Electron Transfer Activity of Mitochondria Neet-Proteins

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ELECTRON TRANSFER ACTIVITY OF MITOCHONDRIA NEET-PROTEINS

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
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in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

In
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By
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ABSTRACT

Mitochondrial NEET proteins are recently discovered iron-sulfur proteins that are localized within mitochondria. There are three NEET proteins: mitoNEET, a type II diabetes drug pioglitazone binding target, a mitoNEET related protein 1 (Miner1 or NAF-1), and a mitoNEET-related protein 2 (Miner2). While both mitoNEET and Miner1 are mitochondrial outer membrane proteins, Miner2 is a mitochondrial matrix protein. All three NEET proteins bind at least one [2Fe-2S] cluster via CDGSH (Cys-Asp-Gly-Ser-His) motif. In this work, we have investigated the electron transfer activity of mitoNEET, and found that the CDGSH-type [2Fe-2S] clusters of mitoNEET can be reduced by the reduced flavin mononucleotide (FMNH$_2$) under anaerobic or aerobic conditions in vitro. We have also found that the reduced mitoNEET [2Fe-2S] clusters can be oxidized by ubiquinone or oxygen. Further studies have demonstrated that the type II diabetes medication pioglitazone may compete for the FMNH$_2$ binding site of mitoNEET, and inhibit the reduction of the mitoNEET [2Fe-2S] clusters by FMNH$_2$. We have also described the inhibitory effect of nitric oxide on the electron transfer activity of the mitochondrial matric protein Miner2. We have found that nitric oxide can bind to the reduced Miner2 [2Fe-2S] clusters without disruption of the cluster, and that visible light excitation can quickly release nitric oxide from the nitric oxide-bound cluster in Miner2. Binding of nitric oxide effectively inhibits the electron transfer activity of the Miner2 [2Fe-2S] clusters. The results suggest that mitochondrial NEET-proteins may play a novel role in energy metabolism in cells, and that nitric oxide may regulate the electron transfer activity of the NEET-proteins and modulate the energy metabolism in mitochondria via directly binding to the [2Fe-2S] clusters in the proteins.
1.1 Iron-sulfur Clusters

Iron-sulfur clusters (ISC) are one of the most ancient and conservative cofactors (1). More than 500 distinct proteins with versatile forms of ISC have been found in archaea, bacteria, and eukaryotes, since ferredoxin, the first known ISC containing protein, was found in 1960s (2-3).

There are various numbers of Fe and S atoms in different forms of ISC. The most common forms of ISC are the [2Fe-2S] clusters and the [4Fe-4S] clusters (Figure 1.1) (4). The [2Fe-2S] and [4Fe-4S] are generally ligated to proteins through cysteine residue. In some cases, aspartate, histidine, or serine can also be the ligands for the iron-sulfur clusters in proteins (5).

The primary biological function of the ISC is electron transfer, as the Fe in ISC can be both electron acceptor and donor (6). For example, a chain of ISC in the complex I of mitochondria transfer electrons from NADH in matrix to the ubiquinone embedded in the mitochondrial inner membrane (7). The second role of ISC is to serve as the active center in an enzyme. For example, fumarase A from Escherichia coli, catalyzes the reversible hydration/dehydration of fumarate holds a [4Fe-4S] in its active center (8). The third function of ISC is the regulation of gene expression. The examples are bacterial transcription factors SoxR, FNR, IRP, IscR (9-11).
Figure 1.1 Structures, core oxidation states, and spin states of crystallographically defined [Fe-S] clusters.
Iron is shown in red, and sulfur is shown in yellow. The spin state, denoted by a question mark, has yet to be determined. The [3Fe-4S] cluster has only been observed as a fragment in heterometallic [M-3Fe-4S]^+ clusters (not shown) in which M is a divalent transition metal ion (3).

1.2 CDGSH-type iron-sulfur domain-containing proteins

The CDGSH Iron-Sulfur domain (CISD) is a type of ancient and widely distributed motif that are found in the three domains of life. This motif is characterized by a highly conserved
seventeen amino acid sequence [C-X-C-X2-(S/T)-X3-P-X-C-D-G-(S/A/T)-H], in which the [2Fe-2S] cluster is coordinated with unique three cysteine residues (3Cys) and one histidine (1His) in proteins (14-18). The most important character of this 3Cys-1His domain is that the ISC attached to it has a higher redox midpoint potential and relatively lower binding stability. Thus, it may serve as an electron donor or the ISC donor (19-31). The versatile function of the CISD proteins may explain why they widely appear in bacteria, archaea, and eukaryotic cells (Figure.1.2).
1.3 Human NEET-protein family and related diseases

There are three CISD proteins, mitoNEET, Miner1, and Miner2, encoded by gene CISD1, CISD2, and CISD3 respectively in human mitochondria. The first member of NEET
family, mitoNEET, was discovered during the screening for the mitochondrial binding targets of the type II diabetes Pioglitazone (32). MitoNEET was named by its mitochondrial localization (mito), and a sequence of amino acid residues ASN-Glu-Glu-Thr (NEET) (15). The rest of the 2 NEET-protein family members were named Miner1/2 meaning mitoNEET related 1/2 initially. The three NEET family proteins share the CDGSH motif that holds a [2Fe-2S] clusters (16). The UV-vis spectra of mitoNEET, Miner1, and Miner2 show great similarity (Figure.1.3) (33).

Further structure studies have shown that mitoNEET and Miner1 are homodimer, with one CISD on each peptide chain, whereas Miner2 has two CISDs, each holding one [2Fe-2S] cluster (17-18) (33). Both mitoNEET and Miner1 are lipid anchored proteins. MitoNEET is anchored to the mitochondrial outer membrane via its N-terminal domain. The C-terminal domain binds a [2Fe-2S] cluster and is exposed to cytosol (32). The mitoNEET homolog Miner1 is mainly associated with the endoplasmic reticulum (ER), with some in the mitochondrial outer membrane (35). Miner2 is a soluble protein in the matrix (36-37). Extensive studies have shown that mitoNEET and Miner1 play key roles in maintaining cellular iron content and production of reactive oxygen species (ROS). MitoNEET was found to have a key role in mitochondrial morphology maintaining, cancer cell proliferation, lipid and glucose homeostasis in obesity and diabetes. The mutations of Miner1 have been associated with the Wolfram Syndrome 2, a genetic neurological disorder (38-48). The biological function of Miner2, however, is currently unknown.
Fig. 1.3 Human NEET protein alignments and UV–vis spectra.
(A) Amino acids conserved in humans are shown in gray. Amino acids that coordinate to the 2Fe–2S clusters are shown in yellow. The 16 amino acid cluster binding domains are boxed in magenta. Note that Miner2 has a repeat of the 16 amino acid 2Fe–2S cluster binding domain, underlined in pink. The transmembrane domains of mitoNEET and NAF-1 are highlighted by a blue box. (B) UV–vis spectra of mitoNEET, NAF-1 and Miner2. Note the similarities of the peak positions in the visible region which are ligand to metal charge transfer bands reflecting the presence of the same type of Fe–S clusters and the similarity of their coordinations (34).

1.4. Structure and biochemical properties of mitoNEET

The whole length of human mitoNEET is 108 amino acid residues. The first 32 residues serve as the hydrophobic lipid anchor, and the rest residues forming the major soluble part facing plasma. The soluble part has two major domains, a hydrophilic β-cap structure at top, and a helix enriched domain cradling [2Fe-2S] clusters close to the mitochondrial outer membrane (Figure 1.4). The [2Fe-2S] is buried in hydrophobic core and coordinated with Cys-72, Cys-74, Cys83, and His-87 (15). Two major binding sites of type II diabetes drugs pioglitazone has been identified. Lys-42 and Ala-43 from chain 1 and Met-44, Leu-47, Arg-76, His-90, and Thr-94 from chain 2 forms site 1, and His-48, Ile-49, Trp-75, Arg-76, Ser-77, and Lys-78 from chain 1
forms site 2 (49). In our studies, we have also identified a unique FMN binding site which is different from, but overlaps with, site 1 and site 2 of mitoNEET (26).

The redox midpoint potential of the mitoNEET [2Fe-2S] cluster is 0 mV at pH 7, which is 300 mV higher than the 4-Cys mutant [2Fe-2S] (H87C) in mitoNEET. Previous studies have proven that the reduced [2Fe-2S] could be oxidized by hydrogen oxide, and the oxidized [2Fe-2S] could be reduced by biological thiols, including reduced glutathione, L-cysteine or N-acetyl-L-cysteine, and dithiothreitol (ref). *E. coli* thioredoxin/thioredoxin reductase system, and NADPH dependent human glutathione reductase could also reduce the mitoNEET ISC in (ref). Pioglitazone could block the mitoNEET [2Fe-2S] cluster from reduction by biological thiols (24-26). Another function of pioglitazone is that it may stabilize the [2Fe-2S] cluster in mitoNEET (15).

