

# Identification of Disulfide Bond Formation between MitoNEET and Glutamate Dehydrogenase 1

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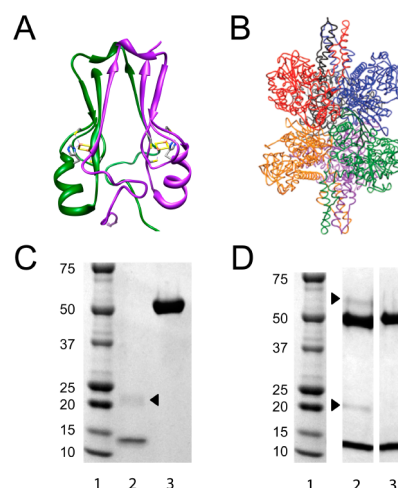
## S Supporting Information

**ABSTRACT:** MitoNEET is a protein that was identified as a drug target for diabetes, but its cellular function as well as its role in diabetes remains elusive. Protein pull-down experiments identified glutamate dehydrogenase 1 (GDH1) as a potential binding partner. GDH1 is a key metabolic enzyme with emerging roles in insulin regulation. MitoNEET forms a covalent complex with GDH1 through disulfide bond formation and acts as an activator. Proteomic analysis identified the specific cysteine residues that participate in the disulfide bond. This is the first report that effectively links mitoNEET to activation of the insulin regulator GDH1.

Glutamate dehydrogenase 1 (GDH1, EC 1.4.1.3) is a key enzyme at the center of cellular catabolism and anabolism. It reversibly converts glutamate to  $\alpha$ -ketoglutarate and ammonia through the use of a NAD<sup>+</sup> or NADP as a cofactor. GDH1 primarily catalyzes the oxidation reaction in mammals to contribute to the pool of  $\alpha$ -ketoglutarate in the Krebs cycle and, as such, is highly regulated by a number of allosteric effectors.<sup>1</sup> The allosteric activator 2-aminobicyclo-2,2,1-heptane-2-carboxylic acid (BCH) has recently been shown to improve  $\beta$ -cell functions under diabetes-like conditions.<sup>3</sup> Conversely, a number of mutations of GDH1 have led to hyperinsulemia/hyperammonemia.<sup>2</sup> We demonstrate here that the mitochondrial protein mitoNEET directly interacts with and covalently modifies GDH1 and acts as a new allosteric activator of GDH1.

MitoNEET is a protein that was discovered in 2004 as a target of the type 2 diabetes drug pioglitazone.<sup>4</sup> The protein is a homodimer with each monomer containing a [2Fe-2S] cluster that is ligated by one histidine and three cysteine residues (Figure 1A).<sup>5</sup> MitoNEET is targeted to the mitochondria and is localized primarily to the outer mitochondrial membrane facing the cytosol. However, a significant portion (up to 14.1%) is localized to the mitochondrial interior.<sup>6</sup>

Since the discovery of mitoNEET, multiple putative functions have been assigned to it, with each suggesting interaction(s) with one or more protein binding partner(s). Proposed functions include redox sensor, regulator of cellular respiration, electron transfer protein, and an iron–sulfur cluster transfer protein.<sup>9–6</sup> The cellular levels of *holo* and *apo* mitoNEET are unknown, and both species may be physiolog-



**Figure 1.** (A) Crystal structure of mitoNEET. (B) Crystal structure of GDH1. (C) SDS-PAGE gel of the molecular mass ladder (lane 1), mitoNEET (lane 2), and GDH1 (lane 3). The arrow denotes the covalent dimer species. (D) SDS-PAGE gel of the covalent complex formation showing the molecular mass ladder (lane 1), mitoNEET with GDH1 without  $\beta$ -mercaptoethanol (BME) (lane 2), and mitoNEET with GDH1 with BME (lane 3). The arrows denote species present only in the absence of BME.

ically relevant. This is the first report that links mitoNEET to the activity of the insulin-regulating protein GDH1.

To identify protein-binding partners of mitoNEET, pull-down experiments conducted with cell lysates isolated from mouse liver (Figure S1 of the Supporting Information) and human hepatoma cells (HepG2) identified GDH1 from an ~62 kDa band by proteomic analysis with 55 ( $q$  value of  $3.37 \times 10^{-6}$ ) and 23 peptides ( $q$  value of  $7.81 \times 10^{-7}$ ) detected, respectively.

