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The superoxide dismutase activity of desulfoferrodoxin from *Desulfovibrio desulfuricans* ATCC 27774

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Desulfoferrodoxin (Dfx), a small iron protein containing two mononuclear iron centres (designated centre I and II), was shown to complement superoxide dismutase (SOD) deficient mutants of *Escherichia coli* [Pianzzola, M.J., Soubes M. & Touati, D. (1996) *J. Bacteriol.* **178**, 6736–6742]. Furthermore, neelaredoxin, a protein from *Desulfovibrio gigas* containing an iron site similar to centre II of Dfx, was recently shown to have a significant SOD activity [Silva, G., Oliveira, S., Gomes, C.M., Pacheco, I., Liu, M.Y., Xavier, A.V., Teixeira, M., Le Gall, J. & Rodrigues-Pousada, C. (1999) *Eur. J. Biochem.* **259**, 235–243]. Thus, the SOD activity of Dfx isolated from the sulphate-reducing bacterium *Desulfovibrio desulfuricans* ATCC 27774 was studied. The protein exhibits a SOD activity of 70 $U \cdot mg^{-1}$, which increases approximately 2.5-fold upon incubation with cyanide. Cyanide binds specifically to Dfx centre II, yielding a low-spin iron species with g-values at 2.27 ($g_{\perp}$) and 1.96 ($g_{\parallel}$). Upon reaction of fully oxidized Dfx with the superoxide generating system xanthine/xanthine oxidase, Dfx centres I and II become partially reduced, suggesting that Dfx operates by a redox cycling mechanism, similar to those proposed for other SODs.

Evidence for another SOD in *D. desulfuricans* is also presented – this enzyme is inhibited by cyanide, and N-terminal sequence data strongly indicates that it is an analogue to Cu,Zn-SODs isolated from other sources. This is the first indication that a Cu-containing protein may be present in a sulphate-reducing bacterium.

Keywords: *Desulfovibrio*; rubredoxin; desulfoferrodoxin; superoxide dismutase; thioredoxin.

Desulfoferrodoxin (Dfx) is one of the several mononuclear nonhaem iron proteins that have been discovered in sulfate-reducing bacteria. These include rubredoxin (Rd) [1–4], desulforedoxin (Dx) [5], rubrerythrin [6], nigerythrin [7] and neelaredoxin [8]. Dfx was first detected in cells of *Desulfovibrio desulfuricans* strain 27774 grown with nitrate as terminal electron acceptor and in *Desulfovibrio vulgaris* Hildenborough [9,10]. Its name comes from the fact that it was first isolated as a half-reduced species: one of its two centres (the Desulforedoxin-like centre I) was oxidized, while the second (centre II) was reduced, in the ferrous state. It was later found that aerobically prepared samples contained both the half-reduced (pink) and the fully oxidized (grey) forms. In following spectroscopic studies, centre II was described as containing mainly nitrogen and/or oxygen ligands involved in an octahedral coordination to the iron [11]. Further resonance-Raman studies indicated possible cysteinyl coordination to centre II [12]. Recently, the crystallographic structure of Dfx has been solved [13,14]. Taking advantage of the previously published amino-acid sequence [11], it was confirmed that centre I was identical to the centre found in *D. gigas* desulforedoxin, whose structure is also known [15]. Centre II has a unique square pyramidal structure, involving one cysteine and four histidine residues.

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Abbreviations: Dfx, desulfoferrodoxin; SOD, superoxide dismutase; Dx, desulforedoxin; Rd, rubredoxin

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Neelaredoxin, a protein so far isolated only from *D. gigas* has been described as containing two iron centres having homology with Dfx centre II [8]. Furthermore, its amino-acid sequence, as deduced from its gene structure (GenBank accession code AF034965) shows that the ligands to Dfx centre II are conserved in neelaredoxin. Neelaredoxin was recently shown to have superoxide dismutase (SOD) activity, thus defining a new type of SOD [16].

The gene coding for Dfx in *D. vulgaris* has been found close to the gene coding for rubredoxin [17,18] and for that reason has been called *rbo*, for rubredoxin oxidoreductase, although evidence for such a function is still lacking. Furthermore, Dfx is not found in *D. gigas* and neither the gene coding for Dx [18] nor the one coding for neelaredoxin [16] are found adjacent to the gene that codes for Rd; rather, the later is adjacent to the gene coding for an Rd : oxygen-oxidoreductase, a protein involved in the reduction of oxygen to water [19,20]. Also, in *D. gigas*, the bona fide NADH : Rd-oxidoreductase is a flavoprotein with no structural relation to Dfx [21]. Finally, the recent data on the complete genomes of the anaerobes *Methanobacterium thermoautothrophicum* [22], *Methanococcus jannaschii* [23] and *Archaeoglobus fulgidus* [24] show that genes coding for Dfx and Dfx-like proteins are not adjacent to those coding for Rd proteins, further indicating that the situation found in *D. vulgaris* and in *D. baarsii* [25] is not common to all anaerobes. Thus, generalizations linking genome organization and function of these proteins are not possible to make in the absence of direct physiological evidence.

A recent observation [25,26] showed that the overexpression of Dfx in mutants of *Escherichia coli* lacking SOD compensates the deleterious effect of these mutations. As Dfx is abundant in

D. desulfuricans cells, it is important to know if this protein has significant SOD activity or if a typical SOD is also present in the cells. In fact, despite being anaerobic micro-organisms, some sulphate reducing bacteria have been shown to contain both catalase and superoxide dismutases [27].