1.5 Iron-Sulfur cluster protein is the sensor of nitric oxide

Nitric oxide (NO) is a hydrophobic molecule that can easily penetrate the cell membrane. The highly reactive NO can then react with ROS, thiols, etc. and generate S-nitrosothiols, nitrogen dioxide, peroxynitrite, dinitrogen trioxide, and nitrite (50). This feature explains why the immune system of higher animals uses NO as cytotoxin in the first line of defense against the invading pathogens. NO also serves as a key signaling molecule in the muscular and nervous system of higher animals (51-54).
Figure 1.4 Overall structural organization and domain topology of dimeric mitoNEET.

(A Upper) The backbone tracing of each protomer colored in green and magenta, respectively, together with the 2Fo − Fc electron density (gray) map contoured at 1.5σ. The protomers pack in a parallel fashion with each protomer harboring a 2Fe–2S cluster, depicted as yellow (sulfur) and red (iron) spheres; N and C termini are indicated. (Lower) The box shows an expanded view of one 2Fe–2S cluster (rotated ≈90° from upper view) and ligands and the corresponding 2Fo − Fc electron density (gray) map contoured at 2.0σ. (B) Ribbon diagram highlighting the two domains of the mitoNEET dimer. A six-stranded β-sandwich forms the intertwined β-cap domain and a larger cluster-binding domain carries two 2Fe–2S clusters. (C) A topology diagram highlighting the organization of the secondary structural units (numbered) illustrates the strand swap between protomers. (D) Coded segments contributing to each domain are highlighted on the primary sequence and block diagram. Protomer sequences within the cluster-binding domain are colored in purple and dark green, and the sequences corresponding to the β-cap domain are given in pink and light green, respectively. The amino acid sequence of the resolved amino acid strand is shown in the box with the cluster and cap regions colored as for protomer A; the numbers indicate the first (Lys-42) and last (Lys-106) resolved amino acid. The ligands to the 2Fe–2S cluster shown in the expanded boxed view in A are indicated in bold and highlighted in gray. The 2Fe–2S binding cradle is located sequentially between two partial β-cap domains. Rendered with Pymol (15).
There are a series of ISC proteins, mainly transcriptional regulators, in *E. coli* that could sense NO and adapt the metabolism of the cells to this stress, including MetR, IscR, Fur, FNR, and SoxR (55-59). The NO molecule interacts with the iron-sulfur cluster in proteins and results in disruption of the cluster and formation of dinitrosyl iron complexes (DNICs) (60). (Figure.1.5.) The NO-modified sensor can then regulate downstream gene transcription (61-63). The DNICs in proteins can be repaired by re-assembly of new iron-sulfur clusters (59) (64). For example, the [2Fe-2S] cluster of SoxR remains reduced *in vivo* under normal condition. Upon NO stress, the SoxR ISC interacts with NO, and produces SoxR containing dinitrosyl iron complex (DNIC) which is active to stimulate the gene expression in cells (65).

![Figure 1.5](image)

Figure. 1.5 Scheme of dinitrosyl iron complexes (DNICs) formation and reparation.
(A) Nitric Oxide could disrupt the [Fe-S] or binds with non-heme iron in cell to form the DNIC. 
(B) Mechanisms in cell to repair the DNIC.

1.6 References


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CHAPTER 2. ELECTRON TRANSFER ACTIVITY OF MITONEET

2.1 Introduction

Pioglitazone is a drug prescribed for the treatment of type II diabetes (1). In addition to acting on the peroxisome proliferator activated receptor gamma (PPARγ) which regulates expression of the genes related to insulin sensitivity (2), pioglitazone also has a rapid pharmacological effect on energy metabolism in mitochondria (3). Search of the pioglitazone binding targets revealed a new protein, mitoNEET, which localizes in the mitochondrial outer membrane (4). In mice, deletion of mitoNEET results in decrease of oxidative phosphorylation capacity in mitochondria by about 30% (5). On the other hand, overexpression of mitoNEET enhances lipid storage and decreases oxidative damage in adipocytes (6,7), and inhibits ferroptosis in human hepatocellular carcinoma cells (8). In beta cells, increased expression of mitoNEET leads to hyperglycemia and glucose intolerance (9). In spinal neurons, microRNA-127 has been shown to specifically target expression of mitoNEET, and depletion of mitoNEET results in neuronal loss and apoptosis (10). In breast cancer cells, mitoNEET is highly expressed, and depletion of mitoNEET inhibits cancer cell proliferation (11,12). It has thus been postulated that mitoNEET is a key regulator of energy metabolism in mitochondria (13), and an attractive chemotherapeutic target for treating the mitochondria-related human diseases (14-17).

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Crystallographic studies have shown that mitoNEET is a homodimer with each monomer hosting a [2Fe-2S] cluster via an unusual ligand arrangement of three cysteine and one histidine residues in its C-terminal cytosolic domain (18-21). The N-terminus of mitoNEET has a transmembrane α-helix which anchors the protein to the mitochondrial outer membrane (4). However, the mechanism by which mitoNEET regulates energy metabolism in mitochondria still remains elusive. One hypothesis stated that mitoNEET may transfer its [2Fe-2S] clusters to apo-proteins such as apo-ferredoxin (22), the iron regulatory protein-1 (IRP-1) (23), or anamorsin, a protein required for iron-sulfur cluster assembly in cytosol (24). Because mitochondria are the primary sites for iron-sulfur cluster biogenesis in cells (25), it is tempting to propose that mitoNEET is a carrier transporting the clusters assembled in mitochondria to target proteins in cytosol (22-24). However, the observed cluster transfer occurs only when the mitoNEET [2Fe-2S] clusters are oxidized (22,23), and the mitoNEET [2Fe-2S] clusters are mostly in a reduced state in cells under normal physiological conditions (26-29). Thus, the exact role of mitoNEET in iron-sulfur cluster biogenesis has yet to be further defined. An alternative hypothesis was that mitoNEET may regulate mitochondrial functions via protein-protein interactions. This hypothesis was based on the observations that mitoNEET interacts with mitochondrial proteins including the Parkinson's disease associated protein Parkin (9), the mitochondrial outer membrane import complex protein 1 (MTX1) (30), glutathione reductase (28), and glutamate dehydrogenase 1 (31). Nevertheless, no direct evidence is available suggesting that the function of mitochondrial proteins are regulated by mitoNEET.

In previous studies, we have found that human mitoNEET has a specific interaction with the reduced flavin mononucleotide (FMNH2), and that FMNH2 can rapidly reduce the mitoNEET [2Fe-2S] clusters (29). Here, we report that the reduced mitoNEET [2Fe-2S] clusters
can be oxidized by oxygen or ubiquinone-2. Compared with oxygen, ubiquinone-2 appears to be more efficient in oxidizing the mitoNEET [2Fe-2S] clusters, indicating that ubiquinone may act as a native electron acceptor of the reduced mitoNEET [2Fe-2S] clusters in mitochondria. In the presence of flavin reductase which reduces FMN to FMNH2 using NADH as the electron donor, mitoNEET mediates oxidation of NADH with a concomitant reduction of oxygen or ubiquinone-2. Furthermore, pioglitazone can effectively inhibit the electron transfer activity of mitoNEET by forming a unique complex with mitoNEET and FMNH2. The results led us to propose that mitoNEET is a redox enzyme that may promote oxidation of NADH to enhance glycolysis in cytosol, and that pioglitazone may regulate energy metabolism in mitochondria by inhibiting the electron transfer activity of mitoNEET.

2.2 Materials and Methods

Protein preparation

Human mitochondrial outer membrane protein mitoNEET33–108 (containing residues 33-108) was purified as described in (28). Escherichia coli flavin reductase (Fre) was prepared using an E. coli strain hosting an expression plasmid encoding Fre from the ASKA library (46). Both mitoNEET and Fre were overproduced in E. coli cells, and purified using Ni-agarose columns, followed by passing through a High-Trap Desalting column. The purity of purified proteins was greater than 95% as judged by electrophoresis analysis on a 15% polyacrylamide gel containing SDS followed by staining with Coomassie Blue. The protein concentrations of mitoNEET and E. coli Fre were measured at 280 nm using the molar extinction coefficients of
8.6 and 26.4 mM-1 cm-1, respectively. The molar extinction coefficients were calculated based on the protein primary sequence.

**Redox state of the mitoNEET [2Fe-2S] clusters**

Reduction and oxidation of the mitoNEET [2Fe-2S] clusters in incubation solutions were monitored using a Beckman DU640 UV-visible absorption spectrometer equipped with a temperature controller. Oxidized mitoNEET [2Fe-2S] clusters have two major absorption peaks at 455 nm and 540 nm, while reduced mitoNEET [2Fe-2S] clusters have the absorption peaks at 420 nm and 540 nm (29). The redox state of the mitoNEET [2Fe-2S] clusters was further determined by EPR measurements. Oxidation of NADH in the incubation solution was monitored at 340 nm after subtracting the basal absorption of proteins the incubation solution. Anaerobic conditions were achieved by purging the incubation solutions with pure argon gas for 15 min in a sealed vial.

**EPR measurements**

The X-band Electron Paramagnetic Resonance (EPR) spectra were recorded using a Bruker model ESR-300 spectrometer equipped with an Oxford Instruments 910 continuous flow cryostat. Routine EPR conditions were: microwave frequency, 9.47 GHz; microwave power, 10.0 mW; modulation frequency, 100 kHz; modulation amplitude, 1.2 mT; temperature, 30 K; receiver gain, 2x10^5.

