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Studies of interaction of homo-dimeric ferredoxin-NAD(P)$^+$ oxidoreductases of *Bacillus subtilis* and *Rhodopseudomonas palustris*, that are closely related to thioredoxin reductases in amino acid sequence, with ferredoxins and pyridine nucleotide coenzymes

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ABSTRACT

Ferredoxin-NADP$^+$ oxidoreductases (FNRs) of *Bacillus subtilis* (YumC) and *Rhodopseudomonas palustris* CGA009 (RPA3954) belong to a novel homo-dimeric type of FNR with high amino acid sequence homology to NADPH-thioredoxin reductases. These FNRs were purified from expression constructs in *Escherichia coli* cells, and their steady-state reactions with [2Fe–2S] type ferredoxins (Fds) from spinach and *R. palustris*, [4Fe–4S] type Fd from *B. subtilis*, NAD(P)$^+$/NAD(P)H and ferricyanide were studied. From the K_m and k_{cat} values for the diaphorase activity with ferricyanide, it is demonstrated that both FNRs are far more specific for NADPH than for NADH. The UV–visible spectral changes induced by NADP$^+$ and *B. subtilis* Fd indicated that both FNRs form a ternary complex with NADP$^+$ and Fd, and that each of the two ligands decreases the affinities of the others. The steady-state kinetics of NADPH-cytochrome *c* reduction activity of YumC is consistent with formation of a ternary complex of NADPH and Fd during catalysis. These results indicate that despite their low sequence homology to other FNRs, these enzymes possess high FNR activity but with measurable differences in affinity for different types of Fds as compared to other more conventional FNRs.

1. Introduction

Ferredoxin (Fd) is a low molecular weight iron–sulfur protein that acts as an electron mediator in a variety of metabolic processes such as photosynthesis, nitrogen fixation, sulfate assimilation, etc. On the other hand, the reduction of Fd is catalyzed by a limited number of processes, such as electron donation from the type I photosynthetic reaction center, some type of hydrogenase, pyruvate-Fd oxidoreductase, and Fd-NAD(P)$^+$ oxidoreductase ([EC 1.18.1.2] and [EC 1.18.1.3], FNR). In oxygenic photosynthetic organisms, FNR plays a pivotal role in the reduction of NADP$^+$ to NADPH by using reduced Fd supplied by the photosystem I photochemical reaction [1], and the NADPH is subsequently used for carbon assimilation by the Calvin–Benson cycle, etc. In many heterotrophs, FNRs or their isoforms, adrenodoxin reductase (AdR) in vertebrates [2,3] and putidaredoxin reductase (PdR) in some bacteria such as *Pseudomonas* species [4], catalyze the reaction in the direction of NAD(P)H oxidation and reduction of iron–sulfur proteins, which are subsequently used as electron donors to cytochrome P450.

FNR catalyzes electron transfer between the two-electron carrier nucleotide, NAD(P)H, and one-electron carrier iron–sulfur proteins such as Fd, adrenodoxin (Ad) and putidaredoxin (Pd), and also a low molecular weight flavoprotein called flavodoxin (Fld) which functions as a one-electron carrier under physiological conditions. FNRs are distributed over a wide variety of organisms and can be classified into four groups based on molecular phylogenetic analysis [5–9]. The first group of FNRs found in plants and cyanobacteria (referred to as the plastid-type FNR in this communication) participate in the redox reaction between [2Fe–2S]-type Fd (or Fld) and NADP$^+$/NADPH, which is used for carbon assimilation, glutamate synthesis, etc. [10]. The second group of FNRs are found in proteobacteria such as *Escherichia coli* [11], *Azotobacter vinelandii* [12] and *Rhodobacter capsulatus* [13,14] (referred to as the proteobacteria-type FNR), and the genes are notably induced under oxidative stress in these bacteria. The Fd reduction activity of proteobacterial FNRs is generally low (typically 0.15–3 s^{-1}) [14,15]. FNR of the above two groups occurs and functions as a monomer.

