Active Site Interactions and Kinetic Characterization of C- And N-Nitroso and Peroxidic Substrates With Alcohol Dehydrogenase: Mechanistic Insights From Studies of the Cobalt-Substituted Enzyme.

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ACTIVE SITE INTERACTIONS AND KINETIC CHARACTERIZATION OF C- AND N- NITROSO AND PEROXIDIC SUBSTRATES WITH ALCOHOL DEHYDROGENASE: MECHANISTIC INSIGHTS FROM STUDIES OF THE COBALT-SUBSTITUTED ENZYME

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In partial fulfillment of the Requirements for the degree of Doctor of Philosophy
in
The Department of Biological Sciences

by
Alexander G. Tkachenko
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Dedication

I dedicate this dissertation to my parents, Gregory and Tamara Tkachenko, who instilled in me the importance of education and lighted my flame of passion for the sciences and better things in life.
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List of Abbreviations

ADH – alcohol dehydrogenase
AMP – adenosine monophosphate
AU – absorbance unit
BQ - benzoquinone
BQI - benzoquinoneimine
CHP – cumene -hydroperoxide
CIS – competitive inhibition and stimulation
DA – dipicolinic Acid
DMNA – p-nitroso-N,N-dimethylaniline
DTT – dithiotreitol
EDTA – ethylenediaminetetraacetic acid
EPR – electron paramagnetic resonance
GSH – glutathione
H$_2$O$_2$ – hydrogen peroxide
HLADH - horse liver alcohol dehydrogenase
ICP – inductively coupled plasma
LMCT – ligand to metal charge transfer
MBHP - p-methylbenzyl hydroperoxide
NDEA – N-nitrosodiethylamine
NDELA – N-nitrosodiethanolamine
NEU – N-nitrosoethylurea
NMOR – N-nitrosomorpholine
NMU – N-nitrosomethylurea

pAP – p-aminophenol

pNSP – p-nitrosophenol

SCE - sister chromatid exchange

SSB - single strand breaks

tBOOH – tert-butyl hydroperoxide

YADH - yeast alcohol dehydrogenase
Foreword

This dissertation is divided into seven chapters. The first chapter provides an introduction to the origin of C- and N-nitroso compounds in our environment, followed by an overview of their metabolism in mammalian cells and the role of alcohol dehydrogenase (ADH) in C-nitroso reduction. The contribution of our laboratory to this understanding of ADH as a major C-nitroso reductase in mammalian liver is discussed.

Chapter two discusses the first use of cobalt-substituted ADH to study the mechanism and kinetics of p-nitrosophenol (pNSP) reduction by horse liver ADH. Also presented in chapter 2 are spectroscopic studies of the interaction of pNSP and N,N'-dimethylnitrosamine (DMNA) with the cobalt active site center. Again, the use of cobalt-substituted ADH has not been employed previously for mechanistic study of nitroso compound metabolism.

In chapter three the first characterization of the role of ADH in the metabolism of potentially carcinogenic N-nitroso compounds is described, with the emphasis on N-nitrosourea and N-nitrosodiethanolamine. Chapters four through six are devoted to the study of a reported peroxidatic activity of ADH.

Chapter four is a preprint of a manuscript entitled "Interaction of alcohol dehydrogenase with tert-butylhydroperoxide: stimulation of the horse liver and inhibition of the yeast enzymes", which identifies tert-butyl hydroperoxide (tBOOH) as both a positive and negative a modulator of ADH activity, depending upon the form of ADH. This chapter presents kinetic characterization of the stimulatory and inhibitory effect of tBOOH on the horse liver and yeast enzymes, respectively. This paper has been accepted for publication in the Archives of Biochemistry and Biophysics, and was

Chapter five is continuation of studies of the effects of peroxide substrates on ADH. The content of this chapter has been submitted for publication to the Journal of Inorganic Biochemistry. The chapter, formatted in the style of this journal, entitled "Inactivation of Alcohol Dehydrogenase by Hydrogen Peroxide: Studies with the Cobalt-substituted Enzyme" presents the first use of cobalt-substituted ADH to investigate the mechanism of peroxidatic attack and subsequent destruction by hydrogen peroxide of the metal coordination sphere.

Finally, the studies presented in chapter six employed the use of cobalt-substituted ADH in an attempt to better understand the nature of the peroxidatic activity of ADH with respect to the organic hydroperoxides tBOOH and cumene hydroperoxide (CHP) and the involvement of the enzyme active site residues in interaction with the ADH metal coordination sphere. Evidence is provided suggesting that distortion of the geometry of the metal coordination sphere, without irreversible destruction of the active site metal ligands, is associated with the stimulation by tBOOH and CHP of ADH-catalyzed ethanol dehydrogenase activity.

Chapter seven summarizes all of the findings presented herein and discusses possible implications of the present research with respect to future basic and applied research directions.
Abstract

The interaction of ADH with C-nitroso, N-nitroso and peroxide substrates was investigated. To better elucidate the mechanism of these interactions, cobalt-substituted HLADH was introduced and compared with native HLADH. Both binary and ternary complexes of p-nitrosophenol with Co(II)ADH caused a blue-shift of the d-d transition region of the Co(II)ADH spectrum, indicating direct interaction of the C-nitroso moiety with the metal active site. In addition to this blue shift with NADH, both pNSP and N,N'-dimethylnitrosamine enhance the absorbance of the 643 nm peak of the ADH/NADH/pyrazole ternary complex, which suggests that these compounds compete with pyrazole for ligand association with the metal center. Enzymatic and nonenzymatic reduction of N-nitrosodiethanolamine, N-nitrosoethylurea, and N-nitrosomethylurea was observed, albeit the reaction rates were relatively slow in comparison to the reduction of C-nitroso compounds; however, the general mechanism involving 2 e-transfer steps in the enzymatic reduction was essentially the same. Spectroscopic studies of Co(II)ADH indicate that N-nitrosodiethanolamine and N-nitrosoethylurea react directly with the coordination sphere of the ADH metal center with strong ternary complex formation. Preincubation of HLADH with H$_2$O$_2$ caused a first-order rate inactivation ($k = 0.11 \pm 0.2$ min$^{-1}$) of the enzyme, whereas tert-butylhydroperoxide (tBOOH) and cumene hydroperoxide (CHP) stimulated HLADH activity by 200 and 170%, respectively. The rate and extent of stimulation by tBOOH were strongly reduced by binary complexes with NAD$^+$ or NADH, whose pyrophosphate groups bind to Arg47 and Arg369. tBOOH irreversibly inactivated yeast ADH (YADH). Similar to H$_2$O$_2$ four $\text{-SH}$ groups per molecule of YADH were modified by tBOOH. Spectral
analysis of Co(II)ADH indicated strong interaction of peroxides with the active site coordination sphere by the loss of LMCT and d-d transition bands. HLADH stimulation is suggested to be due to destabilization of the catalytic Zn-coordination sphere via indirect interaction of tBOOH with the metal center, while inactivation by H$_2$O$_2$ results from direct interaction with the metal center via irreversible modification of active site ligands Cys46 and Cys174 and modification of an anion-binding site formed by Lys228.
Chapter 1: Introduction

Metabolism of chemicals by both mammalian and bacterial enzymes is important in the toxic expression of certain chemicals, including nitroso compounds. For those drugs or other chemicals that bear a functional group susceptible to reduction or oxidation, a thorough understanding of the mechanism of action and hence, the risk assessment may require a careful dissection of mammalian metabolism. The strong association between alcohol ingestion and chemical carcinogenesis is recognized (Caballeria et al., 1997). Many studies have established that chronic alcohol consumption alters the metabolism, toxicity and mutagenicity of a wide variety of xenobiotics substances in animal models. Epidemiological evidence also shows a link between alcohol consumption and human cancer risk (Lieber, 1990).

Our laboratory has shown that horse liver ADH, human class I (β1, β2, a), class II (τ-) and class IV (σ-) ADH catalyze NADH-dependent reduction of potentially carcinogenic C-nitrosoarenes. ADH is identified as a C-nitroso-reducing enzyme to be considered in the mutagenic sequelae of nitro and nitrosoarenes (Maskos and Winston, 1993). The substitution of the zinc ion by the more informative cobalt ion in the active site of ADH is used in the present study to further elucidate the role of ADH in biotransformation of xenobiotics, particularly in the reduction of C-nitroso substrates.

FUNCTIONAL DIVERSITY OF ALCOHOL DEHYDROGENASE FAMILY

Alcohol dehydrogenase [1.1.1.1] is an NAD⁺-dependent oxidoreductase that acts on primary and some secondary alcohols by a mechanism involving both a hydride transfer to NAD⁺ and a proton transfer from substrate to solution (Henehan and Oppenheimer, 1993; Pocker, 1988; Zeppezauer et al., 1984) (Equation 1).
ADH proceeds by an ordered bi-bi mechanism in which the binding of the cofactor near the active site of the enzyme precedes binding of a substrate (Equation 2). Catalysis occurs instantly with hydride transfer from ethanol to the bound cofactor. This reaction is reversible, with reduction of acetaldehyde using NADH as a cofactor (Pocker, 1988).

Aside from numerous alcohols, aldehydes and steroids, C-nitroso compounds, which are intermediates in the metabolism of arylamines and nitroarenes, are good substrates for ADH (Dudley and Winston, 1995; Dunn and Bernhard, 1971; Hajos and Winston, 1992; Kuwada et al., 1980; Maskos and Winston, 1993) presumably due to the presence of a large hydrophobic binding domain. The physiological role of ADH, although related to the metabolism of the alcohols appears to be more diverse.

The multiplicity within the ADH family is impressive, like those for the cytochrome P450 family and the glutathione transferases. The most recent tree, based on 79 different Zn-containing ADH structures was published in 1996 by Shafqat (Shafqat et al., 1996). The complex nature of all these enzymes, with multiple forms and repeated duplications appear similar in pattern and suggest that they all participate in important basic cellular defense mechanisms, where each form has distinctive functions with some overlapping activities.

Mammalian ADH represents an enzyme family of multiple isoforms, from which horse liver ADH (HLADH) appears to be most studied. HLADH is a dimeric enzyme (MW=80kDa) comprising two identical subunits, each containing 374 amino
acids and two zinc ions. Binding studies and steady-state kinetic measurements have failed to provide evidence for any nonequivalence of the two subunits in the ADH molecule. One zinc ion is buried deep in the catalytic domain and is the site of substrate binding. It is coordinated by two sulfurs (Cys-46, Cys-176), one nitrogen (His-67), and one oxygen (water) in a distorted tetrahedral arrangement (Al-Karadaghi et al., 1994). Ser48 forms a hydrogen bond to the metal bound oxygen atom. (Eklund et al., 1994). The noncatalytic zinc ion is liganded by four cysteines 97, 100, 103, and 111 in a near perfect tetrahedral geometry and located about 20Å from the catalytic metal ion site (Cannon and McCay, 1969). The function of this zinc ion is primary structural (Pocker, 1988).

HLADH represents the only zinc enzyme examined by x-ray crystallography so far in which both zinc and a coenzyme are required for activity. The X-ray structure of the metal-depleted enzyme from horse liver has been solved at 1.8 Å resolution (Al-Karadaghi et al., 1994) and that of the holoenzyme at 2.9 Å resolution (Eklund et al., 1994). The active site, which must accommodate the coenzyme differs from that of all other zinc enzymes in the nature of the cysteine ligands and the length of the spacer between Cys-46 and His-67 (19 and 106 amino acids residues respectively). A key property of the enzyme, established by X-ray data, is the existence of two protein domains in each monomer that are relatively free to rotate with respect to each other (Cedergren-Zeppezauer et al., 1985). There is a deep cleft between the domains where NAD$^+$ and substrate binds. The apo- and holo-enzymes exist in the so-called open form, whereas binding of coenzyme induces rotation of the catalytic domains by 10° relative to the coenzyme binding domain, thereby tightening the interactions with NAD$^+$ and
excluding water from the central part of the active site resulting in the closed form (Pocker, 1988). The general fold is very similar in both the closed and open forms. Occupation of the cofactor-binding site appears to be required to stabilize the closed form. In solution, there may be an equilibrium distribution of species in open, closed, and partially closed states (Vallee and Auld, 1990).

Crystallographic data are neither in accord with an outer sphere coordinated substrate nor with a water molecule bound to a penta-coordinated zinc. Crystallographic studies of the enzyme show that the catalytic zinc ion generally exhibits four-coordination (Al-Karadaghi et al., 1994; Eklund et al., 1994; Maret et al., 1979; Schneider et al., 1983), which is supported by several spectroscopic investigations (Dietrich and Zeppezauer, 1982; Maret, 1989; Schneider-Bernlohr et al., 1988). On the other hand, there is strong crystallographic and spectroscopic evidence showing that binding to zinc of certain inhibitors is five-coordinate (Makinen et al., 1983; Makinen and Yim, 1981). Spectroscopic studies of metal-substituted alcohol dehydrogenase have indicated some binary and ternary complexes as five-coordinate (Ryde, 1996). Some investigators have proposed that this complex or the resulting five-coordinate alkoxide-water complex is the intermediate undergoing the catalytic hydride transfer (Ryde, 1996). Resonance Raman spectra of complexes between Cu(II) enzymes and pyrazole (Chen et al., 1987) are in accord with the tetrahedral structure of the catalytic metal ion (Pocker, 1988), whereas perturbed angular correlation spectroscopy on the Cd(II) enzyme suggests a five-coordinated complex (Andersson et al., 1982). Solvent magnetic resonance data for the Cu(II) enzyme suggest that the metal ion accepts pyrazole as a fifth ligand in the absence of bound NAD$^+$ (Andersson et al.,
1981). The converse was drawn from EPR spectral parameters determined with the Co(II) enzyme (Werth et al., 1995). Evidence from kinetic studies has been taken to favor four-coordination and five-coordination (Ryde, 1996) of zinc in the catalytic ternary complexes.

Theoretically, zinc (II), which has a d^{10} the electronic configuration, can be four-, five-, or six-coordinate. In coordination compounds, there is no ligand-field stabilization energy, and a balance between bonding energies and repulsions among the ligands determines the coordination number. In enzymes Zn(II) usually has a coordination number smaller than six leaving available binding sites in their coordination spheres. Theoretically substrates can bind to zinc by substituting for coordinated water or by increasing the coordination number. This behavior would be typical of Lewis acids, and the zinc atom in ADH acts as a Lewis acid. Only changes in the coordination sphere may occur on the side exposed to solvent. Although ADH is a redox enzyme and not a hydrolytic one, it catalyzes nucleophilic attack at alcohol carbon (Pocker, 1988). Therefore, zinc could substitute for protons polarizing a substrate bond by accepting a substrate atom as a ligand.

In many biological systems Zn complexes show facile four- to five-coordinate interconversion. The low barrier between these coordination geometries is quite important, because the substrate may add to the coordination sphere in order to replace the solvent or to be coordinated together with the solvent. Recently, Ryde (Ryde, 1996) published an extensive series of quantum-chemical calculations on models of the active site of alcohol dehydrogenase with different ligands. These calculations indicated that four-coordinate structures were about 100-200 kJ/mol more stable than a five-
coordinate one, depending on the ligands. The only stable binding site for a fifth ligand at the zinc ion is opposite to the normal substrate site, in a small cavity buried behind the zinc ion, and the zinc coordination sphere has to be strongly distorted to accommodate a ligand in this site.

Considering all the controversy, it seems that structural alterations that occur in the protein upon binding of NAD$^+$ and NADH must be responsible for the change in the coordination number of the active site metal ion stabilization of the metal-bound water molecule (Pocker, 1988). Their identification remains a challenging problem in the study of the structural basis of ADH action.

ENVIRONMENTAL OCCURRENCE OF C-NITROSO COMPOUNDS IN VIVO

C-nitroso compounds are intermediates in the metabolism of arylamines and nitroarenes and are substrates for ADH (Dunn and Bernhard, 1971; Horie et al., 1982; Leskovac et al., 1996; Maskos and Winston, 1993). Nitroarenes are ubiquitous contaminants in urban air formed predominantly by incomplete combustion and present in large variety of complex mixtures including the emission of power plants, motor vehicles, incinerators, wood-burning stoves, cigarette smoke, grilled foods, residential home heaters (Rosenkranz and Mermelstein, 1983). Over 60 nitroarenes have been detected in diesel effluent to date (Mermelstein et al., 1985). They are present in the environment as complex mixtures of other nitropolycyclic aromatic hydrocarbons and polychlorinated biphenyls; therefore we can consider nitroarenes being present in urban and rural atmosphere.

There are numerous lines of evidence that nitroarenes are biotransformed by bacteria to the corresponding hydroxylamines, which are then capable of forming
adducts with cellular macromolecules (Oda et al., 1993; Rickert, 1985; Tokiwa et al., 1987). Conversion of nitroarenes to hydroxylamines occurs through nitroso intermediates, which are capable of reacting with the base moieties of DNA (Fuji et al., 1994). There are three potential free radical intermediates of enzymatic nitroreduction: the nitro anion, nitroxyl and aminoxyl radicals (Equation 3).

\[
\begin{align*}
R-\text{NO}_2 & \rightarrow R-\text{NO}_2^- \rightarrow R-\text{NO} \rightarrow R-\text{NO}^- \rightarrow R-\text{NHOH} \rightarrow R-\text{NH}_2^+ \rightarrow R-\text{NH}_2
\end{align*}
\]  
Equation 3

Contrary to the relatively stable nitrocompounds, nitroso compounds (the two-electron reduction product) can be reduced to the corresponding hydronitroxide even by mild reducing agents. Various C-nitroso compounds are intermediates of the arylamine oxidation or the nitroarene reduction; and the reduction of the C-nitroso compounds to their corresponding hydroxylamines is a necessary step in the activation of these compounds to mutagenic end points (Maskos and Winston, 1994).

Research from our laboratory showed, that DT-Diaphorase (NAD(P)H-quinone oxidoreductase) is the major NADPH-C-nitrosoreductase in liver and ADH is the major NADH-C-nitrosoreductase (Hajos and Winston, 1992). Both these enzymes have important toxicological implications. The ratio of DTd to ADH in liver might be an important consideration in the competition for C-nitroso substrates between these enzymes. Moreover, NADH dependent C-nitroso reductase activity in liver cytosol was shown to be due mainly (85%) to ADH (Dudley and Winston, 1995). DTd is the major NADPH-C-nitrosoreductase in rat liver and ADH is the major NADH-C-nitrosoreductase (Hajos and Winston, 1992). Both these enzymes have important toxicological implications.
It was recently shown that HLADH could catalyze both the reduction of 2-NOF (nitrosofluorene) and the rearrangement of N-hydroxy-2-aminofluorene (Maskos and Winston, 1993). This rearrangement most likely proceeds via the highly electrophilic fluorenylnitrenium ion, in equilibrium with its resonance forms, 1- 3-fluorenylecarbocations; and enhanced mutagenicity of 2NOF by ADH in the Ames and umu assays (Maskos and Winston, 1993).

p-Nitrosophenol (pNSP) has been extensively used in our laboratory as a model compound for C-nitrosoreductase activity of ADH. pNSP is readily converted to pAP by ADH in the presence of ethanol and NAD\(^+\) and, the rate of pAP formation is seen to increase with increasing concentration of ethanol (Maskos and Winston, unpublished). The unique feature of the NADH-dependent pNSP reduction by HLADH is the formation of a short-lived spectral intermediate between 253 and 260 nm (Maskos and Winston, 1994), which appears concomitantly with loss of absorbance associated with the nitroso parent compound (400 nm), and an increase in absorbance due to p-AP formation (236 nm).

Analyses of pNSP reduction by ADH indicate that there are two alternative mechanisms (Fig. 1.1). In one mechanism, pNSP is directly reduced to p-aminophenol (pAP) via two enzymatic steps. A product of the first step of reduction, p-N-hydroxyaminophenol (pN-OHAP), remains at the active site of enzyme and undergoes subsequent reduction to pA. In the second mechanism, which is proposed for the unprotonated form of pNSP, a reduction of pNSP is limited to the first step only, and the dehydrated form of pN-OHAP, i.e. benzoquinoneimine (BQI) is the product. Since ADH-dependent reduction of BQI to pAP is relatively slow BQI can be subsequently
Figure 1.1  Reaction scheme for the formation of products during the enzymatic (solid arrows) and nonenzymatic (dashed arrows) pathways of pNSP reduction. (1) first, irreversible, two-electron reduction of pNSP to pNH-hydroxyaminophenol; (2) nonenzymatic dehydration of pN-OHAP to benzoquinoneimine (BQI); (3) second two-electron reduction and subsequent formation of p-aminophenol (pAP); (4) formation of p-hydroxybenzylnitrenium ion from BQI; (5) nonenzymatic reduction of p-hydroxybenzylnitrenium ion to pAP; (6) nonenzymatic hydrolysis of p-hydroxybenzylnitrenium ion to benzoquinone (BQ) (adopted from (Maskos and Winston, 1994)).
converted to the highly electrophilic arylnitrenium ion, which can then undergo NADH-dependent reduction to pAP or hydrolysis to 1,4-benzoquinone.

These two alternative mechanisms have been described for horse liver ADH so far. Elucidation of the involvement of the metal center of ADH in nitroso compound metabolism could lead to better understanding of the basis for such mechanisms as well as extend our knowledge of C-nitroso reduction to the human ADH model.

ENVIRONMENTAL OCCURRENCE OF N-NITROSO COMPOUNDS IN VIVO

N-nitroso compounds are pervasive in the environment. They are used in rubber and metal manufacturing industries, tobacco and tobacco smoke, some alcoholic beverages, cosmetics and toiletries (Eisenbrand et al., 1986; Guttenplan, 1993; Hecht, 1984). The presence of nitrosamines have also been reported in some foods in which their presence would be far from obvious, such as dried milk, cheese and vegetable oils (Lijinsky, 1984). The principal source of exposure of humans to N-nitroso compounds seems to be their formation in the gastrointestinal tract, especially the stomach (Lijinsky, 1984). The nitrite can come from the raw or processed foods in which it is present, or from saliva in which nitrite is formed by bacterial reduction of nitrate in the mouth. In either case it is likely that the nitrite enters the stomach and reacts with secondary or tertiary amines to form nitroso compounds (Lijinsky, 1984). A number of likely candidates for precursors of carcinogenic N-nitroso compounds could also be formed in vivo, and thereby posing a possible risk to man, including arginine, methylguanidine, piperidine, piperine, and piperazine (Lijinsky, 1984).

Several reports (Denkel et al., 1986; Denkel et al., 1987; Eisenbrand et al., 1984; Eisenbrand et al., 1986) showed involvement of ADH in oxidation of various
environmentally common N-nitroso compounds such as N-nitrosodiethanolamine (NDELA). Most of the research on N-nitroso compounds suggested that carcinogenic species were produced via oxidation, sulfation or transamination reactions (Eisenbrand et al., 1984; Farrelly et al., 1986). Nitroso compounds (the two electron reduction product of nitro compounds) can be reduced to their corresponding hydronitroxide even by mild reducing agents; thus, the reduction of N-nitroso compounds in vivo is also feasible (Rickert, 1985).

A comprehensive study of the activation of N-nitroso compounds by ADH was done by Eisenbrand's group during the period 1984-1987 (Denkel et al., 1986; Denkel et al., 1987; Eisenbrand et al., 1984; Eisenbrand et al., 1986), using NDELA as a model compound. NDELA is one of the most widespread N-nitroso compounds in the human environment. It has been detected in exceptionally high concentrations, up to 3%, in industrial metal grinding fluids, and in urine of metal grinders (Tunick et al., 1982; Vohra and Harrington, 1981).

A characteristic of NDELA metabolism is that up to 95% of administered NDELA is excreted unchanged in urine of the rat model (Farrelly et al., 1986). Even with 5% of NDELA metabolized in the body, this compound showed a strong mutagenic response (Farrelly et al., 1986). N-nitroso-2-hydroxyethylglycine (NHEG) and N-nitroso-2-hydroxyethylmethyl-formylilamine were found in urine after p.o. administration of NDELA (Hecht, 1984). N-nitroso-2-hydroxymorpholine (NHMOR) has been detected as a metabolite of NDELA in rat liver S9 fractions. In vivo, NHMOR is oxidized to NHEG, which is a urinary metabolite of NDELA. The detection of NHMOR as a metabolite of NDELA, and its mutagenicity in Salmonella typhimurium,
with or without activation, suggested that it might be a proximate carcinogen of NDELA (Hecht et al., 1989). As cytosolic oxidase, ADH was proposed to oxidize NDELA to ethanolnitrosaminoethanal with the subsequent formation of NHMOR and N-nitrosomorpholine (NMOR) (Hecht, 1984; Hecht et al., 1989). NMOR is one of the most carcinogenic of the N-nitroso compounds, and preferentially exists in the N-Nitroso-2-hydroxymorpholine (NHMOR) hemiacetal form (Hecht et al., 1989). In contrast to NDELA, NMOR is extensively metabolized in rats (1-2% dose unchanged in urine) (Hecht et al., 1989). Established pathways of metabolism include α-hydroxylation and β-hydroxylation to respectively, NHMOR and NDELA, and denitrosation.

The finding of Eisenbrand and coworkers (Denkel et al., 1986; Denkel et al., 1987) showed that NDELA is activated in the rat liver by a two-step metabolic transformation sequence involving ADH and sulfotransferase. When ethanol was added to the diet, induction of single strand breaks (SSB) in DNA by NDELA and NHEEA (N-nitroso(2-hydroxyethyl)ethylamine) in rats was observed, whereas breaks induced by NHMOR were only partially reduced. Apparently, ethanol behaved as a competitive inhibitor of NDELA oxidation by ADH.

6-Alkylnitrosamino ethanols, including NDELA, undergo efficient liver ADH-catalyzed oxidation to their corresponding α-nitrosamino aldehydes (Loeppky et al., 1987). Involvement of ADH in the activation of NDELA to mutagenic endpoints was also supported by Henn et al. (Henn et al., 1989), who reported that ADH from yeast and horse liver induced chromosomal mutations and sister chromatid exchange (SCE) by NDELA in human lymphocyte cultures. No significant chromosome aberrations
were observed. However, the SCE frequency per cell was significantly increased by adding NAD⁺. The addition of 220 units of ADH from yeast as well as 1.8 units of HLADH also significantly raised the number of SCE.

Although the importance of ADH in the activation of these compounds has been established, no mechanistic or kinetic data are available. Most of intermediates are short lived and their existence was only proposed in many cases (Eisenbrand et al., 1986). The reduction of C-nitroso compounds by ADH produces strong electrophiles capable of causing DNA damage as well (Fuji et al., 1994; Maskos and Winston, 1993).

The wide distribution of N-nitroso compounds in the environment coupled with the evidence implicating ADH in the activation of NDELA to its mutagenic endpoints, introduced an opportunity to explore the role of ADH in the reduction of the nitroso group of major industrial N-nitroso compounds. Our hypothesis was that ADH-dependent reduction of N-nitroso substrates would lead to formation of N-hydroxylamines, which analogous to C-hydroxylamines, could be proximate mutagens.

