure 6-7. Power saturation behavior of the g=1.93 EPR signal of E. coli fumarate reductase complex. (Δ) succinate-reduced, (X) dithionite-reduced. Enzyme concentration 47 μM, buffer mixture as in Table 6-2, footnote f. Signal amplitudes correspond to peak to trough of the g=1.93 feature. Conditions: 16K, 0.63 mtesla modulation amplitude, frequency 8.99 GHz.
Figure 6-8. Comparison of EPR spectra for dithionite-reduced samples of (a) E. coli soluble fumarate reductase, (b) E. coli fumarate reductase complex, and (c) beef heart Complex II. Buffer mixture and condition of dithionite reduction as in Table 6-2, footnote f, except for absence of ethylene glycol in all three samples and absence of Triton X-100 from soluble fumarate reductase. Conditions: 10K, 5 mW microwave power, 0.63 mtesla modulation amplitude, frequency 8.99 GHz. Multiplication factors indicate the relative gains for the two spectra for each individual sample.
Figure 6-9. Room temperature CD spectra for *E. coli* fumarate reductase complex: (a) as isolated enzyme; (b) succinate-reduced enzyme; (c) dithionite-reduced enzyme. Enzyme samples identical to those used in MCD and EPR studies.
Figure 6-10. MCD and room temperature absorption spectra for *E. coli* fumarate reductase, as prepared. Upper spectrum: room temperature absorption spectrum. Middle spectra: MCD spectra at 1.65, 4.22, and 7.9K (intensity of all transitions increasing with decreasing temperature) at magnetic field of 4.5 tesla, pathlength 0.171cm. Lower spectrum: MCD spectrum at 1.65K and 4.5 tesla at reduced sensitivity. Enzyme concentration 47 μM; buffer mixture as in Table 6-2, footnote b.
Figure 6-11. MCD spectra of the cytochrome and high potential iron-sulfur center in E. coli fumarate reductase complex, as prepared, and the oxidized [3Fe-4S] center in T. thermophilus Fd. Upper panel: MCD at 1.65K and 4.5 tesla of cytochrome in fumarate reductase complex after subtraction of transitions due to the high potential iron-sulfur center. Middle panel: MCD spectra at 1.65K and 4.5 tesla of fumarate reductase complex after subtraction of transitions due to cytochrome. Lower panel: MCD spectra 1.5K and 4.5 tesla of T. thermophilus Fd., as isolated.
Figure 6-12. MCD magnetization plot for *E. coli* fumarate reductase complex, as prepared. Wavelength=465 nm; 1.74K (X), 4.22K (Δ), magnetic fields between 0 and 4.5 tesla. Solid line represents theoretical magnetization curve for $g_\parallel$=2.015, $g_\perp$=1.955, $m_z$/$m_+ = -1$. 
Figure 6-13. MCD and room temperature absorption spectra for dithionite-reduced, *E. coli* fumarate reductase complex. Enzyme concentration 47 μM, buffer mixture as in Table 6-2. Upper spectrum: room temperature absorption spectrum. Lower spectra: MCD spectra at 1.53, 4.22, and 12.4 K (intensity of all transitions increases with decreasing temperature) at a magnetic field of 4.5 tesla, pathlength 0.175 cm. Arrows indicate sharp derivative due to low spin Fe(II) cytochrome impurity.
Figure 6-14. MCD magnetization plots for dithionite-reduced, *E. coli* fumarate reductase complex. Wavelengths: 715 nm (+), 680 nm (X), 320 nm (O). Temperature 1.53K, magnetic fields between 0 and 4.5 tesla. Solid line is theoretical magnetization curve for $g_\parallel=8.0$ and $g_\perp=0.0$. Broken line is theoretical magnetization curve for $g_\parallel=2.026$, $g_\perp=1.927$, $m_2/m_4=-1$. 
Figure 6-15. MCD and room temperature absorption spectrum of succinate-reduced, E. coli fumarate reductase complex. Enzyme concentration 47 μM, buffer mixture as in Table 6-2, footnote f, except no dithionite. Upper spectrum: room temperature absorption spectrum. Lower spectra: MCD spectra at 1.57, 4.22, and 8.5K (intensity of all transitions increases with decreasing temperature) at magnetic field 4.5 tesla, pathlength 0.1681 cm.
Figure 6-16. MCD magnetization curves for succinate-reduced, E. coli fumarate reductase complex. Wavelengths: 715 nm (O), 680 nm (A), and 320 nm(+). Temperature 1.55K, magnetic field 0 to 4.5 tesla. Solid line is theoretical curve for \( g_{||} = 8.0 \), \( g_{\perp} = 0.0 \). Broken line is theoretical curve for \( g_{||} = 2.026 \), \( g_{\perp} = 1.927 \), \( m_2/m_+ = -1 \).
Figure 6-17. MCD and room temperature absorption spectra for dithionite-reduced, soluble fumarate reductase from E. coli. Enzyme concentration 57 μM, buffer mixture as in table 6-2, footnote g. Upper spectrum: room temperature absorption. Lower spectra: MCD spectra at 1.53, 4.22, and 7.7K (intensity of all transitions increases with decreasing temperature) at a magnetic field of 4.5 tesla, pathlength 0.168 cm.
Figure 6-18. MCD magnetization curves for dithionite-reduced, soluble fumarate reductase from E. coli. Wavelengths: 715 nm (+), 680 nm (X), 550 nm (O), 406 nm (●), and 320 nm (○). Temperature 1.53K, magnetic fields between 0 and 4.5 tesla. Solid line is theoretical magnetization curve for $g_\parallel=8.0$ and $g_\perp=0.0$. Broken line is theoretical magnetization curve for $g_\parallel=2.026$, $g_\perp=1.927$, $m_\perp/m_\parallel=-1$. 
Figure 6-19. Comparison of low temperature MCD spectra of dithionite-reduced soluble fumarate reductase (upper spectrum) and iron-treated, dithionite-reduced soluble fumarate reductase (lower spectrum). Conditions: temperature 4.22K, magnetic field 4.5 tesla.
Figure 6-20. EPR spectra of whole cells of *E. coli* grown anaerobically on a glycerol-fumarate medium. Cells harvested anaerobically and suspended in 50 mM potassium phosphate buffer, pH 7.8. Conditions: 70K (upper panel), 12K (lower panel), 1 mW microwave power, 0.63 mT modulation amplitude, frequency 8.990 GHz.
Figure 6-21. Comparison of EPR spectra of fumarate-oxidized whole cells of _E. coli_ and fumarate reductase complex, as prepared. Whole cells prepared as in Figure 6-20, except that 50 mM fumarate was added to the buffer solution. EPR spectra of fumarate-oxidized whole cells at 70K (a) and 8.5K (b) and fumarate reductase complex, as prepared at 13K (c). Conditions: 1 mW microwave power, 0.63 mtesla modulation amplitude, frequency 8.99 GHz.
Figure 6-22. Comparison of cysteine clusters of the iron-sulfur protein subunit of SDH with the corresponding subunit of fumarate reductase (FRD) and some bacterial ferredoxins. The sequences are in one-letter code and arranged to align the cysteine residues of potential iron-sulfur cluster binding sites. The numbers in parentheses refer to residues not shown, and those at the end denote the amino acid positions of the adjacent residues. From reference 25.
Figure 6-23. Comparison of 70K EPR spectra of as-isolated E. coli cells plasmid-amplified for fumarate reductase subunits. Buffer and conditions as in Figure 6-20. E. coli amplified for subunits: (a) ABCD; (b) AB; (c) A and first part of B. Spectrum (d) corresponds to chromosomal fumarate reductase in E. coli. Multiplication factors indicate the relative gains for each spectrum.
Figure 6-24. Comparison of 15K EPR spectra of fumarate-treated E. coli cells plasmid-amplified for fumarate reductase subunits. Buffer and conditions as in Figure 6-21. E. coli amplified for subunits: (a) ABCD, (b) AB, and (c) A plus part of B. Spectrum (d) corresponds to chromosomal fumarate reductase in E. coli. Multiplication factors indicate the relative gains for each spectrum.
Figure 6–25. Comparison of 8.5K EPR spectra of dithionite-treated E. coli cells plasmid-amplified for fumarate reductase subunits. E. coli amplified for subunits (a) AB and (b) ABCD. Buffer is as in Figure 6–20 except for presence of 40mM glycerol and 20mM dithionite. Conditions: 8.5K, 5mW microwave power, 10mT modulation amplitude, frequency 8.975. Multiplication factors indicate relative gains for each spectrum.
VII. ELECTRON TRANSFER FLAVOPROTEIN DEHYDROGENASE