**Chemicals**

NADH, NADPH, Isopropyl-β-D-1-thiogalactopyranoside, kanamycin, and ampicillin were purchased from Research Product International co. FMN, FAD, ubiquinone-2, pioglitazone, NL-1, and other chemicals were purchased from Sigma co. The molar extinction coefficients of
6.2 mM-1 cm-1 at 340 nm, 12.5 mM1 cm-1 at 445 nm, and 11.3 mM-1 cm-1 at 450 nm were used for measuring the concentration of NADH/NADPH, FMN and FAD, respectively (43). The molar extinction coefficient of 14.9 mM1 cm at 278 nm was used for ubiquinone-2.

2.3 Results

Reduction of the mitoNEET [2Fe-2S] clusters under Anaerobic and Aerobic conditions

Previous studies have shown that human mitoNEET has a specific interaction with FMNH2, and that FMNH2 can quickly reduce the mitoNEET [2Fe-2S] clusters (29). As oxygen would be present in mitochondria, we asked whether oxygen will have any effects on the FMNH2-mediated reduction of the mitoNEET [2Fe-2S] clusters.

To test this idea, human mitoNEET was preincubated with FMN and NADH under anaerobic or aerobic conditions. The reaction was initiated by adding a catalytic amount of E. coli flavin reductase (32) to reduce FMN using NADH as the electron donor (29). Figure 2.1A shows that under anaerobic conditions, the oxidized mitoNEET [2Fe-2S] clusters (indicated by the absorption peaks at 455 nm and 540 nm (27)) were quickly reduced upon addition of flavin reductase, as reported previously (29). About 10 \( \mu \)M NADH was oxidized when 10 \( \mu \)M mitoNEET [2Fe-2S] clusters were reduced in the incubation solution under anaerobic conditions (Figure 2.1C). Under aerobic conditions, the mitoNEET [2Fe-2S] clusters were also fully reduced after addition of flavin reductase (Figure 2.1B). However, NADH was continuously oxidized even after the mitoNEET [2Fe-2S] clusters were fully reduced (Figure 2.1C). The observed oxidation of NADH was mitoNEET-dependent, as only a very small amount of NADH was oxidized in the same incubation solution without mitoNEET under aerobic conditions.
Thus, mitoNEET is able to promote oxidation of NADH in the presence of flavin reductase and FMN under aerobic conditions.

Figure 2.1 Reduction of the mitoNEET [2Fe-2S] clusters by FMNH2 under aerobic and anaerobic conditions. MitoNEET (containing 10 μM [2Fe-2S] clusters) was incubated with FMN (0.1 μM) and NADH (fig. caption cont'd.)
(60 μM) under anaerobic (A) and aerobic (B) conditions. Flavin reductase (Fre) (0.1 μM) was then added to the solutions to initiate the reaction. UV-visible absorption spectra were taken every 80 seconds after addition of Fre for 560 seconds. Absorption peaks at 455 nm and 540 nm represent the oxidized mitoNEET [2Fe-2S] clusters. C), time courses of NADH oxidation in the incubation solutions containing mitoNEET, Fre and FMN under anaerobic (A) (closed circles) or aerobic (B) (open circles) conditions. The amount of NADH in the incubation solutions was measured from the absorption peak at 340 nm after subtracting the absorption of proteins in the incubation solutions. D), same as in B) except no mitoNEET was included. The data were representatives of three independent experiments.

**The reduced mitoNEET [2Fe-2S] clusters are oxidized by oxygen under aerobic conditions**

In the presence of flavin reductase, FMN and excess NADH, the mitoNEET [2Fe-2S] clusters were fully reduced under aerobic conditions (Figure 2.1B). One explanation could be that the reduced mitoNEET [2Fe-2S] clusters are oxidized by oxygen and rapidly re-reduced by FMNH2/NADH/flavin reductase under aerobic conditions. To test this idea, mitoNEET was incubated with a limited amount of NADH and FMN under aerobic conditions. As shown in Figure 2.2, the mitoNEET [2Fe-2S] clusters were fully reduced upon addition of a catalytic amount of flavin reductase. However, when NADH in the incubation solution was completely oxidized, the reduced mitoNEET [2Fe-2S] clusters were gradually re-oxidized under aerobic conditions, demonstrating that the reduced mitoNEET [2Fe2S] clusters are indeed oxidized by oxygen under aerobic conditions.
Figure 2.2 Oxidation kinetics of the reduced mitoNEET [2Fe-2S] clusters by oxygen. MitoNEET (containing 10 μM [2Fe-2S] clusters) was incubated with FMN (0.1 μM) and NADH (20 μM) under aerobic conditions. Flavin reductase (Fre) (0.1 μM) was then added to the incubation solution to initiate the reaction. The amount of NADH in the incubation solution was measured from the absorption peak at 340 nm. The amount of the reduced mitoNEET [2Fe-2S] clusters was measured from the different absorption at the absorption peaks of 455 nm and 420 nm. The amounts of NADH and the reduced mitoNEET [2Fe-2S] clusters in the incubation solution were plotted as a function of incubation time. The data were representatives of three independent experiments.

The reduced mitoNEET [2Fe-2S] clusters can also be oxidized by ubiquinone

2 under anaerobic conditions—in mitochondria, the NADH-ubiquinone oxidoreductase (complex I) oxidizes NADH and reduces ubiquinone via FMN and a chain of iron-sulfur clusters (33). Ubiquinone is a key electron transport component in respiratory chain (34), and is present in both mitochondrial inner and outer membranes (35). As mitoNEET is a mitochondrial outer membrane protein (4), and the [2Fe-2S] clusters in mitoNEET are positioned close to the
membrane (18-21), we postulated that the reduced mitoNEET [2Fe-2S] clusters may also reduce ubiquinone in the mitochondrial outer membrane.

In the experiments, the mitoNEET [2Fe-2S] clusters were pre-reduced with flavin reductase, FMN, and an excess amount of NADH under anaerobic conditions. Ubiquinone-2, a ubiquinone-10 analog, was then injected to the incubation solution anaerobically using a gastight syringe. Figure 2.3 shows that the reduced mitoNEET [2Fe-2S] clusters were immediately oxidized upon injection of ubiquinone-2 under anaerobic conditions. In control, injection of degassed water had no effect on the reduced mitoNEET [2Fe-2S] clusters. After further incubation, the ubiquinone-2- oxidized mitoNEET [2Fe-2S] clusters were fully re-reduced in the incubation solution (Figure 2.3A), suggesting that oxidation of the mitoNEET [2Fe2S] clusters by ubiquinone-2 is reversible.

We also tested whether the reduced mitoNEET [2Fe-2S] clusters could be oxidized by other oxidants. In the experiments, the mitoNEET [2Fe-2S] clusters were pre-reduced with flavin reductase, FMN, and an excess amount of NADH under anaerobic conditions, followed by injection of oxidants anaerobically. We found that DCIP (dichlorophenolindophenol), K₃Fe(CN)₆ or menadione could all quickly oxidize the reduced mitoNEET [2Fe-2S] clusters, suggesting that the reduced mitoNEET [2Fe-2S] clusters may also be oxidized by other oxidants in cytosol. Oxidation of the mitoNEET [2Fe-2S] clusters by DCIP or K₃Fe(CN)₆ was very similar to that of ubiquinone-2. However, menadione appeared to be less active to oxidize the mitoNEET [2Fe-2S] clusters under the experimental conditions. Since ubiquinone is a physiological oxidant and is in the vicinity of the mitoNEET [2Fe-2S] clusters in mitochondria, we propose that ubiquinone could be a native electron acceptor for the reduced mitoNEET [2Fe-2S] clusters in mitochondria.
Figure 2.3 Oxidation of the reduced mitoNEET [2Fe-2S] clusters by ubiquinone-2 under anaerobic condition.

A), oxidation of the reduced mitoNEET [2Fe-2S] clusters by ubiquinone-2 under anaerobic conditions. MitoNEET (containing 10 μM [2Fe-2S] clusters) was reduced with FMN (0.1 μM) and NADH (50 μM) and flavin reductase (0.1 μM) under anaerobic conditions. Ubiquinone-2 (Q2) (20 μM) was injected into the incubation solution anaerobically as indicated. The reduced mitoNEET [2Fe-2S] clusters were measured from the different absorption at the absorption peaks of 455 nm and 420 nm, and plotted as function of the incubation time. B), UV-visible absorption spectra of mitoNEET. Spectra were taken before (spectrum 1) and after (spectrum 2) addition of NADH (50 μM). Spectrum 3, right after anaerobic addition of ubiquinone-2 (20 μM). The experiments were repeated three times. Similar results were obtained.

