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Superoxide Reductases

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Reactive oxygen species (ROS), when in excess, are among the most deleterious species an organism can deal with. The physiological effects of ROS include amino acid chain cleavage, DNA degradation and lipid oxidation, among others. They can be formed in the cytoplasm in a variety of ways, including autooxidation reactions (FMN- and FAD-containing enzymes) and Fenton reactions as a result of the cytoplasmatic pool of iron ions. The superoxide anion ($O_2^{\cdot-}$), despite its short half-life in solution, is particularly pernicious as it can form other reactive ROS (such as the strong oxidant peroxynitrite) or oxidize and/or reduce cellular components. For strict anaerobic or microaerophilic bacteria it is of particular importance to be able to dispose of ROS in a controlled manner, especially if these organisms are temporarily exposed to air. This review aims to describe the structural characteristics of superoxide reductases (SORs) and mechanistic aspects of biological superoxide anion reduction. SORs can be considered the main class of enzymes behind the oxygen detoxification pathway of anaerobic and microaerophilic bacteria. The geometry of the active site (three classes have been described), the possible electron donors in vivo and the current hypothesis for the catalytic mechanism will be discussed. Some phylogenetic considerations are presented, regarding the primary structure of SORs currently available in genome databases.

Introduction

Reactive oxygen species have been implicated in different metabolic pathways. ROS can be divided into radical (superoxide, hydroxyl, peroxyl, alkoxyl and hydroperoxyl) and nonradical (hydrogen peroxide, ozone, singlet oxygen, peroxynitrite and hypochlorous acid) entities. At higher concentrations they present problems to the normal cellular functions by, for example, oxidizing lipids, reducing sugars and amino acids or causing a large number of purine and pyrimidine modifications.[1–3] Reactions with proteins can lead to protein cleavage, cross-linkage or the loss of structural and/or catalytic function. Cellular protein degradation systems may, or may not, recognize these abnormal proteins. When not recognized, abnormal proteins will accumulate and, over time, will cause harmful effects in the cells or organisms. Although it is usual to think of ROS species as byproducts of cell metabolism, at physiological levels ROS can serve other purposes, for example, being involved in cell signalling and other important functions. At this level, ROS can be valuable or even required for a particular physiological reaction. More than 30 years ago, it was shown that the superoxide anion stimulated the formation of cGMP.[4] Interestingly, a recent study unveiled one beneficial role of ROS.[5] According to the authors, ROS are implicated in otoconia (small calcium carbonate crystals present in the inner ears of mammals) formation and could be required for interactions between otoconial components. As a result, ROS seem crucial to the mechanism by which vertebrates are able to sense gravity and maintain balance. For all the above reasons, understanding enzymes that can control ROS concentration is extremely important.

One significant ROS is the superoxide anion ($O_2^{\cdot-}$). The superoxide anion is generated by a one-electron reduction of molecular oxygen.[6–12] Some of the known sources of superoxide are: (1) Electron leakage, which can promote the reduction of O_2 to $O_2^{\cdot-}$. For example, it is known that several components of the mitochondrial electron transport chain (*b*-type cytochromes, coenzyme Q) can leak electrons. Also, in fatty acid desaturation, electron donors of the desaturase enzyme (flavoproteins and cytochrome b_5) can produce $O_2^{\cdot-}$. (2) Heme-containing proteins, namely hemoglobin and myoglobin, will produce $O_2^{\cdot-}$ by conversion of the oxy form into the met form of the protein. (3) Uncoupling of P450 oxidase activity with concomitant production of $H_2O_2/O_2^{\cdot-}$. (4) Enzymatic production of $O_2^{\cdot-}$ by xanthine oxidase, peroxidases and other oxidases. (5) The so-called autooxidation reactions where several molecules (reduced forms of FMN and FAD, adrenalin, thiol-containing mole-

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cules and others) can slowly reduce O_2 to $O_2^{\cdot-}$, most probably in the presence of catalytic amounts of metal ions. (6) Fenton reactions where the products (ferric ions, hydroxyl radical) can react with hydrogen peroxide to produce $O_2^{\cdot-}$. (7) Iron(II) oxidation by ferritin produces H_2O_2 as a secondary product. (8) Intentional production of $O_2^{\cdot-}$, for example in the plasma membrane of macrophages by an enzymatic complex (containing FAD and *b*-type cytochrome) that uses NADPH as the reducing agent.

How long-lived is the superoxide anion after its generation? In aqueous solutions, the superoxide anion readily dismutates into hydrogen peroxide and molecular oxygen. This is a pH-dependent phenomenon, which probably occurs by protonation of at least one superoxide anion. It was calculated that, at pH 7.0, the dismutation reaction has an approximate rate constant of 5×10^5 M^{-1} s^{-1}, which means that superoxide will only subsist for a few microseconds. Nevertheless, during that time superoxide can react with some cellular components to give rise to other potentially toxic species. Chemically, the superoxide anion can act as an oxidizing or a reducing agent. At low levels, the superoxide anion will not react with proteins unless there are accessible thiol groups. In such a case, the thiol groups are oxidized, and thiol-rich or-containing enzymes can be targeted and their functionalities modified. The superoxide anion can also be involved in enzyme reduction such as cytochrome *c* or ferritins. One of the most striking examples of superoxide damage is the oxidation of the [4Fe-4S] cluster of aconitase, a Krebs' cycle enzyme, with concomitant inactivation of the enzyme and release of reactive iron.[13] Other small molecules can also react with superoxide. For instance, NO reacts with superoxide to give rise to peroxynitrite, which is recognized as a potent oxidizing agent. These reactions have deleterious effects and will sequester the superoxide anion from solution and prevent the dismutation reaction.[14,15] For a complete panorama on superoxide, and ROS in general, the reader is advised to consult the excellent book on the matter by Halliwell and Gutteridge.[16]

To avoid the pernicious effects described above and, in a controlled manner, to dispose of intracellular superoxide anions, organisms possess one (or in rare cases, both) of two different enzymatic systems: superoxide dismutases and superoxide reductases. Under optimal conditions these enzymes will sequester the superoxide anion prior to dismutation or its reaction with other cellular components.[17]

In this review we aim to provide a concise, yet all-embracing, focused review on superoxide reductases. Hopefully, we will be able to communicate to the reader the his-

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Table 1. Numbering of functionally important residues in different SOR enzymes.

Organism	Glutamate	Lysine	Histidines	Isoleucine	Cysteine
D. vulgaris Hildenborough	47	48	49, 69, 75, 119	77	116
D. baarsii	47	48	49, 69, 75, 119	77	116
D. desulfuricans ATCC 27774	46	47	48, 68, 74, 118	76	115
T. pallidum	48	49	50, 70, 76, 122	78	119
T. maritima	15	16	17, 45, 51, 118	53	115
P. furiosus	14	15	16, 41, 47, 114	49	111

torical aspects of this interesting class of enzymes and at the same time provide the information needed to build an up-to-date knowledge in this research area.

The reader should note that throughout this article we will refer to SORs by the initial, "historical" names given to the enzymes before their enzymatic activity was characterized; hence, desulfoferrodoxin (Dfx) refers to class I SORs and neelaredoxin (Nlr) to class II SORs. Class III SORs have no official historical name, although they are frequently also called neelaredoxins owing to the UV/Vis spectrum similarities to class II SORs. These enzymes were also once called rubredoxin oxidoreductases (Rbo), when it was discovered that rubredoxin (Rd) could provide electrons to SORs, although this terminology is scarcely used anymore, except to denominate their coding gene (see below).

Furthermore, the reader is advised that in this review residue numbering is always quoted with regard to the numbering established in the work being cited. For the convenience of the readers, Table 1 and Appendix I (in Supporting Information) contain the number of homologous residues in different protein sequences

Desulfoferrodoxin – Class I SOR

In 1990, the purification and characterization of a protein containing a new type of nonheme iron from crude extracts of the sulfate and nitrate reducer *Desulfovibrio (D.) desulfuricans* ATCC 27774 as well as from extracts of *D. vulgaris* Hildenborough was reported.[18] Currently, five Dfx proteins were isolated and characterized from *D. desulfuricans* ATCC 27774, *D. vulgaris* Hildenborough, *D. baarsii*, *Archaeoglobus (A.) fulgidus* and *Methanobacterium (M.) thermoautotrophicum* (Table 2).

The protein from *D. desulfuricans* ATCC 27774 was subjected to extensive biochemical and spectroscopic characterization. This dimeric protein (2×14 kDa) contained two iron atoms per monomer, and the as-isolated form showed a visible spectrum dominated by maxima at 495, 368 and 279 nm, thus exhibiting a pink colour. The EPR spectrum of this form showed a set of resonances (g = 7.7, 5.7, 4.1 and 1.8) characteristic of a high-spin ferric ion (S = 5/2) with an E/D value of 0.08. Mössbauer spectroscopy clearly indicated the presence of two high-spin iron centres, one being ferric and the other ferrous. All these spectroscopic data pointed to the fact that this protein contained one iron centre (centre I) of the FeS_4 type, closely resembling the one found in desulforedoxin (Dx) from *D. gigas*,[19–22] and a second ferrous centre (centre II). Owing to the unusual combination of iron centres, the protein was denominated desulfoferrodoxin. Although initially purified in a semireduced state, named pink form, it was also possible to isolate the fully oxidized state of the protein.[23] In this form, named gray form according to its colour, the UV/Vis spectrum shows additional bands at 335 and 635 nm, the EPR spectrum has additional resonances at g = 4.3 and 9.6 and the Mössbauer spectral component attributed to centre II is characteristic of a high-spin ferric ion with E/D of approximately 1/3. Potentiometric redox titrations of Dfx showed that centre II has a rather high midpoint redox potential (ca. +240 mV at pH 7.6) that is in agreement with the fact that the protein could be aerobically isolated in the semireduced state.