**INTERACTION OF ADH WITH HYDROGEN PEROXIDE**

The application of ozone as an alternative to the use of chlorine for drinking water pretreatment and treatment has received increasing attention in recent years; however, the amount of information available with regard to the formation of ozone by-products during drinking water disinfection and the ultimate fate of these by-products is limited (Evans, 1998). Some of the known potential ozonation by-products include aldehydes, ketones, organic acids, nitrosamines, chloramines, organic peroxides, quinones, bromate and chlorate (Evans, 1998).
It is well known that unsaturated lipids, proteins and DNA are the components of the cell that are most sensitive to oxidative damage by organic peroxides. Unsaturated fatty acids are particularly susceptible to attack by hydroxyl free radicals generating lipid peroxides, which makes them more hydrophilic and consequently, alters the structure and the function of the membrane.

Recently, a peroxidase activity of ADH was identified in which hydrogen peroxide when used as a co-substrate with NAD$^+$ was converted to a stable product referred to as NADX, which is structurally distinct from NADH (Favilla et al., 1980a; Favilla et al., 1988; Favilla et al., 1980b). Apparently, this peroxidatic reaction involved the same enzyme active site at which alcohols are oxidized. In addition to being a substrate for ADH, H$_2$O$_2$ irreversibly inactivated ADH, which was partially prevented by pretreatment of the enzyme with NAD$^+$.

It was suggested that the high Km value for H$_2$O$_2$ (>0.1M) in the peroxidatic activity of ADH (Favilla et al., 1980a; Favilla et al., 1988; Favilla et al., 1980b) reflected the poor tendency of the ternary complex to reach the transition state, which is counterintuitive to a physiological role. However, studies of an organic derivative of H$_2$O$_2$, p-methylbenzyl hydroperoxide (MBHP) by Skursky and his coworkers (Skursky et al., 1992a; Skursky et al., 1992b), which completely inhibited both the activity of pure HLADH and of homologous rabbit blood serum enzyme prompted our present studies.

MBHP showed different mechanism of interaction with ADH than H$_2$O$_2$ with 10,000 times smaller Km than for H$_2$O$_2$ (Skursky et al., 1992a). MBHP caused 50%
inhibition of HLADH at the same order of magnitude as the concentration of enzyme active sites.

The very high affinity of ADH for MBHP was explained partially by hydrophobic interaction between the MBHP and the hydrophobic pocket in the enzyme substrate-binding site. Beside the differences in kinetics, \( \text{H}_2\text{O}_2 \) and MBHP appear to have a different mechanism of action since ADH inhibition by MBHP was reversible, suggesting that active site metal coordination was not destroyed.

We thought that the zinc ion at the active site of ADH might, through reactivity with organic peroxides, modify nearby active site residues. The active site metal ion is directly involved in the binding of substrate and in the case of peroxidic substrates may cause oxidative destruction of active site residues. We have found cobalt-substituted ADH to be useful in assaying the mechanism of peroxidatic reaction.

**BENEFITS OF METAL-SUBSTITUTED ADH**

Direct spectroscopic investigation of zinc enzymes is limited, because Zn(II) is a \( d^{10} \) diamagnetic ion with uninformative electronic and magnetic properties. The \( >80\text{kD} \) molecular weight makes ADH too large for full signal assignment using present state of the art \( ^1\text{H} \) NMR. At the moment the major source of information comes from X-ray data (Bertini et al., 1987). Metallosubstitution of ADH appears to be a valuable research tool for characterizing the role of the metal and its interactions with residues in the active site (Bertini et al., 1987). Co(II)ADH appears to be an exceptional probe to monitor the structure and reactivity of ADH. The specific replacement of the active site zinc ion in horse liver ADH by other divalent metal ions with spectral and magnetic properties that are responsive to changes in the metal coordination sphere is of the main
interest of this research. Zeppezauer and co-workers (Dietrich et al., 1979; Dunn et al., 1982; Formicka-Kozlowska et al., 1988; Koerber et al., 1983; Maret et al., 1979; Sartorius et al., 1987; Schneider-Bernlohr et al., 1988; Werth et al., 1995) demonstrated that the catalytic zinc ion can be specifically removed from the crystalline enzyme without changing the content of the noncatalytic zinc and that the catalytic activity can be reconstituted in the crystalline state with other divalent metal ions. Cobalt (II), which is generally high spin (S=3/2) in tetra coordinate and penta coordinate environments with well-characterized electronic properties, has been particularly useful in this regard. The study of the cobalt substituted at the catalytic site horse liver ADH shows that the protein structure and the coordination of the metal in the active site are essentially unperturbed compared to the native enzyme (Schneider et al., 1983); therefore, the study of the effects of the removing and the substitution of the zinc of the active site of the enzyme with cobalt on the reduction of the C-nitroso compounds by the horse liver ADH are presented here (Chapter 2). Cobalt(II)ADH has also been implicated in our studies of reduction of N-nitroso compounds by ADH (Chapter 3) as well as interaction of ADH with hydrogen peroxide (Chapter 5) and tert-butylhydroperoxide (Chapter 6).
Chapter 2: Spectral Analysis of Cobalt-Substituted ADH Interaction with C-Nitroso Substrates and Comparison of the Kinetics of \( p \)-NSP Reduction by the Native and Cobalt-Substituted Enzymes

INTRODUCTION

Alcohol dehydrogenase (ADH, EC 1.1.1.1) is the primary enzyme responsible for the oxidation of ethanol, with the production of acetaldehyde and NADH being the first step of ethanol elimination (Lieber, 1991). Besides numerous alcohols, aldehydes and steroids, C-nitroso compounds, which are intermediates in the metabolism of arylamines and nitroarenes, are good substrates for ADH (Dunn and Bernhard, 1971; Kuwada et al., 1980) presumably due to the presence of a large hydrophobic binding domain. The potent mutagenic and carcinogenic potential of nitro- and nitrosoarenes involves their facile reduction to N-hydroxyarylamines (Fifer et al., 1986). ADH catalyzes both the reduction of 2-nitrosofluorene and the rearrangement of N-hydroxy-2-aminofluorene, which provides cogent evidence for the production of the electrophilic intermediate arylnitrenium ion (Maskos and Winston, 1993). The ability of ADH from various species to reduce \( p \)-nitroso-N,N-dimethylaniline and \( p \)NSP has been reported recently (Leskovac et al., 1996; Maskos and Winston, 1994; Pantelic et al., 1996). The physiological role of ADH, although clearly related to the metabolism of the alcohols appears to be more diverse.

Direct spectroscopic investigation of zinc enzymes is limited by the fact that Zn is a d\(^{10}\) diamagnetic atom with uninformative electronic and magnetic properties. Metallosubstitution of ADH has been a valuable research tool for probing the chemistry and enzymatic nature of the ADH coordination sphere (Bertini et al., 1987); Co(II)ADH appears to be an exceptional probe to monitor the structure and reactivity of ADH.
In this respect the active site-specific Co(II)-reconstituted enzyme characterized by Zeppezauer and co-workers (Maret et al., 1979) is a particularly good model, as it has been demonstrated that the structure of the enzyme and especially the active site metal ion region are essentially identical with the native enzyme (Schneider et al., 1983). To further explore the mechanism of C-nitrosoreduction by ADH in the toxicity of C-nitroso compounds we used the cobalt-substituted enzyme.

Substitution of the open-shell Co(II) atom for the closed shell Zn(II) atom may be expected to result in a detectable decrease in the pKa of a metal bound ligand. This will alter the Lewis acidity of the metal center with the subsequent alteration of the kinetic parameters of nitroso reduction.

Analysis of pNSP reduction by HLADH indicates that there are two alternative mechanisms (Maskos and Winston, 1994). In one mechanism, pNSP is directly reduced to p-aminophenol (pAP) via two enzymatic steps. A product of the first step of reduction, p-N-hydroxyaminophenol (pN-OHAP), remains at the active site of enzyme and undergoes subsequent reduction to pAP. In the second mechanism, which is proposed for the unprotonated form of pNSP, a reduction of pNSP is limited to the first step only, and the dehydrated form of pN-OHAP, i.e. benzoquinoneimine (BQI) is the product. Since ADH-dependent reduction of BQI to pAP is relatively very slow, BQI may be converted to the electrophilic arylnitrenium ion, which can then undergo NADH-dependent reduction to pAP or hydrolysis to 1,4-benzoquinone. These two alternative mechanisms have been proposed for HLADH, which appear to be different from various human isozymes (Maskos and Winston, 1994). Herein, we compare the effects of pyrazole, acetaldehyde and pH on the C-nitrosoreductase activities of
Co(II)ADH and native HLADH. We show that cobalt-substitution decrease $k_{cat}$ and $K_m$ of C-nitrosoreduction and lowers the $pK_a$ of the metal-bound water, which in turn alter the rate of hydride transfer.

**MATERIALS AND METHODS**

**Chemicals and Enzymes**

Crystalline horse liver ADH and $\beta$NAD$^{+}$ (> 99 % purity) were purchased from Boehringer-Mannheim (Indianapolis, IN). The suspension of the enzyme was centrifuged at 5000 x g for 20 min, and the enzyme was dissolved in 0.1 M phosphate buffer, pH 7.4. Insoluble material was removed by centrifugation and supernatant was dialyzed for 24 hours against 50 mM Tris buffer pH 8.4 to eliminate residual ethanol. Enzyme concentration was calculated using the extinction coefficient 36.4 mM$^{-1}$cm$^{-1}$ at 280 nm (Chen et al., 1987). Pyrazole, NADH, benzaldehyde and potassium ferricyanide were obtained from Sigma (St. Louis, MO). $p$AP, $p$NSP, and phenol were obtained from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of the highest grade commercially available.

**Preparation of Active site substituted ADH**

Active site substitution followed procedure by Maret (Maret et al., 1979) with some modifications. HLADH was dialyzed by equilibrium dialysis against 50 mM Tes/Na+ buffer at pH 6.9 with 20% (v/v) tert-butanol as precipitant. Concentration of tert-butanol was slowly increased with 2% steps in order to obtain stable crystals. White crystals appeared after reaching ~ 10-12% concentration of tert-butanol in the solution.
Figure 2.1  **The enzymatic activity of ADH during metal substitution.** Columns correspond to ethanol oxidation and the line corresponds to pNSP reduction. Dipicolinic acid (5 mM) (DA) was used as a chelating agent for the removal of Zn from ADH.
Rigorous anaerobic conditions are critical for the preparation; therefore, all following steps are performed under argon atmosphere at 4°C. The crystal suspension was treated against 5 mM of dipicolinic acid (chelating agent) for 48 hours or until activity of the enzyme decreased to less than 1%. Then the suspension is freed from the chelating agent by subsequent dialysis against the original buffer with 20% tert-butanol. Insertion of Cobalt ions in crystalline zinc-depleted ADH is done by dialysis against 4-5 mM Cobalt Acetate for 72 hours. The total ionic strength of the buffer was maintained the same as during the crystallization by appropriate reduction of the buffer substance. The formation of blue crystals would confirm the metal substitution. Excess of cobalt was removed by subsequent dialysis against mother buffer with 5 changes of buffer over 3 days period. The obtained crystals were resolved in 150 mM Na₂SO₄ as recommended by Adolph in personal communication. The degree of metal substitution was checked by the absorbance at 340 and 650 nm as well as by ICP analyses on Leeman Labs PS 3000. The decrease of activity after removal of zinc ions from the active site was also used as a marker of the metal substitution (Fig.2.1). Based on the metal analyses the level of substitution for active site substituted ADH was at least 85% and for complete substituted ADH at least 90%.

**Protein Assay**

Protein determination was based on the reported extinction coefficients $A_{280} = 0.49 \text{ mg}^{-1}\text{cm}^2$ for the fully substituted cobalt enzyme, $A_{280} = 0.45 \text{ mg}^{-1}\text{cm}^2$ for the enzyme depleted of the catalytic zinc ions and $A_{280} = 0.49 \text{ mg}^{-1}\text{cm}^2$ for the blue enzyme (Maret et al., 1979).
Enzyme Assays

The extent of \( pNSP \) reduction was determined by tracing the loss of absorbance at 400 nm assuming \( e_{400} = 24 \text{ mM}^{-1}\text{cm}^{-1} \) for the unprotonated form of \( pNSP \). The reactions were initiated with addition of the enzyme and followed spectrophotometrically at 1 min intervals for 5 min time periods. The reaction was conducted in a cuvette with a path length of 0.1 cm and in a final volume of 330 \( \mu l \).

Formation of \( pAP \) from \( pNSP \) was determined essentially by the method of Horie et al. (Horie et al., 1980) with modifications (Hajos and Winston, 1992). \( pNSP \) (0.05-1mM) was incubated for 15 minutes in the presence of 200 \( \mu M \) NADH or NADPH and 1 \( \mu g/ml \) purified HLADH in 50 mM potassium phosphate buffer, pH 7.4 or 0.1 M zwitterionic buffer (MES/NEA/DEA), at specified pH, in a final volume of 2 ml. The reaction was initiated by addition of cofactor and terminated by addition of 300 \( \mu l \) of 1 M \( \text{Na}_2\text{CO}_3 \). \( pAP \) formation was detected spectrophotometrically by subsequent addition of 300 ml of 5% (w/v) aqueous phenol and 200 ml of 0.2% (w/v) potassium ferricyanide. The color was allowed to develop for at least 15 min and the absorbance was read at 630 nm on a Biotek P750 Microplate reader. The concentration of \( pAP \) formed was calculated from a standard curve of commercially available \( pAP \).

ADH activities were determined according to the method of Vallee and Hoch (Vallee and Hoch, 1955) from initial velocities of NADH production at 340 nm with a Hitachi U3110 UV-Vis spectrophotometer at 25°C. Reactions were conducted in quartz cuvettes of 1 cm light-path containing 2.5 mM NAD\(^+\) and 0.4 M ethanol in 1 ml of 32 mM sodium pyrophosphate buffer, pH 8.8. The concentration of ADH active sites was determined by pyrazole titration (Theorell and Yonetani, 1963).
RESULTS

Spectral properties of binary and ternary Co(II)ADH complexes

The spectral properties of metalloenzymes can provide valuable information regarding both the nature of ligands and the geometry of the coordination complexes. The protein spectra of Co(II)ADH are influenced by the LMCT transitions between the metal and sulfur, which appear at 280 nm and 320 nm in model complexes with cobalt (Schneider-Bernlohr et al., 1988) and at 225 nm and 275 nm in complexes with zinc (Schneider-Bernlohr et al., 1988). A broad absorption band appears around 340 nm ($\varepsilon > 4000 \text{ M}^{-1} \text{ cm}^{-1}$), which reflects charge transfer between the cobalt ion and sulfur ligands at both the catalytic and noncatalytic metal-binding sites (Fig. 2.2). Unfortunately, the broad LMCT transition does not discriminate between cobalt bound to the catalytic or noncatalytic center; however, in addition to the strong LMCT band at 340 nm, active site-substituted cobalt ADH produces a relatively strong d-d transition band at 645 nm ($\varepsilon \sim 1000 \text{ M}^{-1} \text{ cm}^{-1}$) (Fig. 2.1b). The complete substituted enzyme in addition to the d-d transition band at 645 nm also shows a d-d transition band at 740 nm ($\varepsilon \sim 700 \text{ M}^{-1} \text{ cm}^{-1}$) (Fig. 2.2), which corresponds to the noncatalytic cobalt ion with perfect tetrahedral geometry ligand coordination. The positions and intensities of the Co(II)ADH absorption bands at 650 and 740 nm are comparable to known tetrahedral Co(II) complexes with three or four thiolate ligands (Zeppezauer, 1983).

Although, it is well established that oxidized coenzyme does not interact with the metal at the active center in binary complexes evidence has been presented that the reduced coenzyme does (Eklund et al., 1982). The coenzyme-induced conformational change of the protein leads to a red shift of the d-d band of Co(II)ADH from 645 nm to
Figure 2.2 UV-Vis spectrum of complete cobalt(II)-substituted ADH.
ADH (20 μM) in Tes buffer, pH 7.4. LMCT- ligand to metal charge transfer band.
673 nm upon binding of NADH (Fig. 2.3, trace 2), which is in agreement with studies by others (Schneider-Bernlohr et al., 1988). The d-d band of Co(II)ADH is sensitive not only to the protein conformation but also to the binding of substrates and substrates analogs.

Pyrazole binding to enzyme/coenzyme binary complexes displaces the metal bound water molecule and results in a highly distorted tetrahedral coordination environment as evidenced by the large splitting of the d-d transition band into two well resolved components centered at 643 and 677 nm (Fig. 2.3, trace 3). The absorption spectrum of the Co(II)ADH/NAD^+/pyrazole ternary complex is almost identical to that of the model compound [Co(S-2,4,6-i-Pr_3C_6H_2)(1-Me-imid)_2], which has a highly distorted tetrahedral structure for the S_2N_2 donor set as a result of the extremely bulky thiolate groups (Werth et al., 1995). The observed bimodal splitting of the d-d transition band is a general feature of ternary complexes formed with strong inhibitors such as pyrazole. The binary complex of ADH with pyrazole resulted in the broadening of the d-d transition band (Fig. 2.4, trace 2) with the formation of barely resolved peaks at 640 and 665 nm. Figure 2.4 (trace 3) shows that binding of NADH to the binary complex ADH-pyrazole results in the classical bimodal splitting of the spectrum; thus, pyrazole binding does not prevent access of the pyridine nucleotide to the coenzyme binding site, hence, ternary complex formation.

The d-d transition region corresponding to the structural metal center in the complete cobalt-substituted enzyme was not affected by addition of either coenzyme or pyrazole (not shown), further substantiating the specific interaction between pyrazole and the active site metal only. This result supports the proposal of Theorell and
Yonetani (Theorell and Yonetani, 1963) that pyrazole is bound to the catalytic zinc in liver alcohol dehydrogenase, and since pyrazole is competitive with alcohol, infers that the alcohol is bound to the metal (Shore and Santiago, 1975).

The involvement of zinc in catalysis requires interaction with substrates or coenzymes or both, in the ternary complexes. Since the addition of saturating concentrations of \( \text{NAD}^+ \) did not strongly affect the 645 nm peak of the cobalt-substituted enzyme, an interaction of oxidized coenzyme with the metal seems improbable. On the other hand, spectral shift due to the ternary complex formation indicates definite interactions (Shore and Santiago, 1975). Based on our experiments with strong inhibitors of ADH we anticipated somewhat similar changes in the spectrum of Co(II)ADH upon addition of C-nitroso substrates, which form Lewis acid complexes with the metal center (Maskos and Winston, 1993; Maskos and Winston, 1994).

While binding of strong ADH inhibitors and reduced coenzyme caused marked spectral changes, the normal substrates of ADH caused only minor if any changes in the Co(II)ADH spectrum (Sartorius et al., 1988). When the classical ADH substrate ethanol was added to Co(II)ADH, the d-d transition region of the spectrum was unaffected (Fig.2.5, trace 2); however, the subsequent addition of \( \text{NAD}^+ \), resulted in a strong shift of the 645 nm peak to 673 nm (Fig.2.5, trace 3). This observed 28 nm red shift reflects the formation of NADH in the ethanol oxidation reaction since \( \text{NAD}^+ \) binding to Co(II)ADH normally causes only broadening of the 645 nm peak with a minor red-shift (Sytkowski and Vallee, 1975). Pyrazole addition showed the classical splitting effect on the Co(II)ADH spectrum (Fig.2.5, trace 4). The small change in the d-d transition region of Co(II)ADH due to ethanol binding corroborates the finding of Shore et. al.
Figure 2.3  Effect of NADH and pyrazole on the d-d transition region of the electronic spectrum of Co(II)ADH.

1) Co(II)ADH (20 μM) in Tes buffer pH 7.4
2) Co(II)ADH + 0.5 mM NADH
3) Co(II)ADH/NADH + 8 mM pyrazole
Figure 2.4  Effect of pyrazole on the d-d transition region of the electronic spectrum of Co(II)ADH.
1) Co(II)ADH (20 µM) in Tes buffer pH 7.4
2) Co(II)ADH + 8 mM pyrazole
3) Co(II)ADH/pyrazole + 0.5 mM NADH
(Shore and Santiago, 1975), which showed that addition of certain ligands (N$_3^-$, SH$^-$, and CN) to Co(II)ADH caused only a small shift of the intensity of d-d band, while the addition of ions (F$^-$, Cl$^-$, or Br$^-$), did not affect the electronic spectra of Co(II)ADH.

Both NADH and the C-nitroso compounds under study absorb strongly in the 340 nm region; therefore, we are only able report their effect on the d-d transition region of cobalt-substituted ADH. When pNSP was added to the Co(II)ADH a 3 nm blue shift of 645 nm peak was observed (Fig.2.6, trace 2). The addition of NADH to the binary complex of ADH with pNSP resulted in the expected red shift of the 643 nm peak; however, the resulting peak was at 669 nm instead of expected 673 nm (Fig. 2.6, trace 3), suggesting the formation of ternary complex of ADH with pNSP and NADH. In this regard, we have been able to show that pNSP is capable of binding to the ADH-NADH binary complex in transient kinetic studies (Maskos and Winston, submitted). The subsequent addition of pyrazole to the ternary complex ADH/NADH/pNSP resulted in the classical bimodal splitting of the spectrum with absorbance maxima at 643 and 678 nm (Fig.2.6, trace 4).

When the order of addition was changed by adding NADH first to Co(II)ADH, the classical red-shift to 673 nm was observed (Fig.2.7, trace 2). The subsequent addition of pNSP caused a 4 nm blue-shift, and the increase in the absorbance of a shoulder that appears at 645 nm (Fig.2.7, trace 3). Apparently the formation of the ternary complex with pNSP resulted in the observed spectral shift from 673 to 669 nm; thus, pNSP appears to interact directly with the coordination sphere of the ADH metal center. The fact that the addition of pNSP to the complex ADH-NADH results in similar spectral changes as does the addition of NADH to ADH-pNSP complex.
Figure 2.5 Effect of ethanol on the d-d transition region of the electronic spectrum of Co(II)ADH.

1) Co(II)ADH (20 μM) in Tes buffer pH 7.4
2) Co(II)ADH + 0.2 mM ethanol
3) Co(II)ADH/ethanol + 2.5 mM NAD$^+$
4) Co(II)ADH/ethanol/NAD$^+$ + 8 mM pyrazole
Figure 2.6  Effect of pNSP on the d-d transition region of the electronic spectrum of Co(II)ADH.
1) Co(II)ADH (20 μM) in Tes buffer pH 7.4
2) Co(II)ADH + 80 μM pNSP
3) Co(II)ADH/pNSP + 0.5 mM NADH
4) Co(II)ADH/pNSP/NADH + 8 mM pyrazole
Figure 2.7  Effect of pNSP on the d-d transition region of the electronic spectrum of the binary complex Co(II)ADH/NADH.

1) Co(II)ADH (20 µM) in Tes buffer pH 7.4  
2) Co(II)ADH + 80 µM pNSP  
3) Co(II)ADH/pNSP + 0.5 mM NADH  
4) Co(II)ADH/pNSP/NADH + 8 mM pyrazole
Figure 2.8  Effect of pNSP on the d-d transition region of the electronic spectrum of the ternary complex Co(II)ADH/NADH/pyrazole.

1) Co(II)ADH (20 µM) in Tes buffer pH 7.4
2) Co(II)ADH + 0.5 mM NADH + pyrazole 8 mM
3) Co(II)ADH/NADH/pyrazole + 80 µM pNSP
Figure 2.9  Effect of DMNA on the d-d transition region of the electronic spectrum of Co(II)ADH.

1) Co(II)ADH (20 µM) in Tes buffer pH 7.4  
2) Co(II)ADH + 1 mM DMNA  
3) Co(II)ADH/DMNA + 0.5 mM NADH  
4) Co(II)ADH/DMNA/NADH + 5 mM pyrazole
indicates that the coenzyme-induced conformational change can occur before or after the binding of substrate. The addition of pyrazole to the ternary complex with pNSP again resulted in the classical bimodal splitting of the spectrum (Fig.2.7, trace 4).

In both experiments, the absorbance of the 643 nm peak of the bimodal spectrum was enhanced by the presence of pNSP. When pNSP was added to the ternary complex of Co(II)ADH/NADH/pyrazole, the absorbance of the 643 nm peak of the bimodal spectrum was increased (Fig.2.8, trace 3), confirming direct interaction of pNSP with the metal center of ADH.

As shown with pNSP, p-nitroso-N,N-dimethylaniline (DMNA) is a substrate for ADH-dependent reduction to the hydroxylamine and the amine (Dunn and Bernhard, 1971; Leskovac et al., 1996). The binary complex of DMNA with ADH showed a 3 nm blue-shift of the 645 nm peak (Fig. 2.9, trace 2). The addition of NADH resulted in the splitting of 645 nm peak into 646 and 672 nm peaks (Fig. 2.9, trace 3), which suggests the formation of stronger ternary complex between ADH and DMNA than between ADH and pNSP. The subsequent addition of pyrazole to the ternary complex ADH/NADH/DMNA resulted in the formation of the classical bimodal spectrum with absorbance maxima at 643 and 673 nm with enhanced absorbance of 643 nm peak (Fig.2.9, trace 4).

Dietrich et al. (Dietrich et al., 1979) suggested that the intensity of the d-d band of Co(II)ADH is proportional to the partial electrical polarizability of the ligand that binds to the binary complex. This was illustrated by an increase in the intensity of the 673 nm band upon binding of isobutyramide to the ADH-NADH complex (Dietrich et al., 1979), and agreed well with our present studies of C-nitroso compounds.
Spectral studies using EPR spectroscopy

Our binding studies confirmed direct interaction between the ADH active site metal atom and C-nitroso compounds. To obtain more detailed information on the mode of binding and coordination of the –N=O group of C-nitrosocompounds to the active site metal, we employed electron paramagnetic resonance (EPR) spectroscopy. High spin cobalt (II) ions are paramagnetic (S=3/2); thus they can be characterized on the basis of their EPR spectra. Resolution of EPR spectra requires that the sample temperature be decreased to liquid helium temperature to increase the electronic relaxation times and sharpen the EPR line widths. Figure 2.10a shows the typical EPR spectrum of active site cobalt-substituted ADH. The initial studies of the ternary complex of Co(II)ADH with NAD⁺ and pyrazole were used to establish conditions for the EPR experiments. The EPR spectrum of the ADH/NAD⁺/pyrazole ternary complex is shown in fig. 2.10b. The g-values were calculated using the formula $g = \frac{h\nu}{\beta B_0}$, where $h$ is Planck’s constant, $\nu$ is the microwave frequency, $\beta$ is the electron Bohr magneton, and $B_0$ is the magnetic field.

The EPR spectrum of this ternary complex showed resonance at $g = 7.1, 2.6,$ and $2.1$. The resonance at the low-field region centered at $g \approx 7.1$ shows hyperfine structure due to the $(I=7/2)^{59}$Co nucleus. In comparison to the corresponding absorption of free Co(II)ADH which is characterized by $g = 6.6, 2.3,$ and $1.6$ (Fig. 2.10a). The observed g-values were in agreement with the results by others (Drott, 1974; Drott et al., 1974; Werth et al., 1995) and could be interpreted in terms of a S=3/2 spin Hamiltonian.