BACKGROUND

Most of the EPR resonances observed in mammalian mitochondria have been assigned to specific enzyme complexes. One of these resonances, a rapidly relaxing ferredoxin-type signal at \( g = 1.886, 1.939, \) and 2.086, was originally ascribed to NADH-UQ oxidoreductase (1). Subsequently, this signal was found to be associated with an iron-sulfur protein of molecular weight 70 KDa containing approximately 4 non-heme iron and 4 acid-labile sulfide atoms and one FAD (2, 3). The amino acid composition of this protein shows the enzyme to be rich in hydrophobic residues, as expected for a membrane-bound protein, with a cysteine content high enough to provide adequate thiol ligands for a [4Fe-4S] core (3, 4).

The redox centers of this iron-sulfur protein equilibrate with NADH-UQ and succinate-UQ oxidoreductase systems (1, 5). But neither the flavin nor the iron-sulfur center are reduced by NADH, NADPH, succinate, glycerol-3-phosphate, or dihydrorotate. The iron-sulfur center is rapidly reduced by the reduced form of electron transfer flavoprotein (ETF) (6-8) from the fatty acylCoA dehydrogenation system (4, 9-11). ETF is involved in \( \beta \)-oxidation of fatty acids, mediating electron transfer between acylCoA dehydrogenase and ETF dehydrogenase (9-11). ETF dehydrogenase is proposed to function as a UQ reductase, and thus is another entry port into the mitochondrial electron transport chain.

Equilibrium spectrophotometric titrations show that ETF dehydrogenase accepts a maximum of two electron from reduced ETF (4).
In contrast, three electrons are accepted from dithionite, or by photochemical reduction with EDTA and deazaflavin as mediators. All electrons can be reversibly transferred to the water-soluble UQ analogue 2, 3-dimethoxy-5-methyl-6-penty1-1, 4-benzoquinone (PB). Reduction of ETF dehydrogenase by PB-hydroquinone is pH dependent (4). The enzyme has a redox potential which decreases by 47mV per pH unit, with an $E_0$ of +38mV at pH 7.3. Therefore, ETF dehydrogenase is thought to bind one or two protons upon reduction.

