Cytochrome *c''* isolated from *Methylophilus methylotrophus*

An example of bis-histidine-co-ordinated Fe³⁺ haem, with near-perpendicular orientation of the ligands

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Cytochrome *c''* (*Methylophilus methylotrophus*) is a soluble protein, *M_r* 15000, possessing one haem which is high-spin in the reduced state but switches to a low-spin form on oxidation. Low-temperature electron-paramagnetic-resonance spectroscopy of the oxidized state shows a low-spin signal at *g_z* = 3.65 with a folded line-shape typical of a haem of low rhombicity, and the near-infrared magnetic-circular-dichroism (m.c.d.) spectra reveal an unusually intense ($\Delta\epsilon = 400 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 5 T, 4.2 K) charge-transfer band at 1560 nm, establishing that the oxidized haem is co-ordinated by two His residues in a near-perpendicular orientation. This conformation is well established for transmembrane *b* cytochromes, but this appears to be the first example in a water-soluble cytochrome. The low-temperature m.c.d. spectra of the reduced form of the protein confirms that the haem contains a high-spin Fe²⁺ ligated by one His residue. The redox-linked spin-state change releases a His group. Since this residue is likely to bind a proton at pH values < 6.5, this cytochrome may provide a useful model of a molecular mechanism of a redox-linked proton uptake and release process.

INTRODUCTION

Methylophilus methylotrophus, an obligate methylotroph, produces a highly unusual, soluble monohaem cytochrome (*M_r* 15000), recently designated *c''* [1]. The optical and n.m.r. spectra establish that the haem is high-spin in the reduced state but switches to a low-spin form on oxidation. Therefore the cytochrome binds CO rapidly in the reduced form but does not react with CN⁻ in the oxidized state except at pH > 12.0. N.m.r. evidence suggested at least one His ligand to the haem iron. The lack of a 695 nm band in the optical spectrum of the oxidized state indicated the absence of Met ligation. In order to clarify the ligation states of the oxidized form of cytochrome *c''*, study has been made of the near-i.r. magnetic circular dichroism (m.c.d.) and the e.p.r. spectrum. It has been shown by using a combination of low-temperature e.p.r. and near-i.r. m.c.d. spectroscopy, that haem axial ligand assignments can be made [2] and in addition, in favourable circumstances, information can be derived about the rhombic crystal-field component generated at the Fe³⁺ ion by the ligation state. This in turn can lead to conclusions about the relative orientation of axial ligands [3].

This study confirms that the Fe³⁺ haem in cytochrome *c''* has bis-His co-ordination and further indicates that the imidazole planes have near-perpendicular relative orientation. The reduced cytochrome loses one His ligand, becoming high-spin Fe²⁺ and five-co-ordinate.

MATERIALS AND METHODS

Cytochrome *c''* was purified from *M. methylotrophus* (N.C.I.B. 10515) grown on methanol under nitrogen-limited conditions as previously described [1]. Protein with a purity index (*A*₄₀₈/*A*₂₈₀) of 8:5 was used. ²H₂O, without buffer, was employed as solvent to give good transmission in the near-i.r. region between 1 and 2 μm . Glycerol was added to a dilution of 50% (v/v) to provide frozen glasses of high transparency. Reduced samples were

prepared by addition of small amounts of solid Na₂S₂O₄ under N₂, care being taken to ensure that the solution did not fall to a pH < 4.3.

E.p.r. spectra were recorded with a Bruker ER 200-D-SRC X-band spectrometer equipped with an Aspect 2000 computer. Temperature regulation of the sample was by means of an Oxford Instruments ESR-900 continuous-flow cryostat with a DTC-2 controller and a Au-Fe/chromel thermocouple. M.c.d. measurements were carried out in the region 450–1050 nm with a JASCO J500-D spectropolarimeter and in the region 800–2000 nm with a home-built instrument [4]. The magnetic field and sample temperature control was provided by a split-coil superconducting magnet (Oxford Instruments, SM-4) with a maximum field strength of 5 T and sample temperature control between 1.5 and 200 K. M.c.d. spectra were recorded by computer using software written locally [5]. M.c.d. spectra are quoted in terms of $\Delta\epsilon = \epsilon_L - \epsilon_R$ where ϵ_L and ϵ_R are the molar absorption coefficients for left and right circularly polarized light.

