Isolation and characterization of a thermostable F_{420}:NADPH oxidoreductase from Thermobifida fusca

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F_{420}H_{2}-dependent enzymes reduce a wide range of substrates that are otherwise recalcitrant to enzyme-catalyzed reduction, and their potential for applications in biocatalysis has attracted increasing attention. Thermobifida fusca is a moderately thermophilic bacterium and holds high biocatalytic potential as a source for several highly thermostable enzymes. We report here on the isolation and characterization of a thermostable F_{420}:NADPH oxidoreductase (Tfu-FNO) from T. fusca, the first F_{420}-dependent enzyme described from this bacterium. Tfu-FNO was heterologously expressed in Escherichia coli, yielding up to 200 mg of recombinant enzyme per liter of culture. We found that Tfu-FNO is highly thermostable, reaching its highest activity at 65 °C and that Tfu-FNO is likely to act in vivo as an F_{420} reductase at the expense of NADPH, similar to its counterpart in Streptomyces griseus. We obtained the crystal structure of FNO in complex with NADP^{\*} at 1.8 Å resolution, providing the first bacterial FNO structure. The overall architecture and NADP^{\*}-binding site of Tfu-FNO were highly similar to those of the Archaeoglobus fulgidus FNO (AF-FNO). The active site is located in a hydrophobic pocket between an N-terminal dinucleotide binding domain and a smaller C-terminal domain. Residues interacting with the 2′-phosphate of NADP^{\*} were probed by targeted mutagenesis, indicating that Thr-28, Ser-50, Arg-51, and Arg-55 are important for discriminating between NADP^{\*} and NAD^{\*}. Interestingly, a T28A mutant increased the kinetic efficiency >3-fold as compared with the wild-type enzyme when NADH is the substrate. The biochemical and structural data presented here provide crucial insights into the molecular recognition of the two cofactors, F_{420} and NAD(P)H by FNO.

Flavins can arguably be regarded as the most extensively studied redox cofactors. One natural flavin analogue is cofactor F_{420} which was first isolated and characterized from methanogenic archaea in 1972 (1). Since then, F_{420} has been found in members of methanogens, Actinomycetes, Cyanobacteria, and some Betaproteobacteria (2). Replacement of the 5′ nitrogen of flavins with a carbon in F_{420}, resulting in a so-called deazaflavin, renders the cofactor nearly unreactive toward molecular oxygen. Hence, F_{420} is an obligate hydride-transfer cofactor similar to the nicotinamide cofactors (Fig. 1). In addition, the 8′-OH group on the isoalloxazine ring in F_{420} has been suggested to slow down the autooxidation of the reduced cofactor (F_{420H_{2}}) in air; thus, the reduced species is much more stable than that of flavins (3).

Many F_{420}(H_{2})-dependent enzymes have been characterized recently, and their potential for applications in biocatalysis has attracted increasing attention (4, 5). F_{420}-dependent enzymes studied so far have been shown to be capable of reducing a wide range of substrates that are otherwise recalcitrant to enzyme-catalyzed reduction (4, 5). However, the commercial availability of cofactor F_{420} remains a bottleneck for studying and applying the respective enzymes. Therefore, it would be attractive to have access to an efficient F_{420H_{2}} cofactor recycling system. In this context, F_{420}:NADPH oxidoreductases (FNOs, EC 1.5.1.40; Fig. 1) could become very valuable as NADPH-driven F_{420H_{2}}-recycling systems. FNOs catalyze the reduction of NADP^{+} using F_{420H_{2}} and have been found in a number of archaea (6–10) and bacteria (11) (Fig. 1). It has been argued that in methanogens, FNO catalyzes mainly the reduction of NADP^{+} using F_{420H_{2}}, whereas bacterial FNOs are supposed to catalyze the reverse reaction (11).

