Crystal structure analysis of *Bacillus subtilis* ferredoxin-NADP⁺ oxidoreductase and the structural basis for its substrate selectivity

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Abstract: *Bacillus subtilis* *yumC* encodes a novel type of ferredoxin-NADP⁺ oxidoreductase (FNR) with a primary sequence and oligomeric conformation distinct from those of previously known FNRs. In this study, the crystal structure of *B. subtilis* FNR (BsFNR) complexed with NADP⁺ has been determined. BsFNR features two distinct binding domains for FAD and NADPH in accordance with its structural similarity to *Escherichia coli* NADPH-thioredoxin reductase (TdR) and TdR-like protein from *Thermus thermophilus* HB8 (PDB code: 2ZBW). The deduced mode of NADP⁺ binding to the BsFNR molecule is nonproductive in that the nicotinamide and isoalloxazine rings are over 15 Å apart. A unique C-terminal extension, not found in *E. coli* TdR but in TdR-like protein from *T. thermophilus* HB8, covers the *re*-face of the isoalloxazine moiety of FAD. In particular, Tyr50 in the FAD-binding region and His324 in the C-terminal extension stack on the *si*- and *re*-faces of the isoalloxazine ring of FAD, respectively. Aromatic residues corresponding to Tyr50 and His324 are also found in the plastid-type FNR superfamily of enzymes, and the residue corresponding to His324 has been reported to be responsible for nucleotide specificity. In contrast to the plastid-type FNRs, replacement of His324 with Phe or Ser had little effect on the specificity or reactivity of BsFNR with NAD(P)H, whereas replacement of Arg190, which interacts with the 2'-phosphate of NADP⁺, drastically decreased its affinity toward NADPH. This implies that BsFNR adopts the same nucleotide binding mode as the TdR enzyme family and that aromatic residue on the *re*-face of FAD is hardly relevant to the nucleotide selectivity.

Keywords: crystal structure; oxidoreductase; electron transfer; protein–protein interaction; conformational change

Additional Supporting Information may be found in the online version of this article.

Abbreviations: AdR, adrenodoxin reductase; AMP, adenosine monophosphate; BsFNR, *B. subtilis* FNR; Fd, ferredoxin; FNR, ferredoxin-NADP⁺ oxidoreductase; GR, glutathione reductase; NMN, nicotinamide mononucleotide; PDB, Protein Data Bank; PdR, putidaredoxin reductase; PEG, polyethylene glycol; RMSD, root-mean-square deviation; TdR, NADPH-thioredoxin reductase; WT, wild type.

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Introduction

Ferredoxin-NADP⁺ oxidoreductases (FNRs, [EC 1.18.1.2], [EC 1.18.1.3]) catalyze a redox reaction of NAD(P)H with ferredoxin (Fd), a low-redox-potential iron-sulfur protein, or with flavodoxin, a low-molecular-weight FMN-containing flavoprotein. FNRs and their isoforms, putidaredoxin reductase (Pdr)¹ and adrenodoxin reductase (AdR),^{2,3} occur in a wide variety of organisms, except for certain strict anaerobes such as Clostridia and methanogens, which instead contain pyruvate-ferredoxin oxidoreductases.⁴ Predictions based on phylogenic and structural analyses and on catalytic properties indicate that FNRs possess multiple phylogenic origins. FNRs are classified into the following three groups with five subclasses⁵⁻⁹: the plastid-type family, including plastid- and bacteria-type subclasses from plastids and γ -proteobacteria, respectively; the glutathione reductase (GR)-type family, including AdR- and Pdr-type subclasses from vertebrate and pseudomonas species, respectively; and the NADPH-thioredoxin reductase (Tdr)-type family. Despite a structural similarity, the plastid- and bacterial-type FNRs are differed in catalytic rate and physiological function.^{5,7} AdR- and Pdr-type FNRs play a role in cyt P450 reduction system. However, they are differed in specificity to NAD(P)H and catalytic rate.⁵ Structural and functional aspects of these FNRs have been precisely reviewed.^{5,7}

Crystal structures of the plastid-type,¹⁰⁻¹⁴ bacteria-type,¹⁵⁻¹⁷ Pdr-type,¹⁸⁻²¹ and AdR-type²²⁻²⁸ FNRs have been determined, leading to detailed understanding of their substrate-binding and catalytic mechanisms. All these FNR families possess separate binding domains for FAD or FMN, and for NAD(P)H,²⁹ as does the disulfide reductase family such as GR³⁰ and Tdr from *Escherichia coli*.^{31,32} Although the folding topology of each nucleotide-binding domain is distinct between the plastid- and GR-type FNRs,^{5,29} crystal structure analyses have indicated that the specificity for NADH versus NADPH is determined by the same basic mechanism, namely, positively charged residues recognize the 2'-phosphate group of the adenosine monophosphate (AMP) moiety of an NADP⁺ in majority of the NADPH-specific enzymes, whereas the corresponding residues of the NAD⁺-binding site often hydrogen bonding with the 2'-hydroxy group of the AMP moiety and negatively charged residues often avoid NADPH binding.^{5,11} Protein engineering studies have shown that these assigned residues are functionally important for nucleotide specificity, but do not absolutely determine specificities to NAD(P)H.³³⁻³⁶ Absolute changes in selectivity for either NADH or NADPH require the substitution of not only these residues but also of the residues required to recognize the pyrophosphate and adenine moieties of NAD(P)H, which is required for the conformational changes to address the nicotinamide mononucleotide (NMN) moiety to the isoalloxazine ring for a hydride ion trans-

