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REPLACEMENT OF METHIONINE AS THE AXIAL LIGAND OF *ACHROMOBACTER CYCLOCLASTES* CYTOCHROME c_{554} AT HIGH pH VALUES REVEALED BY ABSORPTION, EPR AND MCD SPECTROSCOPY

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Cytochrome c_{554} from the denitrifying bacterium *Achromobacter cycloclastes* is a monoheme class II *c*-type cytochrome with a His-Met axial coordination at neutral pH. The amino acid composition and the N-terminal sequence of the cytochrome have been determined. Subsequent determination of the pH-dependence of the redox potential and examination of the EPR and MCD spectra of ferricytochrome c_{554} revealed a new form at high pH values made apparent with both spectroscopies. These observations are consistent with the presence of lysine as the axial ligand for which methionine substitutes at high pH values.

Denitrifying bacteria *Achromobacter cycloclastes* IAM 1013 cells yield monoheme cytochrome c_{554} (13 kD) with a majority of low-spin form (methionine-histidine coordination) coexisting with a minor high-spin form present at all pH values. According to a pH-dependent spin equilibrium studied by NMR, three forms, designated I, II and III, were attributed to the ferricytochrome. At low pH, forms I and II predominate and are related by a pK_a of 5.0, with a methionyl residue identified by NMR spectroscopy as the sixth ligand. Elevation of the pH above 8.9 reveals in the NMR spectrum an apparent alteration of the heme environment structure, and form III develops. From ^1H NMR studies, the pK_a associated with the transition from form II to III was estimated to be 8.9. We have previously suggested (1) that, in form III, methionine is no longer an axial ligand, but is replaced by a strong field ligand such as an arginine or a lysine.

Abbreviations:
EPR: Electron Paramagnetic Resonance; MCD: Magnetic Circular Dichroism; NMR: Nuclear Magnetic Resonance; NIR: Near Infrared.

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In the present study, we investigated in greater detail the modification occurring in the pH range associated with the equilibrium between the forms II and III. EPR and MCD spectroscopy were used to identify the nature of the new ligand in form III. We have also determined the change in the redox potential with pH.

MATERIAL AND METHODS

A. cycloclastes IAM 1013 was grown as previously described (2) and the cytochrome c_{554} was purified as previously indicated (1). Visible electronic spectra for the cytochrome were recorded with a Shimadzu UV-260 spectrophotometer. The N-terminal sequence was determined with an Applied Biosystem 477A protein sequencer coupled to an Applied Biosystem 120A analyser. Electron Paramagnetic Resonance spectroscopy (EPR) was carried out with a Bruker ESP 380 spectrometer, equipped with a continuous flow helium cryostat (Oxford Instruments Co., Oxford) and a computer for carrying out mathematical manipulations. The redox titrations of anaerobic solutions of the protein (7.4 μM) were performed in a optical redox cell, using a Crison Micro pH 2002 digital pH meter. Buffer exchange, when necessary, was achieved using an Amicon ultra filtration unit, fitted with a PM10 membrane, operating at a pressure of 55 psi. Protein concentration of cytochrome c_{554} was determined from the absorption spectrum using an extinction coefficient of the Soret band ε_{412}=94290 $M^{-1}cm^{-1}$ of ferricytochrome. Reoxidation of auto-reduced samples was achieved by the addition of 10 μl aliquots of 60 mM $K_3Fe(CN)_6$ solution. Quoted pH* values indicate meter readings uncorrected for the isotope effect. Deuterated buffers employed were: pH* 4.5-50 mM sodium acetate ; pH* 7.2-50 mM (N-[2-hydroxyethyl]piperazine-N´-[2-ethanesulfonic acid]) (HEPES); pH* 10 and 11.9-50 mM (3-[cyclohexylamino]-1-propane-sulfonic acid) (CAPS). For sample preparation for Magnetic Circular Dichroism (MCD) studies, approximately 3 mg of lyophilised protein were dissolved in 50 mM deuterated HEPES buffer, pH*7.2, and spun at 4000 rpm for 15 minutes. The magnetic field used to obtain the MCD spectra was 5 T and the temperature 4.2 K. MCD spectra were recorded as described earlier (3).