Thermobifida fusca is a moderately thermophilic soil bacterium with high G+C content. This actinomycete holds high biocatalytic potential as it has already served as a source for several highly thermostable enzymes, e.g. catalase, Baeyer-Villiger monooxygenase, and glycoside hydrolases (12–14). Interestingly, a recent bioinformatic study predicted that the T. fusca genome contains 16 genes encoding for F_{420}-dependent enzymes (15). Nevertheless, there has been so far no biochemical evidence for such enzymes. Here, we describe the identification and characterization of a dimeric thermostable F_{420}:NADPH oxidoreductase from T. fusca (Tfu-FNO), con-

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The atomic coordinates and structure factors (code 5N2I) have been deposited in the Protein Data Bank (http://wwpdb.org).

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firming the presence of \(F_{420}\)-dependent enzymes in this mesophilic bacterium. Despite the high GC content (67%) of the gene sequence, Tfu-FNO is readily expressed in *Escherichia coli*. Notably, Tfu-FNO is a thermostable enzyme and shows a clear substrate preference toward NADP(H) instead of NAD(H). By solving the three-dimensional crystal structure of Tfu-FNO, we set out site-directed mutagenesis to corroborate the role of residues that interact with the phosphate moiety at 2' position of NADP+.

**Results**

**Purification of Tfu-FNO**

A BLAST search for Af-FNO homologs in *T. fusca* resulted in the identification of the Tfu_0907 gene (TFU_RS04835). The encoded protein shares 40 and 70% sequence identity to FNOs from *Archaeoglobus fulgidus* and *Streptomyces griseus*, respectively (Fig. 2). The Tfu-fno gene, with a high GC content (67%), was amplified from the genomic DNA of *T. fusca* and transformed into *E. coli* TOP10 as a pBAD-fno construct. Purification of the respective protein, Tfu-FNO, was achieved through ammonium sulfate precipitation followed by anion exchange chromatography. DNase I treatment during the first steps of protein purification was found to be essential to remove residual DNA. Tfu-FNO was obtained in pure form with a relatively high yield: 120–200 mg/liter culture. It is worth noting that the amount of purified Tfu-FNO obtained in our system is significantly higher than that of Af-FNO when heterologously expressed in *E. coli* (2 mg/liter culture; Ref. 16).

**Effects of pH and temperature on activity**

FNOs are known to catalyze the reduction of NADP+ at higher pH, whereas at lower pH it catalyzes the reverse reaction. Fig. 3 shows the effect of pH on Tfu-FNO activity. The reduction rate of NADP+ is highest at pH 8.5–9.0, whereas the reverse reaction is optimal between pH 4.0 and 6.0. From the \(k_{\text{obs}}\) values of both the forward and backward reactions, it can be concluded that FNO catalyzes NADP+ reduction more efficiently (Fig. 3). This is in line with the redox potential of \(F_{420}\) (−340 mV) being lower when compared with that of NADP+ (−320 mV) (3).

Because FNO originates from the mesophilic organism *T. fusca*, the enzyme is expected to be stable at relatively high temperatures. Measuring the activities at temperatures between 25 and 90 °C revealed that the enzyme displays highest activity between 60 and 70 °C (Fig. 4). The activity at 65 °C is almost 4× higher than that at 25 °C. The apparent melting temperature of Tfu-FNO was found to be 75 °C, as measured by the Thermofluor® method (17). All the generated Tfu-FNO mutants had melting temperatures similar to the wild-type enzyme (data not shown). This indicates that FNO is remarkably thermostable and is most active at elevated temperatures.

**Steady-state kinetics**

The steady-state kinetic parameters were measured for NADPH and \(F_{420}\) as substrates by following absorbance of these two cofactors at either 340 nm or 400 nm, respectively. The concentration of one substrate was varied while keeping the other substrate at a constant, saturated concentration. The kinetic data fitted well to the Michaelis-Menten kinetic model when the observed rates (\(k_{\text{obs}}\)) were plotted against substrate concentrations. Tfu-FNO had a \(K_m\) value of 7.3 \(\mu\)M and 2.0 \(\mu\)M for NADPH and \(F_{420}\) respectively, at pH 6.0 and 25 °C (Table 1). Thus, Tfu-FNO has a significantly lower \(K_m\) for NADPH (2.0 \(\mu\)M) compared with the values featured by Af-FNO (40 \(\mu\)M) and FNO from *S. griseus* (19.5 \(\mu\)M) (8,11). The \(k_{\text{cat}}\) (3.3 s\(^{-1}\)) of Tfu-FNO was somewhat lower when compared with that of Af-FNO (5.27 s\(^{-1}\)) (18).

