Y25S variant of *Paracoccus pantotrophus* cytochrome cd\(_1\) provides insight into anion binding by d\(_1\) heme and a rare example of a critical difference between solution and crystal structures.

Richard S. Zajicek\(^\dagger\), Myles R. Cheesman\(^\ddagger\), Euan H. J. Gordon\(^\xi\) and Stuart J. Ferguson\(^\ұ\)

\(^\dagger\)Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, United Kingdom.

\(^\ddagger\)School of Chemical Sciences, University of East Anglia, Norwich, NR4 7TJ, United Kingdom.

\(^\xi\)Department of Chemistry and Bioscience, Chalmers University of Technology, Gothenburg, Sweden

*Correspondence to: Tel.: 01865275240; Fax: 01865275259; E-mail: stuart.ferguson@bioch.ox.ac.uk

Keywords: *Paracoccus pantotrophus*; heme coordination; nitrite reductase; magnetic circular dichroism; crystal/solution structural differences; anion binding.

Tyr25 is a ligand to the active site d\(_1\) heme in as prepared, oxidized cytochrome cd\(_1\) nitrite reductase from *Paracoccus pantotrophus*. This form of the enzyme requires reductive activation, a process that involves not only displacement of Tyr25 from the d\(_1\) heme but also switching of the ligands at the c heme from bis-histidinyl to His/Met. A Y25S variant retains this bis-histidinyl coordination in the crystal of the oxidized state which has sulfate bound to the d\(_1\) heme iron. This Y25S form of the enzyme does not require reductive activation, an observation previously interpreted as meaning that the presence of the phenolate oxygen of Tyr25 is the critical determinant of the requirement for activation. This interpretation now needs re-evaluation because, unexpectedly, the oxidized as prepared Y25S protein, unlike the wild type, has different heme iron ligands in solution at room temperature, as judged by magnetic circular dichroism and electron spin resonance spectroscopies, than in the crystal. In addition, the binding of nitrite and cyanide to oxidized Y25S cytochrome cd\(_1\) is markedly different from the wild type enzyme, thus providing insight into the affinity of the oxidized d\(_1\) heme ring for anions in the absence of the steric barrier presented by Tyr25.

**Introduction**

There are two strikingly different forms of bacterial nitrite reductase used in denitrification to catalyse the one electron reduction of nitrite (NO\(_2^–\)) to nitric oxide (NO) and water, namely cytochrome cd\(_1\) (NirS) and the copper-containing enzyme NirK. Cytochrome cd\(_1\) is a soluble tetraheme periplasmic homodimer. Each monomer contains a covalently bound c-type heme and a non-covalently bound d\(_1\) heme (a cofactor unique to this class of enzyme). The c heme is the site of electron entry whereas the d\(_1\) heme binds and reduces nitrite. In addition, each monomer is divided into two distinct domains. The N-terminal domain is predominantly \(\alpha\)-helical and binds the c heme, whereas the C-terminal domain forms an 8-bladed \(\beta\)-propeller in which the d\(_1\) heme sits (1). The crystal structure of the oxidized “as isolated” *P. pantotrophus* cytochrome cd\(_1\) nitrite reductase had unexpected ligands at each of the heme centers (1). The c heme centers on each monomer are bis-histidinyl, coordinated by His69 (belonging to the CXXCH motif) and His17. The d\(_1\) heme has His200 as an axial ligand along with Tyr25; the latter is from the c domain on the same extended N-terminal polypeptide loop as His17. The possibility that the axial ligands His17 and Tyr25 were an artifact of crystallization was eliminated by spectroscopic (magnetic circular dichroism (MCD) and electronic paramagnetic resonance (EPR)) studies in solution; these unambiguously confirmed the bis-histidinyl coordination of the c heme and were entirely consistent with the presence of tyrosine at the d\(_1\) heme (2). Upon reduction, the crystal structure demonstrated a
switch in the heme ligands of both heme centers. The His17 ligand to the oxidized c heme was displaced by Met106 and there was loss of the Tyr25 ligand to the d₁ heme, leaving the latter pentacoordinate and available for NO₂⁻ binding (3).

Subsequent to the initial spectroscopic and crystallographic characterization of the enzyme, it was discovered that significant catalytic activity of P. pantotrophus cytochrome cd₁ required activation by reduction (4). This could be understood in terms of the bis-histidinyl ligands to the oxidized c heme resulting in this center having an $E^\infty$ of approximately +60 mV. The latter value is not obviously compatible with acceptance of electrons from the physiological electron donors, cytochrome $c_{550}$ and pseudoazurin, both of which have $E^\infty$ values of approximately +250 mV. In addition, Tyr25 would present a steric barrier against nitrite binding to the oxidized enzyme. An oxidized form of the enzyme with His/Met coordination at the c heme and absence of Tyr25 binding at the d₁ heme can be generated (5,6). This conformation of cytochrome cd₁ is catalytically active, not least because of the His/Met coordinated c heme center with an estimated $E^\infty$ value of approximately 250 mV (7), very similar to those of either the two physiological electron donor proteins; also the d₁ heme is available to bind substrate in this conformation (6). These observations cast doubt on the proposal that binding and dissociation of Tyr25 and the bis-histidinyl coordination at the c heme played roles in the catalytic activity of the protein (1,3). On the other hand, the finding that Tyr25 readily displaces cyanide from the d₁ heme upon oxidation of the enzyme in the crystal, or in solution, provided further evidence for the strong tendency for this tyrosine to bind to the d₁ heme iron (8).

