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Identification of the ligand-exchange process in the alkaline transition of horse heart cytochrome *c*

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Magnetic-circular-dichroism (m.c.d.) spectra over the wavelength range 300–2000 nm at room temperature and at 4.2 K of horse heart cytochrome *c* are reported at a series of pH values between 7.8 and 11.0, encompassing the alkaline transition. The effect of glassing agents on the e.p.r. spectrum at various pH values is also reported. Comparison of these results with spectra obtained for the n-butylamine adduct of soybean leghaemoglobin support the hypothesis that lysine is the sixth ligand in the alkaline form of horse heart cytochrome *c*. The m.c.d. and e.p.r. spectra of horse heart cytochrome *c* in the presence of 1-methylimidazole have also been examined. These studies strongly suggest that histidine-18, the proximal ligand of the haem, is the ionizing group that triggers the alkaline transition. Low-temperature m.c.d. and e.p.r. spectra are also reported for *Pseudomonas aeruginosa* cytochrome c_{551}. It is shown that no ligand exchange takes place at the haem in this species over the pH range 6.0–11.3.

INTRODUCTION

Cytochromes of the *c* type are low-M_r (80–130 amino acids) electron-transfer proteins that occur widely in respiratory and photosynthetic systems. They are relatively well characterized in terms of both molecular and electronic structures (Mathews, 1985). Crystallographically determined structures are available for several of them (Adman, 1979). They all contain a single protohaem covalently linked via two thioether bonds to the polypeptide chain. The low-spin haem is co-ordinated by two ligands provided by the protein, which at neutral pH are histidine and methionine in both the ferri and ferro states. It is assumed that the haem retains this co-ordination geometry throughout its redox cycle. However, it has been shown by numerous spectroscopic studies that in the ferri state the methionine–histidine co-ordination of the haem is not the only ligand arrangement possible. Variations in pH, ionic strength and temperature can bring about ligand exchange at the haem group (Greenwood & Wilson, 1971). Several low-spin, as well as some high-spin, states are formed, especially at elevated pH values. Much experimental effort has been expended in attempting to prove the structures of these other states. In particular, a high-pH state of horse heart cytochrome *c* produced with a pK_a of 9.3 has been intensively studied. This state, which retains its stable globular nature (as judged by viscosity measurements), was first identified by kinetic studies of its reduction with ascorbate, dithionite and ferrocyanide (Greenwood & Palmer, 1965; Brandt *et al.*, 1966). Two forms of cytochrome *c* at alkaline pH have been identified: the lower-pH form, type III, is ascorbate-reducible; the other, type IV, is ascorbate-irreducible. The alkaline transition has also been observed with a number of spectroscopic techniques, including optical absorption (Theorell & Akesson, 1941*a*,*b*,*c*; Greenwood & Wilson, 1971), and e.p.r. (Lambeth *et al.*, 1973; Brautigan *et al.*, 1977), n.m.r. (Gupta & Koenig, 1971; Wooten *et al.*, 1981) and by resonance-Raman (r.R.) (Kitigawa *et al.*, 1977) spectroscopies. All these techniques have yielded a pK_a of 9.0–9.4 when fitted to a single-proton transition. However, kinetic studies have shown that the alkaline transition is not a simple one-proton ionization. Davis *et al.* (1974) proposed that the transition is due to a single ionization of ferricytochrome *c* at a pK_a of 11 that is coupled to a conformational change. The slow conformational change displaces the pK_a of 11 to produce an apparent pK of approx. 9.

Interest in the alkaline state has centred on the structure of the species formed and also on the nature of the group with an ionization at pK_a of 11. Most of the studies of the structure of the alkaline form have reached the conclusion that this state has lost the methionine-80 haem ligand. Since this form is completely low-spin, it is presumed to be six-co-ordinate. Although histidine-18 is assumed to remain as the proximal ligand, the identity of the sixth ligand is controversial. The ϵ-amino group of one of the nineteen lysine residues has been suggested, on the basis of spectroscopic results from e.p.r., n.m.r. and r.R. Chemical modifications of lysine designed to inhibit its ability to ligand the haem iron have been carried out by using acetylation, amidination, trifluoroacetylation and (trifluoro)phenylcarbonylation (Bosshard, 1981; Smith & Millett, 1980; Wilgus & Stellwagen, 1974). However, the results from the chemical studies are rather contradictory. Wilgus & Stellwagen (1974), for example, purport that lysine-79 is the haem ligand above pH 9, whereas Bosshard (1981) concludes definitely that this residue cannot be the ligand. We therefore re-examined the problem, employing yet another spectroscopic technique, namely near-i.r. m.c.d. spectroscopy.

In a wide-ranging study, Brautigan *et al.* (1977) used low-temperature e.p.r. spectroscopy to identify several low-spin forms of ferricytochromes *c* of various eukaryotic and prokaryotic species. An attempt was made to establish a correlation between e.p.r. principal

Abbreviations used: m.c.d., magnetic c.d.; r.R., resonance Raman; CT, charge transfer; hh, horse heart; Lb, leghaemoglobin; Caps, 3-(cyclohexylamino)propanesulphonic acid; Ches, 2-(cyclohexylamino)ethanesulphonic acid.

g-values (and parameters derived from them) and the state of ligation of the haem. This technique has since been applied to more complex proteins containing *c*-type cytochromes and cytochromes of unknown structure, as a form of diagnosis for the nature of axial ligands. However, this method is not always secure, especially as it has recently been shown that haem groups with the same axial ligation type can have significantly different e.p.r g-values (Salerno, 1984). There is therefore interest in having a different type of spectroscopic probe which can assist in axial-haem-ligand assignments and make the e.p.r. method more secure. Furthermore, since the e.p.r. spectrum can be detected only at cryogenic temperatures, the possible presence of thermal-spin equilibria or temperature-dependent ligand changes may be missed.

It has been pointed out by several authors that the energies of the porphyrin (π)-to-ferric (d) charge-transfer (CT) bands are sensitive to the ligand field created at the metal ion by the groups which surround it (Smith & Williams, 1970). The lowest-energy CT bands lie in the wavelength range 800–2000 nm. These can be difficult to detect by conventional absorption spectroscopy because the wavelength region beyond $\sim$ 1400 nm is partially obscured by the vibrational overtone bands, of both the solvent and the protein. The use of deuterated solvents and protein samples which have been exchanged in ^2H_2O substantially diminish this background, but it is not entirely eliminated. However, m.c.d. spectroscopy gives signals whose intensities depend on ground- and excited-state g-values as well as electric dipole transition moments. Since vibrational g-values are smaller than electronic g-values by a factor of $\sim 10^3$, the vibrational overtones give exceedingly weak m.c.d. signals in comparison with electronic absorption bands (Keiderling, 1981). Hence m.c.d. spectroscopy allows the unambiguous detection of low-energy electronic states in the presence of overlapping vibrational absorption bands, that is, between 1000 and 2500 nm. The m.c.d. spectra of paramagnetic molecules are temperature-dependent, increasing in magnitude as the temperature is lowered. Therefore an additional sensitivity advantage accrues to measurements of m.c.d. spectra carried out at cryogenic temperatures. Nevertheless, the technique does allow CT bands to be detected at all temperatures between 1.5 and 300 K and so gives a means of monitoring changes in thermal equilibria.

In order to use the technique of near-i.r. m.c.d. spectroscopy for the diagnosis of the axial ligands of a low-spin ferric haem, it is necessary to construct an empirical scale of the energies of the CT bands in haemoproteins of well-defined states of axial ligation. The energy of the band is also expected to depend upon the nature of the porphyrin ring, since the donor orbitals are the highest occupied π levels of the ring. A limited number of experiments have been carried out by ourselves (Thomson *et al.*, 1985) and other groups of workers (Stephens *et al.*, 1974; Rawlings *et al.*, 1977) in order to construct empirical scales, and also to investigate the potential range of energies with a defined axial-ligand set.

In the present paper we report the room-temperature and 4.2 K m.c.d. of horse heart (hh) cytochrome *c* over the pH range of the alkaline transition. It has also been necessary to carry out a systematic e.p.r. study of the effect on the axial ligation of adding the glassing agents ethanediol and glycerol, which are required for the low-temperature m.c.d. experiments to enable good-optical-quality samples to be obtained. We find that, in the case of hh cytochrome *c*, the glassing agent has a pronounced effect on the species observed at a given pH value. The spectra have been recorded as a function of pH in order to observe the changes that occur at high pH when the axial ligand, methionine, is presumably lost.