To the best of our knowledge all of the EPR spectroscopic studies with Co(II)ADH reported to date used only unproductive ternary complexes such as
Figure 2.10  

a) EPR spectrum of Co(II)ADH;  


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ADH/NAD⁺/pyrazole. Unfortunately, we were unsuccessful in our attempts to obtain distinguishable EPR spectra of the productive ternary complex of ADH with pNSP and NADH using similar conditions.

**Determination of Kinetic Constants for pNSP Reduction by Co(II)ADH.**

The kinetic constants for the C-nitroso reductase activity of purified horse liver native Zn-containing ADH and the cobalt-substituted enzyme toward pNSP were determined and presented below. The active site-substituted enzyme was used for most of the studies and referred to as Co[c]Zn[n]ADH when used for comparison with the complete cobalt-substituted enzyme (Co[c]Co[n]ADH).

Both forms of the cobalt-substituted enzyme had essentially identical enzymatic activities; the specific activities for ethanol oxidation were about 50 % of that obtained with the native enzyme (Table 2.1), which was in good agreement with previous studies of cobalt-substituted ADH (Maret et al., 1979; Sytkowski and Vallee, 1975). Both complete and active site cobalt-substituted enzymes showed similar activity toward pNSP reduction as well (Table 2.1).

**Table 2.1. Enzymatic properties of Zn(II)ADH and Co(II)ADH.**

<table>
<thead>
<tr>
<th>ADH Samples</th>
<th>Metal Content</th>
<th>Specific Activity</th>
<th>pNSP Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zn²⁺</td>
<td>Co²⁺</td>
<td>EtOH oxidation</td>
</tr>
<tr>
<td>Native ADH</td>
<td>4.0</td>
<td>0</td>
<td>4.9</td>
</tr>
<tr>
<td>Co[c]Zn[n]ADH</td>
<td>2.1</td>
<td>1.8</td>
<td>2.5</td>
</tr>
<tr>
<td>Co[c]Co[n]ADH</td>
<td>0.2</td>
<td>3.7</td>
<td>2.3</td>
</tr>
</tbody>
</table>

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Kinetic constants for the enzymatic reduction of pNSP by NADH were determined at pH 7.4, under conditions pNSP > NADH > E; this ratio of reactants was optimal for precise estimation of kinetic constants at neutral pH. Figure 2.11 shows comparative Lineweaver-Burk plots for pNSP reduction by native Zn(II)ADH vs. Co(II)ADH obtained by monitoring pNSP disappearance at 400 nm, from which the following constants were calculated. Calculated Km and Vmax values for each are shown in Table 2.1.

When high concentrations of pNSP were used the bending of the double reciprocal plots upward was observed, suggesting substrate inhibition. Co(II)ADH showed a similar pattern when pNSP was in excess of 400 μM (not shown). Substrate inhibition was noted previously by Kuwada et al. (Kuwada et al., 1980) by an excess of pNSP (400 μM or more) and by excess of NADH (500 μM or more).

Substrate specificity is usually expressed in terms of kcat/Km, which represents the overall bimolecular rate constant for binding of substrate, hydrogen transfer, and release of product. This parameter is a measure of catalytic efficiency, and is not affected by non-productive binding, which would decrease kcat and Km proportionately (Sigman, 1967).

The kcat/KM of native ADH toward pNSP reduction was 3-fold greater than kcat/KM toward acetaldehyde reduction (115 mM^-1s^-1 vs. 38 mM^-1s^-1, respectively) (Dudley, 2000). Co(II)ADH appeared to be less efficient in catalyzing pNSP reduction than Zn(II)ADH (55 mM^-1s^-1 vs. 115 mM^-1s^-1, respectively). The lower efficiencies are attributable to much lower Vmax values associated with pNSP reduction.
Figure 2.11  **Double reciprocal plot of pNSP reduction by Zn(II)ADH and Co(II)ADH.** The reaction was assayed in the presence of 0.5 mM NADH and variable pNSP concentration (50-700 μM) in phosphate buffer, pH 7.4. The reaction was monitored by the disappearance of pNSP at 400nm.
We measured the production of \( pAP \) by an alternative method for measuring \( pNSP \) reduction that described in methods and materials and obtained slightly different data compared to direct measurements of \( pNSP \) disappearance. The kinetic constants for both methods are compared in Table 2.2. Nevertheless, the rate constants and efficiencies are of the same order of magnitude, all kinetic constants were lower for \( pAP \) formation by the native enzyme, whereas \( k_{\text{cat}} \) of \( pNSP \) reduction catalyzed by Co(II)ADH was not notably different. The difference in the rate of \( pAP \) formation as compared to \( pNSP \) disappearance for native ADH could be attributed to the alternative pathways of \( pNSP \) reduction by ADH (see discussion) (Maskos and Winston, 1994). The small changes in the reaction rates between the two methods for Co(II)ADH may be due to the higher substrate affinity of Co(II)ADH (see discussion).

<table>
<thead>
<tr>
<th>ADH</th>
<th>( pNSP ) disappearance</th>
<th>( pAP ) formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( k_{\text{cat}} ) s(^{-1})</td>
<td>( \text{Km} ) ( \mu \text{M} )</td>
</tr>
<tr>
<td>Zn(II)ADH</td>
<td>26.4 ± 2.1</td>
<td>230 ± 31</td>
</tr>
<tr>
<td>Co(II)ADH</td>
<td>7.2 ± 1.3</td>
<td>139 ± 18</td>
</tr>
</tbody>
</table>

It was anticipated that higher Lewis acidity of Co(II)ADH would have enhanced the rate of \( pNSP \) reduction because the excessive electron withdrawal by the metal ion should favor hydride transfer from NADH to \( pNSP \). However, a lower \( k_{\text{cat}} \) and efficiency for \( pNSP \) reduction was observed. Thus, factors other than Lewis acid strength govern \( pNSP \) reduction catalyzed by ADH.
**Effect of pH on pNSP reduction by Co(II)ADH**

A direct resolution of the molecular origins of pKa values can be achieved by identifying the ionizations of metal-bound ligands. Metal substitution provides an experimental basis for metal-sensitive ionizations. For instance, substitution of the open-shell Co(II) ion for the closed shell Zn(II) ion may be expected to result in a detectable decrease in the pKa of the metal bound ligand (Maret and Makinen, 1991).

Initial velocities of the irreversible reduction of pNSP were measured in zwitterionic buffer over the pH range of 5.8 - 8.9. In this range of pH two tautomeric forms can exist in the reaction mixture, the quinone monoxime and the nitroso forms; equilibrium between these two forms involves the mesomeric common ion (Maskos and Winston, 1999). Maskos and Winston have determined that the pK value for the ionization of free pNSP is 6.20 (Maskos and Winston, 1999). The total concentration of pNSP during steady-state experiments was varied from 50 to 700 μM, with NADH kept constant at 500 μM. The v vs. S plots shown in figure 2.12 show that each of the ADH enzymes approximated Michaelis-Menten kinetics at all pH values studied.

The reaction between the enzyme and pNSP showed strong pH dependence. The influence of pH on the kinetic parameters kcat and kcat/Km in the reduction of pNSP is compared in Fig. 2.13 for Zn(II)ADH and Co(II)ADH.

The ionizations pK₁ and pK₂ are observed in the pH profile of kcat, whereas pKₐ and pKₐ are observed in the pH profile of kcat/Km. Although the profiles are similar for the two enzymes, the limiting values of kcat/Km are lower for Co(II)ADH than for the native enzyme in the higher pH range. The acidic portion of the pH profiles of kcat shows a distinct ionization with pKa ~ 6.3-6.6 for both metalloenzymes (Fig.2.13a).
This is consistent with the pK for single protonic ionization of pNSP, pK = 6.20 (Maskos and Winston, 1999). pK₂ values, could not be determined accurately for Zn(II)ADH; however, they appeared to be at least one pH unit lower than for Co(II)ADH with pK₂ around 8.3.

The pH profile of log kcat/Km for both metalloenzymes is essentially identical in the acidic range. In the alkaline range, the profile for Co(II)ADH is similar in shape to that of Zn(II)ADH but is markedly shifted to lower pH values (Fig. 2.13b). The pKₐ value of 6.8, which is not related to protonation of the substrate, is presumed to be related to a group in the NADH-ADH complex that requires protonation for the formation of the productive NADH-ADH-pNSP complex. The observed pK values of 6.80 is very close to the value expected for the imidazolium moiety of histidine, which has a pK = 6.9.

We observed that an ionization with pK₂ \sim 9, which governs kcat and an ionization with pKₐ > 9, which governs kcat/Km were decreased by substitution of Co(II) for the active site Zn(II). The similar pattern for metal-dependent ionizations was observed by Makinen et al. (Makinen et al., 1983) and was ascribed to metal bound water.

In the oxidation of 2-propanol and benzyl alcohol (Maret and Makinen, 1991) the ionizing groups governing kcat and kcat/Km were altered by metal substitution as well, whereas the value of pK₁ and pKₐ were insensitive to metal substitutions, which corroborates our results with pNSP.
Figure 2.12  Time course of pNSP reduction by Co(II)ADH at various pH. Reactions were assayed in the presence of 0.5 mM NADH and variable pNSP concentrations (50-700 μM) in zwitterionic buffers (0.1 M MES, 0.051 M NEA, 0.051 M DEA). The reaction was monitored by formation of pAP.
Figure 2.13 pH-dependence of kinetic constants $k_{cat}$ (a) and $k_{cat}/K_m$ (b) for pNSP reduction by native and cobalt substituted ADH. Reactions were assayed in the presence of 0.5 mM NADH and variable pNSP concentration (50-700 µM) in zwitterionic buffers (0.1 M MES, 0.051 M NEA, 0.051 M DEA three component buffer system). The reaction was monitored by formation of pAP.
NADH-Dependent pNSP Reduction by Native and Cobalt-Substituted ADH: Effect of Acetaldehyde and Pyrazole

Because the C-nitroso moiety is isosteric and isoelectronic with the aldehyde functional group (Dunn and Bernhard, 1971) it was anticipated that acetaldehyde would competitively inhibit pNSP reduction by ADH. Inhibition by acetaldehyde was indeed competitive with respect to pNSP (Dudley, 2000).

Fig. 2.14 shows a Lineweaver-Burk plot for Co(II)ADH-catalyzed pNSP reduction at pH 7.4 in the absence and presence of 4.75 mM acetaldehyde. Changes in $K_m$ without concurrent changes in $V_{max}$ support the competitive nature of inhibition of pNSP reduction by acetaldehyde. Similar to acetaldehyde, pyrazole competitively inhibited the NADH-pNSP reductase reaction catalyzed by native ADH (Dudley, 2000).

In the present studies with Co(II)ADH the addition of 5 mM pyrazole competitively inhibited NADH-pNSP reduction as well. Figure 2.15 shows the effect of pyrazole on the C-nitrosoreductase activity of Co(II)ADH by monitoring the formation of $p$AP. However, the pattern of inhibition was changed when the concentration of pyrazole was varied. Competitive inhibition was observed only at high pyrazole concentrations (0.1-15mM), while classical noncompetitive inhibition was observed at low pyrazole concentrations (< 50μM) (Fig.2.16).

We envision this process as follows: In the presence of pNSP, the pyrazole, at low concentration, binds to E or to ES to form either EI or ESI complex and presents noncompetitive inhibition. The combination of the pyrazole, pNSP and enzyme alters the conformation of the active site geometry, which becomes more apparent with increasing pyrazole concentration. This distorts the substrate binding site and thereby

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Figure 2.14  Double reciprocal plot of the inhibitory effect of 5 mM acetaldehyde on pNSP reduction by Co(II)ADH. The reaction was assayed in the presence of 0.5 mM NADH and variable pNSP concentrations (50-700 μM) in phosphate buffer, pH 7.4. The reaction was monitored by pAP formation.
Double reciprocal plot of the inhibitory effect of 5 mM pyrazole on pNSP reduction by Co(II)ADH. The reaction was assayed in the presence of 0.5 mM NADH and variable pNSP concentrations (50-700 μM) in phosphate buffer, pH 7.4. The reaction was monitored by pAP formation.
Figure 2.15  **Double reciprocal plot of the inhibitory effect of variable pyrazole concentration on pNSP reduction by Co(II)ADH.** The reaction was assayed in the presence of 0.5 mM NADH and variable pNSP concentrations (50-700 μM) in phosphate buffer, pH 7.4. The reaction was monitored by pAP formation.
hinders substrate binding giving the appearance of competitive inhibition at higher pyrazole concentrations. The resulting complex would become catalytically inactive, since inhibitor might prevent the proper positioning of the catalytic center. The ability of pyrazole to cause a strong change in the coordination sphere of Co(II)ADH was shown in figure 2.4.

DISCUSSION

Herein, the effects of C-nitroso compounds on the electronic spectra of Co(II)ADH are evaluated. C-nitroso reductase activities of the native and cobalt substituted ADH are compared using pNSP as an aldehyde-substrate analogue.

The catalytic metal of ADH is coordinated through a 2 Cys, 1 His and 1 water ligand motif, which results in a distorted tetrahedral coordination geometry and the corresponding d-d transition band at 645 nm in the visible spectrum of Co(II)ADH. The structural cobalt center, which is coordinated by four cysteines in near perfect tetrahedral geometry, is reflected in the d-d transition band at 740 nm.

Our results showed that both binary and ternary complexes of pNSP with Co(II)ADH resulted in a blue shift of the d-d transition region of Co(II)ADH spectra. The spectral changes were relatively small compared to the marked changes caused by the unproductive ternary complex with NADH and pyrazole, but they were stronger when compared to the productive complexes with ethanol. This suggests a stronger influence of nitrogen bound oxygen over carbon bound oxygen on the active site geometry.

Identification of the ligands that comprise the coordination sphere is not obtainable from the electronic spectrum of the cobalt enzyme. However, changes in the
d-d transition region of cobalt-substituted ADH upon binding of either pNSP or DMNA to the binary complex of ADH with NADH indicate a direct interaction of the C-nitroso moiety with the enzyme metal active site.

In addition to the blue shift of the d-d spectrum of the binary complex with NADH, both pNSP and DMNA enhance the absorbance of the 643 nm peak of the ADH/NADH/pyrazole ternary complex, which suggest that C-nitroso compounds interact with the metal center even when pyrazole is occupying the binding site, via simple competition with pyrazole or via the formation of an additional ligand on the metal center. Pentacoordinate coordination usually results in attenuation of the absorbance in d-d transition region; therefore, the observed increase in the absorbance upon pNSP or DMNA binding apparently does not contribute an additional ligand.

The binding of the coenzyme, which has to precede the binding of the substrate, leads to considerable red shift of the d-d band. The coenzyme-triggered conformational change of the protein involving the catalytic metal ions results in a modulation of the Lewis acid strength of the metal, which is able to coordinate and activate the substrate (Dietrich et al., 1979).

Our studies established that C-nitroso substrates of ADH can enter the ADH catalytic site regardless of the presence for the coenzyme; however, the coenzyme is necessary for productive substrate binding during the subsequent reduction by ADH.

In these studies pNSP was used as an aldehyde-substrate analog to compare the C-nitroso reductase activities of the native and cobalt-substituted ADH. The strength of the metal ion as a Lewis acid is directly correlated with the rate of hydride transfer in aldehyde reduction. Shore and Santiago (Shore and Santiago, 1975) showed that the rate
of hydride transfer in alcohol oxidation is slower for the Co(II) substituted enzyme than for the native ADH. This is consistent with the slower rate of pNSP reduction by Co(II)ADH compared to the native Zn-containing enzyme.

Replacement of Zn(II) by Co(II) undoubtedly changes the specific rate constants for certain steps in the mechanism of pNSP reduction, and results in the decrease of both kcat and kcat/Km values. The lower K_m for Co(II)ADH may be attributed to the ability of the enzyme to bind pNSP more productively; therefore, the complex ADH-NADH-pNSP is favored with Co(II)ADH over native ADH. These differences are similar to the kinetic properties exhibited by the cobalt-substituted ADH in the reaction of trans-4-dimethylamino cinnamaldehyde with the enzyme-bound NADH (Morris et al., 1980).

It is of more significance that the rate of pNSP reduction catalyzed by Co(II)ADH is not significantly different to that of pAP formation. The difference in the rate of pAP formation over pNSP disappearance for native ADH can account for the formation of other products of pNSP reduction, which was suggested by Maskos and Winston (Maskos and Winston, 1994) for HLADH. Small changes in the reaction rates for Co(II)ADH suggest that higher substrate affinity of Co(II)ADH prefers a direct mechanism of pNSP reduction as described in Chapter 1. Two alternative pathways of pNSP reduction were shown only for the horse liver enzyme, while some human isozymes (π- and αα- ADH) appear to proceed by only one pathway leading to pAP formation without release of BQI from the active site (Maskos and Winston, submitted). Therefore, the substitution of Co(II) for Zn(II) appears to alter the mechanism of the reaction between NADH and pNSP.
As in alcohol oxidation, the overall \( p_{\text{NSP}} \) reduction rate is likely dependent on coenzyme-binding residues since the rate-limiting step has been shown to be the dissociation of coenzyme. Both \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_m \) values for \( p_{\text{NSP}} \) showed significant variations with pH. The data shows that as pH is increased there is a concurrent decrease in \( p_{\text{AP}} \) formation, i.e. C-nitrosoreduction.

The acidic portion of the pH profiles of \( k_{\text{cat}} \) shows a distinct ionization with pKa ~ 6.3 for both metalloenzymes, which is consistent with the pKa for single protonic ionization of \( p_{\text{NSP}} \) (pK = 6.2) reported by Maskos (Maskos and Winston, 1999). Although the profiles are similar for the two enzymes, the limiting values of \( k_{\text{cat}}/K_m \) are lower for Co(II)ADH than for the native enzyme in the higher pH range, which shows that the alkaline ionization, ascribed to the metal bound water, has been shifted to a lower pKa value of 8.3 due to a metal-substitution. The interpretation of this change is that the metal geometry at high pH having a negatively charged ligands is different from the geometry at lower pH.

Kvassman and Pettersson (Kvassman and Pettersson, 1979), have proposed that the pH-dependence of NADH binding is attributable to electrostatic effects of the coenzyme pyrophosphate group on the ionization of zinc-bound water, which has pK value around 9.2. Therefore the metal substitution reduced the pKa value of water ionization by about one pH unit. Similar results were obtained for the pH dependence of benzyl alcohol oxidation catalyzed by Co(II)ADH (Maret and Makinen, 1991). Cobalt-substituted ADH is proven to be a valuable tool for the study of the involvement of the metal center of ADH in the catalytic mechanism of novel substrate, such as C-nitroso and N-nitroso compounds reported herein.
Chapter 3: Reduction of N-Nitroso Compounds by Alcohol Dehydrogenase in Their Activation to Mutagenic and Carcinogenic Endpoints

INTRODUCTION

Aside from ethanol, ADH catalyzes the transformation of many other substrates such as bile compounds, testosterone, neurotransmitters, congeners, retinol, peroxidic aldehydes, mevalonate, norepinephrine, fatty acids and C-nitroso compounds (Edenberg et al., 1996; Holmes, 1994; Jörnvall, 1994; Jörnvall et al., 1996; Kedishvili et al., 1996). The ADH family of enzymes catalyzes a variety of xenobiotics; thus may be considered as phase I oxidases analogous to cytochrome P450 and flavine monooxygenase (Amdur et al., 1991). In fact, some investigators have suggested that the contribution of ADH to alcohol metabolism may have been previously overestimated (Bradford et al., 1993). In studies of ADH +/- deer mice it was noted that the contribution of catalase to ethanol oxidation was about 50% at low doses of ethanol and approached 100% as the alcohol concentration was elevated (Bradford et al., 1993). Recently Swensson et al. (Svensson et al., 1996) showed an aldehyde dismutase activity catalyzed by human liver ADH. It was also shown that C-nitroso compounds could be reduced by different isozymes of ADH to their corresponding amines (Hajos and Winston, 1992; Maskos and Winston, 1993; Maskos and Winston, 1994). Some of possible intermediates of this reduction, which arises from a novel rearrangement of hydroxyl amines catalyzed by horse liver ADH result in the formation of the extremely potent ultimate carcinogen, aryl nitrenium ion (Maskos and Winston, 1993). Therefore, the evaluation of ADH activity toward different substrates will aid in understanding of not only its physiological role, but also its role in xenobiotic transformation reactions. In light of the ability of ADH to
biotransform and activate certain C-nitroso compounds (Horie et al., 1980; Horie et al., 1982; Leskovac et al., 1996; Maskos and Winston, 1994; Novak et al., 1993), the primary objective of this chapter is to characterize the reduction of carcinogenic N-nitroso-compounds by horse liver alcohol dehydrogenase (HLADH).

Most of the research on N-nitroso compounds suggested that carcinogenic species were produced via oxidation, sulfation or transamination reactions (Eisenbrand et al., 1984; Farrelly et al., 1986). Nitroso compounds (the two electron reduction product of nitro compounds) can be reduced to their corresponding hydronitroxide even by mild reducing agents; thus, the reduction of N-nitroso compounds in vivo is also feasible (Rickert, 1985).

The reduction of C-nitroso compounds by ADH is well established (Hajos and Winston, 1992; Horie et al., 1980; Maskos and Winston, 1993; Maskos and Winston, 1994). Therefore, the activation of N-nitroso compounds, such as N-nitrosodiethanolamine (NDELA) by the ADH pathway could proceed via at least two different mechanisms. The first is via oxidation of the hydroxyl group to corresponding aldehyde, and the second is via reduction of the nitroso group to the corresponding amine. Both reactions could produce numerous short-lived active intermediates, with potential toxicity to cells.

A characteristic of NDELA metabolism is that up to 95% of administered NDELA is excreted unchanged in urine of the rat model (Farrelly et al., 1986). Even with 5% of NDELA metabolized in the body, this compound showed a strong mutagenic response (Farrelly et al., 1986). N-nitroso-2-hydroxymorpholine (NHMOR) has been detected as a metabolite of NDELA in rat liver S9 fractions.
NMOR is one of the most carcinogenic of the N-nitroso compounds, and preferentially exists in the 2-hydroxy-N-nitrosomorpholine (NHMOR) hemiacetal form (Hecht et al., 1989) (Fig. 3.1). In contrast to NDELA, NMOR is extensively metabolized in rats (1-2% dose unchanged in urine) (Hecht et al., 1989). Established pathways of metabolism include α-hydroxylation and β-hydroxylation to respectively, NHMOR and NDELA, and denitrosation.

6-Alkylnitrosaminoethanols, including NDELA, undergo efficient liver ADH-catalyzed oxidation to their corresponding α-nitrosamino aldehydes (Loeppky et al., 1987). Involvement of ADH in the activation of NDELA to mutagenic endpoints was also supported by Henn et al. (Henn et al., 1989), who reported that ADH from yeast and horse liver induced chromosomal mutations and sister chromatid exchange (SCE) by NDELA in human lymphocyte cultures.

Although the importance of ADH in the activation of these compounds has been established, no mechanistic or kinetic data are available. Most of intermediates are short lived and their existence was only proposed in many cases (Eisenbrand et al., 1986). The reduction of C-nitroso compounds by ADH produces strong electrophiles capable of causing DNA damage as well (Fuji et al., 1994; Maskos and Winston, 1993).

The wide distribution of N-nitroso compounds in the environment coupled with the evidence implicating ADH in the activation of NDELA to its mutagenic endpoints, we explored the possible role of ADH in the reduction of the nitroso group of NDELA and other major industrial N-nitroso compounds. Our hypothesis was that ADH-dependent reduction of N-nitroso substrates would lead to formation of N-hydroxylamines, which analogous to C-hydroxylamines, could be proximate mutagens.
Figure 3.1  Chemical structure of related N-nitroso compounds.
In addition to N-nitrosodiethanolamine (NDELA), which carries two functional groups susceptible to both oxidation (hydroxyl group) and reduction (nitroso group), we studied the structurally analogous N-nitrosodiethylamine (NDEA), and two nitrosoureas, i.e. N-nitroso-N-methylurea (NMU) and N-nitroso-N-ethylurea (NEU). All these compounds are carcinogens in vivo and carry a –N=O group susceptible to reduction (Guttenplan, 1993). The chemical structures of these compounds are shown on Fig. 3.1.

MATERIALS AND METHODS

Enzymes and Reagents

Horse liver alcohol dehydrogenases were obtained from Boehringer Mannheim, Germany. The concentration of ADH was determined from the absorbance at 280 nm (for HLADH ε=35.7×10³ M⁻¹cm⁻¹ (Chen et al., 1987)), and for YADH ε=189×10³ M⁻¹cm⁻¹ (Magonet et al., 1992)). βNAD⁺ (> 99% purity) was purchased from Boehringer Mannheim, Germany. NADH (98% purity) was purchased from Sigma Chemical Co., St. Louis, MO. Coenzymes were used without further purification. Concentrations of the coenzyme solutions were determined spectrophotometrically from the extinction coefficients of 18.0×10³ M⁻¹cm⁻¹ at 260 nm for NAD⁺ and of 6.22×10³ M⁻¹cm⁻¹ at 340 nm for NADH. N-nitroso compounds were obtained from Aldrich. All other chemicals were of analytical grade.

Enzyme Assay

Spectrophotometric assays were conducted using Perkin-Elmer Lambda 6 UV-Vis spectrophotometer. Reactions were conducted in quartz cuvettes of 1 cm light path.
HPLC Analysis

The analysis of the reduction of N-nitroso compounds was performed on Rainin Rabbit-HP reverse-phase HPLC system, equipped with an Alltech Econosphere C-8 5 μ column (150 x 4.6 mm). The solvent conditions are noted for each experiment. The eluent was monitored by “Knauer” UV/Vis detector at specified wavelength.

RESULTS

Reduction of N-Nitrosodiethanolamine (NDELA)

The UV/Vis spectrum of NDELA has an absorbance maximum at 344 nm ($\varepsilon_{344} = 103 \text{ M}^{-1}\text{cm}^{1}$). Spectrophotometric studies of NDELA reduction kinetics by ADH are complicated by the overlap of its 344 nm absorbance band with the strong 340 nm absorbance band of NADH ($\varepsilon_{340} = 6220 \text{ M}^{-1}\text{cm}^{1}$). Therefore, spectroscopy was used only to monitor the initial steps of the reaction, which was accomplished by measuring the concomitant disappearance of the 340 nm band represented by both NADH and NDELA. The dissociation constant for NADH is 1 μM (Plapp, 1973), therefore all reactions contained at least a 10-fold excess of NADH. Since an overlap of the spectra occurs, the concentrations of NDELA and NADH were adjusted to give equal absorbance at 340 nm (1-3mM of NDELA and 25-100 μM of NADH). In this manner the changes associated with the consumption of both species could be monitored. The 344 nm absorbance band of NDELA was not significantly altered over the pH range 5.9-9.0 (not shown).