Tyr25 was subsequently mutated to a serine residue (9) to probe the consequences of the loss of this tyrosine ligand on protein conformation and activity. The visible absorption spectrum of the Y25S variant cytochrome cd₁ showed the loss of all peaks associated with signal from a high spin d₁ heme species. This was to be expected, following the loss of Tyr25 which puts the d₁ heme in a state of high/low spin thermal equilibrium (2). Upon removal of Tyr25, there was only a signal associated with a low spin d₁ heme species (2,9). A 1.4 Å structure of this variant protein showed only very localized changes in the immediate vicinity of residue 25 (9). The protein still had bis-histidinyl coordination at the c heme and the serine had effectively replaced Tyr25 in the d₁ heme pocket, but a sulfate ion (presumably from the precipitant) was bound to the d₁ heme rather than the –OH of Ser25. Kinetic assays revealed this enzyme did not require pre-reduction for maximal substrate turnover, unlike wild type cytochrome cd₁ (4,9), possibly as a result of the increased accessibility of the d₁ heme permitting nitrite to bind and subsequently raise the reduction potential of that heme (9). The latter might allow electron transfer from a donor protein even with an energetically uphill initial step due to His/His coordination of the c heme (9,10). It was assumed that onset of catalytic turnover could also trigger the switching of the c-type center to His/Met coordination. An alternative possibility, that the Y25S enzyme had different heme ligands in solution (e.g. His/Met at the c heme center) compared with the crystal, was deemed very improbable because, as discussed above, the wild type enzyme retained the same heme ligands in the crystal and solution for the oxidized as prepared state. Furthermore, the Y25S protein retained essentially all those structural features in the crystal which can be recognized to stabilise the binding of His17 to the c-type center (9). However, further spectroscopic studies of the Y25S protein have, as described in the present paper, unexpectedly undermined this conclusion.

Owing to Tyr25 binding to the d₁ heme in the oxidized wild type enzyme, studies of cyanide binding to the d₁ heme of P. pantotrophus cytochrome cd₁ have only been possible in the reduced form (8). Oxidation of the reduced cyanide bound complex results in the displacement of CN⁻ by Tyr25. It was thought that P. pantotrophus Y25S cytochrome cd₁ might allow the study of cyanide and nitrite binding to ferric d₁ heme without interference from Tyr25, thus providing insight into the relative affinities of the ferrie and ferrous forms of d₁ heme for these two anions. Nitrite will bind only to the activated form of oxidized wild type cytochrome cd₁ (6); the binding affinity of nitrite to ferrous d₁ heme could not be determined because the physiological catalytic reaction would reduce nitrite to nitric oxide and water.
Materials and Methods

Protein purification

The *P. pantotrophus* Y25S cytochrome cd₁ variant was expressed from *P. pantotrophus* strain EG6202 complemented with a Y25S nirS expression vector; pEG760 (9). Cells were grown for 16 - 24 hours, on minimal media supplemented with 20 mM succinate and 20 mM nitrate under anaerobic conditions to ensure maximum cd₁ heme production and incorporation into the protein (9). A standard *P. pantotrophus* cytochrome cd₁ protocol was employed to obtain the periplasmic fraction and purify Y25S cytochrome cd₁ from the cell pellets (9,11). Concentrations of oxidized Y25S cytochrome cd₁ were assayed spectrophotometrically using the absorbance at 410 nm (ε = 255000 M⁻¹ · cm⁻¹). *P. pantotrophus* pseudoazurin was purified from *Escherichia coli* XL1-Blue (Stratagene) which contained the plasmid pJR2 (12). Concentrations of oxidized *P. pantotrophus* pseudoazurin were determined at 590 nm (ε = 1360 M⁻¹ · cm⁻¹).

Spectroscopy

EPR spectra were recorded on an X-band ER-200D spectrometer (Brüker spectrospin) interfaced to a computer and fitted with a liquid helium flow cryostat (ESR-9, Oxford Instruments, Oxford, U.K.). Magnetic circular dichroism (MCD) spectra were recorded on either a circular dichrograph, JASCO-J-500D, for the wavelength range 280-1000 nm, or a laboratory-built dichrograph for the range 800-2500 nm. Samples were mounted within an Oxford Instruments SM4 split-coil superconducting solenoid capable of generating magnetic fields of up to 5 T for low-temperature measurements and in an Oxford Instruments SM1 6 T superconducting solenoid with an ambient-temperature bore for room temperature MCD measurements. All solutions used in this study were dissolved in deuterated 50 mM potassium phosphate buffer at pH* values specified in the text (pH* is the apparent pH of D₂O solutions measured using a standard glass pH electrode). Samples for MCD included deuterated glycerol (C₃H₅O₃D₃) in a 1:1 (v/v) ratio, as a glassing agent. Electronic absorption spectra were recorded on a Perkin Elmer lambda-2 spectrophotometer or a Hitachi instrument.

Titrations with anionic ligands

Equilibrium binding parameters of cyanide were determined by titrating potassium cyanide into an air-tight cuvette containing a solution of oxidized *P. pantotrophus* Y25S cytochrome cd₁ essentially as described in Jafferji et al. (8). Using an excess of potassium cyanide it was shown that only the cd₁ heme significantly bound cyanide at the range of concentrations of cyanide used in the titration. On addition of cyanide to the cuvette, using a Hamilton syringe, the solution was allowed to equilibrate and then the cyanide binding affinity was monitored spectroscopically using the absorption at 672 nm. Note the visible absorption spectrum of the Y25S cytochrome cd₁ variant is different from that of the wild type and therefore it was not appropriate to use the absorption at 632 nm as described in Jafferji et al. (8). The same method was used when titrating dithionite-reduced Y25S cytochrome cd₁ which had been passed down a PD10 desalting column (Pharmacia). Measurements of the equilibrium binding parameters of nitrite were measured in the same fashion using potassium nitrite.