The primary structure of Dfx determined by automated Edman degradation and mass spectrometry of the composing peptides confirmed not only the expected homology with Dx but also revealed a high homology to another iron-containing protein from *D. gigas* named neelaredoxin, which possesses one iron centre similar to centre II of Dfx, but no centre I (described in detail in the next section).[24] In conjunction with Mössbauer and Resonance Raman spectroscopic data, the sequence data indicated that centre

Table 2. List of SOR enzymes isolated and characterized.

Source	Type	Number of Iron Centres	Origin	PDB	Reference
A. fulgidus	Nlr (Class II)	1	Native/Recombinant	–	[58]
A. fulgidus	Dfx (Class I)	2	Recombinant	–	[86]
D. baarsii	Dfx (Class I)	2	Recombinant	1VZG, 1VZH, 1VZI	[34,47]
D. desulfuricans ATCC 27774	Dfx (Class I)	2	Native	1DFX	[18,29]
D. gigas	Nlr (Class II)	1	Native/Recombinant	–	[37,95]
D. vulgaris Hildenborough	Dfx (Class I)	2	Native/Recombinant	–	[18,72]
M. thermautotrophicus	Dfx (Class I)	2	Recombinant	–	[31]
P. furiosus	Nlr (Class II)	1	Native/Recombinant	1DO6, 1DQI, 1DQK	[39,54]
T. maritima	Nlr (Class II)	1	Recombinant	2AMU	[96]
T. pallidum	Class III	1	Recombinant	1Y07	[46,47,50]

II should have only one cysteinyl S ligand in an octahedral coordination of nitrogen and/or oxygen ligands. Sequence comparison also revealed that Dfx from *D. desulfuricans* ATCC 27774 was highly homologous with the product of the *D. vulgaris* Hildenborough *rbo* gene that encodes for Dfx,[25] whereas the N-terminal sequence of the *D. vulgaris* Hildenborough Dfx was identical to the deduced amino acid sequence. It was also found that a single transcript contained both *rub* and *rbo* genes (enconding for rubredoxin and Dfx, respectively), pointing to the fact that Rd could be a redox partner of Dfx. The origin of the Dfx coding gene, *rbo*, was also questioned.[26] It was shown that in *D. vulgaris* the *rbo* gene is expressed as a 14 kDa protein whereas in *D. gigas* the *dsr* gene coding for Dx and the gene coding for Nlr are expressed independently. Thus, Dx is not formed by post-translational modification of Dfx, but most probably the *rbo* gene originated from a gene-fusion event between the genes coding for the Dx and Nlr proteins. So, the Dfx monomer can be described as a 14 kDa polypeptide chain with an approximately 4 kDa N-terminus similar to Dx and a C-terminal region of ca. 10 kDa homologous to Nlr. In fact, two constructs were engineered where either the N-terminal or the C-terminal region was cloned and expressed separately in *Escherichia (E.) coli*.[27] The N-terminal variant consisted of a DNA fragment encoding the first 39 amino acids from *D. vulgaris* Hildenborough Dfx (DfxN), sharing approximately 50% sequence identity with *D. gigas* Dx. The C-terminal domain consisted of a DNA fragment encoding the last 92 amino acids of *D. vulgaris* Hildenborough Dfx (DfxC). Both recombinant fragments were able to bind iron. Biochemical and spectroscopic characterization showed that DfxN and DfxC exhibited properties similar to Dx and Nlr, respectively.

The first crystallographic structure of a superoxide reductase was obtained for *D. desulfuricans* ATCC 27774 Dfx.[28,29] The structure was solved by MAD phasing, using the iron centres present in the protein as anomalous scatterers, up to 1.9 Å resolution (see Figures 1 and 2). The crystallographic model showed the expected two domains: spherical domain I is notably similar to that of Dx (rms deviation of 0.59 Å for the backbone atoms), and the larger domain II harbours an iron site with square-pyramidal coordination to four histidine equatorial ligands and one cysteine axial ligand. The coordination geometry is such that three histidine ligands (His48, His68 and His74) coordinate to the iron ion through their N2 atom, with the exception of His118, which bonds through Nδ1. Cys115 binds in the axial position. A possible sixth ligand was not found, and an axial position is easily accessible to the solvent, which is thus available for substrate binding. Another important feature is that several β-sheets extend from monomer to monomer, which supports the existence of a functional dimer. Domain I and II are linked by a region of polypeptide chain (residues 34–39), which creates an elongated cavity with an approximate volume of 430 $Å^3$. A calcium ion was also found at the dimer interface coordinated by eight oxygen atoms (Ser87, Thr89 from both monomers and two water molecules). The dimeric nature of *D. desulfuricans*

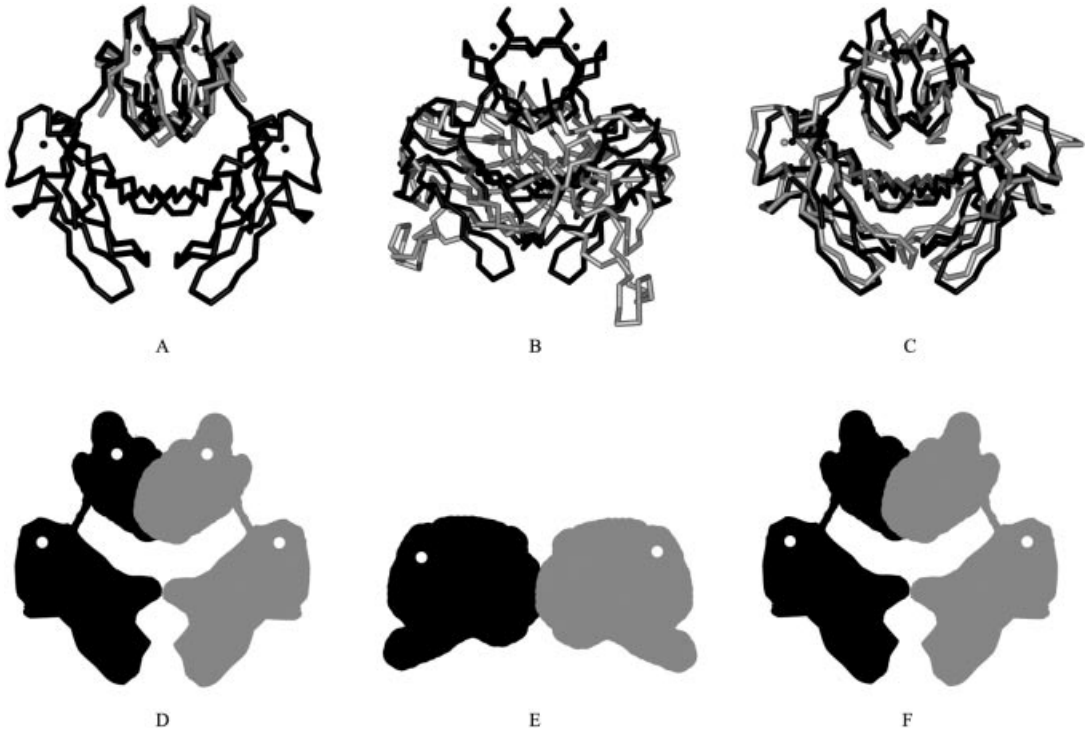

Figure 1. Structural fit of SOR- and SOR-related proteins structures compared to *D. desulfuricans* ATCC 27774 Dfx (represented in black). In grey: (A) *D. gigas* Dx; (B) *P. furiosus* Nlr; and (C) *T. pallidum* SOR. Schematic representation of the three SOR classes: (A) class I, Dfx dimer; (B) class II, Nlr dimer and (C) class III SOR dimer. Alignments were performed by using Chemera 3.0 (http://www.cqfb.fct.unl.pt/bioin/chemera/Chemera/Intro.html).

