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Feature article

Multinuclear NMR and UV–Vis spectroscopy of site directed mutants of the diabetes drug target protein mitoNEET suggest that folding is intimately coupled to iron–sulfur cluster formation

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ABSTRACT

Further understanding of the mitochondrial protein mitoNEET could lead to advancements in drug design for the treatment of mitochondrial diseases. Previous studies have shown that mitoNEET's iron–sulfur cluster plays a key role in its biochemistry. In order to gain insight into the structural stability and folding of mitoNEET's iron–sulfur clusters, mutants were created using site-directed mutagenesis. NMR and UV–Vis spectroscopic analysis of these mutants suggested that half had successfully hindered protein folding that in turn, increased lability and eventually loss of functional iron–sulfur clusters. Understanding the significance of this coupling of lability to misfolding could be key to learning mitoNEET's mode of action in mitochondria. These findings may allow mitoNEET to be utilized for therapeutics and a better understanding of mitochondrial-based diseases.

Contents

1. Background

MitoNEET, a homodimeric iron sulfur cluster (2Fe–2S) containing protein associated with the outer mitochondrial membrane, was initially discovered by an independent study of glitazones interacting with peroxisome proliferator-activated receptor gamma (PPAR-γ). Cross-linking of pioglitazone to the proteome revealed mitoNEET as a site of thiazolidinedione (TZD) interaction [1]. Elucidation of the independent action of PPAR-γ led to the discovery of mitoNEET as a PPAR-γ nuclear receptor subtype. This outer mitochondrial membrane protein has been found to bind to pioglitazone, a TZD that is used in the treatment of type II diabetes [2,3]. In addition, mitoNEET is expressed in many insulin-responsive tissues [3]. This protein also has been determined to possess a role in energy metabolism. Deletion of mitoNEET in mice

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reduced oxidative phosphorylation potential of mitochondria [4]. Locations and general ligand binding interaction models for binding sites led to the discovery of novel mitoNEET ligands. The discovery of mitoNEET has resulted in the identification of many different homologs (Fig. 1). These have been collectively referred to as CDGSH –type iron sulfur domains based upon their characteristic iron sulfur cluster sequence. Our study of mitoNEET is based on screening of fold-critical residues *via* site-directed mutagenesis and heterologous expression to modify this protein and purify six different mutant forms.

Accumulating evidence indicates that many of the clinical effects of TZDs are a result of the drugs interacting with mitoNEET [2].The discovery of mitoNEET was partially driven by the fact that TZDs had effects that could not be explained solely by PPAR-γ binding [5]. MitoNEET knockouts showed reduced effects of TZDs as well as increased reactive oxygen species, while PPAR-γ knockouts did not block the effects of TZDs [6]. Conversely mitoNEET knockdown in mice showed an increase in reactive oxygen species and a decrease in glucose tolerance, while overexpression of mitoNEET leads to low levels of ectopic fat deposition and low levels of reactive oxygen species [7]. MitoNEET's ability to reduce reactive oxygen species from complex I may be useful to treat diseases caused by oxidative tissue damage such as Parkinson's disease, stroke, and Alzheimer's [5]. It is possible to design drugs targeted for mitoNEET that are not structurally similar to TZDs that could reduce the undesirable side effects associated with TZDs [2].This is due to the fact that compounds lacking the TZD ring moiety bind to mitoNEET with similar or higher affinity as compared to TZD ligands such as pioglitazone and rosiglitazone that are used in conjunction with PPAR-γ [5].

The mitoNEET 2Fe–2S has a unique structure compared with other iron sulfur clusters. The two most common 2Fe–2S are ferredoxin clusters, composed of four cysteine residues, and Rieske type clusters, composed of two histidine and two cysteine residues (Fig. 2). The presence of a single histidine residue (H87) is crucial to many of the unique properties of the mitoNEET cluster. H87 is in the same conformation as the histidines in the Rieske type cluster [8]. Protonation of H87 is important in the ability of the protein to transfer its cluster, as well as determining the redox potential [9,10,11]. For 2Fe–2S clusters there are two common redox states: reduced as $Fe(III)_2$ and oxidized as Fe(III)Fe(II). It has been found that at a pH of 7, mitoNEET's 2Fe–2S had a redox potential of +25 mV, which is 180 mV higher than the cytoplasm, indicating a reduced 2Fe–2S cluster under physiological conditions [10]. The protonation of H87 at a pH lower than 7 causes the cluster to become unstable [12]. Both TZDs and the H87C mutation affect this by making the oxidized form more stable [13]. By mutating the H87 residue into a cysteine, the redox potential of the cluster can drop by as much as 300 mV making a stabilized oxidized form [10,13]. The redox state of mitoNEET's cluster can be regulated by thiols and hydrogen peroxide as hydrogen peroxide reversibly oxidizes the cluster while thiols reduce the cluster [14]. In addition to thiols, NADPH is also capable of reducing the cluster [15]. Furthermore, NADPH has been shown to disrupt the interactions between the monomer subunits of the protein. NADPH binds to two residues, K55 and H58, where they interact with coordinating residues on the opposing monomer, H87 and R73, without directly binding to the cluster loop [16]. It has been suggested that the variable redox state of mitoNEET and its large cohort of interacting molecules make it a perfect candidate for a molecular level redox sensor [13]. The transfer of its iron–sulfur cluster could be a key to such function [17]. Thus studies of the structural and biophysical details of cluster stabilization and transfer may be critical to understanding its mechanism of action.