RESULTS

Oxidized form

The e.p.r. spectrum of the oxidized state of cytochrome *c''* (Fig. 1) shows a ramp-shaped signal at *g* = 3.65, and derivative-shaped features at *g* = 4.3 and 6.0. The spectrum also reveals some weak features at *g* ≈ 2.0 but is otherwise remarkably devoid of signals. The *g* = 6.0 signal arises from a small percentage (< 5%) of high-spin Fe³⁺ haem. However, after addition of 50% (v/v) glycerol to the sample this signal disappears and the ramp-shaped signal sharpens. A minor amount of adventitious iron is present as indicated by the signal at *g* = 4.3.

The signal at *g* = 3.65 is the *z*-component of the rhombic trio of a low-spin ferric haem in which the iron is subject to a small rhombic and a large axial distortion [6]. Neither the *g_x* nor the *g_y*

Abbreviations used: m.c.d., magnetic circular dichroism; CT, charge transfer; Lb, leghaemoglobin.

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Fig. 1. E.p.r. spectrum of oxidized *M. methylotrophus* cytochrome *c''*

The sample was 240 μM in distilled water. Microwave frequency = 9.39 GHz, microwave power = 2.01 mW, modulation frequency = 0.50 mT, sample temperature = 5.0 K.

Fig. 2. Low-temperature m.c.d. spectrum of oxidized *M. methylotrophus* cytochrome *c''*

The sample was 240 μM in water/glycerol (50%, v/v). Magnetic field = 5.0 T, sample temperature = 1.6 K, path length = 1.00 mm.

of the *g*-tensor have been detected. This is unsurprising, since they are predicted to lie at very low *g* values.

The m.c.d. band stretching from ~ 880 to 1700 nm arises from range 450–2000 nm at 1.6 K and 5 T (Fig. 2). There are three conclusions to be drawn from these data. First, the m.c.d. spectrum in the region of the α - and β -bands, 450–650 nm, is typical of those of low-spin Fe^{3+} haem [7]. Secondly, the region between 650 and 800 nm is devoid of any features. This confirms the earlier optical absorption spectrum which lacks a 695 nm band. High-spin Fe^{3+} haem gives rise to electronic transitions across the entire wavelength range to beyond 1000 nm [8]. The lack of such electronic transitions is consistent with the absence of high-spin Fe^{3+} haem and in agreement with the evidence from the e.p.r. spectrum in 50% (v/v) glycerol.

The m.c.d. band stretching from ~ 880 –1700 nm arises from porphyrin-to- Fe^{3+} charge-transfer transitions [2]. The wavelength position of this band is diagnostic of haem axial ligand type. The peak position of 1560 nm is in the region expected for ligation by two His residues. An important feature of this band is the high intensity of the peak, $\Delta\epsilon = 520 \text{ M}^{-1}\cdot\text{cm}^{-1}$ at 1.6 K and 5 T [3].

We have shown that the intensity of the near-i.r. charge-transfer (CT) band in the m.c.d. spectrum is inversely correlated with the rhombic distortion parameter, *V*, of the low-spin Fe^{3+} haem [3]. The CT band results from an electronic transition from the highest filled porphyrin orbital to the hole in the d_{xz} , d_{yz}

Fig. 3. Low-temperature m.c.d. data of dithionite-reduced *M. methylotrophus* cytochrome *c''*

M.c.d. spectrum recorded at 1.6 K and 5 T (top). M.c.d. magnetization curves recorded at the indicated temperatures, with a field sweep of 0–5 T at a wavelength of 559.5 nm (bottom). The sample was 240 μM in water/glycerol (50%, v/v). The path length was 1.00 mm.