ATCC 27774 Dfx was also probed in equilibrium unfolding experiments.[30] By using the concentration dependence of aromatic residue emission, the dissociation constant of Dfx was determined to be ca. 1 μM. With the use of guanidine hydrochloride as a denaturating agent and with short incubation times, it was possible to observe one reversible transition (without loss of secondary structure) independent of the Dfx concentration. Only at longer incubation times was complete irreversible unfolding, with iron centre dissociation, observed. On the basis of these studies, a model was proposed where the equilibrium unfolding involves a monomeric intermediate with a native-like secondary structure. Such a finding is in agreement with the observation that mass spectroscopy and gel filtration results of *D. desulfuricans* ATCC 27774 protein are not consistent with a dimeric form.[18,24] However, in the case of the recombinant Dfx of *M. thermoautotrophicum*, more than two oligomeric species exist, with the smallest stable form being the dimeric one.[31]

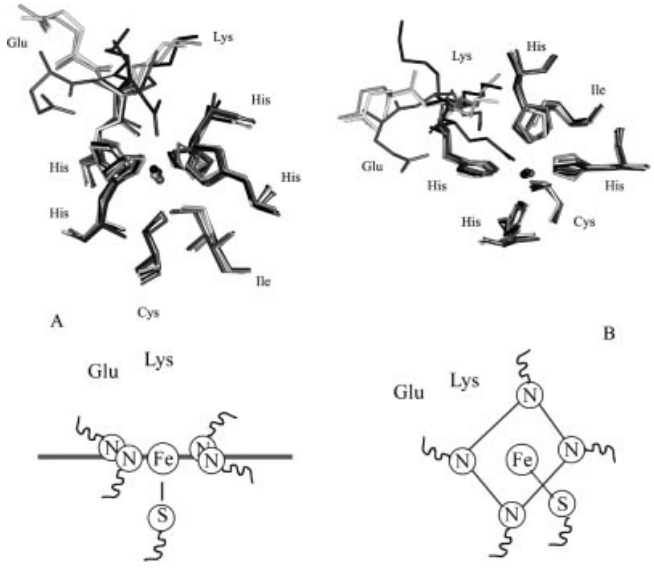

Figure 2. SOR active site showing the iron ligands and other functionally important/conserved residues. Overlapping of five different structures (aligned on the basis of the iron atom) obtained for *D. desulfuricans* ATCC 27774 (white), *T. pallidum* (light grey), *T. maritima* (grey), *P. furiosus* (dark grey), and *D. baarsii* (black). Cartoons below each set of structures intent to explain the view angle: (A) histidine ligands plane oriented transversal to the plane of the paper; (B) histidine ligands plane coincident with plane of the paper.

The accessibility of centre II towards external molecules was also evident by the characterization of a stable cyano-bridged dinuclear iron cluster.[32–34] This adduct is formed upon oxidation of ferrous centre II with potassium ferricyanide to afford the mixed-valence cyano-bridged [Fe^{III}–NC–$Fe^{II}(CN)_5$] complex. As proven by spectroscopic and crystallographic studies, this cyano-bridged diiron complex adopts a bent geometry. In the case of the enzyme of *D. baarsii*, the determined crystallographic structure of the complex shows both electrostatic and steric complementarities between the active site and the ferricyanide anion.

Two different types of studies showed the possibility of structural changes in centre II. In one particular work, the redox-induced structural changes were monitored by using FTIR coupled to electrochemical techniques.[35] The use of both the wild type and mutant proteins led to the proposal that centre II can bind a sixth ligand, namely a glutamate, in its ferric state [supporting the different coordination states observed in the crystallographic structure of *Pyrococcus (P.) furiosus* enzyme, see below]. The same study ruled out any relevant role, other than electrostatic interaction with substrate molecules, of a nearby lysine residue. In a different type of study, with the use of variable temperature UV/Vis, MCD, CD and EPR spectroscopy, it was shown that major electronic–vibrational changes in both centres result from the conversion of the all-ferric, grey form, to the semireduced, pink form.[36] It is suggested that an extended environmental reorganization of the region between centre I and centre II controls the electron transfer. Nonetheless, up to the moment this review was written, no direct proof of such a transfer existed, and its viability is always hindered by the large iron-to-iron distance (ca. 22 Å).

Neelaredoxin – Class II SOR

In 1994, Chen and coworkers reported the isolation, from extracts of *D. gigas*, of an iron-containing blue protein that was named neelaredoxin.[37] Since then, this protein was also purified from other organisms, including *P. furiosus*, and extensive spectroscopic studies were performed.[38] The crystallographic structures were also determined for the oxidized and reduced states (at resolutions of 1.7 and 2.0 Å, respectively).[39] The primary structures of *D. gigas* and *P. furiosus* Nlrs have ca. 50% identity, and both are highly homologous to the C-terminal domain of Dfx proteins. As expected, the observed spectroscopic properties are similar to those obtained for Dfx centre II. In practice, one can consider Nlr to be a short Dfx, only containing the equivalent of the Dfx domain II (see Figure 1).

P. furiosus Nlr is a homotetrameric protein with a subunit molecular mass of about 14 kDa. Each subunit contains one mononuclear iron centre that is similar to the Dfx centre II. The 3D structure of the protein in the reduced state [Protein Data Bank (PDB) entry 1DQK] shows that the ferrous iron centre is coordinated to the imidazole nitrogen atoms from four histidine ligands that occupy the equatorial positions and a cysteine ligand that occupies the axial position. This square-pyramidal coordination leaves a sixth position that is vacant or occupied by a solvent molecule. However, in two subunits of the oxidized Nlr structure (ferric state) a carboxylate group from a nearby glutamate becomes the sixth ligand (see Figure 2). This finding implies that the glutamate residue might have an important role in catalysis. A possible explanation is that the glutamate residue regulates the accessibility of the substrate to the active site. The vacant sixth position in the reduced state would allow substrate binding to centre II with concomitant substrate reduction and release. However, in the ferric state the coordinated glutamate would either slow down or even pre-

vent substrate binding and enzymatic catalysis. Although this is an important finding, one should be aware that this is the only structure available that shows fully occupied coordination of the iron centre and that some mutant studies challenged the proposed gating mechanism (see below). The presence of a glutamate residue as the sixth ligand was shown to be pH-dependent. Despite Resonance Raman spectra not showing it clearly, UV/Vis spectroscopic data present significant changes, which strongly suggest that at higher pH levels or in the E14A mutant protein the glutamate can be substituted for a hydroxide functionality or by Lys15.[40]

Also important is that a potential Rd binding site is found in the vicinity of the solvent-exposed residues that are close to the iron centre. The proposed site contains iron ligands (Glu14, His47 and His114) in addition to adjacent residues (Trp11, Ile39, Pro40, Pro42, Thr44 and Ile113). Binding at this site will result in an iron-to-iron distance of approximately 8 Å.

Possible electron-transfer pathways were identified in a Resonance Raman study of the *P. furiosus* enzyme.[40] In this work, an analogy was made with type 1 copper proteins and an almost-conserved Tyr-Cys-X-X-His motif in class I and II SORs. This hypothesis is especially attractive to class I enzymes where the Tyr residue lays between domains I and II, in a direct path between iron centres. However, the *P. furiosus* structural data shows that the proposed Tyr residue is not responsible for mediating the electron transfer from Rd to Nlr because in this case the residue is located at a subunit interface of the homotetramer and buried inside the protein.

It is noteworthy to mention that in class I and III enzymes [*D. baarsii* and *Treponema (T.) pallidum*, respectively], a high-spin ferric hydroxide species in the active site was identified at basic pH values.[41] This hydroxide species is postulated to occur at the end of the catalytic process and in a pH-dependent equilibrium with the glutamate-coordinated species. Small molecules such as cyanide or nitric oxide can also bind to the Nlr active site. Nitric oxide was used as a substrate analogue to probe the structural and electronic environment of the active site.[42] It was shown that NO binds reversibly to the ferrous centre. The NO adduct is hexacoordinate, with NO binding in a bent mode *trans* to the cysteinyl S ligand, which gives rise to an EPR signal that is characteristic of a near-axial (S = 3/2) spin system (E/D = 0.06). Such a system was rationalized to result from the antiferromagnetic coupling between a high-spin ferric iron (S = 5/2) and a NO^- ion (S = 1). The addition of CN^- to class I and II enzymes in the oxidized form results in the conversion of the native high-spin ferric state (S = 5/2) into a heterogeneous low-spin ferric state (S = 1/2).[43] Combined spectroscopic data (Resonance Raman, FTIR and ENDOR) showed that the bound cyanide adopts three distinct conformations and that this heterogeneity is observed at room temperature. Also, the heterogeneity is not present in ferrous class II enzyme adducts, where a homogeneous near-linear conformation is observed. The use of mutant proteins with the replacement of either glutamate or lysine residues (present in the vicinity of the substrate binding site) by alanine did not reveal any significant spectroscopic difference. Thus, the mutated residues do not interact with the bound cyanide and therefore most probably do not play a role in the orientation and/or protonation of ferric reaction intermediates.

It is also curious to note that it was possible to express the *P. furiosus* enzyme in plant cells, in which case the survival at high temperatures is enhanced.[44] Such experiments are useful to demonstrate that the Nlr gene can produce functional recombinant proteins in plants, altering the eukaryotic metabolism.

Class III SOR

Class I and II enzymes were found in anaerobic bacteria (or, at the most, microaerotolerant). Interestingly, a third class of SOR was found in a microaerophilic obligate pathogen of humans. *T. pallidum* is the agent responsible for venereal syphilis and during infection disseminates hematogenously to invade several tissue types. Throughout this process the bacteria must tolerate significant oxidative stress.[45] An analysis of *T. pallidum* genome does not show the presence of enzymes typically needed to deal with ROS, such as superoxide dismutases, catalases or peroxidases. However, it is possible to find a gene highly homologous to Dfx, the expression of which was shown through reverse transcriptase–polymerase chain reaction.[46,47] Gene sequence analysis showed that the gene product should be a ca. 14 kDa protein containing two domains homologous to Dfx domain I and II. In this case, domain I lacked three of the four cysteine residues responsible for centre I iron binding. The gene was cloned and expressed in *E. coli*, and the gene product was subjected to biochemical and spectroscopic characterization. The protein was found to be a homodimer containing a sole iron site per monomer. This iron site had almost equal visible, EPR and Mössbauer spectra to those of Dfx centre II, and exhibited a midpoint redox potential of approximately +210 mV (pH 7.8).