fer.^{29,35,36,37} Additionally, the aromatic residues stacked on the *re*-face of the isoalloxazine ring of an FAD are responsible for nucleotide specificity in the bipartite reaction mechanisms of plastid-type FNRs and of flavocytochrome P450 BM3.³⁸⁻⁴²

Tdr-type FNRs and their homologs are widely distributed among bacteria, including anaerobic photoautotrophs,⁴³ thermophiles,^{8,44} gram-positive bacteria, and proteobacteria.^{9,45} Tdr-type FNRs occur as homodimers and show less amino acid sequence homology with other FNR types than with bacterial NADPH-thioredoxin reductases. Despite their low sequence homology to plastid-type and GR-type FNRs, Tdr-type FNRs exhibit comparable catalytic properties to these other FNR types such as enzymatic rate and binding affinity for NAD(P)H.⁴⁵ The Tdr-type FNRs and Tdr superfamily include NADPH-specific and NADPH/NADH-bispecific enzymes. Because different bacterial species adopt a variety of metabolic processes depending on their habitat, comparing the structural basis of nucleotide specificity of these FNRs may provide important insights into the physiological function and evolutionary aspects of Fd- and FNR-related metabolic processes. Recently, a crystal structure of FNR from the photoautotrophic anaerobe *Chlorobaculum tepidum* (CtFNR) has been reported.⁴⁶ This structure revealed structural similarity to *E. coli* Tdr as well as unique configurations related to its enzymatic function. However, attempts to crystallize the enzyme complexed with NAD(P)⁺ using a cocrystallization or soaking methods have been unsuccessful. The crystal structure of a thioredoxin reductase-like protein from *Thermus thermophilus* has been deposited in the Protein data bank (PDB; 2ZBW) (45% sequence identity). It was annotated as Tdr-like protein; however, the di-cysteine motif (CXXC) essential for the catalytic function of Tdr is also absent. The *T. thermophilus* Tdr-like protein is likely to be Tdr-type FNRs.

In this study, we have succeeded in crystallizing the NADP⁺ complex of FNR from the heterotrophic gram-positive bacterium *Bacillus subtilis* (BsFNR). The resulting crystal structure revealed that the mode of NADP⁺ binding corresponds to that in Tdr, and that a unique C-terminal extension, which is not conserved in Tdr, covers the *re*-face of the isoalloxazine moiety of FAD as observed in CtFNR⁴⁶ and Tdr-like protein from *T. thermophilus* HB8. To confirm the residues involved in nucleotide selectivity, we prepared Arg186, Arg190, and His324 mutants of BsFNR and examined their spectroscopic characteristics and steady-state kinetics. These studies demonstrated roles for Arg186 and Arg190 in selectivity for NADPH by recognizing the 2'-phosphate of NADP⁺, whereas His324, which is stacked on the isoalloxazine ring as observed in plastid-type FNR, had little effect on reactivity with NAD(P)H. Replacement of these residues did not significantly

Table I. Summary of Crystallographic Data

	Form I	Form II
Space group	<i>P</i> 2 ₁ 2 ₁ 2	<i>C</i> 2
Unit-cell parameters		
<i>a</i> (Å)	63.90	207.47
<i>b</i> (Å)	135.72	64.85
<i>c</i> (Å)	39.19	61.12
β (°)		105.82
Resolution (Å)	50.00–1.80 (1.86–1.80)	50.00–1.90 (1.97–1.90)
No. of reflections	32,363	59,771
Multiplicity	7.0 (6.1)	3.6 (3.0)
Completeness (%)	99.9 (99.5)	96.9 (80.4)
< <i>I</i> >/<σ(<i>I</i>)>	23.0 (2.8)	22.6 (3.1)
<i>R</i> _{merge} (%)	7.1 (38.8)	5.1 (22.0)
<i>R</i> -value (%)	17.0 (20.6)	18.3 (21.4)
<i>R</i> -free (%)	19.6 (20.7)	21.1 (26.4)
No. of nonhydrogen atoms	2913	5860
No. of residues	329	658
No. of solvent molecules	242	518
Average <i>B</i> -factor (Å ²)		
Protein (FAD, NADP ⁺ domains)	20.2 (14.4, 29.2)	24.9 (22.9, 28.0)
Solvent	29.5	32.0
Root-mean-square deviations		
Bond lengths (Å)	0.007	0.009
Bond angles (°)	1.253	1.325
Ramachandran plot		
Favored residues (%)	97.5	96.9
Allowed regions (%)	2.5	3.1

intensify the reactivity with NADH, suggesting that this is controlled by other factors.

