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Crystallization and preliminary X-ray diffraction studies of the prototypal homologue of mitoNEET (*Tth*-NEET0026) from the extreme thermophile *Thermus thermophilus* HB8

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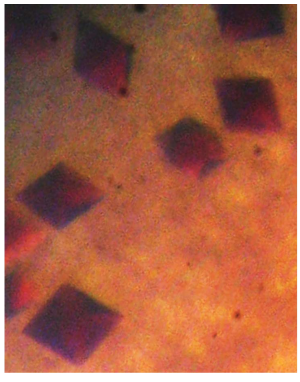
MitoNEET (a mammalian mitochondrial outer membrane protein) is a potential pharmacological and clinical target of the insulin-sensitizer pioglitazone. The thermophilic homologue of mitoNEET (TTHA0026) from *Thermus thermophilus* HB8 has been heterologously overproduced in *Escherichia coli* and purified as a water-soluble prototypal protein containing the mitoNEET-like [2Fe–2S] cluster. The resultant recombinant protein, named *Tth*-NEET0026, has been crystallized in its oxidized form by the hanging-drop vapour-diffusion method using 17%(*w*/*v*) polyethylene glycol 4000, 8.5%(*v*/*v*) 2-propanol, 15%(*v*/*v*) glycerol and 0.085 *M* HEPES–NaOH pH 7.2. The dark reddish crystals diffracted to 1.80 Å resolution and belonged to the tetragonal space group $P4_32_12$, with unit-cell parameters a = 45.51, c = 84.26 Å. The asymmetric unit contains one protein molecule.

1. Introduction

The mammalian mitochondrial outer membrane protein, named 'mitoNEET' [on the basis of its subcellular location on the outer membrane of mitochondria (Wiley *et al.*, 2007) and the internal amino-acid sequence stretch of the mouse and human proteins, Asn-Glu-Glu-Thr (NEET)], was identified as a possible target protein of pioglitazone by cross-linking with a photoaffinity probe (Colca *et al.*, 2004). The drug pioglitazone is a member of the thiazolidinedione class of insulin sensitizers for the treatment of type II diabetes, a complex metabolic disease of prevailing public health concern that is characterized in the initial stage by insulin resistance (Wiederkehr & Wollheim, 2006). Deficiency of mitoNEET expression in mice results in a compromise in the respiratory capacity of cardiac mitochondria (Wiley *et al.*, 2007). Recent crystallographic studies on the recombinant soluble CDGSH-type zinc-finger-like domain of human mitoNEET at 1.5–1.8 Å resolution (Lin *et al.*, 2007; Hou *et al.*, 2007; Paddock *et al.*, 2007; PDB codes 2qd0, 2r13 and 2qh7) have revealed that the recombinant mitoNEET soluble domain is a homodimer with each subunit binding a unique [2Fe–2S] cluster (but no Zn^{2+}) in a three-cysteine plus one-histidine ligand environment (Fig. 1). Despite its potential importance in pharmacology and clinical medicine, the *in vivo* function of this mitochondrial outer membrane protein remains elusive.

Thermus thermophilus strain HB8 (JCM 10941^T) is a Gram-negative thermophilic bacterium isolated from the Mine hot springs, Izu, Japan (Oshima & Imahori, 1974). The whole genomic DNA sequence has been analyzed (http://www.thermus.org) and genetic manipulation systems for the introduction, disruption and homologous expression of intrinsic/foreign genes have been developed (Tamakoshi *et al.*, 1995; Hashimoto *et al.*, 2001). A homology search of the deduced human and rat mitoNEET sequences against the complete *T. thermophilus* HB8 genome database using the NCBI BLAST (http://blast.ncbi.nlm.nih.gov/) and KEGG (Kanehisa *et al.*, 2008; http://www.genome.jp/kegg/) servers indicated at least two hypothetical



