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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-

sured. The amount of oxidized Rd was calculated using an extinction coefficient of 8.8 mM^{-1} cm^{-1} at 495 nm. The data were analyzed using Biokine v3.29 software.

Redox titrations

Redox titrations were performed at 25 °C, and pH 7.2, under an argon atmosphere, and were monitored by visible spectroscopy (400–700 nm), in a Shimadzu Multispec-1501 spectrophotometer. The reaction mixture contained 6 μM Rd in 50 mM Tris–HCl buffer and also the following mediators: 0.1 μM final concentration of 1,2-naphthoquinone-4-sulfonic acid (+215 mV) and 1,2 naphthoquinone (+180 mV); 0.2 μM phenosafranine (−255 mV) and neutral red (−325 mV); 0.5 μM of 2-hydroxy-1,4-naphthoquinone (−152 mV); 1 μM of 1,4 naphthoquinone (+60 mV), tetramethyl-*p*-benzoquinone (+5 mV), menadione (0 mV), plumbagin (−40 mV), and phenazine (−125 mV). Sodium dithionite was used as reductant. The redox potential measurements were performed with a combined silver/silver chloride electrode, calibrated with a quinhydrone saturated solution at pH 7.0. The redox potentials are quoted against the standard hydrogen electrode.

Results and discussion

Physical-chemical characterization of rubredoxins from A. fulgidus

Archaeoglobus fulgidus has two rubredoxin genes in its genome: *rd-1* (AF0880) and *rd-2* [23], and the alignment of the deduced amino acid sequences is represented in Fig. 1. When compared with other rubredoxins, the deduced sequence of Rd-2 has a longer N-terminal constituted by 19 extra amino acids. To clarify this point, the wild-type Rd-2 was purified from *A. fulgidus* cells, and the N-terminal sequencing (AKYECQV) showed that the putative extra fragment is not expressed at all or cleaved during the expression of the protein. Therefore, Rd-2 was expressed without the additional N-terminal fragment.

Archaeoglobus fulgidus Rd-2 is highly homologous with Rd-1 (80% identity, 89% similarity), and also with rubredoxins from other organisms (e.g., *Clostridium pasteurianum* Rd: 58% identity, 70% similarity, *Desulfovibrio gigas* Rd: 67% identity, 67% similarity). *A. fulgidus* Rds can be classified as type I rubredoxins, with two C–X_2–C motifs binding the iron.

The UV/visible and EPR spectra of wild-type Rd-2 were similar to those of recombinant Rd-2. Therefore, results here presented refer only to recombinant rubredoxins. These proteins have ~6 kDa, as determined by SDS–PAGE (data not shown), in accordance with the molecular mass determined by their amino acid sequence: 6.0 and 6.1 kDa for Rd-1 and Rd-2, respectively. The UV/visible spectrum of the oxidized form of Rd-2 (Fig. 2) is similar to that of Rd-1 (not shown) and confirms the presence of a (Fe-Cys_4) center, showing features common to those of the already described type I Rds from other organisms (see [30] for an updated review). It has three main bands, one centered at 368 nm, which is the sum of two different contributions (350 and 385 nm), one band at 495 nm and a third at 575 nm. All the bands disappear upon reduction with sodium dithionite, or with NADH in the presence of *E. coli* NADH:flavorubredoxin oxidoreductase, which was found to catalyze Rd reduction (Fig. 2). The EPR spectrum of both Rds is typical for a type I Rd center, with resonances at $g \approx 9.5$ and 4.3 (not shown), ascribed to the spin transitions of the $|M_s = \pm 1/2\rangle$ and $|M_s = \pm 3/2\rangle$ doublets, respectively, of a ferric high-spin ($S = 5/2$) species with an axial distortion (E/D) of ~0.3. Redox titrations monitored by visible spectroscopy (Fig. 3) allowed us to determine reduction potentials of +75 and +40 mV for Rd-1 and Rd-2, respectively, which are