RESULTS

Amino acid composition and N-terminal sequence. The amino acid composition of *A. cycloclastes* cytochrome c_{554} is presented in Table 1. This cytochrome was also subjected to sequential Edman degradation on an automatic sequencer and the N-terminal sequence of 44 amino acid residues was obtained. (Fig. 1). Sequences for homologous cytochromes are also shown. The N-terminal sequence of cytochrome c_{556} from *Agrobacterium tumefaciens* strain Apple exhibits 57% similarity and that of cytochrome c_{556} from *Rhodopseudomonas palustris* shows 39% similarity with *A. cycloclastes* cytochrome c_{554} (4). In these two cytochromes the covalent bonding of the heme to the polypeptide chain occurs at residues 126 and 130, *i.e.*, near the C-terminal. The similarity between these cytochromes and *A. cycloclastes* cytochrome c_{554} permits us to conclude that cytochrome c_{554} belongs to class II. One interesting observation is that in all three cytochromes a lysine appears one residue after the axial methionine residue at position 11.

Redox potential determinations for cytochrome c_{554}. The redox potentials at different pH values were obtained by following the absorption changes for the band at 554 nm relative to the

Table 1
Amino acid composition of cytochrome c_{554} from *A. cycloclastes*

Amino acid	Number of residues per mol of citocromo c_{554}
Cys	2
Asp+Asn	14
Thr	6
Ser	5
Glu+Gln	12
Pro	6
Gly	13
Ala	21
Val	9
Met	1
Ile	0
Leu	5
Tyr	2
Phen	0
His	3
Lys	21
Trp	1
Arg	4
Total	125

absorbance at 563 nm (isobestic point); the experimental data were fitted with the Nernst equation for one electron (Table 2). Over the pH range examined, the curve for E_m versus pH can be fitted with theoretical curves defined by one proton ionisation equilibrium in the reduced form (K_{red})

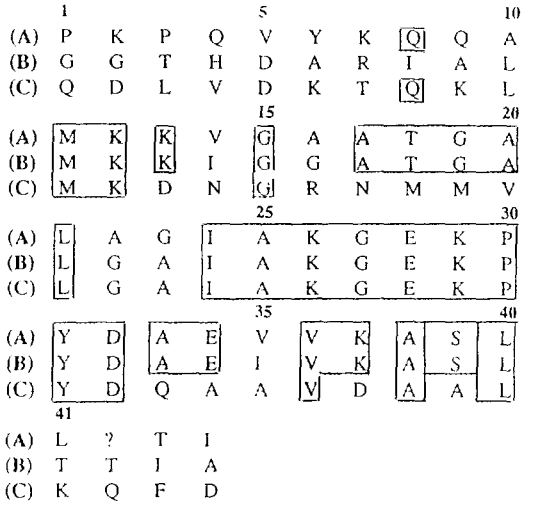

Figure 1. Comparison of the N-terminal sequences of cytochromes: **(A)** cytochrome c_{554} of *A. cycloclastes*; **(B)** cytochrome c_{556} of *Agrobacterium tumefaciens* Apple; **(C)** cytochrome c_{556} of *Rhodopseudomonas palustris*.

Table 2
Redox potentials (relative to NHE) of cytochrome c_{554} from *A. cycloclastes*, at different pH values

pH	E_m(mV)
5.6	288
6.6	221
7.5	190
8.2	177
9.1	175
9.6	155
10.3	100
10.5	102
11.1	46

and two proton ionisation equilibrium in the oxidised form (K_{ox1}, K_{ox2}) (5). The values, pK_{ox1}=5.3±0.2, pK_{red}=7.2±0.1, pK_{ox2}=8.9±0.1 and $E_{m,0}$=300±5.0 mV (Fig. 2) were the selected apparent ionisation constants and redox potential that best fit the experimental results. The pK_{ox} values obtained by this fitting are very similar to those already determined by ^{1}H NMR (1).

EPR spectra of the cytochrome c_{554}. In the oxidised (native) form the 8 K EPR spectrum of the cytochrome shows a signal typical of a low-spin heme (4) with g_{max}=3.55 and a minor species with g_{max}=3.02 (Fig. 3). The two other g values for the main species must be very low, since $g^2_x+g^2_y+g^2_z$=16, and are probably too broad to be detected. Measurement of the pH