genes, *ttha0026* and *ttha1309*, that code for mitoNEET-like soluble proteins (Y. Hayashi-Iwasaki & T. Iwasaki, unpublished results). The *ttha1309* gene is tentatively annotated as a short antiframe (ORF) located within a hypothetical gene cluster coding for a putative radical SAM enzyme family (data not shown). The *ttha0026* gene is a part of the (hypothetical) *ttha0025–ttha0026* cluster coding for a putative leucine-rich membrane protein (TTHA0025; 157 amino acids) and a mitoNEET-like water-soluble protein (TTHA0026; 69 amino acids) (Fig. 1). Moreover, the four ligand residues (Cys37, Cys39, Cys48 and His52) in the [2Fe–2S] cluster-binding motif of human mitoNEET (Lin *et al.*, 2007; Hou *et al.*, 2007; Paddock *et al.*, 2007; PDB codes 2qd0, 2r13 and 2qh7) are strictly conserved in the TTHA0026 protein (Fig. 1), even though TTHA0026 is tentatively categorized as a hypothetical CDGSH-type zinc-finger protein. It is therefore of particular interest to investigate whether TTHA0026 is indeed a bacterial prototypal model of mitoNEET carrying a mitoNEET-like [2Fe–2S] cluster and to address its functionalities through elucidation of its structure at atomic resolution and analyses of the phenotypes and the *in vivo* network of genetically manipulated *T. thermophilus* strains. Here, we present the crystallization of the recombinant TTHA0026 protein (named *Tth*-NEET0026) in a form suitable for high-resolution X-ray studies.

2. Methods and results

2.1. Protein preparation and characterization

The *ttha0026* gene coding for the mitoNEET-like protein (TTHA0026; named *Tth*-NEET0026) from *T. thermophilus* HB8 (JCM 10941^{T}) has been sequenced by whole genomic DNA-sequence analysis of this organism (http://www.thermus.org/) and identified by a homology search of the deduced human and rat mitoNEET sequences against the genomic databases (Fig. 1). The polymerase chain reaction (PCR) was carried out to amplify the *ttha0026* gene using the *T. thermophilus* HB8 genomic DNA and the following oligonucleotide primers (designed based on the reported nucleotide sequence): TthNEET0026NheF primer, 5′-GTT CAA GCT AGC AGT GGA ATG CGG CTG GAG TTC CTG-3′, and TthNEET0026XhoR primer, 5′-GTT CAA CTC GAG TCA GTC GCT TTC CAC CTC CAA GAC-3′. The PCR primers included six extra bases (coding for an engineered Ser-Gly linker) immediately upstream of the initiation codon of the *ttha0026* gene. The PCR product thus amplified was subcloned into an *Nhe*I/*Xho*I site in a pET28a vector (Novagen) and the nucleotide sequences were confirmed for both strands using an ABI PRISM 310 Genetic Analyzer automatic DNA sequencer (PE Biosystems) with a vector-specific T7 promoter and T7 terminator. The resultant vector was named pET28a-TthNEET0026SG.

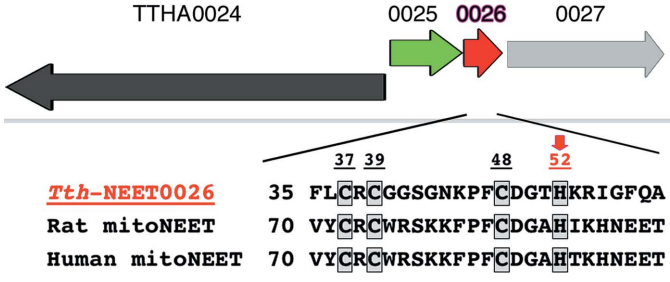


Figure 1
Schematic organization of the *ttha0025–ttha0026* gene cluster and its adjacent regions in the genomic DNA sequence of *T. thermophilus* HB8 (http://www.thermus.org). There is no direct information available concerning the specific function of the products of the *ttha0024–ttha0027* genes [TTHA0025 is a putative (leucine-rich) membrane protein, TTHA0026 is a water-soluble homologue of the mammalian mitoNEET soluble domain (*Tth*-NEET0026) and TTHA0027 is a putative potassium channel β-subunit homologue]. Multiple sequence alignment of selected mitoNEET-like proteins indicates the conservation of one histidine (indicated by a red arrow) and three cysteine residues, which provide the [2Fe–2S](Cys)$_3$(His)$_1$ cluster-binding motif (boxed) in the recombinant human mitoNEET soluble-domain fragment (Lin *et al.*, 2007; Hou *et al.*, 2007; Paddock *et al.*, 2007). The overall sequence identity between *Tth*-NEET0026 (69 amino acids) and the human mitoNEET soluble domain of known structure (76 amino acids) is 20.3%. Accession numbers: *T. thermophilus* HB8 *Tth*-NEET0026, ORF annotation/Kyoto Encyclopedia of Genes and Genomes (KEGG) code TTHA0026, NCBI GeneID code 3168947; *R. norvegicus* (rat) mitoNEET (Cisd1), NCBI GeneID/KEGG code 294362; *Homo sapiens* (human) mitoNEET soluble-domain fragment, PDB codes 2qd0, 2r13 and 2qh7.

