HHS Public Access

Author manuscript

*Free Radic Biol Med*. Author manuscript; available in PMC 2021 August 20.

Published in final edited form as:

*Free Radic Biol Med*. 2020 August 20; 156: 11–19. doi:10.1016/j.freeradbiomed.2020.05.004.

# Exploring the FMN binding site in the mitochondrial outer membrane protein mitoNEET

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## Abstract

MitoNEET is a mitochondrial outer membrane protein that hosts a redox active [2Fe-2S] cluster in the C-terminal cytosolic domain. Increasing evidence has shown that mitoNEET has an essential role in regulating energy metabolism in human cells. Previously, we reported that the [2Fe-2S] clusters in mitoNEET can be reduced by the reduced flavin mononucleotide ($FMNH_2$) and oxidized by oxygen or ubiquinone-2, suggesting that mitoNEET may act as a novel redox enzyme catalyzing electron transfer from $FMNH_2$ to oxygen or ubiquinone. Here, we explore the FMN binding site in mitoNEET by using FMN analogs and find that lumiflavin, like FMN, at nanomolar concentrations can mediate the redox transition of the mitoNEET [2Fe-2S] clusters in the presence of flavin reductase and NADH (100 μM) under aerobic conditions. The electron paramagnetic resonance (EPR) measurements show that both FMN and lumiflavin can dramatically change the EPR spectrum of the reduced mitoNEET [2Fe-2S] clusters and form a covalently bound complex with mitoNEET under blue light exposure, suggesting that FMN/lumiflavin has specific interactions with the [2Fe-2S] clusters in mitoNEET. In contrast, lumichrome, another FMN analog, fails to mediate the redox transition of the mitoNEET [2Fe-2S] clusters and has no effect on the EPR spectrum of the reduced mitoNEET [2Fe-2S] clusters under blue light exposure. Instead, lumichrome can effectively inhibit the $FMNH_2$-mediated reduction of the mitoNEET [2Fe-2S] clusters, indicating that lumichrome may act as a potential inhibitor to block the electron transfer activity of mitoNEET.

## Graphical Abstract

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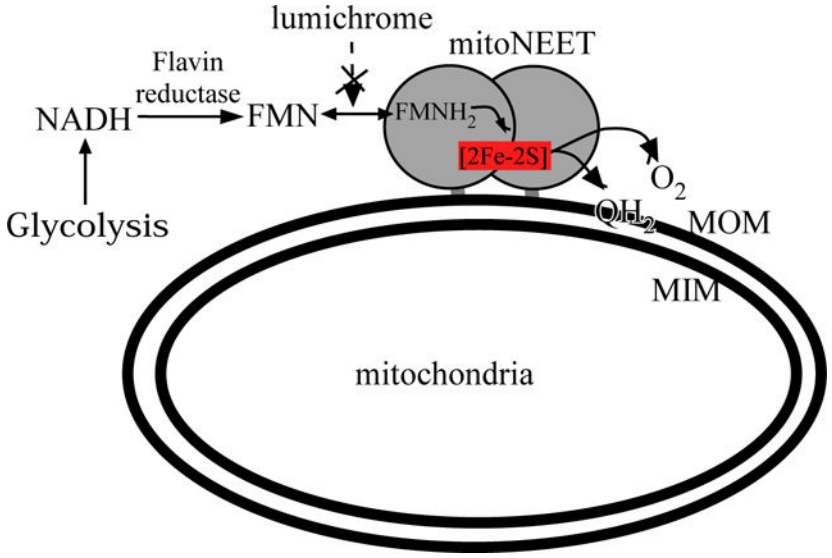
Competing interests

The authors declare no competing interests.




**Keywords**

mitoNEET; FMN; lumiflavin; lumichrome; electron transfer activity; mitochondria

## Introduction

The mitochondrial outer membrane protein mitoNEET was initially identified as a binding target of the type II diabetes drug pioglitazone [1]. 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 [2, 3]. For example, in breast cancer cells, mitoNEET is highly expressed, and deletion of mitoNEET decreases proliferation of the cells [4]. In primary cultured spinal neurons, knockdown of mitoNEET leads to apoptosis [5]. In mice, mitoNEET is proposed to act as a mediator of $Ca^{2+}$-facilitated mitochondrial dysfunction [6] and to regulate the function of the mitochondrial outer membrane protein voltage-dependent anion channel 1 (VDAC1) [7]. Mice with deletion of mitoNEET has signs of striatal mitochondrial dysfunction and a Parkinson's disease phenotype [8]. Because of its role in broad physiological functions, mitoNEET has become a novel target for treating diseases of mitochondrial dysfunction [9–12].