NADH-dependent reduction of NDELA was determined in the presence and absence of ADH by scanning over the range of 250-500 nm at 30 seconds intervals. The decrease in absorbance of the 340 nm band would correspond to substrate
Figure 3.2  **NADH-dependent NDELA reduction by HLADH.** 3 mM NDELA was incubated with 100 μM NADH and ADH was added. The rate of disappearance of 340 nm absorbance was obtained from 30 sec interval scans over the range of 250 – 500 nm in Tes buffer pH 7.4, 1) Nonenzymatic reaction, 2) Addition of 0.075 μM ADH; 3) addition 0.75 μM ADH.
consumption. It was anticipated that within this range of the spectrum, product formation would be seen as newly formed chromophores. Upon addition of 100 μM NADH to 3 mM NDELA in the absence of ADH, a steady decrease in the 340 nm absorbance of 0.5 mAU/min/ml over the first 5 minutes of the reaction was observed. When 0.075 μM ADH was included in the assay the rate of 340 nm loss was increased 3-fold, i.e. to 1.5 mAU/min/ml. When the ADH concentration was increased to 0.75 μM, the reaction rate increased to only 2.4 mAU/min/ml (Fig. 3.2).

Because of the overlap in the NDELA and NADH spectra, the rate of substrate consumption was calculated assuming that the loss of absorbance at 340 nm was due solely to the disappearance of only one of the two substrates by applying the extinction coefficient for each of the substrates independently. Based on this assumption, the nonenzymatic rates of 5 nmol/min/ml for NDELA or 0.08 nmol/min/ml for NADH were obtained. The addition of enzyme doubled the rate of the absorbance loss, which corresponds to a calculated rate of about 10 nmol/min/ml for NDELA or 0.16 nmol/min/ml for NADH. Since the enzymatic activity of ADH toward NDELA was confirmed, in order to more rigorously assign a rate to the catalytic reaction we performed HPLC analysis of NDELA reduction. A single peak corresponding to NDELA with a retention time of 3.5 min was well separated from the NADH peak (RT 1.9 min) (Fig. 3.3a). The area under the curve of the NDELA peak was quantitatively related to the amount of NDELA injected into the system and was used to calculate NDELA consumption. When 0.3 mM NDELA was incubated with 0.65 mM NADH, a slow disappearance of the NDELA peak corresponding to a rate of 0.007 nmol/min/ml was observed (Fig. 3.4). When 0.75 μM ADH was included in the reaction the rate of
Figure 3.3  HPLC analysis of NDELA reduction by ADH after 5 min (a) and after 60 min (b). A mixture of 0.3 mM NDELA, 0.65 mM NADH was separated on a reverse phase C-8 column using 95% water and 5% isopropanol as a mobile phase at flow rate 1 ml/min, and detection at 235 nm.
Figure 3.4  **HPLC analysis of NDELA reduction by ADH.** A mixture of 0.3 mM NDELA, 0.65 mM NADH and 0.85 μM ADH was separated on a reverse phase C-8 column using 95% water and 5% isopropanol as a mobile phase at flow rate 1 ml/min, and detection at 235 nm.
NDELA disappearance during the first 15 min increased by 7-fold to 0.049 nmol/min/ml (Fig. 3.4).

Comparing the rates from HPLC and spectroscopic experiments, the change of absorbance at 340 nm appeared to be mainly due to NADH disappearance, which proceeded at the estimated rate of ~ 0.12 nmol/min/ml; while NDELA was consumed at the rate of ~ 0.05 nmol/min/min. The stoichiometry for enzymatic reduction of NDELA indicates that about two molecules of NADH are consumed during the reduction of one NDELA molecule. This is consistent with subsequent two 2-electron reductions, presumably reduction of the nitroso group to a hydroxylamine followed by reduction of the hydroxylamine to the amine (Maskos and Winston, 1994). The eluent with 1.6 min RT was formed during the enzymatic reduction of NDELA and corresponds to NAD⁺ formation (Fig. 3.3b). The other eluent with 4.6 min RT was also formed during the enzymatic reduction of NDELA (Fig. 3.3b). The peak was undetectable at the beginning of the experiment (Fig. 3.3a) and become more apparent as NDELA was consumed for up to 60 min (Fig. 3.3b) and then slowly decreased (Fig. 3.4). The identity of the 4.6 min RT peak remains to be established.

Among the C-nitroso compounds studied, (Dunn and Bernhard, 1971; Maskos and Winston, 1993; Maskos and Winston, 1994; Pantelic et al., 1996) the kinetic constants were influenced by specific proton ionizations as indicated by pH vs. activity and/or binding measurements. Therefore, the rate of NDELA reduction by ADH was studied as a function of pH in a reaction containing 3 mM of NDELA with 50 μM NADH and addition of 1.5 μM of ADH in Tes buffer at pH 6.6, 7.3, 7.9 and 9. All reactions were linear for at least 1 minute. The rates of the reactions were calculated...
from the velocity determined in first 60 seconds of the reaction. The data are presented as the pH-dependence of the absorbance change at 340 nm (Fig. 3.5). The pH profile was bell-shaped with a maximum at pH 7.9, suggesting the presence of an ionizable group on NDELA with pK value of about 7.9.

Our studies showed the enzymatic nature of NDELA reduction by ADH. Previous results from our laboratory indicated Lewis acid complex formation between the nitroso oxygen of 2-nitroso fluorine and the Zn atom of horse liver ADH (Maskos and Winston, 1993). This complex was essential in the catalytic reduction of the C-nitroso moiety of the substrate. To determine whether such a complex could be visualized during ADH-dependent reduction of NDELA, we synthesized cobalt(II)-substituted ADH (Co(II)ADH) to monitor possible coordination of NDELA to the active site metal atom. Such information cannot be ascertained from the Zn-enzyme because Zn, a d\(^{10}\) element, is neither optically nor magnetically active. The cobalt-substituted enzyme on the other hand is paramagnetic and has an optically active electronic spectrum that reflects ligand to metal charge transfer (LMCT) and d-d orbital transitions (Werth et al., 1995).

When NDELA was added to Co(II)ADH the d-d transition region of the Co(II)ADH spectrum with the absorption maximum at 645 nm remained essentially unchanged (Fig. 3.6). The addition of NADH to the binary complex ADH-NDELA gave the red shift of the d-d transition band characterized by a splitting of the d-d transition band into two chromophores with maxima at 625 and 674 nm (Fig. 3.6, trace 3). The typical spectrum of the d-d transition region of the binary complex ADH-NADH is usually represented with two peaks at 642 and 677 nm (Chapter 2). The small
Figure 3.5  The rate of NDELA reduction by ADH as a function of pH. The reaction mixture contained 3 mM of NDELA, 50 μM NADH, and 1.5 μM of ADH. in Tes buffer at pH 6.6, 7.3, 7.9 and 9. All reactions were linear for at least 1 minute. The data are presented as the pH-dependence of the absorbance change at 340 nm.
Figure 3.6 Effect of NDELA on d-d transition region of the electronic spectrum of Co(II)ADH.
1) Co(II)ADH (20 μM) in Tes buffer pH 7.4
2) Co(II)ADH + 2 mM NDELA
3) Co(II)ADH/NDELA + 1 mM NADH
4) Co(II)ADH/NADH/NDELA + 10 mM pyrazole
blue shift of 677 nm peak to 674 nm peak along with the strong blue shift of 642 nm peak to 625 nm suggests the influence of NDELA on the metal coordination sphere and the ternary complex formation.

The subsequent addition of the ADH inhibitor pyrazole to the ternary complex ADH-NDELA-NADH resulted in the formation of the bimodal spectrum with absorbance maximums at 635 and 677 nm (Fig. 3.6, trace 4). The observed splitting in the d-d transition region of Co(II)ADH is usually observed with the formation of the ternary complex ADH-NAD^-Pyrazole (Sytkowski and Vallee, 1975); however, the classical shift is usually represented by formation of 640 and 677 nm. Apparently formation of the ternary complex with NDELA resulted in the observed spectral shift from 640 to 635 nm; thus, the coordination sphere of the ADH metal center was affected by NDELA, albeit the nitroso group of NDELA did not alter the integrity of the coordination sphere of the oxygen sensitive cobalt.

**Reduction of N-nitrosodiethylamine (NDEA)**

As in the UV/Vis spectrum of NDELA, the absorbtion maximum of NDEA is near 340 nm, i.e. 336 nm (ε₃₃₆ = 79 M^⁻¹ cm⁻¹) and causes a similar spectroscopic interference with NADH. Again the concominant disappearance of 340 nm absorbance, reflecting both NADH and NDEA, was monitored and the concentrations of substrate and coenzyme were adjusted to give equal absorbance (2 mM of NDEA and 100 μM of NADH). The reaction was monitored by scanning the range of 200-700 nm to detect possible product formation, and by scanning between 300 and 400 nm at 30 seconds intervals for kinetic measurements.
Figure 3.7  NADH-dependent NDEA reduction by HLADH. 2 mM NDEA was incubated with 100 μM NADH for 2 minutes and 0.75 μM ADH was added. The rate of disappearance of 340 nm absorbance was obtained from 30 sec intervals scans over the range of 250 – 500 nm in Tes buffer pH 7.4.
Upon addition of 100 μM NADH to 2 mM of NDEA, the $A_{340}$ remained essentially unchanged; thus, NDEA apparently was not reduced by NADH in the absence of ADH. When 0.75 μM ADH was added to the reaction mix the $A_{340}$ decreased rapidly within 30 sec at a rate of 41 mAU/min/ml (Fig. 3.7). The reaction was linear for 30 sec and plateaued after 1 min. The spectral analysis did not show formation of any chromophoric products in the 200-700 nm region. The short burst phase was observed at all ADH and NDEA concentrations studied suggesting the nonspecific reaction with NADH with NDEA by-products. The subsequent HPLC analysis did not confirm NDEA consumption, suggesting that the loss of absorbance at 340 nm was due solely to NADH consumption in the nonspecific reaction.

**Reduction of N-nitroso ethylurea (NEU)**

The UV/Vis spectrum of NEU has an absorbance maximum at 395 nm ($\varepsilon_{395}=500$ M$^{-1}$cm$^{-1}$), which only partially overlaps the NADH spectrum. Thus, we were able to measure the concomitant disappearance of the 340 nm and 395 nm bands, respectively reflecting the cofactor and the substrate. In order to achieve the optimum spectral separation and prevent overlapping of the spectra the concentrations of substrate and coenzyme were used within the concentration range allowing to monitor changes in the NADH and NEU spectra simultaneously. The reaction course was scanned over the range of 280-500 nm at 20 sec intervals.

When NADH was added to NEU, the small decrease in absorbance of both bands at 340 and 395 nm was observed at a rate of 0.2 and 0.5 mAU/min/ml respectively (Fig. 3.8). Subsequent addition of 0.075 μM ADH increased the rate of $A_{340}$ disappearance over 25-fold, to 5.3 mAU/min/ml during the first minute of the
reaction, which was followed by a slow linear phase at a rate of 0.3 mAU/min/ml (Fig. 3.8). The decrease in A\textsubscript{395} was linear throughout the entire reaction course at 0.7 mAU/min/ml (Fig. 3.8). The apparent enzymatic rate of substrate disappearance was estimated as 0.82 nmol/min/ml for NADH and 0.44 nmol/min/ml for NEU.

Hence, the stoichiometry of the enzymatic reduction of NEU was about two molecules of NADH consumed for each NEU molecule reduced. This is consistent with subsequent 2e\textsuperscript{-} reduction steps, presumably reduction of the nitroso group to a hydroxylamine followed by reduction of the hydroxylamine to the amine. Spectroscopic analysis revealed no changes in the 200-700 nm region that could be attributed to formation of products of the NEU reduction.

To better resolve potential products of NEU reduction by ADH, we performed HPLC analysis of this reaction. NEU eluted as a single peak with a R.T. of 5.1 min and was well separated from the peak corresponding to NADH, which has a R.T. of 1.3 min (Fig. 3.9). The area under the curve of the NEU peak was directly related to the amount of NEU injected into the system and was used to calculate NEU consumption. When 0.2 mM NEU was mixed with 0.1 mM NADH in Tes buffer, pH 7.5, the NEU peak disappeared at a rate of 0.04 nmol/min/ml (Fig. 3.10). In a parallel reaction addition of 0.38 μM ADH resulted in 3-fold increase in the rate of NEU disappearance. The reaction was characterized by a fast phase during the first 20 min, in which NEU was consumed at a rate of ~ 0.14 nmol/min/ml and a slow phase (~0.02 nmol/min/ml) after that (Fig. 3.10).

Several peaks were formed in the HPLC spectrum during the course of the reaction with R.T. between 2 and 3 min. Their intensity increased as NEU was
Figure 3.8  NADH-dependent NEU reduction by HLADH. 1 mM NEU was incubated with 10 μM NADH for 5 min followed by addition of 0.075 μM ADH. The absorbance was monitored over 250 – 500 nm in 30 sec intervals scans. The relative rate of absorbance loss at 340 and 395 nm are shown.
Figure 3.9  HPLC chromatogram of NEU reduction mixture with NADH. A mixture of 0.2 mM NEU and 0.1 mM NADH was separated on reverse phase Alltech C-8 column using an isocratic mobile phase of 95% water and 5% isopropanol with a flow rate of 1.5 ml/min, and detection at 235 nm.
Figure 3.10  **HPLC analysis of NEU reduction mixture with NADH.** A mixture of 0.2 mM NEU, 0.1 mM NADH and 0.38 μM was separated on reverse phase Alltech C-8 column using an isocratic mobile phase of 95% water and 5% isopropanol with a flow rate of 1.5 ml/min, and detection at 235 nm.
consumed; however, they were at the detection limits of the instrument, which did not permit accurate quantification.

Our studies showed a clear dependence of NEU reduction on the enzymatic reaction of ADH. Previous results from our laboratory indicated a Lewis acid complex formation between the nitroso oxygen of 2-nitroso fluorine and the Zn atom of HLADH (Maskos and Winston, 1993). Such a complex was essential in the catalytic reduction of the C-nitroso moiety of the substrate. To determine whether such a complex could be visualized during ADH-dependent reduction of NEU, we used Co(II)ADH to monitor possible coordination of NEU to the active site metal atom.

The addition of NEU to the Co(II)ADH caused a blue shift of the d-d transition region of the spectrum from 645 to 642 nm (Fig. 3.11a, trace 1). When NADH was added to the binary complex ADH-NEU the red shift of the d-d transition maximum was observed, characterized by a splitting of the d-d transition band into two chromophores with maxima at 642 and 674 nm (Fig. 3.11a, trace 3). The small blue shift of both peaks compared to the ADH-NADH binary complex (Fig. 3.11b, trace 2) suggests the interaction of NEU with the active site ligands of ADH due to the formation of the ternary complex. The subsequent addition of pyrazole to the ternary complex ADH-NEU-NADH resulted in the formation of the classical bimodal spectrum with absorbance maximums at 640 and 677 nm (Fig. 3.11a, trace 4).

When the addition order was changed and NADH was added first to Co(II)ADH, the classical red shift with bimodal splitting was observed with absorbance maximums at 643 and 677 nm (Fig. 3.11b, trace 2). The subsequent addition of NEU
Figure 3.11a  Effect of NEU on d-d transition region of the electronic spectrum of Co(II)ADH.

1) Co(II)ADH (20 μM) in Tes buffer pH 7.4
2) Co(II)ADH + 1.7 mM NEU
3) Co(II)ADH/NEU + 1 mM NADH
4) Co(II)ADH/NADH/NEU + 10 mM pyrazole
Figure 3.11b  Effect of NEU on d-d transition region of the electronic spectrum of Co(II)ADH/NADH binary complex.

1) Co(II)ADH (20 μM) in Tes buffer pH 7.4
2) Co(II)ADH + 1 mM NADH
3) Co(II)ADH/NADH + 2 mM NEU
did not shift the spectrum; however, the increase in the absorbance of 640 nm peak over expected decrease was observed (Fig. 3.11b, trace 3). The observed increase of the absorbance of 643 nm peak has been usually observed with strong active site binding reagents such as pyrazole (Fig. 3.11a, trace 4) and support the formation of the strong ternary complex between ADH-NADH and NEU.

Reduction of N-nitroso methylurea (NMU)

As in the UV/Vis spectrum of NEU, the absorption maximum of NMU is 390 nm ($\varepsilon_{390}=63 \text{ M}^{-1}\text{cm}^{-1}$) and only partially overlaps the NADH spectrum. In order to achieve the optimum spectral separation and prevent overlapping of the spectra the concentrations of substrate and coenzyme were used within the concentration range allowing to monitor changes in the NADH and NMU spectra simultaneously. The reaction was monitored by scanning the range of 200-700 nm to detect possible product formation, and by scanning between 280 and 500 nm at 20 seconds intervals for kinetic measurements. Due to the low extinction coefficient of NMU and strong influence of NADH absorbance, in the actual reaction mixture the absorbance maxima of NADH and NMU were shifted to 350 and 380 nm respectively. Thus, we monitored the absorbance change at 350 and 380 nm, and the absorbance change at 340 and 390 nm are shown in parenthesis, respectively for each substrate.

Upon incubation with NADH, the $A_{350}$ decreased at a rate of 0.6 mAU/min/ml (0.4 mAU/min/ml for 340 nm), while the $A_{380}$ decreased with a rate of 1.5 mAU/min/ml (1.4 mAU/min/ml for 390 nm) (Fig. 3.12). Addition of 0.15 µM ADH increased the rate of $A_{350}$ and $A_{380}$ disappearance to 1.4 and 1.6 mAU/min/ml respectively (Fig. 3.12).
Figure 3.12  **NADH-dependent NMU reduction by HLADH.**  1.5 mM NMU was incubated with 10 μM NADH for 4 minutes and then 0.15 μM ADH was added. The absorbance was monitored over 250 – 500 nm in 30 sec interval scans. The relative rate of absorbance lost at 350 and 380 nm are shown.

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Figure 3.13  HPLC chromatogram of NMU reduction mixture with NADH. A mixture of 0.3 mM NMU and 0.1 mM NADH was separated on reverse phase Alltech C-8 column using an isocratic mobile phase of 90% water and 10% isopropanol with a flow rate of 1.0 ml/min and detection at 235 nm.
**Figure 3.14**  **HPLC analysis of NMU reduction by ADH.** A mixture of 0.3 mM NMU, 1.3 mM NADH and 0.38 mM ADH was separated on reverse phase Alltech C-8 column using an isocratic mobile phase of 90% water and 10% isopropanol with a flow rate of 1.0 ml/min and detection at 235 nm.
Addition of 0.15 μM ADH increased the rate of $A_{350}$ disappearance to 1.4 and 1.6 mAU/min/ml, while the rate of $A_{380}$ disappearance has no significantly increase over nonenzymatic rate (Fig. 3.12). The apparent enzymatic rate of substrate disappearance was estimated as 0.12 nmol/min/ml for NADH. The observed spectral changes suggest the presence of relatively strong nonenzymatic consumption of NMU, probably reflecting a limited stability of NMU in dilute solutions. Spectroscopic analysis revealed no changes in the 200-700 nm region that could be attributed to formation of products of NMU reduction.

HPLC analysis of NMU reduction shows that NMU eluted as a single peak with a R.T. of 2.8 min and was well separated from NADH (R.T. = 1.4 min) (Fig. 3.13). The area under the curve of the NMU peak was directly related to the amount of NMU injected into the system and was used to calculate NMU consumption.

When 0.3 mM NMU was mixed with 1.3 mM NADH in Tes buffer, pH 7.3, the NMU peak disappeared at a steady state rate of 0.06 nmol/min/ml over two hours (Fig. 3.14). In a parallel reaction addition of 0.38 μM ADH resulted in 5-fold increase in the rate of NMU disappearance. The reaction was characterized by a fast phase during the first 15 min, in which NMU was consumed at a rate of ~ 0.31 nmol/min/ml and a slow phase (~0.04 nmol/min/ml) after that (Fig. 3.14). Figure 14 shows that the amount of NMU consumed in enzymatic reaction within 45 min. equals to the amount of NMU consumed within 120 min. of nonenzymatic reaction.

Both spectral and HPLC analysis showed strong nonenzymatic consumption of NMU; however, the rate of NMU disappearance was strongly affected by the amount of NADH present. When amount of NADH was decreased 10-fold in the reaction mixture
the nonenzymatic rate of NMU disappearance increased 5-fold; however, enzymatic rate of NMU reduction was essentially at the same level (0.25 ± 0.05 nmol/min/mg). NADH disappeared only during enzymatic reaction with the pattern similar to NMU reduction by ADH with the fast phase during the first 15 minutes and the plateau to follow. The apparent rate of NADH consumption was estimated at 0.5 nmol/min; thus, about two molecules of NADH consumed for each NMU molecule reduced, which is consistent with subsequent 2e⁻ reduction steps.

**DISCUSSION**

The discovery of unique ADH-dependent reactions originating from its catalysis of reduction of C-nitroso compounds (Dudley and Winston, 1995; Maskos and Winston, 1993) prompted the present extension of our research to study of N-nitroso compounds. Based on the analysis of the cytosolic fractions and the chemistry of metabolites detected, the N-nitroso compound NDELA had been implicated as a substrate for ADH, with a subsequent activation of NDELA to mutagenic endpoints as shown in cellular assays (Denkel et al., 1987; Hecht et al., 1989). However, the activation of NDELA was only related to NAD⁺ dependent oxidation by ADH. The competitive inhibition of NDELA mutagenicity caused by ethanol treatment of the S-9 fraction used suggested a role for an alcohol-metabolizing enzyme in NDELA mutagenicity. The reduction of the nitroso group of C-nitroso compounds by HLADH produces strong electrophilic intermediates, which can bind to DNA (Maskos and Winston, 1993).
The study presented herein deals with the ability of ADH to catalyze reduction of N-nitroso substrates. The specific activities of ADH toward reduction of studied N-nitroso compounds are summarized in table 3.1.

The nitroso moiety of NDELA was reduced nonenzymatically in the presence of NADH, and the addition of ADH increased the rate of the reduction by 3-5 fold (Table 3.1). The stoichiometry of the enzymatic reaction indicated that 2 molecules of NADH were consumed per each molecule of N-nitroso substrate reduced.

<table>
<thead>
<tr>
<th>Compound</th>
<th>NE rate nmol/min/ml</th>
<th>Enzymatic Rate nmol/min/ml</th>
<th>ADH present µM</th>
<th>Specific Activity nmol/min/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDELA</td>
<td>~ 0.007</td>
<td>0.042</td>
<td>0.75</td>
<td>0.71</td>
</tr>
<tr>
<td>NDEA</td>
<td>~ 0</td>
<td>~ 0</td>
<td>0.75</td>
<td>~ 0</td>
</tr>
<tr>
<td>NEU</td>
<td>0.04</td>
<td>0.14</td>
<td>0.38</td>
<td>4.6</td>
</tr>
<tr>
<td>NMU</td>
<td>0.06</td>
<td>0.24</td>
<td>0.38</td>
<td>7.9</td>
</tr>
</tbody>
</table>

The data are consistent with our previous studies of C-nitroso compounds, where the stoichiometry of C-nitroso reduction was accompanied by 2-e transfer and formation of the amine through formation of a hydroxylamine intermediate. Possible metabolic pathways for NDELA reduction and oxidation are shown on Figure 3.15.

NDEA is structurally similar to NDELA, but appeared to react differently with ADH. Neither nonenzymatic nor enzymatic reduction of NDEA were noticeable when NDEA was incubated with NADH. Similar to NDELA, NEU was reduced nonenzymatically; however the addition of ADH resulted in at least 3-fold increase of the NEU reduction rate. In contrast to NEU, NMU appears to have limited stability in
the diluted solutions, and only HPLC analysis allowed to monitor NMU reduction. Strong nonenzymatic decay of NMU was greatly reduced when NADH concentration was used in excess of 1 mM. In spite of strong nonenzymatic reaction, the enzymatic rate was similar to the rate of NEU reduction, and the stoichiometry of NMU reduction was consistent with that of NEU and NDELA.

Our studies showed the enzymatic nature of the reduction of NDELA, NEU and NMU by ADH. Previous results from our laboratory indicated Lewis acid complex formation between the nitroso oxygen of 2-nitroso fluorine and the Zn atom of horse liver ADH (Maskos and Winston, 1993).

This complex was essential in the catalytic reduction of the C-nitroso moiety of the substrate. In our present studies we used Co(II)ADH to monitor possible coordination of N-nitroso substrates to the active site metal atom. Both NDELA and NEU affected the coordination sphere of the ADH metal center, suggesting the formation of a strong ternary complex between ADH-NADH-NDELA(NEU). The incubation of oxygen-sensitive Co(II) with strong oxygen carriers such as NDELA and NEU did not cause any oxidative damage to the active site metal similar to nitro oxide which could destroy the integrity of the active site metal (Crow et al., 1995).

The results show that ADH could reduce N-nitroso compounds, albeit the reaction rates were relatively low in comparison to the rates of reduction of certain studied C-nitroso compounds by ADH. High concentrations of ADH were needed to obtain noticeable rates of reduction of N-nitroso groups with all of the compounds studied (Table 3.1); however, the general mechanism involving 2 e- transfer of the enzymatic reduction remains essentially identical.
The activation of N-nitroso compounds by ADH was species dependent (Bonfanti et al., 1987), analogously large differences in substrate specificity among different purified human isozymes of ADH for C-nitroso compounds were observed (Dudley, 2000). The class II π-isozyme catalyzed a reduction of the nitroso group of pNSP 100-fold greater than horse liver ADH. Therefore, we cannot exclude the possibility that various isozymes of ADH other than the horse liver enzyme used herein would reduce N-nitroso substrates more efficiently.

N-nitroso compounds are easily reduced by chemical reductants, such as LiAlH₄ or electrochemically (Lijinsky, 1984). These processes offer the most convenient way of preparing N,N-dialkylhydrazines; with strong reagents reduction to the amines is achieved (Lijinsky, 1984). The rocket fuel 1,1-dimethylhydrazine has long been prepared by reduction of nitrosodimethylamine (Lijinsky, 1984). The hydrazines have totally different properties from the nitrosamines. They are bases and form stable salts. Biologically, their activities are quite different from the nitrosamines as well. Hydrazines are considerably less potent toxins and carcinogens than the analogous nitrosamines, and are not mutagenic in the usual systems (Lijinsky, 1984). This makes it implausible that the carcinogenic activities of N-nitroso compounds are mediated through reduction to hydrazines, or through further reduction, which gives rise to amines. It seems quite unlikely therefore, that metabolic oxidation to nitrosamines or reduction to hydrazines plays any role in the carcinogenic or mutagenic action of N-nitroso compounds, although both types of product might be formed to some extent in vivo.
Figure 3.15  Possible metabolic pathways for NDELA reduction and oxidation. Red – reduction, Ox – oxidation
In this regard the reduction of N-nitroso compounds by ADH may have a detoxification function in vivo. In keeping with the report cited above (Lijinsky, 1984) ethanol consumption could have direct effect on the function of ADH in activation of different xenobiotics by competitively inhibiting the activation of N-nitroso compounds via the oxidation pathway and by shifting the balance of NADH/NAD\(^+\) toward NADH. Therefore, ethanol consumption could prevent activation of xenobiotics, contributing to the positive effects of moderate alcohol consumption.
Chapter 4: Interaction of Alcohol Dehydrogenase with tert-Butylhydroperoxide: Stimulation of the Horse Liver and Inhibition of the Yeast Enzymes

INTRODUCTION

ADH is one of the most well studied NAD⁺-dependent dehydrogenases [EC 1.1.1.1]. Aside from ethanol oxidation, ADH has several alternative catalytic activities (Holmes, 1994; Höög and Svensson, 1996). Among them, a recently described peroxidatic activity is particularly interesting. Utilizing hydrogen peroxide as a co-substrate, ADH converted the oxidized coenzyme NAD⁺ to a stable product referred to as NADX, which can be distinguished from the normal redox partner NADH by its spectral properties (Favilla et al., 1988; Favilla et al., 1980b). In addition to being a substrate for ADH, H₂O₂ irreversibly inactivates ADH, which can be partially prevented by pretreatment of the enzyme with NAD⁺ (Favilla et al., 1980a; Favilla et al., 1988; Favilla et al., 1980b).

