REDOX TRANSITION OF IRON-SULFUR CLUSTERS IN MITONEET

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REDOX TRANSITION OF IRON-SULFUR CLUSTERS IN MITONEET

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science in The Department of Biological Sciences

by
Homyra Tasnim
M.B.B.S., Dhaka Medical College, Bangladesh (2016)
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<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E&lt;sub&gt;m&lt;/sub&gt;</td>
<td>redox midpoint potential</td>
</tr>
<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>[Fe-S]</td>
<td>iron-sulfur</td>
</tr>
<tr>
<td>H87C</td>
<td>mitoNEET histidine 87 substituted with cysteine</td>
</tr>
<tr>
<td>Miner1/NAF-1</td>
<td>mitoNEET-related protein 1/ Nutrient-deprivation autophagy factor</td>
</tr>
<tr>
<td>Miner2/miNT</td>
<td>mitoNEET-related protein 2</td>
</tr>
<tr>
<td>mT</td>
<td>milli Tesla</td>
</tr>
<tr>
<td>mV</td>
<td>millivolts</td>
</tr>
<tr>
<td>O.D.</td>
<td>optical density</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>TZD</td>
<td>thiazolidinedione</td>
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Abstract

Iron-sulfur proteins are ubiquitous in nature, and they play a crucial role in multifarious biological processes such as electron transfer, enzymatic catalysis, and homeostatic regulation. MitoNEET is the first discovered iron-sulfur protein in the outer mitochondrial membrane. Previous studies have shown that the [2Fe-2S] clusters of mitoNEET can be reduced by FMNH$_2$ in the presence of NADH and Flavin reductase (Fre) under aerobic conditions suggesting that mitoNEET is novel redox enzyme catalyzing electron transfer from NADH via FMN to oxygen.

The molecular docking models suggest that mitoNEET has a specific binding site for FMN close to its [2Fe-2S] cluster in each monomer. Since the closest distance between the bound FMN and the [2Fe-2S] cluster is approximately 10 Å, it may promote rapid electron transfer from FMNH$_2$ to the [2Fe-2S] clusters. In the first part of this work, we have explored the FMN binding site in mitoNEET utilizing two FMN analogs: lumiflavin and lumichrome. We found that Lumiflavin, as FMN, can reduce the mitoNEET [2Fe-2S] clusters in the presence of NADH and Fre under aerobic conditions. Additionally, lumiflavin can change EPR spectrum of the reduced mitoNEET [2Fe-2S] clusters and under blue light exposure form a covalently bound complex with mitoNEET. Lumichrome, instead, inhibits FMNH$_2$ mediated electron transfer activity indicating lumichrome’s potential as an antagonist of electron transfer activity of mitoNEET.

Ubiquinone-10 is a unique electron carrier found in both inner and outer mitochondrial membrane. Ubiquinone-10 accepts electrons from NADH and FADH$_2$ of several different redox pathways. In the second part of this work, we investigated the oxidation kinetics of the reduced [2Fe-2S] clusters of mitoNEET with a fusion protein, YneM-mitoNEET in nanodisc (To mimic function of native
mitoNEET in biological membrane) by ubiquinone-10 under anerobic conditions. Our result shows that under anerobic conditions, ubiquinone-10 oxidizes the reduced [2Fe-2S] clusters of YneM-mitoNEET in nanodisc. The results indicate that ubiquinone-10 may be an intrinsic oxidant for the reduced [2Fe-2S] clusters of the native mitochondrial outer membrane protein mitoNEET (containing both membrane and soluble portion) and that native mitoNEET act as a redox enzyme that catalyzes electron transfer from FMNH₂ to ubiquinone-10 in mitochondria.
1.1. Iron-sulfur proteins: diverse and versatile

Iron-sulfur clusters are one of the ancient co-factors and ubiquitous electron carriers involved in many fundamental and diverse biochemical processes[1]. Proteins containing iron-sulfur clusters have been found in all three domains of life, including bacteria, archaea, and eukaryotes[2]. In addition, many iron-sulfur cluster containing proteins are conserved throughout evolution and participate in numerous critical functions such as respiration, photosynthesis, nitrogen fixation, energy metabolism, DNA repair, and gene expression regulation[1, 3].

![Figure 1.1. Structure and functions of iron-sulfur proteins[4].](image)

The unique chemical properties of iron and sulfur make them one of the most versatile enzymatic co-factors. Iron-sulfur clusters in proteins can be present in many different combinations of iron and sulfur. However, among several kinds of iron-sulfur clusters most common forms include [2Fe-2S] and [4Fe-4S] (Figure1.1). The first type, designated as [2Fe-2S], contains two iron ions,
two inorganic sulfides, and the second kind denoted by [4Fe-4S] contains four iron ions and four inorganic sulfides. In most cases, the coordinating residue for these clusters is cysteine residue, although more rarely, His, Arg, Asp, and Thr may act as ligand coordinating iron atoms[5, 6]. Besides these large number of iron-sulfur cluster combination, conversion between clusters and in the same cluster have also been reported[7-10].

1.2. Electron transfer: Primary function of iron-sulfur proteins

Iron-sulfur proteins participate in a wide array of biological processes[11]. In most iron-sulfur proteins the main function of the iron-sulfur cluster is to serve as electron transfer groups in one-electron redox processes[12]. The redox potential of iron-sulfur clusters in these proteins ranging from -500mV to +300mV also suggest/indicate their potential role as electron donors/acceptors in fundamental biochemical reactions involving electron transfer[13]. Iron ions of these iron-sulfur clusters alternate between a reduced ferrous state (+2) and an oxidized ferric state (+3) during electron transfer reactions[14]. These properties make the iron-sulfur cluster containing proteins an indispensable part of respiratory and photosynthetic electron-transfer chain[12]. A recently discovered iron-sulfur cluster containing protein, mitoNEET also demonstrated similar electron transfer property. Previous studies reported that redox active iron-sulfur clusters of mitoNEET can catalyze transfer of electron from NADH via FMNH₂ to oxygen or ubiquinone-2, which is an analog of ubiquinone-10[15].

1.3. NEET family: a novel family of iron-sulfur proteins

NEET family comprises a evolutionary conserved group of iron-sulfur proteins that was discovered last decade across all kingdom of life including archaea, bacteria, algae, plant and human[16]. In humans, NEET family consists of three distinct proteins which can be divided in
two different classes (Figure 1.2): 1) NEET Proteins containing one CDGSH domain in each monomer: includes mitoNEET and nutrient deprivation autophagy factor-1 (NAF-1), membrane proteins (*CISD1* and *CISD2* genes encode mitoNEET and NAF-1 respectively), and 2) NEET proteins containing two CDGSH domains in each monomer: mitochondrial inner NEET (MiNT) protein, soluble protein (*CISD3* gene encode MiNT)[16]. MitoNEET and NAF-1 both are present in the outer mitochondrial membrane[17]. MiNT, on the contrary is located in the mitochondrial matrix[18].

![Class I: mNT, NAF-1, At-NEET](image1.png)

![Class II: MiNT](image2.png)

**Figure 1.2. NEET family of proteins[2]**

### 1.4. MitoNEET: electron transfer and beyond

Recent genetic and biochemical analysis revealed that mitochondrial outer membrane protein mitoNEET is plays a critical role in energy metabolism in mitochondria via oxidative phosphorylation for ATP synthesis[16] and beta oxidation of lipid[19]. MitoNEET is also proposed to play an important role in regulation of iron and maintaining ROS balance by possible
transfer of iron-sulfur clusters to potential acceptor proteins including anamorsin and iron regulatory protein 1 (IRP-1)[20, 21].

Disruption of expression of mitoNEET showed increased accumulation of mitochondrial labile iron levels[16]. Increase in mitochondrial iron causes increased generation of ROS and other free radicals[22, 23] and thus results in imbalance of mitochondrial dynamic and causes disturbances in the balance between mitochondrial fission and fusion[24]. On the other hand, decrease in expression of mitoNEET causes abnormalities in mitochondrial membrane potential[16, 25, 26] and loss of function of mitoNEET results in formation of intra mitochondrial junction and abnormalities in network contacts[27]. Recent studies revealed that there may be a redox-sensitive binding interaction between mitoNEET and voltage dependent anion channel1(VDAC1)[28].

1.5. Properties of Flavin mononucleotide (FMN)

Flavin mononucleotide (FMN) is a co-enzyme form of riboflavin and produced because of phosphorylation of riboflavin by riboflavin kinase[29]. FMN is a component of respiratory complex 1 in mitochondria[30].

![Diagram of FMN synthesis from Riboflavin](image-url)

Figure 1.3. FMN synthesis from Riboflavin[33].
The flavin cofactor can be present in aqueous solution in three different redox states: the oxidized state (FMN), the semiquinone state-one-electron reduced state (FMNH\textsuperscript{•}), or the fully reduced state(FMNH\textsubscript{2})[31]. In biological system one electron oxidation or reduction of FMN is observed when one electron at a time is transferred to Fe-S centers whereas two electron reduction is observed when accepting electrons from NADH in complex 1[32].

1.6. Ubiquinone-10: structure and function

Ubiquinone-10 also known as Coenzyme Q is a unique lipid cofactor of the electron transport chain.

![Redox forms of ubiquinone-10](image)

Figure 1.4. Redox forms of ubiquinone-10. Oxidized form of Ubiquinone-10 (ubiquinone) can be reduced to ubiquinol (CoQH\textsubscript{2}) in a two-step reaction in which one electron is transferred in each step through semiquinone form, or by a one-step reaction where two electrons are transferred, without formation of semiquinone intermediate[35].
It participates in many redox reactions involved in bioenergetics, and antioxidant activity. Structurally, ubiquinone-10 consists of a benzoquinone ring attached to a species specific polyisoprenoid side chain[34]. For example, human isoform of ubiquinone has 10 isoprene units, whereas E. coli has eight isoprene units[35]. The reduced form of Ubiquinone-10 is ubiquinol (upon receiving two electrons) and the oxidized form is ubiquinone. Another intermediate form, semiquinone is observed in respiratory chain when only one electron is accepted by ubiquinone-10 (Figure 1.4).