Results

Visible absorption spectroscopy

Figure 1 shows the oxidized and reduced visible absorption spectra of Y25S *P. pantotrophus* cytochrome cd₁. The Soret band of the Y25S cytochrome cd₁ spectrum is red shifted from 406 nm for the wild type to 410 nm, a value consistent with, but not reliably diagnostic for, His/Met coordination of the c heme as in other cytochromes cd₁ (2,13,14) including activated *P. pantotrophus* cytochrome cd₁ (6,15). As seen in Gordon et al. (9), the Y25S spectrum has a single broad peak above 600 nm at ~ 640 nm; this is consistent with the disappearance of the high/low spin thermal equilibrium at the cd₁ heme caused, in the wild type enzyme, by Tyr25 coordination (2), leaving only a low-spin species. The spectra of reduced *P. pantotrophus* wild type and Y25S cytochrome cd₁ are identical, leading to the assumption they have the same structure in the vicinity of the absorbing cofactors. The absorbance maximum at ~ 650 nm, in the reduced spectra of both proteins, allows direct comparison of cd₁ heme content with the wild type protein. Y25S consistently contains less cd₁ heme per dimer. Exact amounts varied from preparation to preparation but were never less than 80% that of the wild type.
Room Temperature Magnetic Circular Dichroism (MCD) spectroscopy

The ultraviolet and visible region room temperature MCD spectrum of oxidized Y25S cytochrome cd1 is shown in Figure 2. As with all cytochromes cd1, the signal between 300 and 580 nm is typical of low-spin ferric protopoheme (16); contributions in this region from ferric d1 hemes are significantly weaker than those of the ferric c hemes (17). In this case, the spectrum is identical to that of P. stutzeri cytochrome cd1 at room temperature (2), a protein with His/Met coordination at the c heme.

Figure 3 shows the room temperature near-infrared MCD spectrum of oxidized ‘as isolated’ wild type and Y25S cytochrome cd1 at room temperature in the region between 700 – 2000 nm. The wild type protein has a predominant peak at 1530 nm whereas the Y25S protein has this peak red shifted to 1775 nm. Both these peaks have higher energy shoulders. The peaks observed for each protein are consistent with the near-infrared charge transfer bands associated with low-spin ferric protopohemes (i.e. the c heme of cytochrome cd1), and can be used as a diagnostic tool to assign heme ligands in b/c type cytochromes (16). The Y25S cytochrome cd1 band at 1775 nm lies in the region indicative of His/Met coordination whereas the wild type cytochrome cd1, 1530 nm band lies in the region diagnostic of His/His coordination. These results indicate that, at room temperature, the c heme of Y25S cytochrome cd1 has His/Met coordination in solution, differing from the crystal structure (9). The near infrared MCD spectrum of P. pantotrophus Y25S cytochrome cd1 is identical to that of the wild type P. stutzeri (2) and P. aeruginosa (14) cytochromes cd1, both of which have His/Met axial ligands to their c hemes. Figure 3 shows a significant broad signal at ~ 1000 nm in the spectrum of the wild type P. pantotrophus cytochrome cd1 which is not present in the Y25S protein. This feature has been attributed to the high-spin ferric d1 heme that is present in thermal equilibrium with the low-spin species at room temperature because of Tyr25 coordination in wild type P. pantotrophus cytochrome cd1 (2).

EPR spectroscopy

The X-band EPR spectrum at 10 K of P. pantotrophus Y25S cytochrome cd1 is shown in Figure 4; there are multiple features. For P. pantotrophus cytochrome cd1, the three features at g = 2.93, 2.34 (possibly 2.25) and ~ 1.4 can be attributed to a low-spin ferric c heme with His/Met axial ligands (5,15). The semi-apo form of cytochrome cd1 (with the d1 heme removed) has near identical g values; this type of cytochrome cd1 has been described as having His/Met ligands to the c heme (15). However, an oxidized, generated by oxidation of the reduced enzyme by hydroxylamine, holo form of wild type cytochrome cd1, displaying a g value of 2.33, was shown to have His/Met ligation at the c heme (5,18). These features are also very similar to those seen in the X-band EPR spectra of P. stutzeri and P. aeruginosa cytochromes cd1, both of which have His/Met coordination at their c hemes (2). In previous work it has been shown that the c-type cytochrome center of wild type P. pantotrophus cytochrome cd1 has a relatively unusual EPR spectrum of the “large gmax type” where g<sub>z</sub> is >3 and the other two g values are not easily detected (2,15). This feature is thought to be due to his-histidinyl axial ligation with the orientation of the imidazoles closer to perpendicular than parallel (19), as is observed in the crystal structure of wild type P. pantotrophus cytochrome cd1 (1). There is no corresponding feature in the spectrum of P. pantotrophus Y25S cytochrome cd1. These EPR observations are absolutely consistent with Y25S cytochrome cd1 having His/Met axial ligands at low temperature, but without low temperature MCD it cannot be said so categorically. It remains possible, but unlikely, the c heme of Y25S cytochrome cd1 may possibly switch from His/Met, at room temperature, to His/His, at low temperature, due to a freezing artifact or thermal equilibrium. Note that the X-ray crystal structure of Y25S cytochrome cd1 was determined at liquid N<sub>2</sub> temperature (9).

