Facile transfer of [2Fe-2S] clusters from the diabetes drug target mitoNEET to an apo-acceptor protein

John A. Zuris1,†, Yael Hariri1,†, Andrea R. Conlan4, Maya Shvartsman3, Dorit Michaeli3, Sagi Tamir3, Mark L. Paddock4, José N. Onuchica2, Ron Mittleman2, Zvi Ioav Cabantchik6, Patricia A. Jennings2, and Rachel Nechushtai1,2

1 Departments of Chemistry and Biochemistry and Physics, University of California at San Diego, La Jolla, CA 92093; 2 The Alexander Silberman Institute of Life Science, Hebrew University of Jerusalem, Edmond J. Safra Campus at Givat Ram, Jerusalem 91904, Israel; 3 Department of Biology, University of North Texas, Denton, TX 76203

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MitoNEET (mNT) is an outer mitochondrial membrane target of the thiazolidinedione diabetes drugs with a unique fold and a labile [2Fe-2S] cluster. The rare 1-His and 3-Cys coordination of mNT’s [2Fe-2S] leads to cluster lability that is strongly dependent on the presence of the single histidine ligand (His87). These properties of mNT are similar to known [2Fe-2S] shuttle proteins. Here we investigated whether mNT is capable of cluster transfer to acceptor protein(s). Facile [2Fe-2S] cluster transfer is observed between oxidized mNT and apo-ferrodoxin (a-Fd) using UV-VIS spectroscopy and native-PAGE, as well as with a mitochondrial iron detection assay in cells. The transfer is unidirectional, proceeds to completion, and occurs with a second-order-reaction rate that is comparable to known iron-sulfur transfer proteins. Mutagenesis of His87 with Cys (H87C) inhibits transfer of the [2Fe-2S] clusters to a-Fd. This inhibition is beyond that expected from increased cluster kinetic stability, as the equivalently stable Lys55 to Glu (K55E) mutant did not inhibit transfer. The H87C mutant also failed to transfer its iron to mitochondria in HEK293 cells. The diabetes drug pioglitazone inhibits iron transfer from WT mNT to mitochondria, indicating that pioglitazone affects a specific property, [2Fe-2S] cluster transfer, in the cellular environment. This finding is interesting in light of the role of iron overload in diabetes. Our findings suggest a likely role of iron overload in diabetes. This finding is interesting in light of the role of oxidative stress. This result is beyond that expected from increased cluster kinetic stability, as the equivalently stable Lys55 to Glu (K55E) mutant did not inhibit transfer. The H87C mutant also failed to transfer its iron to mitochondria in HEK293 cells. The diabetes drug pioglitazone inhibits iron transfer from WT mNT to mitochondria, indicating that pioglitazone affects a specific property, [2Fe-2S] cluster transfer, in the cellular environment. This finding is interesting in light of the role of iron overload in diabetes. Our findings suggest a likely role of iron overload in diabetes. This finding is interesting in light of the role of oxidative stress.

Results

Oxidized mNT, but Not Reduced, Can Transfer Its [2Fe-2S] Cluster to an Apo-Acceptor Protein. We chose a-Fd for cluster transfer experiments because it efficiently transfers its [2Fe-2S] cluster to a-Fd. Thus, His87 is critical to efficient cluster transfer. In addition, the transfer rate is orders of magnitude faster than expected from simple release and capture and is quantitative with no apparent loss to degradation that is commonly observed with FeS clusters in solution (16). These results indicate that specific protein–protein interactions between mNT and a-Fd facilitate cluster transfer. As mNT is a target of TZDs we tested its cluster transfer ability in the presence/absence of pioglitazone in permeabilized human embryonic kidney (HEK293) cells. Pioglitazone inhibits transfer suggesting a possible antiadibetic mode of action for the drug under oxidizing conditions.

The authors declare no conflict of interest.

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†To whom correspondence may be addressed. E-mail: rachel@vms.huji.ac.il or pajennings@ucsd.edu or jonuchic@ucsd.edu.

