

The Outer Mitochondrial Membrane Protein mitoNEET Contains a Novel Redox-active 2Fe-2S Cluster^{*[S]}

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The outer mitochondrial membrane protein mitoNEET was discovered as a binding target of pioglitazone, an insulin-sensitizing drug of the thiazolidinedione class used to treat type 2 diabetes (Colca, J. R., McDonald, W. G., Waldon, D. J., Leone, J. W., Lull, J. M., Bannow, C. A., Lund, E. T., and Mathews, W. R. (2004) *Am. J. Physiol.* 286, E252–E260). We have shown that mitoNEET is a member of a small family of proteins containing a 39-amino-acid CDGSH domain. Although the CDGSH domain is annotated as a zinc finger motif, mitoNEET was shown to contain iron (Wiley, S. E., Murphy, A. N., Ross, S. A., van der Geer, P., and Dixon, J. E. (2007) *Proc. Natl. Acad. Sci. U. S. A.* 104, 5318–5323). Optical and electron paramagnetic resonance spectroscopy showed that it contained a redox-active pH-labile Fe-S cluster. Mass spectrometry showed the loss of 2Fe and 2S upon cofactor extrusion. Spectroscopic studies of recombinant proteins showed that the 2Fe-2S cluster was coordinated by Cys-3 and His-1. The His ligand was shown to be involved in the observed pH lability of the cluster, indicating that loss of this ligand via protonation triggered release of the cluster. mitoNEET is the first identified 2Fe-2S-containing protein located in the outer mitochondrial membrane. Based on the biophysical data and domain fusion analysis, mitoNEET may function in Fe-S cluster shuttling and/or in redox reactions.

Recent studies have revealed a critical role for mitochondrial function in glucose-stimulated insulin secretion (1). There is

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also a growing body of evidence implicating mitochondrial dysfunction in the development of insulin resistance and type 2 diabetes (2). Diabetic patients demonstrate evidence of oxidative stress (3) and have reduced mitochondrial mass and oxidative capacity in skeletal muscle (4, 5). Pioglitazone is a member of the thiazolidinedione class of insulin-sensitizing drugs frequently used to treat type 2 diabetes (6, 7). The thiazolidinedione drugs have traditionally been thought to function as ligands for the peroxisome proliferator-activated receptor γ (8, 9), although it is unclear whether peroxisome proliferator-activated receptor γ is the sole target of this family of drugs (10). Using tagged derivatives of pioglitazone incubated with mitochondrial lysates, Colca and colleagues identified a single cross-linked 17-kDa protein (11). They named the protein mitoNEET based on its location in the mitochondria and the presence of the amino acid sequence Asn-Glu-Glu-Thr (NEET) near the C terminus (11).

We have shown that mitoNEET is an integral outer mitochondrial membrane protein (12). It is localized to the mitochondria by an N-terminal targeting sequence, which acts as a membrane tether, resulting in the majority of the protein being exposed to the cytoplasm (12). mitoNEET belongs to a small family of proteins whose hallmark is the presence of a unique CDGSH domain. We refer to the other members of this family as Miner1 and Miner2 (for mitoNEET-related 1 and 2) (12). Both mitoNEET and Miner1 have one CDGSH domain, whereas Miner2 contains two. Although originally annotated as a zinc finger, the CDGSH domain, which has the consensus sequence CXCX₂(S/T)₃PXCDG(S/A/T)H, was shown to preferentially bind iron (12). Mitochondria from the hearts of mitoNEET-null mice demonstrated reduced oxidative capacity, suggesting a critical role for mitoNEET in proper mitochondrial function (12).

Here we investigated the properties of the recombinant mitoNEET protein. We found that the CDGSH domain of mitoNEET harbors a redox-sensitive 2Fe-2S cluster that is surprisingly pH-labile. Although there are more than 100 families of Fe-S-containing proteins, mitoNEET is unusual in having a Cys-3–His-1 coordination sphere for the 2Fe-2S cluster.