We present evidence that Dfx has SOD activity and that this activity is most probably due to its centre II. In addition, both a copper-zinc SOD and a thioredoxin-like protein are shown to be present in *D. desulfuricans* 27774.

EXPERIMENTAL PROCEDURES

Growth of micro-organism and preparation of cell-free extract

D. desulfuricans ATCC 27774 was grown on a lactate/nitrate medium as previously described [28]. The cell-free extract was prepared under argon to avoid exposure to air, as described in [4].

Purification of desulfoferrodoxin

All purification procedures were performed at 4 °C and pH 7.6, in a Coy anaerobic chamber model 1-2463. The anaerobically purified soluble extract (700 mL) was loaded on a DEAE-52 column (6 × 34 cm) previously equilibrated with 10 mM Tris/HCl, and a 2-L continuous gradient of 10–400 mM Tris/HCl was applied. To detect the fractions containing Dfx, an aliquot of each fraction was left to oxidize under air and the UV/Vis spectrum was recorded. The Dfx fraction, eluted at ≈200 mM, was concentrated in a Diaflo apparatus (Amicon) with a YM3 membrane. After concentration, the fraction was loaded on a DEAE Biogel column (4.5 × 30 cm), and eluted under the same conditions as used in the previous step. Dfx eluted at ≈50 mM and was then applied to a hydroxylapatite column (3 × 25 cm), equilibrated at the same ionic strength. The column was then eluted using a 0.5-L descending linear gradient (50 mM–5 mM) of Tris/HCl, followed by a 1-L ascending linear gradient of 10–350 mM potassium phosphate buffer. The Dfx containing fraction, eluted at ≈50 mM, was finally applied to a second DEAE-Biogel column (4.5 × 28 cm). A continuous gradient (10–200 mM) of Tris/HCl was applied from which fractions containing pure Dfx were obtained (eluted at ≈50 mM). This anaerobic purification of Dfx yielded 120 mg of pure protein from 560 g of cells (wet weight).

Purification of Cu,Zn-SOD

From the first DEAE column a fraction also having SOD activity, detected both by spectroscopic and gel assays, was eluted at 20 mM Tris/HCl. After concentration, this fraction was loaded on a hydroxylapatite column (4 × 25 cm) equilibrated with a Tris/HCl buffer of the same ionic strength, and eluted with an ascending linear gradient of 10–350 mM potassium phosphate buffer (1 L). The fraction with SOD, eluted at 20 mM, was finally applied, under aerobic conditions, to a Superdex75 (XK26/60) column, equilibrated with 50 mM Tris/HCl and 100 mM NaCl. The SOD containing fraction was contaminated with a *c*-type cytochrome. The extremely small amount of protein obtained did not allow further purification. During this procedure another band was detected; analysing the N-terminal sequence of the protein corresponding to this band showed homologies with thioredoxins from other organisms.

D. gigas desulforedoxin and *D. desulfuricans* rubredoxin-1 were purified as in [5] and [29], respectively.

Assays for SOD activity

Spectrophotometric assays. Superoxide dismutase activity was assayed using the xanthine oxidase/cytochrome *c* system as described in [30]. The effect of cyanide was tested in two ways: firstly, by adding cyanide to the reaction mixture, and secondly, by incubating the samples with 10 mM cyanide for 24 h. Appropriate controls were performed for each assay and the pH was monitored after cyanide addition. At this concentration, cyanide had no effect on the rate of cytochrome *c* reduction by the xanthine/xanthine oxidase system. The potassium cyanide solution was buffered at pH 7.6 in 1 M Tris/HCl.

Gel assays. Nondenaturing PAGE gels were used according to [31], using riboflavin in both the resolving and concentrating gels. SOD activity was detected as in [32]; the gels were first soaked in 2.45 mM nitroblue tetrazolium for 20 min, then in a 10-fold diluted solution containing 28 mM tetramethylenediamine, 2.8 mM riboflavin and 36 mM potassium phosphate pH 7.8 for 15 min. The gel was then rinsed with distilled water and illuminated for 10 min. Cyanide effect was determined by adding 10 mM KCN (pH 7.6) to both solutions.

Analytical methods

SDS-polyacrylamide gel electrophoresis was performed according to [33]. The protein molecular masses were determined by 12% SDS/PAGE using Pharmacia low-range protein standards. Protein concentration was determined as in [34]. For N-terminal sequencing, the proteins were electroblotted to poly(vinylidene difluoride) membranes as described in [35]. The N-terminal sequences were determined using an Applied Biosystem Model 477A protein sequencer.

Spectroscopic methods

Visible spectra and SOD activity assays were performed on a Shimadzu UV-3100 spectrophotometer. EPR spectra were recorded on a Bruker ESP 380 spectrometer, equipped with an ESR 900 continuous-flow helium cryostat. Spin quantitations were performed by double integration of experimental spectra obtained under nonsaturating conditions (7.4 μW) at 16 K, as well as through theoretical simulations of the spectra. Myoglobin azide (980 μM) prepared as in [36] was used as standard.

Preparation of Dfx in different states of oxidation

Prior to preparation, Dfx was in the pink, half-reduced state. The fully oxidized grey form, was obtained by oxidation of the pink form with H_2O_2 at a concentration of 120 μM, followed by purification in a gel filtration column (Pharmacia PD-10). Reduced Dfx was obtained by reduction with dithionite, followed by purification in a gel filtration column inside a Coy anaerobic chamber.