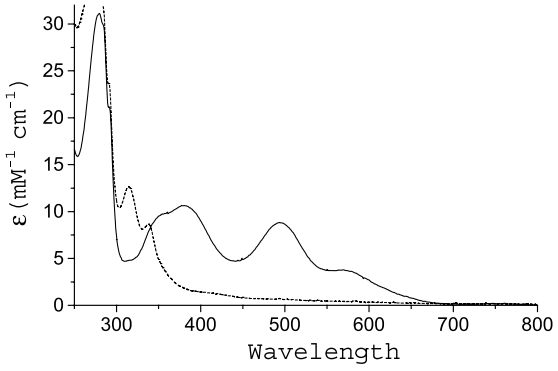


Fig. 2. UV/visible spectrum of the oxidized *A. fulgidus* recombinant Rd-2 (solid line) and reduced by NADH in the presence of *E. coli* NADH:flavorubredoxin oxidoreductase (dashed line).

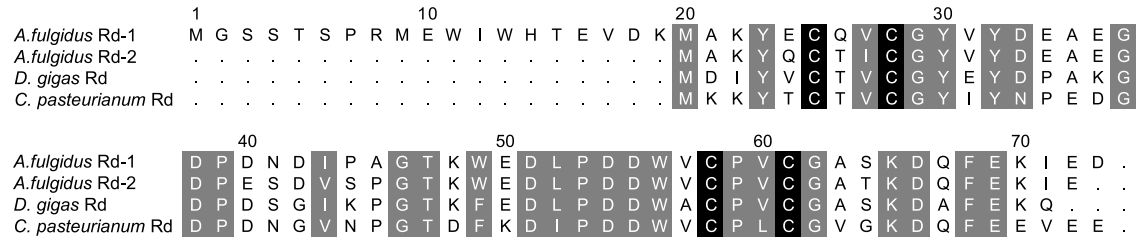


Fig. 1. Comparison of amino acid sequences of Rds from *A. fulgidus* and two canonical type I Rds from the sulfate reducer *D. gigas* [34] and from *C. pasteurianum* [35]. Conserved amino acids are denoted in gray, and the cysteine ligands in black. Multiple sequence alignments produced in Clustal W 1.60 [36], protein weight matrix: BLOSUM series.

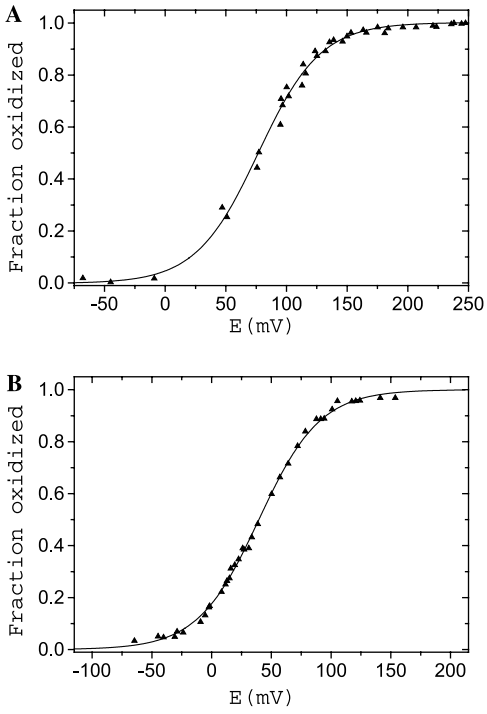


Fig. 3. Redox titration of *A. fulgidus* recombinant (A) Rd-1 and (B) Rd-2. The titration curves were obtained measuring the absorbance at 495 nm; the solid lines correspond to a Nernst equation with $E_o = +75$ mV ($n = 1$) and $E_o = +40$ mV ($n = 1$), for Rd-1 and Rd-2, respectively.

within the values usually found for type I rubredoxins. These reduction potentials are adequate for electron donation to *A. fulgidus* Nlr, which has a reduction potential of +230 mV [15].

