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Superoxide Reductase as a Unique Defense System Against Superoxide Stress in the Microaerophile*Treponema Pallidum*

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Running title : Superoxide reductase from *Treponema pallidum*

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SUMMARY

Aerobic life requires the presence of antioxidant enzymes, such as superoxide dismutase, catalase, peroxidase to eliminate deleterious oxygen derivatives. *Treponema pallidum*, a microaerophilic bacterium responsible for venereal syphilis, is an interesting organism because it lacks all the above-mentioned enzymes, as deduced from its recently sequenced genome. In this paper, we describe a gene in *Treponema pallidum* with sequence homologies to a new class of antioxidant systems, named superoxide reductases, recently isolated from sulfate reducing bacteria [Lombard, M., Fontecave, M., Touati, D., and Nivière, V. (2000) Journal of Biological Chemistry, 275, 115-121]. We report that : (i) expression of the*Treponema pallidum* gene fully restored to a superoxide dismutase-deficient *Escherichia coli* mutant the ability to grow under aerobic conditions ; (ii) the corresponding protein displays a strong superoxide reductase activity ; (iii) the *Treponema pallidum* protein contains only one mononuclear non-heme ferrous center, able to reduce superoxide selectively and efficiently, whereas previously characterized superoxide reductase from *Desulfoarculus baarsii* contains an additional rubredoxin-like ferric center. These results suggest that *Treponema pallidum* antioxidant defenses rely on a new class of superoxide reductase and raise

the question of the importance of superoxide reductases in mechanisms for detoxifying superoxide radicals.

INTRODUCTION

Superoxide radical ($O_2^{.-}$) is the univalent reduction product of molecular oxygen. It belongs to the group of the so-called toxic oxygen derivatives, which also include hydrogen peroxide and hydroxyl radicals (1). For years the only enzymatic system known to catalyze the elimination of superoxide was the superoxide dismutase (SOD) [1], discovered in 1969 by McCord and Fridovich (2). Four classes of SOD have been characterized so far (3, 4), depending on the nature of metal ion of their active sites. They all catalyze the same reaction, e.g. dismutation of superoxide radical anions to hydrogen peroxide and molecular oxygen:

$$O_2^{.-} + O_2^{.-} + 2\ H^+ \rightarrow H_2O_2 + O_2$$

Very recently, a new concept in the field of the mechanisms of cellular defense against superoxide has emerged. It was discovered that elimination of $O_2^{.-}$ could occur by reduction, a reaction catalyzed by an enzyme thus named superoxide reductase (SOR) :

$$O_2^{.-} + 1\ e^- + 2\ H^+ \rightarrow H_2O_2$$

Up to now, two examples of superoxide reductase have been described (5, 6). The first one is a small protein found in anaerobic sulfate reducing bacteria called desulfoferrodoxin (Dfx). Dfx is a homodimer of 2x14kDa, which has been well studied (7-9) and structurally characterized (10). The monomer is organized in two protein domains, each with a specific mononuclear iron site, named center I and center II, respectively. Center I contains a mononuclear ferric iron coordinated by four cysteines in a distorted rubredoxin-type center. Center II has an oxygen stable ferrous iron with square pyramidal coordination to four nitrogens from histidines as equatorial ligands and one sulfur from a cysteine as the axial ligand. We have shown that the iron center II of Dfx from *Desulfoarculus baarsii* is the active site for the SOR activity and that it reduces superoxide very efficiently, without significant SOD activity (5). That Dfx could act as a true SOR enzyme was further supported by the fact that *E.coli* extracts contain NAD(P)H dependent reductase activities able to provide electrons to Dfx, allowing then catalytic cycles for reduction of superoxide (5). Whether center I was participating to the electron transfer and then be essential for a full SOR activity could not be concluded from this study. Although Dfx is not naturally present in *E.coli*, Pianzzola et al. demonstrated that expression of Dfx in this bacterium could totally replace the classical SOD enzymes to overcome a superoxide stress (11). That Dfx was also an antioxidant protein in sulfate reducing bacteria was further shown when the *dfx* gene was deleted in the chromosome of *Desulfovibrio vulgaris*. This deletion increased the oxygen sensitivity of *D.vulgaris* during transient exposure to microaerophilic conditions (12).