The remaining EPR features at g = 2.57, 2.44, 2.25, 1.87 and 1.61 (Figure 4) are consistent with previously observed characteristics of low-spin ferric d1 heme. The triplet of g = 2.57, 2.44 and 1.61 compares well with the g values of the oxidized P. stutzeri d1 heme and are distinct from the values of P. pantotrophus wild type cytochrome cd1 (2). In addition, the triplet at g = 2.44, 2.25 and 1.87 is virtually identical to that seen following oxidation by oxygen of reduced wild type P. pantotrophus cytochrome cd1 (15). These g values are thought to represent His/OH coordination at the d1 heme, which is highly
likely in oxidized Y25S cytochrome cd1 because the d1 heme is exposed. It is clear that there are two different species of low-spin ferric d1 heme possibly sharing a g value of 2.44. These two species could be caused by different ligands (e.g. OH-, H2O or SO42-) or a change in the orientations/protonations of active site residues, and hence hydrogen bonding patterns within the active site; two different orientations of OH- is a further possibility.

Features at g = 6.95 and 4.99 are characteristic of rhombically distorted high-spin ferric d1 heme present in trace amounts. These g values have been seen in varying amounts in all wild-type P. pantotrophus cytochrome cd1 preparations (2,5).

Low Temperature MCD (LTMCD) spectroscopy
It was clear from the experiments described above that at room temperature the ligands to the oxidized c heme were His/Met in solution for the Y25S protein. In addition, the evidence from the EPR measurements also strongly suggested that the oxidized c heme ligands were His/Met. However, although the room temperature near-infrared MCD (NIRMCD) showed that the coordination was His/Met, the EPR cannot be taken as certain evidence for retention of this coordination at the much lower temperature used with the latter technique. Consequently, low temperature NIRMCD analysis was undertaken, using two pH values such that any influence of proton concentration on the c-cytochrome center could be assessed at the same time. The visible LTMCD spectra of samples at pH 6.5 (a pH close to physiological pH and that (pH 5.8) which produced maximal activity (4)) and pH 8.5 are shown in Figure 5. In both spectra it is clear there is no signal above 700 nm. Cheesman et al. (1997) showed that any signal > 700 nm is due to high-spin ferric d1 heme that is in thermal equilibrium with low-spin ferric d1 heme in wild type P. pantotrophus cytochrome cd1 at room temperature. This observation confirms that the EPR features, mentioned previously, of possibly the two species of d1 heme are both from low-spin hemes. The low temperature NIR MCD spectra of Y25S cytochrome cd1 at pH 6.5 and 8.5 are shown in Figure 6. It is clear that the sample at pH 8.5 is close to 100% His/Met, based on the presence of a band at 1775 nm and the absence of a band at 1530 nm. However, the sample at pH 6.5 has a heterogeneous spectrum, with bands characteristic of both His/Met (75%) and His/His (25%). The absence of a “large gmax” species in the EPR spectrum (pH 7.0) signifies (unless there is dramatic difference in the amount of this form at pH 7.0 compared with 6.5) that this species of bis-histidinyl coordinated c heme does not have perpendicular imidizole rings, thus implying that they are in a parallel conformation. The crystal structure of P. pantotrophus Y25S cytochrome cd1 shows the histidine residues are perpendicular to each other. This highlights another difference between the solution and crystal forms of the protein.

The difference between the low temperature NIRMCD spectra at pH values of 6.5 and 8.5 prompted further examination of the visible absorption spectra under the same conditions. In each case the Soret band maximum was at 410 nm (as in Figure 1), rather than at 406 nm as for the wild type protein. Although this wavelength cannot be used in a general sense to assess coordination at heme, the fact that there is no difference between pH 6.5 and 8.5 indicates that for Y25S cytochrome cd1 there is an insignificant increase in His/His coordination on going from 8.5 to 6.5 in solution at room temperature. Thus on the basis of the data described in the present paper, it seems that the His/His coordination is favoured by low temperature and lower pH values.

Reduction with physiological electron donor
Given the ligands to the c heme of oxidized Y25S cytochrome cd1 in solution had changed from those in wild type cytochrome cd1, it was important to investigate whether the in vivo electron donors to cytochrome cd1 (4,20) could, unlike the wild-type (4) reduce Y25S cytochrome cd1 before substrate binding. To determine whether reduced pseudoazurin could pass electrons to oxidized Y25S cytochrome cd1 an excess of reduced pseudoazurin (from which the excess reductant, ascorbate, had been removed by gel filtration) was mixed with oxidized Y25S cytochrome cd1 under anaerobic conditions and in the absence of nitrite. Pseudoazurin was used because its absorbance is small at the relevant wavelengths in comparison to those of the alternative electron donor; cytochrome c550. Figure 7 shows the resultant spectrum of the mixture 30 seconds after mixing. The appearance and magnitude of the split ferrous c heme α-band at ~ 550 nm indicates the Y25S cytochrome cd1 has been completely
reduced by the pseudoazurin. The peak at 460 nm, characteristic of reduced high-spin $d_1$ heme, also illustrates the change in the oxidation state of cytochrome $c_d$. The peak assigned to reduced $d_1$ heme at 650 nm is difficult to identify because of the broad pseudoazurin absorbance at ~590 nm.