Very little is known about ETF dehydrogenase. The iron-sulfur center in ETF dehydrogenase is believed to be a [4Fe-4S] center based on its EPR characteristics and iron/sulfide analysis. A preliminary resonance Raman study of ETF dehydrogenase was consistent with the presence of a [4Fe-4S] center based on the position of a major band centered about 331cm$^{-1}$. However, the quality of the data was poor. In addition, the positions of bands attributed to the flavin suggests that there is no direct complexation between the iron-sulfur center and the flavin. Extensive hydrogen bonding between the flavin and the protein backbone was indicated.

In this work the low temperature MCD spectra of mammalian ETF dehydrogenase were obtained and compared with those of typical protein-bound [4Fe-4S] centers. In addition, EPR experiments are used to investigate the differences in oxidation state of the two redox centers present in enzymatically reduced versus dithionite-reduced ETF dehydrogenase.
RESULTS AND DISCUSSION

EPR data for dithionite-reduced ETF dehydrogenase

At temperatures near 10K, dithionite-reduced ETF dehydrogenase exhibits a rhombic EPR signal with $g = 2.084, 1.939, 1.883$, and a spin intensity of $1.05$ spins per enzyme molecule (Figure 7-1). The signal was not observed at temperatures above 30K and begins to undergo microwave power saturation at 10K and 10mW. The spin relaxation properties of the rhombic EPR signal are typical of those observed for reduced protein-bound [4Fe-4S] clusters. Except for the resonance at $g = 2.001$, attributed to the flavin semiquinone, no additional EPR signals were observed.

MCD data for dithionite-reduced ETF dehydrogenase

Figure 7-2 shows the room temperature absorption and low temperature MCD spectra at 1.68, 4.22, and 8.0K for dithionite-reduced ETF dehydrogenase. The temperature dependence of the MCD bands indicate the presence of paramagnetic iron sulfur clusters. The relatively low $\Delta \varepsilon$ value and the concentration of the sample (55$\mu$M) resulted in very weak signal. Since the signal to noise ratio was high, MCD magnetization data were not collected. To increase the signal-to-noise ratio for the spectra, each spectrum is displayed as the average of three scans.

Low temperature MCD spectra have been recorded for a number of proteins which contain well characterized tetranuclear clusters. Examples include [4Fe-4S] clusters in the ferredoxins from Clostridium pasteurianum (14) and Desulfovibrio africanus (15), and the nitrogenase iron protein from Azotobacter vinelandii (16) and C. pasteurianum (17). All reduced tetranuclear iron sulfur clusters
Investigated thus far exhibit broad positive bands between 350 and 500 nm, and negative features around 625 and 320 nm. Under the conditions in Figure 7-2, the MCD intensities of the most intense transitions range between 50 to 75 M$^{-1}$cm$^{-1}$ for a reduced [4Fe-4S] cluster. A comparison of dithionite-reduced ETF dehydrogenase with the reduced eight-iron ferredoxin from Clostridium pasteurianum and the reduced [4Fe-4S] cluster in nitrogenase from C. pasteurianum is illustrated in Figure 7-3. The low temperature MCD spectrum of ETF dehydrogenase exhibits the pattern of bands associated with a reduced [4Fe-4S] cluster. In addition, the intensity of the 4K MCD spectrum indicates the presence of one reduced [4Fe-4S] center in the enzyme.

**EPR data for enzymatically reduced ETF dehydrogenase**

The EPR spectrum of the enzymatically-reduced ETF dehydrogenase at 10.5K showed a substantial amount of FAD semiquinone radical, g=2.001, in addition to the rhombic EPR resonance corresponding to the reduced [4Fe-4S] (Figure 7-4). Dithionite reduction of this sample resulted in a decrease in the size of the flavin semiquinone signal. At 70K, only the EPR resonance corresponding to the FAD semiquinone is observed in reduced ETF dehydrogenase (Figure 7-5). A comparison of EPR signal intensities shows that, in enzymatically reduced ETF dehydrogenase, the amount of semiquinone is approximately five times greater than that present in the dithionite reduced enzyme. The radical signal remains microwave power saturated at 1 mW up to temperatures of 150K. Therefore, spin quantitation of the flavin semiquinone must be performed at room temperature using an aqueous
solution EPR cell. Unfortunately, the repeated freeze-thaw cycles caused the enzyme sample to degrade and precipitate. Therefore, spin quantitation of the flavin radical signal was not possible. Further experiments required more enzyme than was available. However, the significant reduction of the FAD semiquinone EPR resonance in the dithionite-reduced enzyme demonstrates that dithionite serves to fully reduce FAD in ETF dehydrogenase to the hydroquinone form.

SUMMARY AND CONCLUSIONS

The MCD and EPR data for ETF dehydrogenase were consistent with the presence of one [4Fe-4S] center per enzyme molecule, in agreement with previous work. The intensity and form of the spectra were similar to those obtained for bacterial ferredoxins known to contain [4Fe-4S] centers.