1.7. MitoNEET: key players in health and disease

Diabetes is declared a worldwide epidemic with a major public health challenge[36]. It is also one of the most frequent causes of morbidity and mortality worldwide[37]. Diabetes can be defined as a group of metabolic disorders determined by hyperglycemia resulting from defective insulin secretion, insulin sensitivity or both[38]. Type 2 diabetes is caused by decreased sensitivity to insulin which results in abnormal metabolism and obesity. Several factors such as oxidative stress and imbalance in iron homeostasis are implicated in the development and advancement of diabetes[36, 39].

The outer mitochondrial membrane protein, mitoNEET has been associated with iron, calcium, and ROS homeostasis in mitochondria. As mitoNEET was first discovered as a target of type-2 anti-diabetic drug Thiazolidinedione (TZD)[40], it has garnered interest of scientists because of its association with diabetes. Pioglitazone, a protein belonging to the Thiazolidinedione family is shown to improve both sensitivity of insulin and metabolic control of glucose [40]. Additionally, [41, 42]Pioglitazone is a direct activator of peroxisome proliferator activated receptor-gamma (PPARγ), which is a nuclear receptor present in differentiating adipocyte[43, 44]. PPARγ can also bind to many different fatty acid intermediates/metabolites and thus can act as a global lipid
sensor[45]. Upon activation, PPARγ can regulate expression of gene responsible for insulin sensitivity and fatty acid and glucose homeostasis[46]. Pioglitazone exerts its antidiabetic effect by binding to PPARγ and causing secretion of insulin sensitizers from adipose cells[46]. However, pioglitazone also showed alarming side effects such as hepatic damage, cardiac failure and probably cancer due to its PPAR binding activity[47, 48]. Since pioglitazone can bind to mitoNEET, it can also be speculated that the antidiabetic effects of pioglitazone are a result of direct interaction of pioglitazone and mitoNEET. The exact function of mitoNEET in vivo is however not clear.

Obesity is a multifactorial disease connected with type 2 diabetes mellitus and metabolic disease[49]. It is identified by an increased free fatty acid level, and deposition of triacylglycerol, resulting in increase in lipotoxicity, oxidative stress and oxidative phosphorylation, which is linked to diabetes[50]. Mitochondria manages energy production by beta oxidation of fat or storage of fat in adipose tissue. It is crucial that mitochondrial energy production is coordinated with TCA cycle and ETS as failure to control this mechanism is shown to be associated with type 2 diabetes and obesity. In ob/ob mice model, which induces adipose tissue expansion and as a result an obesity phenotype, expression of mitoNEET was shown to be downregulated[51]. Similarly, expression of mitoNEET was also diminished in both subcutaneous (SAT) and visceral (VAT) adipose tissues of obese human patients [52]. In morbidly obese patients, expression of mitoNEET shows a positive correlation with sensitivity of insulin and expression of Sirtuin 1 protein in both adipose tissue type[52]. Diminished expression of SIRT1 is linked to the development of obesity phenotype and metabolic disorders[53].

Development of obesity and associated metabolic diseases is connected with the level of mitoNEET expression and insulin resistance in type 2 diabetes is linked with development of
obesity and is dependent on mitoNEET expression level in both mice models and humans. It can therefore be speculated that mitoNEET can affect mitochondrial dysfunction related metabolic diseases such as obesity and type 2 diabetes. Hence, mitoNEET may be a novel target for development of drug to treat metabolic diseases along with obesity and type 2 diabetes[54].

While mitoNEET deleted mice shows significant reduction of (about 30%) oxidative phosphorylation capacity in mitochondria[55], overexpression of mitoNEET increases lipid storage and reduces oxidative damage in adipocyte[51, 56]. Additionally, overexpression of mitoNEET in mice was also shown to inhibit ferroptosis in human hepatocellular carcinoma cells[57]. In β cells of pancreas, when mitoNEET is overexpressed, it results in increased blood glucose level and glucose intolerance[58]. In spinal neuron cells, microRNA targeting the expression of mitoNEET encoded by CISD1 and resulting depletion of mitoNEET led to apoptosis and neuronal loss[59]. Breast cancer cells show over-expression of mitoNEET and depletion of mitoNEET leads to inhibition of proliferation of cancer cell[17]. Moreover, altered expression of mitoNEET has also been implicated in other pathological condition such as the development of neurodegenerative disease[60] and cardiovascular disease[61], and browning of white adipose tissue[56]. Therefore, it has been proposed that mitoNEET has a central role in regulation of energy metabolism and thus, it has become an attractive drug target for scientists to treat mitochondria-related human disease.

1.8. Statement of research objective

The mitochondrial outer membrane protein mitoNEET plays a critical role in modulating energy metabolism, iron homeostasis, and the production of reactive oxygen species in mitochondria [1,2]. Its unique location, redox-active[2Fe-2S] clusters, and widespread physiological functions make it an ideal target for treating mitochondrial dysfunction-related diseases. The redox state of
the mitoNEET has been implicated for these physiologic functions; however, the interaction of other reducing/oxidizing agents with mitoNEET remains unclear. Previous studies reported that mitoNEET might act as an enzyme catalyzing electron transfer from cytosolic NADH via FMN to oxygen or ubiquinone in the mitochondrial outer membrane. The molecular docking model also suggested an FMN binding site in mitoNEET near (10Å) the [2Fe-2S] clusters promoting rapid electron transfer between them. This study aims to further investigate the FMN binding site utilizing two FMN analogs: Lumichrome and Lumiflavin.

The second aim of this work is to investigate the electron transfer activity of membrane bound mitoNEET using fusion protein YneM-mitoNEET in nanodisc to mimic the electron transfer kinetics of native mitoNEET in the biological membrane. The third aim of this work is to explore the oxidation kinetics of the reduced [2Fe-2S] clusters of YneM-mitoNEET in nanodisc by ubiquinone-10 under anerobic conditions.
CHAPTER 2. EXPLORING THE FMN BINDING SITE IN THE MITOCHONDRIAL OUTER MEMBRANE PROTEIN MITONEET

2.1. Introduction

The mitochondrial outer membrane protein mitoNEET was named so because of its mitochondrial association and presence of amino acid sequence Asn-Glu-Glu-Thr (NEET) in the c terminal domain of the protein. It was initially identified as a binding target of the type II diabetes drug pioglitazone[40]. Since, it has been demonstrated that mitoNEET has an essential role in regulating energy metabolism, iron homeostasis, and production of reactive oxygen species in mitochondria [16, 25]. For example, in breast cancer cells, mitoNEET is highly expressed, and deletion of mitoNEET decreases proliferation of the cells [17]. In primary cultured spinal neurons, knockdown of mitoNEET leads to apoptosis [59]. In mice, mitoNEET is proposed to act as a mediator of Ca2+-facilitated mitochondrial dysfunction [62] and to regulate the function of the mitochondrial outer membrane protein voltage-dependent anion channel 1 (VDAC1) [28]. Mice with deletion of mitoNEET has signs of striatal mitochondrial dysfunction and a Parkinson's disease phenotype [63]. Because of its role in broad physiological functions, mitoNEET has become a novel target for treating diseases of mitochondrial dysfunction [60, 64-66].

Human mitoNEET is a homodimer with each monomer hosting a [2Fe-2S] cluster in its C-terminal cytosolic region [67-69]. The N terminus of mitoNEET contains a transmembrane alpha-helix domain that anchors mitoNEET to the mitochondrial outer membrane [40]. The specific function of the [2Fe-2S] clusters in mitoNEET, however, still remains largely elusive. Because mitochondria are the primary sites for iron-sulfur cluster biogenesis [70], it has been proposed that

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mitoNEET may act as a carrier that transfers iron-sulfur clusters assembled in mitochondria to target proteins in cytosol or nucleus [21, 71-74]. However, the observed cluster transfer occurs only when the mitoNEET [2Fe-2S] clusters are in an oxidized state [71, 72]. Since the mitoNEET [2Fe-2S] clusters are in a reduced state in cells under normal physiological conditions [75, 76], mitoNEET may help repair the damaged iron-sulfur clusters in proteins when cells are under oxidative stress conditions [71]. Alternatively, mitoNEET may contribute to the antioxidative stress activity [25, 77], as over-expression of mitoNEET prevents the oxidative damage in A375 melanoma cells [78] and in perivascular adipose tissue [79]. Regardless, the redox state of the [2Fe-2S] clusters have a crucial role for the proposed physiological functions of mitoNEET. The redox midpoint potential (Em7) of the mitoNEET [2Fe-2S] clusters has been determined to be about 0 mV at pH 7.0 [80]. However, the physiological components responsible for reducing or oxidizing the mitoNEET [2Fe-2S] clusters were not fully understood. In the previous studies, we found that the mitoNEET [2Fe-2S] clusters can be rapidly reduced by FMNH2 (but not NADH or NADPH) and oxidized by oxygen or ubiquinone [75, 81]. Because FMN can be reduced by flavin reductase using NADH as electron donor [82], we further proposed that mitoNEET may act as a redox enzyme catalyzing the electron transfer from NADH in cytosol to oxygen or ubiquinone in the mitochondrial outer membrane [83]. Since oxidation of NADH in cytosol is linked to the glycolysis activity [84, 85], mitoNEET may indirectly regulate glycolysis by promoting oxidation of cytosolic NADH[75, 83].