Results

Crystal structure of BsFNR

BsFNR complexed with NADP⁺ was crystallized in two space groups: Form I (*P*2₁2₁2: *a* = 63.90, *b* = 135.72, and *c* = 39.19 Å) and Form II (*C*2: *a* = 207.47, *b* = 64.85, *c* = 61.12 Å, and β = 105.82°). The two crystal structures were determined by the molecular-replacement method, and final models for Forms I and II were refined to a *R*-factors of 0.170 and 0.183 at 1.8 and 1.9 Å resolution, respectively (Table I).⁴⁷ Although three C-terminal residues (Glu330, Gln331, and Lys332) were disordered in both crystal forms, 97% of all residues occupied the favored regions of the Ramachandran plots. In the Form I crystal, two molecules related to a crystallographic twofold rotation axis formed a stable dimer. In contrast, in the Form II crystal, two molecules in the asymmetric unit formed a dimer. The total contact area of the dimer interface is about 1700 Å², which is stabilized by 28 direct hydrogen bonds as well as hydrophobic contacts. In both crystal forms, each protomer contained two Rossmann-type nucleotide-binding folds for FAD and NAD(P)H. The FAD-binding domain was composed of two discontinuous regions that included amino acid residues 1–124 and

250–332, whereas residues 125–249 formed the NAD(P)H-binding domain (Figs. 1 and 2). Although the two FAD-binding domains of the dimer in the Form II crystal were related by a noncrystallographic twofold axis, the NAD(P)H-binding domains were not related by this symmetry. The structure of the BsFNR dimer in the Form II crystal was essentially the same as that in the Form I crystal, except for the orientation of the NAD(P)H-binding domain relative to the FAD-binding domain. When the FAD-binding domain of the Form I crystal was superimposed onto that of one protomer (molecule A) of the Form II crystal, the NAD(P)H-binding domains did not overlap well [Fig. 2(d)]. The NAD(P)H-binding domains could be brought together by rotating one domain ~16° around the connecting region formed by two β-strands, β 9 and β 18, a movement permitted by the absence of specific interactions between the two domains. Separate superimposition of each domain yield root-mean-square deviation (RMSD)s of 0.28 and 0.35 Å for the FAD- and NAD(P)H-binding domains, respectively, demonstrating the high structural similarity between the corresponding domains. Hereafter, all structural description of BsFNR will be based on Form I crystal structure.

The topology of each nucleotide-binding domain in BsFNR agreed with the corresponding domain in *E. coli* TdR (PDB code 1TDF) (Fig. 3). The RMSDs of the main chain of the BsFNR FAD-binding domain (162 Cα atoms) and the NAD(P)H-binding domain (124 Cα atoms) against those in *E. coli* TdR were 1.54 and 1.53 Å, respectively. The FAD-binding domain of BsFNR involved four helices (α1–α3 and α6) and 12 strands (β1–β8 and β19–β22) forming three β-pleated sheets, and the arrangement of these secondary structures was almost identical to the FAD-binding domain of *E. coli* TdR³¹ (Fig. 3). The dimer interface between the BsFNR molecules was composed of the α1, α3, and α6 helices and a loop region between strands β3 and β4 with each of these components interacting with its counterpart in the other molecule. A conspicuous difference between the FAD-binding domains of BsFNR and *E. coli* TdR occurs in the loop region where Tyr50–Arg65 is positioned at the *si*-face of the isoalloxazine ring of FAD. In this region, BsFNR Tyr50 stacked almost parallel to the isoalloxazine ring with a distance of 3.3 Å between the Tyr50 hydroxy oxygen and N10 of the isoalloxazine ring, whereas no residues contact the *si*-face of the isoalloxazine ring in *E. coli* TdR.³¹

The NAD(P)H-binding domain of BsFNR contained two helices (α4 and α5) and eight strands (β10–β17) forming two β-pleated sheets, and the arrangement of the secondary structures was basically identical to that of *E. coli* TdR (Fig. 3). In BsFNR, no direct interaction of amino acid residues between the NAD(P)H- and FAD-binding domain was observed, providing flexibility between the two domains.³² As a result, the relative orientation of