Another example of SOR has been isolated from the anaerobic archaea, *Pyrococcus furiosus* (6). The protein presented strong homologies to neelaredoxin (Nlr), a small protein containing a single mononuclear center, earlier characterized from sulfate reducing bacteria (13). Very recently, the three dimensional structure of the *P.furiosus* SOR has been determined at high resolution (14). The protein fold and the unique mononuclear iron center are similar to those of the second domain of Dfx (containing center II), but the first protein domain, chelating the iron center I, in Dfx is missing, as expected from earlier studies of neelaredoxin (13). The protein is a homotetramer, in contrast with the dimeric structure reported for Dfx (10). In *Pyrococcus furiosus*, an electron transferring chain, including NADH, NADH rubredoxin oxidoreductase and rubredoxin, was proposed to provide the electrons necessary for the reaction (6). However, there is no evidence that neelaredoxin function as an antioxidant system *in vivo*, so far.

Whether SOR activity in anaerobic microorganisms, which have to face transitory exposure to air, would present a selective advantage with regard to SOD activity is still an open question. Although some hypothesis have been already proposed elsewhere (5, 6), careful analysis of bacterial genomes pointed out that several anaerobic bacteria possess both genes encoding for putative SORs and SODs, which makes the real physiological function of SOR puzzling. Analysis of the complete genome of the bacterium *Treponema pallidum* (15) the causative agent of venereal syphilis, a microaerophilic bacteria optimally growing at 5% oxygen tension (16), reveals that this organism does not possess the classical antioxidant enzymes, such as SOD, catalases and peroxidases. However a gene encoding a protein with strong sequence homology to Dfx, but lacking cysteine residues involved in the chelation of the iron center I, was found (Fig. 1).

Consequently, we have overproduced, purified and characterized this putative Dfx protein from *T.pallidum*. Here we report that this protein, in spite of the lack of iron center I, has powerful SOR activity and provides a protection from superoxide radicals comparable to SOD. *T.pallidum* is thus a unique microorganism in that its superoxide scavenging capacity might only rely on SOR.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmid Constructs. *E. coli* strain QC 2375 (*sodA sodB recA*) was previously described (17). pVN10-2 construction: a 492bp DNA fragment containing the *dfx* gene of *T.pallidum* was amplified from pGTPEC10 (15) by PCR, using the oligonucleotides 5'-ACGGAATTCACGCGGAGGCACGACAG and 5'-CGCGGATCCCCCAATCTCCTGCTCC, with an *Eco*RI and a *Bam*HI restriction site (underlined), respectively. The amplified fragment was digested with *Eco*RI and *Bam*HI and inserted into the corresponding sites of pJF119EH (5) under ptac promoter control and the resulting plasmid, pVN10-2 transformed in DH5α. The construct was verified by sequencing.

Biochemical and Chemical Reagents. 1-2 mM KO_2 stock solutions were prepared in anhydrous Me_2SO as described in (5). Xanthine oxidase Grade IV from milk (0.24 U/mg), catalase from *Aspergillus niger* (6600 U/mg), cytochrome c from bovine heart, CuZn-SOD from bovine erythrocytes (5800 U/mg) were from Sigma.

Purification of the Recombinant Dfx and analytical determination. *E.coli* DH5α/pVN10-2 cells were grown aerobically at 37 °C in Luria-Bertani (LB) medium complemented with 0.1 mM $FeCl_3$ and 100 μg/ml ampicilin. 1mM IPTG was added at OD 600 nm 0.3. At OD 600 nm of about 2.2 cells were chilled and collected by centrifugation. All the following operations were carried out at 4 °C and pH 7.6. The cell pellet (20 g, wet weight) was suspended in 60 ml of 0.1 M Tris/HCl and sonicated. After ultracentrifugation at 45,000 rpm during 90 min in a Beckman 50.2 Ti rotor, the supernatant was treated with streptomycin sulfate and then precipitated with ammonium sulfate (final concentration 80 % w/v). The pellet was dissolved in 12 ml of 25 mM Tris/HCl and loaded onto an ACA 54 column (360 ml) equilibrated with 25 mM Tris/HCl. A fraction (100 mg) corresponding to the volume of elution of low molecular weight protein was collected. Protein fractions of 10 mg were further chromatographied using a Bio-Rad Biologic system equipped with an anion exchange column, Uno Q-1 (Bio-Rad), and equilibrated with 10 mM Tris/HCl. A linear gradient was applied (0-0.15 M NaCl) in 10 mM Tris/HCl, with a flow rate of 1 ml min^{-1} during 65 min. A fraction (7 mg), eluted with about 40 mM NaCl, contained only one polypeptide of about 16 kDa, as shown by SDS-PAGE analysis (15%, acrylamide). The native molecular mass of the protein was determined with a Superdex 75 gel filtration column (120 ml, Amersham Pharmacia Biotech), as described in (5). Protein concentration was determined using the Bio-Rad protein assay reagent (18). Protein-bound iron was determined by atomic absorption spectroscopy. EPR measurements were made on a Bruker EMX 081 spectrometer equipped with an Oxford Instrument continuous flow cryostat. N-terminal sequence and mass spectra were obtained as described in (5).