The molecular docking models revealed that mitoNEET has a specific FMN binding site in the vicinity of the [2Fe-2S] cluster in each mitoNEET monomer [81]. The shortest distance between the [2Fe-2S] cluster and the bound FMN in mitoNEET is about 10 Å, which could facilitate rapid electron transfer from FMNH2 to the [2Fe-2S] cluster within mitoNEET. To further explore the
FMN binding site in mito-NEET, here we have utilized two FMN analogs: lumiflavin and lumichrome. Lumiflavin and lumichrome are the photolysis products of FMN and retain an alloxazine chromophore of FMN [86]. Our results show that lumiflavin, like FMN, can also mediate the reduction of the mitoNEET [2Fe-2S] clusters in the presence of flavin reductase and NADH under aerobic conditions. Furthermore, incubation with FMN or lumiflavin can dramatically change the electron paramagnetic resonance (EPR) spectrum of the reduced mitoNEET [2Fe-2S] cluster and form a covalently bound complex with mitoNEET under blue light exposure. In contrast, lumichrome fails to mediate the reduction of the mitoNEET [2Fe-2S] clusters in the presence of flavin reductase and NADH or form a covalently bound complex with mitoNEET under blue light exposure. Instead, lumichrome can effectively inhibit the FMNH2-mediated reduction of the mitoNEET [2Fe-2S] clusters, suggesting that lumichrome may compete with FMN for the FMN binding site in mitoNEET and inhibit the electron transfer activity of mitoNEET.

2.2. Materials and methods

Protein preparation

Human mitochondrial outer membrane protein mitoNEET33–108 (containing residues 33–108) encoded by CISD1 gene was prepared as described previously [87]. Human CISD2 gene encoding human Miner157-135 (or NAF-157-135) (containing residues 57–135), CISD3 gene encoding Miner242-134 (or MiNT42-134) (containing residues 42–134), and FDX2 gene encoding ferredoxin 261-186 (containing residues 61–186) were also synthesized (GenScript co.) and cloned into an expression plasmid pET28b+. Recombinant human proteins were purified as described for mitoNEET [34]. The E. coli flavin reductase (Fre) was prepared using an E. coli strain hosting an expression plasmid encoding Fre from the ASKA library [88]. 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. Purified proteins were dissolved in buffer containing NaCl (500 mM) and Tris (20 mM, pH 8.0) for the experiments. To estimate concentration of purified proteins, the extinction coefficients of 8.6, 7.5, 20.3, 7.8, 26.4 mM$^{-1}$ cm$^{-1}$ at 280 nm were used for mitoNEET, Miner1, Miner2, ferredoxin-2, and Fre, respectively.

**Reduction and oxidation of the mitoNEET [2Fe-2S] clusters**

The redox state of the mitoNEET [2Fe-2S] clusters was monitored using a Beckman DU640 UV–visible absorption spectrometer equipped with a temperature controller. The oxidized mitoNEET [2Fe-2S] clusters have major absorption peaks at 458 nm and 540 nm. When the oxidized mitoNEET [2Fe-2S] clusters are reduced, the absorption peak at 458 nm is shifted to a much small absorption peak at 420 nm, while the absorption peak at 540 nm is not significantly changed[81]. Thus, we used the amplitude of the absorption peak at 458 nm to monitor the redox state of the mitoNEET [2Fe-2S] clusters in the reaction solution.

**Blue light exposure**

An LED blue light source (Submersible LED light blue) was purchased from petSolutions.com. Samples in glass vials were exposed to blue light at room temperature. The distance between the sample and the blue light source was about 5 cm to minimize heating from the light source. A Digital Lux Meter (LX1330B, Dr. Meter) was used to measure the intensity of the blue light exposure.

**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, 1.0 mW; modulation frequency, 100 kHz; modulation amplitude, 1.2 mT; temperature, 20 K; receiver gain, $2 \times 10^5$.

**Fluorescence measurements**

A spectrofluorometer (FP-6300, JASCO Co., Japan) was used for the fluorescence spectrum measurements of FMN covalently bound in mitoNEET. The emission spectra of FMN were taken at room temperature using an excitation wavelength of 450 nm. The amount of FMN in the samples was quantified as described in Ref. [89].

**Chemicals**

NADH, isopropyl-β-D-1-thiogalactopyranoside, kanamycin, and ampicillin were purchased from Research Product International co. FMN (flavin mononucleotide), lumiflavin, lumichrome, and other chemicals were purchased from Sigma co. The concentrations of NADH and FMN were determined using extinction coefficients of 6.2 mM-1cm-1 at 340 nm and 12.5 mM-1cm-1 at 445 nm, respectively [90].

**2.3. Results**

**Kinetics of the FMN-mediated reduction of the mitoNEET [2Fe-2S] clusters under aerobic conditions**

Previous studies have shown that the mitoNEET [2Fe-2S] clusters can be reduced by FMNH2 under aerobic conditions[75, 81, 83]. Since the cytosolic concentration of FMN in typical mammalian cells is in the nanomolar range [90], we decided to re-evaluate the reduction kinetics of the mitoNEET [2Fe-2S] clusters at different concentrations of FMN in the presence of NADH and flavin reductase under aerobic conditions. In the experiments, purified human mitoNEET
(containing 10 μM [2Fe-2S] clusters) was pre-incubated with NADH (100 μM) and FMN in buffer containing NaCl (500 mM) and Tris (20 mM, pH 8.0). The reduction of the mitoNEET [2Fe-2S] clusters was initiated by adding a catalytic amount of flavin reductase (Fre) (at a final concentration of 0.25 μM) and monitored by continuously taking the UV–visible absorption spectra of the mitoNEET [2Fe-2S] clusters.

Figure 2.1. Electron transfer activity of mitoNEET at different concentrations of FMN under aerobic conditions. A), reduction and oxidation of the mitoNEET [2Fe-2S] clusters. MitoNEET (containing 10 μM [2Fe-2S] clusters) was incubated with NADH (100 μM) and FMN (50 nM) in buffer containing NaCl (500 mM) and Tris (20 mM, pH 8.0) under aerobic conditions. The reaction was initiated by adding flavin reductase (Fre) (at a final concentration of 0.25 μM). The redox state of the mitoNEET [2Fe-2S] clusters in the reaction solutions was monitored by taking UV–visible absorption spectra every 2 min for 20 min. B), kinetics of reduction and oxidation of the mitoNEET [2Fe-2S] clusters. MitoNEET (containing 10 μM [2Fe-2S] clusters) was incubated with NADH (100 μM) and FMN (0–250 nM as indicated) in buffer containing NaCl (500 mM) and Tris (20 mM, pH 8.0) under aerobic conditions. The reaction was initiated by adding flavin reductase (Fre) (at a final concentration of 0.25 μM). The relative amounts of the oxidized mitoNEET [2Fe-2S] clusters were monitored at the absorption peak of 458 nm and plotted as a function of time after addition of flavin reductase. The data are representatives of three independent experiments.

Fig. 2.1A shows that the oxidized mitoNEET [2Fe-2S] clusters had a major absorption peak at 458 nm [91]. Upon addition of flavin reductase, the absorption peak at 458 nm of the mitoNEET [2Fe-
2S] cluster was quickly diminished and shifted to a much small absorption peak at 420 nm of the reduced mitoNEET [2Fe-2S] clusters. Under the experimental conditions, 50 nM of FMN was sufficient to reduce 10 μM of the mitoNEET [2Fe-2S] clusters within 3 min (Figure 2.1B). As the ratio of FMN to the mitoNEET [2Fe-2S] clusters in the reaction solution was about 1:200, the results suggest that FMN is highly efficient in mediating the reduction of the mitoNEET [2Fe-2S] clusters. Importantly, once the NADH in the reaction solution was fully oxidized (disappearance of the absorption peak at 340 nm), the reduced mitoNEET [2Fe-2S] clusters were re-oxidized (re-appearance of the absorption peak at 458 nm) by oxygen in the solution (Fig. 2.1A), indicating that the FMNH2-mediated reduction of the mitoNEET [2Fe-2S] clusters is reversible as described previously [75, 81, 83].

To further explore the kinetics of the redox transition of the mitoNEET [2Fe-2S] clusters, we carried out the same experiments using different concentrations of FMN. Figure 2.1B shows the redox transition of the mitoNEET [2Fe-2S] clusters at different concentrations of FMN (0–250 nM) in the presence of NADH (100 μM) and flavin reductase under aerobic conditions. Without addition of FMN, there was very little reduction of the mitoNEET [2Fe-2S] clusters. When the concentration of FMN increased from 0 to 250 nM, the reduction rate of the mitoNEET [2Fe-2S] clusters quickly increased. At 250 nM of FMN, the mitoNEET [2Fe-2S] clusters were reduced in less than 1 min and fully re-oxidized in 4 min (Figure 2.1B). Thus, FMN not only mediates the reduction of the mitoNEET [2Fe-2S] clusters, but also promotes the reoxidation of the reduced mitoNEET [2Fe-2S] clusters under aerobic conditions.

**The FMN analog lumiflavin has a similar activity as FMN in mediating redox transition of the mitoNEET [2Fe-2S] clusters under aerobic conditions**

Previous molecular docking models indicated that human mitoNEET has a distinct FMN binding site in the vicinity of the [2Fe-2S] cluster in each monomeric mitoNEET [75, 81, 83]. To further
explore the FMN binding site in mitoNEET, we have used two FMN analogs: lumiflavin and lumichrome (Figure 2.2A). Both lumiflavin and lumichrome are the photolysis products of FMN and retain the alloxazine chromophore of FMN [33]. In the experiments, purified human mitoNEET (containing 10 μM [2Fe-2S] clusters) was pre-incubated with NADH and one of the FMN analogs. The reduction of the mitoNEET [2Fe-2S] clusters was initiated by adding a catalytic amount of flavin reductase and monitored by taking UV–visible absorption spectra of the mitoNEET [2Fe-2S] clusters.

Figure 2.2. Lumiflavin can substitute FMN in mediating the redox transition of the mitoNEET [2Fe-2S] clusters under aerobic conditions. A), structure of FMN, lumiflavin, and lumichrome. B), reduction and oxidation of the mitoNEET [2Fe-2S] clusters mediated by FMN, lumiflavin, and lumichrome. MitoNEET (containing 10 μM [2Fe-2S] clusters) was incubated with NADH (100 μM), and FMN (250 nM) in buffer containing NaCl (50 mM) and Tris (20 mM, pH 8.0) under
aerobic conditions. The reaction was initiated by adding flavin reductase (Fre) (at a final concentration of 0.25 μM). C), same as B), except FMN was replaced with lumiflavin (250 nM). D), same as B), except FMN was replaced with lumichrome (250 nM). The data are representatives of three independent experiments.