EPR studies show that in enzymatic reduction of ETF dehydrogenase, one electron is accepted by FAD and one electron is accepted by the iron-sulfur center (4). In the presence of dithionite, the flavin accepts a third electron to yield the hydroquinone form. It is unknown as to why the two electrons from the acyl CoA system are split at ETF, The two electron reduced form of ETF dehydrogenase is capable of transferring both equivalents to a non-physiological quinone acceptor (4). Interestingly, the currently accepted model for electron transfer through Complex III, the Q-cycle, involves a one electron reduction by a dehydrogenase (18, 19) (Figure 7-6). Therefore, conversion of the n=2 redox processes of the acyl CoA pathway into two n=1 redox steps at ETF dehydrogenase is necessary. There is tentative evidence that in succinate-
dehydrogenase, FAD functions to split the electron pair of the Kreb's cycle. The mechanism by which this occurs is unknown.

There are other unanswered questions concerning the mechanism of electron transfer in ETF dehydrogenase. For example, the enzyme is known to exhibit a pH-dependent redox midpoint potential, consistent with the binding of one to two protons upon reduction. It is unknown as to whether the proton binding site is associated with the iron-sulfur cluster, the flavin, or the protein backbone. Recent studies with inorganic model compounds suggest that iron-sulfur clusters may be directly involved in proton binding (20). Experiments show that synthetic Fe$_4$S$_4$(SR)$_4$ clusters are capable of transporting electrons and protons in a directional electron transport system by a mechanism of reduction-linked proton binding. Since the low temperature MCD spectrum is very sensitive to changes in the electronic structure of the chromophore, MCD experiments with ETF dehydrogenase at variable pHs may be able to determine whether the [4Fe-4S] center is the site of proton binding.

REFERENCES


Biochemistry 21, 6963-6942.


16 Johnson, M.K., Onate, Y., and Hales, B.J., unpublished work.


Figure 7-1. EPR spectrum of dithionite-reduced ETF dehydrogenase. Protein concentration is 55 μM. Buffer is 10 mM Tris-HCl, 10 mM potassium phosphate, 8 mM CHAPS, pH 7.8, and 40% glycerol. Conditions: 13K, 1 mW microwave power, 0.63 mtesla modulation amplitude, frequency 8.99 GHz.
Figure 7-2. MCD and absorption spectra of dithionite-reduced ETF dehydrogenase. Enzyme concentration and buffer as in Figure 7-1. Upper spectrum: room temperature absorption spectrum. Lower spectrum: MCD spectrum at 1.68, 4.22, and 8.0K (intensity of all transitions increases with decreasing temperature) at magnetic field of 4.5 tesla, pathlength 0.1605 cm.
Figure 7-3. Comparison of 4K MCD spectra for (a) dithionite-reduced ETF dehydrogenase, (b) dithionite-reduced eight-iron Fd from *C. pasteurianum* (Bennett, D.E., and Johnson, M.K., unpublished work), (c) dithionite-reduced iron protein from *C. pasteurianum* nitrogenase (Ornate, Y., and Johnson, M.K., unpublished work). (Δε is per [4Fe-4S] cluster.)
Figure 7-4. Comparison of low temperature EPR spectra of ETF dehydrogenase. Enzyme concentration 38.6 μM. Buffer as described on page 56. (a) EPR spectrum of enzymatically reduced ETF dehydrogenase at 10.5K. (b) EPR spectrum of dithionite-reduced ETF dehydrogenase at 9.5K. Conditions: 1 mW microwave power, 0.63 mtesla modulation amplitude, frequency 8.98 GHz.
Figure 7-5. Comparison of 70K EPR spectra of ETF dehydrogenase: (a) enzymatically reduced; (b) dithionite-reduced. Enzyme as used in Figure 7-3. Conditions: 70K, 1 mW microwave power, 0.63 mtesla modulation amplitude, frequency 8.98 GHz. A multiplication factor indicates the relative gains.
Figure 7-6. Protonmotive Q cycle scheme for electron transfer through Complex III showing the one electron reduction of the enzyme complex by a dehydrogenase (deH). (From reference 15.)
VIII. RIESKE PROTEIN

BACKGROUND

The ubiquinol-cytochrome c oxidoreductase of mitochondria (1, 2), many bacteria (3, 4), and the plastoquinol-cytochrome f oxidoreductase of chloroplasts (5-8), contain a distinctive iron-sulfur center known as the Rieske center. Apparently functioning as an oxidant for a quinol and as a reductant for cytochrome c, the Rieske center plays an important role in electron transport in the mammalian respiratory chain.

**Complex III**

In mitochondria, the Rieske center is associated with a unique complex of cytochrome b and c1. This bc1 complex is called Complex III and functions in respiration (9). Complex III catalyzes electron transport coupled to the synthesis of ATP. Several models for this process have been proposed. The currently accepted model is a cyclic scheme known as the double Q cycle (10).

Complex III from a number of organisms has been isolated, characterized, and resolved into 8 to 10 polypeptides. The composition of the enzyme complex is very similar in all organisms thus far examined (9). In addition to the iron-sulfur center, Complex III contains two types of cytochrome b and one cytochrome c1. Although the spatial organization of the components has been determined (10-14), the functions of the individual peptides are still unclear. The sequence of electron flow through the redox components has been established (15).