Kinetic parameters associated with oxidation of the iron center by $O_2^{.-}$. The kinetics of the oxidation of Dfx by $O_2^{.-}$, generated by the xanthine-xanthine oxidase system, was followed spectrophotometricaly at 644 nm, in the absence or in the presence of different amounts of CuZn-SOD, as reported previously (5). In these conditions, the reciprocal of the initial rate of oxidation of Dfx (v_{ox}) should be linear versus CuZn-SOD concentrations, according to Eq.1 :

$$1/v_{ox} = 1/ (k_{ox} [XO]) + k_{SOD} [SOD] / (k_{ox} [XO]\ k_{Dfx} [Dfx]) \quad \text{(Eq.1)}$$

where k_{XO}, is the rate constant of production of $O_2^{.-}$ by xanthine oxidase (XO), k_{Dfx} and k_{SOD} are the second order rate constants of the reaction of Dfx and SOD with $O_2^{.-}$, respectively. At the concentration of CuZn-SOD which decreases by 50% the rate of oxidation of Dfx, one can write : k_{SOD} [SOD] = k_{Dfx} [Dfx] (Eq. 2) (5). Taking into account the known second order rate constant of the reaction of $O_2^{.-}$ with CuZn-SOD at low [$O_2^{.-}$], 2 10^9 M^{-1} s^{-1} (19), the second order rate constant of the oxidation of Dfx by $O_2^{.-}$, k_{Dfx}, was calculated using Eq. 2.

Assays for SOD and reductase(s) activities. The SOD activity was measured as described in (5) using the cytochrome c reduction assay modified from McCord and Fridovich (2). All kinetics, in the absence or presence of different amounts of the purified Dfx, were linear for at least 4 min. One unit of SOD is defined as the amount of protein, which inhibits the rate of the reduction of ferricytochrome c by 50%. *E.coli* crude extracts were prepared as previously described (5). Reduction of Dfx was followed spectrophotometrically at 650 nm, in a cuvette (0.1 ml final volume) containing 110 μM of fully oxidized Dfx, 50 mM Tris/HCl pH 7.6 and 600 μM of NADPH or NADH. The reaction was initiated by adding 5-20 μg of cell extract, anaerobically at 17 °C. Initial velocities of reduction of the iron center were calculated from the decrease of absorption at 650 nm. One unit of activity is defined as the amount of cell extract catalyzing the reduction of 1 nmol of the iron center per min.

RESULTS

The product of the *dfx* gene from *T. pallidum* contains only one mononuclear iron center. The gene encoding for the putative Dfx from *T.pallidum* was cloned under the control of the ptac promoter of the expression vector pJF119EH and overexpressed in *E.coli*. The gene product was identified as a 16 kDa protein on SDS-PAGE analysis and purified using a two-step purification protocol (gel filtration and anion exchange chromatographies). The 16 kDa polypeptide had a GRELSFFLQK N-terminal amino acid sequence, identical to the N-terminal translated sequence of the *T.pallidum dfx* gene (15), but lacking the N-terminal Met residue. A minor amount of the polypeptide with the N-terminal Met residue was also detected. Electrospray mass spectrometry analysis of the solution showed two ionic species, a minor one at 13,801 Da and a major one at 13,671 Da, corresponding to the molecular weights expected from the *dfx* gene sequence with and without the N-terminal Met residue, respectively (15). These data show that the purified 16 kDa protein is the product of the *dfx* gene. Gel-filtration experiments on a Superdex 75 column with the purified protein gave an apparent molecular mass of 27,800 Da (data not shown), showing that the Dfx from *T.pallidum* is a homodimer.

The iron content of Dfx was determined by atomic absorption spectroscopy. A value of 0.8 Fe/polypeptide chain (13,801 Da) was found.