We also report the characterization of the oxidized states of hh and *Pseudomonas aeruginosa* cytochrome c_{551}, by a combined e.p.r. and m.c.d. study. The m.c.d. spectra are recorded throughout the Soret, visible, red and near-i.r. regions, over the wavelength range 300–2000 nm, to provide a set of data diagnostic of haem axial ligation of cytochromes *c*. These two proteins were selected for study because they are both crystallographically defined and they represent one example each of a mammalian and a bacterial class of proteins. The main structural difference between these proteins is a deletion of 15 residues in the bacterial protein.

EXPERIMENTAL PROCEDURES

Hh cytochrome *c* (type III) was obtained from Sigma and used without further purification. *Pseudomonas aeruginosa* cytochrome c_{551} was extracted and purified as described previously (Parr *et al.*, 1976). 1-Methylimidazole and ^2H_2O (99.8 atom% ^2H) were obtained from Aldrich. Glycerol and ethanediol were of AnalaR grade and were purchased from BDH.

Derivatives of hh cytochrome *c* were prepared by adding the exogenous ligand until no further change was observed by absorption spectroscopy.

E.p.r. spectra were recorded by using a Bruker ER 200D spectrometer fitted with an ESR-9 flow cryostat (Oxford Instruments). M.c.d. spectra at low temperature were measured either with a JASCO J-500D dichrograph fitted with an SM 4 superconducting solenoid operating at 5 T (Oxford Instruments) or in a near-i.r. dichrograph constructed in this laboratory. Room-temperature m.c.d. was measured as described above, except that the magnet used was capable of generating a field of 6 T.

Samples for near-i.r. m.c.d. measurement were prepared in ^2H_2O and the p^{2}H values quoted are the meter readings corrected for the small isotope effect by addition of 0.4 pH unit.

RESULTS AND LIGAND ASSIGNMENTS

hh cytochrome *c*

pH titration at room temperature. The room-temperature m.c.d. of hh cytochrome *c*, measured at 6 T in the near-i.r. region (800–2000 nm), is shown in Fig. 1. The spectra presented cover the p^{2}H range 7.8–11.0. At low p^{2}H the spectrum consists of a single peak at 1725 nm and a shoulder at $\sim$ 1550 nm with $\Delta\epsilon$ values of 0.8 and 0.51 $M^{-1}\cdot cm^{-1}\cdot T^{-1}$, in close agreement with previous reports (Rawlings *et al.*, 1977). As the p^{2}H is raised through the alkaline pK_a the intensity of the peak at 1725 nm decreases, with a concomitant increase of intensity at 1465 nm. At p^{2}H 11.0 this transition is virtually complete, producing a single low-spin ferric haem with a peak at 1465 nm and a shoulder at 1280 nm, with $\Delta\epsilon$ values of 1.28 and 0.56 $M^{-1}\cdot cm^{-1}\cdot T^{-1}$ respectively. The p^{2}H-dependence of the 695 nm absorption intensity and the intensity of the 1725 nm and 1465 nm

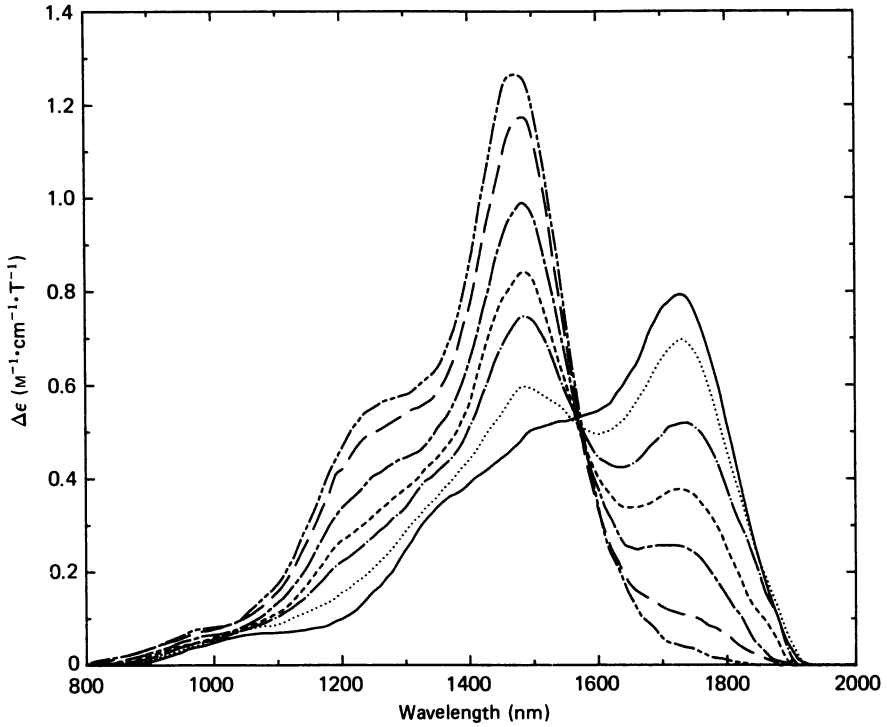


Fig. 1. Near-i.r. m.c.d. spectra of hh cytochrome *c* at room temperature, showing the effect of change of p²H

——, p²H 7.8; · · · · ·, p²H 8.6; —·—, p²H 9.1; -----, p²H 9.3; — - —, p²H 9.7; ——, p²H 10.2; —— - ——, p²H 11.0. All samples were in 50 mM-Caps + 50 mM-Ches + 50 mM-Taps buffer. The pathlength was 2 mm; the magnetic field was 6T.

peaks in the near-i.r. m.c.d. are shown in Fig. 2. The curve plotted through these data is a theoretical curve for a single proton ionization with a pK of 9.3. Clearly the close similarity between the absorption and m.c.d. data shows that the disappearance of the 695 nm band and the 1725 nm peak in the m.c.d. spectrum must have a common origin. Therefore we ascribe the spectrum in Fig. 1, showing a peak at 1465 nm, to the alkaline form of hh cytochrome *c*. The pK_a of 9.3 observed by m.c.d. is in excellent agreement with that determined by other techniques.

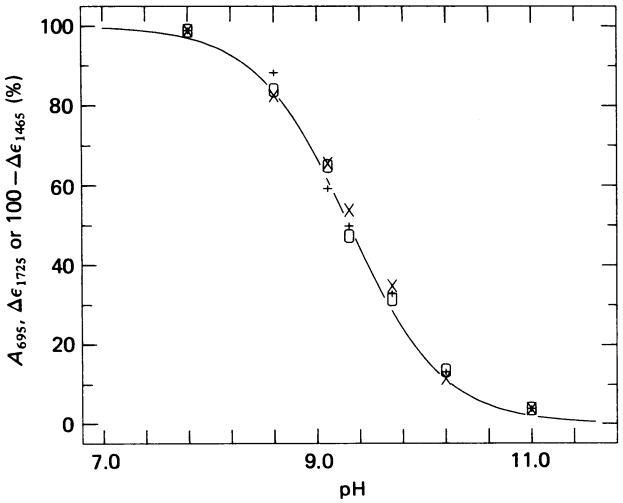


Fig. 2. pH-dependence of the 695 nm absorption and of the near-i.r. m.c.d. intensity of hh cytochrome *c* at room temperature

+, A_{695}; ○, % $\Delta\epsilon_{1725}$; ×, 100 − % $\Delta\epsilon_{1465}$. The smooth curve is a theoretical curve plotted for a transition with a pK of 9.3.

pH titration at low temperature. One of the surprising properties of hh cytochrome *c* is its tendency to form multiple species, as detected by e.p.r. spectroscopy, depending on pH, buffer and ionic strength. In order to obtain m.c.d. spectra of well-defined protein structures, conditions were sought under which a single species exists. This was not successful in all cases. The presence of glassing agents is required for low-temperature m.c.d. studies. Thus a series of e.p.r. studies were carried out in the neutral and alkaline pH range, and in the presence of two glassing agents, namely ethanediol and glycerol.