The interactions of mitoNEET and GDH1 were characterized by gel electrophoresis. MitoNEET forms a homodimer (Figure 1A) in crystal structures with a monomeric mass of ~12 kDa, whereas GDH1 exists as a hexamer (Figure 1B) with a monomeric mass of ~56 kDa.<sup>1</sup> The mass of both proteins was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing and nonreduc-

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ing conditions. Because there are no disulfide bonds in the available crystal structure of either protein, the presence or absence of a reductant should make no difference in the gel results.

Interestingly, under nonreducing conditions, mitoNEET migrated at two different positions in the gel. One band was in agreement with the expected size of ~12 kDa; another band had an apparent molecular mass of ~24 kDa (Figure 1C, lane 2), while GDH1 was resolved as one band at ~50 kDa (Figure 1C, lane 3). The ~24 kDa band was confirmed by proteomic analysis to be a covalently linked mitoNEET homodimer not observed in crystal structures.

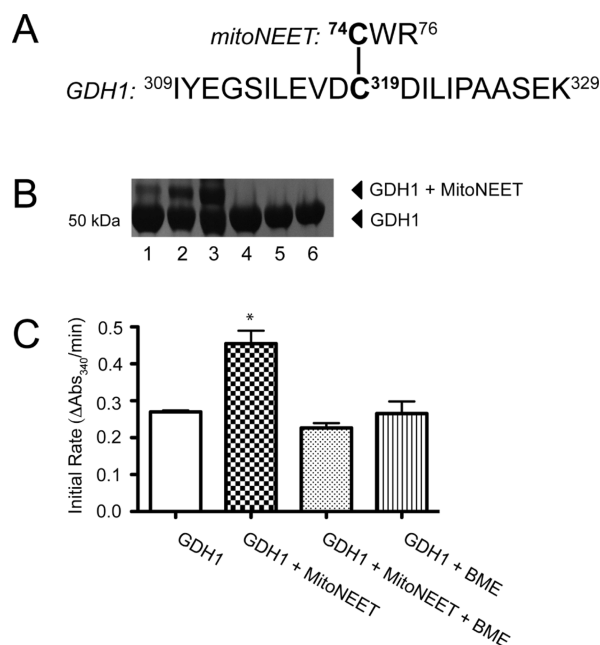
Next, we incubated GDH1 with *holo* mitoNEET to investigate possible covalent bond formation between the two proteins. An ~62 kDa band present on a SDS–PAGE gel was dependent on the presence of both mitoNEET and GDH1 and the absence of a reductant (Figure 1D, lane 2). The band was sequenced using proteomic analysis and identified as a mitoNEET–GDH1 complex. Because the ~62 kDa band was not present in samples that included  $\beta$ -mercaptoethanol (BME) (Figure 1D, lane 3), we concluded the mechanism of mitoNEET–GDH1 cross-linking is the formation of a disulfide bond.

The only three cysteine residues in mitoNEET, Cys72, Cys74, and Cys84, are three of the four residues that ligate the [2Fe-2S] cluster.<sup>5</sup> At least one of the cysteine residues must be liberated from the coordinate covalent bond to an iron atom of the [2Fe-2S] cluster to form an interdimer disulfide bond or a mixed disulfide bond with GDH1. The shortest distance between any two cysteine residues in crystal structures of mitoNEET across the dimer interface is 12.6 Å (Figure S2A of the Supporting Information).

The reactivity of the cysteine residues to form disulfide bonds with themselves and/or other protein(s) suggests mitoNEET is an important redox sensor in both the cytosol and the mitochondrial matrix. The extent of dimer formation at pH 8.5, analyzed by SDS–PAGE without a reductant, increased with increasing concentrations of mitoNEET. With increases in the concentration of mitoNEET from 97.8  $\mu$ M to 195  $\mu$ M to 390  $\mu$ M, the covalent dimer band became more pronounced (Figure S2B of the Supporting Information) and the bands were quantified and showed a ratio of 1.2–1.5 monomers per covalent dimer (Table S1 of the Supporting Information). The covalent dimer band did not form under reducing conditions with BME at any concentration that was investigated. The occurrence of the covalently linked mitoNEET dimer is not due to nonselective polymerization because no higher-order oligomers were observed in the SDS–PAGE analysis. Proteomic analysis of the ~24 kDa band determined that the disulfide bond forms between residues Cys83 and either Cys72 or Cys74 of mitoNEET (Figure S2D of the Supporting Information). The same concentrations of mitoNEET were also analyzed by native gel electrophoresis (Figure S2C of the Supporting Information), and two higher-order oligomeric bands (oligomer 1 and oligomer 2) were observed only in samples not treated with BME. The bands were quantified (Table S2 of the Supporting Information), and the noncovalent dimer/(oligomer 1 + oligomer 2) ratio decreased as the concentration of mitoNEET increased.