Enzymatic studies revealed that the protein was able to catalyze superoxide reduction to hydrogen peroxide and that the rubredoxin of the same organism is capable of functioning as an electron donor in this catalytic reaction.[46–48]

The crystal structure of *T. pallidum* SOR was recently obtained (Figures 1 and 2).[49,50] The enzyme shows a structure similar to Dfx, with two domains. As expected, the N-terminal domain does not contain an iron centre, but it is able to maintain the β-barrel topology in an overall arrangement that is very similar to the one found in Dfx. It is noted that the absence of cysteine residues does cause a decrease in the cohesion of the domain and that in the region corresponding to the one where centre I is present in Dfx some disorder is observed. The C-terminal region contains a pentacoordinate iron atom with four histidine and one cysteine residues in a square-pyramidal geometry. This iron centre is highly exposed to the solvent. The axial posi-

tion *trans* to the cysteine residue is vacant, with a water molecule located at approximately 4.3 Å from the iron centre. However, in one of the molecules present in the asymmetric unit it was found that the same water molecule was located closer to the iron atom (2.6 Å), similar to what was found in the *P. furiosus* enzyme for the reduced iron centre. In contrast to what was found for Dfx, calcium does not seem to be essential for dimerization.

Discovery of New Enzymatic Activity – Superoxide Anion Reduction

The function of these proteins was partially unveiled in 1996 when Touati and coworkers reported that Dfx from sulfate-reducing bacterium *D. baarsii* could suppress all deleterious effects caused by superoxide dismutase (SOD) deficiency in *E. coli*.[51] The authors were trying to isolate the superoxide dismutase gene from *D. baarsii* when they noticed that a DNA fragment containing two open reading frames with homologous sequences to the *rbo* and *rub* genes of *D. vulgaris* Hildenborough functionally complemented an *E. coli* mutant deficient in cytoplasmic SODs. The experiments proved that the *E. coli* mutant regains the ability to grow on minimal medium (without the addition of branched amino acids), and that its growth on gluconate and succinate as the sole carbon sources was no longer impaired. It was also observed that iron–sulfur cluster containing enzymes were protected from inactivation and that oxygen-dependent spontaneous mutagenesis returned to values closed to those observed in the wild type strain. It was possible to ascertain that Dfx centre II was in fact the responsible agent for this effect as neither Rd nor Dx, when expressed independently, were able to significantly raise the aerobic survival of the *E. coli* mutant. In contrast, expression of Dfx would allow complete avoidance of superoxide stress. Subsequently, Liochev and Fridovich proposed that Dfx would be involved in a "direct" or "indirect" mechanism of $O_2^{\cdot-}$ scavenging,[52] but without exhibiting significant SOD activity. Similar to the previous study, these authors were able to confirm that overexpression of Dfx complemented the SOD defects, preventing inactivation of iron–sulfur-containing fumarases.

Additional evidence that Dfx was involved in an oxygen defense mechanism was reported by Voordouw and Voordouw.[53] In this work, the *rbo* gene was deleted from the *D. vulgaris* Hildenborough genome and the resultant mutant was able to grow normally under anaerobic conditions, but it clearly became more sensitive to oxygen inactivation (with 100-fold drop in CFU by exposure to air for 24 hours, when compared to the wild type strain).

The definitive identification of the enzymatic activity of Nlr and Dfx was reported in 1999.[54,55] In an attempt to purify a putative superoxide dismutase from cell-free extracts of *P. furiosus*, Adams and coworkers isolated, for the first time, the above-described *P. furiosus* Nlr. This protein exhibited high activity in the standard SOD assay (values comparable to bovine SOD). In this assay, a constant flux of $O_2^{\cdot-}$ was produced by using a mixture of xanthine and xanthine oxidase under aerobic conditions. This mixture also contained horse heart cytochrome *c*, which could be directly reduced by $O_2^{\cdot-}$, and the reduction rate of cytochrome *c* would thus be representative of $O_2^{\cdot-}$ production (ca. 14 μM min^{-1}).[56,57] The addition of SOD to the reaction mixture would decrease the cytochrome *c* reduction rate as a result of the competition towards $O_2^{\cdot-}$. Excess SOD would completely avoid cytochrome *c* reduction. Replacement of SOD by *P. furiosus* Nlr would have a similar effect, except that for excess amounts of enzyme cytochrome *c* oxidation was observed. Such a fact can only be explained if Nlr can use cytochrome *c* as an electron donor for a redox reaction that involves $O_2^{\cdot-}$ as the substrate. It was then proposed that this reaction would be the direct reduction of $O_2^{\cdot-}$ to peroxide:

$$O_2^{\cdot-} + 2H^+ + D_{red} \rightarrow H_2O_2 + D_{ox}$$

where D_{red} and D_{ox} represent the reduced and oxidized forms of an electron donor. The activity was designated by cytochrome *c* superoxide oxidoreductase or SOR activity. Additionally, it was observed that Rd from the same organism was able to replace cytochrome *c* as the electron donor. This distinct catalytic reaction was further confirmed by testing the SOD activity of Nlr in assays with different superoxide detection methods where no Nlr electron donor was present (pyrogallol, epinephrine or nitroblue tetrazolium oxidation and acetylated cytochrome *c* reduction). In this case, SOR activity was reduced by at least 95% relative to that of SOD. Subsequent works confirmed that Dfx, as well as other Nlr proteins, possessed SOR activity,[46,58,59] that Rd is the putative physiologic electron donor in this reaction[48,57,60] and that the electron transfer between Rd and SOR was in fact the rate-limiting step of the overall reaction in the case of *D. vulgaris* Hildenborough, as shown in an in vitro study by Kurtz and coworkers.[17] These studies definitively pointed to the fact that SOR enzymes are deeply involved in oxygen detoxification systems of either strict anaerobic, microaerotolerant or microaerophilic bacteria. Other work with *D. vulgaris* Hildenborough mutant strains also showed that SOR plays a key role in oxygen defense when this organism is subjected to fully aerobic conditions and for superoxide anions generated in the cytoplasm.[61,62] For microaerophilic conditions, SOD seems to be important for protection of periplasmic components.[63] It was also found that overproduction of Dfx might interfere with the reducing pathway that keeps SoxR in its active form.[64] SoxR is a [2Fe-2S]-containing transcription factor that is oxidized as a response to oxidative stress and that activates global response to oxidative stress.[65–67] However, Wildschut and coworkers reported that rubredoxin:oxygen oxidoreductase (ROO) is also an important component of this system as it can enhance the survival of *D. vulgaris* Hildenborough by itself under microaerophilic conditions.[68]

It was also pointed out that SOR is engineered to perform one reaction (superoxide reduction) whereas SOD can perform both superoxide reduction and oxidation, effec-

tively becoming a superoxide dismutase.[69] Interestingly, it was also proposed that *A. fulgidus* Nlr can be a bifunctional enzyme, and the possibility of ferric Nlr being reduced by the superoxide anion was put forward.[70]

Recently, it was reported that *D. baarsii* Dfx cyano-bridged [Fe^{III}–NC–$Fe^{II}(CN)_5$] complex could react with the superoxide anion.[71] In this case, the authors proposed that superoxide suffers a one-electron reduction with concomitant oxidation of an organic substrate to form alkyl peroxide and a water molecule, and that this catalysis was carried out by the ferrocyanide moiety of the complex.

The Catalytic Mechanism of Superoxide Anion Reduction

How do SORs reduce $O_2^{\cdot-}$? To address this question several researchers tried to identify catalytic intermediates of the reduction process. Several studies based on pulse radiolysis were able to identify different intermediates[70,72–77] and some characterization was done with the aid of UV/Vis, EPR, Resonance Raman and Mössbauer spectroscopy.[41,75,78–81] In these studies, relevant mutants were used, in particular point mutations in glutamate (*D. baarsii* E47A) and lysine (*D. baarsii* K48I) residues located at the active site. While the emergent picture is still not complete (with some discrepancies in the number and nature of the intermediates that depend on the source of the enzyme under study), it is reasonable to describe the catalysis in the following way. The active enzyme (Figure 3, form a) is in the ferrous, pentacoordinate state, which is in redox equilibrium (pH dependent) with the hexacoordinate resting ferric state. As described previously, the active iron centre can have either a hydroxide (Figure 3, form f) or a glutamate (Figure 3, form h) as the sixth ligand. This coordination is pH dependent, with the hydroxide ligand being favoured at higher pH values ($pK_a \approx 9.0$). Catalysis proceeds with substrate binding to the ferrous site. The superoxide anion might be attracted, owing to electrostatic interaction, by a lysine residue (*D. vulgaris* Hildenborough K48 homologue). The first intermediate formed seems to be a ferric–peroxo species that forms with a rate constant $k_1 = 10^9\ M^{-1}\,s^{-1}$ and a characteristic maximum at 600 nm (Figure 3, form b). A