**Cyanide and nitrite binding titration**

Oxidized ‘as isolated’ wild type *P. pantotrophus* cytochrome $c_d$ is unable to bind CN$^-$ at either the $c$ or $d_1$ hemes, but the reduced enzyme binds the ligand at the $d_1$ heme ($K_d = 0.7 \times 10^{-6} \text{ M}$) (8). It was thought, in the absence of Tyr25, that oxidized *P. pantotrophus* Y25S cytochrome $c_d$ would be able to bind CN$^-$. The enzyme was exposed to 10 mM KCN to assess whether it could bind the CN$^-$. In contrast to the wild type protein, oxidized Y25S cytochrome $c_d$ was able to bind CN$^-$. The $d_1$ heme $\alpha$-band at 640 nm in the oxidized ‘as isolated’ *P. pantotrophus* Y25S cytochrome $c_d$ shifted to 631 nm upon addition of cyanide. The position of this $d_1$ heme band is the same as that for oxidized wild type *P. aeruginosa* cytochrome $c_d$ with cyanide bound (14). The visible absorption spectrum of reduced Y25S cytochrome $c_d$ with cyanide bound was identical to that of reduced wild type protein with cyanide bound (8). Upon oxidation with $K_3[\text{Fe(CN)}_6]$, the reduced $d_1$ heme peak of Y25S cytochrome $c_d$ remained at 631 nm rather than returning to 640 nm (Y25S cytochrome $c_d$ oxidized peak), which would have been expected if CN$^-$ had been displaced from the $d_1$ heme by Ser25; cyanide displacement by Tyr25 is seen in wild type cytochrome $c_d$ (8).

Potassium cyanide was titrated into a solution of oxidized ‘as isolated’ *P. pantotrophus* Y25S cytochrome $c_d$, at 25 °C, to determine equilibrium binding parameters. The dissociation constant ($K_d$) was found to be $4.4 \times 10^{-5}$ M at pH 7.0 using a Hill plot; this value is an order of magnitude larger than that of the reduced wild type cytochrome $c_d$ (~ $1 \times 10^{-6}$ M (8)). It is noteworthy that only the $d_1$ heme was affected by the addition of cyanide over this titration range. In addition, a titration of cyanide against reduced Y25S cytochrome $c_d$ was also performed. This showed that the reduced Y25S protein-cyanide complex had a similar dissociation constant to the reduced wild type protein, in each case ~ $1 \times 10^{-6}$ M at pH 7.0.

The ‘as isolated’ oxidized form of wild type *P. pantotrophus* cytochrome $c_d$ will not bind nitrite due to the steric barrier presented by Tyr25. However, if the alternative active form, i.e. with His/Met coordination at the $c$ heme, of the oxidized enzyme is prepared then substrate addition of nitrite results in a stable oxidized enzyme with nitrite bound; the effective $K_d = 2 \text{ mM}$ (6). It was therefore of interest to test whether the oxidized Y25S protein would also bind nitrite. Using the same technique for cyanide binding, nitrite was titrated into a solution of oxidized Y25S cytochrome $c_d$. From this experiment, it can be calculated that the $K_d$ for nitrite dissociation from the enzyme is ~ $7 \times 10^{-5}$ M.

In previous work, the activated ferric form of wild type enzyme was, when complexed with nitrite, unexpectedly EPR silent in respect of the $d_1$ heme (6). The absence of an EPR spectrum was also seen in the present work for the nitrite complex with oxidized Y25S cytochrome $c_d$. The explanation for this EPR silence is not obvious and requires further study, although the present results reinforce the idea that the observation reflects an intrinsic feature of the ferric $d_1$ heme-nitrite complex.

**Discussion**

MCD and EPR spectroscopies categorically identify the axial ligands at the $c$ heme of *P. pantotrophus* Y25S cytochrome $c_d$ as His/Met in solution at room temperature and the majority population at two different pH values at very low temperature. It is reasonable to assume the methionine residue is Met106, an axial ligand to the reduced wild type cytochrome $c_d$ $c$ heme (3). This conserved residue is also an axial ligand to the oxidized $c$ heme of *Pseudomonas aeruginosa* cytochrome $c_d$ (21). This result is in clear disagreement with the 1.4 Å crystal structure of oxidized Y25S cytochrome $c_d$ that showed *his*-histidinyl axial ligation of the $c$ heme (9). The reasons for, and implications of, these results are discussed below.

There have been many discussions since the advent of protein crystallography as to whether solution and crystal structures are the same. In general they are. This paper, therefore, reports a relatively rare case of a protein that is crucially different in structure between the solution and crystalline states. As with most proteins, the crystallization conditions (here including 2.3M
ammonium sulfate and high protein concentration) are rather different from those used for functional studies in solution. Thus we cannot distinguish between high salt/protein concentration or formation of crystals per se as being the determining factor. However, high protein concentration may not be a major factor as our spectroscopic studies were carried out at comparable enzyme concentrations (120 µM) to those used for crystallization (22).

There is no doubt that the solution form of oxidized Y25S cytochrome cd₁ does not have bis-histidinyl coordination of the c heme but rather His/Met. The loss of essentially just one interaction between the phenolate oxygen of Tyr25 and the d₁ heme iron appears to alter the protein folding energy sufficiently such that in the solution conditions used for functional studies, the position of the N-terminal arm, and, consequently, the appropriate position of His17 as a heme ligand for the c-type cytochrome center, is lost. There are, for crystalline wild type cytochrome cd₁, 19 (subunit A) or 20 (subunit B) direct hydrogen bonds and salt bridges, plus the Tyr25 to d₁ heme interaction, between the N-terminal domain (up to residue 135 which comprises the N-terminal arm and the cytochrome c domain) and the d₁ (C-terminal) domain (1,23). Of these interactions, 10 and 11 respectively are between the N-terminal arm (residues 9-48) and the d₁ domain. Thus loss of one interaction, Try25 to d₁, out of nineteen or twenty for each subunit is sufficient to switch the Y25S enzyme from the bis-histidinyl coordination of the c heme to the His/Met state on going from the crystalline to the solution conditions used in the present work. The coordination of the hemes in this enzyme is evidently very delicately balanced. The bis-histidinyl coordination in the Y25S cytochrome cd₁ crystal could be a consequence of the crystallisation conditions and/or the phase change to the crystalline state. However, both low temperature and pH also promote this conformation. This view is supported by the low temperature MCD at pH 6.5 and 8.5. At pH 6.5 there is ~ 25 % of the protein in the bis-histidinyl coordination; a figure that appears to decrease upon an increase in pH. Therefore, if this effect was predominantly temperature dependent, the crystal structure would be expected to be His/Met in the majority form with < 25 % having bis-histidinyl coordination because the structure was determined at pH 7.0 (9).