**Oxidation of the reduced mitoNEET [2Fe-2S] clusters by ubiquinone-2 under aerobic conditions**

Compared with oxygen (Figure 2.2), ubiquinone-2 is much more efficient in oxidizing the reduced mitoNEET [2Fe-2S] clusters (Figure 2.3A). To further explore the reactivity of the reduced mitoNEET [2Fe-2S] clusters with oxygen and ubiquinone-2, we incubated mitoNEET with FMN, NADH and ubiquinone-2 under aerobic conditions. Figure 2.4A shows that NADH was continuously oxidized after flavin reductase was added to the incubation solution. The
observed NADH oxidation was mitoNEET-dependent, as only a very small amount of NADH was oxidized in the incubation solution without mitoNEET under aerobic conditions (Figure 2.4B). Importantly, despite oxidation of NADH in the incubation solution containing ubiquinone-2, the mitoNEET [2Fe-2S] clusters remained oxidized during the incubation process (Figure 2.4A). This result indicated that the reduced mitoNEET [2Fe-2S] clusters might be rapidly oxidized by ubiquinone2 which subsequently transferred electron to oxygen under aerobic conditions. Indeed, when the incubation solution in Figure 2.4A was purged with pure argon gas to remove oxygen, the mitoNEET [2Fe-2S] clusters were re-reduced upon addition of NADH (Figure 2.4C and D). Thus, the results further suggest that ubiquinone in mitochondria may act as an intrinsic electron acceptor for the mitoNEET [2Fe-2S] clusters under anaerobic or aerobic conditions.
Figure 2.4 Oxidation of the reduced mitoNEET [2Fe-2S] clusters by ubiquinone-2 under aerobic condition.

A), mitoNEET (containing 10 μM [2Fe-2S] clusters) was pre-incubated with FMN (0.1 μM), NADH (50 μM), and ubiquinone-2 (20 μM) under aerobic conditions. UV-visible absorption spectra were taken every 40 seconds after addition of Fre (0.1 μM) for 320 seconds. The amount of NADH in the incubation solution was measured from the absorption peak at 340 nm. B), same as A), except mitoNEET was not included in the incubation solution. C), the incubation solution in A) was purged with pure argon gas for 10 min, followed by injection of NADH (50 μM) (fig. caption cont'd.)
anaerobically. UV-visible absorption spectra were taken every 40 seconds for 320 seconds. D), redox transition of the mitoNEET [2Fe-2S] clusters under aerobic and anaerobic conditions. The data were from panels A) and C). The amount of the reduced mitoNEET [2Fe-2S] clusters was measured from the different absorption at the peaks of 455 nm and 420 nm in each spectrum. The data were representatives of three independent experiments.

A pioglitazone analog NL-1 inhibits the electron transfer activity of mitoNEET

MitoNEET was identified as a target of the type II diabetes drug pioglitazone (1). However, pioglitazone has a very low solubility in water (<1 mg/ml), making it difficult to characterize its biochemical activity (28). A new pioglitazone analog, NL-1 or 5-(3,5-di-tert-butyl-4-hydroxybenzyl)-4-hydroxythiazol-2(5H)-one, was designed and synthesized by Geldenhuys, et al. (36). NL-1 has been improved for its solubility in water and binding specificity to mitoNEET. It has similar pharmacological activities as pioglitazone to decrease the maximal respiration rate of mitochondria by 45%, reduce the production of reactive oxygen species (36), and improve the cell survival of cardiac stem cells during oxidative stress (37).

Here, we explored the effect of NL-1 on the electron transfer activity of mitoNEET. In the experiments, mitoNEET was pre-incubated with increasing concentrations of NL-1 under aerobic conditions, followed by addition of flavin reductase and FMN. The reaction was initiated by adding NADH. The amounts of the reduced mitoNEET [2Fe-2S] clusters in the incubation solutions were monitored spectroscopically, and plotted as a function of incubation time. Figure 2.5 shows that as the concentration of NL-1 increased, the reduction of the mitoNEET [2Fe-2S] clusters in the incubation solution gradually decreased. At 250 µM NL-1, the reduction of the mitoNEET [2Fe-2S] clusters (10 µM) in the incubation solution was inhibited by about 70%, indicating that NL-1 can effectively block the electron transfer activity of mitoNEET.
Figure 2.5 Inhibition of the electron transfer activity of mitoNEET by the pioglitazone analog NL-1.

MitoNEET (containing 10 µM [2Fe-2S] clusters) was pre-incubated with increasing concentrations of NL1 (from 0 to 250 µM) at room temperature for 30 min under aerobic conditions, followed by addition of FMN (0.1 µM) and flavin reductase (0.1 µM). NADH (50 µM) was then added to the incubation solutions to initiate the reaction. The amount of the reduced mitoNEET [2Fe-2S] clusters in the incubation solutions was measured from the different absorption at the absorption peaks of 455 nm and 420 nm and plotted as function of the reaction time after injection of NADH. The data are representatives from three independent experiments.

Pioglitazone/NL-1 and FMNH2 forms a unique complex with the reduced mitoNEET [2Fe-2S] cluster

To further assess the effects of pioglitazone and its analog NL-1 on the electron transfer activity of mitoNEET, mitoNEET was incubated with FMN, pioglitazone, NL-1, or the combinations at room temperature for one hour under aerobic conditions. The samples were then reduced with sodium dithionite for the electron paramagnetic resonance (EPR) measurements of the reduced mitoNEET [2Fe-2S] clusters. Figure 2.6A shows that incubation with FMNH2 results in a small EPR signal at g = 1.85 of the reduced mitoNEET [2Fe-2S] clusters (spectrum
2), as reported previously (29). However, unlike FMNH2, incubation with pioglitazone (4) (spectrum 3) or NL-1 (36) (spectrum 4) did not change the EPR spectrum of the reduced mitoNEET [2Fe-2S] clusters. Nevertheless, incubation of mitoNEET with FMNH2 and pioglitazone completely shifted the typical EPR spectrum at \( g = 1.94 \) of the reduced mitoNEET [2Fe-2S] clusters to a new EPR spectrum with \( g = 1.85 \) (spectrum 5). Same result was also observed when mitoNEET was incubated with FMNH2 and NL-1 (spectrum 6). A small EPR signal at \( g = 1.86 \) of the reduced mitoNEET [2Fe-2S] clusters was previously noticed (38). Although the interpretation of the EPR signal at \( g = 1.85 \) remains elusive, the dramatic change of the EPR spectrum suggests that FMNH2 and pioglitazone/NL-1 may have a close interaction with the reduced [2Fe-2S] clusters in mitoNEET, or that binding of FMNH2 and pioglitazone/NL-1 may result in conformational changes that alter the structural orientation of the cluster ligands in mitoNEET.

MitoNEET was further incubated with a fixed concentration of FMN and increasing concentrations of NL-1 at room temperature for one hour under aerobic conditions, followed by reduction with sodium dithionite. Figure 2.6B shows that as the concentration of NL-1 increased, the EPR signal at \( g = 1.85 \) also gradually increased. About 200 \( \mu \)M NL-1 was sufficient to change the EPR spectrum of the \( g = 1.94 \) to that of the \( g = 1.85 \) of the reduced mitoNEET [2Fe-2S] clusters. The results suggested that FMNH2 and pioglitazone/NL-1 have a synergistic effect on the reduced mitoNEET [2Fe-2S] clusters.
Figure 2.6 Synergistic effect of FMNH2 and pioglitazone/NL-1 on the reduced mitoNEET [2Fe-2S] clusters.

A), EPR spectra of the reduced mitoNEET [2Fe-2S] clusters. MitoNEET (containing 20 μM [2Fe-2S] clusters) (spectrum 1) was incubated with FMN (50 μM) (spectrum 2), pioglitazone (Pio) (500 μM) (spectrum 3), NL-1 (200 μM) (spectrum 4), FMN + pioglitazone (Pio) (spectrum 5), FMN + NL-1 (spectrum 6) at room temperature for 60 minutes. After incubation, samples were reduced with freshly prepared sodium dithionite (4 mM), transferred to EPR tubes, and immediately frozen in liquid nitrogen for EPR measurements.

B), titration of NL-1. MitoNEET (containing 20 μM [2Fe-2S] clusters) was incubated with FMN (50 μM) and increasing concentrations of NL-1 (0 to 200 μM) (spectra 1-5) at room temperature for 60 min. After incubation, samples were reduced with freshly prepared sodium dithionite (4 mM), transferred to EPR tubes, and immediately frozen in liquid nitrogen for EPR measurements. The data were representatives of three independent experiments.
2.4 Discussion

MitoNEET is a founding member of a small family of proteins that contain a CDGSH (CysAsp-Gly-Ser-His) motif (39,40). Although it has been reported that mitoNEET is a key regulator of energy metabolism in mitochondria (13), the underlying mechanism has not been fully understood. Several research groups proposed that mitoNEET may transfer its [2Fe-2S] clusters for maturation of iron-sulfur proteins in cytosol (22-24). Others suggested that mitoNEET may regulate mitochondrial functions via specific protein-protein interactions (9). Here, we report that mitoNEET is a novel redox enzyme that transfers electron from FMNH2 to oxygen or ubiquinone in the mitochondrial outer membrane via its [2Fe-2S] clusters. In this process, FMNH2, which may be reduced by flavin reductase (41) and NADH in cytosol, binds to mitoNEET and reduces the [2Fe-2S] clusters in mitoNEET (29). The reduced mitoNEET [2Fe-2S] clusters are readily oxidized by oxygen or ubiquinone (Figure 2.7). Together with flavin reductase (41) and FMN, mitoNEET may effectively promote oxidation of NADH in cytosol with a concomitant reduction of oxygen or ubiquinone in the mitochondrial outer membrane.

