Axial Ligation and Heme Environment in Cytochrome *c*-555 from *Prosthecochloris aestuarii*. Investigation by Absorption and Solvent Perturbation Difference Spectroscopy†

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ABSTRACT: The near-IR absorption spectrum indicated that methionine is the sixth axial heme iron ligand in *Prosthecochloris aestuarii* cytochrome *c*-555. The heme environment has been investigated by the technique of solvent perturbation difference spectroscopy. The heme octapeptide from cytochrome *c* plus added imidazole was used as a model compound for the fully exposed chromophore. The heme was found to be minimally exposed to solvent. A comparison was made with cytochrome *c*, as to the possible causes of the difference in redox potentials between these two cytochromes.

The *P. aestuarii* cytochrome *c*-555 has one heme binding site (-Cys-Ala-Ser-Cys-His-) (Van Beeumen et al., 1976) which by analogy to other *c*-type cytochromes indicates that His-27 is the fifth ligand to the heme iron. This cytochrome has been shown to exhibit a split α peak in the ferrous state at room temperature (Shioi et al., 1972) as does cytochrome *c*-555 from *Chlorobium thiosulfatophilum* (Yamanaka & Okunuki, 1968). The origin of this splitting has been attributed to some peculiar arrangement of the iron ligands and on this basis it has been suggested that such monoheme cytochromes may be categorized as a separate class of bacterial cytochromes (Dickerson & Timkovich, 1975). It has also been proposed that the above cytochromes *c*-555 be classified with cytochromes c_5 and *c*-553 (*f*) on the basis of the number of prolines found near the methionine close to the carboxyl end (Dickerson Timkovich, 1975). *P. aestuarii* cytochrome *c*-555, however, exhibits a redox potential of +103 mV (Shioi et al., 1972) which is significantly lower than the +350 mV exhibited by the algal *f*-type cytochromes (Yamanaka & Okunuki, 1968). These observations indicate a need for further investigation into the nature of the ligand arrangement and the origin of such a large potential difference. Kassner (1972, 1973) has proposed a relationship between the hydrophobicity of the heme environment and the redox potential.

In the present work, we have assigned the sixth axial ligand to the heme iron and explored the heme environment using the technique of solvent perturbation difference spectroscopy (Herskovits, 1967). This latter technique has been used previously to probe the heme environments of other cytochromes (Stellwagen, 1967; Cabral & Love, 1974).

Materials and Methods

Materials. Equine heart cytochrome *c* (Sigma type VI) was used without further purification. The heme octapeptide was prepared from cytochrome *c* by a modification of the procedure of Harbury & Loach (1960) using a silica gel column for the preparative chromatography. Imidazole was purchased from Aldrich.

Isolation of Cytochrome. Cytochrome *c*-555 was isolated from the mixed culture "*C. ethylica*" strain 2K (Gray et al., 1973; Probst et al., 1977), grown as previously described (Bose, 1963). Two hundred grams of frozen cell paste was thawed and suspended in 0.05 M potassium phosphate buffer (pH 7.0) to give a final volume of 600 mL. The cells were broken either by sonication using a Bronwill Biosonik III or by means of a Vibrogen cell mill (Rho Scientific) at 4 °C. The broken cell suspension was centrifuged for 1 h at 16 000*g*, after which the supernatant was centrifuged for 2 h at 95 500*g*. The brown supernatant solution was passed through a DEAE-cellulose column (4.5 × 30 cm) equilibrated with 0.05 M potassium phosphate buffer (pH 7.0). The eluate containing the unadsorbed cytochrome was fractionated with ammonium sulfate. The red precipitate obtained between 40% and 100% saturation was dissolved in 0.05 M Tris buffer (pH 7.5) containing 0.2 M NaCl and applied to a G-75 Sephadex column (4.5 × 100 cm) equilibrated with the same buffer. The cytochrome fraction was dialyzed vs. 1 mM NH_3 and lyophilized. Final purification was achieved by isoelectric focusing on a 110-mL column (LKB-Produkter AB, Stockholm-Bromma, Sweden) using a wide pH range (3–10), followed by isoelectric focusing in the pH range 4–6. The cytochrome was shown to be homogeneous under these conditions, exhibiting an isoelectric point of 5.27.

