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Rubredoxin acts as an electron donor for neelaredoxin in *Archaeoglobus fulgidus*

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Abstract

Archaeoglobus fulgidus neelaredoxin (Nlr) is an electron donor:superoxide oxidoreductase. The reaction of superoxide with reduced Nlr is almost diffusion-limited, but the overall efficiency for detoxifying superoxide in vivo depends on the rate of reduction of Nlr by electron donors. Here, we report the purification and characterization of the two type I rubredoxins from *A. fulgidus* (AF0880 and AF1349) and show that they act as efficient electron donors for neelaredoxin, in vitro, with a second-order rate constant of 10^7 M^{-1} s^{-1} at 10 °C and pH 7.2.

Keywords: Superoxide detoxification; Oxygen metabolism; Rubredoxin; Neelaredoxin; Superoxide reductase

In the last few years, a considerable amount of data has been accumulating concerning the role of three small mononuclear non-heme iron proteins in oxygen detoxifying pathways in anaerobic organisms: rubredoxin (Rd), desulfoferrodoxin (Dfx), and neelaredoxin (Nlr).

Rubredoxins (Rd) are small iron proteins (ca. 6 kDa), containing one iron center coordinated in a tetrahedral geometry to the sulfur atoms of four cysteinyl residues. Typically they have reduction potentials of around 0 mV. These proteins are found in many anaerobic bacteria and archaea where, as one-electron carriers, they have been shown to be involved in several electron transfer chains. So far, two types of Rds have been described, based on the amino acid spacing between the cysteine ligands to the iron: (i) type I Rd, the most common, which have a $CX_2C\cdots CX_2C$ motif binding the iron, and (ii) type II Rd, having two extra amino acid residues between the two first cysteines ($CX_4C\cdots CX_2C$) [1,2]. Rubredoxin-like sites are also found in desulfoerodoxin (Dx), rubrerythrin (Rr), desulfoferrodoxin (Dfx), and flavorubredoxin (Flrd). Desulforedoxin, a protein even smaller than rubredoxins, contains a Rd-like center with a tetrahedral distorted geometry [3], due to the lack of two amino acid residues in its iron binding motif (C–C···C–X_4–C) [4]. Desulfoferrodoxin shares this Dx-like center (Dfx center I) in its N-terminal domain [5], fused to a C-terminal domain that contains another iron center (center II), present also in neelaredoxin [5,6]. In this center, the iron is coordinated with four equatorial histidines and an axial cysteine (Fe-His_4Cys), in the reduced state; in the ferric state, an extra sixth position is occupied by a glutamate. Rr has a domain containing a type I Rd-like iron center and a domain containing a bi-nuclear iron center (Fe_2-His_5Glu) [7]. Flavorubredoxin, a soluble NO-reductase, has a Rd-like C-terminal domain [8–10].

Dfx and Nlr are enzymes involved in the elimination of superoxide in anaerobes and in microaerobes [11–17]. They constitute a new fascinating family of enzymes that instead of dismutating superoxide, like the canonical superoxide dismutases (SOD), can reduce superoxide

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to hydrogen peroxide without the formation of dioxygen [14–16,18–20]. These enzymes exhibit rates for the reduction of superoxide of ca. $10^9\ M^{-1}\ s^{-1}$, which are comparable to those of the most efficient SODs. This latter activity requires a continuous and efficient supply of electrons to maintain SOR in the reduced state in order to decrease the superoxide concentration to sub-lethal levels in cases of oxidative stress. Rubredoxin has been proposed as the electron donor to SOR, and recently it was shown that *Desulfovibrio vulgaris* Rd can efficiently reduce *D. vulgaris* C13S Dfx (a mutant lacking center I) with a second-order rate constant of $\sim 1 \times 10^6\ M^{-1}\ s^{-1}$ at 25 °C and pH 7.5 [21]. Reduction of Nlr by Rd was already shown for *Pyrococcus furiosus* and *Treponema pallidum* neelaredoxins, by steady state measurements [18,22], but no data for the electron transfer rates exist, which are essential to establish the physiological competence of Rd as electron donor. *Archaeoglobus fulgidus* genome [23] has two genes encoding for putative Rds, *rd-1* (AF0880) and r*d2* (AF1349), identified by homology, which can be potential electron donors for neelaredoxin. In this article, we report the purification and characterization of both *A. fulgidus* rubredoxins, and show in a direct way that they donate electrons efficiently to *A. fulgidus* neelaredoxin.

Materials and methods

Purification of wild-type rubredoxin

Cell growth. Archaeoglobus fulgidus (strain VC16, DSMZ 4304) was grown as previously described [24]. Batch cultures were grown at pH 7.0, in a 100 L reactor at 80 °C with stirring (60 rpm) and continuous gassing with N_2/CO_2 (80:20, by vol.; 0.5 L/min), with lactate as the carbon source.