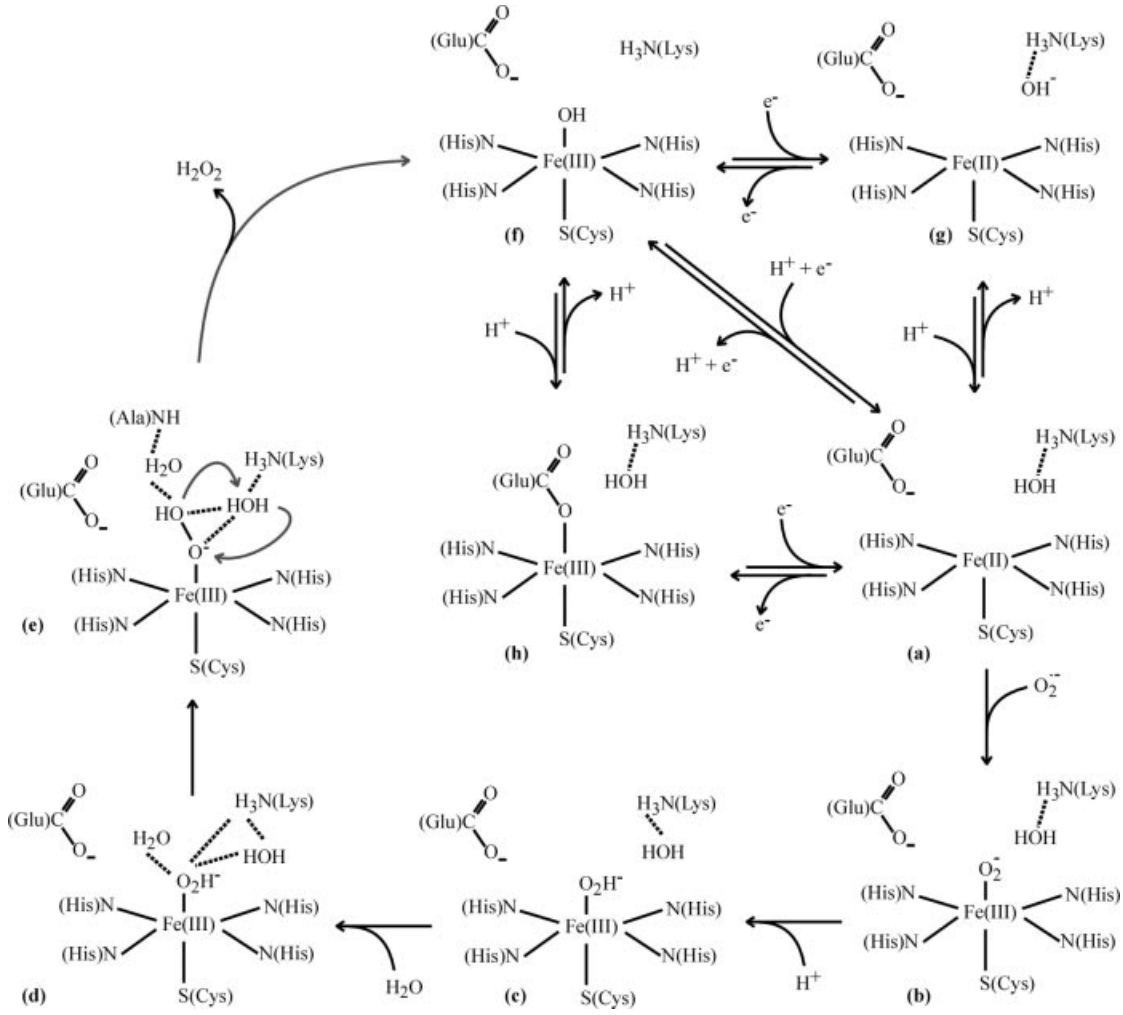

Figure 3. Catalytic scheme of superoxide anion reduction. The following enzyme forms were considered: (a) ferrous active state; (b) ferric peroxo intermediate; (c) ferric hydroperoxo intermediate; (d) an extra water molecule is recruited; (e) rearrangement leading to protonation; (f) ferric hydroxyl-bound resting state and (h) ferric carboxylate-bound resting state.

probable analogue of this intermediate can be obtained by reacting a mutant protein (*D. vulgaris* Hildenborough E47A homologue) with excess H_2O_2. The absence of the glutamate residue does not affect the natural substrate binding, but does stabilize a ferric–peroxo species. This species was characterized by Resonance Raman and Mössbauer spectroscopy and is best described as a high-spin Fe^{3+}-η^2-peroxo derivative. Although some authors claim that in the *D. vulgaris* Hildenborough and *A. fulgidus* enzymes this first intermediate undergoes direct protonation to yield the final products,[75] there is enough evidence (collected for *D. baarsii* and *T. pallidum* SORs) that a second intermediate is formed (Figure 3, form c).[73,74,76] The second intermediate has a characteristic peak at 625 nm and, contrary to the first intermediate, the rate constant for its formation is proportional to the proton concentration of the solvent ($k_2 = k_1 \cdot [H^+]$), which indicates that the protonation process occurs directly from the solvent.[76] As a result of this fact, proton transfer seems to be the rate-limiting step, and thus it is fair to assume that this second intermediate is a ferric hydroperoxo species. Subsequently, protonation of the second intermediate leads to H_2O_2 release. It was proposed that the proton donor could be a water molecule that is hydrogen-bonded to a nearby lysine residue, which would result in a high-spin ferric hydroxide species. Lysine interaction can lower the apparent pK_a of water molecules, which makes it a suitable proton donor to the ferric hydroperoxo intermediate. Experimental evidence supports this conclusion. In the crystallographic structure of *T. pallidum*, it was possible to model a H-bonding network through water molecules that interact with the conserved lysine residue,[50] and, in *D. baarsii*, Resonance Raman studies showed the formation, at basic pH, of a ferric hydroxide species. At more acidic pH values, the conserved glutamate residue replaces the hydroxide moiety. Very recently, a study by Katona et al. was published[82] in which an elegant methodology combining Resonance Raman spectroscopy and kinetic crystallography was used to study *D. baarsii* SOR crystals soaked with H_2O_2. This study revealed that in a E114A mutant (known to stabilize ferric peroxide complexes) the superoxide anion binds in a side-on mode (η^1) and is subsequently reduced by the iron atom. The authors further proposed that upon protonation and coordination to two water molecules, which were recruited and activated by nearby lysine and alanine residues, a second protonation step yields hydrogen peroxide which is then released, leaving the iron centre coordinated to a hydroxy group. This work clearly identifies the role of the water molecules that are present in the active site, whose action avoids the formation of an oxo–ferryl species by enabling the protonation of the proximal oxygen atom (Figure 3, forms d and e).

Computational studies were conducted in an effort to explain this $O_2^{\cdot-}$ reduction mechanism.[83] In this work the use of DFT and ZINDO/S-CI results support the existence of a peroxo or hydroperoxo intermediate and point to the importance of the protonation of this intermediate in the production of H_2O_2, in contrast to possible cleavage of the O–O bond found in heme enzymes.

For purposes of clarity, we did not include in the above discussion the oxidation state of Dfx centre I. The electrons necessary for $O_2^{\cdot-}$ reduction can be donated by Rd either directly to the active site or through centre I, and under physiological conditions it might be possible that this donation will prevent the formation of the glutamate-coordinated species because reduction can occur rapidly after H_2O_2 release, as observed in pulse radiolysis experiments where excess reductant was used.[77] One curious observation is that in a *D. vulgaris* Hildenborough Dfx mutant where one cysteine residue, which was responsible for the iron binding in the Dx-like centre I, was replaced by a serine residue, the resulting protein lacked centre I but possessed a normal centre II and its catalytic properties were almost identical to those of the wild type protein.[84] Thus, this *D. vulgaris* Hildenborough Dfx variant functions in a similar manner to class III SOR. Electron transfer studies, in the absence or presence of substrate, between Rd and the different classes of SOR showed that there is a trend in the specificity for the electron donor and acceptor from the same organism.[48,57] One interesting result was that in *D. gigas*, Dx was able to transfer electrons to SOR more efficiently (with an apparent three times greater rate constant) than Rd. Clearly more studies need to be performed to unveil the role of centre I in catalysis as well as to understand the interaction between redox partners.

In *P. furiosus*, it was possible to reconstitute, in vitro, the reduction pathway.[85] In this case it was shown that Rd is reduced by NAD(P)H by an NAD(P)H rubredoxin oxidoreductase (NROR) and that the reconstitution of the full

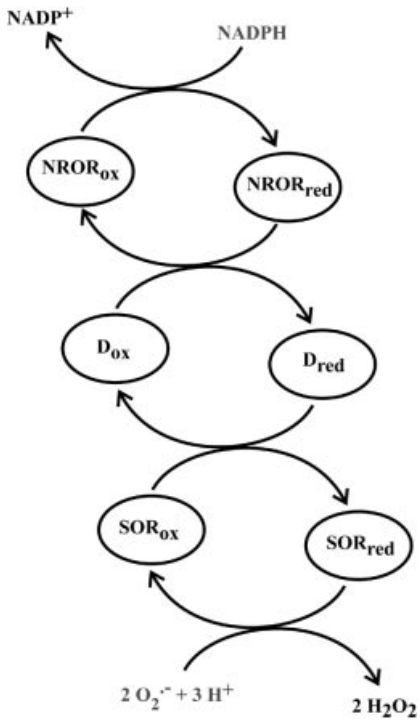

Figure 4. Proposed electron-transfer pathway in superoxide anion reduction. NROR–NADPH: rubredoxin oxidoreductase; D–Rd or Dx; SOR–Dfx, Nlr or class III SOR.

pathway allowed the reduction of the superoxide anion to hydrogen peroxide by using NAD(P)H as the electron donor (Figure 4).