AdR [2,3], PdR [4] and some FNRs from bacteria such as *Mycobacterium tuberculosis*, *Rhodopseudomonas palustris* RPA3782 [16–18] make up the third FNR group (referred to as the mitochondria-type FNR), which perform the reduction of cytochrome P450 via the iron sulfur proteins (Ad and Pd) and generally occur as monomers, except for BphA4 from *Pseudomonas* sp. strain KKS102 which occurs as a

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dimer [19]. Because of the higher redox potentials of Ad and Pd, the reaction is directed toward oxidation of NAD(P)H and the reaction is practically irreversible. The amino acid sequences and three-dimensional structure of the third group FNRs are distinct from those of the FNRs of the former two groups and related to those of glutathione reductases that generally occur as homo-dimers [5–7,20].

More recently, the fourth type of FNRs have been discovered in the green sulfur bacterium *Chlorobaculum tepidum* (syn. *Chlorobium tepidum*) [8] and the low-GC content Gram-positive bacterium *Bacillus subtilis* [9]. These FNRs occur as homo-dimers, and show high amino acid sequence identity with thioredoxin reductases, but lack the two-cysteine motif essential for the catalysis of the latter enzymes. From database searches for genes encoding proteins with high amino acid sequence identity to these types of FNR, homologous genes are found in many Gram-positive bacteria (Firmicutes), green sulfur bacteria, some α-proteobacteria and archaea [9]. These FNRs will be referred to as the Firmicutes and green sulfur bacteria-type FNR. Some bacteria such as *R. palustris* CGA009 have three FNRs, the plastid-type (RPA1578), the mitochondria-type (RPA3782) and the fourth type (RPA3954).

Although the four FNR group enzymes differ from each other in the overall amino acid sequence, they possess a common two-nucleotide-binding-domain architecture, one for FAD or FMN and the other for $NAD(P)^+$/NAD(P)H in the N-terminus and C-terminus, respectively. This architecture is typical of the $NAD(P)^+$/NAD(P)H-linked flavoenzyme family including disulfide reductases such as glutathione reductase and thioredoxin reductase [5–7,20]. Because FNRs have multiple phylogenetic origins, comparative studies of different FNR group enzymes with respect to the mechanisms regulating substrate binding and catalytic properties are necessary for a deeper understanding of the structure–function relationships of FNR. With monomeric FNRs of the plastid-type and the mitochondria-type, substrate binding and reaction mechanisms have been extensively studied (for reviews, see [6,7,10,21]). However, few such studies have been reported on the proteobacteria-type [14,15] and no such studies have been reported on the Firmicutes and green sulfur bacteria-type FNRs.

In the present communication, we have chosen two FNRs from the fourth group, namely, *B. subtilis* FNR (YumC) which has previously been purified and biochemically characterized [9] and *R. palustris* FNR (RPA3954) which has not yet been purified. We studied their reactivity with $NAD(P)^+$/NAD(P)H and Fds by steady-state kinetic analysis and by measuring difference spectra induced by addition of these substrates. In contrast with the plastid-type FNRs which react with both [2Fe–2S] and [4Fe–4S] type Fds at comparable rates [22], the reduction rates of YumC and *R. palustris* FNR for [2Fe–2S] type Fds from spinach and *R. palustris* (RPA3956) were much lower than those for *B. subtilis* [4Fe–4S] Fd. From steady state reaction data analyses reported here, a redox reaction mechanism involving ternary complex formation is suggested for the YumC–*B. subtilis* Fd system.

2. Materials and methods

2.1. Preparation of FNRs and Fds

The following gene products were overexpressed in *E. coli*, and purified as described in Supplementary data section: FNR gene *yumC* of *B. subtilis* subsp. *subtilis str. 168* (NCBI GeneID 936577, [23]), FNR gene of *R. palustris* CGA009 (RPA3954, NCBI GeneID 2690535, [24]), [4Fe–4S] type Fd gene of *B. subtilis* (*ypbA*, Pub-Med ID 938968 [23]), [2Fe–2S] type Fd gene of *R. palustris* CGA009 (RPA3956, NCBI GeneID 2689967 [24]). Spinach [2Fe–2S] type Fd was purified according to the procedure described in [25].