Human mitoNEET is a homodimer with each monomer hosting a [2Fe-2S] cluster in its C-terminal cytosolic region [13–15]. The N-terminus of mitoNEET contains a transmembrane alpha-helix domain that anchors mitoNEET to the mitochondrial outer membrane [1]. 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 [16], it has been proposed that mitoNEET may act as a carrier that transfers iron-sulfur clusters assembled in mitochondria to target proteins in cytosol or nucleus [17–21]. However, the observed cluster transfer occurs only when the mitoNEET [2Fe-2S] clusters are in an oxidized state [17, 19]. Since the mitoNEET [2Fe-2S] clusters are in a reduced state in cells under normal physiological conditions [22, 23], mitoNEET may help repair the damaged

iron-sulfur clusters in proteins when cells are under oxidative stress conditions [17]. Alternatively, mitoNEET may contribute to the anti-oxidative stress activity [3, 24], as over-expression of mitoNEET prevents the oxidative damage in A375 melanoma cells [25] and in perivascular adipose tissue [26]. Regardless, the redox state of the [2Fe-2S] clusters has a crucial role for the proposed physiological functions of mitoNEET. The redox midpoint potential ($E_{m7}$) of the mitoNEET [2Fe-2S] clusters has been determined to be about 0 mV at pH 7.0 [27]. 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 $FMNH_2$ (but not NADH or NADPH) and oxidized by oxygen or ubiquinone [22, 28]. Because FMN can be reduced by flavin reductase using NADH as electron donor [29], 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 [30]. Since oxidation of NADH in cytosol is linked to the glycolysis activity [31, 32], mitoNEET may indirectly regulate glycolysis by promoting oxidation of cytosolic NADH [22,30].

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 [28]. 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 $FMNH_2$ to the [2Fe-2S] cluster within mitoNEET. To further explore the FMN binding site in mitoNEET, 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 [33]. 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 $FMNH_2$-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.

## Materials and methods

### Protein preparation—

Human mitochondrial outer membrane protein $mitoNEET_{33–108}$ (containing residues 33–108) encoded by CISD1 gene was prepared as described previously [34]. Human CISD2 gene encoding human $Miner1_{57–135}$ (or $NAF\text{-}1_{57–135}$) (containing residues 57–135), CISD3 gene encoding $Miner2_{42–134}$ (or $MiNT_{42–134}$) (containing residues 42–134), and FDX2 gene encoding ferredoxin $2_{61–186}$ (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

[35]. 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 [28]. 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, $2\times10^5$.

### 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 [36].

### 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 $mM^{-1}cm^{-1}$ at 340 nm and 12.5 $mM^{-1}cm^{-1}$ at 445 nm, respectively [37].

## Results

### 1. 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 $FMNH_2$ under aerobic conditions [22, 28, 30]. Since the cytosolic concentration of FMN in typical mammalian cells is in the nanomolar range [37], 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 1A shows that the oxidized mitoNEET [2Fe-2S] clusters had a major absorption peak at 458 nm [38]. 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 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 (Figure 1A), indicating that the $FMNH_2$-mediated reduction of the mitoNEET [2Fe-2S] clusters is reversible as described previously [22, 28, 30].

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 1B shows the redox transition of the mitoNEET [2Fe-2S] clusters at different concentrations of FMN (0 to 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 minute and fully re-oxidized in 4 min (Figure 1B). Thus, FMN not only mediates the reduction of the mitoNEET [2Fe-2S] clusters, but also promotes re-oxidization of the reduced mitoNEET [2Fe-2S] clusters under aerobic conditions.

### 2. 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 [28]. To

further explore the FMN binding site in mitoNEET, we have used two FMN analogs: lumiflavin and lumichrome (Figure 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 2C shows that lumiflavin had essentially the same activity as FMN (Figure 2B) in mediating the reduction and oxidation of the mitoNEET [2Fe-2S] clusters under aerobic conditions. In contrast, lumichrome had no such an activity under the experimental conditions (Figure 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 $g_{av}$ = 1.94 [28, 39]. 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.

### 3. FMN forms a covalently bound complex with mitoNEET under blue light exposure

Because flavin can be excited by blue light (at around 450 nm) [40], it has been reported that blue light excitation may result in formation of a covalently bound protein-FMN complex in FMN binding proteins [41, 42]. If mitoNEET binds FMN as predicted in the molecule docking models [28], 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 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 [28]. 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 3, spectrum 3), but not with lumichrome (Figure 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.

As blue light has been shown to induce dimerization in the FMN binding protein aureochrome-1 [43], we asked whether FMN could also promote dimerization of mitoNEET under blue light exposure. After mitoNEET was incubated with different concentrations of

FMN under blue light exposure for 10 min, the samples were subjected to the SDS-PAGE analyses. Figure 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. Re-purified mitoNEET samples were then subjected to the UV-visible absorption measurements. Figure 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 re-purified mitoNEET was further subjected to analysis of FMN in the protein using the fluorescence spectrometry as described in [36]. Upon excitation at 450 nm, FMN has a fluorescence peak at around 520 nm [36]. Figure 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 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 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 4C, spectrum 1). Using the FMN solution as a standard, we estimated that at least 0.2 moles 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 3), but also form a covalently bound complex with mitoNEET (Figure 4) under blue light exposure.