orbitals of the low-spin Fe^{3+} . If the field at the Fe^{3+} were completely axial, that is the rhombic parameter, *V*, is zero, the two *d*-orbitals are equally occupied by the hole and the CT transition is 50% *x*- and 50% *y*-polarized. However, if *V* is large the hole becomes strongly localized in one of the *d*-orbitals, say d_{yz} . Then the CT transition is *y*-polarized. For an optical transition to absorb circularly polarized radiation, that is, to give a non-zero m.c.d. signal, it must have two components polarized perpendicular to one another and to the direction of light propagation. Hence large *V* implies a CT transition polarized predominantly in one direction giving rise to low m.c.d. intensity. The m.c.d. intensity depends therefore upon the imbalance between the *x*- and *y*-polarized transitions and tends to a maximum value as *V*/ λ becomes smaller. The $\Delta\epsilon$ value obtained for the near-i.r. CT band of cytochrome *c''* is consistent with a *V*/ λ value < 1 . This is in agreement with the form of the e.p.r. spectrum. Salerno [6] has shown that as *V* $\rightarrow$ 0 the g_z approaches a maximum limiting value determined by the magnitude of the axial parameter Δ/λ . This results in a typical folded or ramp line-shape. The other two components of the *g*-tensor, g_x and g_y drop

towards zero making their detection impossible in a derivative mode e.p.r. spectrum. Spectra with this line-shape have been called HALS (highly anisotropic low-spin) spectra, although it has been pointed out that this is a misnomer since the iron actually experiences a crystal field of very low rhombicity and high axiality. HALS might better refer to highly axial low-spin spectra.

Reduced form

After reduction with sodium dithionite the e.p.r. spectrum was devoid of signals. The m.c.d. spectrum was recorded between 450 and 850 nm (Fig. 3, top). The spectrum is temperature-dependent in all regions. The magnetic-field-dependence of the band of 559.5 nm at temperatures of 1.6, 4.2 and 10 K is also shown (Fig. 3, bottom). The magnetization properties and the form of the spectrum are very similar to that of high-spin Fe³⁺ haem [9]. The electronic transition between 600 and 850 nm arises from metal-based orbitals and are the *d-d* transitions of the high-spin Fe²⁺ haem [10]. The peak at 743 nm varies in wavelength with protein environment. It occurs at 757 nm in deoxymyoglobin (horse heart) and at 766 nm in the photolysed form of CO-myoglobin [11].

The m.c.d. results show that, at liquid-He temperatures, the haem group in the reduced state of the protein is in the high-spin ferrous (*S* = 2) state and hence five-co-ordinate. The reduction of the haem has brought about a loss of one His ligand.

DISCUSSION

The combined data from low-temperature e.p.r. and m.c.d. spectroscopy confirm the earlier report that the haem group in cytochrome *c*^o undergoes a redox-linked change from low-spin in the Fe³⁺ to high-spin in the Fe²⁺ forms. The m.c.d. data clearly show that the haem co-ordination changes from six to five on reduction. The evidence from the Fe³⁺-state CT band at 1560 nm points unambiguously to co-ordination by two His residues. However, the orientation of the two ligands is highly unusual for a protein environment. The evidence for this assertion comes from two sources. First, the e.p.r. signal from this haem at *g*_z = 3.65 has a folded form or ramp-shape. *g*_x and *g*_y are undetectable. The value of *g*_z is approaching the theoretical limit of *g*_z = 4.0, *g*_x = *g*_y = 0, predicted to arise when the rhombic distortion, *V*/*λ*, falls to zero and the axial parameter *Δ*/*λ*, becomes infinitely large.