Figs. 3(*a*), 3(*b*) and 3(*c*) show the e.p.r. spectra of hh cytochrome *c* at three different p²H values, namely 6.6 (50 mM-sodium phosphate), 10.8 (100 mM-sodium tetraborate) and 11.0 (50 mM-Caps). Only at p²H 6.6 is a single low-spin ferric species observed, with the *g*-values 3.05, 2.25 and 1.26 in reasonable agreement with values of 3.06, 2.24 and 1.24 previously reported by Salmeen & Palmer (1968). At the two higher p²H values, multiple species are observed that are clearly different from the neutral-pH form characterized by a g_z value of 3.05. At p²H 10.8 in tetraborate buffer (Fig. 3*b*), a complex spectrum is obtained showing a major species with $g_z = 3.42$, a shoulder at $g_z = 3.17$ and a weak shoulder at $g_z = 3.6$. This spectrum is identical with that reported by Lambeth *et al.* (1973). The peak at $g = 3.42$ is presumably the alkaline species, but that with $g_z = 3.17$ is difficult to assign. Caps buffer at p²H 11.0 generates two low-spin ferric species with g_z values at 3.50 and 3.33.

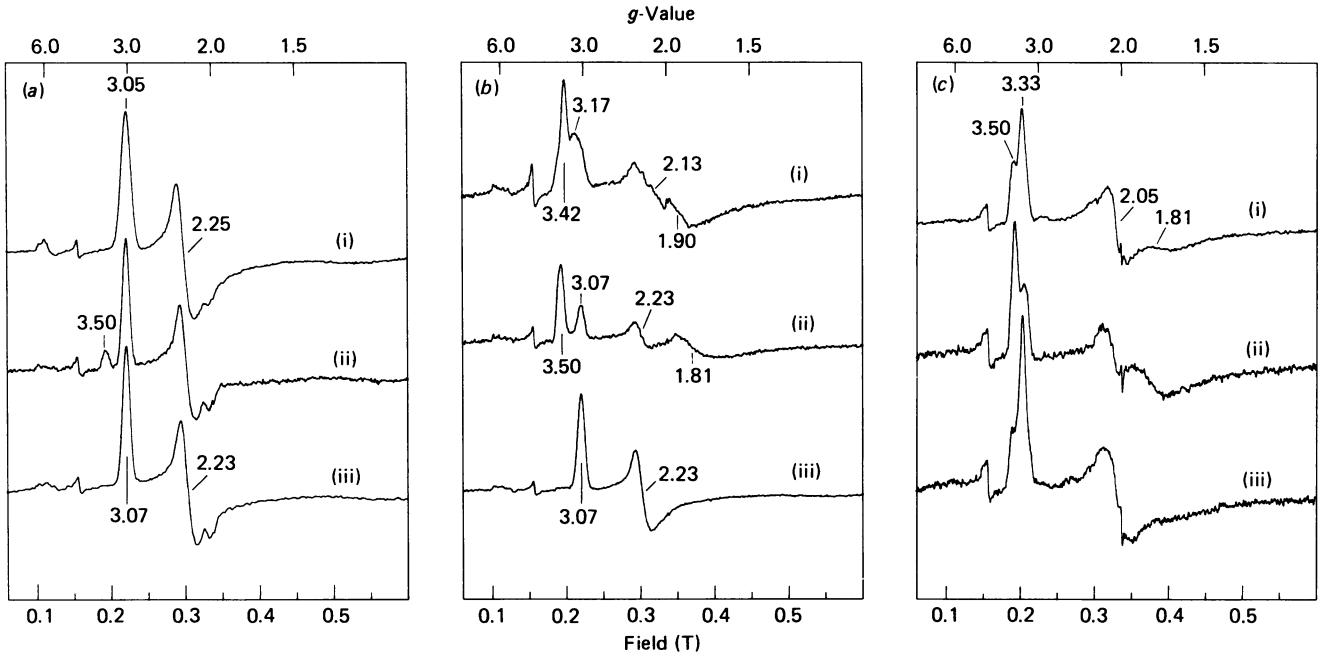


Fig. 3. E.p.r. spectra of hh cytochrome *c* at p²H 6.6 (*a*), p²H 10.8 (*b*) or (*c*) p²H 11.0

(*a*): (i) 50 mM-Phosphate buffer; [cytochrome *c*] = 1.2 mM; gain, 1.6×10^5; (ii) 25 mM-phosphate buffer containing 50% ethanediol; [cytochrome *c*] = 600 μM; gain, 3.2×10^5; (iii) 25 mM-phosphate buffer containing 50% glycerol; [cytochrome *c*] = 600 μM; gain, 3.2×10^5. (*b*): (i) 100 mM-Tetraborate buffer; [cytochrome *c*] = 1.0 mM; gain, 3.2×10^5; (ii) 50 mM-tetraborate buffer containing 50% ethanediol; [cytochrome *c*] = 500 μM; gain, 6.4×10^5; (iii) 50 mM-tetraborate buffer containing 50% glycerol; [cytochrome *c*] = 500 μM; gain, 6.4×10^5. (*c*) (i) 50 mM-Caps buffer; [cytochrome *c*] = 1.0 mM; gain, 3.2×10^5; (ii) 25 mM-Caps buffer containing 50% ethanediol; [cytochrome *c*] = 500 μM; gain, 6.4×10^5; (iii) 25 mM-Caps buffer containing 50% glycerol; [cytochrome *c*] = 500 μM; gain, 6.4×10^5. Conditions for (*a*), (*b*) and (*c*) were: temperature 10 K; microwave frequency 9.40 GHz; power, 2.01 mW.

The effect of the addition of 50% (v/v) ethanediol and of 50% (v/v) glycerol to these three buffers is also shown in Figs. 3(*a*), 3(*b*) and 3(*c*). At p²H 6.6, addition of ethanediol leads to the generation of an additional low-spin species at $g_z = 3.50$, whereas glycerol merely sharpens the spectrum somewhat. Therefore the latter solvent system was employed in the m.c.d. measurements. Addition of the glassing agents to the tetraborate buffer at p²H 10.8 leads to simplification of the e.p.r. spectra. For example, 50% glycerol causes the spectrum to revert to that of a species similar to that of hh cytochrome *c* at p²H 6.6, namely that with *g*-values of 3.07 and 2.23. On the other hand, 50% ethanediol gives a mixture of this form and another with *g*-values of 3.50 and 1.81. In the case of Caps buffer at p²H 11.0 (Fig. 3*c*), addition of 50% ethanediol causes a change in the relative concentrations of the two species observed in the absence of glassing agent. Thus the species with $g_z = 3.50$ increases sharply in concentration compared with the species having a *g*-value of 3.33. Glycerol (50%) causes the latter species to dominate almost totally. The two species with g_z values of 3.50 and 3.33 appear to be interconvertible at this pH in a manner dependent upon the nature of the solvent. Exact reproduction of these spectra is difficult, as these two forms apparently interconvert very readily. This phenomena may depend on the freezing rate of the sample and could be due to polymerization of the protein (Margoliash & Schejter, 1966). The m.c.d. spectrum of hh cytochrome *c* at p²H 11.0 in Caps was measured in the presence of 50% ethanediol, since this contained the highest concentration of the species with $g_z = 3.50$.

The m.c.d. spectra at 4.2 K of three of the samples characterized by e.p.r. spectroscopy are shown in Figs. 4(*a*) and 4(*b*) in the visible and near-i.r. regions respectively. The samples were chosen to enable the m.c.d. characterization of the neutral form of hh cytochrome with $g_z = 3.05$–3.07 and the high-pH form with $g_z = 3.50$. Hence the samples at p²H 6.6 in 50% glycerol, at p²H 10.8 in 50% ethanediol and at p²H 11.0 in 50% ethanediol were selected.