## 4. 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) [44, 45], the mitoNEET-related protein 2 (Miner2 or MiNT) [46, 47], and the mitochondrial ferredoxin 2 [48]. Miner1 is a homolog of mitoNEET with 54% identity and 69% similarity [44], and localizes in the mitochondrial outer membrane and endoplasmic reticulum membrane [45]. 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 [46, 47]. Mitochondrial ferredoxin 2 is a key member of the iron-sulfur cluster biogenesis machinery in mitochondria [48], 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 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 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 5, spectrum 7) or the reduced ferredoxin2 [2Fe-2S] clusters (Figure 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.

### 5. Lumichrome inhibits the $FMNH_2$-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 2D) and has no effect on the EPR spectrum of the reduced mitoNEET [2Fe-2S] clusters under blue light exposure (Figure 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 to 250 nM) in the presence of NADH (100 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 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 $FMNH_2$-mediated reduction of the mitoNEET [2Fe-2S] clusters was inhibited under the experimental conditions (Figure 6D), suggesting that lumichrome is a potent inhibitor to block the electron transfer activity of mitoNEET.

## Discussion

MitoNEET is the first iron-sulfur protein identified in the mitochondrial outer membrane [1]. 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 $FMNH_2$ and oxidized by oxygen or ubiquinone [22, 28, 30], and suggested that mitoNEET may act as a novel redox enzyme to catalyze electron transfer from $FMNH_2$ 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 (Figure 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 [13–15]. 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 [28]. The short distance between FMN and the [2Fe-2S] clusters in mitoNEET could facilitate the rapid electron transfer from $FMNH_2$ to the [2Fe-2S] cluster [28]. Lumiflavin has the same isoalloxazine group as FMN (Figure 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 2C), changing the EPR spectrum of the reduced mitoNEET [2Fe-2S] clusters under blue light exposure (Figure 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 (Figures 2 and 3). Instead, lumichrome effectively inhibits the $FMNH_2$-mediated reduction of the mitoNEET [2Fe-2S] clusters (Figure 6). The subtle difference between lumiflavin and lumichrome (a methyl group on isoalloxazine, Figure 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) [44, 45] and the mitoNEET-related protein 2 (Miner2 or MiNT) [46, 47, 49] in human mitochondria. Like mitoNEET [13–15], 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 [44]. Recent studies further indicated that mitoNEET and Miner1 may share similar physiological functions [4, 50], have specific interactions with the type II diabetes drug pioglitazone and other thiazolidinediones [51], and may even form a mitoNEET-Miner1 dimer [52]. Mutations of Miner1 have been attributed to Wolfram Syndrome 2, a metabolic disease associated with diabetes, blindness, deafness, and a shortened lifespan [50, 53]. In consistent with these observations, here we have found that mitoNEET and Miner1 have similar interactions with FMN (Figure 5). On the other hand, Miner2, which does not have the sequence similarities to mitoNEET or Miner1 [47], does not have specific interactions with FMN under blue light exposure (Figure 5, spectrum 7). Likewise, human ferredoxin-2, which does not have any sequence similarities to mitoNEET or Miner1[48], does not have interactions with FMN under blue light exposure (Figure 5, spectrum 8). Thus, mitoNEET and its homolog Miner1 may have a

specific binding site for FMN to facilitate rapid reduction of the mitoNEET [2Fe-2S] clusters by $FMNH_2$.

Together with cytosolic flavin reductase which catalyzes reduction of FMN and oxidization of NADH [29], mitoNEET may effectively promote oxidation of NADH in cytosol with reduction of oxygen or ubiquinone (Figure 7) [30]. Because oxidation of NADH in cytosol is required for glycolysis activity [31, 32], mitoNEET on the mitochondrial outer membrane may indirectly regulate glycolysis via promoting oxidation of cytosolic NADH [22, 30]. This notion is consistent with the findings that deletion of mitoNEET decreases the oxidative phosphorylation activity [54], and overexpression of mitoNEET increases ATP synthesis in cells [55]. 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 [22, 28]. The docking modelling indicated that the pioglitazone binding site in mitoNEET [11] has a partial overlap with the FMN binding site [22], indicating that binding of pioglitazone may block the access of $FMNH_2$ 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 7), and modulate overall energy metabolism in cells.

## Acknowledgements

This research was supported in part by the National Institutes of Health [Grant number R15GM129564].

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## Highlights

- MitoNEET is a mitochondrial outer membrane protein with a [2Fe-2S] cluster
- MitoNEET catalyzes the electron transfer from $FMNH_2$ to $O_2$ or ubiquinone.
- Lumiflavin has the same activity as FMN in mediating electron transfer in mitoNEET.
- Lumichrome acts as an inhibitor to block the $FMNH_2$ binding in mitoNEET.