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Fig. 1. Facile transfer of the [2Fe-2S] cluster from mitoNEET (mNT) to apo-ferredoxin (a-Fd). The presence of the [2Fe-2S] cluster in mNT can be observed by UV-Vis spectroscopy with a signature peak at 458 nm and in Fd at 423 nm. Frames A through D show the cluster transfer reaction progress. The observed spectra for the combined species are shown as a black line at the top of each frame while the deconvoluted spectra of holo-mNT (red) and holo-Fd (orange) are shown below. (A) Upon approaching completion, the visible spectrum resembles that of Fd, as all a-Fd has been converted to the holo form and mNT has been converted to a-Fd lacking apo-Fd. The upper right corner in each spectrum indicates the percent completion for the cluster transfer reaction. (B) Transfer of mNT’s [2Fe-2S] cluster to a-Fd is also observed by native-PAGE. Upon incubation with a-Fd, mNT shows diminished color, indicating cluster loss over time as the [2Fe-2S] cluster is transferred to a-Fd and the presence of a smaller molecular weight red-colored band indicating formation of holo-Fd. (C) The UV–Vis spectra of the [2Fe-2S] cluster from which the transfer of mNT’s [2Fe-2S] cluster to a-Fd could be easily followed. The UV-Vis spectra of the [2Fe-2S] cluster being transferred changes over time upon mixing of prereduced a-Fd and oxidized mNT under oxidizing conditions as it switches its ligand environment. Deconvolution of the overall spectra show the reaction at 10%, 25%, 50%, and 90% completion (Fig. 1 A–D, respectively). These percentages are directly measured from ratio of the peak intensities at 423 nm (Fd) and 458 nm (mNT). Based on initial concentrations of mNT and the final concentration of Fd, we conclude that the [2Fe-2S] cluster transfer was complete and suffers no appreciable loss to solution. This finding was corroborated by native gel electrophoresis (Fig. 1 E and F, Upper), followed by colorimetric and 55Fe-labeled [2Fe-2S] detection (Fig. 1F, Lower). Holo-mNT’s red-colored band intensity decreased over time when incubated with a-Fd, and the latter is concomitantly converted to the red-colored holo-Fd band. The levels of mNT and a-Fd/Fd remained unchanged as shown by Coomassie staining (Fig. 1E, Lower).

UV-Vis-monitored cluster transfer experiments show that transfer readily occurs under oxidizing conditions as both the initial mNT spectra (Fig. 1A) and final Fd spectra (Fig. 1D) reflect those of each of the respective oxidized species. As mNT’s cluster binding domain on the outer mitochondrial membrane faces the cytosol (7), we wanted to see if transfer was inhibited under reducing conditions like those normally found in this cellular environment. Because both oxidized and reduced mNT have distinct spectra both from each other and Fd we could follow transfer both under oxidizing as well as reducing conditions. Reduced mNT shows no cluster transfer to a-Fd over a period of 500 min (Fig. S1A). Upon addition of oxygen mNT becomes oxidized, as seen by the characteristic oxidized spectra (Fig. S1B) and readily transfers its [2Fe-2S] cluster to a-Fd (Fig. 2). These results show that mNT’s oxidation state regulates whether it can transfer its cluster to a-Fd, and that this cluster transfer capability may normally be inhibited in the reduced cytosolic environment.

His87 Is Critical for Cluster Transfer Between mNT and a-Fd. A projection of mNT’s cluster binding site shows the rare 3-Cys-1-His ligand environment around each of the [2Fe-2S] clusters in mNT (Fig. 3A). We replaced the coordinating His87 with Cys (H87C) to test the role of this residue in cluster transfer. In addition, we replaced Lys55 with Glu (K55E) because this change dramatically alters the properties of the cluster (31).

Time-dependent spectroscopic changes measured by UV-Vis absorption spectroscopy allowed us to quantitatively measure the rate of cluster transfer for WT mNT and the K55E and H87C mutants. Cluster transfer is slowed only slightly in the K55E mutant whereas the transfer rate was significantly retarded by replacement of the single-coordinating His87 with Cys (Fig. 3B and C and Table 1). The transfer rates are distinct from passive decay because (i) the transfer rate to a-Fd is concentration dependent, whereas passive decay is not (Fig. S2A and B), and (ii) the transfer rate is many orders of magnitude faster than passive cluster release (Table 1), hence the reaction is catalyzed. We observed no correlation between kinetic cluster stability and cluster transfer rate because the K55E shows transfer rates similar to WT mNT.
similar to the well-characterized FeS assembly/transfer protein rhodamine B-[\{(1,10-phenanthrolin-5-yl)-aminocarbonyl\} benzyl calcein-green (CALG) as tracer for the cytosolic iron, and red introduction of mNT. We used gently permeabilized HEK293 [2Fe-2S] cluster to an apo-acceptor, we asked if it could be a player in facilitating cluster transfer and provide direct evidence of the cluster binding site shows directly coordinating Cys and His ligands (Table 1), yet its kinetic cluster stability is similar to that of the H87C mutant (Table 1 and Fig. S3). The observed cluster transfer rate for mitoNEET is protein concentration dependent with a measured rate constant of 185 ± 11 M⁻¹ min⁻¹, a rate constant similar to the well-characterized FeS assembly/transfer protein ISA (170 M⁻¹ min⁻¹, Table 1) (35).

