An investigation of the ligand-binding properties of *Pseudomonas aeruginosa* nitrite reductase

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The low-temperature e.p.r. and m.c.d. (magnetic-circular-dichroism) spectra of *Pseudomonas aeruginosa* nitrite reductase, together with those of its partially and fully cyanide-bound derivatives, were investigated. The m.c.d. spectra in the range 600–2000 nm indicate that the native axial ligands to haem *c* are histidine and methionine, and furthermore that it is the methionine ligand that must be displaced before cyanide binding at this haem. The m.c.d. spectra in the range 1000–2000 nm contain no charge-transfer bands arising from low-spin ferric haem d_1 , a chlorin. New optical transitions in the region 700–850 nm were found for the cyanide adduct of haem d_1 . The *g*-values of haem d_1 in the native enzyme are 2.51, 2.43 and 1.71, suggesting co-ordination by two histidine ligands in the oxidized state. There is clear evidence in the e.p.r. data of an interaction between the *c* and d_1 haem groups. This is not apparent in the optical spectra. The results are interpreted in terms of haem groups that are remote from each other, their interaction being mediated through protein conformational changes. The possible implications of this in relation to reduction processes catalysed by the enzyme are considered.

INTRODUCTION

Nitrite reductase (ferrocytochrome *c*-551:O₂ oxidoreductase, EC 1.9.3.2) is a terminal electron carrier in the bacterium *Pseudomonas aeruginosa*. Accepting electrons from azurin and/or cytochrome *c*-551, it catalyses the four-electron reduction of dioxygen to water (Horio *et al.*, 1958), the two-electron reduction of hydroxylamine to ammonia (Singh, 1973) and the physiologically important single-electron reduction of nitrite to nitric oxide (Yamanaka *et al.*, 1963). The enzyme is purified as a dimer of M_r 120 000. Each monomeric unit contains one *c*-type haem and one haem designated d_1 (Kuronen *et al.*, 1975; Silvestrini *et al.*, 1979). As isolated, both types of haem have been shown to be low-spin ferric species (Oriei *et al.*, 1977; Vickery *et al.*, 1978; Walsh *et al.*, 1979, 1981a,b; Johnson *et al.*, 1980; Muhoberac & Wharton, 1983). Previous binding studies have suggested that in this oxidized form both the *c* and the d_1 haem groups will undergo substitution with a variety of ligands, such as cyanide, imidazole and nitric oxide, but that conversely only haem d_1 shows any reactivity with azide and fluoride (Barber *et al.*, 1978a,b; Silvestrini *et al.*, 1979; Walsh *et al.*, 1981a; Muhoberac & Wharton, 1983).

The near-i.r. m.c.d. spectra of low-spin ferric systems have been shown to be diagnostic of the state of axial ligation (Eglinton *et al.*, 1983; Sievers *et al.*, 1983). Consequently we thought it important to obtain such spectra for nitrite reductase and its cyanide adducts in an attempt to identify the native axial ligands and to check the conclusions of other workers concerning the sites of substitution. After preliminary work, mainly at room temperature, which failed to identify near-i.r. m.c.d. bands attributable to haem d_1 , subsequent experiments were performed exclusively in the near-red region of the spectrum at 4.2 K and below. Also, in order that the results of these experiments could be evaluated in relation

to previous studies, absorption, e.p.r. and near-u.v.-visible-region m.c.d. spectra of samples were recorded.

EXPERIMENTAL

Nitrite reductase was purified from cells of *Pseudomonas aeruginosa* (N.C.T.C. 6750) by the method of Parr *et al.* (1976). The ratios A_{410}/A_{280} and A_{640}/A_{520} for the fully oxidized (resting) enzyme were 1.18–1.20 and 1.16–1.20 respectively. Concentration of samples and, for near-i.r. work, exchange into deuterated buffer, was achieved with the aid of a Minicon-B15 Macrosolute Concentrator (Amicon Corp.). ²H₂O (99.8%) was purchased from Aldrich Chemical Co. All other reagents were of analytical grade and were obtained through either Sigma Chemical Co. or BDH Chemicals. All solutions were buffered at pH 8.0 (or p²H 8.4) in 0.1 M-Hepes (before addition of glassing agent).