The intracellular concentration of FMN in human cells is in nanomolar range (42). Here we find that 100 nM FMN is sufficient to reduce 10 μM mitoNEET [2Fe-2S] clusters in the presence of flavin reductase and NADH in less than 2 min (Figure 2.1), indicating that FMN may act as an electron shuttle reducing the mitoNEET [2Fe-2S] clusters (29). In cytosol, FMN is reduced to FMNH2 by flavin reductase (41) using NADH as the electron donor. Because glycolysis will produce NADH which must be oxidized in order to sustain glycolysis activity (43), mitoNEET may enhance glycolysis by promoting oxidation of NADH in cytosol (Figure 2.7). In primary and metastatic cancer cells, glycolysis is highly upregulated, resulting in increased glucose consumption (44). Interestingly, mitoNEET is also highly expressed in cancer
cells and over expression of mitoNEET is essential for cancer cell proliferation (11,12). In this context, we propose that overproduced mitoNEET may enhance glycolysis in cytosol by promoting oxidation of NADH in cancer cells. Accordingly, when mitoNEET is deleted, oxidation of NADH in cytosol could be diminished, leading to decrease of glycolysis in cytosol and oxidative phosphorylation in mitochondria. This notion is consistent with the previous report showing that deletion of mitoNEET decreases oxidative phosphorylation capacity in mitochondria by about 30% (5) and inhibits cancer cell proliferation (11,12). Nevertheless, additional experiments are required to elucidate the physiological link between the electron transfer activity of mitoNEET and glycolysis in cells.

Iron-sulfur clusters in proteins are often sensitive to oxygen and reactive oxygen species (45). However, the mitoNEET [2Fe-2S] clusters are highly resistant to oxygen and hydrogen peroxide (27). In fact, the reduced mitoNEET [2Fe-2S] clusters can be oxidized by oxygen without disruption of the cluster (Figure 2.2). Importantly, compared with oxygen, ubiquinone2 is more efficient in oxidizing the reduced mitoNEET [2Fe-2S] clusters (Figures 2.3 and 2.4). Since mitoNEET is a mitochondrial outer membrane protein (4), and the [2Fe-2S] clusters in mitoNEET are positioned close to the membrane (18-21), we propose that ubiquinone could be an intrinsic electron acceptor for the reduced mitoNEET [2Fe-2S] clusters (Figure 2.7). Upon the single-electron reduction by the reduced mitoNEET [2Fe-2S] cluster, ubiquinone is converted to semiquinone which may be further reduced to ubihydroquinone, or transfer the electron to oxygen to produce superoxide or hydrogen peroxide (34). While other oxidants in cytosol may also oxidize the reduced mitoNEET [2Fe-2S] clusters in cytosol, the proposed electron transfer path from NADH to ubiquinone catalyzed by the mitoNEET [2Fe-2S] clusters (Figure 2.7) is reminiscent of the electron transfer path in complex I in which electrons in NADH are
transferred to ubiquinone via FMN and a chain of iron-sulfur clusters (33). The observed reduction of oxygen by the mitoNEET [2Fe-2S] clusters may reflect the possible electron transfer leak in mitoNEET, as reported in complex I (33). The final products of the electron transfer reaction catalyzed by mitoNEET in mitochondria remain to be determined.

Pioglitazone has two major targets in cells: PPARγ that regulates the expression of genes involved in insulin sensitivity (2) and mitoNEET (4) that modulates energy metabolism in mitochondria (3). It was previously reported that binding of pioglitazone stabilizes the mitoNEET [2Fe-2S] clusters (21) and shifts the redox midpoint potential of the [2Fe-2S] clusters by about 100 mV (26). Here, we find that pioglitazone and its analog NL-1 (36) can inhibit the electron transfer activity of mitoNEET (Figure 2.5) by forming a unique complex with mitoNEET and FMNH2 which has an unusual EPR signal at g = 1.85 (Figure 2.6). Based on the molecular docking modeling (29,36), FMN and pioglitazone/NL-1 have distinct binding sites but with significant overlap in mitoNEET, suggesting that pioglitazone/NL-1 may interfere the FMNH2 binding in mitoNEET and inhibit the electron transfer activity of mitoNEET in mitochondria. As mitoNEET is proposed as a chemotherapeutic target for treating type II diabetes (4), breast cancer (16,17), and neurodegenerative diseases (10), a high-throughput screening approach combining the electron transfer activity assay and EPR measurements of mitoNEET may help identify new drugs that specifically target the mitoNEET [2Fe-2S] clusters in mitochondria.
Figure 2.7 A proposed model for the electron transfer of mitoNEET in mitochondria outer membrane.
In cytosol, FMNH2 is reduced by flavin reductase using NADH as the electron donor. FMNH2 then reduces the mitoNEET [2Fe-2S] clusters which in turn transfer electrons to oxygen or ubiquinone in the mitochondrial outer membrane. Together with flavin reductase and FMN, mitoNEET promotes NADH oxidation with a concomitant reduction of oxygen or ubiquinone in mitochondria.

2.5 References


CHAPTER 3. LIGHT-INDUCED RELEASE OF NITRIC OXIDE FROM THE NITRIC OXIDE-BOUND CDGSH-TYPE [2Fe–2S] CLUSTERS IN MITOCHONDRIAL PROTEIN MINER2

3.1 Introduction

Iron-sulfur proteins are involved in diverse physiological functions ranging from energy metabolism to DNA replication and repair [1]. Most iron-sulfur clusters in proteins are ligated via cysteine residues. However, other amino acid residues such as histidine, glutamine, serine, or arginine may also provide ligands for iron-sulfur clusters in proteins [2]. A new group of iron-sulfur proteins that contain the CDGSH (Cys-Asp-Gly-Ser-His) motifs have recently been identified [3]. Each CDGSH motif in the protein hosts a [2Fe–2S] cluster via a unique ligand arrangement of three cysteine and one histidine residues. In human mitochondria, there are three CDGSH-type iron-sulfur proteins [3,4]: mitoNEET [5], a mitoNEET-related protein 1 (Miner1 or NAF-1) [6], and a mitoNEET-related protein 2 (Miner2) [7,8]. MitoNEET was initially identified as a target of the type II diabetes drug pioglitazone [5]. Miner1 and mitoNEET are homologues with 54% identity and 76% similarity. Mutations of Miner1 have been attributed to causing type II Wolfram Syndrome [9,10]. Both mitoNEET and Miner1 contain a transmembrane alpha helix in the N-terminus and a CDGSH motif in the C-terminal domain that hosts a [2Fe–2S] cluster [6,[11], [12], [13]]. While mitoNEET is a mitochondrial outer

membrane [5], Miner1 is localized in the mitochondrial outer membrane and the endoplasmic reticulum membrane [14]. Increasing evidence suggests that mitoNEET and Miner1 have a crucial regulatory role in cellular redox state, iron homeostasis, and production of reactive oxygen species in mitochondria [15,16]. Interestingly, unlike mitoNEET and Miner1, Miner2 is a soluble mitochondrial matrix protein and hosts two [2Fe–2S] clusters via two CDGSH motifs [7,8]. While it has been proposed that Miner2 could be involved in iron-sulfur cluster biogenesis in mitochondria [8], the physiological function of Miner2 remains largely elusive.

Iron-sulfur clusters in proteins are among the primary targets of nitric oxide (NO), as NO has a specific reactivity with ferrous iron in proteins [[17], [18], [19], [20]]. A number of iron-sulfur proteins have previously been characterized as NO sensors in cells [20]. It has been shown that iron-sulfur clusters in proteins can be readily modified by NO forming the protein-bound dinitrosyl iron complexes [18,21,22], the thiolate-bridged diiron tetranitrosyl complexes [23], or the octa-nitrosyl clusters [24], depending on specific proteins and NO concentrations used in the experiments. Unexpectedly, we have found that the CDGSH-type [2Fe–2S] clusters in Miner2 are able to bind NO and form the stable NO-bound [2Fe–2S] clusters without disruption of the clusters [7]. In the present study, we report that the NO-bound Miner2 [2Fe–2S] clusters can readily release NO upon light excitation in the visible range. The UV–visible and Electron Paramagnetic Resonance (EPR) measurements show that the NO-bound Miner2 [2Fe–2S] clusters are converted to the reduced Miner2 [2Fe–2S] clusters after NO is released upon the light excitation, suggesting that NO binding in the reduced Miner2 [2Fe–2S] clusters is reversible. Furthermore, NO binding effectively inhibits the redox transition of the Miner2 [2Fe–2S] clusters. We propose that NO may modulate the function of Miner2 in mitochondria via directly binding to the CDGSH-type [2Fe–2S] clusters in the protein.
3.2 Materials and methods

Protein preparation

The gene encoding the human Miner2 (containing residues 34–127) with the N-terminal His tag was synthesized (Genscript co.) and cloned into pET28b + plasmid for protein expression in E. coli cells. The cloned DNA sequence was confirmed by direct sequencing (Eurofins MWG Operon). Overnight E. coli cells containing expression plasmid were diluted 1:100 in freshly prepared LB (Luria-Bertani) medium and incubated at 37 °C with aeration (250 rpm) for 3 h, followed by protein induction by adding Isopropyl β-d-1-thiogalactopyranoside (200 μM) overnight at 18 °C. The recombinant protein was purified as described previously [25]. The purity of purified proteins was greater than 95% as judged by electrophoresis analysis on a 15% polyacrylamide gel containing SDS followed by staining with Coomassie Blue. The NO-bound Miner2 [2Fe–2S] clusters were prepared by treating the E. coli cells expressing human Miner2 with NO gas (20 μM) or treating the reduced Miner2 [2Fe–2S] clusters with NO gas under anaerobic conditions as described previously [7]. Nitric oxide gas (Praxair Co.) was first passed through a soda-lima column to remove NO2 and higher nitrogen oxides before being used to bubble pre-degassed water in a sealed 50-ml flask for 5 min. The concentration of nitric oxide in the NO-saturated solution was measured using a nitric oxide electrode (World Precision Instrument.) [26]. The UV–visible absorption spectra were recorded in a Beckman DU640 UV–visible spectrometer equipped with a temperature controller. The extinction coefficient of 11.88 cm−1mM−1 was used to determine the concentration of apo-Miner2.