In the visible region of the m.c.d. spectrum (Fig. 4*a*) there are a series of positive and negative peaks which are typical of a six-co-ordinate low-spin ferric haem at this temperature (Eglinton *et al.*, 1983). At p²H 6.6 there is a positive m.c.d. peak at 695 nm that is the counterpart of the band in the absorption spectrum at the same wavelength. At higher p²H values minor changes occur in the visible-region m.c.d. that are not very useful diagnostically. However, the disappearance of the 695 nm band at p²H 11.0 (Fig. 4*a*, spectra iii) and its presence at p²H 10.8 at a level of ~ 25% of the intensity at p²H 6.6 (Fig. 4*a*, spectra i and ii) agree well with the e.p.r. spectra presented in Fig. 3, on the assumption that only the native methionine–histidine form of hh cytochrome *c* has a 695 nm band and is also characterized by a g_z value of 3.05.

The near-i.r. m.c.d. (Fig. 4*b*) shows a single low-spin ferric species giving a positive peak at 1750 nm and a shoulder at 1450 nm. At p²H 10.8 in 50% ethane-

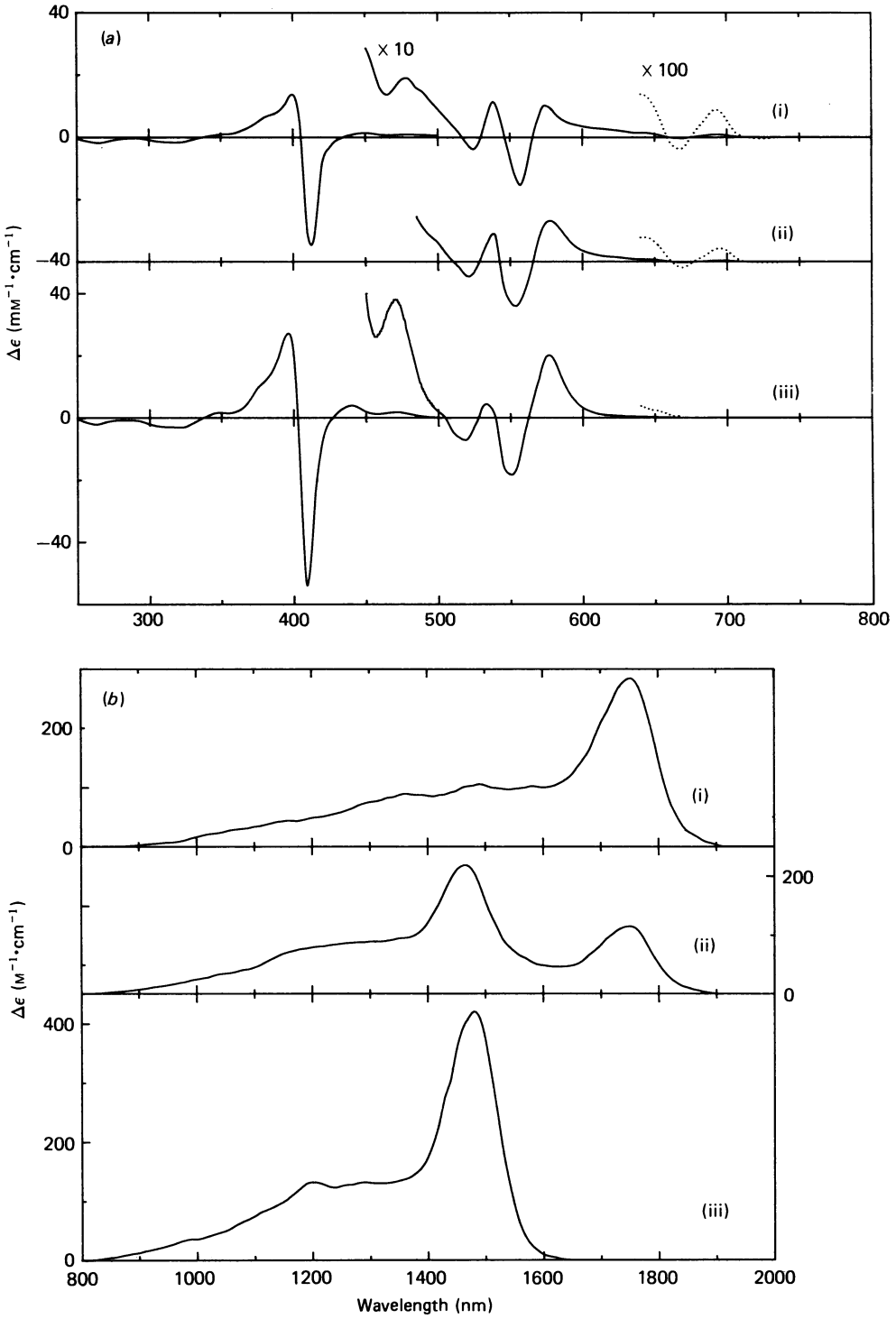


Fig. 4. (*a*) Near-u.v.–visible-region and (*b*) near-i.r. m.c.d. spectra of hh cytochrome *c* at 4.2 K and 5.0 T

(*a*): (i) p²H 6.6; 25 mM-phosphate buffer containing 50% glycerol; [cytochrome *c*] = 235 μM and 19.6 μM: (ii) p²H 10.8; 50 mM-tetraborate buffer containing 50% ethanediol; [cytochrome *c*] = 900 μM; (iii) p²H 11.0; 25 mM-Caps buffer containing 50% ethanediol; [cytochrome *c*] 270 μM and 13.6 μM. (*b*) Sample conditions were as for (*a*).

diol/tetraborate buffer the near-i.r. m.c.d. spectrum consists of two species. One, with a peak at 1750 nm, is clearly the neutral form and corresponds to the e.p.r. species with a g_z of 3.07 in Fig. 3(*b*) (ii). The second species shows a peak at 1480 nm and must correspond to the e.p.r. resonance at $g_z = 3.50$. In Caps buffer at p²H 11.0 in the presence of 50% ethanediol the e.p.r. spectrum exhibits two g_z resonances at 3.50 and 3.33, the former being the more intense.

The near-i.r. m.c.d. (Fig. 4*b*), however, shows only one peak at 1480 nm, with a shoulder at 1200 nm, suggesting the presence of a single type of axial ligation at the haem in this sample.