RESULTS

D. desulfuricans soluble extract

Superoxide dismutase activity was detected in the soluble extract by gel staining. Two bands of SOD activity were observed, with only one band inhibited by cyanide. These bands were assigned by running the gels of the purified proteins (see below). It was impossible to measure SOD activity of the anaerobically prepared extract by the xanthine oxidase/cytochrome *c*

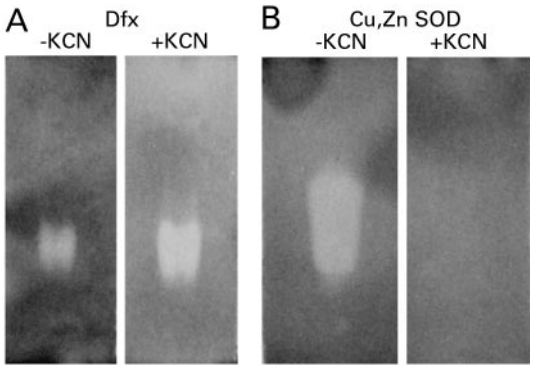

Fig. 1. Dfx and putative Cu,Zn-SOD PAGE gels stained for SOD activity. Panel A, Dfx in the absence and presence of added KCN. Panel B, putative Cu,Zn SOD purified from *D. desulfuricans* ATCC 27774

system, using cytochrome *c* as the electron acceptor, because a quite significant cytochrome reductase activity was detected. Thus, a soluble extract was aerobically prepared for which a SOD activity of 2 ± 0.3 U·mg^{-1} was measured; this activity increased ≈3.5-fold upon addition of 10 mM cyanide.

Desulfoferrodoxin

Although Dfx can be purified in the fully reduced state anaerobically, this form is very unstable because centre I (the Dx-like centre) reacts immediately with oxygen. In contrast, center II is not reactive towards oxygen, so that long time exposure of Dfx to air exclusively gives the 'pink' form of the protein, that is to say centre I oxidized, centre II reduced. Hydrogen peroxide is capable of oxidising centre II, and was used to routinely prepare the grey form. It is notable that mammalian cytochrome *c* was also capable of stoichiometrically oxidizing centre II. This specific reaction explains the lack of observed SOD activity with anaerobically prepared soluble extracts, because they have considerable potential intrinsic reducing power. In addition, the atmosphere of the anaerobic box contains 2% hydrogen, so that the hydrogenase contained in the extract ensures Dfx remains in a reduced state.

Pure Dfx has a clear SOD activity on NBT gels which is not inhibited by cyanide. Instead, a sharp activation is observed (Fig. 1, panel A). This activity could also be determined spectrophotometrically, using sufficiently diluted Dfx solutions so that there was no interference due to the intrinsic cytochrome *c* reductase activity. Using this procedure, an activity of 70 ± 10 U·mg^{-1} was measured for the pink form of Dfx, while a lower activity of ≈25 U·mg^{-1} was obtained for the fully oxidized grey form. In both cases, the activity increased ≈2.5-fold in the presence of 10 mM cyanide. Cyanide binding seems to be rapid, as the increase in activity is observed immediately after adding cyanide to the reaction medium. Samples incubated with 10 mM cyanide to up to 48 h showed the same activity.

The spectroscopic properties of desulfoferrodoxin iron centres have been extensively studied [9,10,12]. The centres in the oxidized, ferric, states have characteristic markers both in EPR and visible electronic spectra; centre I yields an EPR signal with features at g = 7.7, 5.7, 1.8 and has a visible spectrum almost identical to that of *D. gigas* desulforedoxin; centre II has an EPR resonance at g = 4.3 and a broad visible band at ≈660 nm [12]. Thus, using both spectroscopies it is possible to unambiguously monitor the redox state of the two centres.

The interaction of Dfx with superoxide was probed by two independent types of experiments, monitored by both visible and EPR spectroscopy, using the partially-reduced pink form and the fully-oxidized grey form. Upon incubation of Dfx in the pink form, with xanthine/xanthine oxidase, centre II becomes oxidized within a few minutes, as shown by the increases in the g = 4.3 EPR resonance (Fig. 2, panel A, trace b) and absorbance at ≈660 nm (Fig. 2, panel B, trace b). However, this effect is completely blocked by adding catalytic amounts of catalase (Fig. 2, trace c in both panels). A second identical experiment was performed using the fully oxidized protein (Fig. 2, trace d in both panels, see Materials and methods). Within minutes, as