MitoNEET and its paralogs are unique from other 2Fe–2S cluster containing proteins in that its 2Fe–2S clusters are liganded within a 3-Cys-1-His cluster cradle [18,20]. The 2Fe–2S clusters of mitoNEET are coordinated by C72, C74, C83, and H87 (Fig. 2b) [21]. These clusters produce absorption maxima at 458 nm, characteristic of typical 2Fe–2S clusters [12]. In order to provide stability between protomers, hydrophobic and aromatic residues reside predominantly within the 2020 $Å^2$ interface of the formed homodimer (Fig. 3, center) [22] however two symmetrical, buried water molecules coordinate hydrogen bond formation within the hydrophobic core. R73 of one protomer interacts with the main-chain oxygen atoms of P81 and the 2Fe–2S coordinating C72 of the other protomer *via* these hydrogen bonds [21]. Each protein is composed of a helical turn, an α-helix, an anti-parallel β-structure with a strand swap, and 11 interconnecting β-turns and loops [22]. The intertwining of a swapped strand (β1) and loop (L1) from each protomer form the β-cap and cluster-binding domains [18,22].

Mutations to amino acid residues crucial to folding of the native structure of mitoNEET may cause disruption in protein stability and

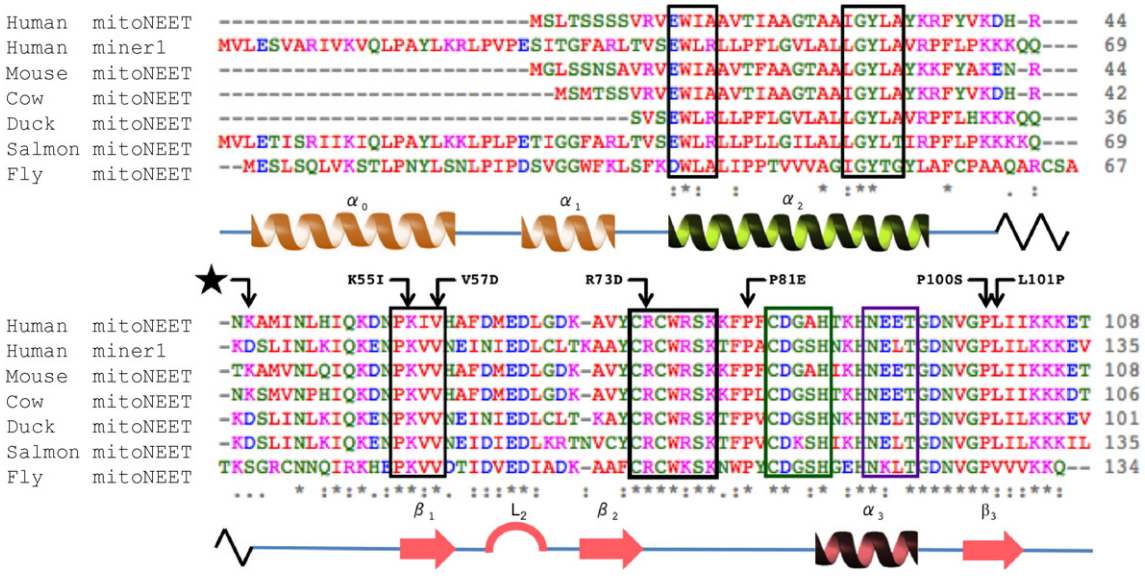

Fig. 1. Aligned are the sequences of seven representative CDGSH motif-containing proteins from vertebrates. The CDGSH motif is boxed and outlined in green. The NEET motif that gives mitoNEET its name is boxed and outlined in purple, and the highly conserved regions are boxed and outlined in black. The ribbon structure depicts the secondary structure beneath the aligned sequences. The frustration loop in the sequence is labeled L_2. The hypervariable region of the sequence is denoted as the black saw-tooth pattern. The n-terminal gold and green alpha helices are predicted using *PredictProtein* and are not present in crystal structures of mitoNEET. The green α_2 helix is predicted to be the transmembrane mitochondrial targeting sequence. The α_0 helix is only present in some NEET proteins. The star represents the start of the known crystal structure as found in *RCSB Protein Data* Bank (PDB: 2QH7). Mutations analyzed in his work are shown by their mutant name and arrows corresponding to their sequence location.