Table 1
Diffraction data statistics.

Values in parentheses are for the outer shell.

Space group	$P4_32_12$
Unit-cell parameters (Å)	a = 45.51, c = 84.26
Resolution range (Å)	19.78–1.80 (1.86–1.80)
No. of measured reflections	105216
No. of unique reflections	8760 (814)
Completeness (%)	99.3 (95.9)
$R_{\rm merge}$† (%)	4.6 (52.2)
$\langle I/\sigma(I)\rangle$	16.4

† $\sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl)\rangle$ are the intensity of measurement i and the mean intensity for the reflection with indices hkl, respectively.

The resultant pET28a-TthNEET0026SG harbouring the *ttha0026* gene was transformed into the host strain *Escherichia coli* BL21-CodonPlus(DE3)-RIL (Stratagene). The transformants were grown overnight at 298 K in Luria–Bertani medium containing 50 µg ml^{-1} kanamycin, 0.4 m*M* $FeCl_3$, 0.2 m*M* L-cysteine hydrochloride monohydrate (Wako Pure Chemicals, Tokyo, Japan) and 1 mg l^{-1} pyridoxal hydrochloride (Sigma). The recombinant holoprotein was induced with 1 m*M* isopropyl β-D-1-thiogalactopyranoside for 24 h at 295 K and the cells were pelleted by centrifugation. The recombinant *Tth*-NEET0026 with a six-His tag and a thrombin cleavage site at the N-terminus was purified essentially as reported previously for archaeal Rieske [2Fe–2S] proteins (Kounosu *et al.*, 2004; Iwasaki *et al.*, 2004), except that the entire purification was performed at 277 K using buffers adjusted to pH 8.0. The heat treatment of the crude cell lysate was also omitted. After proteolytic removal of the six-His tag from the purified recombinant *Tth*-NEET0026 for 16–22 h at 277 K using a Thrombin Cleavage Capture Kit (Novagen), the sample was further purified to electrophoretic homogeneity by gel-filtration chromatography (Sephadex G-75; GE Healthcare Sciences) eluted with 10 m*M* HEPES–NaOH, 500 m*M* NaCl pH 8.0 at room temperature. The purified protein was concentrated to ∼17 mg ml^{-1} with a Centriprep-10 apparatus (Amicon) and stored frozen (193 K) until use.

The construct, with a deduced molecular weight of 8419 Da, contains eight extra residues at the N-terminus: Gly-Ser-His-Met-Ala-Ser-Ser-Gly. These eight residues were added to facilitate purification and are absent from the deduced sequence of this hypothetical protein. The purified recombinant *Tth*-NEET0026 is a homodimer like the human mitoNEET [2Fe–2S] cluster-binding domain of known structure and was a dark reddish colour. The room-temperature visible absorption spectrum of the recombinant *Tth*-NEET0026 was very similar to that for the recombinant soluble-domain fragment (residues 32–108) of *Rattus norvegicus* (rat) mitoNEET (A. Kounosu & T. Iwasaki, unpublished results; see Fig. 1) and shows the typical mitoNEET-type [2Fe–2S](Cys)$_3$(His)$_1$ cluster (Fig. 2*a*).