Sample concentrations were determined spectrophotometrically by using $\epsilon_{410} = 282 \pm 15 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for the dimeric resting protein containing two haem *c* and two haem d_1 moieties (Silvestrini *et al.*, 1979). In keeping with previous communications, absorption coefficients are given on a per-dimer basis. Absorption spectra were recorded with a Cary 118C spectrophotometer.

E.p.r. spectra were obtained with a Bruker ER-200D spectrometer, fitted with an Oxford Instruments ESR-900 flow cryostat and DTC-2 temperature controller. The instrument was calibrated with reference to 1,1-diphenyl-2-picrylhydrazyl.

M.c.d. measurements were made with a Jasco J-500D spectropolarimeter in the range 250–1050 nm and with a dichrograph constructed in our laboratory (Eglinton *et al.*, 1980) in the range 900–2000 nm. Experiments at around room temperature were performed with the use of a superconducting solenoid (with a room-temperature

Fig. 1. X-band e.p.r. spectra of *Pseudomonas aeruginosa* nitrite reductase derivatives at 12 K and 2 mW microwave power

(a) Resting enzyme (180 μM dimer concentration) in 0.1 M-Hepes buffer, pH 8.0, 0.63 mT modulation amplitude, 6.3×10^4 receiver gain. (b) Resting enzyme (90 μM dimer concentration) in 0.05 M-Hepes buffer, pH 8.0, containing 50% (v/v) glycerol, 0.63 mT modulation amplitude, 10×10^4 receiver gain. (c) Fully cyanide-bound derivative (90 μM dimer concentration), solid KCN added to excess, 0.63 mT modulation amplitude, 2×10^5 receiver gain. (d) Partially cyanide-bound derivative (215 μM dimer concentration), 0.63 mT modulation amplitude, 2×10^5 receiver gain.

bore) operating at 4.7 T. At low temperature an Oxford Instruments SM4 split-pair cryomagnet, capable of generating magnetic fields of up to 5.0 T, was employed. In order that good-quality glasses be obtained upon freezing, samples intended for low-temperature optical work were diluted to 50% (v/v) with glycerol before immersion in liquid He. Millimolar $\Delta\epsilon$ values are quoted on a per-monomer basis throughout, so that comparisons with intensities of signals reported for other haemoproteins may readily be made.

RESULTS

Fig. 1(a) shows the e.p.r. spectrum of the enzyme as isolated. This is very similar to the spectra previously published by others (Walsh *et al.*, 1979; Muhoberac & Wharton, 1983). In keeping with these previous studies, we attribute the resonances at $g = 2.51$, 2.43 and 1.71 to low-spin ferric haem d_1 and those at $g = 3.01$ and ~ 2.29 to low-spin ferric haem c . We did not observe the third

component of the latter species reported to be at $g = 1.40$ (Gudat *et al.*, 1973; Muhoberac & Wharton, 1983). The broad signals around $g = 2.0$ are found to a variable degree in all preparations of *Pseudomonas* proteins and are most probably due to adventitious copper(II). Upon addition of glassing agent, namely glycerol, the spectrum sharpens somewhat (Fig. 1b), leading to better resolution of the features at $g = 2.51$ and $g = 2.43$. (Subsequent e.p.r. spectra were all recorded on samples containing glycerol in order to take full advantage of the 'improved resolution'.) Also, a new set of signals arises at $g = \sim 2.92$, 2.25 and 1.83. This is a minority species that appears to be a derivative of haem c , since haem d_1 derivatives tend to have rather more axial spectra. In addition, there is evidence for a change in the axial-ligand environment of certain c -type haem groups in the presence of glassing agent (J. Peterson, P. M. A. Gadsby, A. J. Thomson, N. Foote & C. Greenwood, unpublished work).