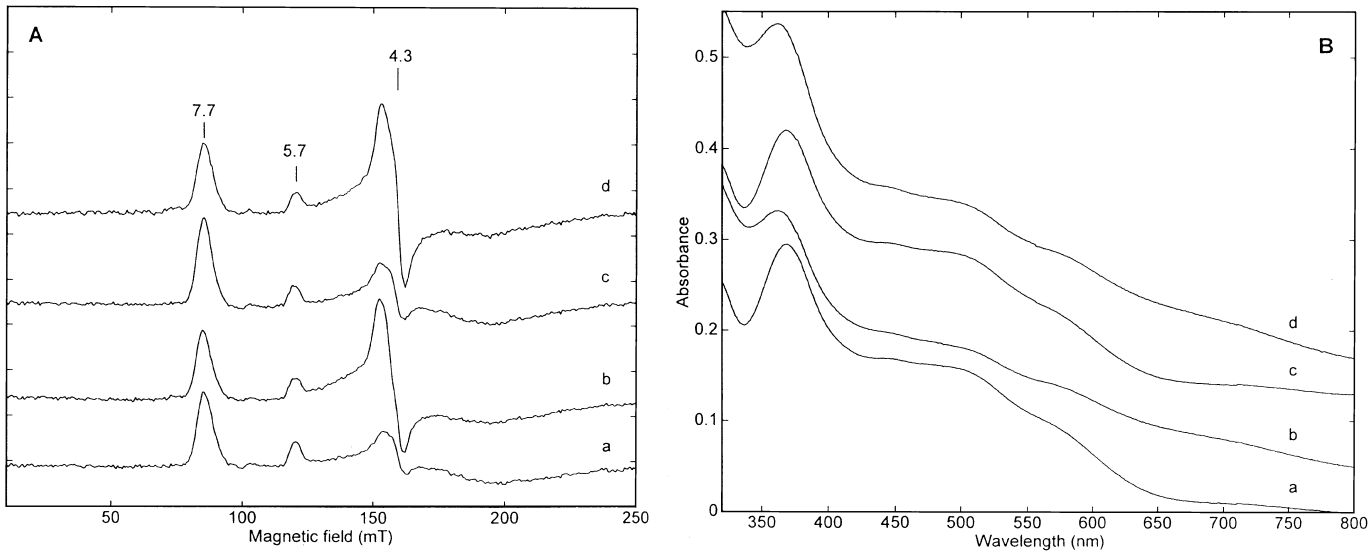

Fig. 2. Spectral changes on Dfx after incubation with xanthine/xanthine oxidase. EPR (panel A) and visible spectra (panel B) were recorded in the following conditions: Trace (a), Dfx as prepared (pink form); Trace (b), same as in trace (a) but after 5 min incubation in the presence of xanthine/xanthine oxidase; Trace (c), same as in trace (b) but in the presence of catalase; Trace (d), Dfx after incubation with H_2O_2 (grey form). The pink form contains a small but variable amount of centre II oxidized, which yields the g = 4.3 EPR signal. EPR conditions: Temperature, 4.6 K; microwave power, 2.4 mW; microwave frequency 9.63 GHz.

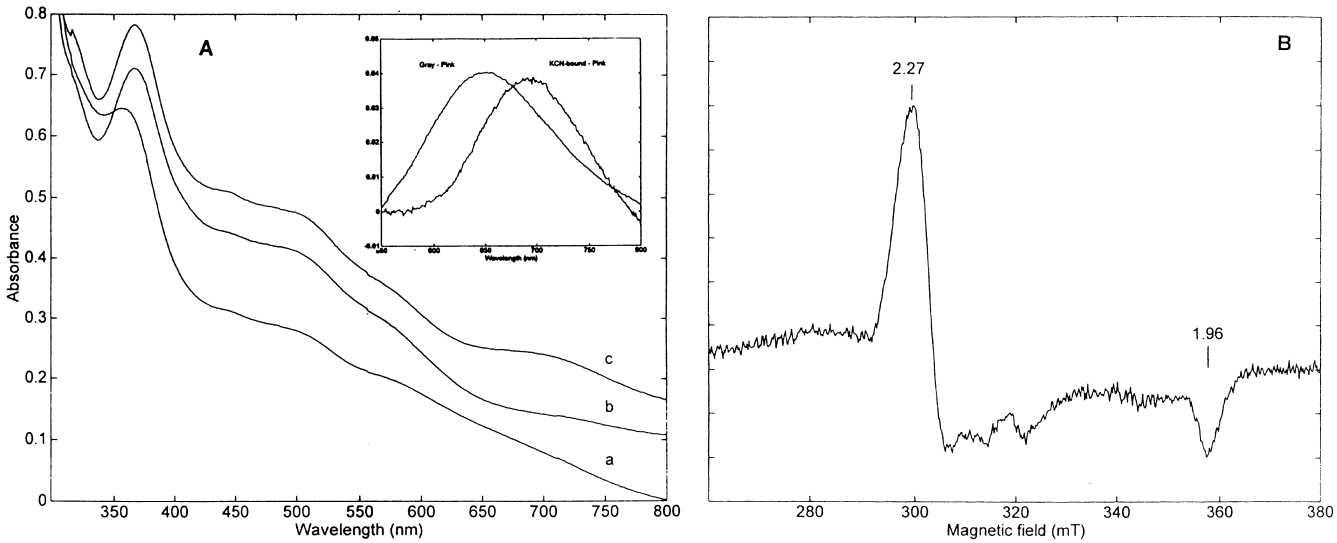

Fig. 3. Effect of cyanide on the visible and EPR spectrum of Dfx. Panel A, Visible spectra of Dfx in the presence of cyanide. Trace (a), grey Dfx; Trace (b), pink Dfx; Trace (c), Dfx (either pink or grey forms) after incubation with 10 mM KCN. Inset: Difference spectra in the 550–800 nm region, between grey and cyanide bound Dfx and the pink form. Panel B, EPR spectrum of cyanide bound Dfx. EPR conditions: Temperature, 11 K; microwave power, 2.4 mW; microwave frequency 9.63 GHz.

followed by both EPR and visible spectroscopies, both Dfx centres become partially reduced indicating that under turnover conditions they are both reduced by the superoxide ion (data not shown).