EXPERIMENTAL PROCEDURES

Construction of Bacterial Expression Plasmids—Construction of the pET28b-MBP-His-mitoNEET^{27–108} plasmid,³ encoding amino acids 27–108 of the mitoNEET protein, was previously described (12). The D84N, H87C, and H87Q mutants were generated in the pET28b-MBP-His-mitoNEET^{27–108} plasmid by site-directed mutagenesis using PCR. Recombinant human mitoNEET^{33–108} (lacking epitope tags) was generated by PCR and cloned into the pET21a⁺ vector. The C72S, C74S, and C83S mutants of mitoNEET^{33–108}

³ The abbreviations used are: MBP, myelin basic protein; EPR, electron paramagnetic resonance; PIPES, 1,4-piperazinediethanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; CHES, 2-(cyclohexylamino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

were generated by site-directed mutagenesis and cloned into the pET21a⁺ vector in-frame with the C-terminal His tag. For simplicity, the tagged and untagged proteins are collectively referred to as Δ -mitoNEET.

Expression and Purification of Δ -mitoNEET Fusion Proteins in *Escherichia coli*—Expression of wild-type MBP-His-mitoNEET^{27–108} and mutants in BL21-CodonPlus-RIL was as described previously (12). The Cys/Ser mutants of Δ -mitoNEET were grown as above, induced overnight at 23 °C, and (Ni-NTA)-purified using nickel-nitrilotriacetic acid agarose following the manufacturer's protocol (Qiagen).

Growth and expression of untagged mitoNEET^{33–108} protein was the same as that of MBP-His-mitoNEET^{27–108}, but the induction was extended to 7 h. The bacteria were lysed in Tb buffer (50 mM Tris-HCl, pH 8.0, 0.1% β -mercaptoethanol (v/v)) and clarified by centrifugation. All centrifugations were at 31,000 $\times g$ for 20 min. The Δ -mitoNEET protein was purified from the lysate using a series of salt precipitations. After an initial 25% (NH₄)₂SO₄ cut, mitoNEET was pelleted with 75% (NH₄)₂SO₄, resuspended in Tb buffer containing 25% (saturating) (NH₄)₂SO₄ and cleared by centrifugation. Following dialysis against Tb buffer, the Δ -mitoNEET solution was loaded onto an SP-Toyopearl 650M cation exchange column and rinsed with Tb buffer until the eluant was free of protein. The Δ -mitoNEET was eluted with 0.2 M NaCl in Tb buffer. At this point, the eluant had a well defined peak at 458 nm and an optical ratio (A_{278}/A_{458}) of \sim 3–4. The sample was dialyzed against TbN buffer (100 mM Tris-HCl, pH 8.0, 0.1% β -mercaptoethanol (v/v), 50 mM NaCl) for storage at 4 °C. The purity of all proteins was evaluated by SDS-PAGE, and concentrations were calculated using the extinction coefficients under denaturing conditions. The integrity of the holo-mitoNEET protein with the cluster was evaluated using UV-VIS spectroscopy.

Optical and Electron Paramagnetic Resonance (EPR) Spectroscopy—The optical spectra of recombinant MBP-His-mitoNEET^{27–108} was measured from the near UV to the near IR (250–1000 nm) on a Cary50 spectrometer (10–20 μ M protein in 50 mM Tris, pH 8.0, and 50 mM NaCl). Chemical reduction of Δ -mitoNEET was achieved by adding 2 mM dithionite to the protein solution. Reoxidation of Δ -mitoNEET was achieved by equilibrating with ambient O₂ for 1 h. The stability of the Δ -mitoNEET Fe-S cluster was monitored at 458 nm. Its decomposition was measured by an absorbance decrease over time under various pH conditions in a buffer mixture (5 mM each of citrate, PIPES, Tricine, CHES, and CAPS). The spectra for MBP-His alone was featureless above 400 nm (data not shown). EPR spectra of MBP-His-mitoNEET^{27–108} were measured in both the oxidized and the dithionite-reduced states using \sim 100 μ M protein in 100 μ l of Tb buffer in a Bruker Elexys E500 spectrometer. Following a change in color due to the addition of a few grains of solid dithionite, samples were submerged in liquid nitrogen until analysis on a Bruker EPR spectrometer at 9 GHz and low temperature (15°K).

