Microbiological Research 188-189 (2016) 9–22

Contents lists available at ScienceDirect

Microbiological Research

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

Biochemical properties and crystal structure of the flavin reductase FerA from *Paracoccus denitrificans*

Vojtěch Sedláček[a], Tomáš Klumpler[b], Jaromír Marek[b], Igor Kučera[a,*]

[a] Department of Biochemistry, Faculty of Science, Masaryk University, Brno, Czech Republic
[b] X-ray Diffraction and Bio-SAXS Core Facility, Central European Institute of Technology, Masaryk University, Brno, Czech Republic

ARTICLE INFO

Article history:
Received 11 January 2016
Received in revised form 15 April 2016
Accepted 20 April 2016
Available online 27 April 2016

Keywords:
FerA
FMN
FAD
Flavin reductase family
Oxidative stress
Paracoccus denitrificans

ABSTRACT

The Pden_2689 gene encoding FerA, an NADH:flavin oxidoreductase required for growth of *Paracoccus denitrificans* under iron limitation, was cloned and overexpressed as a C-terminally His6-tagged derivative. The binding of substrates and products was detected and quantified by isothermal titration calorimetry and fluorometric titration. FerA binds FMN and FAD with comparable affinity in an enthalpically driven, entropically opposed process. The reduced flavin is bound more loosely than the oxidized one, which was confirmed by a negative shift in the redox potential of FMN after addition of FerA. Initial velocity and substrate analogs inhibition studies showed that FerA follows a random-ordered sequence of substrate (NADH and FMN) binding. The primary kinetic isotope effects from stereospecifically deuterated nicotinamide nucleotides demonstrated that hydride transfer occurs from the pro-*S* position and contributes to rate limitation for the overall reaction. The crystal structure of FerA revealed a twisted seven-stranded antiparallel β-barrel similar to that of other short chain flavin reductases. Only minor structural changes around Arg106 took place upon FMN binding. The solution structure FerA derived from small angle X-ray scattering (SAXS) matched the dimer assembly predicted from the crystal structure. Site-directed mutagenesis pinpointed a role of Arg106 and His146 in binding of flavin and NADH, respectively. Pull down experiments performed with cytoplasmic extracts resulted in a negative outcome indicating that FerA might physiologically act without association with other proteins. Rapid kinetics experiments provided evidence for a stabilizing effect of another *P. denitrificans* protein, the NAD(P)H:acceptor oxidoreducase FerB, against spontaneous oxidation of the FerA-produced dihydroflavin.

1. Introduction

The common mechanisms of iron assimilation in microorganisms include the reduction of ferric (Fe(III)) to ferrous (Fe(II)) ion (Miethke, 2013). We have previously demonstrated that the cytoplasm of the soil bacterium *Paracoccus denitrificans* constitutively contains two enzymes capable of reducing a number of Fe(III) complexes by NADH which we have named ferric reductase A and B (FerA and FerB) (Mazoch et al., 2004). Based on experiments in vitro and in vivo using a FerA-deficient mutant strain, a dual role for FerA was suggested with the idea that it both participates in reductive release of siderophore-bound Fe(III) and promotes siderophore biosynthesis (Sedlacek et al., 2009). The biological function of FerB remains unclear, although recent work has indicated its involvement in scavenging excessive superoxide anion radicals ($O_2^{\bullet-}$) (Sedlacek et al., 2015).

Substrate analysis of the purified FerA enzyme showed it to act primarily as a flavin reductase and the enzymatically formed dihydroflavin was validated as the actual reducing agent for Fe(III) (Mazoch et al., 2004). Flavin reductases (FRs) catalyze the reduction of oxidized flavin (FMN, FAD or riboflavin) by NADH or NADPH. They function either alone or in conjunction with other proteins, most notably with flavin-dependent monooxygenases (Huijbers et al., 2014). The designations FRD, FRP and FRG, respectively, are used for the NADH-preferring FRs, the NADPH-preferring FRs and the general FRs, which utilize NADH and NADPH with similar efficiencies. Furthermore, FRs are assigned to class I (flavoproteins) or II (non-flavoproteins) depending on whether they contain or

Abbreviations: FAD, flavin adenine dinucleotide; FerA, ferric reductase A from *Paracoccus denitrificans*; FerB, ferric reductase B from *Paracoccus denitrificans*; FMN, flavin mononucleotide; ITC, isothermal titration calorimetry; KIE, kinetic isotope effect; SOD, superoxide dismutase.