2.2. Enzymatic activity assays

In the following assays, blanks consisted of all assay reagents except FNRs.

NAD(P)H diaphorase activity was assayed with ferricyanide (ε_{420}=1.02 mM^{-1} cm^{-1}, or ε_{440}=0.59 mM^{-1} cm^{-1}) as the electron acceptor in 100 mM potassium phosphate buffer (pH 7.0). The reaction mixture (1 ml) contained 3 mM potassium ferricyanide for YumC or 1 mM for *R. palustris* FNR (RPA3954), 5 mM glucose 6-phosphate (G6P, G7250, Sigma Chemical Co.), 5 U of glucose-6-phosphate dehydrogenase (G6PDH, *Leuconostoc mesenteroides*, G8404, Sigma) and 5–10 nM FNRs together with NAD(P)H as indicated in the figure legends and table.

NAD(P)H oxidase assay was performed according to the procedure described in [9].

NAD(P)H-cytochrome *c* reductase activity was assayed under aerobic conditions by monitoring the increase in the absorbance at 550 nm with horse heart cytochrome *c* ($\Delta\varepsilon_{550}$=21 mM^{-1} cm^{-1}, Sigma). The reaction mixture (1 ml) contained 0.1 mM cytochrome *c* from horse heart, 5 mM G6P, 5 U G6PDH, 20 μM NADPH and 10 nM FNRs in 100 mM potassium phosphate buffer (pH 7.0).

For the Fd-dependent cytochrome *c* reduction activity under aerobic conditions, the assay mixtures (0.5 or 1 ml) contained 0.1 mM cytochrome *c* from horse heart, 5 mM G6P, 5 U/ml G6PDH, and 1–10 nM FNRs together with NAD(P)H and Fds in 100 mM potassium phosphate buffer (pH 7.0). Enzymatic activities are expressed by subtraction of the values of the respective assay blank containing all the assay reagents except FNRs.

Turnover rates are expressed as the number of NAD(P)H molecules oxidized by one molecule of native-form FNRs per second. Each data point in Figs. 4–6 is the average of 3 to 4 independent measurements.

2.3. Spectral measurements

The UV–visible (UV–Vis) absorption spectra were measured with a double beam spectrophotometer (V-560, JASCO, Tokyo, Japan). The spectra of the reduced YumC (18 μM in 100 mM potassium phosphate buffer (pH 7.0)) and *R. palustris* FNR (RPA3954) (14 μM in 100 mM potassium phosphate buffer (pH 7.0)) were obtained after a few minutes of incubation in the presence of a 10-fold excess amount of NADPH, or after 30 min of incubation at 4 °C with an excess amount of dithionite under anaerobic conditions. For K_d determination, each 1–10 μl of either $NADP^+$ (0.01–100 mM) or [4Fe–4S] type of Fd of *B. subtilis* (1.22 mM) stock solutions were added to the cuvettes containing YumC or *R. palustris* FNR as indicated in 2 ml of 100 mM potassium phosphate buffer (pH 7.0). Spectra were measured after a few minutes of incubation at room temperature. The experimentally obtained spectra were corrected for the volume changes, and difference spectra were calculated by subtracting the control spectrum recorded prior to addition of substrates from the corrected ones. For determinations of the relationship between the temperature and the reaction rate, a thermoelectrically temperature controlled cell holder was used.

2.4. Data analysis

Non-linear regression data analysis was performed with Igor 5.0.2 software (WaveMetrics). The K_d values for $NADP^+$ and Fd were determined by fitting to the equation described in Batie and Kamin [26]. The steady-state reaction data in Fig. 6 were fitted to Eq. (1) for a rapid equilibrium bi-reactant system.

$$v = \frac{V_{max}[NADPH][Fd]}{K_s K_m^N + K_m^F[NADPH] + K_m^N[Fd] + [NADPH][Fd]}. \quad (1)$$

In Eq. (1), v is the initial steady-state reaction velocity, V_{max} is the maximum reaction velocity, K_m^F and K_m^N are the concentrations that give a half velocity of V_{max} for Fd and NADPH (the Michaelis–Menten constants), respectively, and K_s is the inhibition constant. Except for