Protein purification. Archaeoglobus fulgidus cells were suspended in 10 mM Tris–HCl, pH 7.1, buffer and broken in a French press, at 6000 psi. The soluble extract was obtained by separation of the membrane pellet through ultracentrifugation at 40,000*g* for 1 h. All purification steps were performed at pH 7.1 and 4 °C. The soluble extract was loaded on a HiLoad 26/10 Q-Sepharose column, equilibrated with 10 mM Tris–HCl (buffer A), and eluted with a 0–1 M NaCl linear gradient, in buffer A. The fraction containing Rd, eluted at 400 mM NaCl, was concentrated on an Amicon ultrafiltration Cell with a YM3 (3 kDa) membrane, loaded on a Superdex 75 column (XK26/50), equilibrated, and eluted with 20 mM Tris–HCl, containing 150 mM NaCl. The Rd fraction, after dialysis against buffer A, in a 3 kDa cut-off tube, was finally applied to a Pharmacia Mono-Q column, equilibrated with buffer A, and eluted with a 0–0.6 M NaCl linear gradient, in buffer A. A purified fraction was obtained, as revealed by SDS–PAGE [25]. The amino acid N-terminal sequence of Rd was obtained by Edman degradation [26] using an Applied Biosystems Procise 491 HT Protein Sequencer.

Expression and purification of recombinant A. fulgidus rubredoxins and neelaredoxin

For expression purposes, homologous oligonucleotides that allowed the introduction of restriction sites at the start codon and downstream of the stop codon were designed: 5′-GAAACATG ATTGCATATGGCAAAGTATC-3′ and 5′-GTATTGTGGAGAAA AGCTTTAACGATTT-3′ for the *rd-1* gene (with *Nde*I and *Hin*dIII restriction sites), and 5′-CGGAGGTTGATCATATGGCGAAGT-3′ and 5′-CTCGGACTGCAGAGGGAATAAAAAAT-3′ for the *rd-2* gene (with *Nde*I and *Pst*I restriction sites). By means of a PCR, using the oligonucleotides, *Pfu* polymerase (Stratagene) and *A. fulgidus* genomic DNA, amplification of the complete *rd-1* gene (237 bp) and *rd-2* gene (209 bp) was achieved. After purification, the DNA fragments were cloned in pT7-7 [27], previously cut with the appropriated restriction enzymes, and transformed in *Escherichia coli* DH5α cells. The resultant recombinant plasmids, pT7AFRd1 and pT7AFRd2, were isolated and sequenced to ensure the integrity of the gene sequences. Cultures of BL21-Gold (DE3) (Stratagene) containing either pT7Rd1 or pT7Rd2 were grown aerobically at 37 °C in M9 minimum media complemented with 0.1 mg/mL ampicillin and 1 mM $FeSO_4$, in a 3 L fermentor. When the culture reached a cell density of $A_{600} = 0.5$, 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added and, after 4 h, the cells were harvested by centrifugation (8000*g*, 10 min) and washed with 10 mM Tris–HCl, pH 7.6.

Protein purification. Both rubredoxins were purified by the same procedures, and all the purification steps were done at 4 °C. Cells were broken in a French press at 6000 psi. The crude extract was ultracentrifuged at 42,000*g* for 16 h. The supernatant (soluble extract) was heated at 80 °C for 30 min and centrifuged at 15,000*g* for 15 min to remove heat sensitive proteins. This heat-treated soluble extract was loaded in a Q-Sepharose column equilibrated with 10 mM Tris–HCl, pH 7.6, and a linear gradient 0–1 M NaCl in the same buffer was applied. The fraction containing rubredoxin was eluted at 450 mM NaCl and was concentrated on a Diaflo apparatus equipped with a YM3 membrane (Amicon). It was then loaded on a Sephadex S75 filtration column pre-equilibrated with 20 mM Tris–HCl, pH 7.6 + 150 mM NaCl and the rubredoxin eluted was judged to be pure by SDS–PAGE [25].

Protein concentration was determined by the Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce) [28], and the total iron content was determined by the 2,4,6-tripyridyl-*s*-triazine (TPTZ) method [29]. Recombinant Nlr and *E. coli* NADH:flavorubredoxin oxidoreductase were obtained as previously described [20,8].

Spectroscopic and enzymatic studies

UV/visible spectra were recorded on a Shimadzu UV-1603 spectrophotometer. EPR spectra were obtained on a Bruker ESP 380 spectrometer, equipped with a continuous flow Oxford Instruments helium cryostat.

NAD(P)H. Rd oxidoreductase activity was tested at room temperature under argon atmosphere by incubating 8 μM Rd in 50 mM Tris–HCl, pH 7.6, with 30 μM NAD(P)H and adding catalytic amounts of *A. fulgidus* soluble extract.

Stopped-flow experiments

The rapid kinetics measurements of Rd electron transfer to Nlr were carried out at 10 °C in 50 mM Tris–HCl, pH 7.2, and 150 mM NaCl, using a Bio-Logic Stopped-flow SM400/S equipped with MOS-200 optical system. Protein solutions were made anaerobic by repeated cycles of vacuum and equilibration with oxygen-free argon, and by supplementing the buffer with 4 U/mL glucose oxidase, 3 mM glucose, and 130 U/mL catalase. Rds were reduced in a cuvette under an argon atmosphere by titration with NADH, in the presence of 0.5 μM *E. coli* NADH:flavorubredoxin oxidoreductase, following the decrease in absorbance at 495 nm. Prior to the experiments, the stopped-flow apparatus was incubated with the glucose oxidase/glucose/catalase system for 15 min to remove oxygen. Solutions of reduced Rd and oxidized Nlr were loaded in two separate drive syringes and after the stopped-flow mixing the absorbance changes at 495 nm were mea-