Secondly, the intensity of the m.c.d. associated with the CT band at 1560 nm is unusually intense for a bis-His-co-ordinated haem. Table 1 shows the parameters of the near-i.r. m.c.d. CT bands of a variety of bis-His-, His-imidazole- and bis-imidazole-co-ordinated haems in proteins and as models. The high *Δε* values of [Fe(OEP)(2-Me-imid)₂]⁺ and cytochrome *c*^o correlate with the high *g*_z values of folded-line shape. The crystal structure of low-spin bis-(2-methylimidazole(*meso*-tetraphenylporphinato))-Fe³⁺ shows that the bulky methyl substituent in the 2-position of the imidazole ligand sterically hinders the formation of the axial Fe-N bond [12]. To relieve this hindrance there is a significant *S*₄ ruffling of the porphinato core and alignment of the imidazole ring plane lies between two iron-porphyrin N bonds. This is an unusual orientation of the imidazole plane which more usually is close to eclipsing a Fe-N pyrrole bond. Even more significant is the fact that the orientations of the two imidazole planes above and below the porphyrin plane are ~ 89°. This mutually perpendicular alignment of the two imidazole planes arises from a need to accommodate the bulky methyl groups. This leads to an iron site which is close to axial with a small or zero rhombic component. The e.p.r. spectrum of the crystalline form of this complex shows a broad signal at *g*_z = 3.56. When dissolved in a

Table 1. Near-i.r. m.c.d. spectra and e.p.r. *g*-values of His. and imidazole co-ordinated ferric haems

The wavelengths (*λ*) of the peaks of the near-i.r. CT bands, as located by low-temperature m.c.d. spectroscopy, of a range of bis-imidazole, imidazole-His and bis-His co-ordinated haems as models and in proteins are shown. Also given are the intensities of the peaks quoted as *Δε* (= *ε*_L - *ε*_R) at 4.2 K and 5 T, and the principal *g*-values determined by e.p.r. spectroscopy. Abbreviations used: Fe(OEP), ferric octaethylporphyrin; 1-Me-imid, 1-methylimidazole; 2-Me-imid, 2-methylimidazole; 4-Me-imid, 4-methylimidazole.

Haem	Reference	<i>Δε</i> (M ⁻¹ cm ⁻¹)*	<i>λ</i> (nm)†	<i>g</i> -values‡
Fe(OEP)(imid) ₂	[28]	180	1575	2.98 2.24 1.47
Cyt <i>c</i> ₃ (Norway)	[2]	234	1548	3.02 2.25 1.50
Cyt <i>c</i> ₃ (<i>D. vulgaris</i>)	[2]	206	1502	2.96 2.29 1.59
Lb(imid)	[2]	215	1630	3.03 2.29 1.50
Lb(1-Me-imid)	[2]	236	1640	2.98 2.76 1.50
Met-Mb (4-Me-imid)	[2]	203	1506	2.92 2.5 1.54
Fe(OEP)(2-Me-imid) ₂	[28]	370	1475	3.53 - -
Cyt <i>c</i> ^o (<i>M. methylotrophus</i>)	(this work)	400	1560	3.65 - -
Cyt <i>b</i> ₁ , formate dehydrogenase (<i>Ps. aeruginosa</i>)	[29]	380	1545	3.77 - -

* *Δε* values measured at 4.2 K and 5 T of near-i.r. CT band.

† *λ*-wavelength of peak maximum in near-i.r. m.c.d.

‡ E.p.r. *g*-values at X-band.

DMF glass the *g*-value is 3.4 with the folded line-shape [13]. Hence it can be concluded both as a result of these experimental findings and theoretical predictions that the high *g*_z value is associated with an almost perpendicular alignment of the two imidazole planes. Given that there is only one example so far whose crystal structure is known, it is not yet possible to describe a quantitative relationship between axial ligand plane orientation, *g*_z values and m.c.d. intensity. Theoretical analysis of the m.c.d. intensity shows that the *Δε* values approach a limit when *λ*/*V* is > 1.0 [3]. In other words, the m.c.d. intensity will be relatively insensitive to variations in angle close to 90°.