The Diabetes Drug Pioglitazone Inhibits Iron Transfer from mNT to Mitochondria. Having demonstrated that mNT can transfer its [2Fe-2S] cluster to an apo-acceptor, we asked if it could be a source of free iron in cells. We used cytosolic and mitochondrial fluorescent indicators to assess changes in free iron levels upon introduction of mNT. We used gently permeabilized HEK293 cells that were double labeled with the fluorescent iron sensors calcine-green (CALG) as tracer for the cytosolic iron, and red rhodamine B-[(1,10-phenanthrolin-5-yl)-aminocarbonyl] benzyl ester (RPA) as a tracer for iron in the mitochondrial matrix (Fig. 4A). The changes in fluorescence in response to the addition of mNT are shown in Fig. 4B. Quenching of RPA, but not CALG, occurred rapidly and was directly proportional to mNT’s concentration (Fig. 4C) indicating transfer of iron to the mitochondrial matrix. Preincubation of mNT with a-Fd prevented iron transfer into mitochondria (Fig. 4D). We tested the importance of increased cluster stability introduced by mutation or drug binding to iron transfer in the cellular system. The K55E mutant is equivalent to the wild type for iron transfer to the mitochondria whereas H87C mutant inhibits transfer (Fig. 5A). Importantly, the addition of pioglitazone to WT inhibited iron transfer (Fig. 5B), indicating a PPARγ-independent effect of TZDs.

Discussion

The rare 3-Cys-1-His ligation geometry of the [2Fe-2S] cluster of mNT (6, 13, 36, 37) is only observed in a few other cases, notably in a mutant version of the FeS cluster transfer protein IscU (38), and because the role of IscU is important in FeS cluster assembly we investigated whether mNT can perform cluster transfer. In order to assess this hypothesis, we examined the cluster transfer potential of mNT using a-Fd, a universal acceptor protein used in FeS studies (35). We demonstrate that mNT’s [2Fe-2S] cluster is transferable to a-Fd (Fig. 1 A–F) at a rate constant (185 ± 11 M⁻¹ min⁻¹) equivalent to the cluster transfer protein ISA (170 ± 8 M⁻¹ min⁻¹) (35). In addition, we find that replacement of the single His ligand to Cys inhibits the process, emphasizing the importance of this coordination in efficient transfer.

We also show that increased levels of mNT lead to Fe accumulation in mitochondria as reported by RPA fluorquinclenching assays in HEK293 cells (Fig. 5A). Fe accumulation is observed with the K55E mutant but not with the H87C mutant protein. Because K55E and H87C have similar cluster stabilities it is clear from this data that His87 is an essential mediator of cluster transfer in cells. The fact that Fe accumulates in the mitochondrial matrix suggests there is a means by which mNT, tethered to the outer mitochondrial membrane, transfers Fe into the matrix. The mechanism in vivo is currently under investigation. Taken together, our findings in the cellular system, which are similar to that observed in vitro, underscore the fact that the conserved His87 ligand facilitates transfer.

TZD cross-linking studies led to the discovery of the mitochondrial target, mNT (1). This target is completely distinct from the accepted paradigm protein, the nuclear transcription factor PPARγ. Here, we report the direct effect of TZD binding to mNT in cellular systems. We show that pioglitazone is capable of regulating Fe accumulation in mitochondria in this study. In addition to providing a cellular readout for TZD binding, this data provides a direct link between previous in vitro observations (6, 8) and our current in vitro and cellular findings. We now present a model describing a possible therapeutic mode of action for pioglitazone (Fig. 6). Because the cytosol is normally highly reducing (39), mNT would be expected to be predominantly in the reduced state. Changes to the cytosolic redox potential, which occur when cells are under oxidative stress (40), could induce transfer of mNT’s [2Fe-2S] cluster. If not carefully regulated, this could lead to Fe overload stress, which is a problem in patients with type II diabetes (41). Pioglitazone, which shows a strong preferential binding to mNT in the oxidized state (8), may act to alleviate this stress.