**The overall structure of Tfu-FNO**

Crystalization of Tfu-FNO was successful, which allowed the elucidation of its crystal structure. This revealed that NADP+ had been co-purified with the native enzyme, as it was found to be bound in the active site (Figs. 5 and 6). All crystal soaking attempts to obtain the \(F_{420}\) cofactor bound in the enzyme active site failed, which can be explained by the tight molecular packing found in Tfu-FNO crystals that would hamper cofactor binding in the same position as found in Af-FNO (Fig. 5A). It is known that, depending on the bacterial species, the number of glutamate moieties of \(F_{420}\) can vary from two to nine, with five to six being the predominant species in mycobacteria (19). Given the crystal arrangement of Tfu-FNO molecules, an oligoglutamate tail of \(F_{420}\) of any length would clash against another subunit interacting through crystal packing (Fig. 5A). Nevertheless, the architecture of the active site is...
Figure 3. pH optimum for the Tfu-FNO-catalyzed F420 reduction using NADPH (dots) or the NADP+/H+ reduction using F420H2 (squares) at 24 °C. $k_{obs}$ (s⁻¹) for the NADP⁺ reduction (pH optima 8–10) was almost 3 × higher than that for the F420 reduction (pH optimum 4–6).

Figure 4. Effect of temperature on Tfu-FNO activity. The reaction mixture of 100 μl contained 1.25 mM NADH, 20 μM F420 in 50 mM KP, pH 6.0. The reaction was started by adding 50 nM FNO. The error bars represent S.D. from two measurements.
highly conserved, and NADP⁺ adopted a virtually identical position with respect to that observed in Af-FNO (Fig. 5B). Therefore, F₄₂₀ was tentatively modeled in Tfu-FNO upon superposition of the archaeal enzyme (Fig. 5C). The modeled F₄₂₀ fit very well into the Tfu-FNO active site without any clashes. Similarly to Af-FNO, F₄₂₀ would bind in Tfu-FNO at the C-terminal domain with its deazaisoalloxazine ring buried deep inside the catalytic pocket and the highly polar oligoglutamyl tail directed toward the exterior of the dimer (Fig. 5, B and C).

As mentioned above, NADP⁺ binds to the N-terminal part of Tfu-FNO in a highly similar manner to that of Af-FNO, which is characteristic for members of the dinucleotide-binding protein family (20, 21). The hydrogen-bonding network between NADP⁺ and the residues that form the active site are illustrated in Fig. 6. In particular, the nicotinamide ring directly docks to the protein by hydrogen-bonding the cofactor amide group to the peptide nitrogen of Ala-155 (corresponding to Ala-137 in Af-FNO). This conserved interaction is believed to be crucial in conferring the trans conformation of the amide group. With this conformation, the pyridine ring of NADP⁺ is maintained planar, which in turn facilitates the hydride transfer between the C4 of the NADP and C5 of F₄₂₀, by shortening the distance of the two atoms (20).