The e.p.r. evidence and the near-i.r. m.c.d. data single out ferricytochrome *c*^o as a clear example of a bis-His co-ordinated haemoprotein in which the relative orientations of the planes of the two His ligands are close to 90° [12]. It is not, however, possible to make a conclusive deduction about the orientation of one His plane relative to the Fe-N pyrrole bond. To generate the particular e.p.r. and m.c.d. spectroscopic parameters the requirement is for a low rhombicity field to be generated at the Fe³⁺. An *S*₄ axis resulting from the perpendicular orientations of two His ligands achieves this.

There has been debate about the axial ligation states of a variety of cytochromes which exhibit high *g*_z values. These are the *b*-cytochromes, *b*-562 and *b*-566, from complex III of the mitochondrion, *b*-568 from Photosystem I, and *b*-558 from *Bacillus subtilis* [14-16]. However, these are all examples of membrane-bound cytochromes. A general model has been proposed for their structure [16]. This comprises several trans-membrane protein *α*-helices which provide two His ligands and thus bind the haem plane parallel to the membrane normal. It remains to be demonstrated conclusively that the ligation state in every case is indeed bis-His. Cytochrome *c*^o is a unique example of a water-soluble protein which exhibits bis-His co-ordination with near-perpendicular ligand orientation.

This protein is the second example of a soluble cytochrome which exhibits the folded line-shape, high *g*_z value in the oxidized state. The other example is cytochrome *c*₄, *Azotobacter vinelandii*,

a two-domain protein containing two haems, both of which are co-ordinated by His and Met [18]. One haem gives, in the oxidized state, a set of typical g -values that is 3.20, 2.10 (1.17) whereas the second haem has a folded line-shape g_z at 3.64. In this case we have proposed that the Met ligand, $-\text{CH}_2-\text{S}-\text{CH}_3$, is oriented close to 90° to the His plane so as to generate a field of low rhombicity at the haem [18].

Ferrocycochrome c'' contains the high-spin ($S = 2$) Fe^{2+} . This is not unambiguous proof that the iron is five co-ordinate. For example, the X-ray structure of the model complex bis-tetrahydrofuran(*meso*-tetraphenylporphinato)- Fe^{2+} shows it to have a six-co-ordinate Fe^{2+} in the porphyrin plane, although magnetic susceptibility and Mössbauer spectroscopy prove that the complex is high-spin, $S = 2$ [19]. Presumably the tetrahydrofuran ligands provide an unusually weak axial crystal field. This is consistent with the very low stability constant of the tetrahydrofuran with haem. His ligation is unlikely to provide such a weak axial field. The best evidence that the Fe^{2+} is five-co-ordinate comes from the m.c.d. spectrum between 600 and 850 nm. This region of the spectrum is similar to that of haemoproteins known to be five-co-ordinate. The transitions in this region are the Fe^{2+} $d-d$ bands whose energies reflect the nature of the axial ligands.

The loss of one His ligand on reduction to the Fe^{2+} state is unusual and unexpected. Evidence from studies of ferroporphyrins in non-aqueous solvents shows that the binding constants for the successive binding of two imidazole ligands is $K_2 > K_1$ [20]. For example, imidazole binding in benzene to deuterohaem and to *meso*-tetraphenylhaem gives $K_1 = 4.5 \times 10^3 \text{ M}^{-1}$ and $8.8 \times 10^3 \text{ M}^{-1}$ and $K_2 = 6.8 \times 10^4 \text{ M}^{-1}$ and $7.9 \times 10^4 \text{ M}^{-1}$ respectively. Hence the second ligand is bound more strongly than the first by a factor of 10. This property frustrates the synthesis in solution of five-co-ordinate Fe^{2+} models and has led to the construction of elaborate synthetic porphyrins which sterically restrict one face with a cap, strap or picket fence [21]. However, only 1 mol of 2-methylimidazole/mol will bind to simple ferrous porphyrins in benzene although with a relatively high stability constant, $K_1 = 1.25 \times 10^4 \text{ M}^{-1}$ and $2.4 \times 10^4 \text{ M}^{-1}$ for deuterohaem and *meso*-tetraphenylhaem [20]. It has been argued that the steric bulk of the methyl group in the 2-position causes the Fe^{2+} to be displaced from the haem plane towards the axial ligand, thus preventing a second ligand from making a bond to the Fe^{2+} on the opposite side of the haem plane [22]. It is now known that, in the case of ferric haem, a pair of 2-methylimidazole ligands can bind the iron and that the steric bulk of the methyl group does not lengthen the $\text{Fe}^{3+}-\text{N}(\text{imidazole})$ bond [12]. Instead the 2-methylimidazole group rotates about the normal to the haem plane to a position of low electron density bisecting the two iron pyrrole N bonds. This orientation of the imidazole plane will lead to a reduction in π -bonding between Fe^{2+} and the ligand and hence may be a factor in the loss of one axial ligand.