Conclusions and Future Directions

We show that oxidized mNT transfers [2Fe-2S] clusters readily and efficiently, and that reduction of the cluster inhibits transfer. In addition, we show that the hallmark His87 ligand is a critical player in facilitating cluster transfer and provide direct evidence for a functional effect of TZD binding to this protein in cells. Our findings raise a set of interesting questions about this class of

Table 1. Comparison of mNT cluster transfer rates with mutants and ISA

<table>
<thead>
<tr>
<th>Cluster decay half-time (min⁻¹)</th>
<th>Initial transfer rate (M⁻¹ min⁻¹)</th>
<th>Transfer rate at 160 μM [mNT] (min⁻¹)</th>
<th>Catalytic enhancement of transfer rate at 160 μM [mNT] (lower limit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>10⁻⁵</td>
<td>185 ± 11</td>
<td>0.03</td>
</tr>
<tr>
<td>K55E</td>
<td>10⁻⁶</td>
<td>115 ± 4</td>
<td>0.02</td>
</tr>
<tr>
<td>H87C</td>
<td>10⁻⁶</td>
<td>&lt;1</td>
<td>NA</td>
</tr>
<tr>
<td>ISA</td>
<td>NA</td>
<td>170 ± 8*</td>
<td>NA</td>
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*Experiment performed at 25°C on iron-sulfur cluster assembly protein (abbreviated as IscA or ISA) (35).
[2Fe-2S] proteins. Notably, do mNT proteins play a role in [2Fe-2S] cluster transfer along with the mitochondrial iron-sulfur cluster assembly (ISC) or cytosolic iron-sulfur cluster assembly (CIA) machinery (17, 42), as mNT’s unique position on the outer mitochondrial membrane suggests it could be useful as a conduit between the cytosol and the mitochondria? Is mNT an [2Fe-2S]/Fe reservoir that provides clusters upon demand (6)? It is possible that mNT acts as a redox sensor and transfers its cluster only when in the oxidized state, such as when cells are stressed. It was long assumed that the TZD class of antidiabetes drugs acted only in a PPARγ-mediated fashion. Because pioglitazone inhibits

![Fig. 4. Transfer of [2Fe-2S]/Fe from mNT to mitochondria in permeabilized HEK293 cells. HEK293 cells labeled with iron sensors RPA (for mitochondria) and calcein-green (CALG) (for cytosol) were permeabilized (A) and used for tracing changes in fluorescence by fluorescence microscopy imaging (B and C) following addition of mNT in succinate medium. (B) A time series of fluorescence [pseudocolor images; RPA fluorescence intensity is given in arbitrary units (a.u.);] following addition of 50 μM mNT and FHQ (6 μM) at different times (indicated by * or ** respectively). (C) The fluorescence traces (reported as a.u.) represent time series taken for cells exposed to different concentrations of mNT. (D) RPA fluorescence 20 minutes after the addition of 10 μM mNT under different conditions. The black bar represents a fluorescence trace from control cells where no additions were made. The red bar represents the fluorescence of cells after mNT addition, the purple bar shows the addition of mNT. In this experiment a-Fd is pretreated with DTT to reduce the free cysteines and prevent inter-disulfides. After 30 min the free DTT is depleted at which point mNT is added. The orange bar represents the fluorescence of cells after addition of a-Fd only. All data are represented as mean ± standard error (SE). The statistical differences between treatments were determined by the paired t test at significance levels *p < 0.05 or **p < 0.01. (N = 14).](image)

![Fig. 5. Transfer of [2Fe-2S]/Fe from mNT’s mutants to permeabilized HEK293 cells labeled with RPA. (A) RPA fluorescence was measured 20 min after the addition of 10 μM of WT mNT or mutants: WT mNT (black), K55E (blue), and H87C (red) bar, respectively. The green bar represents a fluorescence trace from control cells with no additions. Data are represented as mean ± standard error (SE). (N = 17). (B) The antidiabetic drug, pioglitazone, inhibits the Fe/Fe-S transfer from mNT to mitochondria in permeabilized HEK293 cells. Permeabilized cells loaded with RPA were examined by fluorescence microscopy as previously described. The data represent RPA levels 20 min after the addition of: 10 μM mNT (black), mNT (10 μM) preincubated with pioglitazone (100 μM), noted as mNT ∗ pio, (purple bar), and a fluorescence trace from control cells with no additions (green bar). Data are represented as mean ± standard error (SE). The statistical differences between treatments were determined by paired t test at significance levels p < 0.01. (N = 4).](image)