Figure 1. Deduced amino acid sequence alignment of BsFNR (NCBI GeneID: 936577), *C. tepidum* FNR (NCBI GeneID: 1006048), *T. thermophilus* TdR (PDB ID: 2ZBW), and *E. coli* TdR (NCBI GeneID: 949054). Conserved regions are highlighted in red. Secondary structures present in BsFNR are indicated above the sequence. Two canonical dinucleotide-binding motifs with sequence signatures of GxGxxG/A occur in both domains (residues 14–19 and 161–166). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the two domains in BsFNR differed from that in *E. coli* TdR^{31,32} (Fig. 3). *E. coli* TdR possesses redox-active cysteine residues in helix N3 (corresponding to η 2 in BsFNR), Cys135 and Cys138, which are required for thioredoxin reduction, and these residues approach the *re*-face of the isoalloxazine ring in the flavin-oxidizing (FO) form.³² In BsFNR, the corresponding helix η 2 was relatively short and was distant from the *re*-face of the isoalloxazine ring of FAD. Instead, the C-terminal extension (Pro322 to Lys332) of the other subunit covered the *re*-face of the isoalloxazine ring, as described below.

The extended C-terminal region showed a unique construction that was not observed in bacterial TdRs but was conserved in TdR-type FNRs and their homologs (Fig. 1). Crystal structure analysis together with sequence alignment revealed that this

region extended beyond helix α 6 in the dimer interface of the FAD-binding domain, raising up an additional helix α 7, and covered the *re*-face of the isoalloxazine ring of FAD bound to the other subunit (Fig. 2). The imidazole group of His324 located at the end of the N-terminal side of helix α 7 stacked nearly parallel to the *re*-face of the isoalloxazine ring, with a distance of 3.6 Å between C ϵ ₁ of His324 and C9 of the isoalloxazine ring. This was in contrast to *E. coli* TdR, in which the *re*-face of the isoalloxazine ring is covered by the redox-active cysteine residues of helix N3 in the FO form or by the nicotinamide ring of the NADPH in the flavin-reducing (FR) form.⁴⁸ As a result, in BsFNR the isoalloxazine ring of FAD was sandwiched between the two aromatic groups of Tyr50 and His324, which lay almost parallel and with a similar spacing as in the plastid-

Figure 2. (a) Overall structure of the BsFNR monomer in complex with NADP⁺ (Form I). FAD- and NAD(P)H-binding domains are shown in green and lightgreen. FAD is in yellow and NADP⁺ in blue. (b) Overall structure of the BsFNR dimer in complex with NADP⁺ (Form I). Molecules A and B are shown in green and cyan, respectively. FAD is in yellow and NADP⁺ in blue. (c) Electrostatic surface of BsFNR (Form I). (d) Comparison of Form I (blue) and Form II (red). Superposition is based on the FAD-binding domain. The N- and C-termini are labeled N and C, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

type FNRs.^{10–12} In addition to His324, the hydroxy groups of Ser325 and Thr326 in the C-terminal extension lay within hydrogen-bonding distance of O4 and N5 of the isoalloxazine ring, respectively.

FAD- and NADP⁺-binding sites

The crystal structure of BsFNR showed one FAD molecule bound in an extended conformation to the surface of the FAD-binding domain in each protomer [Fig. 4(a)]. Temperature factors of FAD were comparable to those of peptide atoms in the FAD-binding domain. The isoalloxazine ring was positioned at the N-terminus of helix $\alpha 6$, such that the dipole moment of the helix may have stabilized the charge distribution of the ring system.⁴⁹ The isoalloxazine ring was almost planar and sandwiched between the phenol ring of Tyr50 on the *si*-face and the imidazole ring of His324 on the *re*-face. The carboxy oxygen of Asp57 and one water molecule interacted with N3 and O2 of the isoalloxazine ring, but unlike *E. coli* TdR, no interaction was observed with C4 in the ring.³² 2'- and 3'-OH groups in the ribityl chain interacted with the hydroxy group of Tyr50 and the carboxy oxygen of Asp285, respectively. The pyrophosphate

group of FAD was positioned close to the N-terminus of helix $\alpha 1$ and was stabilized by the dipole moment of the helix.⁴⁸ The pyrophosphate oxygen atoms interacted with residues Asp285, Val18, and Gln45 and the 2'- and 3'-OH groups of the AMP ribose interacted with the carboxy oxygen of Glu37 at the C-terminus of strand $\beta 2$, as reported for *E. coli* TdR.³²

The electron density map clearly showed the atom positions for NADP⁺ in an extended conformation [Fig. 4(b)]. The average temperature factor of the assigned NADP⁺ atoms was within the range of those of the peptide atoms in the NADPH-binding domain. The nicotinamide ring was bound to the N-terminus of helix $\alpha 4$ with the amide N7 within hydrogen-bonding distance of the Asp168 carboxy oxygen atom. In the NMN portion of the nucleotide, the ribose C2 hydroxy group interacted with the Asp164 carboxy oxygen atom, and the phosphate oxygen atom interacted with the backbone amino nitrogen atom of Ser165. As proposed by Wierenga *et al.*,⁵⁰ the pyrophosphate portion was placed at the N-terminus of helix $\alpha 4$ with a water-mediated hydrogen bond. It should be noted that the NMN portion of NADP⁺ made contact only