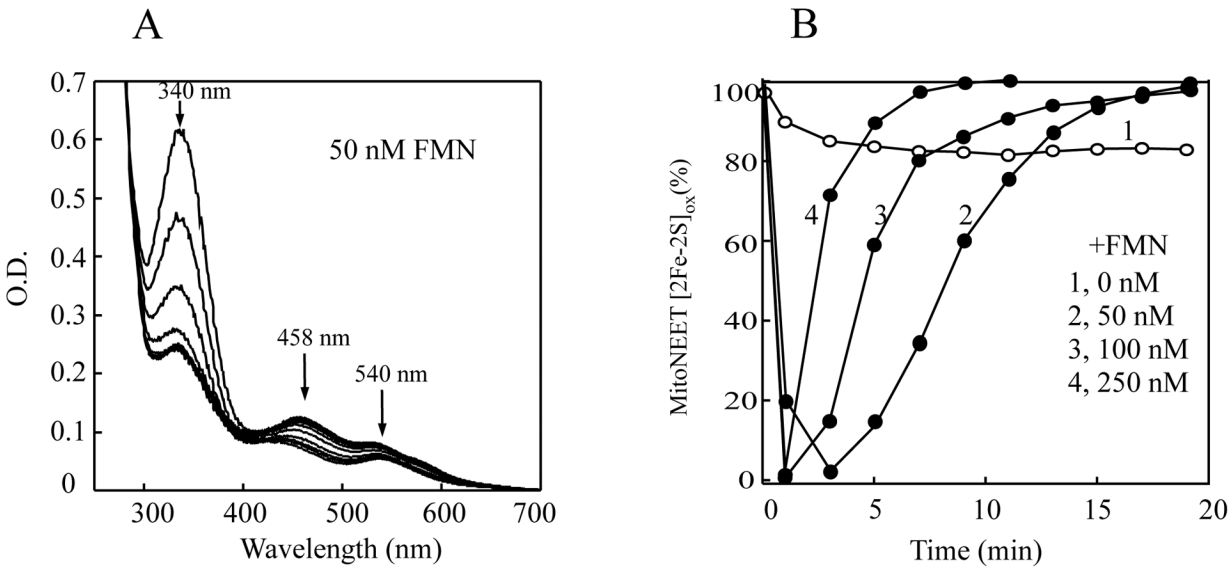


**Figure 1. Electron transfer activity of mitoNEET at different concentrations of FMN under aerobic conditions.**

**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 two minutes 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 to 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 were representatives from three independent experiments.