### NADP⁺-binding site

The residues involved in binding the ADP moiety are also conserved in Tfu-FNO (Fig. 6). Analogous to Af-FNO, the negatively charged group of the ribose 2’-phosphate interacts with the side chains of Thr-28, Ser-50, Arg-51, and Arg-55 (corresponding to Thr-9, Ser-31, Arg-32, and Lys-36 in Af-FNO). These residues are highly conserved in other known FNOs (Fig. 2). These residues, therefore, appear to be crucial for substrate recognition and help to discriminate between NADP⁺ and NAD⁺ (20). To get more insights into the role of these residues, they were mutated into amino acids with different charge and/or size and tested for the cofactor specificity toward the two nicotinamide cofactors. Table 1 shows the kinetic parameters for both NADH and NADPH as substrate. For wild-type Tfu-FNO, the $K_m$ value for NADH (14 mM) is several orders of magnitude higher than that for NADPH (7.3 μM), clearly confirming that the enzyme prefers NADP(H) over NAD(H). For all mutants, the $K_m$ value for NADPH significantly increased (from 2.6- to >68-fold) compared with that of the wild-type enzyme, which verified the crucial role of these residues in binding NADP(H). Intriguingly, recognition of NADH remained the same or improved in all mutants (see Table 3), with a $K_m$ value ranging from 0.23 to 2.3× that of the wild type. Noticeably, R55N and R55S variants have a significantly improved affinity toward NADH. In the case of mutant R55N, $K_m$ of NADH decreased almost 4-fold. The S50E mutant was the best among the tested mutants with a $K_m$ of almost 5× lower and a $K_m$ of ~100-fold higher as compared with wild-type Tfu-FNO. Interestingly, the T28A mutant showed an increased activity toward both NADPH and NADH, with a 4-fold increase in catalytic rate ($k_{cat} = 14 \text{ s}^{-1}$) for NADPH and a 2.8-fold decrease in $K_m$ value (5 mM) for NADH when compared with the wild-type enzyme. This resulted in significantly improved $k_{cat}/K_m$ values for both NADPH and NADH, respectively. Unfortunately, combinations of the mutations did not show significant additive effects (Table 1).
nomycete by cloning and characterization of a thermostable F$_{420}$:NADPH oxidoreductase (Tfu-FNO), which catalyzes the reduction of NADP$^+$ using reduced F$_{420}$ and the reverse reaction.

**The role of FNO in generating reduced F$_{420}$**

F$_{420}$ cofactor provides microorganism alternative redox pathways. The deazaflavin cofactor seems especially equipped for reduction reactions, as it displays a redox potential that is lower when compared with the nicotinamide cofactor. Two enzymes have been identified in previous studies that serve a role in reducing F$_{420}$:FNO and F$_{420}$-dependent glucose-6-phosphate dehydrogenase (FGD) (4). Using *T. fusca* cell-free extract and heterologously expressed Tfu_1669 (a putative *M. tuberculosis* FGD homolog), we could not detect any FGD activity. This suggests that the *T. fusca* proteome indeed does not include an FGD. In fact, it has been shown before that not all actinomycetes have an FGD (22). Therefore, FNO may be the primary enzyme in actinomycetes for providing the cells with F$_{420}$.H$_2$. Nevertheless, at physiological pH (7.0–8.0; Fig. 3) Tfu-FNO performs reduction of NADP$^+$ slightly better than reduction of cofactor F$_{420}$, which is different from the FNO from *S. griseus* (11) and more similar to the archaeal FNOs (7, 8). This can partly be explained by the experimental condition (24°C) differing from the optimum temperature at which the bacteria grow (55°C) and the intercellular environment (e.g. cofactor concentrations, salt concentrations). Several lines of evidence suggest that in other actinomycetes, such as *Rhodococcus opacus* and *Nocardioides simplex*, FNO is also the main source of F$_{420}$.H$_2$. In these bacteria, the *fno* gene was embedded in the same operon with genes encoding for the F$_{420}$-dependent reductases, which are involved in the metabolism of picrate and 2,4-dinitrophenols (23–25). FNO-catalyzed regeneration of F$_{420}$.H$_2$ was also proposed to be crucial for the reductive steps in the biosynthesis of tetracycline by *Streptomyces* (26).

**Structure and NADP(H) binding site of Tfu-FNO**

FNO is believed to be the only F$_{420}$-dependent enzyme known so far that is conserved between archaea and bacteria (4). Except for a 19-amino acid extension loop at the N terminus, Tfu-FNO largely shares the overall topology and cofactor binding site with that from *A. fulgidus* (Figs. 2 and 5B). The residues that interact directly with the 2'-phosphate group of NADP(H) are also highly conserved (Fig. 6) and have proven to

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be essential for binding this cofactor. Upon disrupting the hydrogen-bonding network by mutagenesis, all the mutants lost virtually all ability to recognize NADPH (Table 1). Intriguingly, the affinity of these variants toward NADH improved, with the S50E mutant being the best in terms of specificity for NADH (5.3-fold higher $k_{cat}/K_m$ than that of WT). Yet, an efficient NADH-dependent FNO has still to be engineered. For this, a newly developed tool could be explored that can guide structure-inspired switching of coenzyme specificity (27).