Figure 2.2C shows that lumiflavin had essentially the same activity as FMN (Figure 2.2B) in mediating the reduction and oxidation of the mito-NEET [2Fe-2S] clusters under aerobic conditions. In contrast, lumichrome had no such an activity under the experimental conditions (Figure 2.2D). We also used the electron paramagnetic resonance (EPR) spectroscopy to verify the redox state of the mitoNEET [2Fe-2S] clusters after incubation. While the oxidized mitoNEET [2Fe-2S] clusters are diamagnetic and EPR silent, the reduced mitoNEET [2Fe-2S] clusters are paramagnetic and have a distinct EPR signal at gav = 1.94 [81, 92].

The EPR measurements confirmed that the mitoNEET [2Fe-2S] clusters were reduced after incubation with FMN or lumiflavin in the presence of NADH and flavin reductase under aerobic conditions, but not with lumichrome (data not shown). Thus, lumiflavin, like FMN, can efficiently mediate the reduction and oxidation of the mitoNEET [2Fe-2S] clusters in the presence of NADH and flavin reductase under aerobic conditions.

**FMN forms a covalently bound complex with mitoNEET under blue light exposure**

Because flavin can be excited by blue light (at around 450 nm) [93], it has been reported that blue light excitation may result in formation of a covalently bound protein-FMN complex in FMN binding proteins [94, 95]. If mitoNEET binds FMN as predicted in the molecular docking models [81], we would expect that blue light excitation may also promote formation of a covalently bound complex between FMN and mitoNEET. In the experiments, mitoNEET was incubated with five-fold excess of FMN under blue light exposure (50,000 Lux) at room temperature for 10 min, followed by adding freshly prepared sodium dithionite to reduce the mitoNEET [2Fe-2S] clusters.
The EPR measurements showed that the reduced mitoNEET [2Fe-2S] clusters had a new EPR signal at $g = 1.85$ after incubation with FMN (Figure 2.3, spectrum 2). A similar but small EPR signal at $g = 1.85$ was previously observed when the mitoNEET [2Fe-2S] clusters were incubated with FMN under room light for 30 min [81]. We postulated that blue light exposure may have generated more light-excited FMN for the interaction with the mitoNEET [2Fe-2S] clusters than room light exposure. Importantly, same EPR signal at $g = 1.85$ was also observed when mitoNEET was incubated with five-fold excess of lumiflavin under blue light exposure (Figure 2.3, spectrum 3), but not with lumichrome (Figure 2.3, spectrum 4). While the nature of the unusual EPR signal at $g = 1.85$ of the reduced mitoNEET [2Fe-2S] clusters is not fully understood, the results strongly suggest that FMN and lumiflavin, but not lumichrome, have distinct interactions with the mitoNEET [2Fe-2S] clusters.

![EPR spectra](image)

Figure 2.3. Effect of FMN analogs on the EPR spectrum of the mitoNEET [2Fe-2S] clusters. MitoNEET (containing 10 μM [2Fe-2S] clusters) (spectrum 1) was incubated with 50 μM of FMN (spectrum 2), 50 μM of lumiflavin (spectrum 3), or 50 μM of lumichrome (spectrum 4) at room temperature for 10 min under blue light exposure (50,000 Lux). The samples were then reduced with freshly prepared sodium dithionite (10 mM), transferred to EPR tubes, and frozen.
immediately in liquid nitrogen until EPR measurements. The reduced mitoNEET [2Fe-2S] clusters have an average $g = 1.94$. The reduced mitoNEET [2Fe-2S] clusters after incubation with FMN or lumiflavin under blue light exposure have a new EPR signal at $g = 1.85$.

As blue light has been shown to induce dimerization in the FMN binding protein aureochrome-1 [96], we asked whether FMN could also promote dimerization of mitoNEET under blue light exposure.

Figure 2.4. MitoNEET forms a covalently bound complex with FMN under blue light exposure. A), the SDS-PAGE analysis of mitoNEET after incubation with different concentrations of FMN under blue light exposure. MitoNEET (containing 10 μM [2Fe-2S] clusters) was incubated with FMN (0–40 μM) under blue light exposure (50,000 Lux) for 10 min. Aliquots were taken from the samples for the SDS-PAGE analysis. Lane 1, mitoNEET. Lanes 2–8, mitoNEET incubated with 0, 1, 2, 5, 10, 20, 40 μM of FMN under blue light exposure for 10 min, respectively. Lane 9, MitoNEET incubated with FMN (40 μM) for 10 min without blue light exposure. On right side of the gel, M, monomeric mitoNEET; D, dimeric mitoNEET. B), UV–visible absorption spectra of mitoNEET after incubation with FMN with or without blue light exposure. MitoNEET protein was re-purified from the incubation solutions by
passing through a Desalting Column. Re-purified mitoNEET samples were subjected to the UV–visible absorption measurements. C), quantification of FMN in the re-purified mitoNEET proteins. Re-purified mitoNEET proteins (5 μM) treated with or without 0.2% SDS were subjected to the fluorescence measurements in a spectrofluorometer (FP-6300, JASCO Co., Japan). The emission spectra of FMN were taken upon excitation at 450 nm. Spectrum 1, re-purified mitoNEET after incubation with FMN under blue light exposure was treated with 0.2% SDS. Spectrum 2, re-purified mitoNEET after incubation with FMN under blue light exposure. Spectrum 3, mitoNEET (not incubated with FMN) was treated with 0.2% SDS. Spectrum 4, mitoNEET (control). Based on the fluorescence intensity of FMN in the mitoNEET incubated with FMN under blue light exposure and treated with 0.2% SDS, we estimated that there was at least 0.2 FMN per mitoNEET monomer (n = 3).

After mitoNEET was incubated with different concentrations of FMN under blue light exposure for 10 min, the samples were subjected to the SDSPAGE analyses. Figure 2.4A shows that mitoNEET indeed became a covalently bound homodimer after incubation with FMN under blue light exposure. The observed mitoNEET dimer (D) could not be separated to monomers by incubation with 10 mM dithiothreitol, indicating that the mitoNEET dimer was not formed via disulfide. Thus, mitoNEET most likely became a covalently bound homodimer after incubation with FMN under blue light exposure.

After incubation with FMN under blue light exposure, mitoNEET was re-purified from the incubation solutions by passing the sample through a High-Trap Desalting column to remove unbound FMN. Repurified mitoNEET samples were then subjected to the UV–visible absorption measurements.

Figure 2.4B shows that incubation of mitoNEET with FMN under blue light exposure resulted in a major absorption peak at 250 nm (spectrum 1). Without FMN or blue light exposure, no absorption change of mitoNEET was observed (spectra 2, 3, 4). The repurified mitoNEET was further subjected to analysis of FMN in the protein using the fluorescence spectrometry as described in Ref. [89]. Upon excitation at 450 nm, FMN has a fluorescence peak at around 520
Fig. 2.4C shows that while the re-purified mitoNEET after incubation without FMN under blue light exposure did not have any fluorescence peaks at 520 nm (Figure 2.4, spectrum 3), the re-purified mitoNEET after incubation with FMN under blue light exposure had a major peak of fluorescence at 520 nm (Figure 2.4C, spectrum 2). Addition of 0.2% SDS to denature mitoNEET further enhanced the fluorescence intensity of FMN in the mitoNEET sample incubated with FMN under blue light exposure (Figure 2.4C, spectrum 1). Using the FMN solution as a standard, we estimated that at least 0.2 mol of FMN were present per each mole of the mitoNEET dimer after incubation with five-fold excess of FMN under blue light exposure for 10 min. Similar results were also observed when mitoNEET was incubated with lumiflavin, but not with lumichrome (data not shown). Thus, FMN can not only change the EPR spectrum of the reduced mitoNEET [2Fe-2S] clusters (Figure 2.3), but also form a covalently bound complex with mitoNEET (Figure 2.4) under blue light exposure.

The interaction between FMN and the mitoNEET [2Fe-2S] clusters is unique

The observed EPR signal at $g =1.85$ of the reduced mitoNEET [2Fe-2S] clusters after incubation with FMN under blue light exposure was not previously reported in any iron-sulfur proteins. It would be of interest to examine whether FMN could also change the EPR spectrum of the [2Fe-2S] clusters in other proteins under blue light exposure.

To test this idea, we prepared three human mitochondrial proteins that contain [2Fe-2S] clusters: the mitoNEET-related protein 1 (Miner1 or NAF-1) [97, 98], the mitoNEET-related protein 2 (Miner2 or MiNT) [18, 99], and the mitochondrial ferredoxin 2 [100]. Miner1 is a homolog of mitoNEET with 54% identity and 69% similarity [97], and localizes in the mitochondrial outer membrane and endoplasmic reticulum membrane [98]. Miner2 is a mitochondrial matrix protein with no sequence similarities to mitoNEET or Miner1 but has the same ligands (three cysteine and
one histidine residues) as mitoNEET for hosting the [2Fe-2S] cluster [18, 99]. Mitochondrial ferredoxin 2 is a key member of the iron-sulfur cluster biogenesis machinery in mitochondria [100], and has no sequence similarities to mitoNEET or Miner2. Three human mitochondrial proteins (Miner1, Miner2 and ferredoxin 2) were expressed in E. coli cells and purified as mitoNEET. Each of purified proteins contained one or two [2Fe-2S] clusters and had their distinct EPR spectra (Figure 2.5A). Each purified protein was then incubated with FMN under blue light exposure for 10 min, followed by reduction with freshly prepared sodium dithionite. The samples were transferred to EPR tubes, frozen immediately in liquid nitrogen until the EPR measurements. Figure 2.5B shows that after incubation with FMN under blue light exposure, the reduced Miner1 [2Fe-2S] clusters had the same EPR signal at g = 1.85 as the reduced mitoNEET [2Fe-2S] clusters (spectra 5 and 6), indicating that the mitoNEET homolog Miner1 has the same interactions with FMN as mitoNEET.