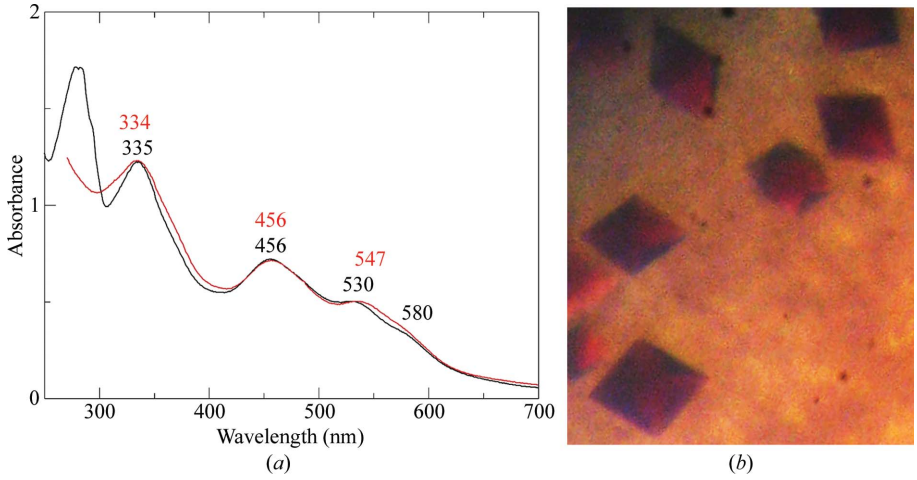


Figure 2
(*a*) The visible–UV absorption spectra of purified *Tth*-NEET0026 (red trace) and rat mitoNEET [2Fe–2S] cluster-binding domain (residues 32–108; black trace) recorded with a Beckman DU-7400 spectrophotometer. The absorption maxima (nm) are indicated in the figure. (*b*) Typical crystals of recombinant *Tth*-NEET0026. The maximum dimensions of the crystals are approximately 0.1 × 0.1 × 0.15 mm.

2.2. Crystallization

Preliminary screening took place by standard hanging-drop vapour diffusion in Linbro plates at 277, 283 and 293 K, with 0.45 ml reservoirs of commercially available sparse-matrix screening kits (Hampton Research Crystal Screen kits I, II and Cryo). Small reddish crystals appeared in 3–14 d (under aerobic conditions without any reducing reagents) in three conditions. Bipyramidal crystals were obtained from one of these conditions when the hanging drops were pretreated for 2–3 d at 293 K to ensure nucleation and then moved to 277 K. Employing this method, optimized crystals were obtained by combining 1.0 µl protein solution with 1.0 µl reservoir solution containing 17%(*w*/*v*) polyethylene glycol 4000, 8.5%(*v*/*v*) 2-propanol, 15%(*v*/*v*) glycerol and 0.085 *M* HEPES–NaOH pH 7.2. Drops were equilibrated against 0.45 ml reservoir solution and the crystals grew to maximal dimensions of approximately 0.1 × 0.1 × 0.15 mm in about 10 d after being moved to 277 K (Fig. 2*b*). They could be flash-cooled successfully in liquid nitrogen without being transferred to a cryoprotectant solution.

2.3. Crystallographic data collection and processing

X-ray diffraction data for recombinant *Tth*-NEET0026 were successfully collected from flash-cooled crystals using a MAR225 CCD detector installed on the SPring-8 BL26B2 beamline. The beamline optics were temporarily equipped with a vertically focusing mirror and a dynamic sagittal focusing monochromator (Yoneda *et al.*, 2001) composed of a newly developed high-accuracy bender and an unribbed monochromator crystal. This setup allowed an approximately fivefold increase in the available flux density at the sample position compared with the ordinary BL26B2 beamline settings. Data collection was performed at a wavelength of 1.0 Å with a total oscillation range of 180° and each diffraction image was taken with an oscillation angle of 1.0° and an exposure time of 5 s. The data were indexed and processed using *HKL*-2000 (Otwinowski & Minor, 1997). The crystals (Fig. 2*b*) were found to diffract to 1.80 Å resolution and belonged to the tetragonal space group $P4_12_12$ or its enantiomorph, with unit-cell parameters a = 45.51, c = 84.26 Å (Table 1). Assuming one protein molecule per asymmetric unit, the Matthews coefficient is 2.8 Å^3 Da^{-1}, corresponding to a solvent content of 57% (Matthews, 1968).

Phase determination was successfully carried out by Fe-SAD using the programs *SHELX* (Sheldrick, 2008) and *HKL2MAP* (Pape & Schneider, 2004), which also established the tetragonal space group $P4_32_12$. Construction, revision and analysis of atomic models using the *Tth*-NEET0026 (TTHA0026) sequence are currently in progress.

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