**Potential applications in biocatalysis**

Tfu-FNO represents a highly attractive candidate for the biocatalytic reduction of F$_{420}$. The enzyme is very thermostable, remains active over a wide range of pH (Figs. 3 and 4), and can be easily expressed in *E. coli* (120–200 mg/liter culture). Tfu-FNO is also a relatively fast enzyme, especially with the T28A mutant displaying a $k_{cat}$ of 14 s$^{-1}$ for NADPH (Table 1). Whereas the majority of current enzymatic F$_{420}$H$_2$ regenerating systems employ FGDs (28, 29), the cost of the expensive, non-recyclable cosubstrate glucose-6-phosphate remains the main bottleneck for the use of such enzyme in large-scale applications. Therefore, an F$_{420}$H$_2$-generating system whose cosubstrate could be recycled, such as T28A Tfu-FNO, would be highly promising. Available, robust NAD(P)H regeneration machineries, such as glucose dehydrogenase or other dehydrogenases, have been thoroughly investigated and widely applied in industry (30). Therefore, by combining Tfu-FNO with an appropriate NAD(P)H recycler, F$_{420}$H$_2$ reductases can be exploited for biocatalytic purposes.

**Experimental procedures**

**Cloning, expression, and purification of Tfu-FNO**

*T. fusca* YX was grown at 55 °C in Hägerdahl medium, and its genomic DNA was extracted using the GeneElute Bacterial Genomic DNA kit (Sigma). The gene Tfu-fno (Tfu_0970, TFU_RS04835) was PCR-amplified from genomic DNA of *T. fusca* using the pair of primers listed in Table 2 with the Ndel and HindIII restriction sites introduced at the 5’ and 3’ positions of the gene, respectively. The purified PCR product and the pBADN/Myc-HisA vector were digested with the restriction enzymes Ndel and HindIII, purified, and ligated (vector to insert ratio ca. 1 to 5 (mol/mol)) using T4 DNA ligase (Promega) with quick ligation buffer. The pBADN/Myc-HisA vector is a variant of the commercial pBAD/Myc-HisA (Invitrogen) where the unique Ncol site at the translation start is replaced with Ndel. The ligation product was transformed into chemically competent *E. coli* TOP10 cells using the heat shock method. Correct transformants were confirmed by sequencing the recombinant plasmid pBAD-fno.

Site-directed mutagenesis was carried out by using the pBAD-fno vector as template and the QuikChange$^{	ext{TM}}$ mutagenesis method with the corresponding pairs of primers listed in Table 2. The primers (200 nm) were used in a 10-μl reaction mixture. In the case of the double mutants, plasmids with a single mutation were used as the template. The remaining parent template vector was digested by incubating with DpnI (New England Biolabs) at 37 °C for 2 h. DpnI was then inactivated at 80 °C for 10 min, and the mutant plasmid was transformed into chemically competent *E. coli* TOP10 cells. Mutations were confirmed by sequencing.