In contrast, incubation with FMN resulted in no EPR signal of the reduced Miner2 [2Fe-2S] clusters (Figure 2.5, spectrum 7) or the reduced ferredoxin 2 [2Fe-2S] clusters (Figure 2.5, spectrum 8) under blue light exposure, likely due to the disruption of the [2Fe-2S] clusters in the proteins by excited FMN or other reactive free radicals under blue light exposure. Thus, not all [2Fe-2S] cluster proteins will have the unique EPR signal at g = 1.85 after incubation with FMN under blue light exposure, and the interaction between mitoNEET and FMN is unique.
Figure 2.5. Effect of FMN on other [2Fe-2S] proteins from human mitochondria. Three human iron-sulfur proteins (Miner1, Miner2 and ferredoxin-2) were prepared and incubated with FMN under blue light exposure (50,000 Lux) for 10 min. A), purified proteins (each containing 10 μM [2Fe-2S] clusters) were reduced with freshly prepared sodium dithionite (4 mM) without incubation with FMN. Spectrum 1, mitoNEET; spectrum 2, Miner1; spectrum 3, Miner2; Spectrum 4, ferredoxin-2. B), purified protein (containing 10 μM [2Fe-2S] clusters) was incubated with FMN (50 μM) under blue light exposure for 10 min, followed by addition of sodium dithionite (4 mM). Spectrum 5, mitoNEET; spectrum 6, Miner1; spectrum 7, Miner2; Spectrum 8, ferredoxin-2. The data are representatives of three independent experiments.

Lumichrome inhibits the FMNH2-mediated reduction of the mitoNEET [2Fe-2S] clusters

Unlike FMN or lumiflavin, lumichrome fails to mediate the reduction of the mitoNEET [2Fe-2S] clusters in the presence of NADH and flavin reductase under aerobic conditions (Figure 2.2D) and has no effect on the EPR spectrum of the reduced mitoNEET [2Fe-2S] clusters under blue light exposure (Figure 2.3, spectrum 4). Since lumichrome has an isoalloxazine group as FMN, we
reasoned that lumichrome may compete with FMN for the FMN binding site in mitoNEET and inhibit the electron transfer activity of mitoNEET.

To test the idea, mitoNEET (containing 10 μM [2Fe-2S] clusters) was pre-incubated with FMN (50 nM) and various concentrations of lumichrome (0–250 nM) in the presence of NADH (100

Figure 2.6. Inhibition of the electron transfer activity of mitoNEET by lumichrome. A), reduction and oxidation of the mitoNEET [2Fe-2S] clusters without lumichrome. Purified mitoNEET (containing 10 μM [2Fe-2S] clusters) was incubated with NADH (100 μM), and FMN (50 nM) in buffer containing NaCl (500 mM) and Tris (20 mM, pH 8.0) under aerobic conditions. The reaction was initiated by adding flavin reductase (Fre) (at a final concentration of 0.25 μM). The redox state of the mitoNEET [2Fe-2S] clusters was monitored by taking UV–visible absorption spectra every 2 min after addition of flavin reductase. B), same as in A), except 50 nM of lumichrome was included in the incubation solution before the reaction was initiated. C), same as in A), except 100 nM of lumichrome was included in the incubation solution before the reaction was initiated. D), same as in A, except 250 nM of lumichrome was included in the incubation solution before the reaction was initiated. The data are representatives of three independent experiments.
μM) under aerobic conditions for 10 min. The reduction of the mitoNEET [2Fe-2S] clusters in the solutions was initiated by adding a catalytic amount of flavin reductase (0.25 μM) as described previously. Figure 2.6 shows that as the concentration of lumichrome increased in the pre-incubation solutions, the FMN-mediated reduction of the mitoNEET [2Fe-2S] clusters gradually decreased. Upon addition of 250 nM of lumichrome, about 50% of the FMNH2-mediated reduction of the mitoNEET [2Fe-2S] clusters was inhibited under the experimental conditions (Figure 2.6D), suggesting that lumichrome is a potent inhibitor to block the electron transfer activity of mitoNEET.

2.4. Discussion

MitoNEET is the first iron-sulfur protein identified in the mitochondrial outer membrane [40]. The unique location of mitoNEET and its redox active [2Fe-2S] clusters have attracted great attentions in the past decade. In the previous studies, we reported that the mitoNEET [2Fe-2S] clusters can be readily reduced by FMNH2 and oxidized by oxygen or ubiquinone [75, 81, 83], and suggested that mitoNEET may act as a novel redox enzyme to catalyze electron transfer from FMNH2 to oxygen or ubiquinone. To further explore the FMN binding site of mitoNEET, here we have utilized two FMN analogs, and found that lumiflavin can substitute for FMN in mediating the reduction of the mitoNEET [2Fe-2S] clusters in the presence of NADH and flavin reductase under aerobic conditions. Under blue light exposure, both FMN and lumiflavin can dramatically change the EPR spectrum of the reduced mitoNEET [2Fe-2S] clusters with an unusual EPR signal at g = 1.85 and form a covalently bound complex with mitoNEET. In contrast, lumichrome, which lacks a methyl group on isoalloxazine (Fig. 2.2A), fails to mediate the reduction of the mitoNEET [2Fe-2S] clusters or change the EPR spectrum of the reduced mitoNEET [2Fe-2S] clusters. Instead,
lumichrome can effectively inhibit the electron transfer activity of mitoNEET, likely by competing with FMN for the FMN binding site in mitoNEET.

In the crystal structure, mitoNEET exists as a homodimer with two mitoNEET monomers associated via a “beta cap” structure, bringing the two [2Fe-2S] clusters close to each other with a closest distance of about 13Å [67-69]. Molecular docking models further revealed a putative FMN binding site in mitoNEET which localizes at the region between the transmembrane α-helix domain and the [2Fe-2S] cluster binding domain [81]. The short distance between FMN and the [2Fe-2S] clusters in mitoNEET could facilitate the rapid electron transfer from FMNH2 to the [2Fe-2S] cluster [81]. Lumiflavin has the same isoalloxazine group as FMN (Figure 2.2A). Like FMN, lumiflavin has almost the same activity as FMN in mediating the reduction of the mitoNEET [2Fe-2S] clusters in the presence of NADH and flavin reductase under aerobic conditions (Figure 2.2C), changing the EPR spectrum of the reduced mitoNEET [2Fe-2S] clusters under blue light exposure (Figure 2.3, spectrum 3), and forming a covalently bound complex with mitoNEET (data not shown), suggesting that both FMN and lumiflavin have specific interactions with mitoNEET. On the other hand, lumichrome fails to mediate the reduction of the mitoNEET [2Fe-2S] clusters and has no effect on the EPR spectrum of the reduced mitoNEET [2Fe-2S] cluster after blue light exposure (Figure 2.2 and 2.3). Instead, lumichrome effectively inhibits the FMNH2-mediated reduction of the mitoNEET [2Fe-2S] clusters (Figure 2.6). The subtle difference between lumiflavin and lumichrome (a methyl group on isoalloxazine, Figure 2.2A) appears to have a critical role in determining their interactions with the mitoNEET [2Fe-2S] clusters.

In addition to mitoNEET, there are two mitoNEET-related proteins, the mitoNEET-related protein 1 (Miner1) or Nutrient-Deprivation Autophagy Factor-1 (NAF-1) [97, 98] and the mitoNEET-related protein 2 (Miner2 or MiNT) [18, 99, 101] in human mitochondria. Like mitoNEET [67-
69], both Miner1 and Miner2 host a [2Fe-2S] cluster via an unusual legend arrangement of three cysteine and one histidine residues. However, mitoNEET and Miner1 are homologous with 54% identity and 69% similarity [97]. Recent studies further indicated that mito-NEET and Miner1 may share similar physiological functions [17, 102], have specific interactions with the type II diabetes drug pioglitazone and other thiazolidinediones [103] and may even form a mitoNEETMiner1 dimer [104]. Mutations of Miner1 have been attributed to Wolfram Syndrome 2, a metabolic disease associated with diabetes, blindness, deafness, and a shortened lifespan [102, 105]. In consistent with these observations, here we have found that mitoNEET and Miner1 have similar interactions with FMN (Figure 2.5). On the other hand, Miner2, which does not have the sequence similarities to mitoNEET or Miner1 [18], does not have specific interactions with FMN under blue light exposure (Figure 2.5, spectrum 7). Likewise, human ferredoxin 2, which does not have any sequence similarities to mitoNEET or Miner1 [100], does not have interactions with FMN under blue light exposure (Figure 2.5, spectrum 8). Thus, mitoNEET and its homolog Miner1 may have a specific binding site for FMN to facilitate rapid reduction of their [2Fe-2S] clusters by FMNH2.

Figure 2.7. A proposed model for the electron transfer activity of mitoNEET and inhibition by lumichrome. In cytosol, NADH generated by glycolysis is oxidized by flavin reductase and FMN is reduced to FMNH2. FMNH2 interacts with mitoNEET via specific binding site and transfers its electrons to the [2Fe-2S] clusters of mitoNEET dimer which localizes on the mitochondrial outer membrane (MOM). MIM, mitochondrial inner membrane. The reduced [2Fe-2S] clusters in
mitoNEET dimer transfer the electrons to oxygen or ubiquinone. Lumichrome, which has an alloxazine group as FMN, may compete with FMN for the FMN binding site in mitoNEET and inhibit the electron transfer activity of mitoNEET.