Chemicals
NADH, isopropyl-β-d-1-thiogalactopyranoside, and kanamycin were purchased from Research Product International co. DAF-FM (4-amino-5-methylamino-2′,7′-difluorofluorescein) was purchased from ThermoFisher Scientific co. The diethylamine NONOate and Griess Reagent were purchased from Cayman Chemical co. FMN and other chemicals were purchased from Sigma co. The extinction coefficients of 6.2 mM−1cm−1 at 340 nm and 12.5 mM−1cm−1 at 445 nm were used for determining the concentrations of NADH and FMN, respectively [27]. FMNH2 was prepared by using NADH and E. coli flavin reductase under anaerobic conditions as described in Ref. [28].

**NO analyses**

The NO-bound Miner2 [2Fe–2S] clusters were exposed to either room light (600 Lux) or a Cole-Parmer 41,720 series Illuminator A (400k Lux) which provides strong visible light source under aerobic or anaerobic conditions. The Cole-Parmer illuminator was equipped with an infrared filter that reduces the transmittance of heat to the sample. The intensity of the light excitation was measured using a Digital Lux Meter (LX1330B, Dr. Meter). Anaerobic conditions were achieved by purging a gas-sealed cuvette with pure Argon gas for 10 min in dark. DAF-FM (4-amino-5-methylamino-2′,7′-difluorofluorescein) was used to detect NO release. DAF-FM interacts with NO to form a benzotriazole product which has fluorescence (ThermoFisher Scientific co). The fluorescence spectra were taken upon excitation at 485 nm in a spectrofluorometer (FP-6300 Spectrofluorometer, Jasco Co.). The fluorescence intensity was also measured in a plate reader (Victor3 1420 Multilabel Counter, PerkinElmer Co.) using the emission wavelength at 535 nm and the excitation wavelength at 485 nm. Similar results were obtained from the spectrofluorometer and the plate reader. The NO-releasing reagent diethylamine NONOate (Cayman Chemicals co.) was used as the NO standard solution. A few
crystals of diethylamine NONOate was first dissolved in buffer containing Tris (20 mM, pH 10.5), and the concentration of the NONOate was determined at 250 nm using an extinction coefficient of 6.5 mM$^{-1}$cm$^{-1}$. The NONOate was then transferred to buffer containing 20 mM Tris (pH 8.0) and 500 mM NaCl for the NO release under anaerobic conditions. Each NONOate molecule releases 1.5 equivalents of NO in buffer containing 20 mM Tris (pH 8.0) and 500 mM NaCl after incubation at 37 °C for 10 min (Cayman Chemicals co.).

**EPR measurements**

The X-band Electron Paramagnetic Resonance (EPR) spectra were recorded using a Bruker model ESR-300 spectrometer equipped with an Oxford Instruments 910 continuous flow cryostat. Routine EPR conditions: microwave frequency, 9.47 GHz; microwave power, 10.0 mW; modulation frequency, 100 kHz; modulation amplitude, 1.2 mT; temperature, 20 K; receiver gain, 2 × 105.

**Mass spectrometry measurements**

For the mass spectrometry measurements, the protein samples were dissolved in 20 mM ammonium acetate (pH 8.0) buffer. The protein samples were either exposed to light excitation (400k lux) or left in dark at room temperature. Mass spectrometry experiments were carried out in the LSU mass spectrometry facility. Protein concentration was about 10 μM. All experiments were performed with an AmaZon speed electron transfer dissociation Ion Trap mass spectrometer (Bruker Daltonics, Billerica, MA, USA). Sample solution was infused via an Apollo II electrospray ion source using a syringe pump at the flow rate of 3 μL/min. MS detection was performed in a full-scan mode in positive ionization enhanced resolution mode with the scan speed of 8100 m/z/s. The parameter settings for ESI-MS were as follows: capillary
voltage, 4500 V; end plate offset, −500 V; nebulizer, 8 psi; dry gas, 4 L/min; dry gas
temperature, 100 °C. MS data were acquired from 300 to 3000 with a target mass of 1200 m/z
and trap drive level of 100%. The software used for data processing and de-convolution was
Compass DataAnalysis (Bruker Daltonics, Billerica, MA, USA). The mass spectrometry data
were analyzed using mMass software [29].

3.3 Results

The NO-bound Miner2 [2Fe–2S] clusters are light sensitive

Purified human Miner2 [2Fe–2S] clusters are in an oxidized state which have an
absorption peak at 458 nm. When the Miner2 [2Fe–2S] clusters are reduced, the absorption peak
at 458 nm is shifted to 420 nm [7]. In the previous studies, we have found that the reduced
Miner2 [2Fe–2S] clusters, but not the oxidized ones, are able to bind nitric oxide (NO) and form
the stable NO-bound Miner2 [2Fe–2S] clusters which have a new distinct absorption peak at
422 nm [7].

In the subsequent studies, we have noticed that when the NO-bound Miner2 [2Fe–2S]
clusters were exposed to room light under aerobic conditions, the absorption peak at 422 nm of
the NO-bound Miner2 [2Fe–2S] clusters was gradually shifted to the absorption peak at 458 nm
of the oxidized Mienr2 [2Fe–2S] clusters (Figure. 3.1A). This observation let us to speculate that
the NO-bound Miner2 [2Fe–2S] clusters could be light sensitive. To test the idea, the NO-bound
Miner2 [2Fe–2S] clusters were subjected to a strong light source (400K Lux) which provides
strong visible light under aerobic conditions. Figure. 3.1B shows that after the strong visible light
excitation, the absorption peak at 422 nm of the NO-bound Miner2 [2Fe–2S] clusters was
quickly changed to the absorption peak at 458 nm of the oxidized Miner2 [2Fe–2S] clusters. The light excitation for about 30 s was sufficient to fully convert the NO-bound Miner2 [2Fe–2S] clusters) to the oxidized Miner2 [2Fe–2S] clusters under aerobic conditions (Figure. 3.1C). Thus, the NO-bound Miner2 [2Fe–2S] clusters are light sensitive, and the NO-bound Miner2 [2Fe–2S] clusters may be converted to the oxidized Miner2 [2Fe–2S] clusters by the light excitation under aerobic conditions.

Figure. 3.1 The NO-bound Miner2 [2Fe–2S] clusters are light sensitive. A), effect of room light on the NO-bound Miner2 [2Fe–2S] clusters. Spectrum 1, the NO-bound (fig. caption cont’d.)
Miner2 [2Fe–2S] clusters (10 μM) were incubated under dark for 1 h under aerobic conditions. Spectrum 2, the NO-bound Miner2 [2Fe–2S] clusters (10 μM) were incubated under room light for 1 h under aerobic conditions. B), effect of the light excitation on the NO-bound Miner2 [2Fe–2S] clusters. Spectrum 1, the NO-bound Miner2 [2Fe–2S] clusters (10 μM) were incubated in dark for 90 s under aerobic conditions. Spectrum 2, the NO-bound Miner2 [2Fe–2S] clusters (10 μM) were exposed to the strong light excitation (400K Lux) for 90 s under aerobic conditions. C), transition from the NO-bound Miner2 [2Fe–2S] clusters to the oxidized Miner2 [2Fe–2S] clusters upon the light excitation. The NO-bound Miner2 [2Fe–2S] clusters were exposed to the strong light excitation (400K Lux) for the indicated time. The absorption spectra were taken after each light exposure. Absorption peaks at 422 nm and 458 nm represent the NO-bound Miner2 [2Fe–2S] clusters and the oxidized Miner2 [2Fe–2S] clusters, respectively.