*E. coli* TOP10 cells with pBAD-fno were grown overnight at 37 °C, 130 rpm in a 5-ml lysogenic broth (LB) containing 50 μg/ml ampicillin. This preculture was used to inoculate 500 ml of the same medium and grown at 37 °C, 130 rpm. When the $A_{600}$ reached 0.4–0.6, the protein expression was induced by the addition of 0.02% (v/v) arabinose followed by incubation at 30 °C, 130 rpm for 12 h. Cells were harvested by centrifugation at 6000 × g for 15 min (JLA 10.500 rotor, 4 °C) and resuspended in 10 ml of 50 mM KPi, pH 7.0, supplemented with 1 μg/ml of DNase I. Cells were sonicated for 7 min (10 s on, 15 s off cycle, 70% amplitude) at 4 °C using a VCX130 Vibra-Cell sonicator (Sonics & Materials, Inc., Newton, CT) and then centrifuged at 15000 × g (JA 17 rotor) for 45 min to obtain the cell-free extract. Tfu-FNO was precipitated by adding 50% saturated ammonium sulfate followed by anion exchange chromatography with a HiTrap$^{	ext{TM}}$ Q HP 5 ml (GE Healthcare) column pre-equilibrated with the same resuspension buffer. Tfu-FNO was eluted by using a linear gradient of 0–1 M NaCl in the same buffer. At around 250 mM NaCl, Tfu-FNO started eluting. Excess salt was removed by using a PD-10 desalting column, and the protein was stored in 50 mM KP, buffer (GE Healthcare). Protein concentration was estimated using Bradford assay (31).

**Temperature, pH optima, and thermostability of Tfu-FNO**

F$_{420}$ was isolated from *M. smegmatis* mc² 4517 as previously published protocol (32). F$_{420}$H$_2$ was prepared by biocatalytic
to 1.8 Å were collected at the ID30B beamline of the European Synchrotron Radiation Facility in Grenoble, France (ESRF). Image indexing, integration, and data collection, crystals were cryo-protected in themother liquor and flash-cooled by plunging them into liquid nitrogen.

**Steady-state kinetic analyses**

To determine the kinetic parameters of the enzyme, initial F_{420} reduction rates were measured using a SynergyMX microplate reader (BioTek) using 96-well F-bottom plates (Greiner Bio-One GmbH) at 25 °C. The reaction was performed in 50 mM KP$_r$ pH 6.0, and was started by adding 25–50 nM enzyme in the final volume of 200 µl. The concentration of one of the substrates was kept constant (250 µM for NADPH and 20 µM for F$_{420}$ respectively) while varying the concentration of the other substrate. All the measurements were performed in duplicate. A decrease of absorption either at 400 nm (F$_{420}$ reduction, $e_{400} = 25.7$ mm$^{-1}$ cm$^{-1}$) or at 340 nm (NADPH oxidation, $e_{340} = 6.22$ mm$^{-1}$ cm$^{-1}$) was followed to determine the observed rates, $k_{obs}$ (s$^{-1}$). $k_w$ and $k_{cat}$ values for NADP$^+$, NADPH, F$_{420}$, and F$_{420}$H$_2$ were calculated by fitting the data into the Michaelis-Menten kinetic model using nonlinear regression with GraphPad Prism 6.00 (GraphPad Software, La Jolla, CA).

**Crystallization, X-ray data collection, and structure determination of Tfu-FNO**

Native Tfu-FNO was crystallized using the sitting-drop vapor diffusion technique at 20 °C by mixing equal volumes of 9.0 mg/ml protein in 10 mM Tris/HCl, pH 7.5, 100 mM NaCl and of the reservoir solution containing 5% (w/v) PEG 3000, 30% (v/v) PEG 400, 10% (v/v) glycerol, 0.1 M HEPES, pH 7.5. Before data collection, crystals were cryo-protected in the mother liquor and flash-cooled by plunging them into liquid nitrogen. X-ray diffraction data to 1.8 Å were collected at the ID30B beamline of the European Synchrotron Radiation Facility in Grenoble, France (ESRF). Image indexing, integration, and data scaling were processed with XDS package (35, 36) and programs of the CCP4 suite (37). The Tfu-FNO structure was initially solved by molecular replacement method with Phaser (38) using the coordinates of FNO from A. fulgidus (PDB ID code 1JAY; Ref. 20), which shares 40% sequence identity with Tfu-FNO as a starting model devoid of all ligands and water molecules. Manual model correction and structure analysis was carried out with Coot (39), whereas alternating cycles of refinement was performed with Refmac5 (40). The figures were generated by using UCSF Chimera (41). Atomic coordinates and structure factors were deposited in the Protein Data Bank under the accession code 5N2I. Detailed data processing and refinement statistics are available in Table 3.

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