![Fig. 6. Model describing a possible therapeutic mode of action for pioglitazone. Because the cytosol is normally highly reducing (39), we expect mNT to be predominantly in the reduced state. Changes to the cytosolic redox potential, which occur when cells are under oxidative stress (40), could induce transfer of mNT’s [2Fe-2S] cluster. If not carefully regulated, this could lead to Fe overload stress, which is a problem in patients with type II diabetes (41). Pioglitazone, which shows a strong preferential binding to mNT in the oxidized state (8), may act to alleviate this stress.](image)
this biochemical process it is clear that determining mNT’s protein partners and a better understanding of how the protein–drug interaction relates to alleviating the negative effects of type II diabetes will be of great importance in the future.

Methods

All the materials used in this work were from best available commercial grade. The acetomethyl ester (AM) of calcein green (CalG) was obtained from Molecular Probes. The mitochondrial metal sensor red rhodamine B-[1,10-phenanthrolin-5-yl] aminocarbonyl] benzyl ester (RPA) was obtained as described elsewhere (43, 44). Pioglitazone (purchased from Shanghai PI Chemicals Ltd.) was dissolved in 100% DMSO and diluted in test medium prior to use.

Expression and Purification of mNT Proteins and a-Fd. The human mNT cDNA encoding the cytoplasmic soluble (mNT) part of the protein (residues 33–108) was amplified by PCR and subcloned into a modified pET28-a(+) vector (Novagen) as described (36). The K55E and H87C mutants of mNT were generated by site-directed mutagenesis of the truncated mNT gene in the pET28a(+) vector. The K55E and H87C mutants of mNT were amplified by PCR and subcloned into a modified pET28-a(+) vector (Novagen) as described (36). The K55E and H87C mutants of mNT were generated by site-directed mutagenesis of the truncated mNT gene in the pET28a(+) vector.

UV-Vis Absorption Spectroscopy Transfer Kinetics and Decays. Absorption spectra were recorded at 350–600 nm (CARY, 300Bio), equipped with a temperature control apparatus set to 37 °C. Special attention was given to changes in absorbance at 458 nm (mNT’s signature [2Fe-2S] absorbance peak) and at 423 nm (characteristic of the [2Fe-2S] cluster in Fd). The extent of cluster transfer was determined from the ratio R = A458/A423 as shown below:

\[ R = \frac{A_{\text{obs}} - A_{\text{initial}}}{A_{\text{final}} - A_{\text{initial}}} \]

In the equation above, \( R_{\text{obs}} \) is the observed A423/A458 ratio at a given time, \( R_{\text{initial}} \) is the initial A423/A458 ratio at time 0, which is equal to 0.85, and \( R_{\text{final}} \) is the A423/A458 ratio at long times when the reaction is considered complete and equal to 1.14. Data is normalized and fit to a single exponential rise.

Initiation transfer rates were determined by taking the tangent of the slope of the fit early into the transfer process (10 min) when concentrations of mNT and a-Fd were still close to their starting amounts. Kinetic measurements were performed using equimolar concentrations of mNT (WT and mutants) and a-Fd in the presence of 50 mM Tris pH 8.0, 100 mM NaCl, 5 mM DTT, and 5 mM EDTA, unless stated otherwise. The a-Fd and DTT were preincubated for 30 min prior to the start of the experiment. Decays were performed at 37 °C and determined by monitoring loss of the 458-nm peak with time. Data were then fit to a single exponential rise. Studies were performed using varying concentrations of mNT in 100 mM citrate 100 mM NaCl for pH 6.5 and in 100 mM Bis-tris 100 mM NaCl for pH 6.5 and 7.0. As a check for possible buffer or salt effects on decay half-time, experiments at pH 6.0 and 6.5 were performed with both buffers and no significant differences in decay half-time were observed. The log-plotted pH-dependent slopes allowed for extrapolation of cluster decay times for pH 8.0, which were estimated to take nearly 105 min for WT and 106 minutes for the K55E and H87C mutants.

Labeling of mNT [2Fe-2S] Cluster. [52FeCl2] (PerkinElmer, Life Sciences Inc.) was diluted with 100 mM sodium citrate pH 8.0 to 1 μl and incubated with mNT at [2Fe-2S]. mNT was a uniquely folded 2Fe-2S outer mitochondrial membrane protein stabilized by pioglitazone. Proc Natl Acad Sci USA 104: 14342−14347.