The effect of cyanide was also probed spectroscopically by studying the binding of cyanide to Dfx, both in the pink and grey forms. The binding of cyanide leads to clear spectroscopic changes (Fig. 3). While the visible and EPR signatures for centre I remain unaltered, centre II acquires a quite distinct electronic structure. The same final state is obtained irrespective of whether the pink or grey Dfx form is used at the start, indicating that upon reaction with cyanide centre II is oxidized by oxygen, in contrast to the native form. In the visible spectrum (Fig. 3, panel A), the 650 nm band from oxidized centre II, readily observed in the difference spectrum between grey and pink forms, shifts ≈40 nm, to 690 nm (Fig. 3, panel A inset). The EPR spectrum of the Dfx reacted with cyanide shows a drastic change; a low-spin species, with a ground state of S = 1/2, is formed, with g-values at $g_{\perp} = 2.27$ and $g_{\parallel} = 1.96$, which is still detectable at 40 K (Fig. 3, panel B). Simultaneously, the EPR signature of native centre II (g = 4.3 resonance) fully bleaches, indicating its full conversion to the low-spin form, which was confirmed by spin quantitation. A value of 1.2 spin·M^{-1} of Dfx was obtained. This value, well within experimental error, together with the fact that the intensity of the EPR and Visible signatures of centre I did not change, indicate that centre II was fully converted to a low-spin cyanide-bound form.

To check whether Dfx centre I could be responsible for the SOD activity, pure *D. gigas* desulforedoxin, which contains a centre analogous to Dfx centre I, was assayed both by the spectrophotometric and the gel assays. SOD activity could not be detected in either case. Also, because Dfx is present in the same operon as rubredoxin-1, the SOD activity of a 1 : 1 mixture of Dfx and Rd-1 was measured – the activity was identical to that of pure Dfx.

Putative Cu,Zn-SOD

The soluble extract gels stained for SOD activity showed the presence of a band additional to the one assigned to Dfx. The protein responsible for this activity was purified and found to be present in small amounts. As determined from activity gels, it is cyanide inhibited (Fig. 1, panel B), a feature typical of Cu,Zn-SODs. It has a molecular mass of ≈23 kDa on SDS/PAGE gels and its N-terminal amino-acid sequence (DSVKVPVNKITDEGVGEAIGFI) has 75% similarity to the Cu,Zn-SOD from *E. coli* [37]. This putative Cu,Zn-SOD copurified with another protein whose N-terminal sequence (AEQVTDATFESVVLKSDLPVLLDF) showed high similarities (79–90%) towards thioredoxins from several organisms (e.g. *Rhodobacter sphaeroides*, *Anabaena* sp., *Haemophilus influenzae*). To our knowledge this is the first report on the presence of such an enzyme in sulphate-reducing bacteria; its physiological role remains to be investigated.

DISCUSSION

Desulfoferrodoxin, an abundant protein in *D. desulfuricans* ATCC 27774, has superoxide dismutase activity, which is detectable both by gel and spectrophotometric assays. The data obtained indicate that the capability of destroying the superoxide ion is mainly due to its unique centre II. This centre has some properties reminiscent of those of canonical Fe-SOD. In fact it has a high reduction potential of about +200 mV [12] and exhibits pentacoordination, dominated by histidinyl ligands [14], leaving an empty coordination site for additional ligand binding. However, in contrast to Fe-SODs, cyanide binds to centre II and, surprisingly, it induces an increase of its SOD

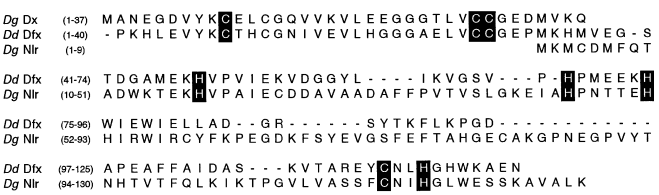

Fig. 4. Sequence alignment of Dfx with neelaredoxin and desulforedoxin. *D. desulfuricans* desulfoferrodoxin (*Dd* Dfx), *D. gigas* neelaredoxin (*Dg* Nlr) and *D. gigas* desulforedoxin (*Dg* Dx). Black boxes indicate the residues involved in the binding of the redox centres.

activity. The binding of cyanide yields a low-spin iron site, whose structure is presently unknown. The observation of the band at 690 nm suggests that the cysteine ligand remains bound. However, it is not possible to judge whether one of the histidine ligands was replaced by cyanide. The same effect was observed for neelaredoxin, although this protein shows a much higher SOD activity [16]. The cyanide effect may be physiologically irrelevant, however it should be pointed out that at least one of the major enzyme families present in the *Desulfovibrio* species, the [NiFe]-hydrogenases, contain cyanide bound at the active site [38]. Neelaredoxin has two mononuclear iron sites, with similar but not identical EPR spectra. Comparison of the amino-acid sequences of Dfx and neelaredoxin (Fig. 4) show that all the ligands of Dfx centre II are conserved in neelaredoxin (residues 48, 68, 74 and 115 by Dfx numbering). However, there is no second set of identical residues in neelaredoxin, indicating that the second centre of neelaredoxin must have a distinct coordination, in agreement with the different EPR signatures observed for its two iron sites [8]. Nevertheless, there are enough extra cysteines and histidines in neelaredoxin to provide a similar coordination environment to the other iron site, again in agreement with the previous observation that the two iron sites have identical electronic spectra, dominated by the cysteine-sulfur-to-iron charge-transfer band at 660 nm [8,12]. It may be speculated that the second centre of neelaredoxin has a higher SOD activity or that it plays an important role in the catalytic cycle of superoxide dismutation. This mechanism remains to be clarified for both neelaredoxin and Dfx. The activity measured for Dfx is lower than that usually found for most SODs, including neelaredoxin, but enzymes with activities similar to that of Dfx have been reported (e.g. *Tetrahymena pyriformis* Fe-SOD [39]). Our data does not fully support the hypothesis recently raised that Dfx functions by reducing superoxide [26]. In fact, in the presence of xanthine/xanthine oxidase, Dfx is initially oxidized, but this oxidation may be prevented by adding catalase, strongly suggesting that the oxidation be mainly due to the reaction product, hydrogen peroxide. Moreover, even in the absence of catalase, after the initial fast oxidation Dfx becomes again partially reduced using xanthine/xanthine oxidase, which, as observed for the canonical Fe-SODs, suggests that under turnover conditions the active centre cycles between the oxidized and reduced forms. One observation remains to be explained; in contrast to neelaredoxin, the fully oxidized form of Dfx has an activity lower than that of the partially-reduced pink form, which may indicate a possible interference of Dfx centre I.