The structures of reduced wild type cytochrome cd₁ crystals soaked in nitrite have been solved (3). Nitrite or nitric oxide was bound to the d₁ heme and His/His coordination of the c heme was observed. In contrast, rapid reaction solution studies, reacting reduced wild type cytochrome cd₁ with nitrite, showed no evidence of His/His coordination of the c heme but only His/Met coordination (24). It is possible, in light of the results presented here, that the same switching of ligands occurred in the nitrite soaked crystals as proposed here for the Y25S cytochrome cd₁ crystals.

Pseudoazurin is unable to reduce oxidized “as isolated” wild type cytochrome cd₁ (4,5), whereas the results in the present paper show pseudoazurin can reduce oxidized Y25S cytochrome cd₁. This result is in good agreement with the reduction potentials of the hemes in each enzyme. In the wild type “as isolated” enzyme electrons must enter the enzyme via a His/His coordinated c heme (1,2) with a midpoint reduction potential of + 60 mV (7). Hence, pseudoazurin cannot act as a stochiometric reductant for the c-type center as it has a higher reduction potential of + 230 mV (11) that makes electron transfer thermodynamically unfavourable. The results presented now show that solution state oxidized Y25S cytochrome cd₁ has His/Met coordination, a change that is thought to raise the redox potential of a c-type heme by approximately 200 mV (25). A reduction potential increased by about this amount would favour electron transfer from pseudoazurin to Y25S cytochrome cd₁. The possibility that the d₁ heme of Y25S cytochrome cd₁ may have a much higher reduction potential than pseudoazurin, making electron flow from pseudoazurin to the d₁ heme sufficiently thermodynamically favourable to overcome an uphill step from pseudoazurin to the cytochrome c heme of the enzyme (9), is not supported by the observation that the c heme became extensively reduced in the presence of reduced pseudoazurin.

Wild type cytochrome cd₁ requires pre-activation to initiate maximal catalytic turnover (4). This is because reduction of the protein induces a conformational change to give the c heme His/Met coordination and make the d₁...
heme pentacoordinate and ready to bind substrate (3). It is the binding of substrate to the \( d_1 \) heme that is thought to provide the thermodynamic driving force for the reaction (4). The model proposed in Gordon et al. (9), which is based on His/His coordination of the \( c \) heme in the Y25S cytochrome \( cd_1 \) crystal structure, suggests that, in the absence of Tyr25, nitrite is able to bind to the \( d_1 \) heme of the oxidized enzyme. The binding of nitrite would then raise the reduction potential of the \( d_1 \) heme to allow electron transfer from pseudoazurin by electron tunnelling (10). Thus reductive preactivation would not have been required for the Y25S variant (9). The results presented now demonstrate the \( c \) heme is His/Met in solution (see (5) for diagrammatic representation), thus explaining why preactivation was not required and raising the possibility of reduction by physiological electron donor before binding of substrate, rather than substrate binding to the oxidized \( d_1 \) heme. It is shown that pseudoazurin can reduce oxidized Y25S cytochrome \( cd_1 \); therefore, substrate most likely binds to a reduced \( d_1 \) heme, which is the assumption in the wild type enzyme (3,26). In the case of Y25S cytochrome \( cd_1 \), it is unlikely that nitrite must bind to oxidized \( d_1 \) heme to give the chain of redox centers its overall driving force. This observation is also in good agreement with the cyanide binding titration that shows cyanide (a model anion) has a higher affinity for ferrous, relative to ferric, \( d_1 \) heme by at least one order of magnitude. Nevertheless, it remains to be seen in future work whether cytochrome \( cd_1 \) has an obligatory mechanistic pathway in which nitrite binds to one oxidation state of the \( d_1 \) heme.

The \( c \) heme Söret band of oxidized ‘as isolated’ wild type cytochrome \( cd_1 \) lies at 406 nm, whereas the Y25S band lies at 410 nm. This would not usually be cause to doubt a 1.4 Å crystal structure, although the Y25S cytochrome \( cd_1 \) crystal structure surprisingly showed no difference in \( c \) heme ligation or ligand orientation from the wild type. The visible absorbance \( c \) heme Söret bands of the oxidized wild type cytochromes \( cd_1 \) from \( P. \) aeruginosa and \( P. \) stutzeri both lie at 411 nm (2,27). It can now be proposed that an oxidized \( c \) heme Söret band of a cytochrome \( cd_1 \) at 410-411 nm is a strong indication of His/Met coordination at the \( c \) heme for cytochrome \( cd_1 \). When oxidized \( P. \) aeruginosa cytochrome \( cd_1 \) was mixed with imidazole the \( c \) heme Söret band shifted from 411 nm to 406 nm (28) reflecting the displacement of methionine by imidazoles; this extends the proposal to include a 406 nm \( c \) heme Söret band as an indication of His/His coordination. 