* Corresponding author at: Department of Biochemistry, Faculty of Science, Masaryk University, Kotlářská 2, 61137 Brno, Czech Republic.
E-mail address: ikucera@chemi.muni.cz (I. Kučera).

http://dx.doi.org/10.1016/j.micres.2016.04.006
0944-5013/

lack a permanently bound flavin cofactor (Tu, 2001). The class I FRs typically follow a ping-pong mechanism in which reduction of the cofactor by NAD(P)H and release of $NAD(P)^+$ precedes electron transfer from the reduced cofactor to the flavin substrate. The members of class II bind both NAD(P)H and flavin forming a ternary complex which then decomposes to release the products, $NAD(P)^+$ and reduced flavin (for specific examples of enzymes, see Ref. (Ellis, 2010)).

In the present study, we have conducted a detailed ligand binding, kinetic and structural characterization of FerA to obtain molecular-level insight into the catalytic mechanism employed by this enzyme. Furthermore, by studying the FerA-catalyzed reaction in the presence of FerB, we uncovered a novel possibility of stabilizing the produced dihydroflavin towards spontaneous oxidation by O_2.

2. Materials and methods

2.1. Gene cloning, protein expression and purification

The coding sequence of FerA was amplified by PCR from the genomic DNA of *P. denitrificans* 1222. The primers were FerAU1 (5′-ACAGGGCGCG*CATATG*AGCCGTCTT-3′) and FerAL3 (5′-ATGCGCGGCTAGCCCC*TCGAG*GCCGGCATC-3′) with restrictase sites for NdeI and XhoI (in italics). The amplified gene was cloned into the expression plasmid pET21a (Novagen) and subsequently transformed into the One Shot BL21(DE3)pLysS *Escherichia coli* strain (Invitrogen). Overnight culture in rich LB (Luria–Bertani) medium inoculated from a single colony of the transformants and verified by PCR using the primers for *ferA* gene and T7 promoter, were diluted 1:25 in an M9 medium containing 0.4% (w/v) glucose, 100 μg ml^{-1} ampicillin and 37 μg ml^{-1} chloramphenicol and grown to OD_{600} of 0.3–0.4 at 30 °C. The expression of a C-terminal hexahistidine tagged recombinant protein was induced by addition of 1 mM isopropyl β-D-thiogalactoside (Duchefa). The overnight grown cells were collected by centrifugation and FerA were purified as described previously (Tesarik et al., 2009).

2.2. Molecular mass determination

One-dimensional SDS gel electrophoresis of FerA was carried out in 12% polyacrylamide gel according to (Laemmli, 1970) using a Mini Protean III (Bio-Rad) vertical electrophoretic system. The mass spectra of FerA were recorded on a Bruker Reflex IV mass spectrometer in a MALDI-TOF mode. Prior analysis, the purified protein was desalted to a final 50 mM sodium phosphate buffer (pH 8.0) by PD-10 column (GE Healthcare). The desalted sample was mixed with a matrix solution in a ratio 1:2 and dried. The spectra were taken in positive-ion linear mode.

2.3. Determination of protein concentration

Protein concentration was determined by QuantiPro BCA Assay Kit (Sigma-Aldrich) using bovine serum albumin as the standard. Proteins eluted from chromatographic columns during purification were also monitored at 280 nm.