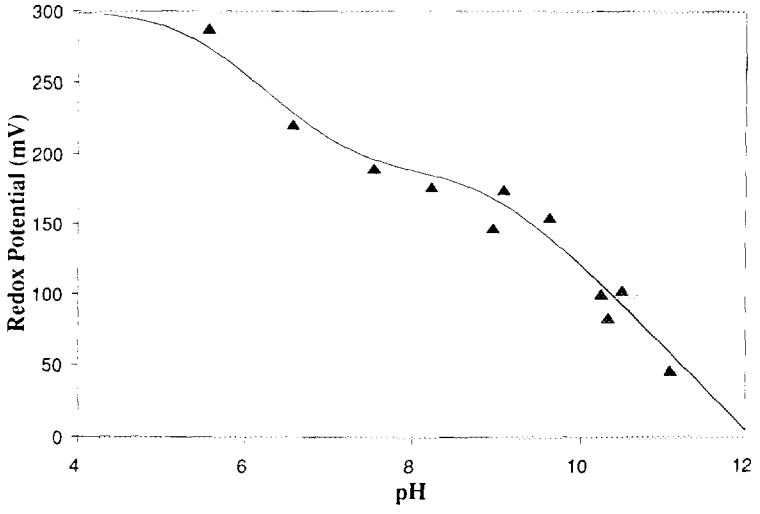

Figure 2. pH dependence of the redox potential of *A. cycloclastes* cytochrome c_{554}. The theoretical curve was obtained through the presented equation with pK_{ox1}=5.3±0.2, pK_{red}=7.2±0.1, pK_{ox2}=8.9±0.1 and $E_{m,0}$=300±5.0 mV.

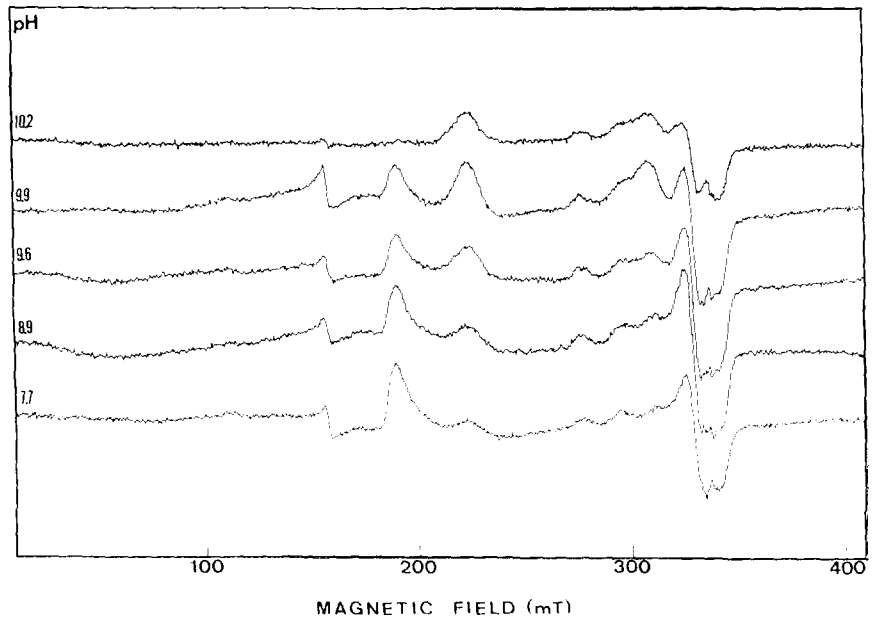

Figure 3. pH dependence of the EPR spectrum of *A. cycloclastes* cytochrome c_{554}. Other experimental conditions: T=10 K, gain $8x10^5$; microwave power 2; modulation amplitude 1 mT and microwave frequency 9.42 GHz.

dependence of the EPR spectra of solutions held between pH 5 and pH 10.2 (Fig. 3) revealed that at high pH values the signal at g_{max}=3.02 begins to increase concomitantly with the decrease of the signal at g_{max}=3.55. Total conversion of this latter form to a form with g_{max}=3.02 occurs at pH 10.2. A pK_a of 8.8 could be estimated from the ratio of the areas of the two signals [corrected with the Aasa-Vangard formula (6)]. Contaminating copper ion (S=1/2) is present in the sample in a total amount of 6% when compared to the amount of heme. Computer manipulations enabled us to determine the g_{med} associated with the signal with g_{max}=3.02 (g_{med}~2.13). These values cannot be fitted in any of the regions of the Blumberg and Peisach truth diagram (7), although they suggest the change of a sulphur ligand to a nitrogen ligand (the tetragonal field value, 3.68, is typical of a nitrogen ligand).

MCD spectroscopy. Fig. 4 shows the 4.2K MCD spectra of cytochrome c_{554} at pH* values of (A) 4.5, (B) 7.2, and (C) 10.0. Optical transitions are present throughout the region of 400-600 nm, and the spectra for all pH* values display shapes typical of a low spin Fe(III) heme coordinated by two strong field ligands. At neutral pH and high pH values (B and C), a small contribution of Fe(II) heme is evident, and this is due to some degree of sample auto reduction upon the addition of glycerol to 50% (v/v). Optical bands mostly originate from π–π^* transitions of the porphyrin ring (4), and as a consequence, the MCD spectrum is not greatly affected by a change of the axial ligation, provided that the Fe(III) remains low spin.