Phylogenetic Considerations

Searching the microbial genomic databases yields a total of 50 SOR analogues, with approximately 12% of the sequenced genomes containing at least one gene coding for this enzyme. A comparison of all 50 amino acid sequences can be found in Appendix I and the phylogenetic relationships among SOR enzymes are displayed in Figure 5. From the 50 different sequences, it was possible to identify 23 Dfx, 20 Nlr and 6 class III enzymes. One sequence (*Geobacter sp.* FRC-32) showed a high homology to the C-terminal part of Dfx proteins but completely lacked the N-terminal part, and so it can be described as a short Nlr protein (please note that for this case the genome is not completely sequenced). Comparison of all sequences reveals that only seven amino acids are completely conserved. These are the five ligands of the active site (four histidine and one cysteine residues), one proline and one isoleucine, both located near the active site and, so far, with no direct implication on the catalytic mechanism. Both the lysine and glutamate residues were judged necessary for catalysis and are highly conserved. In one instance the lysine residue

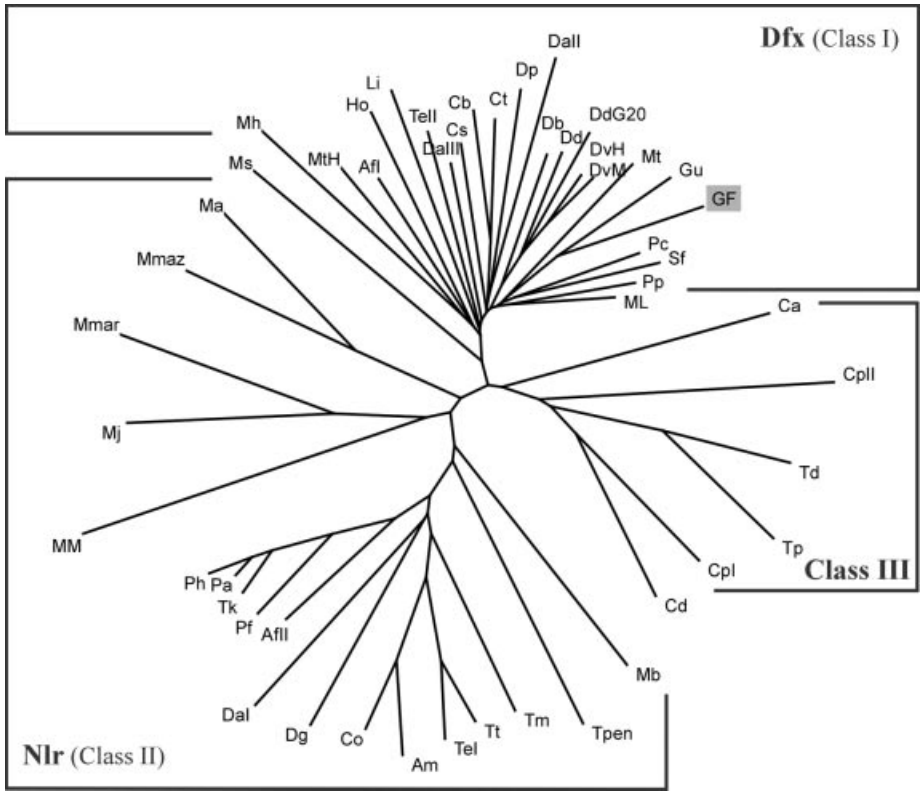

Figure 5. Phylogenetic relationship among SOR enzymes considering the three classes described in the text. Sequences were obtained in the integrated microbial genomes, DOE Joint Genome Institute (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi), The Institute for Genomic Research (http://www.tigr.org/), RCSB PDB database (http://www.rcsb.org/pdb/home/home.do) or through the NCBI website (http://www.ncbi.nlm.nih.gov/). Sequence alignment and tree construction were performed with ClustalX[94] and the tree displayed with TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). The grey box denotes the special case of class I SORs, *Geobacter sp.* FRC-32. The following abbreviations were used: **AfI**, *Archaeoglobus fulgidus* DSM4304; **AfII**, *Archaeoglobus fulgidus*; **Am**, *Alkaliphilus metalliredigens* QYMF; **Ca**, *Clostridium acetobutylicum* ATCC 824; **Cb**, *Clostridium beijerinckii* NCIMB 8052; **Cd**, *Clostridium difficile* 630; **Co**, *Clostridium sp.* OhILAs; **CpI**, *Clostridium phytofermentans* ISDg; **CpII**, *Clostridium phytofermentans* ISDg; **Cs**, *Caldicellulosiruptor saccharolyticus* DSM 8903; **Ct**, *Clostridium tetani* E88; **DaI**, *Desulfuromonas acetoxidans* DSM 684; **DaII**, *Desulfuromonas acetoxidans* DSM 684; **DaIII**, *Desulfuromonas acetoxidans* DSM 684; **Db**, *Desulfovibrio baarsii*; **Dd**, *Desulfovibrio desulfuricans* ATCC 27774; *Desulfovibrio desulfuricans* G20; **Dg**, *Desulfovibrio gigas*; **Dp**, *Desulfotalea psychrophila* LSv54; **DdG20**, *Desulfovibrio desulfuricans* G20; **DvH**, *Desulfovibrio vulgaris subsp. vulgaris* str. Hildenborough; **DvM**, *Desulfovibrio vulgaris* Myazaki; **GF**, *Geobacter sp.* FRC-32; **Gu**, *Geobacter uraniumreducens* Rf4; **Ho**, *Halothermothrix orenii* H 168; **Li**, *Lawsonia intracellularis*; **Ma**, *Methanosarcina acetivorans* C2A; **Mb**, *Methanococcoides burtonii* DSM 6242; **Mh**, *Methanospirillum hungatei* JF-1; **Mj**, *Methanocaldococcus jannaschii* DSM 2661; **ML**, delta proteobacterium MLMS-1; **MM**, *Magnetococcus sp.* MC-1; **Mmar**, *Methanococcus maripaludis* strain S2; **Mmaz**, *Methanosarcina mazei* strain Goe1; **Ms**, *Methanosphaera stadtmanae* DSM 3091; **Mt**, *Moorella thermoacetica* ATCC 39073; **MtH**, *Methanobacterium thermoautotrophicum* delta H; **Pa**, *Pyrococcus abyssi* GE5; **Pc**, *Pelobacter carbinolicus* DSM 2380; **Pf**, *Pyrococcus furiosus* DSM 3638; **Ph**, *Pyrococcus horikoshii shinkaj* OT3; **Pp**, *Pelobacter propionicus* DSM 2379; **Sf**, *Syntrophobacter fumaroxidans* MPOB; **Td**, *Treponema denticola* ATCC 35405; **TeI**, *Thermoanaerobacter ethanolicus* ATCC 33223; **TeII**, *Thermoanaerobacter ethanolicus* ATCC 33223; **Tk**, *Thermococcus kodakaraensis* KOD1; **Tm**, *Thermotoga maritima* MSB8; **Tp**, *Treponema pallidum subsp. pallidum* str. Nichols; **Tpen**, *Thermofilum pendens* Hrk 5; **Tt**, *Thermoanaerobacter tengcongensis* MB4;.

seemed to be replaced by an arginine residue whereas glutamate can be either located one residue further away from the active site (primary sequence wise) or be replaced by an aspartate residue (in one case). They are, however, highly conserved as one would expect from the mechanistic studies referred above. It is noteworthy that some organisms contain more than one gene coding for different SOR enzymes, such as the case of *Clostridium phytofermentans* where two different class III putative SOR enzymes exist and *Desulfuromonas acetoxidans* with three putative SOR coding genes. In the case of *A. fulgidus*, *Desulfuromonas acetoxidans* and *Thermoanaerobacter ethanolicus* it is even possible to find genes coding for both Dfx and Nlr enzymes. In the case of *A. fulgidus*, both gene products were already isolated.[70,86]

Another striking feature is that genes coding for Dx, one of the possible redox partners of SOR enzymes, could be found in *D. gigas*, where it was originally identified, but also in *Methanosarcina mazei* and *Methanospirillum hungatei*. Whereas the first two organisms contain genes coding for Nlr enzymes, the last one only seems to contain a gene for the Dfx enzyme.

Another striking example is the one found for *P. horikoshii*, where it was not possible to find any Rd or Dx coding gene. In this case, it will be necessary to consider a different electron donor.

Outlook

SORs are now structurally well-characterized proteins, with a considerable amount of biochemical, spectroscopic and crystallographic data on all three classes of enzymes. The mechanism of superoxide anion reduction is, however, still under evaluation. It is not yet completely understood why some enzymes of the same class will have two reaction intermediates (peroxo and hydroperoxo species) whereas others (of different classes) seem to have a single intermediate that undergoes direct protonation with concomitant H_2O_2 release.