Figure 3. (a) Comparison of BsFNR and the FO form of *E. coli* TdR (PDB ID 1TDF) with the FAD-binding domains superimposed. BsFNR molecules A and B are shown in green and cyan, respectively, and *E. coli* TdR is in orange. (b) Comparison of BsFNR and the productive (FR) form of *E. coli* TdR (PDB ID 1F6M) with the FAD-binding domains superimposed. Molecule A of BsFNR is shown in green, the C-terminal region of BsFNR molecule B is in cyan, and the productive form of *E. coli* TdR is in magenta. NADP⁺ bound to BsFNR and to the productive form of *E. coli* TdR is shown in blue and black, respectively. FAD is shown in yellow. (c) The NAD(P)H-binding domains of BsFNR and the productive form of *E. coli* TdR are superimposed, showing the relative 60° rotation of the BsFNR NAD(P)H-binding domain. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

with the residues in helix $\alpha 4$. In the AMP portion, the ribose 2'-phosphate interacted with two positively charged residues, Arg186 and Arg190, in the loop region between strand $\beta 12$ and helix $\alpha 5$, as observed in *E. coli* TdR.³² In contrast, the corresponding positions in NADH/NADPH bispecific CtFNR were occupied by Gly195 and Gln199 (Fig. 1), suggesting the

involvement of these residues in nucleotide selection. A notable difference from *E. coli* TdR was the presence in BsFNR of the Tyr246 phenol ring near the hinge region, which stacked almost parallel to the adenine ring with a 3.5 Å distance between Cz of Tyr246 and C2 of the adenine ring. An aromatic residue was conserved at the position corresponding to BsFNR Tyr246 among the majority of TdR-type FNRs, including *Rhodopseudomonas palustris* FNR (Phe249), whereas nonaromatic residues occurred in the sequences of *E. coli* TdR and CtFNR (Ile244 and Ile259, respectively) (Fig. 1).

Effects of Arg186, Arg190, and His324 mutations on the interaction with NAD(P)H

The crystal structure revealed that Arg186 and Arg190 interacted with the 2'-phosphate group of the AMP ribose of NADP⁺, and that His324 stacked on the *re*-face of the FAD isoalloxazine ring (Fig. 2). To confirm the functional properties attributed to these residues, we performed site-directed mutagenesis of NADPH-specific BsFNR to replace Arg186, Asp187, Arg190, and His324 with the residues occurring in NADH/NADPH-bispecific *C. tepidum* FNR (Gly, His, Gln, and Phe, respectively). In addition, His324Ser mutant was also prepared.

Replacement of Arg186 with glycine led to drastically reduced amounts of recombinant protein.

Figure 4. Stereoviews of the FAD-binding site (a) and NADP⁺-binding site (b) of BsFNR. NADP⁺ omitted map (contoured at 3 σ) are shown in cyan. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 5. UV-vis difference spectra for BsFNR upon binding of NADP⁺. Spectra before nucleotide addition were subtracted from those recorded after the addition of 1 mM NADP⁺. Reaction mixtures contained 8–19 μ M enzymes in 100 mM potassium phosphate buffer (pH 7.0) at 25°C. (a) Spectra at a saturated amount of NADP⁺ estimated with K_d values of 14 μ M for WT, 44 μ M for H324F mutant, and 18 μ M for H324S mutant: black line, WT; red line, H324F; blue line, H324S. (b) Difference spectra induced by the addition of 1 mM NADP⁺ to the solutions containing indicated amounts of enzymes: black line, WT (16 μ M); green line, R186G/H187D/R190Q (19 μ M); blue line, R190Q (15 μ M); red line, R186G/H187D (12 μ M). [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://www.interscience.wiley.com).]

Simultaneous replacement of both Arg186 and Asp187 with glycine and histidine, respectively, yielded satisfactory amounts of the recombinant protein, and this latter variant was used for further experiments. The other recombinant proteins were successfully expressed and purified to apparent homogeneity on SDS-PAGE.⁴⁵ The apparent molecular masses of mutated BsFNR polypeptides were $\sim$ 40 kDa on SDS-PAGE. WT and mutated BsFNR repre-

sent only one fraction with an apparent molecular mass of $\sim$ 120 kDa on a gel-permeation chromatography (Supporting Information Fig. 1).

UV-vis absorption spectra of air-oxidized FNRs exhibited three absorption bands at 273–5, 381, and 457–9 nm for WT, R186G/D187H, R190Q, R186G/D187H/R190Q, and H324S variants. The absorption bands of the H324F variant were slightly red-shifted to 274, 382, and 460 nm, but not to same extent as the shifting of bands (274, 388, and 466 nm) that has been observed for the F337H mutation of the corresponding residue in *C. tepidum* FNR.^{43,46} Absorption coefficients of WT and mutated BsFNR at the λ_{max} near 460 nm exhibited similar values of 12.0–12.2 $\text{mM}^{-1} \text{cm}^{-1}$, except for 12.8 $\text{mM}^{-1} \text{cm}^{-1}$ in H324S and 11.4 $\text{mM}^{-1} \text{cm}^{-1}$ in R186G/H187D and in H324F. Interactions of WT BsFNR with NADP⁺ induced a spectral shift,⁴⁵ producing troughs at 452 and 480 nm and peaks at 469 and 508 nm in the difference spectrum [Fig. 5(a)]. These shifts were drastically decreased in R190Q and nearly undetectable in R186G/H187D/R190Q, suggesting that recognition of the 2'-phosphate depended heavily on Arg190 [Fig. 5(b)]. Notably, the intensities of the absorption bands in the difference spectrum of the H324S mutant were more intense than those of the WT enzyme. This is presumably because replacement of histidine by the less bulky serine favored the interaction. Addition of 1 mM NAD⁺ to the WT BsFNR solution induces slight decrease in absorbance around 480 nm (Supporting Information Fig. 2). In the case of H324F, H324S, R186G/D187H, R190Q, and R186G/D187H/R190Q mutants, a significant change was not observed.

K_m values for NADPH measured in ferricyanide reduction assays were 5- and 40-fold higher in the R186G/D187H and R190Q mutants, respectively, than in the WT, but k_{cat} values are barely affected (Table II). In contrast to the plastid-type FNRs, replacement of His324 in BsFNR had little effect on its K_m for NAD(P)H, and its catalytic efficiencies increased slightly. Similar results have been obtained for the F337H, F337S, and F337Y mutations of *C. tepidum* FNR.⁴⁶ Likewise, replacement of BsFNR His324 slightly affected NADH-dependent ferricyanide reduction activity, which was also contrary to results for the plastid-type FNRs.^{38–42}

Discussion

The crystal structure of the BsFNR-NADP⁺ complex clearly denoted the positions of both the NMN and AMP portions of the bound NADP⁺ molecule. The binding mode of this cofactor was predicted to be similar to those in *E. coli* TdR based on the conserved sequence motifs for NADPH binding, G¹⁶¹GGXXA and H¹⁸⁵RRXXFR (in BsFNR numbering, Fig. 1).⁵⁰ Both protomer contains one molecule of NADP⁺ in almost same position in two crystal

Table II. *NAD(P)H-Ferricyanide Reductase Activity, Spectroscopic Properties, and K_d Values for NADP^+ of BsFNR and Its Mutants*

	WT	R186G/D187H	R190Q	R186G/D187H/R190Q	H324F	H324S
NADPH K_m (μM)	10.9 ± 0.2^a	55 ± 6	430 ± 20	1300 ± 600	13.1 ± 1.8	7.9 ± 1.2
NADPH k_{cat} (s^{-1})	900 ± 40^a	1080 ± 30	1460 ± 30	870 ± 170	1320 ± 40	1070 ± 30
k_{cat}/K_m ($\text{s}^{-1} \text{M}^{-1}$)	8.3×10^7	2.0×10^7	3.4×10^6	6.8×10^5	1.0×10^8	1.4×10^6
NADH at 1 mM k_{obs} (s^{-1})	15 ± 3	130 ± 30	8 ± 6	92 ± 6	24 ± 6	30 ± 10
ϵ ($\text{mM}^{-1} \text{cm}^{-1}$)/ λ_{max} (nm)	12.2/458	11.4/459	12.3/458	12.0/458	11.4/460	12.8/457
K_d for NADP^+ (μM)	14 ± 3^a	—	—	—	44 ± 8	17.5 ± 1.2

^a Values are obtained from Seo *et al.*⁴⁵

forms, indicating a functionality of the dimer is not relevant in the reaction with NADP^+/H . The binding mode of NADP^+ found in the NADP^+ -BsFNR crystal structure appears to be nonproductive because the nicotinamide ring is over 15 Å away from the isoalloxazine ring system. As such a form may not provide a perturbation of an absorption spectrum of FAD, obtained crystal structure is not corresponded to the form in the presence of an excess amount of NADP^+ in solution. In the crystal structure of the FR form of *E. coli* TdR, the nicotinamide ring of NADPH is in close contact with the *re*-face of the isoalloxazine ring system, allowing the stereospecific transfer of a hydride ion.⁵¹ Therefore, in the case of BsFNR, the NADPH-binding domain must rotate 60° relative to the FAD-binding domain in order for hydride transfer to take place. It is evident from the different conformations observed in the two crystal forms that a large domain movement can indeed occurred, and flexibility of BsFNR was demonstrated in the bending of its hinge region [Fig. 2(d)]. In CtFNR, replacement of the Gly residues at the hinge region with proline diminished its reactivity with NADPH, supporting the catalytic indispensability of this bending motion.⁴⁶ The relative orientation of one protomer *T. thermophilus* TdR-like protein (TtFNR) is almost same as that of BsFNR Form I (The RMSD value, 1.28 Å for 317 C α atoms). The relative orientation of NAD(P)H-binding domain in the other protomer of TtFNR was different (the relative 23° rotation). The flexibility between the two domains will be a common feature for the TdR-type FNRs. This type of pronounced and essential domain movement may be regarded as a feature that distinguishes the TdR-type enzymes from other FNR family members.

BsFNR and CtFNR differ in their spectroscopic properties and nucleotide specificities.^{43,45} The NADPH- and FAD-binding domains of BsFNR can be superimposed with molecule A of homodimeric form of CtFNR (PDB code: 3AB1⁴⁶), whereas NADPH-binding domain of CtFNR homodimer molecule B is rotated ~30° from that in BsFNR [Fig. 6(a)]. Superposition of the NADPH-binding domains [Fig. 6(b)] provides a larger RMSD value, 1.28 Å (for C α atoms of 125–248 in BsFNR vs. 133–261 in molecule B of CtFNR) than that for the FAD-binding

domains, 0.95 Å (see below). A distinctive configuration is found in the nucleotide-binding loop between the strand β 12 and the helix α 5 (Arg185 to Glu193 in BsFNR numbering; Fig. 1). Among the BsFNR mutants examined in this report, R186G and D187H regained reactivity with NADH by a factor of nine compared with the WT enzyme (Table II). Yet, the turnover rate for NADH was still only one-tenth that for NADPH and fell short of the level seen in WT CtFNR.⁴⁶ Further investigation of the amino acid residues in this region will be required to fully understand the nucleotide selectivity toward NADH, the importance of local structural motion, and how these relate to the NAD(P)H selectivity of plastid-type FNR³⁶ and human GR.³⁷

When superposed, the FAD-binding domains of BsFNR and CtFNR [Fig. 6(c)] exhibit subtle differences in main-chain configuration; the RMSD value is 0.95 Å for C α atoms of 6–124 and 250–329 in BsFNR against 13–132 and 263–342 in molecule B of CtFNR. Both chain configuration and amino acid residues at the *si*-face of the isoalloxazine ring (Tyr50-Val58 in BsFNR) are highly conserved between these two FNRs, but differ completely from those in TdRs which are Ser and Thr rich (Fig. 1). One conspicuous difference between the FAD-binding domains of BsFNR and CtFNR is at the C-terminal helical extension [Figs. 1 and 6(c)]. Among TdR-type FNRs, BsFNR has comparatively short (eight residues) C-terminal helix, whereas CtFNR has a rather long (23 residues) helix that is rich in Lys and Ala residues. This helix is sufficiently long to form a helix dipole field, and variations in its length and amino acid composition should affect its dipole field intensity.⁴⁹ In contrast to the plastid-type FNRs, the aromatic residues at the N-terminus of the helix α 7, which stacks on the *re*-face of the isoalloxazine ring, vary widely in different bacterial species. For example, the helix N-terminal residue is His in the Firmicute enzyme, Tyr in α -proteobacteria, and Phe for green sulfur bacteria. However, the next two to three residues of the helix are conserved serines and threonines, which hydrogen bond with the isoalloxazine ring of FAD.

Substitutions of His324 by Phe or Ser in BsFNR had little effect on either reactivity with NADPH or nucleotide selectivity toward NAD(P)H (Table II).

Figure 6. (a) Comparison of BsFNR (green) and CtFNR (red) (PDB code: 3AB1). FAD bound to BsFNR and CtFNR is shown in yellow and orange, respectively. NADP⁺ bound to BsFNR is shown in blue. (b) Comparison of NADP⁺-binding sites of BsFNR (green) and CtFNR (magenta). (c) Comparison of FAD-binding sites of BsFNR (green) and CtFNR (magenta). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Nevertheless, the alteration of the UV–vis difference spectra and K_d values for nucleotide binding indicated that replacement of His324 with Ser actually

does participate in nucleotide binding and subsequent conformational changes. Therefore, the presence of unique hydrogen bonds in BsFNR between Ser325 and O4 and between Thr326 and N5 of FAD may keep the extended region in its original position and related to the reactivity. Among other enzymes in the FNR and disulfide reductase families, replacement of Tyr197 in human GR drastically affected the enzyme turnover rate rather than its nucleotide specificity,⁵² whereas replacement of Y303 in *Anabaena* FNR altered nucleotide selectivity but catalytic rates remained largely unaffected. The contrasting results of mutational analysis of the aromatic residues on the *re*-face of the ring between BsFNR and CtFNR and these other FNR types and human GR^{38–42} suggest a functional difference. Because the presence of the C-terminal extension imparts the unique homodimer configuration observed in TdR-type FNR enzymes, further investigation of this region is needed to understand structure–function relationships of the TdR-type FNRs.