It has been reported (Barber *et al.*, 1978b) that the addition of cyanide to nitrite reductase results in slow binding processes of equivalent velocity at both haem groups, leading to a 'fully' cyanide-bound species. The e.p.r. spectrum of this derivative (Fig. 1c) is again very similar to published spectra (Walsh *et al.*, 1979; Muhoberac & Wharton, 1983), in which the resonances at around $g = 2.47$, 2.34 and 1.67 have been assigned to the cyanide adduct of haem d_1 . The feature at $g = 3.33$ may then be recognized as the g_z component of the spectrum of the haem c -cyanide adduct. Passing the fully cyanide-bound nitrite reductase down a Sephadex G-25 column leads to a new species, which we call the 'partially' cyanide-bound derivative. The e.p.r. spectrum of this system (Fig. 1d) contains signals at $g = 2.46$, 2.34 and 1.68, which are nearly identical with the haem d_1 -cyanide features of the fully cyanide-bound form of the enzyme (Fig. 1c; Walsh *et al.*, 1979; Muhoberac & Wharton, 1983). We conclude that the column treatment results in removal of the bound cyanide from haem c only. However, more interestingly, the spectrum of haem c in this partially cyanide-bound derivative is different to that of the cyanide-free system (Fig. 1b). There is no minority species with g -values at 2.92, 2.25 and 1.83 and the g_z component of the haem c species at $g = 3.01$ has moved to $g = 2.95$. This is good evidence that the two

Fig. 2. Absorption spectra of *Pseudomonas aeruginosa* nitrite reductase derivatives at 20 °C with 10 mm pathlength

—, Resting enzyme (6 μM dimer concentration);
 ----, partially cyanide-bound derivative (4 μM dimer concentration);
 ·····, fully cyanide-bound derivative (6 μM dimer concentration), 4 mM in KCN.

Fig. 3. Near-u.v.-visible-region m.c.d. spectra of *Pseudomonas aeruginosa* nitrite reductase derivatives at 4.2 K at 5.0 T with 1 mm pathlength

(a) Resting enzyme (27 μM monomer concentration). (b) Partially cyanide-bound derivatives (48 μM monomer concentration). (c) Fully cyanide-bound derivatives (27 μM monomer concentration), solid KCN added to excess.

haem species in nitrite reductase are in some form of communication, the binding of cyanide at haem d_1 being detectable by changes in the haem c spectrum. We were unable to find the g_x and g_y components of the haem c signal of Fig. 1(d).

The room-temperature absorption spectra of the

Fig. 4. Near-i.r. m.c.d. spectra of *Pseudomonas aeruginosa* nitrite reductase derivatives

(a) At 20 °C at 4.7 T with 2 mm pathlength: —, resting enzyme (330 μM monomer concentration); ---, partially cyanide-bound derivative (430 μM monomer concentration); ·····, fully cyanide-bound derivative (315 μM monomer concentration); the spectrum of the partially cyanide-bound derivative has been displaced by +0.05 $\Delta\epsilon$ for clarity. (b) At 4.2 K at 5.0 T with 1.0 mm pathlength: resting enzyme (180 μM monomer concentration).

resting enzyme (continuous line) and its partially (broken line) and fully (dotted line) cyanide-bound derivatives are shown in Fig. 2. Binding of cyanide to haem d_1 leads to spectral changes at approx. 480 and 640 nm. The differences between the spectra of the partially and fully cyanide-bound species are slight. This is not unexpected, since a comparison of the absorption spectrum of the resting enzyme and that of extracted ferrihaem d_1 (Walsh *et al.*, 1981b) establishes that haem d_1 in the resting enzyme is low-spin ferric at room temperature. The e.p.r. and low-temperature m.c.d. spectra of the resting state show that haem d_1 remains low-spin at liquid-He temperature (Johnson *et al.*, 1980; Walsh *et al.*, 1979, 1981a,b).

The 4.2 K m.c.d. spectrum of the resting enzyme (Fig. 3a) and the fully cyanide-bound derivative (Fig. 3c) are analogous to those reported previously (Walsh *et al.*, 1979). Surprisingly, the ligation of cyanide at haem d_1 results in minimal change in the m.c.d. spectrum (cf. Figs. 3a and 3b). On the other hand, the binding of cyanide to haem c can be observed by a slight increase in the intensity of the Soret band (cf. Figs. 3b and 3c).

Perhaps more remarkable than any differences between the spectra of the individual species in Figs. 2 and 3 is the overall similarity. The absorption spectrum seems to be more sensitive to ligand substitution at haem d_1 and the m.c.d. spectrum to substitution at haem c . Consequently,

Fig. 5. Near-red m.c.d. spectra of *Pseudomonas aeruginosa* nitrite reductase derivatives at 4.2 K at 5.0 T with 1.0 mm pathlength

—, Resting enzyme (180 μM monomer concentration); ---, partially cyanide-bound derivative (430 μM monomer concentration); ·····, fully cyanide-bound derivative (180 μM monomer concentration), solid KCN added to excess.

in order to distinguish between the resting, partially cyanide-bound and fully cyanide-bound enzyme by their near-u.v.-visible-region optical properties, one needs to consider both types of spectrum.