Whatever the actual operating catalytic mechanism is, our data explain the results obtained in [25], that overexpression of Dfx in *E. coli* mutants lacking SOD compensates for the deleterious effect of these mutations, and that overexpression of desulforedoxin had no complementation effect. A rationale for the presence of the Rd gene in the same operon coding for Dfx, in *Desulfovibrio*, is not yet clarified – direct electron transfer between Rd and Dfx has not been demonstrated, and the presence of Rd in the SOD assay had no effect. However, under physiological conditions, Rd may be important to maintain Dfx in its reduced form, thus increasing its activity.

Whether the actual physiological function of Dfx is to act as a vestigial superoxide dismutase remains to be determined. Dfx and Dfx-like proteins appear to be widely spread in anaerobic bacteria and archaea, such as *Methanobacterium thermoautotrophicum* [22], *Archaeoglobus fulgidus* [24] and *Methanococcus jannaschii* [23]. None of these archaea genomes have genes coding for classical superoxide dismutases, although SOD activity was detected in *A. fulgidus* and *M. jannaschii* (unpublished data). Our results suggest that these activities were due to the presence of desulfoferrodoxin-like proteins. The recently published genome of *Treponema pallidum* contains a gene coding for a Dfx-like protein (ORF tp0823) [40] with only the ligands to centre II, but otherwise lacks any gene coding for the classical SODs.

Another superoxide dismutase is also expressed in *D. desulfuricans,* which is inhibited by cyanide, a characteristic of Cu,Zn-SODs. Its N-terminal sequence is homologous to that of *E. coli* Cu,Zn-SOD. This result constitutes the first indication that a copper protein may be present in a sulphate-reducing bacterium. However, the level of expression of this enzyme seems to be very low, under the growth conditions used, most probably due to the very low solubility of reduced copper ions. The increase in activity of the soluble extract in the presence of cyanide suggests that most of the SOD activity present in the extract is due to Dfx, as the putative Cu,Zn-SOD is fully inhibited by cyanide.

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REFERENCES

1. Le Gall, J. & Dragoni, N. (1966) Dependence of sulphate-reduction on a crystallized ferredoxin from *Desulfovibrio gigas*. *Biochem. Biophys. Res. Commum.* **23**, 145–149.
2. Bruschi, M. & Le Gall, J. (1972) Purification et propriétés d'une rubrédoxine isolée à partir de *Desulfovibrio vulgaris* (Souche NCIB 8303). *Biochem. Biophys. Acta* **263**, 279–282.
3. Bruschi, M., Hatchikian, E.C., Golovleva, L. & Le Gall, J. (1977) Purification and characterization of cytochrome c_3, ferredoxin and rubredoxin isolated from *Desulfovibrio desulfuricans* Norway. *J. Bacteriol.* **129**, 30–38.
4. Le Gall, J., Liu, M.Y., Gomes, C.M., Braga, V., Pacheco, I., Regalla, M., Xavier, A.V. & Teixeira, M. (1998) Characterisation of a new rubredoxin isolated from *Desulfovibrio desulfuricans* 27774: definition of a new family of rubredoxins. *FEBS Lett.* **429**, 295–298.
5. Moura, I., Bruschi, M., Le Gall, J., Moura, J.J.G. & Xavier, A.V. (1977) Isolation and characterization of desulforedoxin a new type of non-heme iron protein from *Desulfovibrio gigas*. *Biochem. Biophys. Res. Comm.* **75**, 1037–1044.
6. Le Gall, J., Prickril, B.C., Moura, I., Xavier, A.V., Moura, J.J.G. & Huynh, B.H. (1988) Isolation and characterization of rubrerythrin, a non-heme iron protein from *Desulfovibrio vulgaris* that contains rubredoxin centers and a hemerythrin-like binuclear center. *Biochemistry* **27**, 1636–1642.
7. Pierik, A.J., Wolbert, R.B., Portier, G.L., Verhagen, M.F. & Hagen, W.R. (1993) Nigerythrin and rubrerythrin from *Desulfovibrio vulgaris* each contain two mononuclear iron centers and two dinuclear iron clusters. *Eur. J. Biochem.* **212**, 237–245.
8. Chen, L., Sharma, P., Le Gall, J., Mariano, A.M., Teixeira, M. & Xavier, A.V. (1994) A blue non-heme iron protein from *Desulfovibrio gigas*. *Eur. J. Biochem.* **226**, 613–618.
9. Moura, I., Tavares, P., Moura, J.J.G., Ravi, N., Huynh, B.H., Liu, M.Y. & Le Gall, J. (1990) Purification and characterization of desulfoferrodoxin. A novel protein from *Desulfovibrio desulfuricans* ATCC 27774 and from *Desulfovibrio vulgaris* (strain Hildenborough) that contains a distorted rubredoxin center and a mononuclear ferrous center. *J. Biol. Chem.* **265**, 21596–21602.