Preparation of Solutions. All cytochrome solutions used for solvent perturbation measurements contained 3 to 5 × 10^{-6} M cytochrome *c*-555 (as determined using the extinction coefficients of Shioi et al., 1972) and 0.1 M potassium phosphate buffer (pH 7.0). The heme octapeptide solutions contained 0.7 to 1 × 10^{-5} M heme octapeptide, 0.1 M potassium phosphate buffer (pH 7.0), and 0.1 M imidazole. For the solvent perturbation measurements, equal volumes of cytochrome (or heme octapeptide) and perturbant stock solutions were added directly to cuvettes using 2-mL Gilmont micrometer burettes. Measurements on the ferrous cytochrome were made on deaerated solutions containing 0.01 M sodium dithionite under argon atmosphere.

Spectrophotometric Measurements. Spectra were recorded on a Cary 14R spectrophotometer at ambient temperature (23–25 °C). Difference spectra were recorded on the expanded scale (0.0 to 0.1 Å). Solvent perturbation difference spectroscopy was performed essentially as described by Herskovits (1967), except that standard 1-cm path length, Teflon-stoppered cuvettes were used instead of tandem cells since the perturbants used do not absorb in the Soret region (400–420 nm). Circular dichroism spectra of ferricytochrome *c*-555 solutions in 20% perturbant were measured at 22 °C on a

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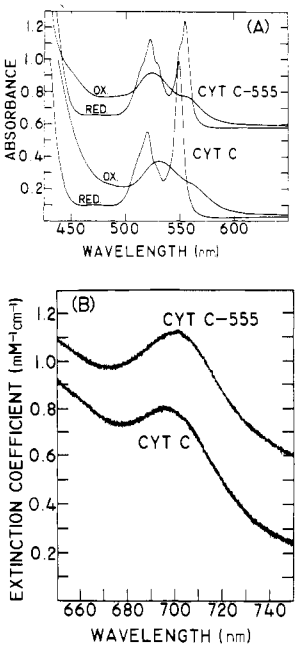

FIGURE 1: Visible and near-IR spectra. Cytochrome *c*, 6.83×10^{-5} M; cytochrome *c*-555, 6.81×10^{-5} M; 0.2 M potassium phosphate buffer, pH 7.00, 25 °C. (A) Visible spectra of the oxidized (ox) and reduced (red) cytochromes *c* and *c*-555 in the α,β region. Reduction was performed by addition of excess sodium dithionite. The cytochrome *c*-555 spectra have been displaced vertically for clarity. (B) Near-IR spectra of ferricytochromes *c* and *c*-555 recorded on a Cary 14R spectrophotometer utilizing an expanded scale slide wire (0.0–0.1 Å).

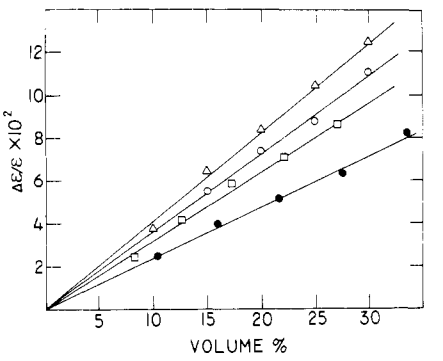

FIGURE 2: $\Delta\epsilon/\epsilon$ values of the heme octapeptide plus imidazole as a function of perturbant concentration. (□) Glycerol; (○) ethylene glycol; (●) sucrose (wt %); (△) Me_2SO. All solutions contained 0.1 M phosphate buffer (pH 7.0) and 0.1 M imidazole. Heme octapeptide concentrations ranged from 0.7 to 1×10^{-5} M.