Based on analysis of total iron and heme content of isolated
Rieske center from yeast (16) and beef heart (17) it is likely that the 24 Kda iron-sulfur protein contains one [2Fe-2S] core. Results of an ENDOR study with $^{57}$Fe-enriched yeast demonstrate that the Rieske center is a binuclear core (18). However, the EPR-determined g values ($g=2.00, 1.90, 1.84$) and redox potential of the Rieske center are not characteristic of a [2Fe-2S] center (2). The midpoint potential varies depending on the organism and type of preparation, but it is 400 to 600mV higher than is typically observed for ferredoxins.

**Primary structure of Rieske protein and other [2Fe-2S] proteins**

There are now thought to be at least three distinct classes of [2Fe-2S] centers typified by spinach ferredoxin, adrenodoxin, and Rieske center. This classification is based on amino acid sequence analysis, as well as spectroscopic and magnetic properties. The amino acid sequences of spinach Fd, beef adrenodoxin (19, 20), and *Neurospora crassa* Rieske protein (21) have been determined. The arrangement of the ligating cysteines for spinach Fd and beef adrenodoxin is similar while yeast Rieske protein exhibits a distinctly different arrangement of cysteine residues (Figure 8-1). In the cluster binding cavities of spinach Fd and beef adrenodoxin, there are three cysteine residues close together, separated by two to four amino acids. The fourth cysteine residue is located about thirty residues away. In contrast, the four cysteines of yeast Rieske protein are arranged as two pairs. One pair is separated by four amino acids, and the other pair by one proline residue.

**Spectroscopic and magnetic properties of Rieske protein and other [2Fe-2S] proteins**

The similarities of many of the physical properties of spinach Fd
and beef adrenodoxin are striking, especially when compared to those of Rieske protein. Reference 22 provides a comparison of many of the spectroscopic and magnetic properties of spinach Fd and adrenodoxin, concluding that variations between the two proteins reflect only slight differences in the clusters. Most of what is currently known about the physical properties of Rieske protein can be found in reference 23.

Neither the optical nor CD spectrum of spinach Fd, in either redox state, differs significantly from the corresponding beef adrenodoxin spectrum (24). In Rieske protein however, the distinct maximum in the optical absorption spectrum is red-shifted some 40nm relative to that of the other [2Fe-2S] proteins (23). In addition, the molar extinction coefficient (per cluster) of Rieske protein is approximately two-thirds of that of spinach Fd and beef adrenodoxin. The CD spectra of oxidized and reduced Rieske protein are also quite different from those obtained for other [2Fe-2S] proteins (23).

Oxidized [2Fe-2S] proteins typically exhibit six Raman active bands in the 280 to 430cm\(^{-1}\) region, with three prominent bands at about 285, 330, and 395cm\(^{-1}\). Oxidized spinach Fd and adrenodoxin exhibit similar resonance Raman spectra, although differences in terminal iron-sulfur frequencies of about 5cm\(^{-1}\) suggest conformational differences in the ligated cysteines (25). The bridging iron-sulfur frequencies are similar in the two proteins. Reduction of adrenodoxin shifts the bridging frequencies 16 to 24cm\(^{-1}\) lower in frequency consistent with an expected weakening of the bridging bond. There has been no resonance Raman work reported for Rieske protein.

The \(^{57}\text{Fe} \) Mossbauer properties of oxidized beef adrenodoxin (26)
are similar to those of oxidized spinach Fd (27), but somewhat different from those of Rieske protein (23). In particular, the isomer shift for the ferrous site is distinctly more positive in reduced Rieske protein compared to that in other reduced [2Fe-2S] proteins, supporting the idea that non-cysteine ligands are coordinated to the ferrous site in Rieske protein. Differences in the electron spin relaxation time for the reduced clusters are also observed. In Rieske protein, as in spinach Fd, the electron spin relaxation time is sufficiently rapid for resolution of the Mossbauer spectrum into quadrupole doublets (23). The relaxation time of reduced spinach Fd is faster than that of reduced beef adrenodoxin, presumably due to the weaker exchange interaction. The strength of the exchange interaction is about 50% stronger in adrenodoxin (28) ($J_{ox} \geq 350 \text{ cm}^{-1}$, $J_{red} \leq 250 \text{ cm}^{-1}$) than in spinach Fd (29).

Perhaps the most conspicuous difference between the three types of iron-sulfur proteins is the anisotropy of the g tensor and the principal g values for the reduced cluster. In most reduced [2Fe-2S] proteins, $g_{av}$ is about 1.94. Reduced spinach Fd has a rhombic EPR spectrum with $g=2.05, 1.95, 1.89$ (30), while that for adrenodoxin is almost isotropic and can be simulated with g values of 2.021, 1.935, 1.93 (31). The EPR spectrum of reduced Rieske protein is quite different with $g_{av}$ of about 1.91. The principal g values for Rieske protein are 2.00, 1.90, 1.84 (32).