The NO-bound Miner2 [2Fe–2S] clusters release NO upon light excitation

Previous studies have shown that when the NO-bound Miner2 [2Fe–2S] clusters were treated with Griess Reagent, nitrite was released from the NO-bound clusters due to denaturation of the protein [7]. Here, we used the Griess Reagent to explore whether the NO was actually removed from the NO-bound [2Fe–2S] clusters in Miner2 upon the light excitation. In the experiments, after the NO-bound Miner2 [2Fe–2S] clusters were exposed to the light excitation, the protein was re-purified by passing through a HiTrap Desalting column (GE Healthcare Life Sciences). Re-purified protein was then treated with Griess Reagents. As shown in Figure. 3.2A, the light excitation almost completely eliminated the acid-labile nitrite of the NO-bound [2Fe–2S] clusters in Miner2. On the other hand, the [2Fe–2S] clusters in Miner2 remained intact after the light excitation, suggesting that the NO-bound Miner2 [2Fe–2S] clusters only release NO upon the light excitation.
Figure. 3.2 NO is released from the NO-bound Miner2 [2Fe–2S] clusters upon light excitation. A), the NO-bound Miner2 [2Fe–2S] clusters release NO after the light excitation. The Miner2 [2Fe–2S] clusters or the NO-bound Miner2 [2Fe–2S] clusters (10 μM each) were either kept in dark or subjected to the light excitation (400K Lux) 60 s, followed by re-purification of the protein by passing through a High-Trap Desalting column. Re-purified proteins were then incubated with Griess Regents to determine the acid-labile nitrite. Sodium nitrite solution (1 mM) was used as a standard. Data were averages ± standard deviation from three experiments. B), mass spectra of the NO-bound Miner2 [2Fe–2S] clusters before and after the light excitation (400K Lux for 60 s). Spectrum 1, the NO-bound Miner2 [2Fe–2S] clusters. Spectrum 2, same as in spectrum 1 except after the light excitation. The mass spectra were deconvoluted using mMass software [29]. C), fluorescence spectra of DAF-FM incubated with the Miner2 [2Fe–2S] clusters under dark (spectrum 1) or after the light excitation (spectrum 2). The Miner2 [2Fe–2S] clusters (10 μM) were incubated with DAF-FM (4-amino-5-methylamino-2’,7’-difluorofluorescein) (10 μM) in dark for 2 min at room temperature under aerobic conditions. The samples were either (fig. caption cont’d.)
kept in dark or exposed to the light excitation (400K Lux) for 60 s. The fluorescence spectra were taken using the excitation wavelength at 485 nm. D), fluorescence spectra of DAF-FM incubated with the NO-bound Miner2 [2Fe–2S] clusters under dark (spectrum 1) or after the light excitation (spectrum 2). Same as in C), except the NO-bound Miner2 [2Fe–2S] clusters (10 μM) were used.

The NO-bound Miner2 [2Fe–2S] clusters were also subjected to the Electrospray Ionization (ESI) mass spectrometry analyses before and after the light excitation. As shown in Figure. 3.2B, the NO-bound Miner2 [2Fe–2S] clusters had a major mass peak at 12,889 before the light excitation. After the light excitation, the mass peak at 12,889 disappeared and was replaced with a new mass peak at 12,827, a mass decrease of 62 Da. The same mass peak at 12,827 was observed in the purified Miner2 [2Fe–2S] clusters which represents the sum of the Miner2 peptide (12,784) with a likely N-terminal acetylation (43) [30] and two [2Fe–2S] clusters (2 × 176). As each Miner2 protein hosts two [2Fe–2S] clusters and each [2Fe–2S] cluster can bind one NO [7], release of two NO molecules from the NO-bound Miner2 [2Fe–2S] clusters by the light excitation would result in the mass decrease by 62 Da. Thus, the mass spectrometry results suggested that the NO-bound Miner2 [2Fe–2S] clusters release two NO molecules upon the light excitation.

To further explore whether NO gas was released from the NO-bound Miner2 [2Fe–2S] clusters by the light excitation, we used the NO probe DAF-FM (4-amino-5-methylamino-2′,7′-difluorofluorescein) [31]. DAF-FM is essentially nonfluorescent until it reacts with NO to form a fluorescent benzotriazole (Thermo Fisher Scientific co). In the experiments, the Miner2 [2Fe–2S] clusters or the NO-bound Miner2 [2Fe–2S] clusters were incubated with DAF-FM under aerobic conditions, followed by the light excitation (400K Lux for 90 s). Figure. 3.2C shows that when the Miner2 [2Fe–2S] clusters were exposed to the light excitation, very little change of the fluorescence intensity of DAF-FM was observed. On the other hand, when the NO-bound
Miner2 [2Fe–2S] clusters mixed with DAF-FM were exposed to the light excitation, the fluorescence intensity of DAF-FM was significantly increased (Figure. 3.2D). Using the diethylamine NONOate as the NO standard solution, we estimated that about 3.4 ± 0.2 μM NO was released from 10 μM of the NO-bound Miner2 [2Fe–2S] clusters after the light excitation.

**The NO-bound Miner2 [2Fe–2S] clusters are converted to the reduced Miner2 [2Fe–2S] clusters by the light excitation under anaerobic conditions**

To determine whether oxygen is required for the light-induced NO release from the NO-bound Miner2 [2Fe–2S] clusters, we measured the UV–visible absorption spectrum of the NO-bound Miner2 [2Fe–2S] clusters before and after the light excitation under anaerobic conditions. Figure. 3.3A shows that after the light excitation, the absorption peak at 422 nm of the NO-bound Miner2 [2Fe–2S] clusters was shifted to the peak at 420 nm. Because the absorption peak at 420 nm reflects the reduced Miner2 [2Fe–2S] clusters [7], the results suggested that the NO-bound Miner2 [2Fe–2S] clusters are converted to the reduced Miner2 [2Fe–2S] clusters by the light excitation under anaerobic conditions.

In previous studies, we have shown that the reduced Miner2 [2Fe–2S] clusters have a distinct Electron Paramagnetic Resonance (EPR) signal at g = 1.92, and the NO-bound Miner2 [2Fe–2S] clusters are EPR-silent [7]. Here, we used EPR to examine whether the reduced Miner2 [2Fe–2S] clusters are actually formed when the NO-bound Miner2 [2Fe–2S] clusters are exposed to the light excitation under anaerobic conditions. Figure. 3.3B shows that the NO-bound Miner2 [2Fe–2S] clusters had an EPR signal at g = 2.04 which represented a small amount of dinitrosyl iron complex formed during the NO treatment of Miner2 [7]. When the NO-bound Miner2 [2Fe–2S] clusters were exposed to the light excitation under anaerobic conditions, a new EPR signal at
g = 1.92 of the reduced Miner2 [2Fe–2S] clusters appeared (Figure. 3.3B). Integration of the EPR signal at g = 1.92 revealed that about 6.2 ± 0.5 μM of the reduced Miner2 [2Fe–2S] clusters were formed after 10 μM of the NO-bound Miner2 [2Fe–2S] clusters were exposed to the light excitation under anaerobic conditions. It should be pointed out that the EPR signal at g = 2.04 of the dinitrosyl iron complex was not changed upon the light excitation (Figure. 3.3B), indicating that dinitrosyl iron complex was not light sensitive. Taken together, the results established that the NO-bound Miner2 [2Fe–2S] clusters are converted to the reduced Miner2 [2Fe–2S] clusters by the light excitation under anaerobic conditions.

Figure. 3.3 Oxygen is not required for the light-induced NO release from the NO-bound Miner2 [2Fe–2S] clusters. A), UV–visible absorption spectra of the NO-bound Miner2 [2Fe–2S] clusters before and after the light excitation under anaerobic conditions. The NO-bound Miner2 [2Fe–2S] clusters (10 μM) were incubated under anaerobic conditions, followed by exposure to the light excitation (400K Lux) for 0, 5, 10, 15, and 20 s. Spectra were taken after each light excitation. B), EPR spectra of the NO-bound Miner2 [2Fe–2S] clusters. Spectrum 1, the NO-bound Miner2 [2Fe–2S] clusters (10 μM). Spectrum 2, same as in spectrum 1 except after the light excitation (400K Lux for 60 s) under anaerobic conditions. The results are representatives from three independent experiments.
NO binding inhibits the redox transition of the Miner2 [2Fe–2S] clusters

Like the mitoNEET [2Fe–2S] clusters [28], the Miner2 [2Fe–2S] clusters can be reduced by the reduced flavin mononucleotide (FMNH2) and oxidized by oxygen. Figure. 3.4A shows that the oxidized Miner2 [2Fe–2S] clusters were readily reduced by FMNH2 under anaerobic conditions, and that the reduced Miner2 [2Fe–2S] clusters were re-oxidized after exposure to air, indicating that the Miner2 [2Fe–2S] clusters can undergo redox transition. In contrast, the NO-bound Miner2 [2Fe–2S] clusters could not be reduced by FMNH2 or oxidized by oxygen, as the absorption peak at 422 nm of the NO-bound Miner2 [2Fe–2S] clusters remained the same upon addition of FMNH2 or exposure to air (Figure. 3.4B). The results suggested that the NO-bound Miner2 [2Fe–2S] clusters are resistant to redox transition. When the NO-bound Miner2 [2Fe–2S] clusters were exposed to the light excitation, the NO-bound Miner2 [2Fe–2S] clusters quickly became the reduced Miner2 [2Fe–2S] clusters under anaerobic conditions, and further exposure to air led to oxidation of the reduced Miner2 [2Fe–2S] clusters (Figure. 3.4C). The results suggested that release of NO from the NO-bound Miner2 [2Fe–2S] clusters restores the redox transition activity of the Miner2 [2Fe–2S] clusters.
Figure. 3.4 NO inhibits the redox transition of the Miner2 [2Fe–2S] clusters.