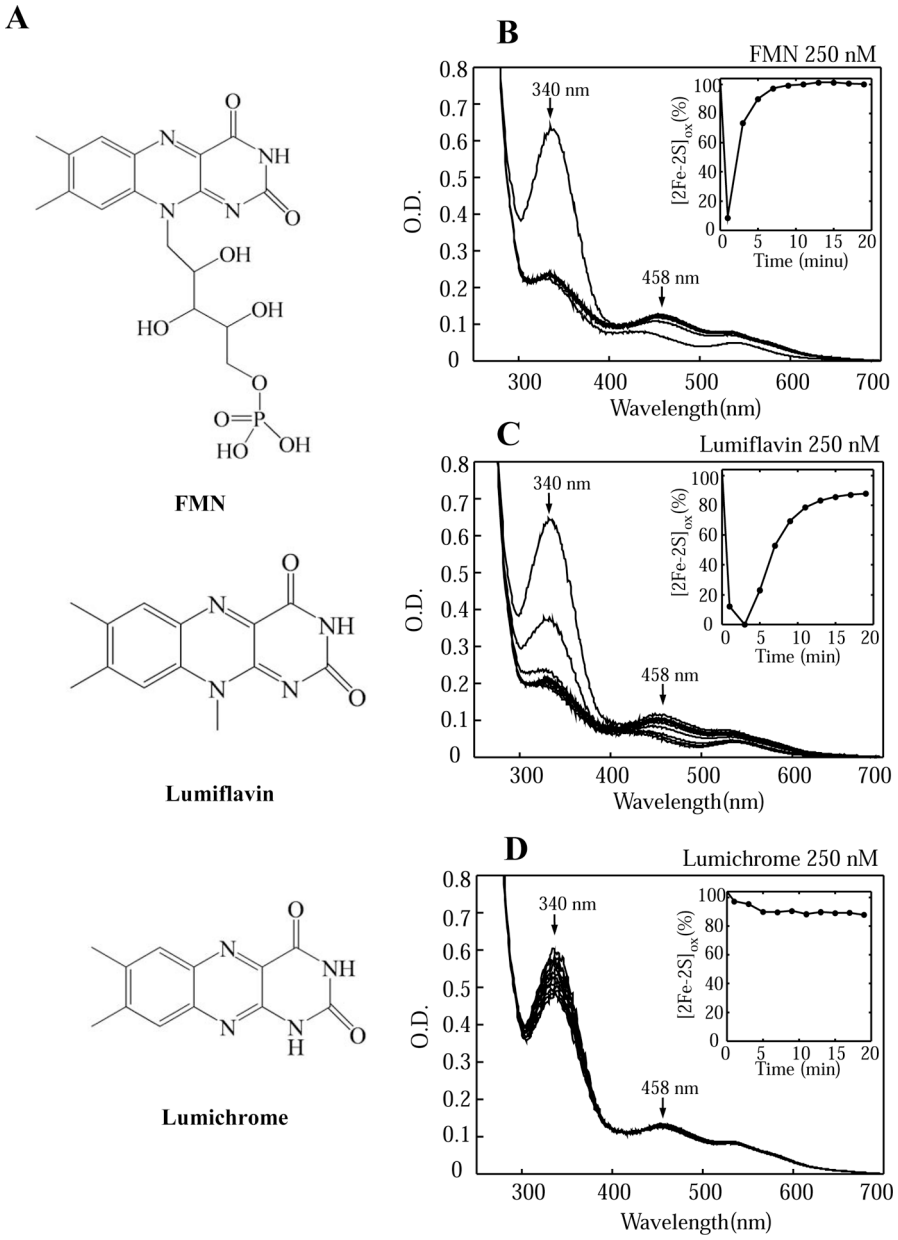


**Figure 2. Lumiflavin can substitute FMN in mediating the redox transition of the mitoNEET [2Fe-2S] clusters under aerobic conditions.**

**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 were representatives of three independent experiments.

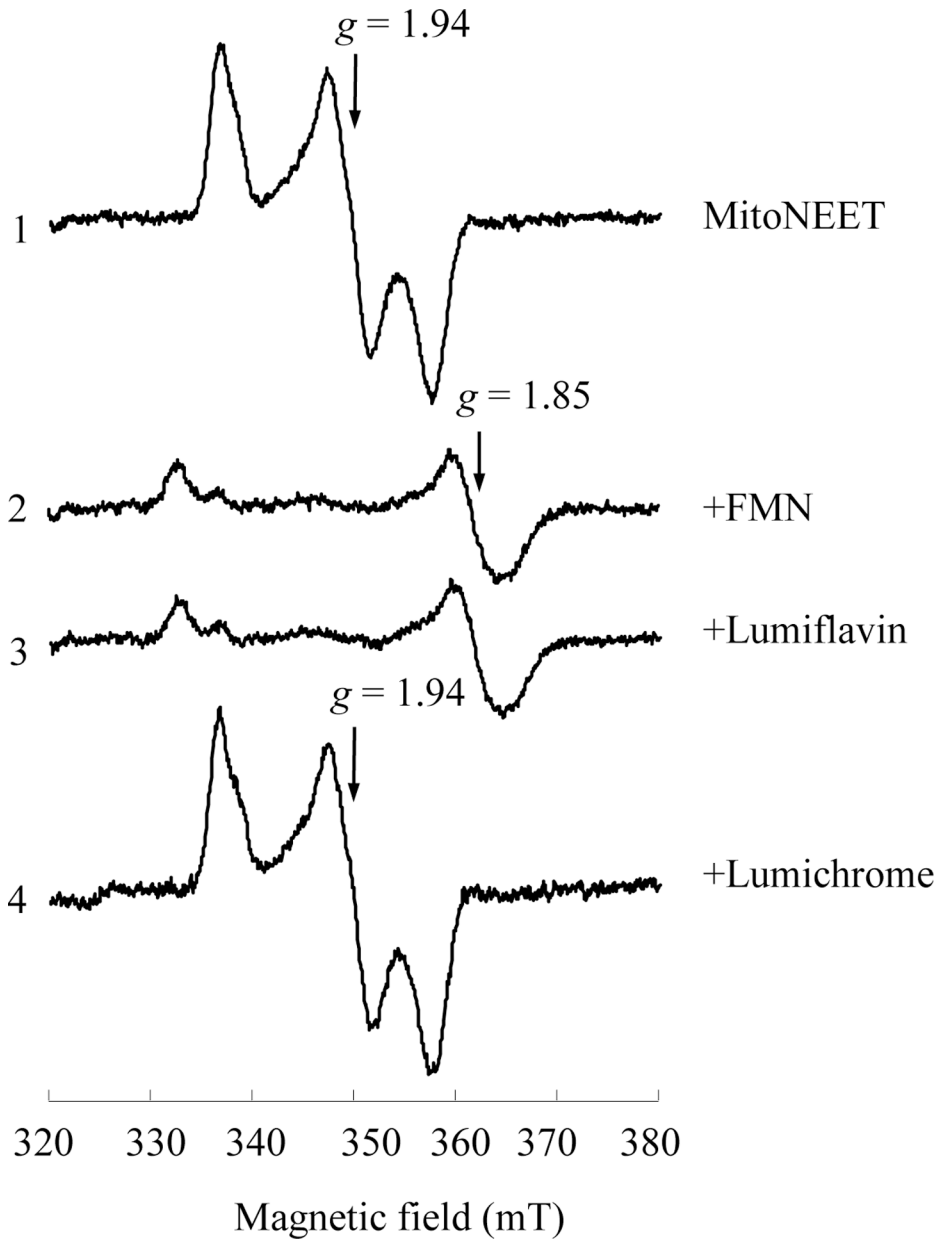


**Figure 3. Effect of FMN analogs on the EPR spectrum of the mitoNEET [2Fe-2S] clusters.**

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 $g = 1.85$.