The g values observed for reduced [2Fe-2S] clusters such as spinach Fd and adrenodoxin have been rationalized by a model involving spin coupled ferrous and ferric ions bridged by sulfide ions, with terminal ligands provided by cysteine residues (33). Mossbauer work
later confirmed this structure. In this model, deviations from \( g=2 \) of the high field resonance frequencies, \( g_x \) and \( g_y \), are primarily attributed to the ferrous site. A ligand field model, developed by Bertrand and Gayda (34), describes rhombic distortion at the ferrous site and can account for \( g \) factor variations for \([2\text{Fe-2S}]\) clusters having a \( g_{av} \) of about 1.94. In an extension of this model to \([2\text{Fe-2S}]\) clusters with \( g_{av} \) of about 1.91, it was shown that the ligand field energies of the ferrous site should be similar within this class of iron-sulfur clusters (35). Differences between the splitting of energy levels for \([2\text{Fe-2S}]\) clusters with \( g_{av}=1.91 \) and those with \( g_{av}=1.94 \) were attributed to one of possible two effects. One possibility is that in iron-sulfur proteins with \( g_{av}=1.91 \), there is a greater inequivalence between the bridging and terminal ligands compared to proteins like spinach Fd. This explanation implies coordination of the \([2\text{Fe-2S}]\) cluster by non-cysteine ligands, e.g., histidine or tyrosine. An alternate rationalization for differences in \( g_{av} \) invokes strong compression of the ligands at the ferrous site. Such compression could be related to the unusual sequence of cysteine residues in Rieske protein. More detailed structural information about the different types of \([2\text{Fe-2S}]\) centers is necessary to distinguish between these two explanations.

**Objective**

Clearly, there are distinct differences between the binuclear cluster in Rieske protein and the \([2\text{Fe-2S}]\) clusters of other ferredoxins. Based on Mossbauer (23) and ENDOR (36) work, it is believed that the \([2\text{Fe-2S}]\) center in *Thermus thermophilus* Rieske protein is coordinated by two cysteine and two non-cysteine ligands.
Low temperature MCD is very sensitive to structural variations of clusters in iron-sulfur proteins and thus is a very powerful tool for addressing the question of coordination of iron-sulfur clusters by non-cysteine ligands. In this work, low temperature MCD spectra and magnetic properties of reduced *T. thermophilus* Rieske protein have been obtained and compared with those of spinach Fd in an attempt to further elucidate differences between protein-bound [2Fe-2S] clusters and to understand the observed variation in redox potentials. Although redox potentials in iron-sulfur proteins are determined by complex factors, the nature of the ligands bound to iron atoms is expected to be a major factor. MCD measurements on oxidized Rieske protein were obtained over a temperature range of 4.22 to 150K in order to determine a lower limit for the electron coupling energy, J.

*Rieske protein from Thermus thermophilus*

Spectral similarities between the membrane-associated electron transport components of *T. thermophilus* and of mitochondria have been noted (37). Work with *T. thermophilus* membranes has shown the presence of a menaquinone-specific segment of the respiratory chain capable of transferring electrons from NADH to partially purified Rieske protein (38). The Rieske protein from *T. thermophilus* has the advantage that it is not nearly as unstable as that isolated from beef heart. Although decidedly different from other [2Fe-2S] ferredoxins, the absorption, CD, and EPR spectra from *T. thermophilus* Rieske protein are indistinguishable from those of the mitochondrial Rieske protein (23).

Chemical analyses of the *T. thermophilus* Rieske protein show that the protein contains four acid-labile sulfides and four non-heme irons
The amino acid composition of the protein indicates the presence of four cysteine residues. Iron analysis and Mossbauer experiments suggest that the *T. thermophilus* Rieske protein contains two [2Fe-2S] cores. Interestingly, the Mossbauer spectrum of Rieske protein is very similar to that of phthalate dioxygenase from *Pseudomonas putrida*. ENDOR and electron spin echo (ESE) experiments show that the binuclear cores associated with *T. thermophilus* Rieske protein and phthalate dioxygenase from *Pseudomonas cepacia* are very similar in structure (36). The pronounced similarity between spectral properties of Rieske center and several NADH-dependent mono- and dioxygenases has been commented on (23, 39-42).

RESULTS AND DISCUSSION

**CD data for spinach Fd and Rieske protein**

Figure 8-2 shows the room temperature CD spectra for oxidized and reduced Rieske protein. As previously observed (23), the oxidized protein has a weak, negative feature centered about 540nm, a negative band at 375nm, and positive bands between 400-500nm and between 250-350nm. The reduced protein has negative bands at 500 and 384nm, and positive bands centered at 300nm and between 400-480nm.

The form of the CD spectra of *T. thermophilus* Rieske protein is quite different from those typically observed for [2Fe-2S] ferredoxins. Figures 8-3a and 8-3b give comparisons of spinach Fd and Rieske protein in reduced and oxidized forms, respectively. The characteristic band at 420nm is absent in oxidized Rieske protein. These differences suggest that the environment of the [2Fe-2S] core in Rieske protein is quite different from that in other two-iron
ferredoxins. As is generally the case for [2Fe-2S] proteins, the CD spectra of *T. thermophilus* Rieske protein is intense relative to CD spectra of other types of iron-sulfur clusters.

**MCD and EPR data for dithionite-reduced spinach ferredoxin**

Figure 8-4 shows the 70K EPR spectrum for dithionite-reduced spinach ferredoxin. The rhombic EPR signal has g values of 2.05, 1.96, and 1.89. The spin quantitation was 1.1 spins per protein molecule, based on a UV-visible determination of the concentration of oxidized spinach Fd ($C_{420}=9400\text{M}^{-1}\text{cm}^{-1}$).