The near-i.r. m.c.d. spectra of resting nitrite reductase at room temperature and at 4.2 K are shown in Figs. 4(a) and 4(b) respectively (continuous lines). The main feature at approx. 1750 nm is characteristic of a low-spin ferric haem that is axially co-ordinated by histidine and methionine, as in cytochromes *c*-551 from *Pseudomonas*, mammalian cytochrome *c* and tuna cytochrome *c* (J. Peterson, P. M. A. Gadsby, N. Foote, C. Greenwood & A. J. Thomson, unpublished work) and in cytochrome *b*-562 from *Escherichia coli* (Moore *et al.*, 1985). This confirms that the haem *c* in *Pseudomonas* nitrite reductase is liganded by histidine and methionine. Moreover, the intensities of the signals at room temperature and 4.2 K are both consistent with a single low-spin haem. There is no evidence for a temperature-dependent spin equilibrium as observed in the high-potential haem of *Pseudomonas* cytochrome *c*-551 peroxidase (Foote *et al.*, 1984).

The room-temperature near-i.r. m.c.d. spectrum of the fully cyanide-bound nitrite reductase (Fig. 4a, dotted line) gives a peak at 1550 nm that is analogous to the spectrum of the cyanide adduct of horse heart cytochrome *c* (P. M. A. Gadsby, J. Peterson, N. Foote, C. Greenwood & A. J. Thomson, unpublished work). This suggests that in the near-i.r. the m.c.d. spectrum of the enzyme is dominated by features arising from the haem *c*. The observation that the spectrum of the partially cyanide-bound derivative (Fig. 4a, broken line) between 1000 and 2000 nm is nearly identical with that of the resting system (continuous line) further supports this argument.

Fig. 5 shows the near-red m.c.d. spectra at 4.2 K of resting (continuous line), partially cyanide-bound (broken line) and fully cyanide-bound (dotted line) nitrite reductase. The m.c.d. in this region of the spectrum unambiguously distinguishes between the three forms of the enzyme. The negative trough at 695 nm is characteristic

Fig. 6. M.c.d. magnetization characteristics of the 820 nm feature in the spectrum of the partially cyanide-bound derivative of *Pseudomonas aeruginosa* nitrite reductase

The sample was the same as in Fig. 5. Data were collected at 1.6 K (●) and 4.2 K (○); —, theoretical curve simulated for a 98% *x*-*y*-polarized transition from an $S = \frac{1}{2}$ ground state with $g_x = 1.68$, $g_y = 2.34$ and $g_z = 2.46$.

of methionine co-ordination (Foote *et al.*, 1984; Moore *et al.*, 1985). Only in the case of the fully cyanide-bound derivative is this feature absent from the spectrum, indicating displacement of methionine by cyanide at haem *c*. The trough at 820 nm in the spectra of the partially and fully cyanide-bound systems may be attributed to a haem d_1 -cyanide species, as may a less well-resolved negative band at approx. 735 nm that is superimposed on a stronger positive feature due to haem *c*.

It is unclear whether the bands at approx. 735 and 820 nm in the spectra of the partially and fully cyanide-bound systems (Fig. 5) represent the iron(III)-chlorin counterparts of the near-i.r. charge-transfer transitions of low-spin iron(III)-protoporphyrin spectra (Cheng *et al.*, 1973; Rawlings *et al.*, 1977). Since high-spin ferric haemoproteins are known to have bands in this region (Eglinton *et al.*, 1983; Foote *et al.*, 1984), it is important to establish that the observed features are due to a low-spin species. This can be conveniently achieved by construction of the m.c.d. magnetization characteristics of the partially cyanide-bound derivative at 820 nm (Fig. 6). The general behaviour of the signal intensity as a function of magnetic field and absolute temperature for an $S = \frac{1}{2}$ system has been reported by Thomson & Johnson (1980). An m.c.d. magnetization curve can be simulated by using a computer program to average over all space for a randomly oriented paramagnet with anisotropic ground-state *g*-values (A. J. Thomson, T. A. Kent, M. R. Cheesman & R. Grinter, unpublished work). The resulting fit is shown in Fig. 6 for a 98% *x*-*y*-polarized transition from an $S = \frac{1}{2}$ ground state with $g_x = 1.68$, $g_y = 2.34$ and $g_z = 2.46$. The fit to the data is good, and therefore we may with confidence assign the 820 nm feature to a low-spin ferric haem d_1 -cyanide complex. Note that the magnetization curves for a high-

spin ferric haem are expected to be nested because the zero-field components of the $S = \frac{5}{2}$ ground state are separated in energy by several wavenumbers.