Mass Spectrometry—Untagged mitoNEET^{33–108} (0.25 μ M) was desalted by applying 20 μ l of sample to a solid phase extraction pipette tip (TopTip, Glygen Corp.) containing 30 μ l of Poros20 reverse phase C18-polymer resin (Applied Biosystems). The sample was washed four times with 100 μ l of 10%

MeOH in H₂O (without buffer). The mitoNEET^{27–108} protein was eluted using four 50- μ l aliquots of 50% MeOH. The eluted protein was injected on the Hewlett-Packard 5989 electrospray mass spectrometer (Agilent) with 50% MeOH as the flow injection solvent. Aliquots 2 and 3 gave the maximum recovery by mass spec abundance. These fractions were combined and divided again, and one was acidified with formic acid (2% final concentration). Approximately 1 min after acidification, the mass spectrum was acquired.

Analysis of Prokaryotic Genomes—Amino acid sequences corresponding to the CDGSH domains of human mitoNEET (AAH59168) and Miner2 (EAW60525) were used to perform protein BLAST searches on the TIGR/CMR web site. The genomic organization of the prokaryotic protein hits were compared using the TIGR Genome Region Comparison feature to identify operons or gene clusters containing CDGSH domain (CD 47973) proteins. Domain composition of proteins was evaluated using the National Center for Biotechnology Information (NCBI) Conserved Domain Search.

RESULTS AND DISCUSSION

mitoNEET Binds a Redox-active 2Fe-2S Cluster—The outer mitochondrial membrane protein mitoNEET is the charter member of a small family of proteins containing a 39-amino-acid CDGSH domain (amino acids 55–93 in mitoNEET, Fig. 1A) (12). Although the CDGSH domain has been annotated as a zinc finger in the NCBI data base, our results indicated that the mitoNEET protein did not contain zinc as expected but instead bound iron (1.6 mol of iron/mol of protein) (12). To obtain a more discriminating view of the bound iron, the optical absorbance spectrum of Δ -mitoNEET (lacking the N-terminal hydrophobic, membrane-localizing portion of the protein, Fig. 1A) was measured. The MBP-His-mitoNEET^{27–108} and the untagged mitoNEET^{33–108} proteins displayed identical spectral properties in the visible region. For simplicity, they will be collectively referred to as Δ -mitoNEET; however, details about the protein constructs used for individual experiments are presented under "Experimental Procedures." The Δ -mitoNEET spectrum had peaks at 458 and 530 nm (Fig. 1B). This pattern resembled that of several types of 2Fe-2S cluster-containing proteins, such as ferredoxin (13) and the Rieske Fe-S protein; (14) (Fig. 1B). This result was the initial indication that the mitoNEET protein may contain an Fe-S cluster.

Because many Fe-S cluster-containing proteins are redox-active and function as electron transfer proteins (15), we tested whether Δ -mitoNEET could be reduced/oxidized *in vitro*. The absorption spectra exhibited changes expected for the reduction of a 2Fe-2S center (14). The peak at 458 nm showed a decrease of \sim 90% in the presence of the reducing agent dithionite (Fig. 1C). Subsequent exposure to oxygen resulted in complete recovery of the 458 nm peak, indicating reoxidation (Fig. 1C). The spectral changes suggested that Δ -mitoNEET contained an Fe-S cluster that can undergo oxidation/reduction.

