MitoNEET is a Uniquely Folded Outer Mitochondrial Membrane Protein Stabilized by Diabetes Drugs

The rise in obesity in the United States parallels a dramatic increase in obesity-associated diseases, most notably type-2 diabetes. This disease is predicted to reach epidemic proportions in the next several decades (Zimmet *et al* 2001, Urek *et al* 2007). Thus, understanding the biochemical processes underlying type-2 diabetes and identifying new targets for therapeutic intervention are critical for national and world health. A drug of choice to treat type-II diabetes is pioglitazone, a thiazolidinedione (TZD) derivative originally thought to exert its effect through activation of the nuclear transcription factor PPARγ. Recently, a novel protein target for pioglitazone was discovered and was called *mitoNEET* (Colca *et al*. 2004). This protein is anchored to the *outer* mitochondrial membrane (OMM) (Wiley *et al* 2007). Contrary to predictions that this was a zinc-finger transcription factor we discovered that *mitoNEET* is a novel 2Fe-2S protein.

In an effort to understand the structural properties of this protein, a soluble form of recombinant human *mitoNEET* was crystallized in an orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 46.81 Å, b = 49.62 Å, c = 59.01 Å (Paddock *et al* 2007). The structure was determined by x-ray diffraction from 1.5 Å resolution data collected from SSRL Beamline 9-2. Initial phasing was obtained from Fe-MAD (multiwavelength anomalous dispersion) datasets collected at wavelengths corresponding to the inflection, high energy remote, and absorption peak of Fe. Data was processed using automated MAD script developed at SSRL. The structural model was refined to an R-factor = 18.2 %.

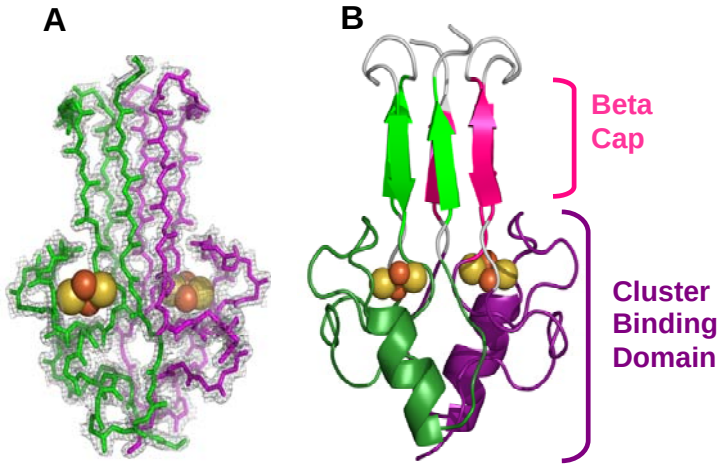

Figure 1. Overall structural organization and domain topology of dimeric *mitoNEET*. (A) The backbone tracing of each protomer colored in green and magenta, respectively, together with the observed 2Fo-Fc electron density (grey) map contoured at 1.5σ. A 2Fe-2S cluster is present in each protomer, and is depicted in CPK rendition as yellow (sulfur) and red (iron) spheres. The protomers pack in a parallel fashion. The N-termini and C-termini are indicated. **(B)** Ribbon diagram highlighting the two domains and protomer interactions within the *mitoNEET* dimer: a six stranded beta sandwich forms the intertwined beta cap domain and a larger cluster binding domain carries two 2Fe-2S clusters (one per protomer).

The crystal structure reveals that *mitoNEET* folds into a unique homodimeric structure with one 2Fe–2S cluster bound to each monomer within the dimmer (Figs. 1 & 2). A structural similarity search revealed that this fold is novel when compared with the >650 known Fe–S proteins, and it is also unique when compared with the >44,200 known members of the structural databases. The protein is folded into two spatially distinct subregions: a β-rich or "β-cap" domain and a helical 2Fe–2S binding or "cluster-binding" domain (Fig. 1*B*). The β-rich domain contains a strand swap from opposite ends of the primary sequence to form the β-cap structure (Fig. 1*B*).