It was suggested that the high Km for H₂O₂ (>0.1M) in the peroxidatic activity of ADH (Favilla et al., 1980a; Favilla et al., 1988; Favilla et al., 1980b) reflected the poor tendency of the ternary complex to reach the transition state, which is counterintuitive to a physiological role. However, an organic derivative of H₂O₂, p-methylbenzyl hydroperoxide (MBHP), completely inhibited the activity of purified horse liver alcohol dehydrogenase (HLADH) at a concentration 10⁴ times lower than H₂O₂ (Skursky et al., 1992a; Skursky et al., 1992b). The high affinity of ADH for MBHP was explained partially by interaction of MBHP with the hydrophobic pocket in

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the enzyme substrate-binding site. H$_2$O$_2$ and MBHP appeared to have different mechanisms of inactivation; ADH inactivation by MBHP was reversible suggesting that the active-site metal coordination sphere was not destroyed by MBHP.

In our studies to investigate the kinetics and mechanisms of alkyl hydroperoxide inactivation of ADH we unexpectedly found that preincubation of HLADH with tert-butyl hydroperoxide (tBOOH) stimulated the ethanol dehydrogenase (oxidase) activity of this enzyme. In stark contrast to the activation of HLADH by tBOOH, the yeast enzyme was markedly inhibited by this oxidant. Binary complexes of YADH with coenzymes afforded only partial protection against inactivation. Thus, in the present communication, we report on the kinetics of interaction of tBOOH with horse liver and yeast ADH. Our studies employing classical ADH binary complexes indicate that tBOOH-dependent stimulation of HLADH is associated with destabilization of the catalytic Zn-coordination sphere, whereas tBOOH causes inactivation of YADH, apparently through oxidation of active site –SH group ligands (Leskovac et al., 1999).

**MATERIALS AND METHODS**

**Enzymes and Reagents**

Horse liver and yeast alcohol dehydrogenases were obtained from Boehringer Mannheim, USA. The concentration of ADH was determined from the absorbance at 280 nm (for HLADH $\varepsilon = 35.7 \times 10^3$ M$^{-1}$cm$^{-1}$ (Chen et al., 1987)), and for YADH $\varepsilon = 189 \times 10^3$ M$^{-1}$cm$^{-1}$ (Magonet et al., 1992)). $\beta$NAD$^+$ (> 99% purity) was purchased from Boehringer Mannheim, USA. NADH (98% purity) was purchased from Sigma Chemical Co., St. Louis, MO. Coenzymes were used without further purification. Concentrations of the coenzyme solutions were determined spectrophotometrically from
the extinction coefficients of $18.0 \times 10^3 \text{ M}^{-1} \text{cm}^{-1}$ at 260 nm for NAD$^+$ and of $6.22 \times 10^3 \text{ M}^{-1} \text{cm}^{-1}$ at 340 nm for NADH. tert-Butylhydroperoxide was also obtained from Sigma as 70% solution. All other chemicals were of analytical grade.

**Enzyme Assay**

ADH-catalyzed ethanol dehydrogenase activity was determined according to the method of Vallee and Hoch (Vallee and Hoch, 1955) from initial velocities of NADH production at 340 nm on a Perkin Elmer Lambda 6 UV-Vis spectrophotometer. Reactions were conducted in quartz cuvettes of 1 cm light-path containing 2.5 mM NAD$^+$ and 360 mM ethanol in 1 ml of 32 mM sodium pyrophosphate buffer, pH 8.8. The concentration of ADH active sites was determined by pyrazole titration (Theorell and Yonetani, 1963).

**Peroxide-Modified Enzyme Studies**

Horse liver or yeast alcohol dehydrogenase was incubated with tert butyl hydroperoxide in 50 mM potassium phosphate buffer over the pH range 5.9-8.4, in 50 mM sodium pyrophosphate buffer over the pH range 8.8-9.6, and in glycine buffer above pH 9.6. The reaction was initiated by adding the peroxide to the enzyme mixture, in either the presence or absence of specified protective agents. The effect of tBOOH on the modification of ADH with time was measured using a Perkin Elmer Lambda 6 UV-Vis spectrophotometer by withdrawing specified aliquots from the incubation mixture, adding these withdrawn aliquots to assay cuvettes, which contained the reactants prescribed above under Enzyme Assay and measuring the initial rate of NADH production.
Sulfhydryl Group Analysis

Total sulfhydryl content before and during incubation of ADH with tBOOH was determined essentially by the method of Ellman (Ellman, 1959). 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB, Ellman's reagent) was prepared by dissolving the solid DTNB in methanol to make a 40 mM stock solution and adding volumes of this stock solution to 1.0 ml reaction solutions to give a final concentration of 0.6 mM. Reaction solutions contained 0.1-0.5 mg of ADH in 0.1 M potassium phosphate buffer, pH 7.9. Derivatization of enzyme –SH groups was achieved by exhaustively reacting native ADH with DTNB under conditions of denaturation with 5.7 M guanidine-HCl. The extinction coefficient at 412 nm for 5-thio-2-nitrobenzoic acid in guanidine HCl, pH 7.9 was experimentally determined by titrating DTNB with a known amount of freshly prepared cysteine in the presence of tBOOH, since this hydroperoxide (0.5-2 mM) affects the stability and hence, the absorptivity of 5-thio-2-nitrobenzoic acid.

RESULTS

Dependence of Stimulation of Ethanol Oxidation on tBOOH Concentration

As prelude to a comprehensive study of the effects of peroxide action on ADH we explored the effects of tBOOH on ethanol oxidation catalyzed by horse liver ADH. Based on previous studies of peroxide effects on ADH (Crow et al., 1995; Favilla et al., 1980a; Favilla et al., 1980b; Skursky et al., 1992a; Skursky et al., 1992b), we anticipated strong inhibition of ADH activity by tBOOH. Surprisingly, tBOOH did not inactivate ADH; rather, when HLADH was incubated with 50 mM tBOOH the enzyme became progressively more active and reached a maximum of two-fold stimulation with respect ethanol oxidation (Fig. 4.1a). A similar effect of tBOOH was observed on the
reverse reaction of HLADH, i.e. NADH-dependent reduction of acetaldehyde (data not shown). The course of the reaction was monitored by assaying 10 μl aliquots withdrawn from the incubation mixture at the indicated time points. This method introduced small amounts of tBOOH (0.01-3 mM) to the ethanol oxidation mixture; therefore, the effect of the presence of tBOOH 0.1-10 mM in the assay mixture was tested, and no significant effect on the ADH activity was observed.

We also considered the possibility that contaminant iron present in phosphate and pyrophosphate buffers might, via Fenton chemistry, have generated alkoxyl radicals (RO°) from tBOOH (Winston et al., 1983), which might have contributed to the kinetic modifications shown in Figure 4.1. Therefore, experiments were conducted using buffers that had been passed through a Chelex-100 column to remove metals. No differences in the kinetics of ADH activation by tBOOH were observed with Chelex-treated as compared to buffers that had not been so treated. This was not surprising since in phosphate buffers devoid of EDTA the iron content is in the form of ferric phosphate, which does not promote the Fenton reaction, even in the presence of reductants (Winston et al., 1984).

The observed stimulation is for relatively high tBOOH concentrations (10-300 mM). When the concentration of tBOOH was below 10 mM stimulation was observed, but the incubation time required to achieve this stimulation was typically three or more hours. The degree of stimulation of ADH was not further increased by concentrations of tBOOH greater than 100 mM. The higher the concentration of tBOOH in the preincubation mix the more rapid was the activity of ADH stimulated. Also, the time
Figure 4.1a  Stimulation of HLADH-dependent ethanol oxidation by tBOOH.
HLADH (3 μM) was incubated at 25°C with 25-100 mM of tBOOH or 100 mM tertiary butanol in 50 mM potassium phosphate, pH 7.4. Aliquots (10μl) from the incubation mix were removed at the times shown and assayed for ethanol oxidation activity in the presence of 2.5 mM NAD⁺ and 360 mM ethanol. 1) 100 mM tBOOH, 2) 50 mM tBOOH, 3) 25 mM tBOOH, 4) 100 mM tert-butanol.
Figure 4.1b Dependence of $t_{\text{max}}$ and $t_{150}$ of ADH stimulation on tBOOH concentration. $t_{\text{max}}$ and $t_{150}$ are respectively, the time required for ADH to reach the maximum stimulated level of 200% and the time required to reach half maximal stimulation when incubated with tBOOH.
required for reversal of this stimulation was inversely related to tBOOH concentration. The reversible phase of the reaction could be envisaged as competing oxidative damage to the enzyme as the peroxide is used in a peroxidatic reaction perhaps, through a free radical-mediated process at the active site.

To eliminate the possibility that the stimulation of HLADH activity was due to a nonspecific interaction of the hydrophobic t-butyl group, rather than a function of the peroxide action of tBOOH, 100 mM tertiary butanol was substituted for tBOOH. Only a negligible increase (4-6%) in ADH activity was observed with tertiary butanol (Fig. 4.1a).

To quantify the stimulatory effect of tBOOH on HLADH the parameters $t_{150}$ and $t_{max}$ are introduced and defined, respectively as the time of incubation with tBOOH to achieve 50% stimulation of HLADH activity above the activity in the absence of tBOOH, and the time needed to achieve maximum stimulation. Figure 4.1b shows that $t_{150}$ and $t_{max}$ were dependent on tBOOH concentration up to 100 mM; above this concentration further stimulation was negligible.

**Dependence of HLADH Stimulation by tBOOH on Ethanol Concentration**

Various reagents that are able to modify HLADH activity depend on the concentration of ethanol in the reaction (Dalziel and Dickinson, 1966b; Kaplan et al., 1956; Plapp, 1970; Theorell and McKinley-McKee, 1961; Theorell et al., 1969). A given reagent could act as a positive effector and stimulate the oxidation of ethanol by HLADH when ethanol concentration is high and as a negative effector inhibiting enzyme activity at low ethanol concentration (Dalziel and Dickinson, 1966b; Kaplan et al., 1956; Theorell et al., 1969). To determine if tBOOH also acted in this dual
Figure 4.2  The effect of ethanol concentration on the stimulation of ADH by tBOOH. HLADH (6 μM) was incubated with 100 mM tBOOH for 120 min. Aliquots (10 μl) were removed from the incubation mixture at various time points and assayed for ethanol oxidation activity in the presence of 2.5 mM NAD$^+$ with the indicated concentrations (4-800 mM) of ethanol. Ethanol oxidation was measured by formation of NADH at 340 nm as described in Methods and Materials.
capacity, HLADH stimulation by tBOOH was studied at five concentrations of ethanol (Fig. 4.2). The results are shown in comparison with the ethanol concentration-dependence of the untreated enzyme. The dependence of HLADH activity on ethanol concentration was similar for the tBOOH-treated and untreated enzymes; in both cases substrate inhibition at the higher concentration of ethanol was seen, however the relative stimulation caused by tBOOH remained essentially unaffected. Thus, even under conditions of substrate inhibition ADH activity was stimulated by tBOOH.

**Effect of Protective Agents on tBOOH Stimulation of ADH**

Several ligands, known to form binary complexes with HLADH (Reynolds and McKinley-McKee, 1969) were studied with respect to their ability to prevent the modification of ADH by tBOOH (Fig. 4.3). tBOOH did not stimulate ethanol oxidation as effectively when certain active site residues of the enzyme were protected by the prior formation of binary complexes. When NAD$^+$ is bound to the enzyme, its pyrophosphate group interacts with Arg-47 and Arg-369 (Chen and Plapp, 1978), thereby preventing tBOOH, the activator, from interacting with these active site residues as shown by the strong decrease in the stimulation caused by tBOOH. The dissociation constants for NAD$^+$ and NADH are 51 μM and 1 μM, respectively (Plapp, 1970). The dissociation constant reflects the stability of the enzyme binary complex; thus, under conditions of our assay, which contained 1 mM NAD$^+$ or 0.2 mM NADH, the ability of tBOOH to interact with the active site of the enzyme is undoubtedly compromised.

AMP selectively binds to an anion binding site formed by Lys-228 (Plapp, 1974). This reagent did not prevent tBOOH from stimulating ADH activity, suggesting
Figure 4.3  Effect of protective agents on stimulation of ADH by tBOOH. HLADH (5 μM) was incubated with either NAD⁺, NADH, AMP, glutathione or dithiothreitol for 5 minutes prior to the addition of 100 mM tBOOH. Aliquots (10 μl) were removed from the incubation mixture and assayed for ethanol oxidation in a 1 ml reaction volume containing 25 mM pyrophosphate buffer, pH 8.8, 2.5 mM NAD⁺ and 360 mM ethanol.
that Lys-228 is not a locus of tBOOH-dependent stimulation of HLADH. Hydrogen peroxide causes inactivation of ADH and this was associated with -SH oxidation in the enzyme (Favilla et al., 1980a). In the present studies, addition of the sulfhydryl group protective agent dithiothreitol (DTT) did not prevent the stimulation of HLADH caused by preincubation with tBOOH and glutathione (GSH) afforded only limited protection (Fig. 4.3). This does not rigorously exclude a role for active site -SH groups or thiolate ligands in the stimulation caused by tBOOH as these reagents might not access the hydrophobic interior of the active site. DTT might protect certain -SH groups, e.g. surface -SH groups from oxidation, but these are not critical in the stimulation caused by tBOOH.

Sulfhydryl Group Analysis of HLADH

The quantitative change in titratable -SH group content of the enzyme was measured as a function of time on removed aliquots from the incubation mixture of ADH with tBOOH and compared with the accompanying process of stimulation by tBOOH of ADH activity. ADH was not denatured with guanidine HCl until after the enzyme was incubated with tBOOH in order to evaluate the change in the number of only those thiol groups modified by the tBOOH. After 5 min of preincubation of 20 μM ADH with 50 mM tBOOH, 4 to 5 -SH groups per molecule of ADH dimer were apparently modified. There are 14 free -SH groups per subunit of ADH (Reynolds and McKinley-McKee, 1969); thus, when completely denatured ADH presents 28 -SH groups per molecule prior to reaction with tBOOH and about 23 to 24 titratable -SH groups remained after tBOOH treatment (data not shown). Nevertheless, the maximum
number of -SH groups modified occurred within five minutes of incubation, while $t_{\text{max}}$ for stimulation by the same concentration of tBOOH was not achieved for up to 2 hours.

**pH-Dependent Stimulation of HLADH**

To determine a possible role for ionizable amino acid residues in tBOOH-dependent stimulation of ADH the interaction of HLADH with tBOOH was studied over the pH range of 5.9 to 8.8; in this pH range the enzyme is stable (Maskos and Winston, 1993). For these studies HLADH was preincubated with 100 mM tBOOH, and aliquots were removed at various time points to permit the $t_{150}$ value for stimulation to be obtained in the manner used to generate the data in Figure 4.1 for each pH studied. The dependence of the $t_{150}$ on pH is shown in Figure 4.4, which shows that two ionizable groups with pKa values of 6.9 and 8.2 are indicated to have a role in tBOOH-dependent stimulation of ADH.

**tBOOH Inhibition of Yeast ADH**

We wondered if the stimulation of ADH by tBOOH was specific for the horse liver enzyme. The enzymatic activities of HLADH and YADH are absolutely dependent on the integrity of the zinc coordination sphere of the catalytic site. In contrast to the stimulation of HLADH by tBOOH, incubation of YADH with 0.5-50 mM tBOOH caused progressive inactivation of the enzyme (Fig. 4.5). A plot of log of the percent of residual YADH activity vs. time of incubation with tBOOH (Fig. 4.6a) is linear to about 10% of the initial activity. This indicates that the inactivation reaction approximates first order kinetics with respect to enzyme at any fixed concentration of tBOOH, which is reminiscent of the decay in ADH activity caused by hydrogen peroxide (Favilla et al., 1980a). The reaction order with respect to tBOOH was
Figure 4.4  pH-dependence of ADH stimulation by tBOOH. HLADH (5 μM) was incubated with 100 mM tBOOH at the indicated pH values. Aliquots (10 μl) were removed from the incubation mixture at various times between 1 and 120 min in order to determine the $t_{150}$ values for each pH. Each aliquot was then assayed for ethanol oxidation in a 1 ml reaction volume containing 25 mM pyrophosphate buffer, pH 8.8, 2.5 mM NAD$^+$ and 360 mM ethanol.
obtained from the slope of the linear portion of the log $k'$ versus log [tBOOH] plot (Fig. 4.6b). The slope equal to 1.2 indicates that at least 1 molecule of tBOOH binds to 1 molecule of yeast ADH during the inactivation process. A double inverse plot of the observed pseudo first-order decay rate constant $k'$ versus [tBOOH] (Fig. 4.6c) was linear with an intercept on the ordinate axis. This indicates that tBOOH-dependent inactivation of YADH obeys saturation kinetics. By extrapolating the linear plot to the ordinate axis, an apparent pseudo-first order rate constant $k_2$ was determined as $0.35 \pm 0.06 \text{ min}^{-1}$. This compares favorably with the analogous rate constant of $0.11 \pm 0.02 \text{ min}^{-1}$ for inactivation of HLADH by $H_2O_2$ (Favilla et al., 1980a). Likewise, by extrapolating the plot to the abscissa, (Fig. 4.6c) the dissociation constant, $K_{E,tBOOH}$, for the YADH-tBOOH complex of $85 \pm 12 \text{ mM}$ was obtained. Comparing this value with that of the dissociation constant for the HLADH-$H_2O_2$ complex ($250 \pm 37 \text{ mM}$) it appears that the YADH-tBOOH complex is the more stable by a factor of three.

The value of the second-order rate constant $k_i$, obtained from the slope of the best fit straight line shown in Figure 4.6d is $3.4 \pm 0.7 \text{ M}^{-1} \text{ min}^{-1}$. This is of the same order as for the HLADH-$H_2O_2$ complex ($7 \pm 1$) (Favilla et al., 1980a).

The zinc thiolate ($Zn_1Cys_2His_1-H_2O$) moiety in yeast alcohol dehydrogenase is structurally similar to the horse liver enzyme, but the thiol groups of yeast ADH are considerably more reactive than those of the liver enzyme (Reynolds and McKinley-McKee, 1969). When binary complexes of YADH with 1 mM NAD$^+$ and 0.2 mM NADH were formed prior to the addition of tBOOH the rate of inactivation was only reduced by 22 and 44%, respectively (Table 4.1).
Figure 4.5  Comparison of the time-dependent modification by tBOOH of yeast and horse liver alcohol dehydrogenase. YADH (75 nM) was incubated with 20 mM tBOOH and HLADH (3 μM) was incubated with 100 mM tBOOH for up to 300 min. At the various time points indicated in the figure, aliquots (10 μl) were removed from the incubation mixture and assayed for ethanol oxidation in a 1 ml reaction volume containing 25 mM pyrophosphate buffer, pH 8.8, 2.5 mM NAD⁺ and 360 mM ethanol.
Figure 4.6a  Semilog plots of ethanol oxidation activity of yeast ADH as a function of incubation time and varying concentrations of tBOOH. YADH (75 nM) was incubated with 0.5, 2, 5, 20 or 50 mM tBOOH and 10 µl aliquots from the incubation mixture were assayed for ethanol oxidation at the indicated time points in a 1 ml reaction volume containing 25 mM pyrophosphate buffer, pH 8.8, 2.5 mM NAD\(^+\) and 360 mM ethanol.
**Figure 4.6b** Secondary log/log plot of the concentration dependent inactivation of YADH by tBOOH.
Figure 4.6c  Double inverse plot of the observed pseudo first-order decay rate constant k' versus [tBOOH].
Figure 4.6d  Relationship between $k'$ of YADH and tBOOH concentration.
Table 4.1  $t_{1/2}$ and $k'$ for inhibition of ethanol oxidation by tBOOH of YADH.

<table>
<thead>
<tr>
<th>Protective Agents</th>
<th>$t_{1/2}$ min</th>
<th>$k' = \ln(2)/t_{1/2}$ min$^{-1}$</th>
<th>Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mM tBOOH</td>
<td>8</td>
<td>0.087</td>
<td>N/A</td>
</tr>
<tr>
<td>1 mM NAD$^+$</td>
<td>13</td>
<td>0.053</td>
<td>22%</td>
</tr>
<tr>
<td>5 mM NAD$^+$</td>
<td>27</td>
<td>0.026</td>
<td>44%</td>
</tr>
<tr>
<td>0.2 mM NADH</td>
<td>28</td>
<td>0.025</td>
<td>48%</td>
</tr>
<tr>
<td>1 mM NADH</td>
<td>55</td>
<td>0.013</td>
<td>70%</td>
</tr>
</tbody>
</table>

YADH (0.13 μM) was incubated with specified concentration of NAD$^+$ and NADH for 5 minutes prior addition of 25 mM tBOOH and 10 μl aliquots from the incubation mixture were assayed for ethanol oxidation.

With the horse liver enzyme the analogous binary complexes afforded much stronger protection against tBOOH stimulation (70 and 81%, respectively; Fig. 4.3). When the concentration of these coenzymes was increased five-fold (5 mM NAD$^+$ and 1 mM NADH) the level of protection afforded YADH increased to 48 and 70%, respectively. In both cases NADH protected more strongly than NAD$^+$. The relatively weaker protection of YADH against tBOOH inactivation by binary complexes with coenzymes suggest that the active site of YADH is more accessible to tBOOH, even at coenzyme concentrations that far exceed the dissociation constants.

**Sulfhydryl Group Analysis of YADH**

We also monitored the concomitant modification of thiol groups of the yeast enzyme and the accompanying inactivation in the manner analogous to experiments with the horse liver enzyme. After 15 min of preincubation of 16 μM YADH with 10 mM tBOOH, at least three cysteine residues per enzyme subunit were modified (presumably oxidized) and YADH was inactivated by about 50%. A similar trend was obtained when HLADH was inactivated by H$_2$O$_2$ (Favilla et al., 1980a; Favilla et al., 1980b) under conditions in which the active site metal bound Cys-46 or Cys-174 were...
oxidized. The rate of modification of –SH groups of YADH increased over time with increasing concentration of tBOOH (Fig. 4.7a). In contrast to HLADH the time-dependent pattern of –SH group oxidation of YADH strongly correlates with the time-dependence of YADH inactivation by tBOOH.

Complete inactivation of YADH corresponded to oxidation of about four thiol groups per ADH subunit; thus, two additional thiol groups were oxidized as compared to HLADH. The time course of thiol group oxidation of YADH showed two distinct phases (Fig. 4.7a). Figure 4.7b shows the relationship between the residual activity of YADH as a function of the number of –SH groups oxidized. In the first faster phase of the time course about two thiol groups per subunit were oxidized within five min of the incubation with 5 mM tBOOH (Fig. 4.7a). This corresponded to a small (10-15%) decrease in YADH activity (Fig. 4.7b), and was reminiscent of the time dependence for thiol group oxidation seen with HLADH, i.e. two thiol groups were modified in about five min. In the second slower phase, which was not seen with HLADH, two additional thiol groups were oxidized in the yeast enzyme (Fig. 4.7b). The oxidation of these thiol groups in the second phase was associated with the observed further loss in catalytic activity of the enzyme (Fig. 4.7b). The first two thiol groups oxidized are most likely Cys residues external to the hydrophobic active site, which do not significantly affect catalysis by YADH. Analogous to studies of ADH inactivation by H₂O₂ (Favilla et al., 1980a; Favilla et al., 1980b), we suggest that the two Cys residues modified (oxidized) in the second phase are likely the active site Zn ligands Cys-46 and Cys-174.
Figure 4.7a  Time-dependent modification of -SH groups of YADH during incubation with tBOOH. YADH (16 μM) was incubated with 2.5, 5 or 10 mM tBOOH. Aliquots (40 μl) were removed and assayed for total -SH group content under denaturing conditions (5.7 M Guanidine) with Ellman’s reagent as described in materials and methods.
Figure 4.7b  Residual YADH Activity during incubation with tBOOH vs. modified —SH group content. The ethanol oxidase activity and —SH group content of YADH were determined as described in materials and methods on aliquots withdrawn from the incubation mixture of tBOOH (5 mM) and YADH (16 µM) at various time intervals.
DISCUSSION

We have shown that when the prooxidant tBOOH is preincubation with either HLADH or YADH the ethanol dehydrogenase activities of these enzymes are altered in two markedly different ways. Ethanol oxidation by HLADH is stimulated by tBOOH and that by YADH is inhibited. The stimulation of HLADH by tBOOH is related to changes in the geometry of the Zn coordination sphere caused by binding of tBOOH to the active site. The Zn ligands Cys-46 and Cys-174 are not oxidized by the peroxide. The inhibition of YADH by tBOOH is related to active site sulfhydryl group oxidation.