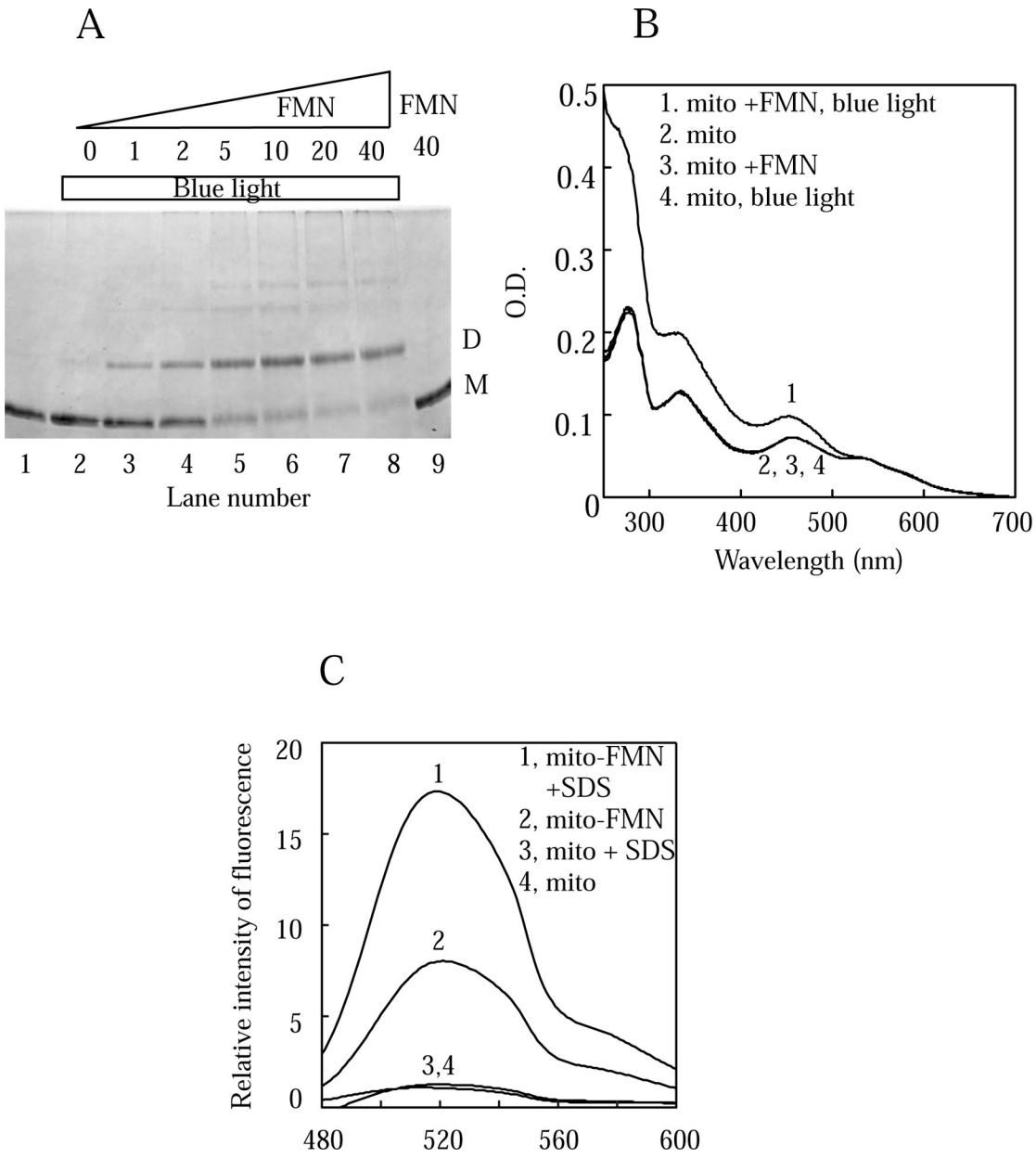


**Figure 4. MitoNEET forms a covalently bound complex with FMN under blue light exposure.**

**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 to 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 (containing 10 μM [2Fe-2S] clusters) was incubated with or without FMN (40 μM) under blue light exposure (50,000 Lux) for 10

min. 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).