A prominent feature of the structure is the presence of two 2Fe–2S clusters that are separated by ≈16 Å from each other within the larger helical cluster-binding domain (≈30 Å across) (Fig. 2). The *mitoNEET* dimer has an unusual distribution of aromatics (Fig. 2B) forming a ring around the central b-sheet region and charges (Fig. 2C) that create an internal macro-dipole. Three Cys residues and one histidine residue on each monomer are ligands to the 2Fe-2S clusters. Interestingly, *mitoNEET* shares this unusual 3Cys cluster coordination with the structurally unrelated cluster scaffold protein IscU (Ramelot *et al* 2004).

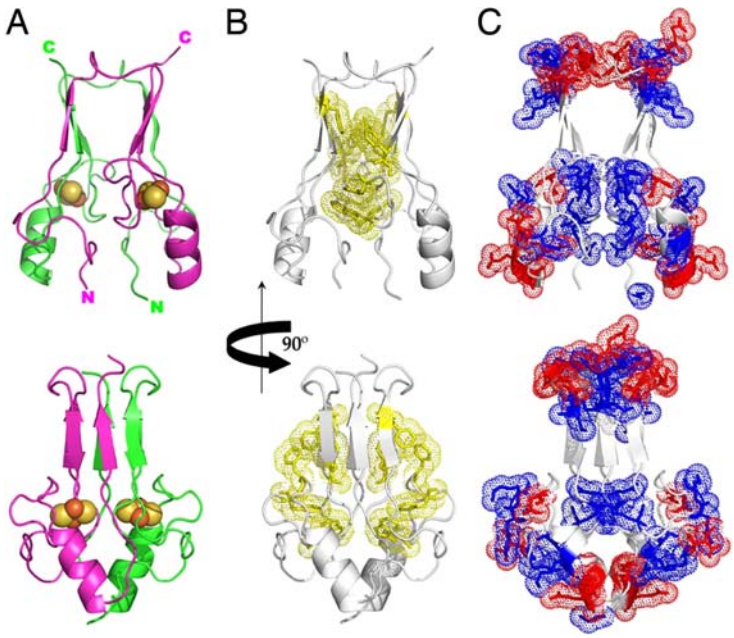

Figure 2. The overall distribution of charges within *mitoNEET* create a macro-dipole separated by hydrophobic belt. **(A)** The ribbon diagram of *mitoNEET* is displayed in two orientations. The bottom view is rotated 90° from the top along the vertical axis shown in the center. The protomers of the unit are colored in green and purple, respectively. Each 2Fe-2S cluster is shown with yellow (sulfur) and red (iron) spheres. **(B)** The ribbons of each protomer are colored grey and the packing of the ten aromatic residues (five from each protomer) are emphasized by yellow dots. Apolar residues are also localized to this region, but are not shown. **(C)** The separation of charged residues in *mitoNEET* indicates segregation of the aromatic and apolar regions of the protein. The negatively charged residues are labeled in red and the positively charged residues in blue. The upper panel emphasizes both the asymmetry of charges within the interior of the molecule and the separation of these charges by the nonpolar residues (panel B).

Complementary biophysical investigations showed that the cluster is redox active and labile below pH 8.0 (Wiley *et al* 2007), characteristics possibly related to its function. Optical and NMR experiments demonstrated that the presence of the diabetes drug pioglitazone increased the stability by ≈10-fold. The unusual lability was associated with the coordinating ligand His-87 (Wiley *et al* 2007), which cannot serve as a stabilizing ligand for the 2Fe–2S when protonated. This unusual characteristic of the protein raises the interesting possibility that *mitoNEET* participates in Fe–S cluster assembly or storage. We predict that the cluster-binding domain is situated near the OMM *in vivo* placing *mitoNEET* in a unique position to receive and transfer clusters that have crossed the outer mitochondrial membrane, a process that is not presently fully understood.

Although TZDs activate peroxisome proliferator-activating receptors (PPARγ), data suggesting alternative modes of action involving mitochondria has accumulated. Our structural results may have important implications for both mechanisms of drug action and future optimization of TZDs, especially in light of the fact that the structures of PPARγ and mitoNEET are nearly completely dissimilar. Whether the beneficial effects of TZDs on mitochondria including biogenesis and normalization of lipid oxidation are mediated through *mitoNEET* is unknown. However, these data, combined with those of Colca *et al.* (1994), suggest that pioglitazone can bind and alter the properties of *mitoNEET* that is expressed in many insulin-responsive tissues. Although further biological and biophysical experiments are

needed to relate *in vitro* binding to *in vivo* effects, *mitoNEET* may prove to be an alternative target for drug actions.

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