Figure 8-5 shows the room temperature absorption and low temperature MCD spectra at 1.61, 4.22, and 7.1K for reduced spinach ferredoxin. The form of the spectrum is the same as previously reported (24). Magnetization data recorded at 325nm were well fit by a theoretical curve for an $S=1/2$ ground state with the EPR-determined g values (not shown), indicating that EPR spectrum and all MCD transitions originate from the same $S=1/2$ ground state.

**EPR and MCD data for dithionite-reduced Rieske protein**

The 70K EPR spectrum of dithionite-reduced Rieske protein is shown in Figure 8-6. The rhombic EPR signal at $g=2.00$, 1.90, 1.84 has a spin concentration of 2.3 spins per molecule, indicating two clusters per enzyme molecule, in agreement with previous work (23).

Figure 8-7 shows the room temperature absorption and low temperature MCD spectra at 1.58, 4.22, and 8.0K for dithionite-reduced Rieske protein. The low temperature MCD spectrum is rich in detail compared to the absorption or room temperature MCD (23) spectra for Rieske center. All temperature-dependent transitions are ascribed to paramagnetic iron-sulfur centers.
The form of the *T. thermophilus* Rieske center MCD spectrum is very different from the low temperature MCD spectra of other [2Fe-2S] proteins (43). Differences are illustrated by a comparison of the 4K MCD spectra of reduced Rieske center and reduced spinach Fd (Figure 8–8).

Paramagnetic [2Fe-2S] centers characteristically exhibit positive MCD bands centered around 700 and 540nm and an intense negative band about 320nm (43). Dithionite-reduced *T. thermophilus* Rieske protein exhibits intense, positive MCD bands centered about 510 and 380nm, two intense negative bands around 340nm, and positive bands at 600, 550, and 510nm. The intensity of the 4K MCD spectrum of *T. thermophilus* Rieske protein is similar to that observed for other [2Fe-2S] cores. In agreement with Mossbauer and EPR data (23), the low temperature MCD spectrum of reduced Rieske protein demonstrates that the iron-sulfur core in this protein is very different from other binuclear centers. Magnetization data, collected between 358 and 292nm, were fit to a theoretical curve for an S=1/2 ground state with the EPR-determined g values for Rieske center (Figure 8–9), demonstrating that the EPR and MCD transitions originate from the same S=1/2 ground state.

**MCD data for oxidized Rieske protein**

The low temperature MCD spectrum of oxidized *T. thermophilus* Rieske center (not shown) has no distinctive features except for a sharp, biphasic band centered about 280nm, indicative of tryptophan (44). The MCD spectrum was temperature independent since there were no significant differences in intensity were noted in the 4.22, 80, or 150K MCD spectra for the oxidized protein. In addition, the spectra were not noticeably different from that reported for the room
temperature MCD of oxidized *T. thermophilus* Rieske center (23), indicating that the separation between the $S=1$ and $S=0$ states is at least several hundred wavenumbers.

**SUMMARY AND CONCLUSIONS**

The results presented here support the idea that the Rieske center is a separate class of [2Fe-2S] cluster. The form of the low temperature MCD spectrum exhibited by reduced *T. thermophilus* Rieske protein is very different from that of reduced spinach Fd. A pronounced similarity of spectral properties of *T. thermophilus* Rieske protein to those of the phthalate dioxygenases from *Pseudomonas* has been previously noted (23, 36). The spectral similarities are believed to indicate similar cluster structure, in particular, coordination by nitrogenous ligands, e.g. histidine. Comparison of the low temperature MCD spectrum and magnetic properties of Rieske protein with those of *Pseudomonas* dioxygenases would be useful in identifying differences in protein-bound [2Fe-2S] clusters and the factors controlling redox potential. Unfortunately, the phthalate dioxygenases were not available for study.

More discriminating use of MCD to analyze differences in [2Fe-2S] clusters requires assignment of the spectra. The MCD charge transfer region of typical oxidized and reduced [2Fe-2S] proteins, e.g. spinach Fd, has been related to the analogous spectrum of rubredoxin (24), which can be assigned with some certainty (45). In this model, the iron atoms were treated as distinct FeS$_4$ chromophores, and thus the MCD spectrum of a [2Fe-2S] cluster was treated as a composite of two rubredoxins. Unambiguous assignments were not possible because of
many parameters of unknown magnitude and the overlapping transitions, however, energy level diagrams for the electronic transitions were developed. A similar approach could be applied to the analysis of the Rieske protein MCD spectrum, using appropriate model compounds.

Analysis of the low temperature MCD spectrum of Rieske protein should result in a better understanding of the physical properties of this iron-sulfur center. For example, coordination by nitrogenous ligands may in part explain the higher redox potential of Rieske protein compared to other [2Fe-2S] ferredoxins. It has been suggested that substitution of the neutral N-ligand for RS⁻ would give a lower formal charge on the cluster, resulting in an increased electrostatic stabilization of the reduced form (36). Differences in the nature of the ligand coordinating the iron-sulfur center would be expected to have a drastic effect on the electronic spectrum of the cluster. Comparative MCD studies of [2Fe-2S] model compounds in which thiolate ligands have been substituted for oxygen- or nitrogen-containing functional groups are necessary for assignment of the Rieske MCD transitions. At this time, there are no reported preparations of appropriate model compounds.