The binary complexes of NAD⁺- or NADH-ADH prevent the stimulation of ADH by tBOOH (Fig. 4.3), suggesting the involvement of an amino acid residue near the binding site of the nicotinamide ring that was perturbed by tBOOH. This residue is probably very close to the catalytic site since the pyrophosphate groups of NAD⁺ or NADH bind to Arg-47 and Arg-369 and thereby prevent access of tBOOH to Cys-46. An anion-binding site within the active-site of HLADH is furnished by the positively charged groups of Arg-47 and Lys-228 (Plapp, 1974). NADH dissociation from the enzyme-substrate complex is the rate-limiting step in ethanol oxidation (Branden, 1974) and modification of Lys-228 is attributed to lowering of the dissociation constant of NADH (Chen and Plapp, 1978). For example, ADH is stimulated by methyl acetimidate, a chemical that modifies Lys-228 (Plapp, 1974) and AMP prevented that stimulation. In the present study AMP did not prevent stimulation of ADH by tBOOH suggesting that modification of Lys-228 is not a principal event in the mechanism by which tBOOH stimulated ADH.
Six -SH groups per ADH subunit interact with either the catalytic or structural Zn atoms, and the modification of only one active site thiol can affect ADH activity (Vallee and Auld, 1990). Our studies show that about two thiol groups per enzyme subunit were modified by tBOOH in the horse liver and about four thiol groups in the yeast enzyme. This appears to be related to the marked differences in the effects on the two ADH of tBOOH; i.e., stimulation of the liver and inhibition of the yeast enzymes. These differences may well reflect significant differences in enzymic properties between the two enzymes. For example, coenzymes bind to the liver always more tightly than to the yeast enzyme, and this is associated with a different charge distribution among the residues within the respective active sites (Leskovac et al., 1999). The time-dependent modification of thiol groups corresponded to the time-dependent inactivation of YADH by tBOOH, indicating that -SH groups are important in the mechanism by which tBOOH inactivates the yeast enzyme. If the ADH surface -SH groups were modified by tBOOH they do not appear to have a significant role in the observed tBOOH-altered ADH kinetics. Inactivation of YADH by tBOOH is likely associated with oxidation of the Zn thiolate ligands. Thus, the protection afforded by pyridine nucleotide coenzymes may reflect protection of Cys-46, which is normally the more reactive of the Zn-bound ligands (Langeland and McKinley-McKee, 1997), from peroxidic attack, whereas Cys-174, the other Zn-bound ligand, is susceptible to attack by tBOOH in a manner analogous to that by alkylating agents (Favilla et al., 1980a). We can only speculate on the involvement of Cys-46 and Cys-174 at this time, nevertheless ADH inactivation by H$_2$O$_2$ has been suggested to involve these ligands (Favilla et al., 1980a).
The inactivation of YADH has a more established basis of reconciliation than the remarkable stimulatory effect on ADH activity by even very high concentrations of the prooxidant tBOOH. The fate of the remaining zinc coordinate (His-67) is not known; however, the pH-dependence of the rate of tBOOH stimulation of HLADH suggested a role for an ionizable proton with a pKa ~ 6.9. Histidine is a plausible residue for tBOOH interaction as it is oxidizable and the pKa associated with stimulation of HLADH by tBOOH is consistent with histidine ionization. Two possibilities are His-67, a ligand of the catalytic zinc atom, and His-51, which is on the surface of the enzyme and participates in a proton relay system. We cannot rigorously establish that histidine residues were modified by tBOOH. The proton relay system of the yeast and liver enzymes differ only in the substitution of Thr-48 in YADH for Ser-48 of HLADH (Leskovac et al., 1999). Therefore, the difference in tBOOH action on the two enzymes is not likely explained by action on His-51. The pH-dependent stimulation of HLADH by tBOOH also indicates an ionizable group with a pKa of 8.3. This is considered to reflect the catalytic Zn-bound water ionization (Branden, 1974), which is supported by the sharp decrease in the t150 value above pH 8.2. The zinc atom is less positive at high pH (Langeland and McKinley-McKee, 1997), which would result in weaker Lewis acid complex formation between substrate and enzyme.

HLADH activation occurs by facilitated coenzyme dissociation caused by the interaction of modifying agents with Lys-228, which forms an anion binding site about 8 Å from the sulfur of Cys-46 (Plapp, 1974). AMP, which selectively binds to an anion-binding site formed by Lys-228 (Plapp, 1974), did not prevent tBOOH from stimulating ADH activity, which suggests that Lys-228 is not affected by tBOOH and
that the purine ring of the coenzyme is not important in preventing the stimulation caused by tBOOH.

Finally the present data suggest that subtle changes in the zinc-thiolate center of ADH may cause marked changes in the enzyme function (stimulation of HLADH as compared to inactivation of YADH). The selective sensitivity of zinc-thiolate centers coupled with their presence in numerous proteins with critical roles in cellular regulation suggests that they may be key targets in oxidant-mediated pathology. Although the stimulation of ADH-dependent ethanol metabolism caused by tBOOH in our in vitro studies required relatively high concentrations, other alkyl hydroperoxides such as lipid hydroperoxides might play a role in the regulation of enzymatic activity at more physiologically relevant concentrations. Further investigation of the molecular mechanism of human ADH interaction with possible physiological peroxides may be of heuristic value.
Chapter 5: Inactivation of Alcohol Dehydrogenase by Hydrogen Peroxide: Studies with the Cobalt-Substituted Enzyme

INTRODUCTION

H$_2$O$_2$ has been shown to mediate an irreversible inactivation of ADH (Favilla et al., 1980b). This inactivation was partially prevented by pretreatment of the enzyme with NAD$^+$ (Favilla et al., 1980a; Favilla et al., 1980b; Mazzini et al., 1980). To better understand the mechanism of inactivation of ADH by H$_2$O$_2$ we used two approaches to this study. The first employed a series of active-site-directed reagents classically used to probe specific loci of interaction of various inhibitors and substrates of ADH (Dalziel and Dickinson, 1966a; Shore and Theorell, 1967; Theorell et al., 1969) to determine the potential loci of H$_2$O$_2$ action on this enzyme. H$_2$O$_2$ was preincubated with ADH in the presence or absence of these reagents, and ethanol oxidation was measured on aliquots of the enzyme incubate taken at selected time periods to access the degree of protection afforded against H$_2$O$_2$-dependent destruction of the enzyme. The second approach was used to study the direct effects of H$_2$O$_2$ on the coordination sphere of the ADH active site and the coenzyme binding site as a function of the changes in the spectral characteristics of ADH in which active site Zn was replaced with cobalt (Co(II)ADH). Co(II)ADH has a distinguishable ligand to metal charge transfer (LMCT) band and a d-d transition band in the electronic spectrum that reflects the catalytic cobalt ion of the enzyme (Werth et al., 1995). We exploited these spectral characteristics to evaluate the chemical interaction of hydrogen peroxide, as these are indicative of perturbances of the metal center of the enzyme. We show that when incubated with Co(II)ADH, H$_2$O$_2$ causes a loss of absorbance of the principal LMCT band and similarly, the d-d transition
band. Moreover, the reagents that prevent inactivation of ADH by H$_2$O$_2$ prevent the spectral transitions caused by H$_2$O$_2$.

**MATERIALS AND METHODS**

**Enzymes and Reagents**

Horse liver alcohol dehydrogenase (ADH) was obtained from Boehringer Mannheim, USA. The concentration of ADH was determined from its molar absorptivity at 280 nm ($\varepsilon = 35.7 \times 10^3$ M$^{-1}$cm$^{-1}$) (Chen et al., 1987). βNAD$^+$ (> 99% purity) was purchased from Boehringer Mannheim, Germany, and NADH (98% purity) was purchased from Sigma Chemical Co., St. Louis, MO. Both coenzymes were used without further purification. Concentrations of the coenzyme solutions were determined spectrophotometrically from the extinction coefficients, $\varepsilon = 18.0 \times 10^3$ M$^{-1}$cm$^{-1}$ at 260 nm for NAD$^+$ and $\varepsilon = 6.22 \times 10^3$ M$^{-1}$cm$^{-1}$ at 340 nm for NADH. H$_2$O$_2$ solutions were prepared fresh from 30% stock and the concentration was determined from the extinction coefficient 19.6 M$^{-1}$cm$^{-1}$ at 253.7 nm (Favilla et al., 1988). All other chemicals were of analytical grade.

**ADH Activity Assay**

ADH activity was determined according to the method of Vallee and Hoch (Vallee and Hoch, 1955) from initial velocities of NADH production at 340 nm on a Perkin Elmer Lambda 6 UV-Vis spectrophotometer. Reactions were conducted in quartz cuvettes of 1 cm light-path containing 2.5 mM NAD$^+$ and 0.4 M ethanol in 1 ml of 32 mM sodium pyrophosphate buffer, pH 8.8. The concentration of ADH active sites was determined by pyrazole titration (Theorell and Yonetani, 1963).
**Peroxyde-Modified ADH Studies**

ADH (3 \(\mu\)M) was incubated in 50 mM potassium phosphate buffer over the pH range 5.9-8.4, in 50 mM sodium pyrophosphate buffer over the pH range 8.8-9.6, and in glycine buffer above pH 9.6. The reaction was initiated by adding \(H_2O_2\) to the mixture of enzyme, in either the presence or absence of specified protective agents. The effect of \(H_2O_2\) on the modification of ADH with time was measured with a Perkin Elmer Lambda 6 UV-Vis spectrophotometer by withdrawing specified aliquots from the incubation mixture, adding these aliquots to assay cuvettes and measuring the initial rate of NADH production.

**Sulfhydryl Group Analysis**

Total sulfhydryl content, before and during incubation of ADH with \(H_2O_2\), was determined essentially by the method of Ellman (Ellman, 1959). 5,5'-Dithio-bis-2-nitrobenzoic acid (DTNB, Ellman’s reagent) was prepared by dissolving solid to 40 mM in methanol and adding 15 \(\mu\)l volumes of this solution to 1.0 ml reaction solutions to give a final concentration of 0.6 mM. Reaction solutions contained 0.5-1 \(\mu\)M of ADH in 0.1 M potassium phosphate buffer, pH 7.9, and 5.7 M guanidine HCl as a denaturant. The extinction coefficient at 412 nm for 5-thio-2-nitrobenzoic acid in guanidine HCl, pH 7.9 was experimentally determined by titrating DTNB with a known amount of freshly prepared cysteine, since the presence of \(H_2O_2\) affects the absorptivity of 5-thio-2-nitrobenzoic acid.

**Preparation of Active-Site-Substituted ADH**

The substitution of Cobalt(II) for active site zinc ion ADH was performed as described by Maret (Maret et al., 1979) with modifications (Chapter 2). Metal analysis
was performed by ICP on Leeman Labs PS 3000, which confirmed that the substitution of Zn by Co was greater than 85%. Metal substitution was also confirmed by EPR and UV/Vis spectra (Maret et al., 1979).

RESULTS

Inhibition of Ethanol Oxidation: Dependence on H$_2$O$_2$ Concentration

The rate of inactivation of ADH (5 μM) as a function of variable concentrations of H$_2$O$_2$ (5-360 mM) was determined initially in the absence of cofactor or other reagents by measuring ethanol oxidation of 10 μl aliquots withdrawn from the preincubation mixture of ADH and peroxide at the indicated time points. H$_2$O$_2$ caused a first order rate decay of ADH activity (Fig. 5.1). The half-life ($t_{1/2}$) of ADH during H$_2$O$_2$-dependent decay was inversely related to the H$_2$O$_2$ concentration (Table 5.1). From the half-life the first order rate constant for ADH decay at a given concentration of H$_2$O$_2$ was determined from the relationship $k = (\ln 2)/t_{1/2}$ (Reynolds and McKinley-McKee, 1969) and is directly related to H$_2$O$_2$ concentration (Table 5.1).

Table 5.1 $t_{1/2}$ and $k'$ for inhibition of ethanol oxidation by ADH pretreated with different H$_2$O$_2$ concentrations.

<table>
<thead>
<tr>
<th>H$_2$O$_2$ Concentration (mM)</th>
<th>0.3</th>
<th>2.5</th>
<th>25</th>
<th>50</th>
<th>100</th>
<th>250</th>
<th>360</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{1/2}$ min</td>
<td>1600</td>
<td>650</td>
<td>95</td>
<td>60</td>
<td>25</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td>$k' = \ln 2/t_{1/2}$ min$^{-1}$</td>
<td>0.0004</td>
<td>0.001</td>
<td>0.007</td>
<td>0.012</td>
<td>0.027</td>
<td>0.043</td>
<td>0.069</td>
</tr>
</tbody>
</table>

The reaction order with respect to H$_2$O$_2$ was obtained from the slope of the linear portion of the plot of log $k'$ versus log [H$_2$O$_2$] (Fig. 5.2) and was consistent with a pseudo first order decay of ADH with respect to H$_2$O$_2$. The observed pseudo first order rate constant for ADH decay ($0.11 \pm 0.2$ min$^{-1}$) and dissociation constant of the ADH-
Figure 5.1  Semilog plots of ethanol oxidation activity of ADH as a function of incubation time and varying concentrations of H₂O₂.  HLADH (5 μM) was incubated with 5-360 mM H₂O₂ and 10 μl aliquots from the incubation mixture were assayed for ethanol oxidation at the indicated time points in a 1 ml reaction volume containing 25 mM pyrophosphate buffer, pH 8.8, 2.5 mM NAD⁺ and 360 mM ethanol.
Figure 5.2 Secondary log/log plot of the concentration dependent inactivation of ADH by H$_2$O$_2$. 

\[
y = 1.1985x + 0.1733 \\
R^2 = 0.9954
\]
H₂O₂ complex (250 ± 37 mM) were determined from a double reciprocal plot of 1/k' vs. 1/H₂O₂ concentration (not shown). Having established these kinetic parameters, the effects of specific active-site-directed reagents could be evaluated in quantitative terms that are reflective of the rate constants and the reaction order for ADH inactivation.

**Effect of Protective Agents on Inactivation of ADH by H₂O₂**

Based on sulfhydryl group titration and limited NAD⁺ protection Favilla et al. (Favilla et al., 1980a; Favilla et al., 1980b) suggested that the oxidation of active site cysteine residues by H₂O₂ was associated with ADH inactivation. We extended these studies by including study of the effects of the reduced and oxidized coenzymes, AMP and other classical ADH protective complexes (Chen and Plapp, 1978; Dahl and McKinley-McKee, 1977; Levy et al., 1963; Plapp, 1973; Reynolds and McKinley-McKee, 1972). Studies by others (Favilla and Cavatorta, 1975; Favilla et al., 1980a) showed that when ADH was preincubated with NAD⁺ the enzyme was protected from inactivation by H₂O₂ and the degree of protection decreased with time due to consumption of the coenzyme during the peroxidatic reaction (Favilla et al., 1980a). This protection by NAD⁺ and measurement of -SH group oxidation by H₂O₂ were the only lines of evidence to indicate the active-site as the locus of peroxide action. Figure 5.1 shows that when ADH was preincubated with 100 mM H₂O₂ the enzyme activity decayed with a t₁/₂ of about 25 min. These conditions were used as the control for study of the effects of the protective agents. To study the effects of the active site-directed reagents on the inactivation of ADH by H₂O₂ the enzyme was preincubated with the reagents for 5 min prior to the addition of 100 mM H₂O₂. The effect of these reagents
on the apparent first-order rate constants and $t_{1/2}$ for inactivation of ADH are presented in Table 5.2.

The presence of 3-6 mM NAD$^+$ was shown previously to protect ADH against inactivation by 20 mM H$_2$O$_2$, presumably because coenzyme binding prevents peroxide access to Cys-46 and Cys-174 (Favilla et al., 1980a). Under the conditions of our assay (5 &mu;M ADH, 8 mM NAD$^+$, 100 mM H$_2$O$_2$) NAD$^+$ afforded relatively little protection against ADH inactivation by H$_2$O$_2$, (~ 13%), even at concentrations ten-fold greater than their dissociation constants (Plapp, 1970). NADH afforded much stronger protection under the same conditions. In the presence of 0.2 mM NADH, $k'$ for ADH inactivation by H$_2$O$_2$ was about 5-times lower than in its absence (Table 5.2).

<table>
<thead>
<tr>
<th>Protective Agents</th>
<th>$t_{1/2}$ min</th>
<th>$k' = \ln(2)/t_{1/2}$ min$^{-1}$</th>
<th>Relative protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM H$_2$O$_2$</td>
<td>25</td>
<td>0.029</td>
<td>---</td>
</tr>
<tr>
<td>0.2 mM NADH</td>
<td>150</td>
<td>0.005</td>
<td>84 %</td>
</tr>
<tr>
<td>8 mM NAD$^+$</td>
<td>29</td>
<td>0.024</td>
<td>13 %</td>
</tr>
<tr>
<td>1 mM AMP</td>
<td>86</td>
<td>0.008</td>
<td>80 %</td>
</tr>
<tr>
<td>1 mM GSH</td>
<td>28</td>
<td>0.025</td>
<td>10 %</td>
</tr>
<tr>
<td>1 mM DTT</td>
<td>23</td>
<td>0.030</td>
<td>0 %</td>
</tr>
<tr>
<td>10 mM Imidazole</td>
<td>7</td>
<td>0.099</td>
<td>-66 %*</td>
</tr>
</tbody>
</table>

* rate of ADH inactivation by H$_2$O$_2$ in the presence of imidazole is stimulated by 66%

A similar effect was reported for bromoacetamido compounds, where the rate of ADH inactivation was four to five times faster in the presence of NAD$^+$ than in the presence of NADH (Chen and Plapp, 1978).
AMP, which binds to an anion-binding site formed by Lys-228, afforded strong protection of ADH from inactivation. This protection, which is reflected in the decreased $k'$ of inactivation (90%) coincides with the reported protection against carboxymethylation of ADH by the Lys-modifying reagent iodoacetic acid (Reynolds and McKinley-McKee, 1969).

GSH and DTT, which protect $-\text{SH}$ groups from oxidation were evaluated for their ability to prevent ADH inactivation by $\text{H}_2\text{O}_2$. Despite the fact that inactivation of ADH by $\text{H}_2\text{O}_2$ was associated with modification of sulfhydryl groups in the active site (Favilla et al., 1980a) 1 mM GSH only slightly protected and 1 mM DTT afforded no protection (actually caused a slight stimulation) of ADH from inactivation by $\text{H}_2\text{O}_2$. These data suggest that GSH and DTT do not prevent oxidation of $-\text{SH}$ ligand within the active site of ADH.

Imidazole, which binds to the active site metal-center of ADH (Dahl and McKinley-McKee, 1977) increased the rate of $\text{H}_2\text{O}_2$-dependent inactivation by 66%. A possible explanation for this increase in the rate of inactivation is that when imidazole binds to ADH one or more of the $-\text{SH}$ ligands of Zn are perturbed; thus, making them more accessible to oxidative attack by $\text{H}_2\text{O}_2$. It is noteworthy that the ternary complex ADH-NADH-imidazole is the only species in which a direct bonding interaction occurs between the coenzyme molecule and the ligand sphere of the catalytic metal ion, i.e. the hydrogen bond between the sulfur atom of Cys-46 and the carboxamide group of NADH (Bertini et al., 1987).
pH-Dependent Modification of ADH by H$_2$O$_2$

To implicate a role for ionizable amino acid residues in H$_2$O$_2$-dependent inactivation of ADH the rate of inactivation was studied over the pH range of 5.9 to 8.8; in this pH range the enzyme is stable (Maskos and Winston, 1993). For these studies aliquots were removed from the incubation mixture of ADH and H$_2$O$_2$ at the various pH, and ethanol oxidation was determined on the removed aliquots as described in materials and methods. Rate constants were calculated from the half-lives of the enzymes from first-order rate decay plots as in Table 5.1. A plot of the rate constants versus pH shows no change in the rate of inactivation of the enzyme over the range of pH 5.9 to pH 8.4 (Fig. 5.3). The rate of inactivation was increased markedly at pH above 8.4. These results suggest that inactivation of the ADH by H$_2$O$_2$ is associated with an ionizable group having a pKa of about 8.4.

SH Group Analysis

Each subunit of ADH contains 14 free -SH groups (Sigman, 1967). Two of them are liganded to the catalytic Zn atom (Fig. 5.4) and their modification can alter the activity of ADH. To implicate a role for -SH group modification in H$_2$O$_2$-dependent inactivation of ADH we monitored the change in -SH group content of the enzyme and the accompanying process of inactivation. Prior to treatment with H$_2$O$_2$ an experimental value of about 13.5 thiol/thiolate groups per ADH subunit (not shown) was obtained. ADH was denatured with guanidine after H$_2$O$_2$ treatment; thus, only the change in number of thiol groups modified by H$_2$O$_2$ was calculated. Oxidation of about 5-6 thiol groups per ADH subunit resulted in greater than 80% inactivation. The time course of thiol group oxidation shows two distinct phases (Fig. 5.5a). Figure 5.5b shows the
Figure 5.3  
**pH-dependence of ADH inactivation by H$_2$O$_2$.** HLADH (5 μM) was incubated with 100 mM H$_2$O$_2$ at the indicated pH values. Aliquots (10 μl) were removed from the incubation mixture at various times between 1 and 120 min in order to determine the k' values for each pH. Each aliquot was then assayed for ethanol oxidation as described in methods and materials.
Figure 5.4 Schematic representation of active site ligands of HLADH.
Figure 5.5a  Time-dependent modification of –SH groups of ADH during incubation with H$_2$O$_2$. ADH (20 μM) was incubated with 50 mM H$_2$O$_2$. Aliquots (40 μl) were removed and assayed for total –SH group content under denaturing conditions (5.7 M Guanidine) with Ellman’s reagent as described in materials and methods.
Residual ADH activity during incubation with of H₂O₂ vs. modified –SH group content. The ethanol oxidase activity and –SH group content of HLADH were determined as described in materials and methods on aliquots withdrawn from the incubation mixture of H₂O₂ (50 mM) and HLADH (20 μM) at various time intervals.
relationship between the residual activity of ADH as a function of the number of –SH groups oxidized. In the first faster phase of the time course at least two thiol groups per subunit were oxidized within ten min of the incubation with 50 mM H2O2 (Fig. 5.5a).

This corresponded to a small (~15%) decrease in ADH activity (Fig. 5.5b). In the second slower phase, additional thiol group oxidation coincided with the observed further loss in catalytic activity of the enzyme (Fig. 5.5b). The first two thiol groups oxidized are most likely Cys residues external to the hydrophobic active site, which do not significantly affect catalysis by ADH.

Favilla et al. (Favilla et al., 1980a) reported that two cysteine residues per ADH subunit were oxidized within 30 min when 10 μM enzyme was treated with 6 mM H2O2, and complete inactivation of ADH required oxidation of at least 3 –SH groups per subunit. From these studies one can assume that –SH groups oxidized by H2O2 correspond not to only active site cysteines as was previously suggested (Favilla et al., 1980a), but also other –SH groups as well.

**Effect of H2O2 on Spectral Transitions of Cobalt-Substituted ADH**

Direct evidence of active site modification by H2O2 can be obtained from studying cobalt-substituted ADH. Co(II)ADH has a distinctive ligand to metal charge transfer band (LMCT) at 340 nm and a characteristic absorbance that reflects d-d-transitions of the cobalt atom at 645 nm in the electronic spectrum (Fig. 5.6). To better understand the effects of H2O2 on the metal center of the enzyme as well as the effects of the protective reagents used in the afore-mentioned kinetic studies, the absorbance of LMCT and d-d transition bands of active-site-substituted Co(II)ADH upon treatment
with H₂O₂ was studied in the absence and presence of protective agents used to generate Table 5.2.

Due to their low extinction coefficients \( \epsilon_{\text{LMCT}} = 1200 \text{ cm}^{-1} \text{ mM}^{-1}, \epsilon_{\text{d-d}} = 700 \text{ cm}^{-1} \text{ mM}^{-1} \), high concentrations of Co(II)ADH were required to effectively visualize changes in the LMCT and d-d transition regions of the spectrum (Fig. 5.6). When 10 mM H₂O₂ was incubated with 50 \( \mu \text{M} \) Co(II)ADH a loss of absorbance of the LMCT and d-d transition bands was observed within 4 min of H₂O₂ addition (Fig. 5.6 inset). These spectral transitions reflect either the direct interaction of H₂O₂ with the metal atom, or with the ligands associated with the coordination sphere of the metal center. The d-d region of the spectrum reflects the distorted tetrahedral geometry of the cobalt coordination sphere (Sytkowski and Vallee, 1975). Thus, the binding of certain ligands to the active site of the enzyme, either at the metal center itself, or close enough for orbital overlap with the corners of the tetrahedral structure of the coordination sphere, is reflected in the spectral transitions of the d-d band observed in figure 5.7 (Huheey et al., 1993). A profound change in the Co(II)ADH spectrum (Fig. 5.7, solid line) is seen in the spectrum of the ternary complex of coenzyme and the ADH inhibitor pyrazole (Fig. 5.7, dashed line). In this case, a splitting of the 645 nm band yields a second band at 678 nm (Fig. 5.7) indicating strong perturbation of the geometry of the Co(II)ADH coordination sphere.

In the binary complex of Co(II)ADH-NADH the absorbance maximum at 645 is shifted to 678 nm (Fig. 5.8a, trace 1), which reflects the coenzyme-triggered
Figure 5.6  Time-dependent changes in LMCT and d-d transition bands of the electronic spectrum of Co(II)ADH by H$_2$O$_2$. 50 µM ADH was incubated with 10 mM H$_2$O$_2$ and the course of the reaction was scanned over the indicated range of wavelengths at 60 seconds intervals; Inserts: Time dependent change in the 340 nm (LMCT) and 645 nm (d-d transition) bands of Co(II)ADH spectra.
Figure 5.7  Spectral splitting of d-d transition band of Co(II)ADH upon addition of NAD\(^+\) and pyrazole. 20 \(\mu\)M ADH was incubated with 2 mM NAD\(^+\) and 10 mM pyrazole in Tes buffer, pH 7.4
conformational change of the protein involving the catalytic metal ion. Addition of 
H₂O₂ to the cuvette containing the NADH-ADH binary complex resulted in a blue shift 
of the absorbance maximum to about 668 nm, which reflects the direct coordination of 
the cobalt (II) to the substrate (Dietrich et al., 1979).

Following the addition of H₂O₂ a small loss of absorbance at 668 nm of 0.001 
AU is seen over a period of 5 min (Fig. 5.8a, traces 2 and 3). When compared to the 0.008 AU loss by the same concentration of H₂O₂ (Fig. 5.6) the data indicate relatively strong protection by NADH from absorbance loss caused by H₂O₂. The typical bimodal 
spectrum of the NADH-pyrazole-ADH ternary complex was observed upon addition of pyrazole after 7 min of incubation with H₂O₂ (Fig. 5.8a, trace 4) suggesting that the integrity of the metal center was not significantly altered by H₂O₂ when the enzyme was protected by NADH.

Pretreatment of Co(II)ADH with 25 mM NAD⁺ i.e., 25 times greater than the concentration of NADH used for Fig. 5.8a, afforded relatively less protection of the enzyme from H₂O₂-dependent loss of absorbance of the d-d transition region (compare the 0.004 AU loss of the d-d band in Fig. 5.8b with the 0.001 AU loss in Fig. 5.8a). This data was in agreement with the limited protection by NAD⁺ against H₂O₂-dependent inactivation of the Zn enzyme (Table 5.2). The addition of 10 mM pyrazole to the cuvette containing ADH, NAD⁺ and H₂O₂ ten minutes after addition of H₂O₂ did not produce the spectrum of the ternary complex as was seen in figure 5.8a. Thus, it appeared that the metal center had been significantly disrupted upon incubation with H₂O₂, even in the presence of NAD⁺.
Figure 5.8a  
Effect of NADH on the time-dependent changes in d-d transition region of the electronic spectrum of Co(II)ADH by H$_2$O$_2$.