Stopped-flow kinetics of electron transfer between Rd and Nlr

The kinetics of reduction of Nlr by Rd was measured by stopped-flow, following the oxidation of Rd by the increase in absorbance at 495 nm. Rubredoxin was reduced prior to the stopped-flow mixing using NADH in the presence of catalytic amounts of *E. coli* NADH:flavorubredoxin oxidoreductase. As both rubredoxins from *A. fulgidus* are readily oxidized by oxygen, the assays were done under anaerobic conditions to ensure that oxidation of Rd was only due to Nlr. The experiments were performed at 10 °C and high ionic strength (150 mM NaCl), in an attempt to slow down the reaction, since at 25 °C, about 75% of the rubredoxin was already oxidized after the mixing dead time. An initial protein concentration of 5 μM was chosen to be low enough to decrease the apparent rate constant, without compromising the amplitude of the absorbance changes and, consequently, the resolution. Even at low temperature, only the last 60% of the reaction could be followed. As a result, pseudo-first-order conditions could not be studied, due to the large error associated with the fitting process in the absence of the initial data, and therefore, the order of reaction in respect to each reagent could not be measured. The kinetic traces obtained by mixing equimolar amounts of reduced Rd with oxidized Nlr are depicted in Fig. 4. The second-order rate constant for the reaction can be calculated if a bimolecular mechanism is assumed, and adjusting the data to the following equation:

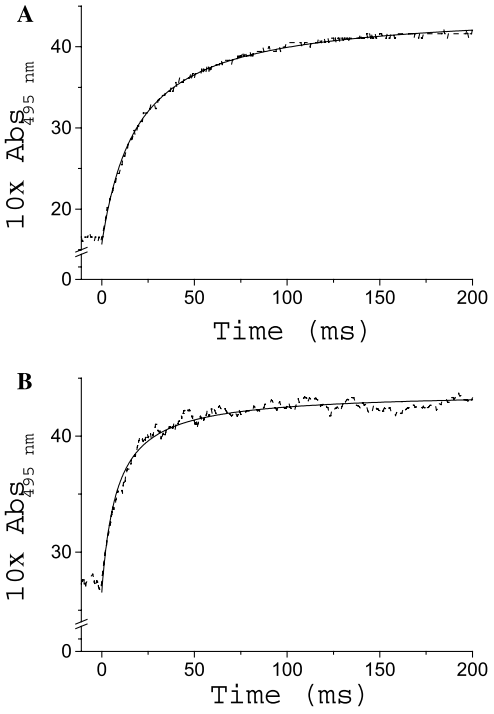


Fig. 4. Stopped-flow kinetics of the oxidation of reduced recombinant (A) Rd-1 and (B) Rd-2 by oxidized Nlr measured by the absorbance changes at 495 nm. Solutions of 10 μM reduced Rd were mixed (1:1) (v/v) with 10 μM oxidized Nlr in 50 mM Tris–HCl, pH 7.6, and 150 mM NaCl containing 4 U/mL glucose oxidase, 3 mM glucose, and 130 U/mL catalase, at 10 °C.

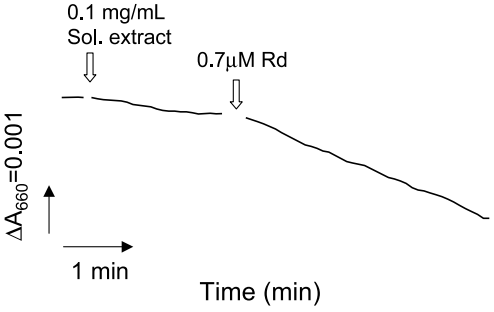
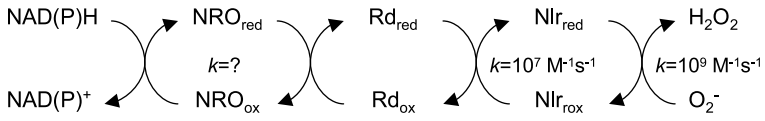


Fig. 5. NADPH:rubredoxin oxidoreductase activity of *A. fulgidus* soluble extract. Neelaredoxin (7 μM) is slowly reduced by 30 μM NADPH in the presence of 0.1 mg/mL soluble extract. An increase of Nlr reduction rate is observed upon addition of 0.7 μM Rd.