It is not the general case that all bis-His co-ordinated haems with high g_z -values in the oxidized state lose one axial ligand on reduction. For example, this does not apparently occur in the case of *Bacillus subtilis* cytochrome *b*-558, which remains low-spin on reduction (H. Friden, L. Hederstedt, M. R. Cheesman & A. J. Thomson, unpublished work). Other membrane-bound cytochromes *b* also appear to remain low-spin, bis-His on reduction [14].

Leghaemoglobin (Lb), the mono-haem protein from the root nodules of leguminous plants, which reversibly binds O_2 , is five-co-ordinate in the Fe^{2+} state but an oxidation acquires a second axial ligand to become low-spin [23]. This ligand is the distal histidine residue [24]. The proportion of low-spin six-co-ordinate haem in the oxidized form depends upon the source of the Lb. For example, oxidized soya-bean Lb *c* has only a small percentage

of low-spin haem whereas proteins from kidney bean [25] and *Sesbania* (C. A. Appleby, D. Bogusz, P. M. A. Gadsby & A. J. Thomson, unpublished work) appear to be almost totally low-spin in the Fe^{3+} state although they are totally five-co-ordinate in the reduced state. This property has been ascribed by Appleby and co-workers [26] to the high mobility of the distal His residue in the haem pocket. He associates the flexibility of this group with the unusually high on rate of O_2 in the deoxy form which gives Lb a very high binding constant for O_2 [27]. It is not known what factors influence the change in the spin state of Lb on oxidation and reduction. It is unlikely to be a mechanism similar to that suggested here for cytochrome c'' because the low-spin ferric form of Lb give e.p.r. signals which are normal, not the fielded line-shape characteristic of near-perpendicular bis-His orientation.

Cytochrome c'' provides an interesting example of a protein containing a haem group which, on reduction, releases a basic group, the His residue. The N-3 position of His in aqueous solution has a $\text{p}K_a = 6.5$. Hence reduction of cytochrome c'' at pH values less than this should cause the protein to bind a proton at His N-3. Oxidation of the haem would lead to loss of this proton. Hence this protein may provide an example of a redox-linked proton uptake/release process. One such case is provided by *Escherichia coli* cytochrome *b*-562, in which a co-ordinated His ligand loses or gains a proton at the N-1 position with a $\text{p}K_a = 8.5$ [30]. Redox-linked proton and release mechanisms are an essential component of a proton pump driven by electron transfer. Cytochrome c'' may provide a simple model of one component of a membrane-bound proton pump.