10. Verhagen, M.F.J.M., Voohorst, W.G.B., Kolkman, J.A., Wolbert, R.B.G. & Hagen, W.R. (1993) On the two iron centers of desulfoferrodoxin. *FEBS Lett.* **336**, 13–18.
11. Devreese, B., Tavares, P., Lampreia, J., Van Damme, N., Le Gall, J., Moura, J.J.G., Van Beeumen, J. & Moura, I. (1996) Primary structure of desulfoferrodoxin from *Desulfovibrio desulfuricans* ATCC 27774, a new class of non-heme iron proteins. *FEBS Lett.* **385**, 138–149.
12. Tavares, P., Ravi, N., Moura, J.J.G., Le Gall, J., Huang, Y.H., Crouse, B.R., Jonhson, M.K., Huynh, B.H., Liu, M.Y. & Moura, I. (1994) Spectroscopic properties of desulfoferrodoxin from *Desulfovibrio desulfuricans* ATCC 27774. *J. Biol. Chem.* **269**, 10504–10510.
13. Coelho, A.V., Matias, P.M., Carrondo, M.A., Tavares, P., Moura, J.J.G., Moura, I., Fülöp, V., Hajdu, J. & Le Gall, J. (1996) Preliminary crystallographic analysis of the oxidized form of a two mono-nuclear iron centers protein from *Desulfovibrio desulfuricans* ATCC 27774. *Protein Sci.* **5**, 1189–1191.
14. Coelho, A.V., Matias, P., Fülöp, V., Thompson, A., Gonzalez, A. & Carrondo & M.A. (1997) Desulfoferrodoxin structure determined by MAD phasing and refinement to 1.9-Å resolution reveals a unique combination of a tetrahedral FeS_4 centre with a square pyramidal $FeSN_4$ centre. *J. Biol. Inorg. Chem.* **2**, 680–689.
15. Archer, M., Huber, R., Tavares, P., Moura, I., Moura, J.J.G., Carrondo, M.A., Sieker, L.C., Le Gall, J. & Romão, M.J. (1995) Crystal structure of Desulforedoxin from *Desulfovibrio gigas* determined at 1.8 Å resolution: a novel non-heme iron protein structure. *J. Mol. Biol.* **251**, 690–702.
16. Silva, G., Oliveira, S., Gomes, C.M., Pacheco, I., Liu, M.Y., Xavier, A.V., Teixeira, M., LeGall, J. & Rodrigues-Pousada, C. (1999) *Desulfovibrio gigas* neelaredoxin: a novel superoxide dismutase integrated in a putative oxygen sensory operon of an anaerobe. *Eur. J. Biochem.* **259**, 235–243.
17. Brumlik, M.J. & Voordouw, G. (1989) Analysis of the transcriptional unit encoding the genes for rubredoxin (*rub*) and a putative rubredoxin oxidoreductase (*rbo*) in *Desulfovibrio vulgaris* Hildenborough. *J. Bacteriol.* **171**, 4996–5004.
18. Brumlik, M.J., Leroy, G., Bruschi, M. & Voordouw, G. (1990) The nucleotide sequence of the *Desulfovibrio gigas* desulforedoxin gene indicates that the *Desulfovibrio vulgaris rbo* gene originated from a gene fusion event. *J. Bacteriol.* **172**, 7289–7292.
19. Chen, L., Liu, M.Y., Le Gall, J., Faraleira, P., Santos, H. & Xavier, A.V. (1993) Rubredoxin oxidase, a new flavo-heme-protein, is the site of oxygen reduction to water by the 'strict anaerobe' *Desulfovibrio gigas*. *Biochem. Biophys. Res. Comm.* **193**, 100–105.
20. Gomes, C.M., Silva, G., Oliveira, S., LeGall, J., Liu, M.Y., Xavier, A.V., Rodrigues-Pousada, C. & Teixeira, M. (1997) Studies on the redox centers of the terminal oxidase from *Desulfovibrio gigas* and evidence for its interaction with rubredoxin. *J. Biol. Chem.* **272**, 22502–22508.
21. Chen, L., Le Gall, J. & Xavier, A.V. (1994) Purification, characterization and properties of an NADH oxidase from *Desulfovibrio vulgaris* and its coupling to adenylyl phosphosulfate reductase. *Biochem. Biophys. Res. Comm.* **203**, 839–844.
22. Smith, D.R., Doucette-Stamm, L.A., Deloughery, C., Lee, H., Dubois, J., Aldredge, T., Bashirzadeh, R., Blakely, D., Cook, R., Gilbert, K., Harrison, D., Hoang, L., Keagle, P., Lumm, W., Pothier, B., Qiu, D., Spadafora, R., Vicaire, R., Wang, Y., Wierzbowski, J., Gibson, R., Jiwani, N., Caruso, A., Bush, D., Safer, H., Patwell, D., Prabhakar, S., McDouglas, S., Shimer, G., Goyal, A., Pietrokovski, S., Church, G.M., Daniels, C.J., Mao, J.I., Rice, P., Nölling, J. & Reeve, J.N. (1997) Complete genome sequence of *Methanobacterium thermoautotrophicum* δH: functional analysis and comparative genomics. *J. Bacteriol.* **179**, 7135–7155.
23. Bult, C.J., White, O., Olsen, G.J., Zhou, L., Fleischmann, R.D., Sutton, G.G., Blake, J.A., FitzGerald, L.M., Clayton, R.A., Gocayne, J.D., Kerlavage, A.R., Dougherty, B.A., Tomb, J.F., Adams, M.D., Reich, C.I., Overbeek, R., Kirkness, E.F., Weinstock, K.G., Merrick, J.M., Glodek, A., Scott, J.L., Geoghagen, N.S.M. & Venter, J.C. (1996) Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. *Science* **273**, 1058–1073.