The importance of Dfx centre I in catalysis is also elusive. Is this centre the primary recipient of electrons, delivering them to the active site in a regulated manner? This is apparently not the case because in Dfx mutants, where centre I was destroyed, activity remained similar to that of the wild type proteins.[84]Also, class III enzymes show a similar overall quaternary structure and are able to function without centre I. Nevertheless, almost half the genes found are ascribed to Dfx-type enzymes, which means that evolution decided to maintain centre I regardless of its apparent lack of function. It was suggested that centre I might have a catalytic role such as reducing molecular oxygen to superoxide that, consequently, could be further reduced in Dfx centre II.[84] While this is certainly an attractive proposal because organisms could in a single enzyme cope with oxygen and superoxide tolerance, no proof of such a catalysis exists.

Could the role of centre I be related to the nature of the physiologic electron donor? Up until now, both Rd and Dx were identified as possible electron donors. Dx was thought to be present only in *D. gigas* and was shown to be able to conduct superoxide-mediated electron transfer to *D. gigas* Nlr and *T. pallidum* SOR. It was now found that a putative Dx gene exists in *Methanospirillum hungatei* that also contains a putative Dfx coding gene. Therefore, it would be interesting to know if Dx could also act as an electron donor for Dfx enzymes.

Finally, the reader should be aware that during the past years some authors have addressed the biological function of superoxide reductase[87–92] as well as the use of biomimetic models to understand the catalytic mechanism.[93]

Supporting Information (see footnote on the first page of this article)**:** Sequence alignment of all SOR and putative SOR proteins found done with ClustalX.[94] Figure was prepared using the ESPript program.[97]

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[1] K. Keyer, A. S. Gort, J. A. Imlay, *J. Bacteriol.* **1995**, *177*, 6782–6790.
[2] B. Halliwell, J. M. Gutteridge, C. E. Cross, *J. Lab. Clin. Med.* **1992**, *119*, 598–620.
[3] J. M. Gutteridge, B. Halliwell, *Baillieres Clin. Haematol.* **1989**, *2*, 195–256.
[4] C. K. Mittal, F. Murad, *Proc. Natl. Acad. Sci. USA* **1977**, *74*, 4360–4364.
[5] P. J. Kiss, J. Knisz, Y. Zhang, J. Baltrusaitis, C. D. Sigmund, R. Thalmann, R. J. Smith, E. Verpy, B. Banfi, *Curr. Biol.* **2006**, *16*, 208–213.
[6] I. Fridovich, *Annu. Rev. Biochem.* **1995**, *64*, 97–112.
[7] H. de Groot, A. Littauer, *Free Radical Biol. Med.* **1989**, *6*, 541–551.
[8] J. Carlsson, G. Nyberg, J. Wrethen, *Appl. Environ. Microbiol.* **1978**, *36*, 223–229.
[9] J. Membrillo-Hernandez, N. Ioannidis, R. K. Poole, *FEBS Lett.* **1996**, *382*, 141–144.
[10] J. F. Turrens, *Biosci. Rep.* **1997**, *17*, 3–8.
[11] J. A. Imlay, *J. Biol. Chem.* **1995**, *270*, 19767–19777.
[12] A. R. Goeptar, H. Scheerens, N. P. Vermeulen, *Crit. Rev. Toxicol.* **1995**, *25*, 25–65.
[13] P. R. Gardner, *Biosci. Rep.* **1997**, *17*, 33–42.
[14] S. I. Liochev, I. Fridovich, *Iubmb Life* **1999**, *48*, 157–161.
[15] F. Auchere, F. Rusnak, *J. Biol. Inorg. Chem.* **2002**, *7*, 664–667.
[16] J. M. Gutteridge, B. Halliwell, *Free Radicals in Biology and Medicine*, 4th ed., Oxford University Press, **2007**.
[17] J. P. Emerson, E. D. Coulter, R. S. Phillips, D. M. Kurtz, *J. Biol. Chem.* **2003**, *278*, 39662–39668.
[18] I. Moura, P. Tavares, J. J. G. Moura, N. Ravi, B. H. Huynh, M. Y. Liu, J. Legall, *J. Biol. Chem.* **1990**, *265*, 21596–21602.
[19] C. Czaja, R. Litwiller, A. J. Tomlinson, S. Naylor, P. Tavares, J. Legall, J. J. G. Moura, I. Moura, F. Rusnak, *J. Biol. Chem.* **1995**, *270*, 20273–20277.
[20] B. J. Goodfellow, F. Rusnak, I. Moura, T. Domke, J. J. Moura, *Protein Sci.* **1998**, *7*, 928–937.
[21] M. Archer, R. Huber, P. Tavares, I. Moura, J. J. Moura, M. A. Carrondo, L. C. Sieker, J. LeGall, M. J. Romao, *J. Mol. Biol.* **1995**, *251*, 690–702.