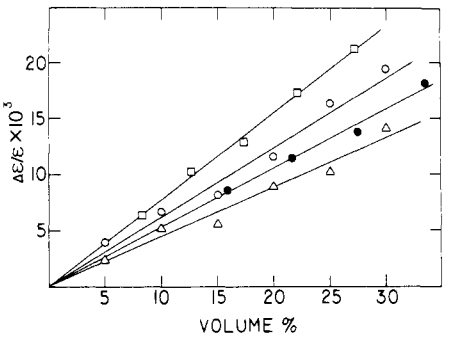

FIGURE 3: $\Delta\epsilon/\epsilon$ values of ferricytochrome *c*-555 as a function of perturbant concentration. (□) Glycerol; (○) ethylene glycol; (●) sucrose (wt %); (△) Me_2SO. All solutions contained 0.1 M phosphate buffer (pH 7.0). Cytochrome concentrations ranged from 3 to 5×10^{-6} M.

Durrum-Jasco ORD/UV-5 spectropolarimeter with the SS-20 CD modification. The solutions were prepared as described for the solvent perturbation difference spectra.

Results

Visible and Near-IR[1] *Spectra.* Figure 1 compares the optical absorption spectra of cytochromes *c* and *c*-555. Ferricytochrome *c* exhibits a weak charge transfer band at 695 nm which has been shown to be due to the binding of methionine-80 as the sixth iron ligand (Schechter & Saludjian, 1967; Sreenathan & Taylor, 1971). Figure 1B shows that ferricytochrome *c*-555 exhibits a similar absorption band at 701 nm. The extinction coefficient of this band is 1.120 mM^{-1} cm^{-1} as compared with 0.801 mM^{-1} cm^{-1} for cytochrome *c*, relative to a water baseline.

Heme Environment. Solvent perturbation difference spectroscopy has been used to determine the extent of exposure of chromophores in proteins to solvent, by comparison with the difference spectra of the model chromophore. In this study we have used the heme octapeptide of ferricytochrome *c* with added imidazole as the model chromophore. This cytochrome fragment contains residues 14 to 21 with the heme moiety covalently attached to cysteinyl residues 14 and 17 via two thioether linkages, and the fifth heme iron coordination position occupied by an imidazole nitrogen of histidine 18 (Harbury & Loach, 1960). The added imidazole coordinates in the sixth position to form a low-spin complex with an absorption spectrum similar to that of ferricytochrome *c*-555. In contrast, addition of *N*-acetylmethionine to the ferric heme peptide, to form a complex corresponding to that found in cytochrome *c*-555, has been found to yield an absorption spectrum indicative of incomplete coordination even at very high ligand concentration (Harbury et al., 1965). In previous solvent perturbation studies of cytochromes (Stellwagen, 1967; Cabral & Love, 1974), the heme hexadecapeptide of cytochrome *c* plus imidazole was used as a model chromophore and it was assumed that this small peptide is incapable of shielding the large heme chromophore from contact with solvent. The $\Delta\epsilon/\epsilon$ values observed for the heme octapeptide in this study are consistently greater than those which have been observed (Cabral & Love, 1974) for the heme hexadecapeptide, indicating that the smaller heme octapeptide may be a more appropriate model for the 100% exposed chromophore.

A linear relationship between $\Delta\epsilon/\epsilon$ and perturbant concentration has been used (Herskovits, 1967) as an indication that solvent perturbation occurs without changes in the native protein conformation. Figures 2 and 3 show that $\Delta\epsilon/\epsilon$ values for both ferricytochrome *c*-555 and the ferric heme octapeptide are proportional to perturbant concentration in the range investigated. In addition, the circular dichroism of ferricytochrome *c*-555 was examined to determine the effect of 20% perturbant on the protein conformation. As shown in Table I there appear to be no significant changes in the molar ellipticities of the major extrema between 250 and 450 nm in the presence of 20% perturbant. In contrast, previous solvent perturbation studies on cytochromes using monohydric alcohols (Kaminsky et al., 1972) showed significant alterations in native protein conformation as shown by circular dichroism measurements. The present results are consistent with the observation (Toniolo et al., 1974) that polyhydric alcohols support peptide secondary structure.