24. Klenk, H.P., Clayton, R.A., Tomb, J.F., White, O., Nelson, K.E., Ketchum, K.A., Dodson, R.J., Gwinn, M., Hickey, E.K., Peterson, J.D., Richardson, D.L., Kerlavage, A.R., Graham, D.E., Kyrpides, N.C., Fleischmann, R.D., Quackenbush, J., Lee, N.H., Sutton, G.G., Gill, S., Kirkness, E.F., Dougherty, B.A., McKenney, K., Adams, M.D., Loftus, B., Peterson, S., Reich, C.I., McNeil, L.K., Badger, J.H., Goldek, A., Zhou, L., Overbeek, R., Gocayne, J.D., Weidman, J.F., McDonald, L., Utterback, T., Cotton, M.D., Spriggs, T., Artiach, P., Kaine, B.P., Sykes, S.M., Sadow, P.W., D'Andrea, K.P., Bowman, C., Fujii, C., Garland, S.A., Mason, T.M., Olsen, G.J., Fraser, C.M., Smith, H.O., Woese, C.R. & Venter, J.C. (1997) The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon *Archaeoglobus fulgidus*. *Nature* **390**, 364–370.
25. Pianzzola, M.J., Soubes, M. & Touati, D. (1996) Overproduction of the rbo gene product from *Desulfovibrio* species suppresses all deleterious effects of lack of superoxide dismutase in *Escherichia coli*. *J. Bacteriol.* **178**, 6736–6742.
26. Liochev, S.I. & Fridovich, I. (1997) A mechanism for complementation of the *sodA sodB* defect in *Escherichia coli* by overproduction of the *rbo* gene product (Desulfoferrodoxin) from *Desulfoarculus baarsii*. *J. Biol. Chem.* **272**, 25573–25575.
27. Hatchikian, C.E., Le Gall & J. & Bell, G.R. (1977) Significance of superoxide dismutase and catalase activities in the strict anaerobes, sulfate reducing bacteria. In *Superoxide and Superoxide Dismutases* (Michelson, A.M., McCord, J.M. & Fridovich, I., eds.), pp. 159–172. Academic Press, London, UK.
28. Liu, M.C. & Peck, H.D. Jr (1981) The isolation of a hexaheme cytochrome from *Desulfovibrio desulfuricans* and its identification as a new type of nitrite reductase. *J. Biol. Chem.* **256**, 13159–13164.
29. LeGall, J. & Forget, N. (1978) Purification of electron transfer components from sulphate-reducing bacteria. In *Methods in Enzymology*, **Vol. LIII**, Biomembranes (Fleischer, S. & Packer, L., eds.), pp. 613–634. Academic Press, NY, USA.
30. McCord, J.M. & Fridovich, I. (1969) Superoxide Dismutase, an enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* **244**, 6049–6055.
31. Hames, B.D. (1990) One-dimensional polyacrylamide gel electrophoresis. In *Gel Electrophoresis of Proteins, a Practical Approach* (Hames, B.D. & Rickwood, D., eds.), pp. 30–50. IRL Press, Oxford, UK.
32. Beauchamp, C. & Fridovich, I. (1971) Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* **44**, 276–287.
33. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
34. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.
35. Choli, T. & Withmann-Liebold (1990) Protein blotting followed by microsequencing. *Electrophoresis* **11**, 562–568.
36. Bolard, J. & Garnier, A. (1972) Circular dichroism studies of myoglobin and cytochrome *c* derivatives. *Biochim. Biophys. Acta* **263**, 535–540.
37. Blattner, F.R., Plunkett,G., IIIBloch, C.A., Perna, N.T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J.D., Rode, C.K., Mayhew, G.F., Gregor, J., Davis, N.W., Kirkpatrick, H.A., Goeden, M.A., Rose, D.J., Mau, B. & Shao, Y. (1997) The complete genome sequence of *Escherichia coli* K-12. *Science* **277**, 1453–1474.
38. Happe, R.P., Roseboom, W., Pierik, A.J., Albracht, S.P. & Bagley, K.A. (1997) Biological activation of hydrogen. *Nature* **385**, 126.
39. Barra, D., Schininà, M.E., Bossa, F., Puget, K., Durosay, P., Guissani, A. & Michelson, A.M. (1990) A tetrameric iron superoxide dismutase from the eucaryote *Tetrahymena pyriformis*. *J. Biol. Chem.* **265**, 17680–17687.
40. Fraser, C.M., Norris, S.J., Weinstock, G.M., White, O., Sutton, G.G., Dodson, R., Gwinn, M., Hickey, E.K., Clayton, R., Ketchum, K.A., Sodergren, E., Hardham, J.M., McLeod, M.P., Salzberg, S., Peterson, J., Khalak, H., Richardson, D., Howell, J.K., Chidambaram, M., Utterback, T., McDonald, L., Artiach, P., Bowman, C., Cotton, M.D., Fujii, C., Garland, S., Hatch, B., Horst, K., Roberts, K., Sandusky, M., Weidman, J., Smith, H.O. & Venter, J.C. (1998) Complete genome sequence of *Treponema pallidum*, the syphilis spirochete. *Science* **281**, 375–388.