No evidence for the presence of Zn or Mn atoms were found. Figure 2 shows the UV-visible spectrum of the as-isolated Dfx, with weak absorption bands centered at 644 and 330 nm. No contributions at 370 and 503 nm, characteristic for iron center I in Dfx from *D.desulfuricans* (7), or *D.baarsii* (5) could be detected, suggesting that Dfx from *T.pallidum* is missing iron center I. When the protein was treated with potassium ferricyanide, the intensity of the bands at 644 and 330 nm greatly increased and a value of 2,300 M^{-1} cm^{-1} was determined for the molar extinction coefficient at 644 nm in the fully oxidized protein. Furthermore, the 4 °K EPR spectrum of the isolated protein displays only a weak resonance at $g = 4.3$, which strongly increased during the treatment with ferricyanide (Fig. 3). This spectrum is similar to that reported for the ferric form of Dfx from *D. desulfuricans* (9) and from *D.vulgaris* (8), and was attributed to the oxidized center II. The iron center of the *T.pallidum* Dfx was thus essentially in the ferrous state and could be fully oxidized by ferricyanide.

Collectively, these data show that Dfx from *T. pallidum* contains only one iron center, equivalent to center II from well-characterized Dfxs from sulfate reducing bacteria, and is missing a second iron center, equivalent to center I, present in the other characterized Dfxs (7-10). These data are in agreement with the absence of three cyteine ligands in the *T.pallidum* Dfx sequence, replaced by a Q, a S and a A (Fig. 1).

Dfx from *T. pallidum* functionally complements *E.coli* SOD-deficient mutants. The capability of the *dfx* gene product from *T.pallidum* to complement *E. coli* SOD deficiency was tested. In fact, the *E.coli sodA sodB recA* mutant cannot grow in the presence of oxygen because of the combined lack of superoxide dismutase activity (*sodA sodB*) and the DNA strand break repair activity (*recA*) which results in lethal DNA oxidative damage (17, 20). As shown in Table I, in the presence of 1 mM IPTG, the plasmid pVN10-2, which encodes the structural *T.pallidum dfx* gene under the control of a *tac* promoter, fully restores aerobic growth to the *sodA sodB recA* mutant, whereas the parental plasmid pJF119EH did not. This clearly showed that production of Dfx from *T.pallidum* efficiently suppresses the deleterious effects due to the lack of SOD in *E.coli* and consequently fully protects against superoxide stress.

Reduction of superoxide by *T.pallidum Dfx.* That *T.pallidum* Dfx could catalyze the elimination of superoxide by reduction and then act as a superoxide reductase was further investigated. First, we have verified that Dfx from *T.pallidum* did not exhibit any significant SOD activity, assayed from its inhibitory effect on the reduction of cytochrome c by $O_2^{\cdot -}$ generated by the xanthine-xanthine oxidase system. Addition of 28 µg of purified Dfx was required to observe 50% inhibition of cytochrome c reduction, corresponding to a value for the specific SOD activity of 35 U

mg^{-1} (data not shown). This value is only about 0.5 % of a standard SOD enzyme specific activity and strongly suggested that Dfx from *T. pallidum* could not function as a SOD enzyme within the cell.

Successive additions of stoichiometric amounts of $O_2^{\cdot-}$ (KO_2 dissolved in Me_2SO), in the presence of catalase, resulted in the oxidation of the iron center, as shown by the increase of the band at 644 nm of the visible spectrum of Dfx (Fig. 4). Spectral changes occurred during the mixing time. A 4-fold molar excess of $O_2^{\cdot-}$ was required for a complete oxidation of the iron center and further addition of KO_2 did not promote additional changes (data not shown). Considering the very rapid spontaneous dismutation of superoxide (21), these data showed that superoxide efficiently oxidized the iron center of Dfx from *T.pallidum*.