Another question to be addressed concerns the similarity of Rieske centers from different sources. Rieske protein from T. thermophilus is different from that of yeast and beef heart in that the protein contains two binuclear iron-sulfur clusters rather than just one. Based on the similarity of other spectral properties, it is reasonable to assume that low temperature MCD spectra of yeast and bovine Rieske protein would be similar to that from T. thermophilus. However, this assumption should be verified.
There are many unanswered questions concerning Rieske protein, including how it functions in electron transport. The present work demonstrates the potential of low temperature MCD in understanding the origin of the unique electronic and redox properties of the Rieske center.

REFERENCES


Figure 8-1. Schematic representation of the [2Fe-2S] binding cavity in (a) spinach Fd, (b) beef adrenodoxin, and (c) N. crassa Rieske protein. Numbers refer to the position of cysteine residues in the polypeptide chain.
Figure 8-2. Room temperature CD spectra of *T. thermophilus* Rieske protein. Protein as used in MCD and EPR studies. Upper spectrum: as-isolated Rieske protein. Lower spectrum: dithionite-reduced Rieske protein.
Figure 8-3. Comparison of room temperature CD spectra of *T. thermophilus* Rieske protein (----) and spinach Fd (-----). Upper spectrum: reduced protein; lower spectrum: oxidized protein. Δε scale in terms of concentration of [2Fe-2S] cluster.
Figure 8-4. EPR spectrum of dithionite-reduced spinach Fd. Protein concentration 232μM, buffer is 50mM potassium phosphate, pH 7.8 containing 50% ethylene glycol. Conditions: 70K, 1 mW microwave power, 0.63 mTesla modulation amplitude, frequency 8.983 GHz.
Figure 8-5. MCD and room temperature absorption spectra of dithionite-reduced spinach ferredoxin. Enzyme concentration and buffer as in Figure 8-1. Upper spectrum: room temperature absorption spectrum. Lower spectra: MCD spectra at 1.61, 4.22, and 7.1K (intensity of all transitions increases with decreasing temperature) at a magnetic field of 4.5 tesla, pathlength 0.1664 cm.
Figure 8-6. EPR spectrum of dithionite-reduced *T. thermophilus* Rieske protein. Protein concentration 218µM, buffer is 75mM Tris-HCl, 0.1mM EDTA, pH 7.8 containing 50% ethylene glycol. Conditions: 70K, 1 mW microwave power, 0.63 mtesla modulation amplitude, frequency 9.018 GHz.
Figure 8-7. MCD and room temperature absorption spectra of dithionite-reduced T. thermophilus Rieske center. Protein concentration 109\( \mu \)M, concentration of [2Fe-2S] cluster 218\( \mu \)M, buffer as in Figure 8-6. \( \epsilon \) and \( \Delta \epsilon \) scales are in terms of concentration of [2Fe-2S] cluster. Upper spectrum: room temperature absorption spectrum. Lower spectrum: MCD spectra at 1.58, 4.22, and 8.0K at magnetic field of 4.5 tesla, pathlength 0.1542 cm.
Figure 8-8. Comparison of low temperature MCD spectra for dithionite-reduced *T. thermophilus* Rieske center and dithionite-reduced spinach Fd. Conditions: 4.22K, magnetic field 4.5 tesla. Upper spectrum: dithionite-reduced Rieske protein. Lower spectrum: dithionite-reduced spinach Fd.
Figure 8-9. MCD magnetization curves for dithionite-reduced Rieske center. Data collected in the peak to trough region between 358 and 232 nm. Temperatures: (+) represents 1.57K, (A) represents 4.22K at magnetic fields between 0 and 4.5 tesla; (-) represents various temperatures between 8 and 84K, at magnetic fields of 4.5 tesla. Solid line is theoretical curve for $g_{||}=2.05$, $g_{\perp}=1.925$. 
Joyce Elaine Morningstar was born in Kansas City, Missouri, on October 18, 1955, the daughter of Frances Martha Hillstrom and Harry Milton Morningstar. While in high school, she attended a National Science Foundation Summer Program in Biochemistry at Purdue University, doing endocrinology research under the direction of Dr. P.V. Malven. She worked with Dr. N. Henderson on plant genetics at the University of Missouri at Kansas City before graduating from North Kansas City High School, Kansas City, Missouri, in 1974.

Joyce attended New College of the University of South Florida, Sarasota, Florida, from 1974 through 1978, receiving a Bachelor of Arts degree in Chemistry. The title of her B.A. thesis, directed by Dr. J. Butler, is "Synthesis of Isomerically Pure Methylene-cyclopropanes via Organometallic Chemistry". Joyce spent the summers of 1976 and 1977 doing research in organic synthesis with Dr. Aldred at Tulane University, New Orleans, Louisiana, and with Dr. J. Gilbert at the University of Texas, Austin, Texas, respectively. After graduating from New College, Joyce worked for a summer at Celanese Corporation as a Research Chemist.

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DOCTORAL EXAMINATION AND DISSERTATION REPORT

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Major Field: Inorganic Chemistry

Title of Dissertation: Spectroscopic Studies of Mitochondrial Iron Sulfur Proteins

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