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REFERENCES

1. Santos, H. & Turner, D. L. (1988) *Biochim. Biophys. Acta* **954**, 237–286
2. Gadsby, P. M. A. & Thomson, A. J. (1990) *J. Am. Chem. Soc.* **112**, 5003–5011
3. Thomson, A. J. & Gadsby, P. M. A. (1990) *J. Chem. Soc. Dalton Trans.* 1921–1928
4. Eglinton, D. G., Gadsby, P. M. A., Sievers, G., Peterson, J. & Thomson, A. J. (1983) *Biochim. Biophys. Acta* **742**, 648–658
5. George, S. J. (1987) Ph.D. Thesis, University of East Anglia
6. Salerno, J. C. (1984) *J. Biol. Chem.* **259**, 2331–2336
7. Cheng, J. C., Osborne, G. A., Stephens, P. J. & Eaton, W. A. (1973) *Nature (London)* **24**, 193–194
8. Smith, D. W. & Williams, R. J. P. (1970) *Struct. Bonding* **7**, 1–45
9. Thomson, A. J. & Johnson, M. K. (1980) *Biochem. J.* **191**, 411–420
10. Eaton, W. A., Hanson, L. K., Stephens, D. J., Sutherland, J. C. & Dunn, J. B. R. (1978) *J. Am. Chem. Soc.* **100**, 4991–5003
11. Sharanov, Y. A., Sharanova, N. A., Figlowsky, V. A. & Grigorjev, V. A. (1982) *Biochim. Biophys. Acta* **709**, 332–341
12. Scheidt, W. R., Kerner, J. F., Hoard, J. L. & Reed, C. A. (1987) *J. Am. Chem. Soc.* **109**, 1963–1968
13. Walker, F. A., Huynh, B.-H., Scheidt, W. R. & Osvath, S. R. (1986) *J. Am. Chem. Soc.* **108**, 5288–5297
14. Siedow, J. N., Power, S., de la Rosa, F. F. & Palmer, G. (1978) *J. Biol. Chem.* **253**, 2392–2399
15. Palmer, G. (1984) in *Iron Porphyrins*, part 2 (Lever, A. B. P. & Gray, H. B., eds.), Addison-Wesley, Reading
16. Andersson, K. K. & Hederstedt, L. (1986) *J. Bacteriol.* **167**, 639–735
17. Widger, W. R., Cramer, W. A., Hermann, R. G. & Trebst, A. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 674–678
18. Gadsby, P. M. A., Hartshorn, R. F., Moura, J. J. G., Sinclair-Day, J. D., Sykes, A. G. & Thomson, A. J. (1989) *Biochim. Biophys. Acta* **994**, 37–46
19. Reed, C. A., Mashiko, T., Scheidt, W. R., Spartalian, K. & Lang, G. (1980) *J. Am. Chem. Soc.* **102**, 2302–2306

20. Brault, D. & Rougee, M. (1974) *Biochemistry* **13**, 4591–4597
21. Hashimoto, T., Dyer, R. L., Crossley, M. J., Baldwin, J. E. & Basolo, F. (1982) *J. Am. Chem. Soc.* **104**, 2101–2109
22. Collman, J. P. & Reed, C. A. (1977) *J. Am. Chem. Soc.* **99**, 2048–2049
23. Appleby, C. A., Blumberg, W. E., Peisach, J., Wittenberg, B. A. & Wittenberg, J. B. (1976) *J. Biol. Chem.* **251**, 6090–6096
24. Wright, P. E. & Appleby, C. A. (1977) *FEBS Lett.* **78**, 61–66
25. Maskall, C. S., Gibson, J. F. & Dart, P. J. (1977) *Biochem. J.* **167**, 435–445
26. Appleby, C. A., Blumberg, W. E., Bradbury, J. H., Fuchsman, W. H., Peisach, J., Wittenberg, B. A., Wittenberg, J. B. & Wright, P. E. (1982) in *Hemoglobin and Oxygen Binding* (Chien, H., ed.), pp. 435–441, Elsevier/North-Holland, New York
27. Gibson, Q. H., Wittenberg, J. B., Wittenberg, B. A., Bogusz, D. & Appleby, C. A. (1989) *J. Biol. Chem.* **264**, 100–107
28. Gadsby, P. M. A. & Thomson, A. J. (1986) *FEBS Lett.* **197**, 253–257
29. Godfrey, C., Coddington, A., Greenwood, C., Thomson, A. J. & Gadsby, P. M. A. (1987) *Biochem. J.* **243**, 225–233
30. Moore, G. R., Williams, R. J. P., Peterson, J., Thomson, A. J. & Mathews, F. S. (1985) *Biochim. Biophys. Acta* **829**, 83–96

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