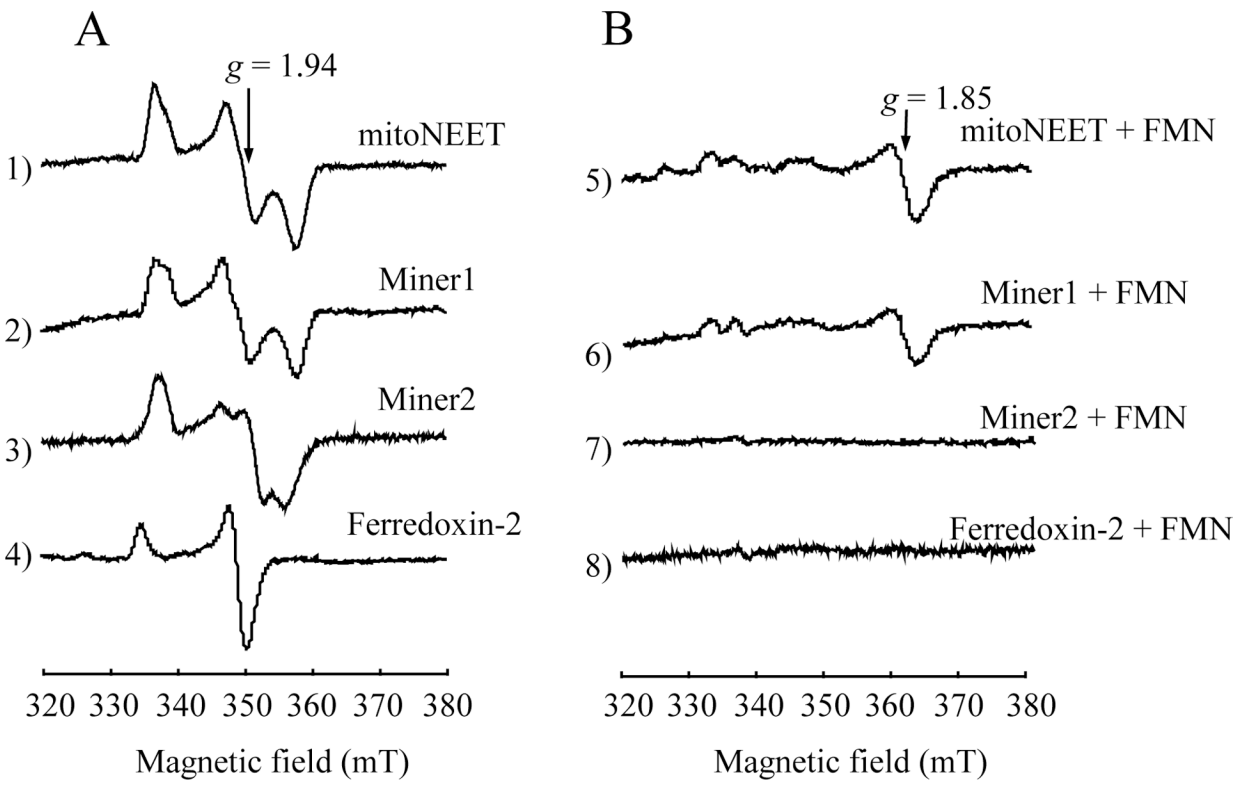


**Figure 5. Effect of FMN on other [2Fe-2S] proteins from human mitochondria.**

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 1, mitoNEET; spectrum 2, Miner1; spectrum 3, Miner2; Spectrum 4, ferredoxin-2. The data are representatives of three independent experiments.