1) Co(II)ADH (10 μM) + 1 mM NADH in Tes buffer pH 7.4
2) Co(II)ADH/NADH + 10 mM H$_2$O$_2$  1 min
3) Co(II)ADH/NADH/H$_2$O$_2$  5 min
4) Co(II)ADH/NADH/H$_2$O$_2$ + 10 mM pyrazole
Figure 5.8b  Effect of NAD⁺ on the time-dependent changes in d-d transition region of the electronic spectrum of Co(II)ADH by H₂O₂.

1) Co(II)ADH (20 μM) in Tes buffer pH 7.4
2) Co(II)ADH + 25 mM NAD⁺
3) Co(II)ADH/ NAD⁺ + 10 mM H₂O₂  1 min
4) Co(II)ADH/ NAD⁺/H₂O₂  2 min
5) Co(II)ADH/ NAD⁺/H₂O₂  3 min
6) Co(II)ADH/ NAD⁺/H₂O₂ + 10 mM pyrazole

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Figure 5.8c  Effect of imidazole on the time-dependent changes in d-d transition region of the electronic spectrum of Co(II)ADH by H$_2$O$_2$.

1) Co(II)ADH (40 μM) in Tes buffer pH 7.4
2) Co(II)ADH + 2 mM imidazole
3) Co(II)ADH/imidazole + 10 mM H$_2$O$_2$ 1 min
4) Co(II)ADH/imidazole/H$_2$O$_2$ 2 min
5) Co(II)ADH/imidazole/H$_2$O$_2$ 3 min
6) Co(II)ADH/imidazole/H$_2$O$_2$ 4 min
Figure 5.8d Effect of AMP on the time-dependent changes in d-d transition region of the electronic spectrum of Co(II)ADH by H₂O₂.

1) Co(II)ADH (10 μM) in Tes buffer pH 7.4
2) Co(II)ADH + 10 mM AMP
3) Co(II)ADH/AMP + 10 mM H₂O₂ 1 min
4) Co(II)ADH/AMP/H₂O₂ 5 min
Pretreatment of Co(II)ADH with imidazole, which binds to the cobalt atom and the -SH group of Cys-46 (Reynolds and McKinley-McKee, 1969) provided no protection against H$_2$O$_2$-dependent loss of the d-d transition band (Fig. 5.8c). In fact imidazole facilitated the loss of the d-d band by H$_2$O$_2$ (compare the 0.008 AU loss of this band in figure 5.6 to the 0.016 AU loss in the presence of imidazole in figure 5.8c). This is reminiscent of the increased inactivation rate constant for ADH in the presence of imidazole (Table 5.2). Preincubation of imidazole with the native enzyme showed no protection as well and even accelerated the rate of ADH inactivation. Pretreatment of Co(II)ADH with AMP strongly prevented loss of the d-d transition region of the Co-substituted enzyme caused by H$_2$O$_2$ treatment (Fig. 5.8d), which is in excellent agreement with the effect of AMP on the ADH inactivation rate constant (Table 5.2).

**DISCUSSION**

Earlier studies by Favilla et al. (Favilla et al., 1980a; Favilla et al., 1980b) have shown that when horse liver ADH is incubated in the presence of H$_2$O$_2$ the enzyme loses catalytic activity over time. The observation that NAD$^+$ protected ADH from inactivation by H$_2$O$_2$ led those authors to suggest an active site-directed mechanism involving oxidation of the Zn ligands Cys-46 and Cys-174 (Favilla et al., 1988). No direct evidence for this mechanism was presented. Herein, we provide more direct evidence that inactivation of ADH by H$_2$O$_2$ was indeed associated with oxidant modification of the -SH metal center ligands and also with the modification of an active site anion hole that accommodates the binding of the pyridine nucleotide cofactor. This evidence is based on the relative protection by several active-site-directed reagents.
against H₂O₂-dependent inactivation of native Zn-ADH and loss of integrity of the metal coordination sphere of cobalt-substituted ADH.

As stated under Results in this manuscript, there are 14 titratable (with Ellman’s reagent) sulfhydryl groups per subunit of ADH, two of which are active site Zn ligands. The fact that strong reducing agents such as DTT (1 mM) and GSH (1 mM) did not prevent inactivation of ADH by H₂O₂ (Table 5.2) suggest that modifications of -SH groups external to the active site of the enzyme have a negligible effect on ADH catalytic activity; therefore, we assume that observed H₂O₂-dependent inactivation reflects interaction of H₂O₂ with amino acid residues inside of the active site.

NADH protects the enzyme from inactivation by H₂O₂, however protection by NAD⁺ was significantly less. The strong protection afforded by NADH corresponds well with the protection against loss of absorbance of the d-d transition band of Co(II)ADH by H₂O₂ (Fig. 5.7). The stronger protection afforded by NADH compared to NAD⁺ against H₂O₂ was also observed in studies of bromoacetamido compounds, in which the rate of ADH inactivation was four to five times faster in the presence of 1 mM NAD⁺ than in the presence of 0.2 mM NADH (Chen and Plapp, 1978). The pyrophosphate group of NADH binds to the guanidinium groups of Arg-47 and Arg-369, but Lys-228 also interacts electrostatically (Sekhar and Plapp, 1988). The positively charged Lys-228 best attracts the negatively charged pyrophosphate of NADH. Bertini et al. (Bertini et al., 1987) based on ¹H-NMR studies suggested that the nicotinamide ring of NAD⁺ has a different orientation within the active site of ADH than NADH. This may account for electrostatic repulsion of the positively charged nicotinamide ring (Adolph et al., 1997), and the observed faster dissociation of NAD⁺.
than NADH. Thus, at pH 7.4, NAD\(^+\) binds more weakly to the coenzyme-binding site, which could be envisaged to permit greater accessibility of H\(_2\)O\(_2\) to the active site coordination sphere.

Further evidence of a role for Lys-228 in peroxide-mediated inactivation of ADH is seen in the protective effect of AMP on this process. AMP, which specifically binds to Lys-228 (Chen and Plapp, 1978) afforded greater than 90% protection against H\(_2\)O\(_2\) inactivation of both native and metal substituted enzyme, an observation that is consistent with the preventive effect of AMP against carboxymethylation of ADH by iodoacetic acid (Reynolds and McKinley-McKee, 1969). The protective effect of AMP against inactivation of ADH by H\(_2\)O\(_2\) suggests that in the absence of coenzyme, H\(_2\)O\(_2\) could bind to an anion-binding site formed by Arg-47 and Lys-228 and from that locus oxidize Cys-46 or Cys-174 bound to the catalytic zinc.

Imidazole is a reversible inhibitor of ADH and has been used to elucidate the role of the catalytic zinc atom, to which it binds (Reynolds and McKinley-McKee, 1969). As does AMP, the binary ADH-imidazole complex protected ADH from carboxymethylation with iodoacetate (Reynolds and McKinley-McKee, 1969). In the absence of coenzyme, imidazole increased the rate of inactivation of ADH by H\(_2\)O\(_2\) by 66% and did not protect against the H\(_2\)O\(_2\)-dependent loss of absorbance of the d-d transition bands of Co(II)ADH. This further implies that the interaction of H\(_2\)O\(_2\) with the metal center most likely occurs through modification of the metal bound ligands. Incubation of the enzyme with 50 mM H\(_2\)O\(_2\) results in modification of at least 4 -SH groups per subunit and 2 of the 4 oxidized -SH groups are indicated to be associated with Cys-46 and Cys-174, which are integral with the metal coordination sphere of the
The loss of LMCT bands of the active site Co(II)-substituted ADH after treatment with H\textsubscript{2}O\textsubscript{2} is highly consistent with oxidation of the active site metal ligands. The catalytic metal is coordinated through a 2 Cys, 1 His and 1 water ligand motif (Fig. 5.4), which results in a distorted tetrahedral coordination geometry and the corresponding d-d transition band at 650 nm in the visible spectrum of Co(II)ADH (Sytkowski and Vallee, 1975). The d-d transition band of the structural cobalt center, which is coordinated by four cysteines in near perfect tetrahedral geometry absorbs at 750 nm (Sytkowski and Vallee, 1978). Disruption of any of four bonds between cobalt and its associated ligands would be reflected in the spectral changes of both the charge transfer and d-d transition bands. Identification of the ligands that comprise the coordination sphere is not obtainable from the electronic spectrum of the cobalt enzyme. The thiol group study indicated oxidation of Cys-46 and Cys-174, but the fate of remaining zinc ligand, His-67, is not known. Histidine coordination alone is not sufficient to maintain the conformation of zinc in its catalytic form in the protein. The pKa for inactivation of ADH by H\textsubscript{2}O\textsubscript{2} of 8.4 (Fig. 5.3) does not support a direct role for His-67, which has a pKa of about 6.9. Enzymes for which a catalytic role exists for His typically display an activity change at pH 6.9 (Hennecke and Plapp, 1983). The rate of inactivation of ADH was associated with a large increase in the value of k' at pH 8.4 suggesting the ionization of the active site-bound water molecule (Brooks and Shore, 1972; Kvassman and Pettersson, 1979; Maret and Zeppezauer, 1986).

Despite inherent differences in the chemistry between Zn\textsuperscript{2+} and Co\textsuperscript{2+}, which result in about a 50% lower catalytic activity of the Co-substituted enzyme, the substitution of Co\textsuperscript{2+} for Zn\textsuperscript{2+} does not alter the coordination geometry of the active site.
of ADH as shown by protein crystallography (Schneider et al., 1983). The data obtained with the cobalt enzyme in the present study clearly establishes the validity of our use of site-directed reagents as evinced by the fact that those agents which protected native ADH from inactivation by \( \text{H}_2\text{O}_2 \) are the same reagents that prevented destruction and perturbation of metal coordination sphere of Co(II)ADH. Regardless of model used it seems reasonable to suggest that the irreversible inactivation of ADH by \( \text{H}_2\text{O}_2 \) in the absence of coenzyme was caused by both the oxidation of the active site –SH groups of Cys-46 and Cys-174 and the modification of an anion-binding site formed by Lys-228.
Chapter 6: Interaction of tert-Butyl Hydroperoxide and Cumene Hydroperoxide with Horse Liver Alcohol Dehydrogenase

INTRODUCTION

Alcohol dehydrogenase [EC 1.1.1.1], an NAD⁺/NADH dependent zinc metalloenzyme (Pocker and Li, 1996), displays broad substrate specificity, and has been suggested to participate in cellular detoxification mechanisms (Estonius et al., 1996). A recently described peroxidatic activity is particularly interesting. Utilizing H₂O₂ as a co-substrate, ADH converted the oxidized coenzyme NAD⁺ to a stable product referred to as NADX, which is different from the normal redox partner NADH (Favilla et al., 1988; Favilla et al., 1980b). In addition to being a substrate for ADH, H₂O₂ caused irreversible inactivation of ADH, which was partially prevented by pretreatment of the enzyme with NAD⁺ (Favilla et al., 1980a; Favilla et al., 1980b). NADX was also formed when horse liver ADH was incubated with NAD⁺ and methylbenzyl hydroperoxide (MBHP) (Skursky et al., 1992b). In attempts to compare the effects of MBPH with that of tBOOH and CHP on HLADH we observed that these peroxides caused stimulation of the enzyme, even when it was preincubated with high (10 - 100 mM) concentrations of these peroxides (Chapter 4). Furthermore, neither tBOOH nor CHP served to generate NADX in the peroxidatic reaction of ADH, NAD⁺ and horse liver ADH.

Hydroperoxides are ubiquitous compounds in nature. Many organic compounds, including ethers, acetals, olefins, ureas, amides, aldehydes, and alcohols can form potentially toxic peroxides (Mirafzal and Baunsgarten, 1988). The potential for human exposure to organic peroxides via by-products of drinking water ozonation and possible metabolites of common fuel additives also exists.
In light of these marked differences that we observed in the behaviors and reactivities of tBOOH and CHP as compared to the effects of MBPH with respect to ADH, we undertook a detailed kinetic study of tBOOH and CHP interactions with horse liver ADH. As part of this study we investigated the effects of organic hydroperoxides on the coordination geometry of the active site and coenzyme binding sites were evaluated by use of cobalt-substituted horse liver ADH (Co(II)HLADH). Co(II)ADH has distinguishable ligand to metal charge transfer and d-d- transition regions in the electronic spectrum for catalytic and structural cobalt ions that permit visualization of active site coordination sphere interactions with the peroxides.

MATERIALS AND METHODS

Enzymes and Reagents

Horse liver alcohol dehydrogenase was obtained from Boehringer Mannheim, USA. The concentration of ADH was determined via the absorbance at 280 nm (for HLADH $\varepsilon=35.7\times10^3 \text{ M}^{-1}\text{cm}^{-1}$ (Chen et al., 1987)). $\beta$NAD$^+$ (> 99% purity) was purchased from Boehringer Mannheim, USA. NADH (98% purity) was purchased from Sigma Chemical Co., St. Louis, MO. Concentrations of the coenzyme solutions were determined spectrophotometrically from the extinction coefficients of $18.0\times10^3 \text{ M}^{-1}\text{cm}^{-1}$ at 260 nm for NAD$^+$ and of $6.22\times10^3 \text{ M}^{-1}\text{cm}^{-1}$ at 340 nm for NADH. tert-Butyl and cumene hydroperoxide were obtained from Sigma as 70-80% solutions. All other chemicals were of analytical grade.

Enzyme Assay

ADH activity was determined according to the method of Vallee and Hoch (Vallee and Hoch, 1955) from initial velocities of NADH production at 340 nm on a
Perkin Elmer Lambda 6 UV-Vis spectrophotometer. Reactions were conducted in quartz cuvettes of 1 cm light-path containing 2.5 mM NAD\(^+\) and 360 mM ethanol in 1 ml of 32 mM sodium pyrophosphate buffer, pH 8.8. The concentration of ADH active sites was determined by pyrazole titration (Theorell and Yonetani, 1963).

**Peroxide-Modified Enzyme Studies**

Horse liver alcohol dehydrogenase was incubated in 50 mM potassium phosphate buffer, pH 7.4. The reaction was initiated by adding the peroxide to the enzyme mixture, in either the presence or absence of specified protective agents. The effect of peroxides on the modification of ADH with time was measured using a Perkin Elmer Lambda 6 UV-Vis spectrophotometer by withdrawing specified aliquots from the incubation mixture, adding these withdrawn aliquots to assay cuvettes and measuring the initial rate of NADH production.

**Sulfhydryl Group Analysis**

Total sulfhydryl content before and during incubation of ADH with tBOOH was determined essentially by the method of Ellman (Ellman, 1959) as described in Chapter 5.

**Preparation of Active-Site-Substituted ADH**

The substitution of Cobalt (II) for active site zinc ion ADH was performed as described by Maret (Maret et al., 1979) with minor modifications as described in Chapter 2. The metal analysis was performed by ICP, which confirmed that the substitution of Zn by Co was greater than 85%. Metal substitution was also confirmed by EPR and UV/Vis spectra (Maret et al., 1979).
RESULTS

Dependence of Stimulation of Ethanol Oxidation by ADH on tBOOH and CHP Concentrations

In contrast to the inactivation of HLADH caused by H₂O₂, when HLADH was preincubated with tBOOH or CHP stimulation of HLADH-dependent ethanol oxidation was observed. tBOOH (100 mM) increased the rate of the reaction by 200 ± 15% and CHP (100 mM) by 170 ± 20% respectively (Fig. 6.1). The course of the reaction was monitored by assaying 10 μl aliquots withdrawn from the incubation mixture at the indicated time points. This method introduced small amounts of tBOOH or CHP (0.01-3 mM) to the ethanol oxidation mixture; therefore, their effect on the assay mixture was tested, and no significant effect on the ADH activity was observed. Direct addition of 50 mM tBOOH or CHP to the reaction mixture did not have any noticeable effect on the rate of the reaction; thus, HLADH stimulation required preincubation of the enzyme with organic hydroperoxides. Addition of CHP introduced small amounts of methanol to the reaction mixture, and a slight inhibitory effect of methanol was observed, therefore methanol was added to controls. We also considered the possibility that contaminant iron present in phosphate and pyrophosphate buffers might, via Fenton chemistry, have generated alkoxy radicals (RO) from hydroperoxides (Winston et al., 1983), which might have contributed to the kinetic modifications shown in Figure 6.1. No differences in the kinetics of ADH stimulation were observed with Chelex-treated as compared to buffers that had not been so treated.
Figure 6.1 Stimulation of HLADH-dependent ethanol oxidation by tBOOH and CHP. HLADH (3 μM) was incubated at 25°C with tBOOH or CHP in 50 mM potassium phosphate, pH 7.4; 10 μl aliquots from the incubation mix were removed at times shown and assayed for ethanol oxidation activity.
Figure 6.2  

a) Secondary plot of the rate of HLADH stimulation by tBOOH.  
b) Secondary plot of the rate of HLADH stimulation by CHP.
The stimulation was observed with relatively high concentrations of tBOOH and CHP (10-300 mM). When the concentration of tBOOH or CHP was below 10 mM stimulation was observed as well, but the incubation time required to achieve this stimulation was typically 3 or more hours. The higher the concentration of tBOOH or CHP in the preincubation mix the more rapid was the stimulation of ADH activity. While the maximum level of stimulation by tBOOH was essentially unaffected by tBOOH concentrations, the maximum level of CHP stimulation was dependent on CHP concentration (Fig. 6.1).

Incubation of the enzyme with tBOOH and CHP for prolonged periods of time led to a progressive loss of the stimulatory effect, which indicates that the observed stimulation was reversible. The reversible phase of the reaction could be envisaged as competing oxidative damage to the enzyme, perhaps through a free radical-mediated process at the active site.

To quantify the stimulatory effects of tBOOH and CHP on HLADH the parameters $t_{150}$ and $t_{max}$ are introduced and defined, respectively as the time of incubation with the peroxides to achieve 50% stimulation of HLADH activity above the activity in their absence, and the time needed to achieve maximum stimulation (Table 6.1). The $t_{150}$ and $t_{max}$ decreased with tBOOH concentration up to 100 mM; above this concentration $t_{150}$ and $t_{max}$ remained relatively constant (Table 6.1). The lower water solubility of CHP limited the concentration range studied to 100mM. The rate of activation was directly dependent on tBOOH or CHP concentration (Fig. 6.2). The apparent bimolecular rate constant of activation, $k_a$, obtained from the slope of the best straight line was 1.0 M$^{-1}$min$^{-1}$ for tBOOH and 2.2 M$^{-1}$min$^{-1}$ for CHP (Fig. 6.2).
Table 6.1  \( t_{150} \) and \( t_{\text{max}} \) for stimulation of ethanol oxidation by HLADH pretreated with tBOOH and CHP.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>10 mM</th>
<th>25 mM</th>
<th>50 mM</th>
<th>100 mM</th>
<th>150 mM</th>
<th>200 mM</th>
<th>300 mM</th>
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<tbody>
<tr>
<td>tBOOH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( t_{150%} ) min</td>
<td>110</td>
<td>70</td>
<td>33</td>
<td>11</td>
<td>7</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>( t_{\text{max}} ) min</td>
<td>360</td>
<td>250</td>
<td>140</td>
<td>62</td>
<td>45</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>Activation Rate min(^{-1})</td>
<td>0.009</td>
<td>0.014</td>
<td>0.03</td>
<td>0.09</td>
<td>0.14</td>
<td>0.17</td>
<td>0.2</td>
</tr>
<tr>
<td>CHP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( t_{150%} ) min</td>
<td>28</td>
<td>14</td>
<td>7</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( t_{\text{max}} ) min</td>
<td>116</td>
<td>72</td>
<td>47</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activation Rate min(^{-1})</td>
<td>0.036</td>
<td>0.071</td>
<td>0.14</td>
<td>0.25</td>
<td></td>
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</table>

Kinetics of Reactions Catalyzed by Native and tBOOH-Modified Enzymes

Various reagents that are able to modify HLADH activity depend on the concentration of ethanol in the reaction (Dalziel and Dickinson, 1966b; Kaplan et al., 1956; Plapp, 1970; Theorell and McKinley-McKee, 1961; Theorell et al., 1969). A given reagent could act as a positive effector and stimulate the oxidation of ethanol by HLADH when ethanol concentration is high and as a negative effector inhibiting enzyme activity at low ethanol concentration (Dalziel and Dickinson, 1966b; Kaplan et al., 1956; Theorell et al., 1969). The kinetic basis for the enhanced activity of the tBOOH-modified enzyme was studied by means of Michaelis-Menten kinetics using variable concentrations of NAD\(^+\) and ethanol. The assays with native and modified enzymes were performed at pH 8.8 (Fig. 6.3). At constant tBOOH concentration and NAD\(^+\) as the variable substrate, the tBOOH-modified enzyme showed a classical noncompetitive activation pattern (Cleland, 1988) with unchanged \( K_m \) at 100 ± 5 \( \mu \)M and increased \( k_{\text{cat}} \) from 0.24 to 0.47 min\(^{-1}\) (Fig. 6.3a).

Preliminary studies show that tBOOH-dependent stimulation of HLADH activity was not affected by the concentration of ethanol (4-800 mM) present in reaction
mixture (Chapter 4). However, additional kinetic studies showed that the stimulatory
effect of tBOOH changed to an inhibitory effect when the concentration of ethanol was
below 1 mM in the reaction mix. An increase in both Km and Vmax for tBOOH-treated
enzyme with respect to ethanol was observed (Fig. 6.3b) and was in agreement with
previous studies of ADH activators, e.g., cyanide, imidazole, cyclohexanol (Dalziel and
Dickinson, 1966b; Plapp, 1970; Theorell et al., 1969). The double reciprocal plot shown
in figure 6.3b is a typical example of competitive inhibition and stimulation (CIS) as
described by Theorell et al. (Theorell et al., 1969).

The fact that tBOOH-modified enzyme had the same Km values for NAD^ suggested that NAD^ binding was not affected by tBOOH, i.e., the affinity of the enzyme for NAD^ was unchanged. The increase of Km and Vmax obtained from double reciprocal plots of 1/v vs. 1/[ethanol] for the native and tBOOH-treated enzyme suggested that both substrate binding and substrate-coenzyme complex dissociation were affected.

**pH Studies of Ethanol Oxidation by ROOH-Treated ADH**

We had previously demonstrated a role for two ionizable groups with pKa values of 6.9 and 8.2 in tBOOH-dependent stimulation of ADH (Chapter 4). In that study tBOOH was incubated with ADH at various pH (5.9-8.9) and ethanol concentration was measured at constant pH (8.8).

In the present study we show the effect of tBOOH modification on ethanol oxidation as a function of pH. In these experiments ADH was incubated with 100 mM tBOOH at a constant pH of 7.4. After reaching t_{\text{max}}, aliquots of the tBOOH-modified ADH were removed and ethanol oxidation was measured in mixtures of variable pH.
Figure 6.3a  Double reciprocal plot of the effect of NAD$^+$ concentration on ethanol oxidation activity of ADH. HLADH (5 μM) was incubated with 100 mM tBOOH. Aliquots (10 μl) were removed from the incubation mixture at tmax and assayed for ethanol oxidation activity in the presence of 360 mM ethanol and variable NAD$^+$ concentration (50-800 μM).
Figure 6.3b Double reciprocal plot of the effect of ethanol concentration on ethanol oxidation activity of ADH. HLADH (5 μM) was incubated with 100 mM tBOOH. Aliquots (10 μl) were removed from the incubation mixture at tmax and assayed for ethanol oxidation activity in the presence of 2.5 mM NAD$^+$ and variable ethanol concentration (0.5 - 800 mM) of ethanol.

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pH profiles for these reactions were essentially identical to that of the native enzyme (Fig. 6.4) and indicated two ionizable groups with pKa values of 7.3 and 9.2. The latter has been ascribed to the zinc-bound water molecule/hydroxyl ion, the pKa of which has been reported as 8.8 (Maret and Zeppezauer, 1986). The stronger stimulation of ADH by tBOOH observed at pH 8.8 as compared to pH 7 is due, at least in part, to the relief from substrate inhibition by ethanol of the native enzyme above pH 8.8 (Kvassman and Pettersson, 1979). The magnitude of ADH stimulation by tBOOH did not appear to be related to pH as the percentage of stimulation of ethanol oxidation by tBOOH-modified enzyme was essentially unchanged over the pH range studied.

**Peroxidatic Activity of HLADH toward Organic Hydroperoxides**

The interaction of H$_2$O$_2$ with HLADH, in which NAD$^+$ is converted to a unique product NADX, which has a distinctive UV-spectra with a $\lambda_{max}$ of 300 nm (Favilla and Cavatorta, 1975; Favilla et al., 1980a; Favilla et al., 1988), prompted the study of whether NAD$^+$ might similarly generate NADX with organic hydroperoxides. NADX is produced in a two-step process involving an enzymatic and a nonenzymatic component. The enzymatic reaction produces compound I with an absorbance maximum of about 285 nm (Skursky et al., 1992b), which reflects the presence of intermediates in the reaction (Fig. 6.5a).

When tBOOH or CHP was substituted for H$_2$O$_2$ in the peroxidatic reaction with HLADH, no chromophores were detected in the range of 285-300 nm. This was surprising in light of the fact that this intermediate was also formed with methylbenzyl hydroperoxide as a peroxidic substrate (Skursky et al., 1992b). When tBOOH or CHP was used as a peroxidatic substrate a time-dependent increase in absorbance at 320 and
Figure 6.4  

pH-dependence of ethanol oxidation by tBOOH-modified HLADH. HLADH (5 μM) was incubated with 100 mM tBOOH at pH 7.4. Aliquots (10 μl) were removed from the incubation mixture at $t_{\text{max}}$ and assayed for ethanol oxidation at the indicated pH.
Figure 6.5a  
Product formation in peroxidatic reaction of HLADH with H$_2$O$_2$. ADH (0.6 μM) was incubated with 5 mM NAD$^+$ and 10 mM H$_2$O$_2$ in Tes buffer pH 7.4. Scans at 60 sec intervals are shown.
Figure 6.5  b) Product formation in peroxidatic reaction of HLADH with tBOOH. ADH (0.6 μM) was incubated with 5 mM NAD and 100 mM of tBOOH in Tes buffer pH 7.4. Scans at 60 sec intervals are shown.

   c) Double reciprocal plot of product formation in peroxidatic reaction with tBOOH.
Figure 6.5  

d) Product formation in peroxidatic reaction of HLADH with CHP. ADH (0.6 μM) was incubated with 5 mM NAD and 10 mM of CHP in Tes buffer pH 7.4. Scans at 60 sec intervals are shown.

e) Double reciprocal plot of product formation in peroxidatic reaction with CHP.
340 nm, respectively was observed (Fig. 6.5b,d). The formation of these chromophores was dependent on the presence of peroxide and HLADH in the reaction mix; thus, tBOOH and CHP behaved as substrates forming absorbing products.

In these experiments the rate of absorbance increase at 320 and 340 nm, respectively produced linear double reciprocal plots (Fig. 6.5c,e). The rate of product formation was calculated from the extinction coefficient of NADH at 340 nm. The reaction with tBOOH was relatively slow (100-fold slower than EtOH oxidation) with an apparent rate (kcat) of ~2 min⁻¹, and an apparent Km of about 4 ± 1 mM. A four-fold higher reaction rate was observed with CHP (kcat = 8 min⁻¹). The apparent Km for CHP in this reaction was 1.43 ± 0.2 mM, which is of the same order of magnitude as the Km for ethanol, but more than a hundred-fold lower than the Km for the reaction of HLADH with hydrogen peroxide (200 mM) (Favilla et al., 1980a).