This was confirmed by the determination of the rate constant for the oxidation of Dfx by $O_2^{\cdot-}$, using a methodology developed earlier (5). The kinetics of the oxidation of the iron center by $O_2^{\cdot-}$, generated by the xanthine-xanthine oxidase system in the presence of catalase, was followed spectrophotometrically at 644 nm, in the absence or in the presence of different amounts of CuZn-SOD. As shown in Fig. 5A, in the absence of SOD, oxidation of the iron center by $O_2^{\cdot-}$ was linear with time and was complete after about 2.5 min reaction. In the presence of large amounts of CuZn-SOD, the rate of oxidation was decreased. Figure 5B shows a linear plot of the reciprocal of the initial rate of oxidation of iron center (v_{ox}) as a function of CuZn-SOD concentration, according to Eq. 1, as described in Materials and Methods. From this plot, the concentration of CuZn-SOD that decreases by 50% the rate of the iron center was determined to be 3.9 µM. The second order rate constant of the oxidation of the iron center by $O_2^{.-}$ can be now calculated using Eq. 2 (Materials and Methods). A value of 1 10^9 M^{-1} s^{-1} was obtained.

The experiments presented above have been carried out in the presence of catalase in order to eliminate a possible effect of H_2O_2 that could be produced during spontaneous $O_2^{.-}$ dismutation. The ability of H_2O_2 to oxidize Dfx was nevertheless tested. The kinetic of the oxidation of the iron center (22 µM Dfx, in 50 mM Tris/HCl, pH 7.6) by 0.3, 0.5, 0.8, 1 and 1.5 mM H_2O_2 was followed spectrophotometrically at 644 nm, at 25 °C. In all cases, the reactions followed a pseudo first order kinetic with a value for the second order rate constant equal to 120 M^{-1} s^{-1} (data not shown). This is almost negligible when compared to the value of the rate constant of the oxidation of the iron center by $O_2^{.-}$.

Dfx from *T.pallidum* can act as a superoxide reductase. In the experiments with *sodA sodB recA E.coli* mutant strain (see above), Dfx was overexpressed. We thus could not a priori exclude a simple $O_2^{.-}$ trapping effect (a non-catalytic elimination process) of an excess of Dfx leading to complementation of the SOD deficiency. However, cytosolic *E.coli* extracts were able to reduce the oxidized form of the Dfx from *T.pallidum* with a specific activity of 22 nmol of iron center reduced /min/ mg in the presence of either NADPH or NADH (data not shown). The membrane fractions presented also some Dfx reductase activities, with a specific activity of 10 nmol of iron center reduced /min/ mg, in the presence of either NADH or NADPH (data not shown). These data demonstrated that both cytosolic and membrane *E.coli* extracts had the potential for catalytic reduction of Dfx from *T.pallidum*. This reaction regenerates the active ferrous center for new cycles of superoxide reduction. This result thus supports the notion that Dfx from *T.pallidum* is a superoxide reductase, which allows aerobic growth of *E.coli sod*$^-$ mutant strains. It further indicates that, at least in *E.coli*, the presence of an iron center I is not required for providing Dfx with a functional SOR activity.

DISCUSSION

We have isolated a protein from *T.pallidum* on the basis of its strong sequence homology with desulfoferrodoxins (Dfxs) from sulfate reducing bacteria (Fig. 1). However, there is a major difference between this protein and the Dfxs previously described. Dfx from *T.pallidum* only chelates one iron center, which has all the spectroscopic characteristics of the so-called ferrous center II in Dfx from *D.vulgaris* (8) and *D.desulfuricans* (9). Accordingly, all the ligands chelating the iron center II in Dfxs are found strictly conserved in the sequence of *T.pallidum*, in addition to the residues surrounding these positions (Fig. 1). The second iron center (center I) is absent in Dfx from *T.pallidum*, in agreement with the absence of three cysteine ligands replaced by a Q, a S and a A (Fig. 1). In that respect, Dfx from *T.pallidum* shows interesting similarities to neelaredoxin (Nlr), a protein initially isolated from the sulfate reducing bacteria *Desulfovibrio gigas* (13) and recently described as a SOR in *Pyrococcus furiosus* (6). Nlr also contains a single mononuclear iron center, with spectroscopic properties similar to those of the iron center II of Dfxs (13, 14). However, although Nlr presents a similar structural fold to the C-terminal domain of Dfxs (14), with conservation of the ligands of the iron center II, it lacks the whole protein domain corresponding to the N-terminal sequence of Dfxs from sulfate reducing bacteria (Fig. 6).

Instead, Dfx from *T.pallidum* can be aligned with the entire sequence of the other Dfxs, including the whole N-terminal domain (Fig. 1 and 6). In addition, Nlr sequences exhibit one major additional loop, which is not present in the C-terminal domain of classical Dfxs and in the sequence of the *T.pallidum* protein (Fig. 6). On the whole, it is correct to classify the protein from *T.pallidum* as a new type of Dfx, rather than a Nlr.