Together with cytosolic flavin reductase which catalyzes reduction of FMN and oxidization of NADH [82], mitoNEET may effectively promote oxidation of NADH in cytosol with reduction of oxygen or ubiquinone (Figure 2.7) [83]. Because oxidation of NADH in cytosol is required for glycolysis activity [84, 85], mitoNEET on the mitochondrial outer membrane may indirectly regulate glycolysis via promoting oxidation of cytosolic NADH [75, 83]. This notion is consistent with the findings that deletion of mitoNEET decreases the oxidative phosphorylation activity [106], and overexpression of mitoNEET increases ATP synthesis in cells [107]. It may be envisioned that inhibition of the electron transfer activity of mitoNEET will decrease oxidation of NADH in cytosol (and therefore glycolysis) and overall energy metabolism in cells. In previous studies, we reported that the type II diabetes drug pioglitazone can inhibit the electron transfer activity of mitoNEET[75, 81]. The docking modelling indicated that the pioglitazone binding site in mitoNEET [65] has a partial overlap with the FMN binding site [75], indicating that binding of pioglitazone may block the access of FMNH2 to reduce the [2Fe-2S] clusters in mitoNEET. In this context, we propose that the FMN analog lumichrome, which has the same isoalloxazine group as FMN, may compete with FMN for the FMN binding site in mitoNEET, inhibit the electron transfer activity of mitoNEET (Figure 2.7), and modulate overall energy metabolism in cells.
CHAPTER 3: OXIDATION OF MITOCHONDRIAL OUTER MEMBRANE PROTEIN MITONEET [2FE-2S] CLUSTERS BY UBIQUINONE-10

3.1. Introduction

MitoNEET, the first identified iron-sulfur protein in the outer mitochondrial membrane, was discovered as a target of type 2 anti-diabetic drug pioglitazone[40, 55]. Since then, mitoNEET has been implicated in many pathophysiological conditions[27]. For example, in breast cancer cells, mitoNEET is over-expressed and deletion of mitoNEET results in decreased proliferation of cells[17, 108, 109]. In spinal neurons, microRNA targeting mitoNEET expression causes depletion of mitoNEET and leads to neuronal loss and apoptosis[59]. In beta cells of pancreas, overexpression of mitoNEET results in hyperglycemia and glucose intolerance[58]. In mice, overexpression of mitoNEET increases lipid storage and reduces oxidative damage in adipocyte[51]. On the contrary, mitoNEET deleted mice shows a decrease in oxidative phosphorylation capacity in mitochondria[106] and signs of striatal mitochondrial dysfunction and a Parkinson’s disease phenotype[110]. MitoNEET has also been proposed to regulate the function of voltage-dependent anion channel 1 (VDAC1) located in the outer membrane of mitochondria [28]. In this context, therefore, it has been postulated that mitoNEET may have a regulatory role in energy metabolism, iron homeostasis, and generation of reactive oxygen species[16]. Because of the unique location of mitoNEET in the outer membrane of mitochondria and its broad physiological functions, mitoNEET has attracted the attention of the scientists for treating mitochondrial dysfunction related diseases[60, 64-66].

Structurally, mitoNEET is a homodimer with each monomer containing a [2Fe-2S] cluster in its C-terminal cytosolic region and a N-terminal trans-membrane α-helical region which anchors the protein in the mitochondrial outer membrane[40, 67-69]. However, the specific functions of the
[2Fe-2S] clusters are not clear yet. For the proposed physiological functions of mitoNEET, the redox state plays a crucial role. The midpoint redox potential of mitoNEET is 0 mV (at pH 7), and the cytosolic redox potential is ~325 mV (pH 7), hence, the mitoNEET [2Fe-2S] clusters are in reduced state in cell in physiological condition. Nevertheless, the physiological components reducing or oxidizing mitoNEET [2Fe-2S] clusters were not fully understood. Previous studies have reported that mitoNEET\textsubscript{33-108} [2Fe-2S] clusters (containing amino acid residue from 33-108) can be reduced by reduced FMN and oxidized by oxygen or a ubiquinone analog, ubiquinone-2[81]. Here, we investigated the electron transfer activity of membrane bound mitoNEET using fusion protein YneM-mitoNEET in nanodisc to study the electron transfer kinetics of native mitoNEET in the biological membrane. We find that YneM-mitoNEET in nanodisc can be rapidly reduced by FMNH\textsubscript{2}, in presence of catalytic amount of Flavin reductase and NADH under both aerobic and anerobic conditions. Here we also explored the oxidation kinetics of the reduced [2Fe-2S] clusters of YneM-mitoNEET in nanodisc by ubiquinone-10 under anerobic conditions. Our result shows that under anerobic conditions, ubiquinone-10 oxidizes the reduced [2Fe-2S] clusters of YneM-mitoNEET in nanodisc. The results suggest that ubiquinone-10 may act as an intrinsic oxidant for the reduced [2Fe-2S] clusters of the mitochondrial outer membrane bound YneM-mitoNEET and that mitochondrial outer membrane bound YneM-mitoNEET is a novel redox enzyme that catalyzes electron transfer from FMNH\textsubscript{2} to ubiquinone-10 in mitochondria.

3.2. Materials and methods

Protein preparation

Two DNA fragments, one encoding human mitoNEET\textsubscript{33–108} (containing amino acid residues 33–108) and another encoding E. coli YneM\textsubscript{1–28} (containing amino acid residues 1-28) were synthesized. The YneM-mitoNEET fusion gene was created by placing the YneM sequence at the
5′ end of mitoNEET\textsubscript{33–108} and a histidine tag at the 3′ end of mitoNEET\textsubscript{33–108} (Genscript Co.) and was cloned into pET28b+. Recombinant YneM-mitoNEET was expressed in E.coli BL21/DE3 strain and purified as described for MalFGK\textsubscript{2} complex\cite{111}. Briefly, YneM-MitoNEET his-tagged at the C-terminus was expressed from pET28b+ in E. coli strain BL21/DE3 and were grown in LB medium at 37°C and induced at OD\textsubscript{600}~0.6 with 0.5M IPTG overnight at 25°C, harvested, and disrupted by passing through a French press once. The crude cell extracts were ultra-centrifuged at 125000g for an hour and the membrane pellets were resuspended in TSG20 buffer (50 mM Tris, 100 mM NaCl, 20% v/v Glycerol, pH 7.9) at a final concentration of 5mg/ml and solubilized with 1% v/v Triton-x-100 for overnight at 4°C with gentle shaking. The YneM-mitoNEET was purified using a Ni-NTA column. Contaminants were washed away with TSG10 buffer (50 mM Tris, 100 mM NaCl, 10% v/v Glycerol, pH 7.9) containing 50mM Imidazole and 0.5% Triton-x-100 and the purified protein was eluted with TSG10 buffer containing 600mM Imidazole and 0.5% Triton-x-100. The membrane scaffold protein MSP1E3 was cloned into an expression plasmid pET28b+ and transformed in E. coli BL21/DE3 strain. Recombinant MSP1E3 was purified as described previously\cite{111}. The E. coli flavin reductase (Fre) was prepared from an E. coli strain with an expression plasmid encoding Fre from the ASKA library\cite{88}. The purity of purified proteins was found more than 95% according to electrophoresis analysis with a 15% polyacrylamide gel containing SDS followed by staining with Coomassie Blue. The extinction co-efficients of 9.89, 29.4, 26.4 mM−1 cm−1 at 280 nm were used to determine the concentration of purified YneM-mitoNEET, MSP1E3, and Fre respectively.

**Nanodisc preparation**

The nanodisc was assembled as described previously\cite{111} with following modifications. Nanodiscs containing YneM-mitoNEET were assembled using MSP1E3 in TSG10 buffer and the
lipid 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) solubilized in 0.5% Triton-x-100. The purified fusion protein was mixed at Protein: MSP: Lipid ratio of 1:3:60 [22] in TSG10 buffer containing 0.5% Triton-X-100 with the final volume of 366.5ul. After incubating for 10 minutes at room temperature, the nanodisc assembly step was initiated by adding of 50 ul BioBead SM-2 suspension and the mixture was incubated overnight on a rocking table at 4°C. The beads were sedimented by gravity and the solution was pipetted through a narrow tip to avoid BioBeads. The freshly prepared nanodiscs were used to perform experiments.

**Reduction and oxidation of the YneM-mitoNEET [2Fe-2S] clusters**

The redox state of the YneM-mitoNEET [2Fe-2S] clusters was observed with a Beckman DU640 UV–visible absorption spectrometer equipped with a temperature controller. The oxidized YneM-mitoNEET [2Fe-2S] clusters present a major absorption peaks at 458 nm and 540 nm. Upon reduction the absorption peak of oxidized YneM-mitoNEET [2Fe-2S] clusters at 458 nm is shifted to a much small absorption peak at 420 nm, but the absorption peak at 540 nm remains unchanged [112]. Hence, the amplitude of the absorption peak at 458 nm was used to monitor the redox state of the YneM-mitoNEET [2Fe-2S] clusters in the reaction solution.

**EPR measurements**

The X-band Electron Paramagnetic Resonance (EPR) spectra were measured utilizing a Bruker model ESR-300 spectrometer equipped with an Oxford Instruments 910 continuous flow cryostat. Routine EPR conditions include microwave frequency, 9.47 GHz; microwave power, 1.0 mW; modulation frequency, 100 kHz; modulation amplitude, 1.2 mT; temperature, 20 K; receiver gain, $2 \times 105$. 
Preparation of the nanodisc sample for TEM analysis

TEM samples were prepared by adding 3 μl of the nanodisc sample (The concentration range for nanodisc was 0.5-1 μM) to a glow discharged 300 mesh carbon filed grid (EMS:CF300-cu). After 2 minutes of incubation, the surplus solution was blotted off with filter paper and the grid was immediately stained with 1% uranyl acetate (TED Pella Cat: 19481) for 1-2 minutes. The grids were evaluated by JEOL JEM-1400 transmission electron microscope (TEM) with 120-200 keV accelerating voltage suffices. The size of the nanodisc stacks was determined using ImageJ (Fiji).[113, 114].