Between 600 nm and 3000 nm, spectral form is dependent not only on the type of the porphyryn ring, but also on the nature of the heme iron axial ligand set (3). Fig. 4 also shows the

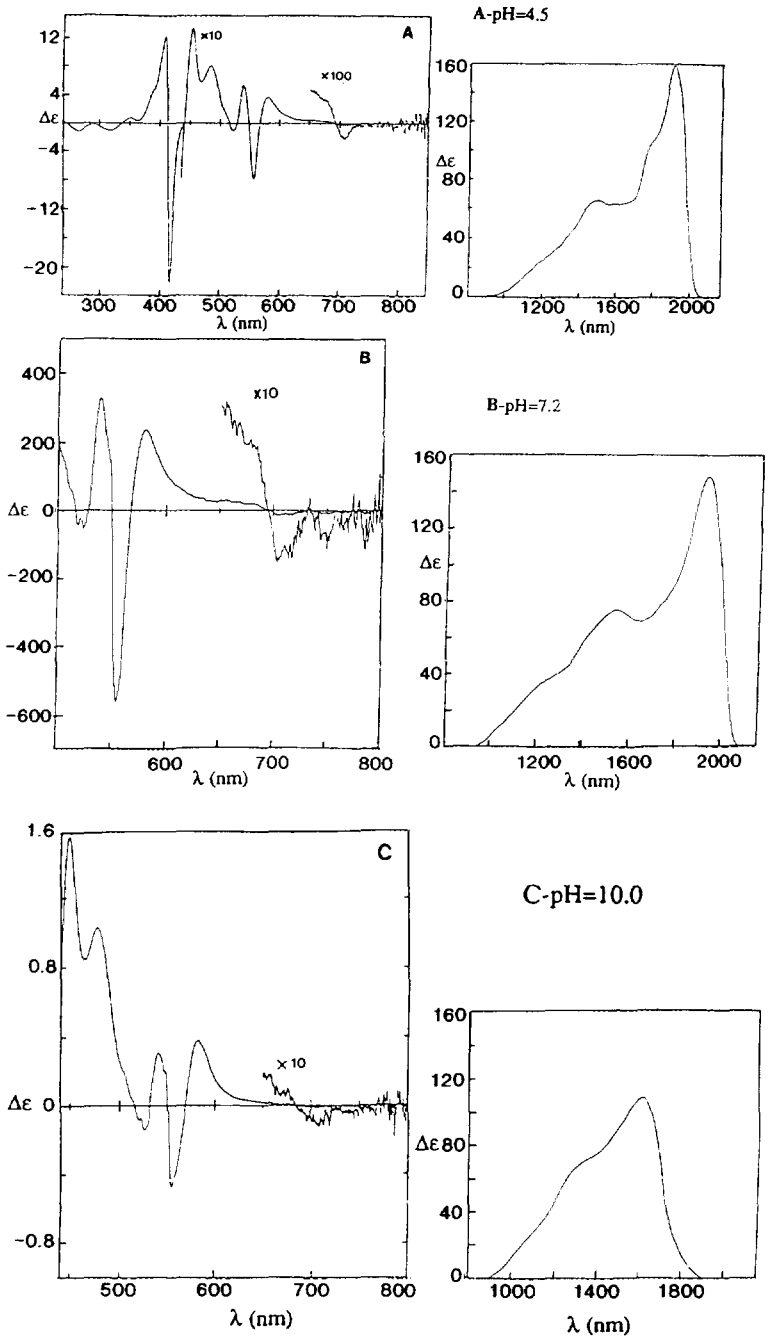

Figure 4. NIR-MCD spectra of *A. cycloclastes* cytochrome c_{554} at the indicated pH values: A- pH=4.5; B- pH=7.2 and C- pH=10.0. Magnetic field 5 T, T=4.2 K and $\Delta\varepsilon$ in $M^{-1}cm^{-1}$.

4.2 K near infrared (NIR) MCD spectra of cytochrome c_{554} at pH* values of (A) 4.5, (B) 7.2, and (C) 10.0. At low and neutral pH a band with a maximum at 1900 nm appears, while at high pH the maximum changes to 1605 nm.