The species with an m.c.d. peak at 1750 nm and a band

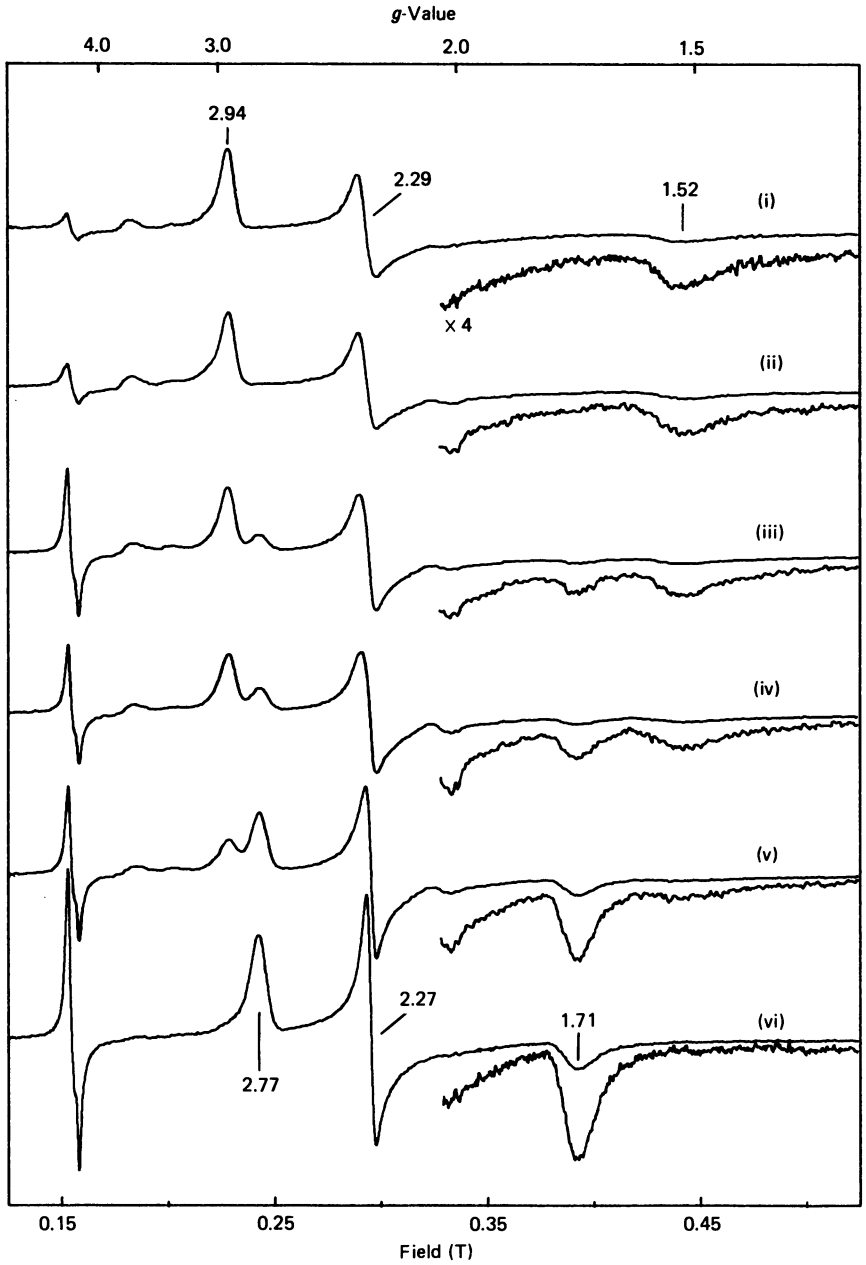


Fig. 5. E.p.r. spectra of the 1-methylimidazole adduct of cytochrome *c*

(i) pH 10.0; (ii) pH 10.5; (iii) pH 11.2; (iv) pH 11.4; (v) pH 11.75; (vi) pH 12.0. All samples were in 50 mM-Caps + 50 mM-Ches + 50 mM-Taps buffer. Conditions: [protein] = 800 μM; [1-methylimidazole] = 800 mM; temperature, 10 K; microwave frequency, 9.40 GHz; power, 2.01 mW; gain, 1.25×10^5.

at 695 nm can be assigned unequivocally to ferric haem co-ordinated by methionine and histidine ligands. The e.p.r. spectrum of this, the native species, has a g_z value of 3.05–3.07. The near-i.r. m.c.d. spectrum detects at high pH a second species characterized by a peak at 1480 nm and lacking a 695 nm band or any bands in the region 600–800 nm. This species can be assigned to the e.p.r. species with $g_z = 3.50$. Although the e.p.r. spectra of the high-pH forms show two low-spin ferric species, it is clear from the relative intensities that the 1480 nm peak belongs to the species with $g_z = 3.50$. For example, in tetraborate buffer (p^{2}H 10.8)/ethanediol, the e.p.r. spectrum shows a mixture of the native form ($g_z = 3.07$) and the form with $g_z = 3.50$. The near-i.r. m.c.d. spectrum shows two species also: the native form at 1750 nm and a band at 1480 nm which may be assigned to the species with $g_z = 3.50$. The interpretation of the nature of the species present in Caps buffer (p^{2}H 11.0)/ethanediol is less clear. The native form is absent, as judged by both e.p.r. and near-i.r. m.c.d. The form with $g_z = 3.50$ predominates and is evident in the near-i.r. m.c.d. The species with $g_z = 3.33$ is difficult to assign, since there are no features in the near-i.r. m.c.d. spectrum that can be unambiguously assigned to it. Therefore this species either has a near-i.r. m.c.d. peak at 1480 nm overlaying that of the $g_z = 3.50$ form or lies at

shorter wavelengths and is lying below the m.c.d. intensity between 800 and 1400 nm. We have no reason to choose between these two possibilities.

1-Methylimidazole complex of hh cytochrome *c*. In order to investigate the possibility that the pK_a of 11 in ferricytochrome *c* observed in kinetic studies by Davis *et al.* (1974) and linked to a conformational change may be due to the ionization of the N-1 of the proximal histidine ligand of the haem iron, a pH titration of the 1-methylimidazole adduct of cytochrome *c* between pH 10 and 12 has been carried out by e.p.r. spectroscopy. 1-Methylimidazole was chosen because it is a ligand that will maintain the haem in the low-spin ferric state and because it is neutral and hence unlikely to shift the pK_a of the histidine group very significantly. The methyl group blocks the N-1 position of the imidazole and avoids the possibility of confusion between the ionization of the N-1 of the added imidazole and the histidine ligand. The results are presented in Fig. 5.

The e.p.r. spectrum is pH-independent between 7 and 10, exhibiting a rhombic spectrum with *g*-values of 2.94, 2.29 and 1.52. Above pH 10 there is a gradual change to a less anisotropic spectrum, exhibiting *g*-values of 2.77, 2.27 and 1.71. The pK_a of this transition is approx. 11.6. A decrease in rhombicity of the e.p.r. spectrum of a bisimidazole-ligated haem with increase in pH has been reported in a number of cases and is interpreted as a deprotonation of an axial imidazole ligand (Quinn *et al.*, 1982). Thus the pH-dependent changes in the e.p.r. of the 1-methylimidazole adduct of cytochrome *c* must be due to deprotonation of the proximal histidine residue, since 1-methylimidazole does not possess an N-1 proton. The possibility that the e.p.r. spectrum at pH 12 arises from state V of cytochrome *c* can be excluded, because this form exhibits *g*-values of 2.89, 2.23 and 1.67 and also appears at a slightly higher pH value. These results suggest that the proximal histidine residue has a pK_a of 11.6. We therefore propose that this is the same ionization observed in native cytochrome *c* with an apparent pK_a of 11 (Davis *et al.*, 1974). The elevation in the pK_a from 11 to 11.6 can be ascribed to the change in the distal ligand from methionine to 1-methylimidazole.

***Pseudomonas aeruginosa* ferricytochrome c_{551}**

In view of the solvent-dependence of the hh cytochrome *c* spectra, a similar study of the solvent-dependency of the spectra of *Ps. aeruginosa* c_{551} was performed. The e.p.r. spectrum of *Ps. aeruginosa* c_{551} in 50 mM-Mes, p^{2}H 5.4, is shown in Fig. 6. This shows two of the *g*-values at $g_z = 3.20$ and $g_y = 2.05$; g_x is not observed. Also observed in the e.p.r. spectrum is a slightly rhombic high-spin ferric peak at $g = 6$ and a sharp derivative peak at $g = 2.05$, arising from contamination with the copper protein azurin. Both of these are present to the extent of only a few per cent. The addition of either ethanediol or glycerol at this p^{2}H had no effect on the e.p.r. spectrum (results not shown). The form of the e.p.r. spectrum is also unchanged at p^{2}H 7.7. Hence the presence of glassing agent has no effect on the protein structure detectable by e.p.r. spectroscopy over this p^{2}H range.

At p^{2}H 10.8 in 50 mM-Caps buffer the e.p.r. spectrum changes slightly (Fig. 6). The predominant form remains the species with *g*-values of $g_z = 3.20$ and $g_y = 2.05$, but a second low-spin ferric species is observed with *g*-values