Scheme 1.

$$[Rd_{ox}] = [Rd_{red}]_0 - [Rd_{red}]_0/(1 + k' \times t)$$
$$\text{and} \quad [Rd_{red}]_0 = [Nlr_{ox}]_0,$$

where $[Rd_{ox}]$ is the amount of oxidized Rd, $[Rd]_0$ and $[Nlr]_0$ are the initial concentrations of reduced Rd and oxidized Nlr, respectively, and k' is an apparent rate constant equal to the product of the second-order constant by the initial concentration of reagents. Consistent with the higher reduction potential obtained for Rd-1 in respect to Rd-2, the calculated second-order rate constant for the electron transfer to Nlr, at 10 °C and pH 7.2, is lower for Rd-1 ($1 \times 10^7\ M^{-1}\ s^{-1}$) than for Rd-2 ($6 \times 10^7\ M^{-1}\ s^{-1}$). At the optimal growing temperature of *A. fulgidus* (83 °C) these reaction rates are expected to be even higher, which means that a pathway for reduction of Nlr by Rd is physiologically competent. In the case of Rd and Dfx from *D. vulgaris*, the calculated rate constant was about one order of magnitude lower [21], suggesting a more efficient electron transfer for Nlr in *A. fulgidus*.

Presence of a NADPH:rubredoxin oxidoreductase in A. fulgidus

It was already shown that Nlr can be slowly reduced by NADPH in the presence of *A. fulgidus* soluble extract [15]. However, the rate of reduction is increased if catalytic amounts of Rd are added (Fig. 5), which suggests the presence of a NADPH:rubredoxin oxidoreductase (NRO) in *A. fulgidus* soluble extract. This activity is specific for NADPH, as no reduction was observed when NADH was used instead. The measured specific activity of the soluble extract for Rd reduction by NADPH obtained at 25 °C and pH 7.6 was 4 μM/min/mg for Rd-2 and 5 μM/min/mg for Rd-1. This clearly shows that reduction of Nlr by NADPH can occur in vivo mediated by NRO and Rd as represented in Scheme 1.

While the electron transfer from Rd to Nlr is an efficient process, as shown in this work, the remaining steps of the superoxide detoxification pathway are not yet characterized.

In addition to donating electrons to SOR, Rds have been described as being components of other redox systems. *D. gigas* Rd is the electron transfer protein between NADH:rubredoxin oxidoreductase (NRO) and rubredoxin:oxygen oxidoreductase (ROO) [8,31,32]. Recently, *P. furiosus* Rd was reported to donate electrons to rubrerythin (Rr), which has peroxidase activity [33]. *A. fulgidus* genome contains also four homologues of Rr, which potentially contribute, together with SORs, to the elimination of oxygen reactive species. In *Desulfovibrio desulfuricans* (ATCC 27774), the type II Rd is the electron donor for bacterioferritin, playing a crucial role in the iron metabolism of the organism [2]. Since the metabolisms of oxygen and iron are intimately related in aerobes, particularly during the formation of reactive oxygen species, it is clear that Rds play important roles as electron transfer proteins involved in the protection of anaerobes against the formation and propagation of these reactive species. The physiological relevance of these detoxifying systems depends on the extent to which an anaerobic organism may have to cope with oxygen radicals, generated from a transient exposure to oxygenated environments. This role can be particularly important upon phagocytosis of bacterial pathogens, during which the pathogen is exposed to toxic concentrations of reactive oxygen species produced by the macrophage.

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