DISCUSSION

This work has been concerned with the identification of the axial ligands of the *c* and d_1 haem groups in the nitrate reductase from *Pseudomonas aeruginosa* both in the native state and in various states of binding with cyanide ion. The near-i.r. m.c.d. spectrum establishes unambiguously that the *c* haem is ligated by histidine and methionine, giving rise both at room temperature and at 4.2 K, in the presence of 50% glycerol, to a band peaking at 1750 nm with $\Delta\epsilon$ values corresponding to one haem. The conclusion is supported by the detection of a negative m.c.d. band at 695 nm, which is the m.c.d. counterpart of the well-known '695' band taken to be diagnostic of methionine co-ordination to a low-spin ferric haem of the protoporphyrin IX class. The *g*-values of 3.01 and 2.29 are also in the range typical for haem co-ordinated by methionine and histidine. Cyanide binds to the *c* haem to displace the methionine ligand and form a cyano histidine-co-ordinated haem. The near-i.r. m.c.d. peak at 1550 nm is assigned to a haem of this ligation state by comparison with data on the cyanide adducts of horse heart metmyoglobin and cytochrome *c*. The *g*-value at 3.33 is also consistent with this view.

No optical bands attributable to haem d_1 have been discovered in the wavelength region 1000–2000 nm in spite of the fact that the e.p.r. spectra show the haem to be low-spin ferric. This spectral region contains porphyrin-to-ferric charge-transfer bands in the case of the protoporphyrin IX class of haems. Haem d_1 is now established to contain a reduced porphyrin ring of the chlorin type with exocyclic ring substituents of a strongly electron-withdrawing character, probably a dioxo-isobacteriochlorin (Timkovich *et al.*, 1984; Chang, 1985). It therefore seems likely that the wavelengths of the charge-transfer bands have been shifted because the energies of the highest-filled donor π orbitals of the haem have been changed. We have been able to assign the features in the m.c.d. spectrum in the region 700–850 nm to the oxidized d_1 haem when liganded by cyanide. It may be that these are porphyrin-to-ferric-ion charge-transfer bands that have been shifted from approx. 1550 nm (6450 cm^{-1}) to approx. 800 nm ($12\,500\text{ cm}^{-1}$) by changes in the π -orbital energies. This finding is one of general interest in connection with the use of near-i.r. spectra of haem groups for axial-ligand assignment. However, on the basis of this one example, we clearly cannot assert that the charge-transfer bands of all chlorins lie in the region 700–850 nm.

In order to reach a conclusion about the nature of the axial ligands of haem d_1 in the native enzyme we consider the *g*-values of 2.51, 2.43 and 1.71. A study of the optical and e.p.r. properties of model iron(III)-chlorin and iron(III)-isobacteriochlorin bis-ligated complexes (Stolzenberg *et al.*, 1981) shows that the bis-histidine models of octa-ethylisobacteriochlorin (OEiBC) and octaethylchlorin (OEC), $[\text{Fe}(\text{OEiBC})(N\text{-MeIm})_2]\text{Cl}$ and $[\text{Fe}(\text{OEC})(N\text{-MeIm})_2]\text{Cl}$, where *N*-MeIm represents *N*-methylimidazole, have *g*-values of 2.49, 2.37 and 1.71 and of 2.51, 2.37 and 1.73 respectively. These values are remarkably similar to those of native haem d_1 and quite different from that of chlorin models ligated by phenolates and

thiolates (Stolzenberg *et al.*, 1981). These studies therefore strongly suggest that the d_1 haem in the oxidized state of nitrite reductase of *Pseudomonas aeruginosa* is ligated by two histidine residues. A similar conclusion (Stolzenberg *et al.*, 1981) has been drawn for the ligation state of the haem d_1 in the nitrate reductase from *Thiobacillus denitrificans* (LeGall *et al.*, 1979).