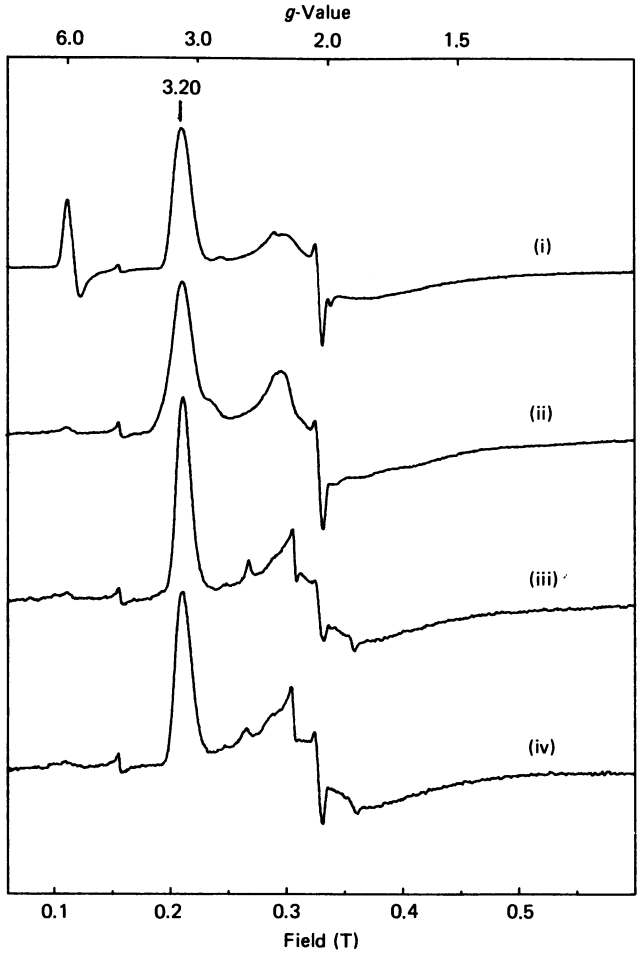


Fig. 6. E.p.r. spectra of *Ps. aeruginosa* cytochrome c_{551}

(i) p^{2}H 5.4 in 50 mM-Mes buffer; [cytochrome] = 1.5 mM; gain, 4×10^4; (ii) p^{2}H 10.8 in 50 mM-Caps buffer; [cytochrome] = 1.5 mM; gain, 4×10^4; (iii) p^{2}H 10.8 in 25 mM-Caps buffer containing 50% ethanediol; [cytochrome] = 750 μM; gain, 8×10^4; (iv) p^{2}H 10.8 in 25 mM-Caps buffer containing 50% glycerol; [cytochrome] = 750 μM; gain, 8×10^4. Conditions: temperature, 10 K; microwave frequency, 9.40 GHz; power 2.01 mW.

of $g_z = 2.92$, $g_y = 2.22$ and $g_x = 1.64$. On addition of ethanediol or glycerol, a slightly different e.p.r. spectrum is obtained. In the presence of glassing agent the main species remains at $g_z = 3.2$, but now the species observed in aqueous solution at $g_z = 2.92$ is absent. A new species is present with *g*-values of $g_z = 2.52$, $g_y = 2.19$ and $g_x = 1.88$, but only at a very low level. From the similarity of the *g*-values of this species to that of metmyoglobin hydroxide (Eglinton *et al.*, 1983), the axial ligands are most likely to be histidine and hydroxide. Over the p^{2}H range 5.4–10.8, however, the e.p.r. spectrum is virtually unchanged, particularly in the presence of glassing agent.

The m.c.d. spectrum in the near-i.r. region (Fig. 7) reflects this invariance with p^{2}H. At p^{2}H 5.4, 7.7 and 10.8 the spectra exhibit a positive peak at 1800 nm, with a shoulder at 1525 nm, typical of a histidine–methionine-ligated haem. The m.c.d. in the visible region of the spectrum also remains unchanged as the p^{2}H is raised

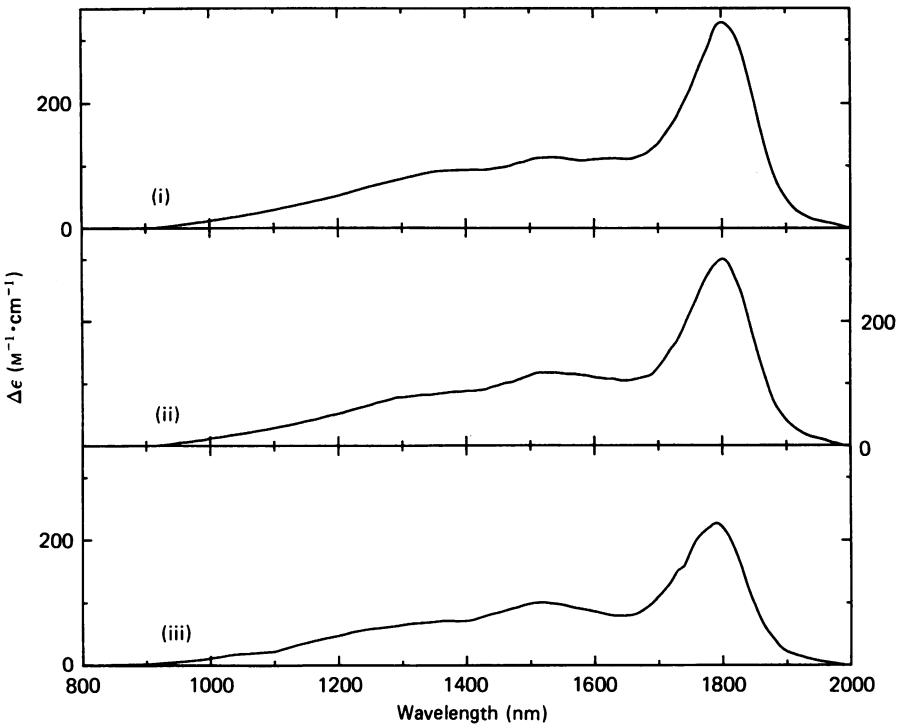


Fig. 7. Near-i.r. region m.c.d. spectra of *Ps. aeruginosa* cytochrome c_{551} at 4.2 K and 5.0 T

(i) p²H 5.4 in 25 mM-Mes buffer containing 50% ethanediol; [cytochrome] = 230 μM; (ii) p²H 7.7 in 25 mM-Mes buffer containing 50% ethanediol; [cytochrome] = 230 μM; (iii) p²H 10.8 in 25 mM-Caps buffer containing 50% ethanediol; [cytochrome] = 840 μM.

Table 1. Wavelength positions of the near-i.r. CT bands, as observed with low-temperature m.c.d. spectroscopy, and *g*-values of bis-co-ordinated low-spin ferric haems

Abbreviation used: n.d., not determined.

Type of co-ordination (and example)	Wavelength maximum (nm)	E.p.r. *g*-values	Reference
Methionine–histidine (cytochrome c_{551}, *Ps. aeruginosa*)	1810	3.19, 2.05, n.d.	Foote *et al.* (1984)
Histidine–histidine (cytochrome c_3, *Desulfovibrio vulgaris*)	1510	2.97, 2.29, 1.56	Foote *et al.* (1984)
Amine–histidine (Lb + n-butylamine)	1550	3.38, 2.05, n.d.	P. M. A. Gadsby, J. Peterson & A. J. Thomson, unpublished work
Amine–amine [Fe(III)-octaethylporphine + n-butylamine]	1320	3.69, 1.69, n.d.	Gadsby & Thomson (1986)
Methionine–histidinate (cytochrome b_{562}, *Escherichia coli*)	1550	2.79, 2.26, 1.67	Moore *et al.* (1985)
Histidine–histidinate (Lb–imidazole adduct)	1350	2.82, 2.29, 1.69	Gadsby & Thomson (1982)

from 5.4 to 10.8 (results not shown). This shows that there is no change in the state of ligation of the haem over this p²H range. There is, however, a pH-dependent change in the redox potential, with a pK_a of 6.2 in the oxidized state of the protein (Moore *et al.*, 1980). The pK_a has been assigned to the ionization of one of the haem propionic acid groups. Although the m.c.d. results cannot confirm this assignment, they do show the absence of a change in axial ligation through this pK_a.

The lack of a pH-dependent change in the axial ligation at a pH similar to that observed in hh cytochrome *c* may reflect the replacement of lysine-72 and lysine-79 by serine and proline respectively (Mathews, 1985). However, preliminary optical studies, in agreement with r.R. studies (Kitagawa *et al.*, 1977), suggest a change of axial ligation at about pH 11.5. This pK_a may well be attributable to the deprotonation of the same residue as that observed in hh cytochrome *c* at

pH 11.1 (Davis *et al.*, 1974). This would support the conclusion that lysine-79 is not the ionizing group in either protein.

It has previously been suggested that cytochromes *c* fell into two groups exhibiting g_z-values of the order of 3.06 (Class I) or 3.20 (Class II) (Brautigan *et al.*, 1977). In this scheme, Class II is believed to have the histidine ligand in the deprotonated form, or to possess enhanced hydrogen bonding. According to this classification, *Ps. aeruginosa* c_{551} should be placed in Class II. The m.c.d. results presented here disagree with this assignment, as it has been shown that the near-i.r. band typical of histidine–methionine ligation is blue-shifted by $\approx 1100\ cm^{-1}$ compared with the protonated form and would therefore exhibit a band at 1550 nm (Moore *et al.*, 1985). In addition, the e.p.r. spectrum of the deprotonated form is less rhombic than that of the protonated form, the opposite of that suggested by Brautigan *et al.* (1977). It seems that a single type of axial ligation, namely histidine–methionine, can show g_z values ranging from 3.05 for hh cytochrome *c* to 3.50 reported for yeast cytochrome c_1 (Siedow *et al.*, 1978). This may reflect subtle changes in ligand orientation or metal-to-ligand bond distance rather than changes in ligation or in the state of ligand protonation.