A), reduction and oxidation of the Miner2 [2Fe–2S] clusters. Purified Miner2 (10 μM) (spectrum 1) was incubated with FMN (0.1 μM), NADH (20 μM) and E. coli flavin reductase (0.1 μM) for 5 min under anaerobic conditions (spectrum 2). The sample was then exposed to air for 10 min (spectrum 3). B), effect of oxygen and FMNH2 on the NO-bound Miner2 [2Fe–2S] clusters. The NO-bound Miner2 [2Fe–2S] clusters (10 μM) (spectrum 1) was incubated with FMN (0.1 μM), NADH (20 μM) and E. coli flavin reductase (0.1 μM) for 5 min under anaerobic conditions (spectrum 2). The sample was then exposed to air for 10 min (spectrum 3). C), oxidation of the NO-bound Miner2 [2Fe–2S] clusters by the light excitation and air. The NO-bound Miner2 [2Fe–2S] clusters (10 μM) (spectrum 1) were exposed to the light excitation (400K Lux) for 30 s under anaerobic conditions (spectrum 2), followed by exposure to air for 10 min (spectrum 3). Absorption peaks at 422 nm, 420 nm, and 458 nm represent the NO-bound Miner2 [2Fe–2S] clusters, the reduced Miner2 [2Fe–2S] clusters, and the oxidized Miner2 [2Fe–2S] clusters, respectively.
3.4 Discussion

In previous studies, we have shown that the mitochondrial matrix protein Miner2 [2Fe–2S] clusters have a unique activity to bind NO and form the stable NO-bound [2Fe–2S] clusters [7]. Here, we report that the NO-bound [2Fe–2S] clusters in Miner2 are light sensitive. Upon the light excitation, the NO-bound Miner2 [2Fe–2S] clusters release NO and become the reduced Miner2 [2Fe–2S] clusters under anaerobic conditions. Additional studies further reveal that binding of NO effectively inhibits the redox transition of the Miner2 [2Fe–2S] clusters, indicating that NO may modulate the physiological function of Miner2 in mitochondria by directly binding to the CDGSH-type [2Fe–2S] clusters.

NO has a high reactivity with heme and iron-sulfur clusters in proteins [32,33]. The binding of NO to heme has been extensively investigated in proteins such as soluble guanylate cyclase [34]. However, much less has been known about the specific interactions between NO and iron-sulfur clusters in proteins. Previous studies from different research groups including ours have shown that iron-sulfur clusters in proteins are readily disrupted by NO, forming the dinitrosyl iron complex [18,21,22], the thiolate-bridged diiron tetranitrosyl complex [23], or the octa-nitrosyl cluster [24]. To the best of our knowledge, the Miner2 CDGSH-type [2Fe–2S] clusters represent the first example that the [2Fe–2S] clusters can bind NO and form stable NO-bound clusters [7]. The unique NO binding activity of the CDGSH-type [2Fe–2S] cluster in Miner2 is likely due to its unusual ligand arrangement of three cysteine and one histidine residues [8]. In the CDGSH-type [2Fe–2S] clusters, one of the iron atoms is ligated via two cysteine residues and the other iron via one cysteine and one histidine residues [8]. The iron center ligated via cysteine/histidine residues in the [2Fe–2S] cluster is most likely redox active [35]. In this context, we propose that NO binds to the ferrous iron in the reduced Miner2 [2Fe–2S] clusters.
2S] cluster (Figure. 3.5), which is reminiscent of the NO binding in the ferrous heme in proteins [34]. The NO binding affinity for the ferrous heme in proteins is extremely high with the binding constant of about 1011 M−1 [36]. On the other hand, the NO binding affinity for the ferric heme in proteins is relatively weaker with the binding constant in the range of 103 to 105 M−1 [37]. Accordingly, we find that the oxidized Miner2 [2Fe–2S] clusters fail to bind NO, and only the reduced Miner2 [2Fe–2S] clusters are able to bind NO with a high binding affinity [7]. Since the redox midpoint potent (Em) of the CDGSH-type [2Fe–2S] clusters in mitoNEET is about 0 mV at pH 7.0 [38], it is most likely that the [2Fe–2S] clusters in Miner2 are in a reduced state in mitochondria [7]. Thus, the Miner2 [2Fe–2S] clusters are capable of binding NO under physiological conditions.

Figure. 3.5 Proposed model for the NO-mediated inhibition of the redox transition of the Miner2 [2Fe–2S] clusters. Mitochondrial protein Miner2 contains two [2Fe–2S] clusters. Only one of the [2Fe–2S] clusters is shown. In the reduced Miner2 [2Fe–2S] cluster, the iron atom ligated with his/cys is in ferrous state and can be oxidized by oxygen. When NO binds to the ferrous iron atom in the reduced [2Fe–2S] cluster, the cluster can no longer be oxidized by oxygen or reduced by FMNH2. When NO is released from ten NO-bound Miner2 [2Fe–2S] clusters by light excitation, the oxidation of the reduced Miner [2Fe–2S] clusters by oxygen is restored.

While the NO-bound Miner2 [2Fe–2S] clusters are stable under aerobic or anaerobic conditions, they are highly sensitive to visible light (Figure. 3.1). The mass spectrometry
analyses showed that two NO molecules may be released from the NO-bound Miner2 [2Fe–2S] clusters by the light excitation (Figure. 3.2B). The EPR and UV–visible absorption measurements further revealed that the NO-bound Miner2 [2Fe–2S] clusters are converted to the reduced Miner2 [2Fe–2S] clusters upon the light excitation under anaerobic conditions (Figure. 3.3), demonstrating that the NO binding in the reduced Miner2 [2Fe–2S] clusters is reversible. We were unable to observe the re-binding of NO to the reduced Miner2 [2Fe–2S] clusters after the light excitation, likely due to the diffusion of NO and/or the reaction of NO with other reactants in the solution. However, addition of extra NO to the reaction solution resulted in re-binding of NO to the reduced Miner2 [2Fe–2S] clusters in dark (data not shown), further supporting a notion that the reduced Miner2 [2Fe–2S] clusters can reversibly bind NO.

Like the mitoNEET [2Fe–2S] clusters, the Miner2 [2Fe–2S] clusters can be readily reduced by FMNH2 and oxidized by oxygen. However, the NO-bound Miner2 [2Fe–2S] clusters are resistant to reduction or oxidation (Figure. 3.4B). When NO is released from the NO-bound Miner2 [2Fe–2S] clusters by the light excitation, the reduced Miner2 [2Fe–2S] clusters can be re-oxidized by oxygen (Figure. 3.4C), suggesting that NO binding effectively blocks the redox transition of the Miner2 [2Fe–2S] clusters (Figure. 3.5). It has been reported that the mononuclear iron center in nitrile hydratase from Rhodococcus sp. N-771 has a strong binding activity for NO [39]. Purified nitrile hydratase is inactive due to NO binding in the mononuclear iron center, and light excitation releases NO from nitrile hydratase and activates the enzyme activity [40]. For the NO-bound Miner2 [2Fe–2S] clusters, release of NO by light excitation appears to also restore the redox transition of the Miner2 [2Fe–2S] clusters. While the mechanism by which the light-induced release of NO from the NO-bound Miner2 [2Fe–2S] clusters is not fully understood, our results strongly suggested that the NO binding in the reduced
Miner2 [2Fe–2S] clusters is reversible, and NO may change the physiological activity of Miner2 in mitochondria by directly binding to the [2Fe–2S] clusters. Since there are many CDGSH-type [2Fe–2S] proteins found in human cells [4], the reversible NO binding in the Miner2 [2Fe–2S] clusters may represent a novel example of NO regulation via the CDGSH-type [2Fe–2S] clusters in proteins.

3.5 References


CHAPTER 4. CONCLUSIONS

Human mitoNEET has a key role in iron homeostasis, production of reactive oxygen species and energy metabolism in mitochondria. Here we find that mitoNEET is a novel electron transfer protein that can catalyze the electron transfer from NADH to oxygen or ubiquinone. We also identify a unique binding site of flavin mononucleotide (FMN) binding site in mitoNEET, which is different from but overlaps with the binding sites of type II diabetes drug Pioglitazone in mitoNEET. Pioglitazone or its homologs can effectively inhibit the electron transfer activity of mitoNEET, apparently by competing with FMN binding in mitoNEET. We propose that mitoNEET may promote glycolysis in cytosol by oxidizing cytosolic NADH and regulate energy metabolism in normal and cancer cells. We also find that the human Miner2 [2Fe-2S] clusters can reversibly bind nitric oxide without disruption of the clusters. Binding of nitric oxide to the [2Fe-2S] clusters in Miner2 effectively inhibit the electron transfer activity of Miner2, demonstrating that nitric oxide may regulate energy metabolism by directly binding to the [2Fe-2S] clusters in the NEET-proteins in mitochondria.
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