[1] Abbreviations used: Me_2SO, dimethyl sulfoxide; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; IR, infrared; ϵ, extinction coefficient; SD, standard deviation.

TABLE I: The Effect of Perturbants on the Circular Dichroism of Ferricytochrome *c*-555.

Perturbant (20%)	$[\theta] \times 10^{-4}$ (deg cm^2 mol^{-1})[a]				
	412 nm[b]	352 nm[c]	305 nm[b]	296 nm[c]	260 nm[b]
No perturbant	11.5	−1.8	1.4	1.2	5.7
Me_2SO	11.7	−1.7	1.7	1.4	6.4
Ethylene glycol	12.0	−1.9	1.4	1.2	6.2
Glycerol	12.6	−2.1	1.5	1.1	6.8
Sucrose	12.8	−1.9	1.4	1.1	6.9

[a] Error limits: $\pm 0.5 \times 10^{-4}$ [b] Maxima. [c] Minima.

The solvent perturbation results for 20% perturbant concentrations shown in Table II were obtained from the least square lines. Relative exposures were calculated using: % relative exposure = $(\Delta\epsilon/\epsilon)_{\text{cytochrome}}/(\Delta\epsilon/\epsilon)_{\text{model}} \times 100\%$.

Discussion

On the basis of the 701-nm absorption band exhibited by ferricytochrome *c*-555, we propose that the sixth ligand to the heme iron is a methionyl residue. This band is red-shifted relative to that of cytochrome *c*, as are the other bands in the visible region. The amino acid sequence of "*C. ethylica*" cytochrome *c*-555 (Van Beeumen et al., 1976), now known to be *P. aestuarii* cytochrome *c*-555, shows four methionines, one of which, Met-73, is followed by a proline residue. This -Met-Pro- sequence also occurs in three algal *f*-type cytochromes, *C. thiosulfatophilum* cytochrome *c*-555, and cytochrome c_5, in a position comparable to that of the -Met-Pro- sequence reported to contain the sixth iron ligand methionine in *Ps. aeruginosa* cytochrome *c*-551 (Ambler, 1974; Fanger et al., 1967). Such a -Met-Pro- sequence does not occur in cytochrome *c*. If Met-73 is the sixth iron ligand in cytochrome *c*-555, then there may be a substantial difference in the folding of the polypeptide chain in this region even though the overall folding pattern of these two cytochromes may be the same (Dickerson et al., 1976). Such differences may contribute to the differences in the position of the absorption maxima of these two proteins.

The solvent perturbation results, summarized in Table II, indicate that the folding of the polypeptide chain about the heme greatly restricts its contact with solvent, as shown by the smaller $\Delta\epsilon/\epsilon$ values observed for the cytochrome relative to the 100% exposed model compound. Larger perturbants do not give a smaller degree of exposure than smaller perturbants, suggesting that the folding of the polypeptide chain does not selectively restrict the access to the heme of the perturbants according to size. These results are similar to those observed for cytochrome *c* (Stellwagen, 1967) and are consistent with a crevice-like structure surrounding the heme with an exposed edge. The lower relative exposure observed for Me_2SO is somewhat difficult to accept in light of the relative sizes of the perturbants. Since the other perturbants, all essentially polyhydroxy alcohols, give more reasonable and consistent results, it is assumed that Me_2SO acts somehow differently as a perturbant. A solvent perturbation study of human luteinizing hormone (Bishop & Ryan, 1975) suggests that Me_2SO may display differential solvation effects with proteins, giving anomalous measures of the extent of solvent exposure. Such effects may also occur with the heme octapeptide, influencing the $\Delta\epsilon/\epsilon$ value observed for this model.