To further elucidate the nature of the bound Fe-S cluster, the EPR spectrum of Δ -mitoNEET was obtained in the oxidized and reduced states (Fig. 1D). The absence of a signal in the oxidized state suggested that the Fe-S cluster has an even number of iron atoms with paired electrons that give rise to a ground

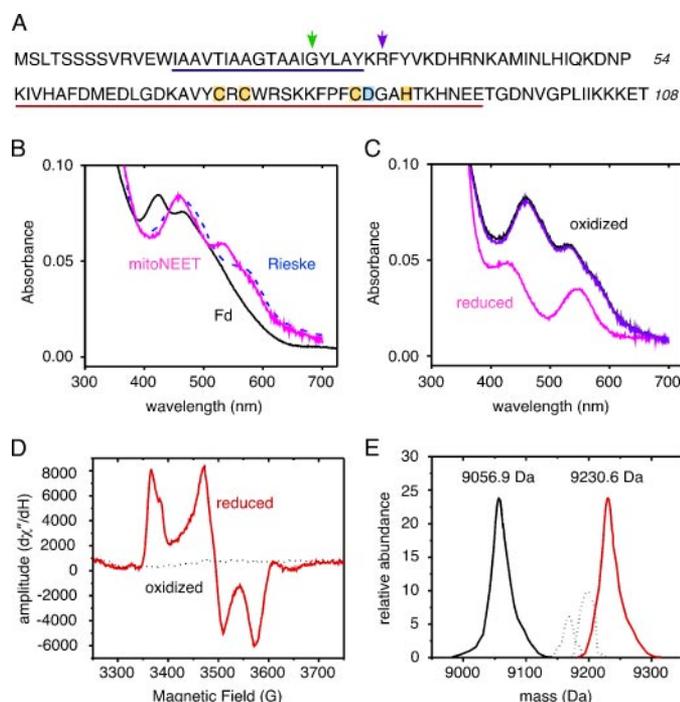


FIGURE 1. The CDGSH domain of mitoNEET binds a redox-active 2Fe-2S cluster. **A**, amino acid sequence of human mitoNEET with the Fe-S cluster ligands indicated in yellow and the Asp-84 indicated in blue. The CDGSH domain is highlighted in rust, and the transmembrane region is highlighted in blue. The N-terminal residue of the mitoNEET^{27–108} constructs is indicated by the green arrow, and that of the mitoNEET^{33–108} constructs is indicated by the purple arrow. **B**, optical spectra of mitoNEET (pink), ferredoxin from *Mastigocladus laminosus* (black), and the Rieske 2Fe-2S protein from *T. thermophilus* (blue) (19), showing the absorption range of Fe-S clusters in proteins. Spectra were scaled to have similar values for their respective peak in the 400–500 nm range. **C**, optical spectra of mitoNEET as isolated (black), after reduction with dithionite (pink) and subsequent oxidation with O₂ (purple). **D**, EPR spectrum of mitoNEET measured at 9 GHz in the oxidized (dotted) and dithionite-reduced (red) states. **E**, superimposed deconvoluted mass spectra of mitoNEET before (red, 9230.6 Da peak) and after acidification (black, 9056.9 Da peak) to release the 2Fe-2S cluster. The minor peaks (dotted) represented intermediates in the degradation of the cluster.

state with $S = 0$. The spectrum observed in the dithionite-reduced protein is indicative of a simple $S = \frac{1}{2}$, 2Fe-2S cluster with a single unpaired electron (Fig. 1D), although similar results could be obtained from a 4Fe-4S cluster (16). Collectively, these data demonstrate that Δ -mitoNEET binds either a 2Fe-2S cluster or a 4Fe-4S cluster that is redox-active.

To remove the ambiguity of the iron and sulfur stoichiometry of the cluster, we employed the accuracy of mass spectrometry. We also made use of the property that the Fe-S cluster of mitoNEET was lost when acidified (see below). The deconvoluted mass spectra of Δ -mitoNEET exhibited a peak at 9230.6 (± 0.2) Da (Fig. 1E). This is consistent with the amino acid sequence of mitoNEET (residues 33–108) and the presence of a bound co-factor containing two iron and two sulfur atoms. Upon acidification, the peak at 9230.6 (± 0.2) Da disappeared, and concomitantly, a peak at 9056.9 (± 0.2) Da appeared (Fig. 1E). The difference in these masses (173.7 ± 0.3 Da) corresponds to that expected for the extrusion of a single 2Fe-2S cluster. Together, these results showed that Δ -mitoNEET bound a single 2Fe-2S cluster.