Chemicals

NADH, isopropyl-β-D-1-thiogalactopyranoside, kanamycin, and ampicillin were purchased from Research Product International co. Ubiquinone-10, 1,2-Dipalmitoyl-sn-glycero-3-phosphochine (DPPC), Flavin mononucleotide (FMN), Lumichrome (LC), and other chemicals were purchased from Sigma co. The concentrations of NADH and FMN were determined using extinction coefficients of 6.2 mM-1cm-1 at 340 nm and 12.5 mM-1cm-1 at 445 nm, respectively[90].

3.3. Results

Construction of fusion protein YneM-mitoNEET and preparation of nanodisc to mimic native mitoNEET in biological membrane.

A fusion protein (Figure 3.1A) with the N terminal transmembrane α-helix of E. coli YneM and the C-terminus of human mitoNEET was constructed to investigate the electron transfer activity of the [2Fe-2S] clusters of native mitoNEET in the biological membrane. Previous studies have reported that the oxidized [2Fe-2S] clusters of mitoNEET33-108 have the absorption peaks at 458
nm and 540 nm[87]. Here, we determined the UV-Visible absorption spectrum of the membrane bound mitoNEET using the fusion protein.

Figure 3.1. Characterization of YneM-mitoNEET fusion protein and nanodisc. A, YneM-mitoNEET fusion gene construct. B, UV-Visible absorption spectrum of YneM-mitoNEET. C, SDS-PAGE analysis of YneM-mitoNEET. D, Transmission electron microscopic image of nanodisc containing YneM-mitoNEET.

YneM-mitoNEET and observed that oxidized [2Fe-2S] clusters of YneM-mitoNEET have similar peaks at 458 nm and 540 nm (Figure 3.1B) as mitoNEET_{33-108}. Purified fusion protein, YneM-mitoNEET (analyzed with 15% SDS PAGE for purity and was found more than 95% pure, Figure 3.1C) was used to prepare nanodisc containing YneM-mitoNEET and TEM image was captured for the nanodiscs (Figure 3.1D).
Reduction of the YneM-mitoNEET [2Fe-2S] clusters in nanodisc by FMNH₂.

To explore the reduction kinetics of YneM-mitoNEET [2Fe-2S] clusters in nanodisc, nanodiscs containing YneM-mitoNEET (containing 6 μM [2Fe-2S] clusters) were incubated with FMN (0.05 μM) and Fre (0.2 μM) and the reaction was initiated by adding NADH (20 μM) anaerobically. Figure 3.2A shows that oxidized [2Fe-2S] clusters of YneM-mitoNEET in nanodisc had a major absorption peak at 458 nm. Once the NADH was added, the major absorption peak at 458 nm was diminished and a new absorption peak for the reduced [2Fe-2S] clusters was observed at 420 nm. Under the experimental condition, 20 μM of NADH was sufficient to reduce 6 μM of YneM-mitoNEET [2Fe-2S] clusters within 3 minutes. Thus, under anaerobic conditions, YneM-mitoNEET [2Fe-2S] clusters in nanodiscs which mimics native mitoNEET [2Fe-2S] clusters in biological membrane can be rapidly reduced by NADH in the presence of FMN and catalytic amount of Fre.

Figure 3.2. Reduction of the YneM-mitoNEET [2Fe-2S] clusters in nanodisc by FMNH₂. A, UV-visible absorption spectra of YneM-mitoNEET in nanodisc after incubation with NADH, FMN, Flavin reductase (Fre) and NADH under anaerobic condition. YneM-mitoNEET in nanodisc (containing 6 μM of [2Fe-2S] clusters) was incubated with FMN (0.05 μM) and Fre (0.2 μM). Then, NADH (20 μM) was added to the solution to start the reaction. UV-visible absorption spectra were taken every 30 seconds after adding NADH for 12 minutes. Absorption peaks at 458 nm and
540 nm represent the oxidized YneM-mitoNEET [2Fe-2S] clusters. O.D., optical density. B, EPR spectra of YneM-mitoNEET in nanodisc, Purified proteins mitoNEET (soluble part) and YneM-mitoNEET in nanodisc (each containing 6 μM [2Fe-2S] clusters) were reduced with freshly prepared sodium dithionite (4mM) (spectrum 1-5 except spectrum 3). Spectrum 1, YneM-mitoNEET in nanodisc; spectrum 2, YneM-mitoNEET in nanodisc incubated with FMN (0.2 μM), Fre (0.2 μM) and NADH (20 μM); spectrum 3, YneM-mitoNEET in nanodisc (reduced); spectrum 4, mitoNEET; spectrum 5, mitoNEET (reduced). The data are representative of three independent experiments.

We also performed EPR experiments to observe the reduction of YneM-mitoNEET [2Fe-2S] clusters by NADH in presence of FMN and catalytic amount of Fre. Interestingly, the nanodiscs containing YneM-mitoNEET shows a unique signal at g=1.85 upon reduction (Both by FMN, NADH, and Fre, spectrum 2; by dithionite, spectrum 3). A plausible explanation for such signal could be that there is an interaction between the reduced [2Fe-2S] clusters and the DPPC used to prepare the nanodisc.

**Redox transition of YneM-mitoNEET [2Fe-2S] clusters in nanodisc under aerobic conditions**

Mitochondria are often under aerobic conditions inside cells[31], hence here we investigated the reduction and oxidation kinetics of the membrane bound mitoNEET using fusion construct YneM-mitoNEET under aerobic conditions. In experiments, nanodiscs containing YneM-mitoNEET (containing 6 μM [2Fe-2S] clusters) were incubated with FMN, Fre and NADH aerobically. The redox states of the [2Fe-2S] clusters of YneM-mitoNEET and the NADH were measured simultaneously at 458nm and 340nm, respectively. Fig 3.3C demonstrates rapid reduction of [2Fe-2S] clusters of YneM-mitoNEET while Figure 3.3D shows the concomitant oxidation of NADH under aerobic conditions. As shown in Figure 3.3C and 3.3D, when the NADH in the solution was completely oxidized (at about 7 minutes of incubation), the reduced [2Fe-2S] clusters of YneM-mitoNEET gradually started to oxidize, suggesting that the YneM-mitoNEET [2Fe-2S] clusters
were continuously reduced by FMNH₂/NADH/Fre and oxidized by oxygen. We also investigated the redox transition of a mutant of YneM-mitoNEET_H87C (His-87 is substituted to Cys) under similar conditions (Figure 3.3F) and the results did not show the significant reduction as observed in Figure 3.3E, confirming what has been observed previously for the mutant of mitoNEET_{33-108} [91].

Figure 3.3. Reduction and oxidation of YneM-mitoNEET [2Fe-2S] clusters in nanodisc under aerobic condition. A and B, Purified YneM-mitoNEET in nanodisc (containing 6 μM [2Fe-2S]
clusters) was incubated with FMN (0.2 μM), and Fre (0.2 μM) in a buffer (pH 7.9) containing 20 mM Tris and 20 mM NaCl under aerobic condition. Then, NADH (at a final concentration of 10 μM) was added to the solution to start the reaction. The redox state of the YneM-mitoNEET [2Fe-2S] clusters (at wavelength 458nm, A) and NADH oxidation (at wavelength 340nm, B) was monitored by taking UV-Visible absorption spectra every 1 minute after adding NADH. C, D same as A, except 100 μM NADH was added in the incubation solution to start the reaction.

We further explored the redox transition of the [2Fe-2S] clusters of YneM-mitoNEET in presence of FMN, Fre and 10 μM NADH under aerobic conditions. Figure 3.3A shows that with 10 μM of NADH, the reduction of [2Fe-2S] clusters under aerobic conditions were not as significant as shown in Figure 3.3C. Thus, under aerobic conditions, in presence of catalytic amount of FMN and Fre, the redox transition of YneM-mitoNEET [2Fe-2S] clusters may be largely dependent on the concentration of NADH and oxygen.

**Lumichrome inhibits the FMNH$_2$- mediated reduction of the [2Fe-2S] clusters of YneM-mitoNEET in nanodisc**

Lumichrome has been previously shown to inhibit FMNH$_2$ mediated reduction of [2Fe-2S] clusters of mitoNEET$_{33-108}$ in aerobic conditions in presence of NADH and flavin reductase (Fre)$_{115}$. Since structurally lumichrome contains the same isoalloxazine group as FMN, it was hypothesized that lumichrome may compete with FMN for the FMN binding site in mitoNEET and thus blocks electron transfer activity of mitoNEET$_{30}$. Here, we investigated if lumichrome would also inhibit FMNH$_2$ mediated electron transfer in YneM-mitoNEET in nanodisc.

For experiment, nanodisc with YneM-mitoNEET (containing 6 μM [2Fe-2S] clusters) was prepared and was incubated with FMN (0.2 μM) and Lumichrome (0.5 μM) in the presence of Fre (0.2 μM) under aerobic conditions.
Figure 3.4. Inhibition of electron transfer activity of YneM-mitoNEET in nanodisc by Lumichrome. A, reduction, and oxidation of the YneM-mitoNEET [2Fe-2S] clusters in nanodisc without lumichrome. Purified YneM-mitoNEET in nanodisc (containing 6 μM [2Fe-2S] clusters) was incubated with Fre (0.2 μM), and FMN (0.2 μM) in a buffer (pH 7.9) containing 20 mM Tris and 20 mM NaCl under aerobic conditions. The reaction was initiated by adding NADH (at a final concentration of 100 μM). The redox state of the YneM-mitoNEET [2Fe-2S] clusters was observed by taking UV-Visible absorption spectra every 1 minute after adding NADH. B, same as in A, except 0.5 μM of lumichrome was included in the incubation solution before the reaction was initiated. The data are representative of three independent experiments.

The reaction was started by adding NADH (100 μM) to the solution. Figure 3.4 shows that 0.5 μM lumichrome effectively inhibited about 60% of the FMNH₂ mediated reduction of [2Fe-2S] clusters of YneM-mitoNEET in nanodisc indicating that lumichrome is a potent antagonist of electron transfer activity of membrane bound mitoNEET construct YneM-mitoNEET.