DISCUSSION

The change in the redox potential of the cytochrome c_{554} with pH permitted the determination of two pK_{ox} values and a pK_{red} value. The pK_{ox1} and pK_{ox2} matched, within experimental error with those already obtained by ^1H NMR (1). It is likely that the pK_{red}=7.2 is linked to the reduced form of the same ionisation group that originated the pK_{ox1}=5.3 in the oxidised form. Similar cases have been observed in other cytochromes, e.g., cytochromes c_{551} from several *Pseudomonas* species and cytochrome c_{550} from *Paracoccus denitrificans* (8-12). The separation of these two pK values indicates that the ionizable group lies close to the heme, as does a propionate or carboxyl group (13). As these pK values are not associated with changes in the visible and EPR spectra of the cytochrome, this ionisation appears not to affect the primary ligand field of the iron. In contrast, the pK_{ox2}=8.9 is associated with the loss of the 695 nm absorbance band in the visible spectrum (14). Such transition also occurs in several other cytochromes (13-15). The disappearance of the 695 nm band could be attributable to either substitution of the methionine residue or a change in conformation. To clarify this point, the interdependence between changes in pH and the EPR and MCD spectra was investigated. The EPR spectrum of the ferricytochrome at neutral pH is highly anisotropic, with a g_{max} value of 3.55 characteristic of a low-spin cytochrome, and an almost axial ligand field (4). When the pH is raised to high values, a signal at g_{max}=3.02 (with g_{med}=2.13) starts to develop. At pH 10.2 the signal at g_{max}=3.55 disappears and is replaced by the new signal. As we have seen, the variation of the g values suggests a change from a sulphur ligand to a nitrogen ligand. In the NIR MCD spectra at low and neutral pH, a band with a maximum at 1900 nm appears, while at high pH the maximum changes to 1605 nm. In order to understand the significance of this shift we must examine the origin of the band. The optical spectra of all low spin protoporphyrin heme species are expected to contain two one-electron Charge Transfer (CT) transitions from the highest filled porphyrin π-orbitals of symmetry, a_{1u} and a_{2u}, into the partially filled Fe(III) d orbital d_{yz} (3). The band observed in the NIR region can be assigned to the lower energy transition, that of a_{1u}-d_{yz}. The higher energy transition has recently been located at 734 nm in the spectrum of *E. coli* bacterioferritin, which contains *b*-type heme groups coordinated by two methionines (16,17). This transition is not observed in cases where methionine is not an axial ligand, most likely occurring in the 400-600 nm region, where it is obscured by the more intense porphyrin π–π^* transitions. The position of the NIR band is known to be dependent on the identity of the axial ligands of the heme iron. This is not unexpected since these ligands affect the energy of the Fe(III) d-orbital set. Furthermore, it has been demonstrated that the band position alters in a systematic fashion depending upon the axial coordination set: changing one of the ligands results in a band shift which is independent of the identity of the other ligand. Hence the band position is characteristic of one or more possible ligation sets and is therefore useful in the identification of the ligand set. In this case, the band position at low and neutral pH and the presence of its derivative shape feature at approximately 695 nm are consistent with the NMR assignment of histidine-methionine. The appearance, at low pH, of a shoulder on the 1900 nm peak may be indicative of an equilibrium mixture of forms I and II, which are related by a pK_a of 5.3. This pK_a is believed to be associated with the protonation of a group which belongs to the heme (propionate), or which lies in the close

vicinity of the heme (4). Data from UV-visible, EPR and NMR spectroscopies (1) suggest that, at high pH, a switch in axial ligation occurs. It is thought that the methionine ligand is replaced by another strong field ligand, thus maintaining the low spin configuration of the heme. The MCD spectra reflect such a change, with a shift of the band to 1605 nm. Reference to the predicted energies of the CT band (18) indicate that there are three possible ligation sets which would give rise to a band at this wavelength, namely: histidine-lysine, histidine-histidine and histidinate-methionine. We have also presented evidence for the loss of the methionine residue at high pH (1). This conclusion is not immediately obvious from UV-visible and MCD data. The MCD band at 717 nm, corresponding to the 696 nm (band due to the presence of methionine) in the optical spectrum, is of low intensity and therefore not well resolved above noise level. Hence the disappearance of such a band is not easily followed. However, the UV-visible and NMR data, together with the fact that the pK_a for the deprotonation of histidine is usually significantly higher than 9, allow us to discount the histidinate-methionine possibility.

In conclusion significant features of the NMR (1), EPR and MCD spectra obtained for solutions at high pH values indicate a change in the heme environment of cytochrome c_{554} and could be explained by the presence of lysine or histidine as the new axial ligand. The examination of the amino acid sequence for cytochrome c_{554} reveals the presence of a lysine next to the methionine residue involved in heme coordination at low pH, strongly implicating lysine as the replacement axial ligand at high pH.

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