The CDGSH Domain Binds the 2Fe-2S Cluster with an Unusual Cys-3–His-1 Coordination—A 2Fe-2S cluster is generally bound to a protein via four ligands (15). The most common coordinations are those seen in ferredoxin (Cys-4) and the Rieske (Cys-2–His-2) proteins. To determine the residues in Δ -mitoNEET that coordinate the 2Fe-2S cluster, we generated three mutant proteins, each with a single Cys to Ser substitution (Cys-72, Cys-74 and Cys-83) (Fig. 1A). In each case, the recombinant protein lacked any visible color, and the absorbance in the 300–500 nm range attributed to the 2Fe-2S cluster was absent (data not shown) as expected. The most probable candidates for the fourth amino acid ligand were His-87 and Asp-84 given their conservation in CDGSH domains (Fig. 1A). Thus, two Δ -mitoNEET mutants, D84N and H87Q, were constructed. The H87Q protein lacked any visible color and lacked any optical signature of a bound 2Fe-2S cluster, whereas the D84N protein exhibited an absorption spectrum similar to recombinant Δ -mitoNEET with a spectral signature characteristic of a 2Fe-2S cluster (Fig. 2A). The absence of stoichiometric iron in the H87Q mutant and presence of nearly 2-fold stoichiometric iron/mol of protein in the D84N mutant were confirmed by metal analysis (data not shown). These results indicated that Cys-72, Cys-74, Cys-83, and His-87 were the coordinating ligands of the 2Fe-2S cluster in Δ -mitoNEET.

Protonation of His-87 Facilitates Release of the 2Fe-2S Cluster of mitoNEET—The observation that the 2Fe-2S cluster absorbance was lost under acidic conditions led us to further investigate the stability of the Δ -mitoNEET protein as a function of pH. The stability of the 2Fe-2S cluster was followed by monitoring the absorbance between 350 and 550 nm. In particular, the integrity of the cluster was assessed at 458 nm, the peak attributed to the mitoNEET 2Fe-2S cluster. When recombinant Δ -mitoNEET was stored at pH 8.3, there was no detectable change in the absorption spectrum with time (data not shown). However, the 2Fe-2S cluster of Δ -mitoNEET was somewhat labile at a pH of 7.0 (Fig. 2B). Lowering the pH accelerated the loss of the cluster, as monitored by a change in the absorption at 458 nm (Fig. 2B). The 2Fe-2S cluster was significantly less stable at pH 4.5 when compared with pH 7.0 (Fig. 2B). The rate of loss of the 458 nm absorption peak was accelerated at pH < 7.0 (data not shown), indicating that a group(s) with a pK_a below 7.0 is important for the stability of the cluster.

To better characterize the unusual lability of the 2Fe-2S cluster, we compared the stability of Δ -mitoNEET, which contains a Cys-3–His-1 architecture, to that of a thermophilic cyanobacterial ferredoxin, which has a Cys-4 ligation. At pH 4.5, 50% of the 2Fe-2S of Δ -mitoNEET was lost within 30 min. We refer to this as the “half-life” of the cluster. Under similar conditions, a half-life of 7000 min was measured for ferredoxin (Fig. 2B). We thought this difference in stability, with respect to ferredoxin, was likely due to Δ -mitoNEET utilizing an amino acid other than Cys as the fourth ligand to coordinate binding of the 2Fe-2S cluster.