Y25S cytochrome \( cd_1 \) gives us the opportunity to study, for the first time, the binding properties of ferric \( d_1 \) heme \textit{in situ} without the effects of Tyr25 as a ligand. It is reasonable to assume Ser25 is not obstructing entry to the \( d_1 \) heme iron because no structures even of wild type cytochrome \( cd_1 \) with His/Met coordination to the \( c \) heme have described Tyr25 bound to the \( d_1 \) heme (3,8,30). In addition, the observed \( K_d \) for the order 10⁻¹ M, for cyanide dissociation from oxidized Y25S cytochrome \( cd_1 \) would imply little or no hindrance from Ser25. The equilibrium dissociation constant for cyanide binding to ferric \( d_1 \) heme of Y25S cytochrome \( cd_1 \) (\( K_d = 4.4 \times 10^{-5} \)) is more than an order of magnitude greater than that for cyanide dissociation from ferrous \( d_1 \) heme in wild type cytochrome \( cd_1 \) \((\sim 1 \times 10^{-9} \) M\) (8)). The result with Y25S cytochrome \( cd_1 \) is in excellent agreement (perhaps coincidentally) with the \( K_d \) for cyanide dissociation from ferric \( d_1 \) heme when the latter was inserted into the heme binding pocket of myoglobin (4.2 \( \times 10^{-5} \) M) (31). The decreased \( K_d \) of cyanide binding to ferrous \( d_1 \) heme, compared to ferric, is in contrast to the usual cyanide binding properties of hemes. Cyanide generally binds very strongly to the ferric form of heme iron, examples of dissociation constants include: 1 \( \times 10^{-9} \) M for human hemoglobin and 2.5 \( \times 10^{-4} \) M for mitochondrial horse heart cytochrome \( c \) (32). These compare to values of 1 M or above for the ferrous forms of the same proteins (32,33). Such vast contrasts are not seen for the \( d_1 \) heme of cytochrome \( cd_1 \). These observations show that
the $d_1$ heme, compared to protoheme, is tailored to bind anions in a ferrous rather than a ferric state. The covalent modifications distinguishing $d_1$ heme from 'normal' heme are electronegative alterations, increasing the positive potential of the $d_1$ heme iron (34). The immediate environment of the $d_1$ heme active site also contains two highly conserved histidine residues (His345 and His388 in P. pantotrophus), which, if protonated, contribute to the positive electrostatic potential of the active site (8). These two factors can be argued to contribute to the increased ability of $d_1$ heme, especially in the ferrous state, of cytochrome $cd_1$ to bind anions.

Allen et al. (6) estimated that the dissociation constant of the complex of nitrite with the activated, oxidized form of wild type cytochrome $cd_1$ from P. pantotrophus was approximately 30-fold higher than that reported here for the analogous complex of the oxidized Y25S enzyme. Presumably the tendency of Tyr25 to religate to the $d_1$ heme, and thus displace the nitrite from the wild type enzyme, is reflected in a lesser affinity i.e. higher dissociation constant, for nitrite than the Y25S variant protein. The results obtained with the latter enzyme are more likely to reflect the intrinsic affinity of the oxidized $d_1$ heme ring, along with the active site ligands such as His345 and His388, for nitrite. The affinity of the oxidized enzyme for nitrite is, therefore, quite high which implies that a reaction mechanism in which nitrite binds to oxidized $d_1$ heme warrants consideration (the $K_M$ (nitrite) with physiological electron donor is $7 – 19 \mu M$ (4)). It is discussed elsewhere in this paper that the affinity of the reduced $d_1$ heme for cyanide is at least an order of magnitude higher than that of the oxidized $d_1$ heme. The affinities of the oxidized $d_1$ heme for nitrite and cyanide are very similar (c/f 65 $\mu M$ and 44 $\mu M$). It could then, by extrapolation, be estimated that the affinity of the reduced $d_1$ heme for nitrite would be at least an order of magnitude higher than that of the oxidized heme. If this is indeed true for nitrite binding, we can conclude that a mechanism involving the binding of nitrite to the reduced $d_1$ heme also warrants careful consideration.

In conclusion, the most important outcome of general significance is that the crystal structure of the Y25S mutant of P. pantotrophus cytochrome $cd_1$ led us to an incorrect interpretation of the properties of the protein. The continuing need for solution spectroscopic techniques to confirm or otherwise aspects of protein crystal structures is clearly very important, especially where the properties of a protein are not readily interpretable in terms of a crystal structure.

Acknowledgements:

We thank the BBSRC for the grant C19430 and a studentship to RSZ. EHJG was supported in part by European Union Biotechnology Structural Biology Project BIO4 CT96-0281.
1. Fülöp, V., Moir, J. W., Ferguson, S. J., and Hajdu, J. (1995) *Cell* **81**, 369-377.
2. Cheesman, M. R., Ferguson, S. J., Moir, J. W., Richardson, D. J., Zumft, W. G., and Thomson, A. J. (1997) *Biochemistry* **36**, 16267-16276.
3. Williams, P. A., Fülöp, V., Garman, E. F., Saunders, N. F., Ferguson, S. J., and Hajdu, J. (1997) *Nature* **389**, 406-412.
4. Richter, C. D., Allen, J. W., Higham, C. W., Köppenhofer, A., Zajicek, R. S., Watmough, N. J., and Ferguson, S. J. (2002) *J Biol Chem* **277**, 3093-3100.
5. Allen, J. W., Watmough, N. J., and Ferguson, S. J. (2000) *Biochemistry* **39**, 4243-4249.
6. Köppenhofer, A., Turner, K. L., Allen, J. W., Chapman, S. K., and Ferguson, S. J. (2000) *Biochemistry* **39**, 4243-4249.
7. Allen, J. W., Watmough, N. J., and Ferguson, S. J. (2000) *Biochem Biophys Res Commun* **279**, 674-677.
8. Walker, F. A., Huynh, B. H., Scheidt, W. R., and Osvath, S. R. (1986) *J. Am. Chem. Soc.* **108**, 5288-5297.
9. Pearson, I. V., Page, M. D., van Spanning, R. J., and Ferguson, S. J. (2003) *J Bacteriol* **185**, 6308-6315.
10. Nurizzo, D., Silvestrini, M. C., Mathieu, M., Cutruzzolá, F., Bourgeois, D., Fülöp, V., Hajdu, J., Brunori, M., and Cambillau, C. (1997) *Structure* **5**, 1157-1171.
11. Fülöp, V., Moir, J. W., Ferguson, S. J., and Hajdu, J. (1993) *J Mol Biol* **232**, 1211-1212.
12. Baker, S. C., Saunders, N. F., Willis, A. C., Ferguson, S. J., Hajdu, J., and Fülöp, V. (1997) *J Mol Biol* **269**, 440-455.
13. George, S. J., Allen, J. W., Ferguson, S. J., and Thorneley, R. N. (2000) *J Biol Chem* **275**, 33231-33237.
14. Feinberg, B. A., Liu, X., Ryan, M. D., Schejter, A., Zhang, C., and Margoliash, E. (1998) *Biochemistry* **37**, 13091-13101.
15. Fülöp, V., Watmough, N. J., and Ferguson, S. J. (2001) *Adv. Inorg. Chem* **51**, 163-204.
16. Walsh, T. A., Johnson, M. K., Greenwood, C., Barber, D., Springall, J. P., and Thomson, A. J. (1979) *Biochem J* **177**, 29-39.
28. Walsh, T. A., Johnson, M. K., Thomson, A. J., Barber, D., and Greenwood, C. (1981) *J Inorg Biochem* **14**, 1-14
29. Doi, M., Shioi, Y., Morita, M., and Takamiya, K. (1989) *Eur J Biochem* **184**, 521-527
30. Sjögren, T., and Hajdu, J. (2001) *J Biol Chem* **276**, 13072-13076.
31. Steup, M. B., and Muhoberac, B. B. (1989) *J Inorg Biochem* **37**, 233-257.
32. Viola, F., Aime, S., Coletta, M., Desideri, A., Fasano, M., Paoletti, S., Tarricone, C., and Ascenzi, P. (1996) *J Inorg Biochem* **62**, 213-222
33. Brunori, M., Antonini, G., Castagnola, M., and Bellelli, A. (1992) *J Biol Chem* **267**, 2258-2263
34. Cutruzzolá, F., Rinaldo, S., Centola, F., and Brunori, M. (2003) *IUBMB Life* **55**, 617-621
Figure Legends

Figure 1. Visible absorption spectra of oxidized ‘as isolated’ (—) and reduced (---) *P. pantotrophus* Y25S cytochrome *cd*1. Reduced sample prepared with dithionite, all samples were in 50 mM potassium phosphate buffer, pH 7.0; spectra were recorded at 25 °C. Protein concentration is 0.5 µM.

Figure 2. Ultra violet and visible region MCD spectrum of *P. pantotrophus* Y25S cytochrome *cd*1. Samples were in 50 mM potassium phosphate buffer, pH 7.0. Spectrum recorded at 25 °C. The protein concentration was 120 µM.

Figure 3. Near-infrared room temperature MCD of oxidized ‘as isolated’ wild type *P. pantotrophus* *cd*1 (Top) (Cheesman *et al.*, 1997) and Y25S cytochrome *cd*1 (Bottom) (This work). Samples were in 50 mM potassium phosphate buffer, pH 7.0. Spectrum recorded at 25 °C.

Figure 4. X-band EPR spectra of oxidized Y25S *P. pantotrophus* cytochrome *cd*1. Sample was in 50 mM potassium phosphate buffer, pH 7.0, spectrum recorded at 10 K. Protein concentration was ~ 250 µM.

Figure 5. Low temperature ultra violet and visible region MCD spectrum of oxidized *P. pantotrophus* Y25S cytochrome *cd*1 at pH 8.5 (—) and 6.5 (---). Buffers were as described in Materials and Methods. Spectra recorded at 4.2 K. Protein concentration was 120 µM for each sample.

Figure 6. Low Temperature near-infrared region MCD of oxidized *P. pantotrophus* Y25S cytochrome *cd*1 at pH 8.5 (—) and 6.5 (---). Buffers were as described in Materials and Methods. Spectra recorded at 25 °C. Protein concentration was 120 µM for each sample.

Figure 7: Spectra of reduced Y25S cytochrome *cd*1 (—) and oxidized Y25S cytochrome *cd*1 mixed with reduced pseudoazurin (---). 1.5 µM anaerobic oxidized Y25S cytochrome *cd*1 was injected into a cuvette containing 250 µM anaerobic reduced pseudoazurin. The spectra of reduced Y25S cytochrome *cd*1 was normalised to the 418 nm Soret band of the Y25S cytochrome *cd*1 and pseudoazurin mixture.
Y25S variant of Paracoccus pantotrophus cytochrome cd1 provides insight into anion binding by d1 heme and a rare example of a critical difference between solution and crystal structures

Richard S. Zajicek, Myles R. Cheesman, Euan H. J. Gordon and Stuart J. Ferguson

J. Biol. Chem. published online May 18, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M501890200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts