Plant Physiology and Biochemistry 81 (2014) 143–148

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Role of the C-terminal extension stacked on the *re*-face of the isoalloxazine ring moiety of the flavin adenine dinucleotide prosthetic group in ferredoxin-NADP$^+$ oxidoreductase from *Bacillus subtilis*

Daisuke Seo [a,*], Tomoya Asano [b], Hirofumi Komori [c], Takeshi Sakurai [a]

[a] Division of Material Science, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma, Kanazawa, Ishikawa 920-1192, Japan
[b] Division of Functional Genomics, Advanced Science Research Center, Kanazawa University, Takaramachi 13-1, Kanazawa, Ishikawa 920-0934, Japan
[c] Faculty of Education, Kagawa University, 1-1 Saiwai, Takamatsu, Kagawa 760-8522, Japan

ARTICLE INFO

Article history:
Received 1 November 2013
Accepted 20 January 2014
Available online 30 January 2014

Keywords:
Ferredoxin
Ferredoxin-NADP$^+$ oxidoreductase
Flavin

ABSTRACT

Ferredoxin-NADP$^+$ oxidoreductase [EC 1.18.1.2] from *Bacillus subtilis* (*Bs*FNR) is homologous to the bacterial NADPH-thioredoxin reductase, but possesses a unique C-terminal extension that covers the *re*-face of the isoalloxazine ring moiety of the flavin adenine dinucleotide (FAD) prosthetic group. In this report, we utilize *Bs*FNR mutants depleted of their C-terminal residues to examine the importance of the C-terminal extension in reactions with NADPH and ferredoxin (Fd) from *B. subtilis* by spectroscopic and steady-state reaction analyses. The depletions of residues Y313 to K332 (whole C-terminal extension region) and S325 to K332 (His324 intact) resulted in significant increases in the catalytic efficiency with NADPH in diaphorase assay with ferricyanide, whereas K_m values for ferricyanide were increased. In the cytochrome *c* reduction assay in the presence of *B. subtilis* ferredoxin, the S325–K332 depleted mutant displayed a significant decrease in the turnover rate with an Fd concentration range of 1–10 μM. The Y313–K332 depleted mutant demonstrated an increase in the rate of the direct reduction of horse heart cytochrome *c* in the absence of Fd. These data indicated that depletion of the C-terminal extension plays an important role in the reaction of *Bs*FNR with ferredoxin.

1. Introduction

Ferredoxin-NAD(P)$^+$ oxidoreductase ([EC 1.18.1.2], [EC 1.18.1.3], FNR) is a member of the dehydrogenase family of the flavoprotein superfamily (Aliverti et al., 2008; Dym and Eisenberg, 2001; Correll et al., 1993). FNR catalyzes the redox reaction between the two electron carrier nucleotides, NAD(P)H, and the one electron carrier iron-sulfur proteins, ferredoxin (Fd), adrenodoxin (Ad) and putidaredoxin (Pd), and also the low molecular weight flavoprotein, flavodoxin. In photosynthesis, FNR catalyzes the reduction of NADP$^+$ to NADPH by photochemically reduced Fd (Sétif, 2001; Knaff and Hirasawa, 1991). In non-photosynthetic processes, FNR and its isoforms catalyze the reduction of Fd, Ad and Pd with NAD(P)H. In the latter case, reduced iron-sulfur proteins play an indispensable role as a low redox potential electron donor in a variety of metabolic processes including cytochrome P450-dependent hydroxylation, nitrogen fixation and the tolerance of active oxygen species (Aliverti et al., 2008; Knaff and Hirasawa, 1991; Bianchi et al., 1993; Munro et al., 2007; Ewen et al., 2011).

FNRs generally consist of two nucleotide-binding domains that are typically found among the FAD-dependent dehydrogenase family (Aliverti et al., 2008; Dym and Eisenberg, 2001; Correll et al., 1993). Phylogenic and structural information on FNR and its isoforms indicates that FNRs are categorized into five groups (Aliverti et al., 2008; Ceccarelli et al., 2004; Seo et al., 2004; Muraki et al., 2010). FNRs from green sulfur bacteria, Firmicutes and thermophiles (designated as TrxR-type FNRs) exhibit significant conservation of structural topology with bacterial NADPH-thioredoxin reductase (TrxR) (Seo et al., 2004; Muraki et al., 2010; Komori et al., 2010), whereas adrenodoxin reductase (AdR) and putidaredoxin

Abbreviations: Ad, adrenodoxin; AdR, adrenodoxin reductase; FAD, flavin adenine dinucleotide; Fd, ferredoxin; FNR, ferredoxin-NAD(P)$^+$ oxidoreductase; G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; GR, glutathione reductase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; K_d, dissociation constant; K_m, Michaelis constant; MALDI TOFMS, Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry; Pd, putidaredoxin; PdR, putidaredoxin reductase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Td, thioredoxin; TrxR, bacterial NADPH-thioredoxin reductase; Tris, tris(hydroxymethyl)aminomethane; WT, wild type.

* Corresponding author. Tel.: +81 76 264 5683; fax: +81 76 264 5742.
E-mail addresses: dseo@se.kanazawa-u.ac.jp, dseo@ruri.waseda.jp (D. Seo).

0981-9428/$ – see front matter © 2014 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.plaphy.2014.01.011

reductase (PdR) exhibit conservation of structural topology with glutathione reductase (GR) (Aliverti et al., 2008; Dym and Eisenberg, 2001). The structural topologies of these FNR groups are distinct from those of FNRs from plastid, cyanobacteria and proteobacteria. The latter FNRs are structurally related to phthalate dioxygenase reductase, NADPH-cytochrome P450 reductase and cytochrome b_5 reductase (Aliverti et al., 2008; Dym and Eisenberg, 2001; Correll et al., 1993; Karplus and Faber, 2004). Despite the differences in structural topology, similar arrangements of amino acid residues are often found around the isoalloxazine ring moiety of the FAD prosthetic group among FNR and its relatives. One such arrangement of amino acid residues involves the aromatic residues stacked on the isoalloxazine ring moiety of the FAD prosthetic group. The functional role of these residues has been extensively studied in plastid-type FNRs. Mutational analyses of FNRs from cyanobacteria (Piubelli et al., 2000; Nogués et al., 2004; Tejero et al., 2005) and Apicomplexa (Baroni et al., 2012), and cytochrome P450 BM3 (Neeli et al., 2005) display altered selectivity towards NADH/NADPH upon replacement of the aromatic residues on the *re*-face of the ring. In the case of TrxR-type FNRs from *Chlorobaculum tepidum* (*Ct*FNR) and *Bacillus subtilis* (*Bs*FNR), replacement of the *re*-face Phe and His, respectively, did not significantly affect its selectivity and reactivity to NAD(P)H (Muraki et al., 2010; Komori et al., 2010).

The crystal structures of *Ct*FNR (Muraki et al., 2010), *Thermus thermophilus* HB8 (PDB code: 2ZBW) (Mandai et al., 2009a) and *Bs*FNR (Komori et al., 2010) have revealed that these FNRs possess homologous structural topology with the TrxR, but only the TrxR-type FNRs possess the two conserved aromatic residues on the *si*- and *re*-face of the ring (Fig. 1). In the case of *Bs*FNR, Tyr50 on the *si*-face and His324 on the *re*-face of the isoalloxazine ring moiety of the FAD prosthetic group stacked almost in parallel to the isoalloxazine ring moiety of the FAD prosthetic group at a distance of approximately 3.5 Å (Komori et al., 2010). The five amino acid residues following His324 form a unique short α-helix in the crystal structure of *Bs*FNR and cover the *re*-face of the ring (Fig. 1). A structurally related TrxR from *Escherichia coli* (*Ec*TrxR) lacks the corresponding aromatic residues and C-terminal short α-helix (Waksman et al., 1994). The role of the C-terminal extension in *Bs*FNR including the *re*-face His324 residue remains unclear. The crystal structure of *Bs*FNR in the $NADP^+$-bound form indicates that *Bs*FNR and *Ec*TrxR share similarities in their $NADP^+$ binding mode (Komori et al., 2010), and accordingly, a drastic domain motion would be required for hydride ion transfer between $NAD(P)^+$/H and the FAD prosthetic group as previously proposed for *Ec*TrxR (Lennon et al., 2000). In such a scenario, the C-terminal extension would prevent the nicotinamide ring moiety of $NADP^+$/H from coming into close contact with the isoalloxazine ring moiety in TrxR-type FNR, thereby participating in the reaction with NAD(P)H. To analyze the functional role of the C-terminal extension of *Bs*FNR during interactions with Fd and NAD(P)H, we performed a reaction analysis of *Bs*FNR mutants with specific alterations designed to impair the C-terminal region.