2.4. Isothermal titration calorimetry

Binding of FMN or FAD by FerA was assessed via ITC measurements using the Auto-iTC 200 isothermal titration calorimeter (Malvern). Experiments were carried out in a buffer containing 50 mM sodium phosphate, 300 mM NaCl and 250 mM imidazole (pH 8.0). During the titration, the reaction mixture was continuously stirred at 750 rpm and at 25 °C. For each step of the titration, the binding heat was determined as the difference between the heat change generated after the injection of the flavins into the protein solution and the corresponding background, which was obtained by injection of the flavins into the sample cell with the buffer. Thermodynamic parameters for the binding were determined from ITC results using Microcal Origin version 8.0 (OriginLab) with the "ITC custom" add-on installed.

2.5. Fluorescence measurement of dissociation constants

The binding of FMN, $FMNH_2$, FAD, $FADH_2$, NAD^+, NADH, AMP, riboflavin and lumichrome by FerA was assessed by protein fluorescence quenching measurements carried out at 30 °C using Luminiscence Spectrometer LS-50B (Perkin-Elmer). A fixed amount of FerA was titrated with aliquots of ligands mentioned above of a known concentration in 50 mM phosphate buffer (pH 7.0) containing 10 mM EDTA. Excitation was at 280 nm, and the emission was recorded at 330 nm. Fluorescence intensities were read 3 min after each addition of quencher. Where appropriate, reduction of flavins was achieved by sodium dithionite and their reduced forms were kept under nitrogen atmosphere for subsequent titration which was also performed under strict anaerobic conditions. Titrations by FerA of FMN and FAD as fluorophores were done in a similar manner but at the excitation and emission wavelength of 450 and 517 nm, respectively. The fluorescence anisotropy of FAD was measured in a FluoroMax-4 spectrofluorometer (Horiba Scientific) equipped with polarizers operated by FluorEssence software version 3.5 and calculated from the intensities at four mutually perpendicular polarizer settings (I_{VV}, I_{VH}, I_{HV}, and I_{HH}) as previously described (Lakowicz, 2006). The dissociation constants K_d were obtained by nonlinear regression analysis according to a 1:1 binding model (Lostao et al., 2000).

2.6. Determination of the standard redox potential

The standard redox potentials of FMN and FMN with FerA were determined at 25 °C according to the method of Massey (Massey, 1990). The reaction mixture contained in 1-ml cuvette 50 μM FMN, 20 μM phenosafranine, 0.4 mM xanthine, 5 μM benzyl viologen in a 50 mM sodium phosphate buffer with 0.1 mM EDTA (pH 7.0). After flushing by argon (99.9999%, v/v), a catalytic amount of a milk xanthine oxidase (Sigma-Aldrich) suspension was added to a final concentration 0.013 mg ml^{-1} still under strict anaerobic conditions. The concentration of FerA was 0.2 mM. Absorbance of the dye and FMN was recorded by UV–vis spectrophotometer UltraSpec 2000 (GE Healthcare). The absorbance readings at 450 nm (FMN) and 521 nm (phenosafranine) were corrected for the contribution of the other component.

2.7. Enzyme kinetics

The bi-substrate kinetic analysis was performed by an UltraSpec 2000 spectrophotometer with a temperature-controlled cuvette holder (30 °C) in 25 mM Tris–HCl (pH 7.4). NADH oxidation was measured at 340 nm; initial velocities were calculated from the linear slope of the progress curves obtained, using a molar absorption coefficient of 6.22 mM^{-1} cm^{-1}. The kinetic parameters were calculated by the Marquardt-Levenberg nonlinear fit algorithm included in the Microcal Origin software. Data conforming to a sequential mechanism were fitted to Eq. (1). Data for competitive and mixed inhibition were fitted to Eqs. (2) and (3), respectively.

$$v = V[A][B]/\{K_a[B] + K_b[A] + [A][B] + K_{ia}K_b\} \quad (1)$$

$$v = V[A]/\{K_a(1 + [I]/K_{is}) + [A]\} \quad (2)$$

$$v = V[A]/\{K_a(1 + [I]/K_{is}) + [A](1 + [I]/K_{ii})\} \quad (3)$$