Cytochrome *c*-555 has a molecular weight of 11 024 based on the amino acid composition (Van Beeumen et al., 1976) as compared with 12 384 for cytochrome *c* (Margoliash, 1962). In view of this difference in size, it might be expected that the heme in cytochrome *c*-555 would be somewhat more exposed to solvent than the heme in cytochrome *c*. Solvent perturbation results, however, indicate the opposite. Table III compares the relative exposure of the heme moieties in ferrocytochrome *c*-555 and ferrocytochrome *c*. The perturbants used indicate that the heme in cytochrome *c*-555 is 75% to 98% exposed relative to the heme in cytochrome *c*. Comparison of the $\Delta\epsilon/\epsilon$ value observed for ethylene glycol shown in Table II with the reported value for ferricytochrome *c* (Stellwagen, 1967) shows that the *c*-555 is 61% exposed relative to the heme in ferricytochrome *c*. Such differences in the extents of exposure between cytochromes could indicate a basis for differences in rates of electron transfer according to the edge-on mechanism (Dickerson & Timkovich, 1975).

Since cytochrome *c*-555 and cytochrome *c* both have the same axial ligands to the heme iron, the question arises as to the cause of the difference in redox potentials between these two cytochromes. It has been proposed (Kassner, 1972) that a more hydrophilic heme environment stabilizes the ferric state relative to a hydrophobic environment and thus results in a lower redox potential. On this basis, one would predict that cytochrome *c*-555 should have a higher redox potential than cytochrome *c*, since a more hydrophobic environment would in general be expected for a smaller degree of exposure to solvent. The observation that the redox potential is actually about 160 mV lower suggests that the degree of solvent exposure does not correlate with the redox potentials. It is possible, however, that, although the heme is less exposed to solvent, the amino acid side chains in the vicinity of the heme may be of a more polar nature in cytochrome *c*-555, such that the heme environment is actually more hydrophilic, resulting in a lower redox potential. This picture, however, is not obvious from a comparison of the amino acid compositions which shows that cytochrome *c*-555 (Van Beeumen et al., 1976) contains 39% nonpolar amino acids (Ala, Val, Leu, Ile, Pro, Phe, Trp, and

TABLE II: Effect of Perturbants on Ferricytochrome *c*-555 and the Heme Octapeptide of Ferricytochrome *c*.

Perturbant 20%	Cytochrome *c*-555		Heme octapeptide		Rel exposure (%)	Effective radius (Å)[a]
	$\Delta\lambda_{max}$ (nm)	$\Delta\epsilon/\epsilon$ (± SD)	$\Delta\lambda_{max}$ (nm)	$\Delta\epsilon/\epsilon$ (± SD)		
Me_2SO	413	0.0089 (±0.0008)	412	0.0823 (±0.0026)	11	1.9
Ethylene glycol	413	0.0125 (±0.0009)	412	0.0725 (±0.0018)	17	2.3
Glycerol	413	0.0156 (±0.0003)	412	0.0642 (±0.0020)	24	2.6
Sucrose	413	0.0106 (±0.0005)	412.5	0.0479 (±0.0018)	22	3.6

[a] Stellwagen, 1967.

TABLE III: Exposure of the Ferrocytochrome *c*-555 Heme Relative to Ferrocytochrome *c*.[a]

Perturbant 20%	$\Delta\epsilon/\epsilon$ (± av deviation)	Rel exposure (%)
Me_2SO	0.0198 (±0.0004)	75
Ethylene glycol	0.0166 (±0.0031)	81
Glycerol	0.0194 (±0.0005)	98
Sucrose	0.0135 (±0.0002)	78

[a] Calculated using the $\Delta\epsilon/\epsilon$ values for ferrocytochrome *c* (Stellwagen, 1967).

Met), compared with 31% for cytochrome *c* (Margoliash & Schejter, 1966). It should be noted though that, among the nonpolar amino acids, cytochrome *c*-555 contains a smaller percentage of those having more bulky side chains, including only one phenylalanine compared to four in cytochrome *c*. The proximity to the heme of these large nonpolar amino acid side chains in cytochrome *c* may provide a more hydrophobic environment (Kassner, 1973) than that in cytochrome *c*-555. Further investigations of the possible correlation of the redox potential with the degree of solvent exposure of the heme in other cytochromes are in progress.

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