All the data reported here strongly suggest that this new type of Dfx functions as a superoxide reductase (SOR) :

i) expression of Dfx from *T.pallidum* is able to fully protect an *E.coli* SOD mutant from oxidative stress (Table I). The data were comparable to the data reported for the Dfx from *D.baarsii* (5) and suggested that, in *E.coli*, the iron center I of Dfx is not important for a functional complementation.

ii) Dfx from *T.pallidum* can reduce $O_2^{\cdot-}$ very efficiently. The second order rate constant of the oxidation of the reduced Dfx from *T.pallidum* by $O_2^{\cdot-}$ has been determined to be $1\ 10^9\ M^{-1}\ s^{-1}$, a value even greater than that reported for the *D.baarsii* enzyme ($6\text{-}7\ 10^8\ M^{-1}\ s^{-1}$) (5). The reaction is specific for $O_2^{\cdot-}$, since H_2O_2 did oxidize the iron center much more slowly (second order rate constant : $120\ M^{-1}\ s^{-1}$). Dfx from *T.pallidum* is also O_2 resistant and the protein was isolated mainly in a stable ferrous iron state.

iii) that reduction of O_2^{-} could be catalytic within the cell depends on the presence of a cellular system able to reduce the oxidized iron center for a complete catalytic cycle. We have found that cell extracts of *E.coli* contained NAD(P)H-dependent reductase activities, which may fulfill this function. Although, these activities are smaller than those reported in the case of *D.baarsii* (5), it still demonstrated that *E.coli* extracts could catalytically reduce Dfx from *T.pallidum*. In addition, because the reductase activities are not specific to membrane or cytosol fractions and to the reduced pyridine nucleotides, it thus appears that *E.coli* extracts do not posses a single specific system to reduce the iron center of SOR. This is in line with the great accessibility of the active site of SORs (10, 14) and their high redox potential (9, 13), which make a large number of reducing agents and reductases potentially good candidates. Consequently, it is very likely that similar activities exist in *T.pallidum* as well.

A question remains as far as the role of iron center I in Dfxs from sulfate reducing bacteria is concerned. The existence of SORs (Dfx from *T.pallidum* and Nlr from *P.furiosus* for example) containing only one iron center would suggest that center I in Dfx from *D.baarsii* does not participate to electron transfer/$O_2^{\cdot -}$ reduction during SOR activity and that this function reside only in iron center II. Further experiments are required to understand the function of center I.

Although SODs from far remains the most widespread defense mechanism against superoxide, several examples of another mechanism, superoxide reductase (SOR) have been reported yet. SORs primarily appeared as a simple mean specific for anaerobic bacteria to eliminate superoxide (5, 6, 11), possibly presenting selecting advantage during transitory exposure to air (12). The benefit of a SOR, compared to a SOD, in these organisms may be in relation with the presence of large amounts of a variety of strongly auto-oxidizable redox proteins, such as redox carriers (cytochromes, ferredoxins, flavodoxins, for example). As illustrated in Fig. 7, by shuttling the electrons from the auto-oxidizable redox proteins to superoxide, SOR could, in a single reaction, eliminate both superoxide and the source of its production. Such a reaction may allow the anaerobic bacteria to shut off transitory $O_2^{\cdot -}$ production from those redox carriers, with no need for sophisticated regulatory systems, such as found in facultative anaerobes. Other authors pointed out that reduction of superoxide does not produce molecular oxygen, as does the dismutation reaction, thus protecting O_2-sensitive cellular species from inactivation (4). However this latter hypothesis is questionable taking into account that from the genome and protein sequences available, it appears that several anaerobic microorganisms, like *D.gigas* (13, 24), *D.desulfuricans* (7, 25), *D.vulgaris* Hildenborough (22, Shenvi, N. V. & Kurtz, D. M., GenBank direct submission, accession number AF034841) *Methanobacterium thermoautotrophicum* (23) or *Clostridium acetobutylicum* (Genome Therapeutics Corporation, completed genome, not published; ORFs CAC2865, CAC2999, CAC1647) contain both *sor* and *sod* genes. Further studies are necessary to determine the respective roles of each enzyme and why there is such an apparent redundancy in mechanisms for elimination of superoxide.