Materials and Methods

Preparation of WT and mutated BsFNR

Site-directed mutagenesis of the *yumC* gene construct was performed according to the QuikChange method using the plasmid pETBlue-*yumC* plasmid⁴⁵ as template and the primers listed in Supporting Information Table I. Proper mutation and absence of second-site mutations in the open reading frame of the *yumC* gene were verified by DNA sequencing (Hitachi SQ5500). Expression and purification of the wild type (WT) and mutant forms of BsFNR were carried out according to the methods described previously.⁴⁵

Crystallization and structure analysis of WT BsFNR

Two crystal forms were obtained at 20°C with a reservoir solution containing 0.1M HEPES buffer (pH 7.5), 30% 1,2-propanediol, and 20% PEG 400 (Form I) and 20% PEG 3350, 0.2M sodium fluoride, and 5% Trehalose (Form II).⁴⁷ Protein droplets prepared by mixing 1 μ L protein solution containing 10 mg mL⁻¹ BsFNR and 2.5 mM NADP⁺ with 1 μ L reservoir solution were equilibrated against 100 μ L reservoir solutions at 293 K. X-ray diffraction data sets were collected with a Jupiter 210 CCD detector (Rigaku Corp., Tokyo, Japan) using a synchrotron radiation source at the BL38B1 beamline at SPring-8 (Japan). Diffraction images were processed with the programs HKL2000⁵³ and CCP4.⁵⁴ The structure was solved by the molecular replacement method implemented in PHASER crystallographic software.⁵⁵ A Form I crystal was used for the initial structure determination with the crystal structure of the *T.*

thermophilus HB8 thioredoxin reductase-like protein (PDB code: 2ZBW) as the primary search model. Further model building and refinement of the structure were carried out with the programs COOT⁵⁶ and refmac.⁵⁷ Progress and validity of the refinement process were checked by monitoring the *R*-free value for 5% of the total reflections.⁵⁸ The structure of Form II was solved by molecular replacement using the structure determined from Form I. Model geometry was analyzed with the program MOLPROBITY.⁵⁹ The data collection and refinement statistics are summarized in Table I. The RMSD was calculated using the program LSQMAN.⁶⁰ The figures were prepared with PyMOL (<http://www.pymol.org/>) using the coordinates from PDB files 1TDF and 1F6M for *E. coli* TdR.

Enzymatic activity assays and spectroscopic analyses

The NAD(P)H diaphorase assay was performed in 100 mM potassium phosphate buffer (pH 7.0) at 25°C with $K_3[Fe(CN)_6]$ ($\epsilon_{420} = 1.02 \text{ mM}^{-1} \text{ cm}^{-1}$ or $\epsilon_{440} = 0.59 \text{ mM}^{-1} \text{ cm}^{-1}$) as electron acceptor.⁴⁵ The reaction mixture (1 mL) contained 3 mM $K_3[Fe(CN)_6]$, 5 mM glucose 6-phosphate (G6P), 5 U of *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase, and 5–10 nM FNR together with NAD(P)H as indicated in Table II. Turnover rates are expressed as the number of NAD(P)H molecules consumed per FNR dimer per second. Each data point represents the average of three to four independent measurements. Michaelis–Menten K_m and k_{cat} values were estimated by nonlinear regression analysis using Igor software (Ver. 5.0.2, Wavemetric, Portland, OR).

The UV–vis absorption spectra were measured in a JASCO V-560 double beam spectrophotometer (Tokyo, Japan). After correction of experimentally obtained spectra for volume changes, difference spectra were calculated by subtracting the control spectrum recorded before substrate addition. K_d values for $NADP^+$ were determined according to the method described by Seo *et al.*⁴⁵ with values of $\Delta A_{507} - \Delta A_{484}$ for H324F mutant and $\Delta A_{506} - \Delta A_{480}$ for H324S mutant in the difference spectra.

Absorption coefficients of WT and mutated BsFNR were determined by heat denaturation method.⁶¹ An $\sim 10 \mu\text{M}$ solution of BsFNR solution in 10 mM HEPES Na buffer (pH 7.0) was incubated at 100°C for 10 min in the dark. Denatured polypeptides were removed by centrifugation at 15,000g for 15 min. The FAD concentration in the supernatant was determined from $\epsilon_{450} = 11.3 \text{ mM}^{-1} \text{ cm}^{-1}$.

PDB accession codes

Atomic coordinates and structure factors have been deposited in the RCSB Protein data bank under accession codes 3LZW and 3LZX.

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