Both Asp-84 and His-87 are conserved in CDGSH domains throughout evolution. To investigate whether either of these amino acids was involved in the pH lability of the cluster, we optically monitored the stability of the 2Fe-2S cluster in the D84N mutant of Δ -mitoNEET and in a H87C mutant. Both the

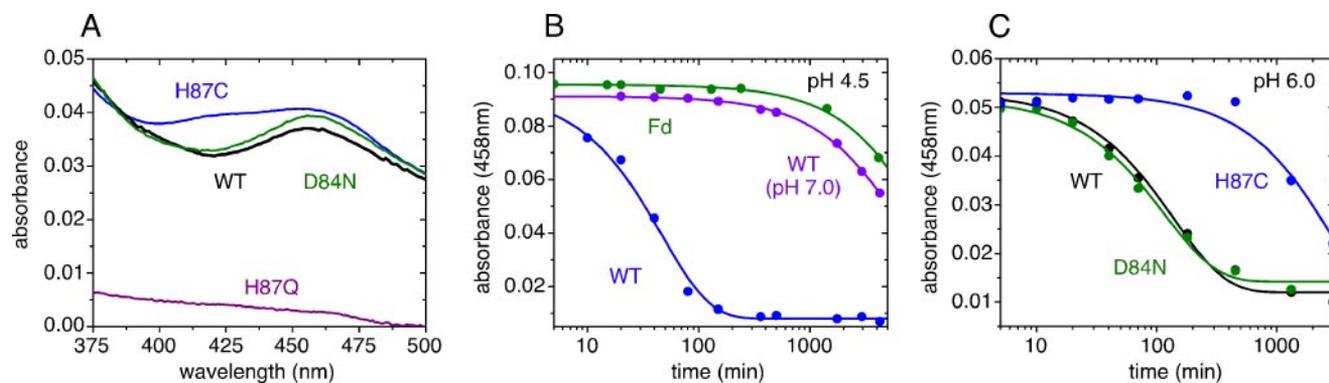


FIGURE 2. **The 2Fe-2S cluster of mitoNEET is pH-labile.** The absorbance and stability of the Fe-S cluster of mitoNEET was monitored at 458 nm in mutant proteins as a function of time at different pH. *A*, optical spectra of wild-type (WT) (black), D84N (green), H87C (blue), and H87Q (purple) mitoNEET proteins. *B*, stability of the 2Fe-2S cluster of mitoNEET at pH 7.0 (purple) and 4.5 (blue) when compared with that of ferredoxin (Fd), monitored at 420 nm at pH 4.5 (green). *C*, stability of the 2Fe-2S cluster of wild-type (black), D84N (green), and H87C (blue) mitoNEET at pH 6.0 as a function of time.

H87C and the D84N mutant proteins bound iron at a stoichiometry similar to the wild type and had a spectral signature characteristic of a protein containing a 2Fe-2S cluster (Fig. 2A). The D84N mutant demonstrated a pH decay profile indistinguishable from that of the wild-type Δ -mitoNEET protein (Fig. 2C). In contrast, the H87C mutation resulted in a profound change in the half-life at pH 6.0, increasing from 70 min for wild-type to 2000 min for the H87C mutant (Fig. 2C). Thus, changing the CDGSH domain of Δ -mitoNEET to a more typical Cys-4 ligation center resulted in a protein with increased stability of its 2Fe-2S cluster at low pH. This experiment indicated that protonation of His-87 upon acidification facilitates release of the 2Fe-2S cluster.

To the best of our knowledge, there are no other known examples of stable and reversibly reducible 2Fe-2S clusters coordinated by Cys-3–His-1 residues. In addition, both Miner1 and Miner2 share the same CDGSH motif, suggesting that all members of this family are likely to have similar residues coordinating the 2Fe-2S clusters and, consequently, similar biophysical properties. Preliminary experiments with recombinant Miner1 and Miner2, which are also red in color, revealed similar absorbance peaks, dithionite reducibility, and cluster lability that were comparable with those of mitoNEET (data not shown). The fact that CDGSH domains are conserved from bacteria to humans suggests that this unique coordination sphere for the 2Fe-2S cluster has been maintained for millions of years.