In this respect, *Treponema pallidum* is a very interesting bacterium. It is a microaerophilic microorganism, with an optimal growth rate in the presence of 5% of molecular oxygen (16). This is the first example of an organism which can grow in the presence of oxygen without expressing a SOD enzyme (with the exeption of Mn SOD-mimic complexes produced by lactic acid bacteria (26)). Here we have shown that *T.pallidum* relies on a simplified version of Dfx, with full SOR activity, as the only mechanism for elimination of superoxide and protection from oxidative stress. This makes *T.pallidum* a unique model for studying the link between superoxide reductase and oxidative stress.

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FOOTNOTES

[1] The abbreviations used are : SOD, superoxide dismutase; SOR, superoxide reductase; Dfx, desulfoferrodoxin; Nlr, neelaredoxin; EPR, electron paramagnetic resonance; PAGE, polyacrylamide gel electrophoresis.

FIGURE LEGENDS

Figure 1. Sequence comparison of the putative Dfx from *T.pallidum* with various Dfxs sequences. From top to the bottom: Dfxs from *T.pallidum* (Tp.), *D.baarsii* (Db.), *D.desulfuricans* (Dd.) and *D.vulgaris* Hildemborough (Dv.). The alignments were produced by Clustal W. Shadowed lines indicate the residues involved in the binding of the two mononuclear iron centers, Center I and Center II (10).

Figure 2. Absorption spectra of the recombinant *T.pallidum* Dfx. A 23.6 µM protein containing 0.72 Fe/polypeptide chain suspended in 50 mM Tris/HCl, pH 7.6 was used. Spectrum of Dfx as isolated (lower trace) and treated with 25 µM potassium ferricyanide (upper trace). The inset shows a blow up of the 400-800 nm region.

Figure 3. EPR spectra of the Dfx from *T.pallidum*. A. Spectrum of the as-isolated Dfx. B. Spectrum of the Dfx oxidized with 200 µM potassium ferricyanide. Experimental conditions : protein concentration 200 µM; microwave power 0.2 mwatt; frequency 9.44 Ghz; modulation amplitude 20 G, receiver gain 5.02×10^5. Spectra were recorded at 4 °K.

Figure 4. Effect of $O_2^{\cdot -}$ on the visible spectra of *T.pallidum* Dfx. The micro-cuvette (100 µl final volume) contains 208 µM of Dfx (150 µM iron center) in 50 mM Tris/HCl pH 7.6, 500 U/ml catalase. Successive additions of 150 µM KO_2, from a 1.5 mM KO_2 stock solution dissolved in 100% Me_2SO (14 M), were performed. After each addition, a spectrum was recorded. From the bottom to the top, no addition, 1 equivalent, 2 equivalents, 3 equivalents, 4 equivalents per iron center.

Figure 5. Kinetics of oxidation of the *T.pallidum* Dfx by $O_2^{\cdot -}$. A. oxidation of the iron center was followed spectroscopically, at 25 °C, by the increase of absorbance at 644 nm. The cuvette contains (300 µl final volume) 10.3 µM Dfx (corresponding to 7.4 µM iron center), 50 mM Tris/HCl pH 7.6, 400 µM xanthine, 500 U/ml catalase and different amounts of CuZn-SOD. The oxidation was initiated by adding 0.013 U of xanthine oxidase. The following traces are presented: CuZn-SOD: (O) 0 µM; (Δ) 2 µM; (□) 3 µM and (◇) 5 µM of CuZn-SOD. B. shows the reciprocal of the initial velocity of the oxidation of the iron center as a function of [CuZn-SOD].

Figure 6. Sequence comparison between Dfxs and Nlrs. From top to bottom: Nlr from *P.furiosus* (Pf.), Nlr from *D.gigas* (Dg.), Dfx from *T.pallidum* (Tp.), Dfx from *D.baarsii* (Db.), Dfx from *D.desulfuricans* (Dd.) and Dfx from *D.vulgaris* Hildemborough (Dv.). The alignments

were produced by Clustal W. Shadowed lines indicate the residues involved in the binding of the two mononuclear iron centers (10, 14).

Figure 7. Scheme for the hypothesis of the detoxification activity of SOR. In the presence of $O_2^{.-}$, formed from the auto-oxidizable redox proteins in the presence of O_2, SOR eliminates both $O_2^{.-}$ and its source of production. In the absence of $O_2/O_2^{.-}$, SOR is not active, and the electrons are shuttled towards the cellular metabolisms.