**Effect of tBOOH on the Electronic Spectrum of Cobalt-Substituted ADH**

Metal-coordination by H₂O₂ was a necessary transition state in the peroxidatic reaction of ADH (Favilla et al., 1980b). The inactivation of ADH by H₂O₂ was associated with oxidation of the Zn-SH ligands Cys-46 and Cys-174 (Chapter 5). Thus, the effects of the peroxidic modifiers on the zinc-binding site and the geometry of the Zn coordination sphere are of importance in the mechanism of enzyme modification by the peroxides.

We used active site Co(II)-substituted ADH (Co(II)ADH) in an effort to exploit the characteristic ligand to metal charge transfer (LMCT) and d-d transition bands of this enzyme to better understand the modulating effects of organic hydroperoxides on HLADH-dependent ethanol oxidation and the effects of the various active-site-directed
probes on tBOOH-stimulated ethanol oxidation (Fig. 6.6). Identification of the coordinating ligands is not obtainable from UV-Vis spectra of the cobalt enzyme, but can indicate the integrity of the tetrahedral coordination sphere (Drum and Vallee, 1970).

The d-d transition region in the electronic spectrum of active site-substituted ADH corresponds to the distorted tetrahedral geometry of the cobalt coordination sphere (Sytkowski and Vallee, 1975); thus, the binding of certain ligands to the active site of the enzyme, either at the metal center itself, or close enough for orbital overlap with the corners of the tetrahedral structure of the coordination sphere is reflected in spectral transitions associated with the d-d band (Huheey et al., 1993). Such transitions also occur in the LMCT band, but are masked by the strong absorbance of the pyridine nucleotides, which overlap with that of the LMCT.

When 2.5 mM tBOOH was incubated with Co(II)ADH (50 μM) a concentration-dependent loss of absorbance of both the LMCT and d-d transition bands was observed within 5 minutes of incubation. These spectral transitions reflect the interaction of tBOOH with the metal center, either directly or with the ligands associated with the coordination sphere of the metal center (Fig. 6.6). In studying the effect of the protective agents we used a tBOOH concentration of 5 mM unless otherwise specified. This concentration caused considerable and rapid disruption of the Co(II)ADH active site geometry when no protective agents were used. If 5 mM tBOOH did not show a significant effect on the d-d transition region of the enzyme, the concentration was increased up to 100 mM, which typically results in instantaneous disappearance of the LMCT and d-d transition regions in the absence of protective
Figure 6.6  Time-dependent changes in LMCT and d-d transition bands of the electronic spectrum of Co(II)ADH by tBOOH. 30 μM ADH was incubated with 2.5 mM tBOOH and the course of the reaction was scanned at the interval of 30 sec. **Insert:** Time dependent change in the 645 nm (d-d transition) band of Co(II)ADH spectra.
agents. The effects of NADH, NAD⁺, DTT and imidazole on the d-d transitions of the Co(II)ADH spectrum are shown on Figure 6.7.

Compared to the d-d spectrum of Co(II)ADH alone, which has an absorbance maximum at 645 nm (Fig. 6.7a, trace 1), that of the binary complex of NADH-Co(II)ADH is red-shifted to 678 nm and displays a small shoulder at 645 nm (Fig. 6.7a, trace 2), which reflects the coenzyme-mediated conformational change of the protein involving the catalytic metal ion (Dietrich and Zeppezauer, 1982). Addition of 10 mM tBOOH resulted in a profound change in the Co(II)ADH spectrum (Fig. 6.7a, trace 3). The 678 nm peak was blue-shifted to 675 nm, while the absorbance of the 645 nm shoulder was greatly increased resulting in the well-resolved peak at 643 nm. Such spectral splitting is reminiscent of classical ternary complex with pyrazole (Chapter 5), which reflects the direct coordination of the substrate to the cobalt atom. Following the addition of tBOOH a small loss of absorbance in the 675 nm band of 0.002 AU is seen over a period of 3 min (Fig. 6.7a, traces 2-5). When compared to the 0.008 AU loss by the 5 mM tBOOH (Fig. 6.6) the data indicate relatively strong protection by NADH from absorbance loss caused by tBOOH. The subsequent addition of 100 mM tBOOH almost completely abolished the 675 nm peak, while 643 nm peak was essentially intact (Fig. 6.7a, trace 6).

Pretreatment of Co(II)ADH with 5 mM NAD⁺, afforded only limited protection of the enzyme from tBOOH-dependent loss of absorbance of the d-d transition region (Fig. 6.7b). Thus, it appeared that the metal center had been disrupted upon incubation with tBOOH, even in the presence of NAD⁺. Similar results were shown for H₂O₂-dependent loss of absorbance of the d-d transition region (Chapter 5).
Figure 6.7a  Effect of NADH on the time-dependent changes in d-d transition region of the electronic spectrum of Co(II)ADH by tBOOH.
1) Co(II)ADH (20 μM) in Tes buffer pH 7.4
2) Co(II)ADH + 2 mM NADH
3) Co(II)ADH/NADH + 10 mM tBOOH  1 min
4) Co(II)ADH/NADH/tBOOH  3 min
5) Co(II)ADH/NADH/tBOOH  5 min
6) Co(II)ADH/NADH/tBOOH + 100 mM tBOOH
Treatment of Co(II)ADH with 1mM DTT resulted in a profound change in the Co(II)ADH spectrum (Fig. 6.7c, trace 2). When 10 mM DTT was added to Co(II)ADH the profound change in the d-d transition region was observed. This change was characterized by a splitting of the d-d band into two chromophores with maxima at 640 and 690 nm. In addition to the splitting of the 645 nm peak an additional peak at 560 nm was formed. To the best of our knowledge such splitting of the d-d transition band has not been reported for binary complexes of Co(II)ADH; the observed splitting is reminiscent of the ADH ternary complex with NADH and pyrazole (Chapter 5), and presumably reflects the direct coordination of DTT to the active site metal.

Following the addition of 5 mM tBOOH a small loss of absorbance of the 690 nm peak of ~ 0.0015 AU is seen over a period of 3 min (Fig. 6.7c, traces 3-4). When compared to the 0.008 AU loss by the same concentration of tBOOH (Fig. 6.6) the data indicate relatively strong protection by DTT from absorbance loss caused by tBOOH. While tBOOH treatment caused an absorbance decrease of the 690 nm peak, the 640 nm peak was essentially intact. The absorbance of 560 nm peak increased concomitantly with the loss of absorbance of the 690 nm peak (Fig. 6.7c).

Pretreatment of HLADH with imidazole, which binds to the catalytic metal atom and the –SH ligands (Reynolds and McKinley-McKee, 1969), showed some protection against tBOOH-dependent perturbation of the Co-center of ADH (Fig. 6.7d).
Figure 6.7b  Effect of NAD$^+$ on the time-dependent changes in d-d transition region of the electronic spectrum of Co(II)ADH by tBOOH.

1) Co(II)ADH (20 μM) + 5 mM NAD$^+$ in Tes buffer, pH 7.4
2) Co(II)ADH/NAD$^+$ + 5 mM tBOOH 1 min
3) Co(II)ADH/NAD$^+$tBOOH 2 min
4) Co(II)ADH/NAD$^+$tBOOH 3 min
Figure 6.7c Effect of DTT on the time-dependent changes in d-d transition region of the electronic spectrum of Co(II)ADH by tBOOH.

1) Co(II)ADH (20 μM) in Tes buffer, pH 7.4
2) Co(II)ADH + 10 mM DTT
3) Co(II)ADH/DTT + 5 mM tBOOH 1 min
4) Co(II)ADH/DTT/tBOOH 3 min
Figure 6.7d  Effect of imidazole on the time-dependent changes in d-d transition region of Co(II)ADH by tBOOH.
1) Co(II)ADH (20 μM) in Tes buffer, pH 7.4
2) Co(II)ADH + 10 mM imidazole
3) Co(II)ADH/imidazole + 5 mM tBOOH  1 min
4) Co(II)ADH/imidazole/tBOOH  3 min
5) Co(II)ADH/imidazole/tBOOH  5 min

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DISCUSSION

The inactivation of horse liver ADH upon pre-or co-incubation of ADH with hydrogen peroxide is associated with oxidation of the -SH groups of Cys-46 and Cys-174, the coordinating ligands of the active site Zn atom [Chapter 5; Favilla, 1980 #201]. The evidence presented herein shows that the ethanol dehydrogenase activity of HLADH is stimulated when preincubated in the presence of tBOOH or CHP and that the Zn ligand -SH groups are not oxidized. Rather, these peroxides, upon binding to the active site appears to alter the geometry of the Zn coordination sphere in a manner that facilitates oxidation of ethanol. The mode of binding of the metal atoms, their environment, and accessibility may combine to account for the observed differences in the effects of H_2O_2 and the organic hydroperoxides on the native vs. the cobalt-substituted ADH.

Most activators of HLADH activity studied to date (Adolph et al., 1991; Dalziel and Dickinson, 1966b; Denkel et al., 1986; Plapp, 1974) expressed their stimulatory effect on the enzyme only with high concentrations of substrates, i.e., ethanol and coenzyme. When the ethanol concentration in the reaction mixture was decreased below a certain threshold concentration the stimulation effects were either diminished or the enzyme was inactivated (Plapp, 1974). These effects are referred to as a competitive inhibition and stimulation kinetic mechanism (CIS) (Theorell et al., 1969). In the present study only when the ethanol concentration was below 1 mM in the reaction mixture was a transition in the effects of tBOOH from a stimulatory effect to an inhibitory effect observed. None of previously reported activators of ADH were able
to affect stimulation of the enzyme at this ethanol concentration. In contrast to ethanol a stimulatory effect was observed at all concentrations of NAD$^+$ studied (Fig. 6.2a).

The CIS kinetics associated with variable ethanol concentration are characterized by an increase in Vmax and Km of the reaction, which suggests that enzyme modification affected both substrate binding and substrate-coenzyme complex dissociation. The data also suggest that the coenzyme-binding region was not affected by tBOOH since the affinity of the enzyme for NAD$^+$ was essentially unchanged.

Binary complexes of NAD$^+$- or NADH-ADH prevent the stimulation of ADH by tBOOH suggesting the involvement of the enzyme active site in this stimulation (Chapter 4). Imidazole, a reversible inhibitor of HLADH has been used to show the role of the catalytic zinc atom to which it binds in the observed CIS kinetics (Reynolds and McKinley-McKee, 1969). In the present study imidazole afforded strong protection against the loss of the d-d transition region of the Co(II)ADH spectrum caused by peroxides. This suggests an interaction of tBOOH with the metal; however, since the -SH groups of the metal ligands are not oxidized by tBOOH as indicated by the quantifiable titration of -SH groups with Ellman’s reagent (Chapter 4) the interaction most likely caused conformational changes in the coordination sphere of the enzyme without destroying the metal bound ligands.

Marked changes observed in the spectral characteristics of the d-d transition region, and particularly in the ligand to metal charge transfer (LMCT) band of the electronic spectrum of cobalt-substituted ADH upon interaction with tBOOH indicate direct effects of tBOOH with the metal active site of the enzyme. Cobalt-substituted ADH is extremely oxygen-sensitive; its preparation requires strict anaerobic conditions.
Because of the oxidizing nature of organic hydroperoxides the loss of the spectral characteristics of Co(II)ADH caused by tBOOH might simply be due to the oxidation of the Co$^{2+}$ ion. If this is true then high concentration of strong reducing agents such as ascorbate or DTT should restore the absorbance lost upon treatment with the peroxide. On the other hand if ligand destruction was caused by peroxide interaction with the active site, the original d-d spectrum would not be restored. When either ascorbate or DTT was added in 5- to 10-fold excess of tBOOH, after the loss of d-d transition band, no restoration of any of the original spectrum was observed. This leads us to conclude that unlike the zinc enzyme, in which ligand destruction did not occur when incubated with tBOOH, that of the cobalt enzyme did. Further evidence for this is from the work of Skursky et al. (Skursky et al., 1992b) who showed that the loss of ADH activity caused by MBHP was reversed when MBHP was removed from the enzyme by size-exclusion chromatography. The effect of protective agents on the spectral transitions of Co-substituted HLADH shows the interaction of H$_2$O$_2$ and tBOOH with the enzyme active site and their apparent ability to alter the metal coordination sphere.

Several studies have indicated that HLADH activation occurs by facilitated coenzyme dissociation caused by the interaction of modifying agents with Lys-228, which forms an anion binding site about 8 Å from the sulfur of Cys-46 (Plapp, 1974). The fact that AMP, which selectively binds to an anion binding site formed by Lys-228 (Plapp, 1974), prevented loss of the d-d-transition region spectrum of Co(II)ADH caused by tBOOH suggests that Lys-228 is a target residue within the active site for tBOOH. In this regard the purine ring of the coenzyme is important in preventing the stimulation caused by tBOOH.
Inactivation of ADH by peroxides has a more established basis of reconciliation than the remarkable stimulatory effect on ADH activity observed even with very high concentrations of the prooxidant tBOOH. Whereas H$_2$O$_2$ requires covalent modification of -SH ligands to affect inactivation of ADH, covalent modification is not obligatory for enhancement of the activity of HLADH by chemicals. Conformational changes within the active site or differences in the geometry of the transition state complex can promote stimulation of an enzyme’s activity. For example, the activity of ADH is about 10-times more active with respect to ethanol oxidation with 3-acetylpyridine, a structural analogue of NAD$^+$, than it is with NADH; 3-acetylpyridine adenine dinucleotide dissociates about 7-times faster from the enzyme than does NADH.

In the native enzyme, the stimulation effect of tBOOH increases as zinc becomes less positive (i.e. at high pH; (Langeland and McKinley-McKee, 1997)), thus the stability of the active site coordination sphere appears to be related to the ionization status of both zinc-H$_2$O and Cys-ligands. Zinc is not redox active in aqueous solution and is quite stable as the divalent cation. It has the highest charge to atomic radius ratio of any element and maintains partial cationic character even in a tetracoordinate complex, which imparts its Lewis acid character. Thus, zinc will attract anionic oxidants and withdraw electron density from these oxidants. A perturbed zinc coordination sphere could destabilize the ternary metal complex resulting in decreased Lewis acidity of the metal and in lower affinity binding of substrate (Sartorius et al., 1988), thereby promoting its faster release from the abortive complex of the enzyme. Compounds such as 2,2'-dipyridyl and cyclohexanol activate HLADH in a similar
fashion by binding to zinc with subsequent destabilization of the coordination sphere (Dalziel and Dickinson, 1966b).

An interesting aspect of the interaction of \( \text{H}_2\text{O}_2 \) with HLADH is the enzyme-catalyzed peroxidatic reaction, in which \( \text{NAD}^+ \) is consumed at the expense of production of NADX (Favilla et al., 1980b). NADX is spectrally distinct from NADH and is a ring-opened product of this peroxidatic reaction of HLADH (Mazzini et al., 1980). Our study of tBOOH and CHP as substrates in the peroxidatic reaction catalyzed by HLADH showed marked differences from the peroxidatic reaction with \( \text{H}_2\text{O}_2 \). When tBOOH or CHP was substituted for \( \text{H}_2\text{O}_2 \) in the peroxidatic reaction with HLADH, no chromophores were detected in the range of 285-300 nm to indicate formation of a product analogous to NADX.

While \( \text{H}_2\text{O}_2 \) appears to have a strong electronegative interaction with the ADH active site resulting in the metal ligand disruption, bulky organic hydroperoxides appear only to destabilize those ligands. The lack of NADX production when tBOOH and CHP are used by HLADH as peroxidatic substrates most likely reflects steric considerations of the organic hydroperoxides, which contain bulky alkyl groups. Nevertheless, the fact that the formation of chromophores at 320 and 340 nm in the \( \text{NAD}^+ \)-dependent reactions with tBOOH and CHP, respectively were dependent on the presence of ADH, peroxide and \( \text{NAD}^+ \) in the reaction indicate that they reflect products of an ADH-catalyzed peroxidatic reaction. It remains to be established whether these products differ structurally from NADH.

The results of our studies of native and metal substituted ADH suggest that tBOOH and CHP act at a locus or at loci near the active site metal center and indirectly
interact with the active site metal center without causing disruption of metal to ligand bonds. This hypothesis is supported by the marked changes in the d-d transition region, and particularly in the ligand to metal charge transfer (LMCT) band in the electronic spectrum of cobalt-substituted ADH upon binding of tBOOH to HLADH. A perturbed coordination sphere around the ADH metal center could be envisaged to decrease the binding affinity of the substrate to the enzyme. The competitive inhibition and stimulation kinetic mechanism supports the fact that enzyme modification affected both substrate binding and substrate-coenzyme complex dissociation; therefore, the increase in activity appears to be due to faster breakdown of the peroxide-modified enzyme-coenzyme complexes.

The diverse effects of hydroperoxide substrates on one representative of the large family of ADH raises some interesting questions concerning the possible biological relevance of the peroxidatic reactions reported herein. The oxidant sensitivity of the zinc-thiolate moiety of ADH (ZnCys$_2$His$_1$), the fact that there are over 300 Zn-containing enzymes, many of which have similar motifs (Crow et al., 1995), and the ubiquitous presence of zinc finger proteins, which contain the ZnCys$_2$His$_2$ motif suggest any of these as key targets for oxidant-mediated functional alteration.

Despite the relatively high concentrations of tBOOH and CHP used for the study of peroxide-dependent stimulation of ADH-catalyzed ethanol oxidation, other alkyl hydroperoxides, including biologically-generated and xenobiotic peroxides might play a role in the regulation of enzymatic activity at more physiologically relevant concentrations. In that regard, methylbenzyl hydroperoxide was reported to be a peroxidatic substrate that can both inactivate ADH in rabbit serum and promote the
production of NADX from NADH as described above at concentrations in the µM range (Skursky et al., 1992a). Taken as a whole, our data provide a framework for further investigation of the mechanism of interaction of ADH isozymes, including human ADH with various organic hydroperoxides.
Chapter 7: Summary

In continuing study of nontraditional activities of ADH, the interaction of horse liver ADH with C-nitroso, N-nitroso and peroxide compounds was investigated. In order to better elucidate the mechanism of these interactions, the cobalt substituted HLADH was introduced and compared with zinc-containing native HLADH.

The catalytic metal of ADH is coordinated through a 2 Cys, 1 His and 1 water ligand motif, which results in a distorted tetrahedral coordination geometry and the corresponding d-d transition band at 645 nm in the visible spectrum of Co(II)ADH. Both binary and ternary complexes of pNSP with Co(II)ADH resulted in a blue shift of the d-d transition region of Co(II)ADH spectra, which indicates a direct interaction of the C-nitroso moiety with the enzyme metal active site and precludes against a second sphere mechanism through metal bound H$_2$O or OH$^-$. In addition to the blue shift of the d-d spectrum of the binary complex with NADH, both pNSP and DMNA enhance the absorbance of the 643 nm peak of the ADH/NADH/pyrazole ternary complex, which suggests that both of these C-nitroso compounds are able to compete with pyrazole for ligand association with the metal center. Our studies established that C-nitroso substrates of ADH can enter the ADH catalytic site regardless of the presence for the coenzyme; however, the coenzyme is necessary for productive substrate binding during the subsequent reduction by ADH.

Replacement of Zn(II) by Co(II) undoubtedly changes the specific rate constants for certain steps in the mechanism of pNSP reduction, and results in the decrease of both $k_{cat}$ and $k_{cat}/K_m$ values. The lower $K_M$ for Co(II)ADH may be attributed to the
ability of the enzyme to bind pNSP more productively; therefore, the complex ADH-NADH-pNSP is favored with Co(II)ADH over native ADH.

The rate of pNSP reduction catalyzed by Co(II)ADH was not significantly different from that of pAP formation, which suggests that higher substrate affinity of Co(II)ADH prefers a direct mechanism of pNSP reduction leading to pAP formation without release of BQI from the active site. Similar mechanism was proposed for various human ADH (Maskos and Winston, submitted).

The discovery of unique ADH-dependent reactions originating from its catalysis of reduction of C-nitroso compounds (Dudley and Winston, 1995; Maskos and Winston, 1993) prompted the present extension of our research to study of N-nitroso compounds. Both enzymatic (ADH) and nonenzymatic reduction of the N-nitroso compounds NDELA, NEU and NMU was observed; however, no appreciable nonenzymatic or enzymatic reduction of NDEA was noticed. The enzymatic rate of NMU reduction was similar to the rate of NEU reduction, despite strong nonenzymatic decay of NMU.

The reduction of the N-nitroso compounds studied here by HLADH did not appear as efficient as the reduction of the C-nitroso substrate pNSP; however, the general mechanism involving 2 e- transfer steps in the enzymatic reduction of the N- or C- nitroso substrates is essentially the same, i.e., via formation of hydroxylamine and amine products. Spectroscopic studies with Co(II)ADH indicates that NDELA and NEU react directly with the coordination sphere of the ADH metal center, suggesting the formation of a strong ternary complex between ADH-NADH-NDELA(NEU). The incubation of oxygen-sensitive Co(II) with strong oxygen carriers such as NDELA and
NEU did not cause any oxidative damage to the active site metal contrary to nitro oxide, which could destroy the integrity of the active site metal (Crow et al., 1995).

In contrast to N-nitroso compounds, herein, we provide direct evidence that H$_2$O$_2$ caused inactivation of ADH by both the oxidation of the active site -SH groups of Cys-46 and Cys-174 and the modification of an anion-binding site formed by Lys-228. While preincubation of HLADH with H$_2$O$_2$ resulted in the first order rate inactivation of the enzyme, whereas tBOOH and CHP stimulated the ethanol oxidation activity by 200 and 170% respectively.

Detailed kinetic studies of stimulation of ethanol oxidation activity of ADH by tBOOH at various ethanol concentration showed that tBOOH interacts with ADH via a competitive inhibition and stimulation kinetic mechanism (CIS) (Theorell et al., 1969). The CIS kinetics associated with variable ethanol concentration are characterized by an increase in Vmax and Km of the reaction, which suggests that enzyme modification affected both substrate binding and substrate-coenzyme complex dissociation.

Active-site-directed reagents and classical ADH binary complexes were used to probe the possible mechanism of this activating effect. The rate and extent of stimulation by tBOOH was strongly reduced by binary complexes with NAD$^+$ or NADH, whose pyrophosphate groups bind to Arg-47 and Arg-369. A similar effect was observed on ADH inactivation by H$_2$O$_2$.

In contrast to the liver enzyme, treatment of yeast ADH with tBOOH irreversibly inhibited its activity with respect to ethanol oxidation. Similar to H$_2$O$_2$ four -SH groups per molecule of YADH were modified by tBOOH, whereas only two -SH groups were modified in HLADH. The time-dependent modification of thiol groups
corresponded to the time-dependent inactivation of YADH by tBOOH, indicating that inactivation of YADH by tBOOH is likely associated with oxidation of the Zn thiolate ligands Cys-46 and Cys-174.

Spectral analysis of Co(II)ADH indicated strong interaction of tBOOH and H2O2 with the metal coordination sphere of the active site by the loss of absorbance of the LMCT and d-d transition regions of the spectrum. The mechanism of stimulation of HLADH is suggested to be due to destabilization of the catalytic Zn-coordination sphere via indirect interaction of tBOOH with ADH metal center, while inactivation by H2O2 results from direct interaction with ADH metal center via irreversible modification of active site ligands Cys-46 and Cys-174 and the modification of an anion-binding site formed by Lys-228.

An interesting aspect of the interaction of H2O2 with HLADH is the enzyme-catalyzed peroxidatic reaction, in which NAD+ is consumed at the expense of production of NADX (Favilla et al., 1980b). Our study of tBOOH and CHP as substrates in the peroxidatic reaction catalyzed by HLADH showed marked differences from the peroxidatic reaction with H2O2. When tBOOH or CHP was substituted for H2O2 in the peroxidatic reaction with HLADH, no chromophores were detected in the range of 285-300 nm to indicate formation of a product analogous to NADX. The lack of NADX production when tBOOH and CHP are used by HLADH as peroxidatic substrates most likely reflects steric considerations of the organic hydroperoxides, which contain bulky alkyl groups.

The present data suggest that subtle changes in the zinc-thiolate center of ADH may cause marked changes in the enzyme function (stimulation of HLADH as
compared to inactivation of YADH). The diverse effects of hydroperoxide substrates on one representative of the large family of enzymes raises some interesting questions concerning the possible biological relevance of the peroxidatic reactions reported herein. The oxidant sensitivity of the zinc-thiolate moiety of ADH (ZnCys$_2$His$_1$), the fact that there are over 300 Zn-containing enzymes, many of which have similar motifs (Crow et al., 1995), and the ubiquitous presence of zinc finger proteins, which contain the ZnCys$_2$His$_2$ motif suggest any of these as key targets for oxidant-mediated functional alteration.

Despite the relatively high concentrations of tBOOH and CHP used for the study of peroxide-dependent stimulation of ADH-catalyzed ethanol oxidation, other alkyl hydroperoxides, including biologically-generated and xenobiotic peroxides might play a role in the regulation of enzymatic activity at more physiologically relevant concentrations. Further investigation of the molecular mechanism of human ADH interaction with possible physiological peroxides may be of heuristic value.

Taken as a whole, our data provide a framework for further investigation of the mechanism of interaction of ADH isozymes, including human ADH with various organic hydroperoxides and N-nitroso compounds.
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Dudley, B. (2000). Activation and Metabolism of p-Nitrosophenol and 2,2'-Thiobisethanol by Horse Liver and Human Alcohol Dehydrogenase:Toxicological Implications., LSU.


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Appendix  Letter of Permission

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May 25, 2000

Alexander Tkachenko
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Re: “Interaction of Alcohol Dehydrogenase with Tert-butylhydroperoxide: Stimulation of the Horse Liver and Inhibition of the Yeast Enzymes” manuscript = AB1200-0163-St010 (in press)

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Vita

Alexander G. Tkachenko was born in Kiev, Ukraine. He graduated from Kiev High School #15 with honors, in May 1985. He was accepted into the School of Veterinary Medicine at Ukrainian Agricultural University, where he received his Doctor of Veterinary Medicine degree in June 1992. He was working at the Institute of Veterinary Medicine in Kiev, Ukraine as a senior veterinary medicine doctor until he received a full academic scholarship to attend the graduate program in Biochemistry at Louisiana State University in August 1994. Alexander hopes to continue working in the pharmaceutical industry after he receives his doctoral degree in biochemistry on August 4, 2000.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Alexander G. Tkachenko

Major Field: Biochemistry

Title of Dissertation: Active Site Interactions and Kinetic Characterization of C- and N-nitroso and Peroxidic Substrates with Alcohol Dehydrogenase: Mechanistic Insights from Studies of the Cobalt-substituted Enzyme

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

May 18, 2000