In comparison, GDH1 has six cysteine residues: Cys55, Cys89, Cys115, Cys197, Cys270, and Cys319. As such, there are 18 possible combinations of cysteines that could form mixed disulfide bonds between Cys<sub>mitoNEET</sub> and Cys<sub>GDH1</sub>.

Proteomic analysis identified only the Cys74<sub>mitoNEET</sub>–Cys319<sub>GDH1</sub> pair as the residues responsible for the mixed disulfide bond formation (Figure 2A) through a mass shift of



**Figure 2.** Characterizing the mitoNEET–GDH1 complex using (A) proteomic identification of the disulfide cysteine residues and (B) SDS–PAGE analysis of the concentration dependence on complex formation without (lanes 1–3) and with (lanes 4–6) the reductant BME. The concentration of mitoNEET monomer was 195.6  $\mu$ M (lanes 1 and 3), 390.2  $\mu$ M (lanes 2 and 4), and 780  $\mu$ M (lanes 3 and 6) with a GDH1 monomer concentration of 180  $\mu$ M. (C) Impact of mitoNEET on GDH1 activity. The asterisk indicates significant differences between rates ( $P < 0.05$ ).

463.55 Da of the GDH1 peptide. The mass shift corresponds to the addition of the mitoNEET peptide <sup>74</sup>CWR<sup>76</sup> (Figure 2A, S3 of Supporting Information). Additionally, the concentration dependence of the mitoNEET–GDH1 complex was investigated by increasing the mitoNEET monomer concentration from 195.6  $\mu$ M to 390  $\mu$ M to 780  $\mu$ M while keeping the monomer GDH1 concentration at 180  $\mu$ M (Figure 2B). The percentage of cross-linked mitoNEET–GDH1 covalent complex increased from 24.51% (195.6  $\mu$ M mitoNEET) to 40.66% (780  $\mu$ M mitoNEET) (Table S3 in the Supporting Information).

The lack of a mixed disulfide bond with GDH1 involving Cys83<sub>mitoNEET</sub> is notable because it readily participates in the homodimer disulfide bond. This result implies that there are directing factors, such as steric or electrostatic interactions, that either preclude Cys83<sub>mitoNEET</sub> or encourage Cys74<sub>mitoNEET</sub> to react with Cys319<sub>GDH1</sub>. Two negatively charged aspartate residues flank Cys319<sub>GDH1</sub>, while Cys74<sub>mitoNEET</sub> is located next to two positively charged arginine residues at positions  $n - 1$  and  $n + 2$ . These charged residues for both proteins, near the covalently bound cysteines, might control the formation of the mixed disulfide bond. Cys319<sub>GDH1</sub> was previously identified as being modified by nucleotide analogues.<sup>10</sup> Additionally, mutagenesis of Cys323<sub>GDH1</sub>, the analogous cysteine residue in the human isoform, decreased  $k_{cat}$  by approximately 10-fold relative to that of the wild-type enzyme.<sup>11</sup> Therefore, we investigated if the reaction between mitoNEET and GDH1 impacts GDH1 activity, making mitoNEET a regulator of GDH1. GDH1

enzyme activity was determined by monitoring the production of NADH at 340 nm ( $\lambda$ ). The addition of mitoNEET in a monomer equimolar amount increased the initial velocity of GDH1 from  $0.270 \pm 0.003$  to  $0.428 \pm 0.002 \Delta\text{Abs}_{340}/\text{min}$  for GDH1 alone (Figure 2C).<sup>12</sup>

The increase in the activity of GDH1 resulted from the formation of a mixed disulfide bond with mitoNEET because the activation was negated with the inclusion of BME to the GDH1–mitoNEET sample ( $0.226 \pm 0.007 \Delta\text{Abs}_{340}/\text{min}$ ). This mirrors the result of the addition of the small molecule BCH to GDH1.<sup>3</sup> MitoNEET is a unique activator of GDH1 in that it is a mitochondrial protein and exerts its action through a redox-sensitive covalent bond. The ability of the cysteine residues of mitoNEET to form disulfide bonds introduces intriguing new evidence of the cellular functions of mitoNEET as a redox sensor in addition to an activator of the insulin regulator GDH1.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

Experimental procedures and additional data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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