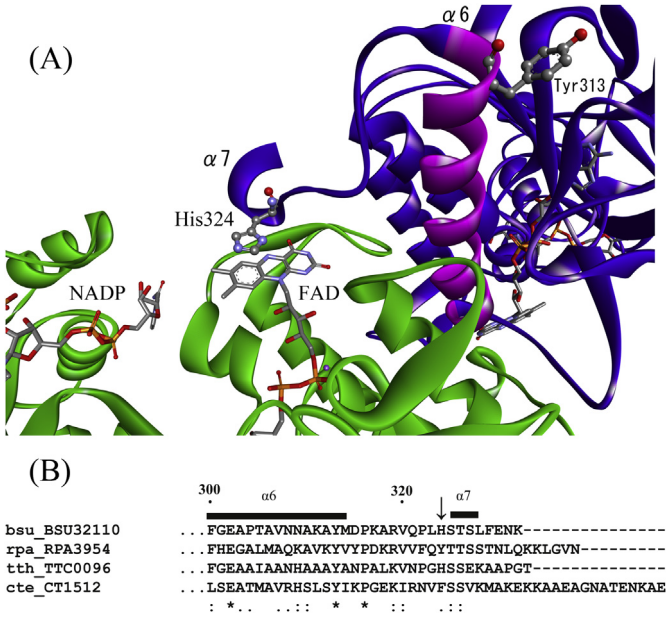

Fig. 1. (A) Close-up view of the C-terminal extension in the crystal structure of *Bs*FNR (PDB code: 3LZX). The figure was prepared using Discovery Studio 3.5 Visualizer (Accelrys Inc., USA). Side chains of Tyr313 and His324 are depicted as ball and stick model. Subunits A and B and helix 6 are colored in blue, green and purple, respectively. (B) Partially aligned amino acid sequences of the C-terminal region of TrxR-type FNRs. The numbers of amino acid residues in *Bs*FNR are indicated. The His324 residue of *Bs*FNR is indicated by an arrow. The positions of the 6th and 7th helices assigned in the crystal structure of *Bs*FNR (Komori et al., 2010) are indicated by rods. BSU_32110: FNR from *Bacillus subtilis* subsp. *subtilis str. 168* (this work), rpa_RPA3954: *Rhodopseudomonas palustris* CGA009 FNR, tth_TTC0096: *Thermus thermophilus* HB27 FNR, cte_CT1512: *Chlorobaculum tepidum* FNR.

2. Results

2.1. Preparation of WT and mutant FNRs

Two C-terminal depleted *Bs*FNR mutants ΔY313–K332 and ΔS325–K332 (hereafter designated as ΔY313 and ΔS325, respectively) were purified to homogeneity using a method for the preparation of recombinant wild type (WT) enzyme (Fig. S1). The molecular mass of the WT *Bs*FNR polypeptide migrated with an apparent molecular mass of 40 kDa on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. ΔS325 *Bs*FNR also migrated with an apparent molecular mass similar to that of WT and the ΔY313 mutant had an apparent molecular mass of approximately 38 kDa on an SDS-PAGE gel. Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOFMS) analysis of the WT *Bs*FNR in native form displayed major peaks with masses of 36749 Da and 37523 Da. The corresponding values calculated from the DNA sequence with or without FAD were 37625 and 36839 Da, respectively. Similarly, mutant protein peak sizes of 34431 Da and 35179 Da (34496 and 35281 Da by sequence) for ΔY313 and 35866 Da and 36624 Da (35932 and 36718 Da by sequence) for ΔS325 mutants correlated with WT peak sizes (Fig. S2). All of the purified FNRs eluted as a single peak during gel-permeation chromatography. The deduced apparent molecular mass of WT *Bs*FNR was approximately 97 kDa (Table 1). ΔY313 and ΔS325 mutants also exhibited a single peak with a deduced

Table 1
Enzymatic, spectroscopic and molecular properties of WT and mutant *Bs*FNRs.

	WT	ΔY313	ΔS325
NADPH diaphorase with ferricyanide[e]			
K_m for NADPH (μM)[a]	20 ± 1	10.7 ± 0.4	8.7 ± 0.4
K_m for ferricyanide (μM)[b]	290 ± 20	990 ± 90	1600 ± 160
k_{cat} (s^{-1})[a]	930 ± 11	872 ± 7	1050 ± 10
K_d for $NADP^+$ (μM)[c,e]	4.6 ± 0.2	1.5 ± 0.2	1.5 ± 0.2
ε (mM^{-1} cm^{-1} per subunit)/at λ_{max} (nm)	12.3/457[d]	12.6/457	12.3/457
App. M_r (gel-permeation/SDS-PAGE, kDa)	97/40	94/38	94/40

[a] At 4 mM ferricyanide.
[b] At 1 mM NADPH.
[c] Obtained with the data in Fig. 2C.
[d] From (Seo et al., 2004).
[e] Each parameter value is represented ± one standard deviation.