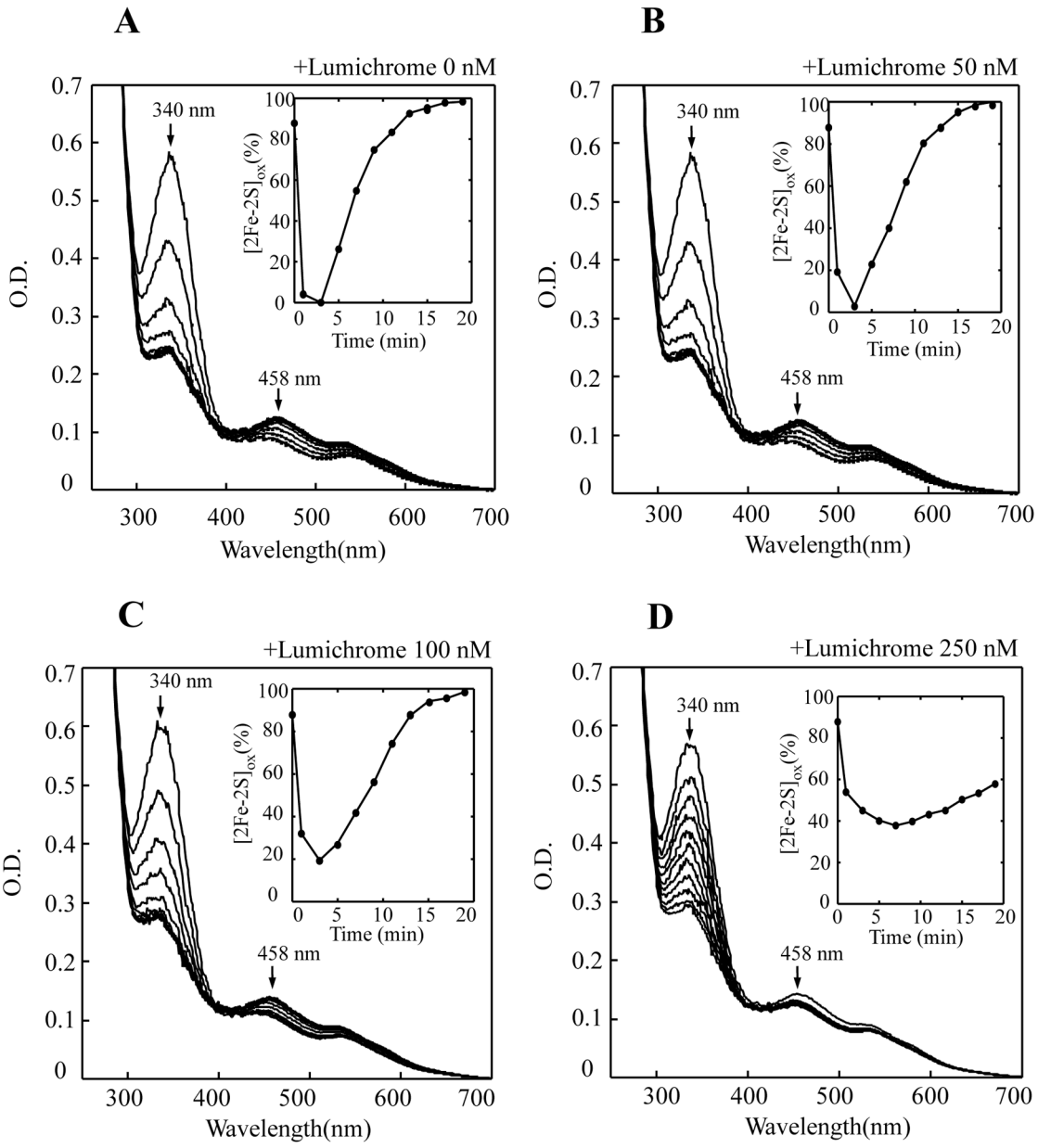


**Figure 6. Inhibition of the electron transfer activity of mitoNEET by lumichrome.**

**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 minutes 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.

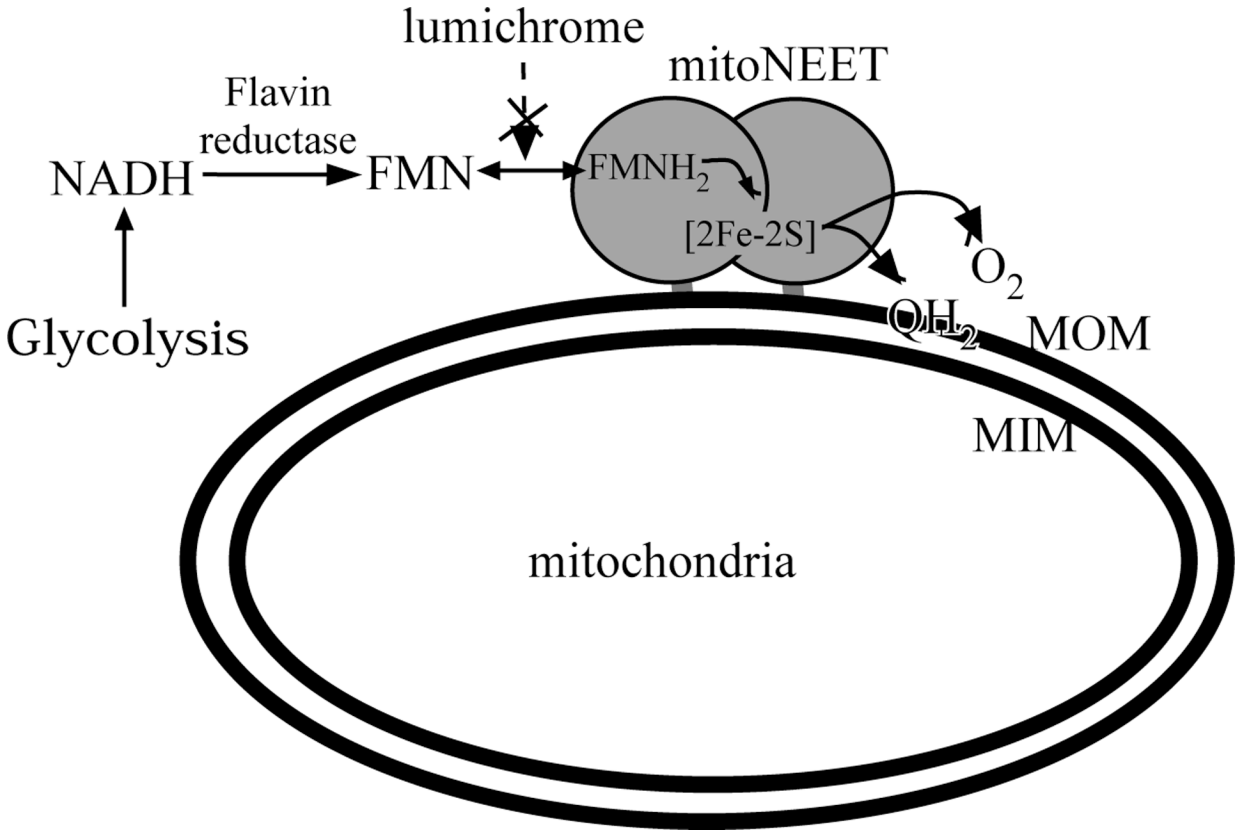


**Figure 7. A proposed model for the electron transfer activity of mitoNEET and inhibition by lumichrome.**

In cytosol, NADH generated by glycolysis is oxidized by flavin reductase and FMN is reduced to $FMNH_2$. $FMNH_2$ 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.