There is good evidence in the data of Fig. 1 that an interaction exists between the haem groups of nitrite reductase; the substitution of a native endogenous ligand at haem d_1 by cyanide results in a small but measurable change in those features of the e.p.r. spectrum attributable to haem *c*. Analogous effects have been observed after the addition to haem d_1 of other ligands such as fluoride (Muhoberac & Wharton, 1983) and nitric oxide (Walsh *et al.*, 1981a). This is unlikely to be a salt effect, since it is observed for both added anionic ligands and added neutral ligands. Significantly, the lack of any measurable difference in the near-i.r. m.c.d. spectra of resting nitrite reductase (Fig. 4a, continuous line) and the species with cyanide bound to haem d_1 only (Fig. 4a, broken line) is strong evidence that haem *c* retains the same set of axial ligands in these two derivatives.

The insensitivity of the haem *c* optical spectrum to cyanide substitution at haem d_1 is also apparent in the near-red region (Fig. 5). The band at 695 nm in the resting enzyme (continuous line) is almost identical with that of the partially cyanide-bound form (broken line). In addition, the 820 nm band, due to cyanide-bound haem d_1 , is not markedly perturbed by the substitution of cyanide at haem *c*. The slight difference between this band in the partially (broken line) and fully (dotted line) cyanide-bound derivatives is mainly attributable to overlap with the haem *c* band to lower energy, which undergoes a blue-shift on ligation with cyanide. This is in accord with the conclusions of Vickery *et al.* (1978), who studied the m.c.d. and absorption difference spectra of the resting, reduced and reduced carbon monoxide-bound forms of the enzyme. These authors state that in the range 250–700 nm the optical properties of nitrite reductase can be interpreted simply as the sum of the individual haem *c* and haem d_1 components, without invoking haem–haem interactions.

It is now quite clear, at least in the case of the cyanide adduct to haem d_1 , that there is no optical counterpart to the changes detected in the e.p.r. spectrum of haem *c*. This can be most easily explained if the displacement of the endogenous ligand by cyanide leads to a conformational change in the protein that may effect a minor reorientation of the axial ligands at haem *c*. It is therefore possible that in the monomeric unit the haem *c* and haem d_1 moieties are remote from each other. This is in agreement with, for example, an estimate of the haem *c*–haem d_1 distance based on a calculation of the inter-haem electron-transfer rate that yielded a separation of 1.3–1.5 nm (13–15 Å) (Schichmann & Gray, 1981). Interestingly, the e.p.r. signals due to cyanide-bound haem d_1 appear to be unaffected by substitution of the methionine by cyanide at haem *c* (cf. Figs. 1c and 1d). Presumably this indicates that the haem–haem interaction is mediated through the protein chain containing the endogenous ligand to haem d_1 that is displaced by prior exogenous ligand binding.

Although this work confirms that of Vickery *et al.* (1978) and Ching *et al.* (1982) that there is no direct electronic interaction between haem groups, kinetic

studies have suggested an indirect interaction between haem *c* and haem *d₁* to explain the effect that carbon monoxide ligation of ferrohaem *d₁* has on the oxidation of haem *c* by *Pseudomonas* azurin (Parr *et al.*, 1977) and ferricyanide (Barber *et al.*, 1978*b*). Moreover, Silvestrini *et al.* (1982) concluded from kinetic data that the binding of carbon monoxide to ferrohaem *d₁* induced a decrease in redox potential of the *c* haem of at least 80 mV. This appears to be in keeping with our present results, which indicate that the effect of ferrihaem *d₁* ligation is to modify the properties of the haem *c*. In addition, co-operativity between the *d₁* haem groups in the reduced dimeric complex has been invoked to explain certain ligand-binding phenomena (Parr *et al.*, 1975; Barber *et al.*, 1978*b*), and therefore there is now documented evidence that all four of the haem groups in the dimer interact. This idea is supported by the observation that the rate of electron transfer from haem *c* to haem *d₁* during the oxidation of nitrite reductase by dioxygen is increased 100-fold relative to the situation where the oxidation of the enzyme is performed in the absence of species that are potential ligands for haem *d₁* (Greenwood *et al.*, 1978).

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