[22] I. Moura, M. Bruschi, J. Le Gall, J. J. Moura, A. V. Xavier, *Biochem. Biophys. Res. Commun.* **1977**, *75*, 1037–1044.
[23] P. Tavares, N. Ravi, J. J. G. Moura, J. Legall, Y. H. Huang, B. R. Crouse, M. K. Johnson, B. H. Huynh, I. Moura, *J. Biol. Chem.* **1994**, *269*, 10504–10510.
[24] B. Devreese, P. Tavares, J. Lampreia, N. VanDamme, J. LeGall, J. J. G. Moura, J. VanBeeumen, I. Moura, *FEBS Lett.* **1996**, *385*, 138–142.
[25] M. J. Brumlik, G. Voordouw, *J. Bacteriol.* **1989**, *171*, 4996–5004.
[26] M. J. Brumlik, G. Leroy, M. Bruschi, G. Voordouw, *J. Bacteriol.* **1990**, *172*, 7289–7292.
[27] C. Ascenso, F. Rusnak, I. Cabrito, M. J. Lima, S. Naylor, I. Moura, J. J. G. Moura, *J. Biol. Inorg. Chem.* **2000**, *5*, 720–729.
[28] A. V. Coelho, P. M. Matias, M. A. Carrondo, P. Tavares, J. J. G. Moura, I. Moura, V. Fulop, J. Hajdu, J. LeGall, *Protein Sci.* **1996**, *5*, 1189–1191.
[29] A. V. Coelho, P. Matias, V. Fulop, A. Thompson, A. Gonzalez, M. A. Carrondo, *J. Biol. Inorg. Chem.* **1997**, *2*, 680–689.
[30] D. Apiyo, K. Jones, J. Guidry, P. Wittung-Stafshede, *Biochemistry* **2001**, *40*, 4940–4948.
[31] Y. K. Chae, *Mol. Cells* **2002**, *13*, 259–263.
[32] M. D. Clay, J. P. Emerson, E. D. Coulter, D. M. Kurtz, M. K. Johnson, *J. Biol. Inorg. Chem.* **2003**, *8*, 671–682.
[33] F. Auchere, P. Raleiras, L. Benson, S. Y. Venyaminov, P. Tavares, J. J. G. Moura, I. Moura, F. Rusnak, *Inorg. Chem.* **2003**, *42*, 938–940.
[34] V. Adam, A. Royant, V. Niviere, F. P. Molina-Heredia, D. Bourgeois, *Structure* **2004**, *12*, 1729–1740.
[35] C. Berthomieu, F. Dupeyrat, M. Fontecave, A. Vermeglio, V. Niviere, *Biochemistry* **2002**, *41*, 10360–10368.
[36] N. H. Andersen, S. E. Harnung, I. Trabjerg, I. Moura, J. J. G. Moura, J. Ulstrup, *Dalton Trans.* **2003**, 3328–3338.
[37] L. A. Chen, P. Sharma, J. Legall, A. M. Mariano, M. Teixeira, A. V. Xavier, *Eur. J. Biochem.* **1994**, *226*, 613–618.
[38] M. D. Clay, F. E. Jenney, P. L. Hagedoorn, G. N. George, M. W. W. Adams, M. K. Johnson, *J. Am. Chem. Soc.* **2002**, *124*, 788–805.
[39] A. P. Yeh, Y. L. Hu, F. E. Jenney, M. W. W. Adams, D. C. Rees, *Biochemistry* **2000**, *39*, 2499–2508.
[40] M. D. Clay, F. E. Jenney, H. J. Noh, P. L. Hagedoorn, M. W. W. Adams, M. K. Johnson, *Biochemistry* **2002**, *41*, 9833–9841.
[41] C. Mathe, V. Niviere, T. A. Mattioli, *J. Am. Chem. Soc.* **2005**, *127*, 16436–16441.
[42] M. D. Clay, C. A. Cosper, F. E. Jenney, M. W. W. Adams, M. K. Johnson, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 3796–3801.
[43] M. D. Clay, T. C. Yang, F. E. Jenney, I. Y. Kung, C. A. Cosper, R. Krishnan, D. M. Kurtz, M. W. W. Adams, B. M. Hoffman, M. K. Johnson, *Biochemistry* **2006**, *45*, 427–438.
[44] Y. J. Im, M. K. Ji, A. M. Lee, W. F. Boss, A. M. Grunden, *FEBS Lett.* **2005**, *579*, 5521–5526.
[45] K. R. Hazlett, D. L. Cox, R. A. Sikkink, F. Auch′ere, F. Rusnak, J. D. Radolf, *Methods Enzymol.* **2002**, *353*, 140–156.
[46] T. Jovanovic, C. Ascenso, K. R. O. Hazlett, R. Sikkink, C. Krebs, R. Litwiller, L. M. Benson, I. Moura, J. J. G. Moura, J. D. Radolf, B. H. Huynh, S. Naylor, F. Rusnak, *J. Biol. Chem.* **2000**, *275*, 28439–28448.
[47] M. Lombard, D. Touati, M. Fontecave, V. Niviere, *J. Biol. Chem.* **2000**, *275*, 27021–27026.
[48] F. Auchere, R. Sikkink, C. Cordas, P. Raleiras, P. Tavares, I. Moura, J. J. G. Moura, *J. Biol. Inorg. Chem.* **2004**, *9*, 839–849.
[49] T. Santos-Silva, J. Trincao, A. L. Carvalho, C. Bonifacio, F. Auchere, I. Moura, J. J. G. Moura, M. J. Romao, *Acta Crystallogr., Sect. F: Struct. Biology. Cryst. Commun.* **2005**, *61*, 967–970.
[50] T. Santos-Silva, J. Trincao, A. L. Carvalho, C. Bonifacio, F. Auchere, P. Raleiras, I. Moura, J. J. G. Moura, M. J. Romao, *J. Biol. Inorg. Chem.* **2006**, *11*, 548–558.
[51] M. J. Pianzzola, M. Soubes, D. Touati, *J. Bacteriol.* **1996**, *178*, 6736–6742.
[52] S. I. Liochev, I. Fridovich, *J. Biol. Chem.* **1997**, *272*, 25573–25575.
[53] J. K. Voordouw, G. Voordouw, *Appl. Environ. Microbiol.* **1998**, *64*, 2882–2887.
[54] F. E. Jenney, M. F. J. M. Verhagen, X. Y. Cui, M. W. W. Adams, *Science* **1999**, *286*, 306–309.
[55] F. E. Jenney, M. F. J. M. Verhagen, M. W. W. Adams, *J. Inorg. Biochem.* **1999**, *74*, 181–181.
[56] J. M. McCord, I. Fridovich, *J. Biol. Chem.* **1969**, *244*, 6049–6055.
[57] F. Auchere, S. R. Pauleta, P. Tavares, I. Moura, J. J. G. Moura, *J. Biol. Inorg. Chem.* **2006**, *11*, 433–444.
[58] I. A. Abreu, L. M. Saraiva, J. Carita, H. Huber, K. O. Stetter, D. Cabelli, M. Teixeira, *Mol. Microbiol.* **2000**, *38*, 322–334.
[59] M. Lombard, M. Fontecave, D. Touati, V. Niviere, *J. Biol. Chem.* **2000**, *275*, 115–121.
[60] J. V. Rodrigues, I. A. Abreu, L. M. Saraiva, M. Teixeira, *Biochem. Biophys. Res. Commun.* **2005**, *329*, 1300–1305.
[61] M. Fournier, Y. Zhang, J. D. Wildschut, A. Dolla, J. K. Voordouw, D. C. Schriemer, G. Voordouw, *J. Bacteriol.* **2003**, *185*, 71–79.
[62] H. L. Lumppio, N. V. Shenvi, A. O. Summers, G. Voordouw, D. M. Kurtz, *J. Bacteriol.* **2001**, *183*, 101–108.
[63] M. N. Davydova, R. Z. Sabirova, *Anaerobe* **2003**, *9*, 39–41.
[64] P. Gaudu, S. Dubrac, D. Touati, *J. Bacteriol.* **2000**, *182*, 1761–1763.
[65] H. Ding, E. Hidalgo, B. Demple, *J. Biol. Chem.* **1996**, *271*, 33173–33175.
[66] P. Gaudu, B. Weiss, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 10094–10098.
[67] E. Hidalgo, H. Ding, B. Demple, *Cell* **1997**, *88*, 121–129.
[68] J. D. Wildschut, R. M. Lang, J. K. Voordouw, G. Voordouw, *J. Bacteriol.* **2006**, *188*, 6253–6260.
[69] S. I. Liochev, I. Fridovich, *J. Biol. Chem.* **2000**, *275*, 38482–38485.
[70] I. A. Abreu, L. M. Saraiva, C. M. Soares, M. Teixeira, D. E. Cabelli, *J. Biol. Chem.* **2001**, *276*, 38995–39001.
[71] F. P. Molina-Heredia, C. Houee-Levin, C. Berthomieu, D. Touati, E. Tremey, V. Favaudon, V. Adam, V. Niviere, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 14750–14755.
[72] E. D. Coulter, J. P. Emerson, D. M. Kurtz, D. E. Cabelli, *J. Am. Chem. Soc.* **2000**, *122*, 11555–11556.
[73] V. Niviere, M. Lombard, M. Fontecave, C. Houee-Levin, *FEBS Lett.* **2001**, *497*, 171–173.
[74] M. Lombard, C. Houee-Levin, D. Touati, M. Fontecave, V. Niviere, *Biochemistry* **2001**, *40*, 5032–5040.
[75] J. P. Emerson, E. D. Coulter, D. E. Cabelli, R. S. Phillips, D. M. Kurtz, *Biochemistry* **2002**, *41*, 4348–4357.
[76] V. Niviere, M. Asso, C. O. Weill, M. Lombard, B. Guigliarelli, V. Favaudon, C. Houee-Levin, *Biochemistry* **2004**, *43*, 808–818.
[77] J. V. Rodrigues, I. A. Abreu, D. Cabelli, M. Teixeira, *Biochemistry* **2006**, *45*, 9266–9278.
[78] C. Mathe, T. A. Mattioli, O. Horner, M. Lombard, J. M. Latour, M. Fontecave, V. Niviere, *J. Am. Chem. Soc.* **2002**, *124*, 4966–4967.
[79] O. Horner, J. M. Mouesca, J. L. Oddou, C. Jeandey, V. Niviere, T. A. Mattioli, C. Mathe, M. Fontecave, P. Maldivi, P. Bonville, J. A. Halfen, J. M. Latour, *Biochemistry* **2004**, *43*, 8815–8825.
[80] C. Mathe, V. Niviere, C. Houee-Levin, T. A. Mattioli, *Biophys. Chem.* **2006**, *119*, 38–48.
[81] P. Raleiras in "Superoxide Reductases, Structural and Functional Aspects", PhD thesis, Universidade Nova de Lisboa, Lisboa, **2005**.
[82] G. Katona, P. Carpentier, V. Niviere, P. Amara, V. Adam, J. Ohana, N. Tsanov, D. Bourgeois, *Science* **2007**, *316*, 449–453.
[83] R. Silaghi-Dumitrescu, L. Silaghi-Dumitrescu, E. D. Coulter, D. M. Kurtz, *Inorg. Chem.* **2003**, *42*, 446–456.

[84] J. P. Emerson, D. E. Cabelli, D. M. Kurtz, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 3802–3807.
[85] A. A. Grunden, F. E. Jenney, K. S. Ma, M. Y. Ji, M. V. Weinberg, M. W. W. Adams, *Appl. Environ. Microbiol.* **2005**, *71*, 1522–1530.
[86] J. V. Rodrigues, L. M. Saraiva, I. A. Abreu, M. Teixeira, D. E. Cabelli, *J. Biol. Inorg. Chem.* **2007**, *12*, 248–256.
[87] J. A. Imlay, *J. Biol. Inorg. Chem.* **2002**, *7*, 659–663.
[88] D. M. Kurtz, *Acc. Chem. Res.* **2004**, *37*, 902–908.
[89] M. W. W. Adams, F. E. Jenney, M. D. Clay, M. K. Johnson, *J. Biol. Inorg. Chem.* **2002**, *7*, 647–652.
[90] D. M. Kurtz, *J. Inorg. Biochem.* **2006**, *100*, 679–693.
[91] D. M. Kurtz, E. D. Coulter, *J. Biol. Inorg. Chem.* **2002**, *7*, 653–658.
[92] V. Niviere, M. Fontecave, *J. Biol. Inorg. Chem.* **2004**, *9*, 119–123.
[93] L. M. Brines, J. A. Kovacs, *Eur. J. Inorg. Chem.* **2006**, 29–38.
[94] J. D. Thompson, T. J. Gibson, F. Plewniak, F. Jeanmougin, D. G. Higgins, *Nucleic Acids Res.* **1997**, *25*, 4876–4882.
[95] G. Silva, S. Oliveira, C. M. Gomes, I. Pacheco, M. Y. Liu, A. V. Xavier, M. Teixeira, J. LeGall, C. Rodrigues-Pousada, *Eur. J. Biochem.* **1999**, *259*, 235–243.
[96] R. G. Zhang, Y. Kim, T. Skarina, S. Beasley, R. Laskowski, C. Arrowsmith, A. Edwards, A. Joachimiak, A. Savchenko, *J. Biol. Chem.* **2002**, *277*, 19183–19190.
[97] P. Gouet, E. Courcelle, D. I. Stuart, F. Metoz, *Bioinformatics* **1999**, *15*, 305–308.

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