**Effect of ubiquinone-10 on redox transition of the YneM-mitoNEET [2Fe-2S] clusters under aerobic conditions**

Previously, it has been reported that both oxygen and ubiquinone analog, ubiquinone 2 can mediate the oxidation of reduced [2Fe-2S] clusters of mitoNEET[83]. However, there has not been any report of the effect of ubiquinone-10 on the redox transition of the YneM-mitoNEET [2Fe-2S] clusters. In this study, we examined the oxidation kinetics of the reduced [2Fe-2S] clusters of
YneM-mitoNEET by ubiquinone-10 under anerobic condition. In the experiments, nanodiscs containing YneM-mitoNEET (containing 6 μM [2Fe-2S] clusters) were prepared in absence (Figure 3.5A) and presence of ubiquinone-10 (determined by ubiquinone-10: DPPC ratio). Nanodiscs with or without ubiquinone-10 were incubated with Fre (0.2 μM) and FMN (0.05 μM). Figure 3.5B shows, at ubiquinone-10: DPPC ratio 1:13, addition of NADH (20 μM) at first rapidly reduced the [2Fe-2S] clusters, and then the reduced [2Fe-2S] clusters were oxidized under anerobic conditions suggesting that ubiquinone-10 in the solution mediated the oxidation of the reduced [2Fe-2S] clusters.

Figure 3.5. Oxidation of the reduced YneM-mitoNEET [2Fe-2S] clusters in nanodisc by ubiquinone-10 (Q₁₀) under anerobic conditions. A, Oxidation kinetics of the reduced YneM-mitoNEET [2Fe-2S] clusters in absence of ubiquinone-10. Purified YneM-mitoNEET in nanodisc (containing 6 μM [2Fe-2S] clusters) was incubated with FMN (0.05 μM), and Fre (0.2 μM) in a buffer (pH 7.9) containing 20 mM Tris and 20 mM NaCl under anerobic conditions. The reaction was started by adding 20 μM NADH anerobically. The redox state of the YneM-mitoNEET [2Fe-2S] clusters were monitored at 458 nm. B, same as A, except ubiquinone-10 was present in the incubation solution at a ubiquinone-10: DPPC ratio of 1:13 in the nanodisc. The data were representative of three independent experiments.
Thus, under anerobic conditions, ubiquinone-10 oxidizes the reduced YneM-mitoNEET[2Fe-2S] clusters in a concentration dependent manner.

3.4. Discussion

MitoNEET, the founding member of the NEET family of proteins has been proposed to play an essential regulatory role in energy metabolism in mitochondria[16]. However, the specific functions of mitoNEET are not fully determined yet. One hypothesis proposed that via specific protein-protein interactions, mitoNEET may regulate mitochondrial functions[58]. On the other hand, other researchers suggested that mitoNEET through its cluster transfer reaction contribute to iron-sulfur protein maturation in cytosol[21, 71, 72]. Previous studies have reported that [2Fe-2S] clusters of mitoNEET33-108 can be reduced by FMNH$_2$ and oxidized by oxygen or ubiquinone-2[75]. Here, we explored the electron transfer activity of membrane bound mitoNEET using fusion protein YneM-mitoNEET in nanodisc. Taking advantage of the fusion protein in nanodiscs, we further investigated the oxidation kinetics of reduced YneM-mitoNEET [2Fe-2S] clusters by hydrophobic ubiquinone-10.

Crystal structure of mitoNEET reveals that it’s a homodimer and each monomer of mitoNEET is associated with a β-cap structure that brings each [2Fe-2S] clusters in each monomer closer with the closest distance being 13 Å[67-69]. As the molecular docking model suggests, the binding site of FMN in mitoNEET is in between transmembrane α-helix domain and [2Fe-2S] cluster domain[81] and the distance between the FMN binding site and [2Fe-2S] cluster is short. This short distance therefore could be the reason behind the rapid reduction of the mitoNEET [2Fe-2S] clusters by FMNH$_2$ [81]. Such electron transfer activity was re-evaluated in this study using membrane bound fusion protein YneM-mitoNEET to comprehend the electron transfer activity of mitoNEET in more native conformation. In consistent with previous studies, membrane bound
mitoNEET (YneM-mitoNEET) also showed reduction of [2Fe-2S] clusters in presence of flavin reductase, FMN, and NADH (donor of electron) in both aerobic (Figure 3.3) and anaerobic (Figure 3.2) conditions suggesting their role as redox carrier in the biological membrane. In this context, membrane bound mitoNEET (YneM-mitoNEET) may effectively facilitate oxidation of NADH (Figure 3.3D) along with cytosolic flavin reductase which reduces FMN and oxidizes NADH[82]. Since NADH oxidation in cytoplasm is crucial for glycolysis[84, 85], mitoNEET on the outer mitochondrial membrane thereby may indirectly promote glycolysis[75, 83] and thus may act as a potent regulator of energy metabolism. This hypothesis is supported by the findings in previous studies that over-expression of mitoNEET enhances ATP synthesis[106, 107] while deletion of mitoNEET downregulates oxidative phosphorylation in cells[10].

In theory, then the inhibition of electron transfer activity of mitoNEET may decrease NADH oxidation in cytoplasm and thus inhibit glycolysis and modulate energy metabolism in cell[30]. Previous studies have reported type-2 antidiabetic drug, pioglitazone is an inhibitor of electron transfer activity of mitoNEET[15, 81]. The molecular docking approach revealed that the reason for this inhibition could be the partial overlapping of FMN and pioglitazone binding site[15] suggesting pioglitazone may block FMNH\(_2\) access to its binding site and thus, interfere with reduction of [2Fe-2S] clusters in mitoNEET. With similar context, Lumichrome, which shares the same isoalloxazine group as FMN, and may compete with FMN for its binding site[30], was used in this study to observe inhibition of electron transfer activity in membrane bound mitoNEET (YneM-mitoNEET). Lumichrome was found to inhibit 60% of electron transfer activity in membrane bound mitoNEET (YneM-mitoNEET) consistent with previous studies[115].
Figure 3.6. A schematic diagram showing the electron transfer activity in YneM-mitoNEET

Ubiquinone-10 is a lipophilic cofactor and a key component of electron transport chain in mitochondria that shuttles electrons for ATP synthesis[116, 117]. It can be found in both inner and outer mitochondrial membranes[118]. MitoNEET’s unique location in the outer mitochondrial membrane[40] and the short distance between [2Fe-2S] clusters in mitoNEET[55, 67-69] and the membrane led us to hypothesize that ubiquinone-10 could be an intrinsic electron acceptor for the reduced [2Fe-2S] clusters of mitoNEET. Using fusion protein YneM-mitoNEET in nanodiscs which resembles native mitoNEET in biological membrane we have observed, YneM-mitoNEET in nanodiscs oxidizes FMNH₂ and reduces ubiquinone-10 under anaerobic conditions (Fig 3.5B, Figure 3.6). The result suggests that ubiquinone in the mitochondrial outer membrane may serve as an intrinsic electron acceptor of the [2Fe-2S] clusters of mitoNEET. However, the underlying mechanism of reduction of ubiquinone-10 by reduced [2Fe-2S] clusters is not fully understood. One possible mechanism could be a single-electron transfer from one reduced [2Fe-2S] clusters of mitoNEET to ubiquinone generating a semiquinone intermediate[31]. Another idea is a two-electron transfer from the two reduced [2Fe-2S] clusters of mitoNEET dimer to ubiquinone-10.
forming ubihydroquinone. As the crystal structure suggests the two [2Fe-2S] clusters in the mitoNEET dimer are close to each other (the closest distance is about 13 Å)[67-69], the possibility of two electron transfer by two [2Fe-2S] cluster to ubiquinone-10 cannot be excluded. Further experiments are required to elucidate the molecular mechanism involved in [2Fe-2S] clusters oxidation in mitoNEET by ubiquinone-10.
CHAPTER 4. CONCLUSION

MitoNEET, located in the outer membrane of mitochondria, contains a redox active [2Fe-2S] cluster in the C-terminal cytosolic domain. It has been proposed that mitoNEET may have a crucial regulatory role in energy metabolism in human cells. Here, we explored the FMN binding site in mitoNEET by utilizing analogs of FMN and we observed that lumiflavin, similar as FMN, at nanomolar concentrations can mediate the redox transition of the [2Fe-2S] clusters of mitoNEET when flavin reductase and NADH (100 μM) is present under aerobic conditions. The electron paramagnetic resonance (EPR) data reveal that both FMN and lumiflavin cause drastic change in the EPR spectrum of the reduced mitoNEET [2Fe-2S] clusters and under blue light exposure can form a covalently bound complex with mitoNEET, indicating that FMN/lumiflavin has specific interactions with the mitoNEET [2Fe-2S] clusters. On the other hand, lumichrome, another analog of FMN, is unable to cause the redox transition of the mitoNEET [2Fe-2S] clusters and shows no effect on the EPR spectrum of the reduced mitoNEET [2Fe-2S] clusters under blue light exposure. Rather, lumichrome effectively blocks the FMNH$_2$-mediated reduction of the mitoNEET [2Fe-2S] clusters, suggesting that lumichrome may act as a potential antagonist to inhibit the electron transfer activity of mitoNEET.

We also investigated the electron transfer activity of membrane bound mitoNEET using fusion protein YneM-mitoNEET in nanodisc with an aim to study the electron transfer kinetics of native mitoNEET in the biological membrane. We found that YneM-mitoNEET in nanodisc can be rapidly reduced by FMNH$_2$, in presence of catalytic amount of Flavin reductase and NADH under both aerobic and anaerobic conditions. Moreover, we found that under anaerobic conditions, ubiquinone-10 oxidizes the reduced [2Fe-2S] clusters of YneM-mitoNEET in nanodisc. The results suggest that ubiquinone-10 may act as an intrinsic oxidant for the reduced [2Fe-2S] clusters.
of the native mitochondrial outer membrane protein mitoNEET and that native mitoNEET is a novel redox enzyme that catalyzes electron transfer from FMNH$_2$ to ubiquinone-10 in mitochondria (Figure 3.6).
Dear Homyra Tasnim

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