DISCUSSION

By using m.c.d. spectroscopy over the wide wavelength range 300–2000 nm we have characterized the ligand state of hh cytochrome *c* and cytochrome c_{551} (*Ps. aeruginosa*) as a function of pH between 7.0 and 11.0. In order to identify the ligation forms it has been necessary to construct a scale that identifies the wavelength position of the near-i.r. CT band with the axial ligands in model proteins of known structure. This scale has been built up, in the main, as a result of work carried out in this laboratory. Table 1 collects together the data from the present and from previous studies. In view of many earlier proposals that the state of ligation of the low-spin alkaline form of hh cytochrome *c* is lysine–histidine, the m.c.d. characteristics of this ligation state were required for this study. No protein is known yet with certainty to have this co-ordination geometry. Therefore this state has been modelled with an amine adduct of a histidine-co-ordinated haem. A report of the near-i.r. m.c.d. of the lysine derivatives of methaemoglobin (Rawlings *et al.*, 1977) suggests that this species exhibits a peak at 1580 nm. However, in the present work we have been unable to produce this complex, either with haemoglobin or with myoglobin. We have been able to produce a satisfactory model of this co-ordination type with the n-butylamine adduct of soybean leghaemoglobin (Lb) (P. M. A. Gadsby, J. Peterson, G. Sievers and A. J. Thomson, unpublished work). Lb isolated from soybean is similar in structure to myoglobin, but possesses a larger haem crevice on the distal side which can readily bind more bulky ligands, especially those in the anionic form, such as nicotinate and long-chain carboxylates (Wittenberg *et al.*, 1974). The addition of n-butylamine to soybean Lb at p^2H 10.4 produces an optical spectrum typical of the formation of a low-spin ferric haem. The e.p.r. and near-i.r. m.c.d. data are quoted in Table 1. The model for co-ordination by two lysine side chains is provided by the bis-n-butylamine adduct of Fe(III)-octaethylporphine (Gadsby & Thomson, 1986) (Table 1). Also included in the summary Table (Table 1) are the e.p.r. *g*-values, since the work of Brautigan *et al.* (1977) has been widely used to assign axial ligand states of cytochrome *c* derivatives. The use of only one technique, either e.p.r. or near-i.r. m.c.d., to make an axial ligand assignment of a low-spin ferric haemoprotein is insecure, since for a given set of ligands the spectral parameters can vary widely. However, the use of two techniques together considerably diminishes the ambiguity. The results can be applied to the problem of the nature of the alkaline transition in hh cytochrome *c* to obtain an unambiguous answer.

The presence of a band at 1800 nm and one at 695 nm that is more distinctive in the low-temperature m.c.d. spectrum than in the absorption spectrum are unequivocal evidence for the presence of methionine–histidine co-ordination. These features are shown in this work for hh cytochrome *c* and for cytochrome c_{551}. They have also been earlier demonstrated in the case of cytochrome b_{562} (*Escherichia coli*) (Moore *et al.*, 1985) and in the dihaem cytochrome *c* peroxidase from *Ps. aeruginosa* (Foote *et al.*, 1984). The alkaline transition with an overall pK_a of 9.3 in hh cytochrome *c* leads to a lysine–histidine co-ordinated haem. This is established clearly and unambiguously by the striking similarity of the m.c.d. spectra, at 4.2 K, of the n-butylamine adduct of Lb and of hh cytochrome *c* at p^2H 11.0 (Fig. 8).

Although we assign the near-i.r. m.c.d. band at 1480 nm at 4.2 K to histidine–lysine ligation, consideration must be given to two alternative possibilities suggested by the data in Table 1. The first is bishistidine ligation, which gives rise to a peak in the range 1500–1600 nm. However, the only residue available is histidine-26, ligation of which would require an extensive conformation change. Also the e.p.r. *g*-values are inconsistent with a normal bishistidine-type ligation; the value of g_z, for instance, is usually in the range 3.0–2.9. Ligation of histidine-33 is also excluded, as this residue is not invariant (Mathews, 1985). A second possible type of axial ligation is methionine–histidinate, brought about by deprotonation of histidine-18, the proximal histidine residue. This would be expected to possess a near-i.r. m.c.d. band at approx. 1470 nm (Gadsby & Thomson, 1982). However, this type of ligation has been observed for cytochrome b_{562} from *E. coli* at alkaline pH, and, significantly, retains a '695 nm' band detectable by m.c.d. (Moore *et al.*, 1985), although the absorption band due to this transition disappears (Myer & Bullock, 1978) by shifting under the α-band. This band is blue-shifted along with the near-i.r. band. These shifts are paralleled by a decrease in the anisotropy of the e.p.r. spectrum. Neither of the above phenomena are observed for hh cytochrome *c*. Thus we can exclude this type of ligation.

Evidence for a lysine ϵ-amino group as the strong-field ligand is then spectroscopic, being based upon the conclusions of the present work and the e.p.r. spectra of Brautigan *et al.* (1977). However, a wide range of chemical-modification studies (in which the co-ordinating ability of the amino group of various lysine residues has been blocked) have been made in an attempt to show that the side chain of lysine is not the ligand of haem above the alkaline isomerization. The most direct approach in this latter category was made by Bosshard (1981), who measured the relative chemical reactivity of lysine residues in oxidized cytochrome *c* towards

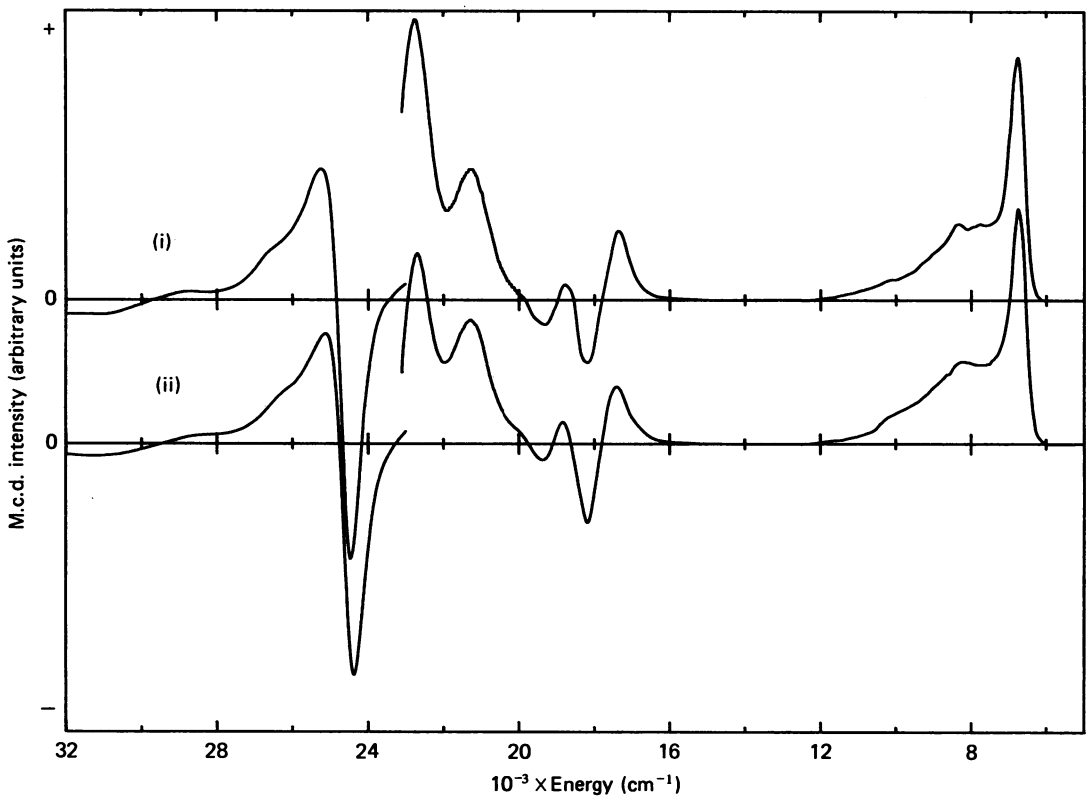


Fig. 8. Comparison of the m.c.d. spectra of 4.2 K of (i) hh cytochrome *c*, p²H 11.0, and (ii) soybean Lb-*c*–n-butylamine adduct, p²H 10.4

The spectra are plotted on an energy scale, and the Lb spectrum is blue-shifted by 300 cm^{-1}.