Possible Functions of mitoNEET—Pioglitazone is an insulin-sensitizing drug used to treat type 2 diabetes (7), and mitoNEET was originally identified as a binding target of pioglitazone. The protein consists of little more than a mitochondrial targeting sequence with a transmembrane tether and a cytoplasmic 2Fe-2S-containing CDGSH domain. Thus, it is reasonable to expect that the behavior of this domain *in vitro* will accurately reflect that of mitoNEET *in vivo*.

Functionally, the mitochondria from mitoNEET-null heart tissue have reduced oxidative capacity (12), although the mechanism by which mitoNEET achieves this effect is unclear. Two possible functions are suggested by its biophysical properties; namely, that mitoNEET may function in Fe-S cluster biosynthesis and/or as a redox protein. The propensity of mitoNEET to release its Fe-S cluster is conspicuous and suggests a possible role for mitoNEET in cytoplasmic Fe-S cluster assembly and/or



FIGURE 3. **Models of CDGSH-FMN fusion proteins.** Shown is a schematic representation of the domain architecture of representative prokaryotic proteins with an FMN-binding domain (cd02802) fused to tandem CDGSH domains (NP_800276), a rubredoxin domain (COG11773, YP_001087305), a ferredoxin domain (COG1146, YP_012500.1), a Rieske domain (cd03474, YP_511063), or a NapF domain (COG1145, YP_359561).

shuttling assembled Fe-S clusters out of the mitochondria. The link to the protonation state of His-87 would provide a convenient trigger for controlled cluster release. Although we protonate His-87 by lowering the pH *in vitro*, we envision that *in vivo*, another protein or cofactor could supply the proton. Although many of the proteins involved in the biosynthesis and shuttling of 2Fe-2S clusters are known, it is recognized that several key mitochondrial components remain to be elucidated (17). It is possible that mitoNEET, the first identified 2Fe-2S protein localized to the outer mitochondrial membrane, participates in these processes.

In addition, the 2Fe-2S cluster of mitoNEET is redox-active, suggesting that it can play a role in redox chemistry. To gain evidence that the CDGSH domain may function in cellular redox reactions, we applied the bioinformatics tools of domain fusion and phylogenetic analysis. This approach involves searching for proteins or domains of interest (e.g. CDGSH) in genomes of lower complexity (18). Often, domains fused into a single protein in prokaryotes are present in distinct interacting proteins in eukaryotes. In addition, prokaryotic genes present in operons or clusters suggest coordinate regulation and/or functional interactions. There are numerous prokaryotic proteins similar to mitoNEET/Miner1 and to Miner2 containing either a single CDGSH domain or tandem CDGSH domains, respectively. Many of the CDGSH domain-containing proteins are in gene clusters with sequences annotated as flavoproteins. Most interestingly, there are at least 17 different species of marine α and γ proteobacteria having tandem CDGSH domains fused to a flavin-binding domain (Fig. 3). This domain is most similar to the FMN-binding domain of glutamate syn-

these (supplemental Fig. 1). Our preliminary conclusion is that such fusions occur for functionally related proteins, which in this case would imply a role in redox reactions. Furthermore, it appears that in some prokaryotes, the CDGSH domain has been replaced in this FMN fusion protein with several other redox centers, including a rubredoxin, ferredoxin, Rieske, or NapF domain (all of which are motifs that bind redox-active Fe-S clusters) (Fig. 3) (19). If one assumes that the eukaryotic mitoNEET/Miner1 and Miner2 function similarly to these prokaryotic proteins, then it is likely that mitoNEET and other members of the family have redox/electron transport capability.

Finally, it is important to recall that mitoNEET was originally identified by cross-linking with a pioglitazone-derived photo-probe (11). Thus, it is tempting to suggest that the interaction with the drug could modulate the redox potential or the function of the CDGSH domain of mitoNEET. We are currently performing studies to investigate the structure of mitoNEET, as well as its *in vivo* function and its role in diabetes.

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