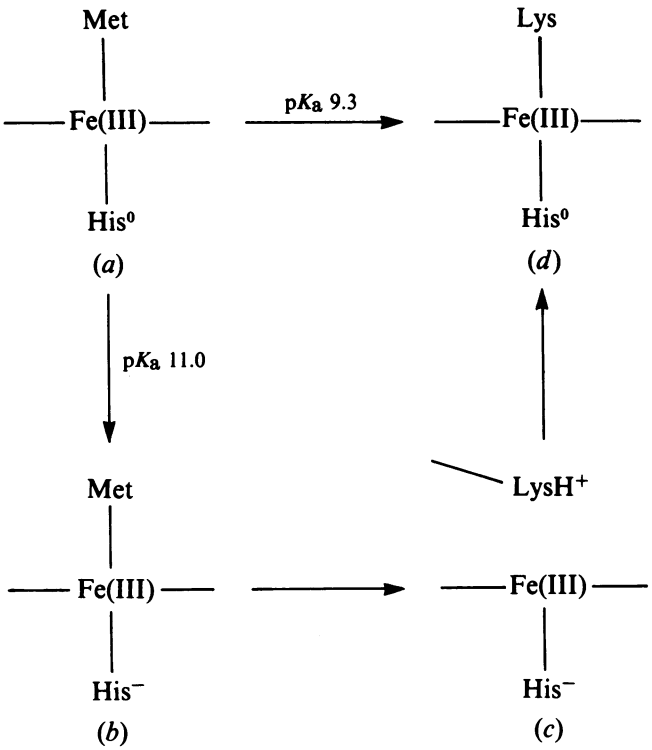
acetylation and amidination. The relative rates of acetylation measured between pH 7 and 11 were compared with those of the peptide fragment 66–80 cut out of the native molecule. There was no significant difference between these rates over this pH range for lysine residues 72, 73 and 79. Hence, it is claimed, these results rule out the possibility of the ϵ-amino group of any of these three lysine residues being the alkaline strong-field ligand. Bosshard (1981) further suggests it is extremely unlikely that any of the remaining 16 lysine residues might compete with methionine-80 as the axial haem ligand unless there is a very large conformational change which, he considers, is extremely unlikely at pH 9.0 in a molecule of the noted conformational stability of cytochrome *c*. He therefore firmly rules out any lysine residue as the axial ligand.

The spectroscopic evidence presented here appears to be conclusively in favour of lysine as the strong-field ligand above pH 9.0. In view of the strong evidence of Bosshard (1981) that lysine residues 72, 73 and 79 are not ligands, it remains to be considered whether any of the 16 other lysine residues could provide an ϵ-amino group as ligand.

All of the 19 lysine residues are located on the surface of the protein. But the side chain carrying the amino group is an alkyl chain {-$[CH_2]_4$-} with a relatively long reach. Although Bosshard (1981) suggests that large conformational rearrangement is unlikely to occur around pH 9, we note that the loss of methionine-80, triggered by the deprotonation of the N-1 of histidine-18, could well lead to considerable movement of the protein backbone on the distal side of the haem plane. Therefore there seems to be no reason to exclude lysine-13, -86 or -87 as potential haem ligands. None of them is excluded by Bosshard's (1981) study. These residues are invariant in all the cytochromes *c* known to undergo the alkaline transition. Therefore we conclude that the weight of spectroscopic evidence favours lysine as the ligand of haem above pH 9. But we are unable to identify which of several lysine residues is the culprit.

Contrasting with these results on hh cytochrome *c* the e.p.r. and m.c.d. spectra of oxidized *Ps aeruginosa* cytochrome c_{551} are independent on pH over the range 5.4–10.8. The position of the near-i.r. CT band at 1800 nm identifies the axial ligation as histidine–methionine, in agreement with the crystal structure (Matsoora *et al.*, 1982) and n.m.r. studies (Keller & Wüthrich, 1978). The pH-invariance of the spectra shows the absence of ligand exchange at the haem, although the midpoint potential is pH-dependent.

Turning to consideration of the mechanism of the alkaline ligand-exchange reaction in hh cytochrome *c*, it has been shown that the process is complex, not a simple displacement of the methionine axial ligand by lysine (Davis *et al.*, 1974). Instead a group ionizing with a p*K* of 11 appears to trigger the process. The nature of this ionizing group has also been the subject of much debate, and various studies have led to a number of different suggestions as to its identity. These include lysine-79, tyrosine, histidine-18 and buried water. Spectrophotometric titrations of the phenolic groups of native and fully trifluoroacetylated cytochrome *c* appear to exclude tyrosine (Stellwagen *et al.*, 1975). We propose here that it is the ionization of the N-1 proton of the



Scheme 1. Interconversion of forms of hh cytochrome *c*

(*a*) Neutral low-spin form of hh cytochrome *c*, state III; (*b*) transient low-spin form; (*c*) transient high-spin form; (*d*) alkaline low-spin form, state IV.

proximal histidine ligand. This is based on the e.p.r. titration of the 1-methylimidazole adduct of hh cytochrome *c*. It is clear, however, that the proximal histidine is not deprotonated in the alkaline state of cytochrome *c*. Thus the deprotonation appears to act as a trigger for the alkaline isomerization. The deprotonation of the proximal histidine residues would produce a pronounced *trans* effect, weakening the iron–methionine bond. This then would lead to the replacement of methionine by lysine, which itself must deprotonate before replacing methionine. The histidine–lysine ligation of the equilibrium alkaline form implies, therefore, that the histidine reprotonates upon lysine ligation in the distal position. These proposals are summarized in Scheme 1. The transient high-spin form has been observed by both kinetic difference spectra (Saigo, 1981) and by the time-resolved r.R. spectroscopy (Uno *et al.*, 1984). This form is assigned as high-spin because of the presence of a 600 nm absorption band typical of high-spin haem. The transient species observed by r.R. is also reported to be similar to the alkaline form of the totally guanidinated cytochrome *c*, in which ligation by lysine is prevented. Ferricytochrome *c* that is totally guanidinated or totally trifluoroacetylated is high-spin at alkaline pH and exhibits pK_a values of 8.8 and 9.9 respectively (Stellwagen *et al.*, 1975), suggesting that lysine is not the ionizing group involved in this pK_a. Fully aminated and fully maleylated cytochrome *c* exhibit transitions to low-spin forms without 695 nm bands, which may correspond to the low-spin transient form in Scheme 1 (Pettigrew *et al.*, 1976). This species is the same as the high-pH form of *E. coli* cytochrome b_{562} and exhibits visible absorption maxima which are slightly red-shifted, as expected from the results obtained with cytochrome b_{562}. A transient low-spin form has also been observed by a rapid-scanning stopped-flow method. This exhibits red-shifted absorption bands and a band in the 695 nm region, although of diminished intensity (Kihara *et al.*, 1976). This suggests that the transient low-spin form retains the methionine ligation, as shown in Scheme 1.

Deprotonation of a residue close to the haem has been implicated in the autoreduction of two cytochromes in the facultative methylotroph *Pseudomonas* AM1 at high pH (O'Keeffe & Anthony, 1980). Thus the deprotonation of the proximal histidine residue could be the event leading to the autoreduction of hh cytochrome c that is often observed as the pH is raised through the alkaline transition. In contrast with ferricytochrome *c*, the axial ligation of ferrocytochrome *c* remains as histidine–methionine in the pH range 4–12 (Margoliash & Schejter, 1966). Thus the deprotonation of the proximal histidine residue must be at a higher pH in ferrocytochrome *c*. This is expected because of the increased electron density at the iron in the ferro form.

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