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Magnetic circular dichroism spectroscopy as a probe of axial heme ligand replacement in semisynthetic mutants of cytochrome *c*

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Horse heart cytochrome *c* with either histidine or cysteine replacing the endogenous axial methionine ligand at position 80 has been characterized with magnetic circular dichroism (MCD) spectroscopy in the UV-visible region. Comparison of the MCD spectra of the mutant proteins in the ferric state to those of authentic bis-imidazole- and imidazole/thiolate-ligated ferric heme proteins clearly shows that the histidine-imidazole and cysteine-thiolate groups of the replacement amino acids at position 80 are coordinated to the heme iron in the mutant proteins. This study demonstrates the power of MCD spectroscopy in identifying axial ligands in mutant heme proteins. Accurate axial ligand assignment is essential for proper interpretation of the altered properties of such novel proteins.

MCD spectroscopy; Active site structure determination; Cytochrome; Cysteine ligation; Histidine ligation; Electron transfer protein

1. INTRODUCTION

With the advent of site specific mutagenesis and the availability of improved synthetic methodologies, it has become quite feasible to prepare mutant metalloenzymes in which one of the amino acid ligands to the metal has been replaced with a different amino acid. If the altered properties of the mutant protein are to be understood, it is essential to establish that the replacement amino acid has in fact become coordinated to the metal. In the present study, mutants of cytochrome *c* in which the axial methionine ligand at position 80 has been replaced with histidine (cyt *c*-His80) or cysteine (cyt *c*- Cys80) [1,2], have been investigated with magnetic circular dichroism (MCD) spectroscopy [3,4] in order to establish the coordination structure of the heme iron in the mutant proteins.

Cytochrome *c* is a heme-containing electron-transfer protein which is a critical component of the mitochondrial electron-transport chain [5]. Because the tertiary structure of a large number of cytochromes *c* have been established [6], the protein has been a favorite target for studies of the role of protein structure in electron transfer [7,8]. In order to explore the role of the axial ligand in electron transfer with heme proteins, Raphael and Gray [1,2] used a semisynthetic procedure to prepare cyt *c*-His80 and cyt *c*-Cys80. We have used MCD spectroscopy to characterize the mutant proteins because the technique has been particularly effective in establishing the coordination structures of native heme proteins as well as synthetic heme model complexes [3,4,9,10]. The results presented herein further demonstrate the utility of MCD spectroscopy as a structure determination method.

Abbreviations: EPR, electron paramagnetic resonance; MCD, magnetic circular dichroism; P-450, cytochromes P-450 from any source; P-450-CAM, the camphor-hydroxylating P-450 from *Pseudomonas putida*; cyt *c*-Cys80, cytochrome *c* having cysteine as an axial ligand in place of methionine at position 80; cyt *c*-His80, cytochrome *c* having histidine in place of methionine at position 80; cyt *c*-Imid, the imidazole adduct of cytochrome *c* with imidazole in place of methionine

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2. MATERIALS AND METHODS

Samples of cyt *c*-His80 and cyt *c*-Cys80 [1,2] were generously supplied by Dr Adrienne L. Raphael and Prof. Harry B. Gray (California Institute of Technology). Native horse heart cytochrome *c* and imidazole were purchased from Aldrich. All additional chemicals and biochemicals were reagent grade and were used without further purification.

All spectral measurements were carried out at 4°C using protein samples (30–150 μM) prepared in 50 mM phosphate buffer at pH 7.0. Native cytochrome *c* samples were prepared in potassium phosphate buffer while the two mutant proteins were examined in sodium phosphate buffer in the presence of 200 mM NaCl. Before spectral studies, all cytochrome *c* samples were fully oxidized by treatment with excess potassium ferricyanide, followed by gel filtration chromatography [11]. The imidazole complex of native cytochrome *c* was prepared by treatment of the native ferric protein with a 50 000-fold excess of imidazole [12,13]. The pH values were measured with a Brinkman 101 pH meter (Metrohm combination electrode). Protein concentrations were determined spectrophotometrically, using published extinction coefficients [1,11,14,15].

Electronic absorption spectra were recorded using Varian/Cary 210 and 2300 spectrophotometers, each of which had been interfaced to an IBM PC for data acquisition. MCD/CD spectra were recorded with a JASCO J-500A spectropolarimeter equipped with a JASCO MCD-

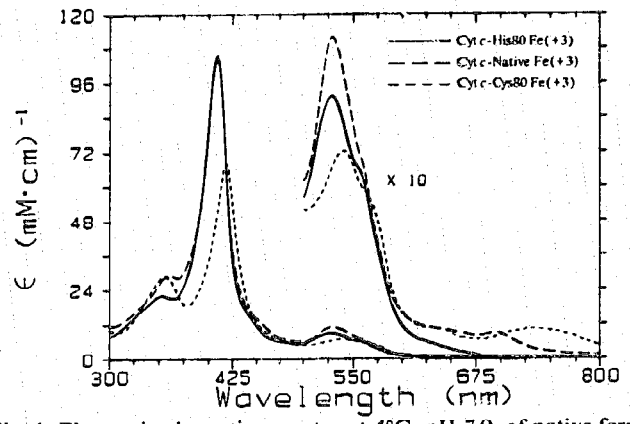

Fig. 1. Electronic absorption spectra at 4°C, pH 7.0, of native ferric horse heart cytochrome *c* and of the ferric cyt *c*-His80 and cyt *c*-Cys80 mutants. The spectra were obtained in 50 mM potassium phosphate buffer for native cytochrome *c* (76 μM) (— — —) and in 50 mM sodium phosphate buffer, 0.2 M NaCl, for cyt *c*-His80 (152 μM) (——) and cyt *c*-Cys80 (31.8 μM) (------).

1B electromagnet operated at a field strength of 1.41 T. The J-500A was interfaced to an IBM PS/2 Model 50 by a JASCO IF-500-2 interface unit. The number of scans signal averaged were 2–4. Data acquisition and handling were carried out as previously described [11,16].

The MCD spectra of cytochrome *c* and of cyt *c*-Imid reported herein closely match those previously reported by Vickery et al. [17]. In comparing the spectrum of cyt *c*-Cys80 to that of the imidazole complex of P-450 (Fig. 2), the cyt *c* spectrum has been red-shifted by 8 nm, as was done by Vickery et al. [17], to make up for the fact that the *c* heme does not have two vinyl substituents.

3. RESULTS AND DISCUSSION

Fig. 1 shows a comparison of the electronic absorption spectra, in the Soret and visible regions, of native ferric horse heart cytochrome *c* and the cyt *c*-His80 and cyt *c*-Cys80 mutants. The UV-visible absorption spectra of native cytochrome *c* and cyt *c*-His80 are very similar, particularly in the Soret region. A bis-imidazole-ligated derivative of cytochrome *c* formed by displacement of methionine-80 by added exogenous imidazole (cyt *c*-Imid) also has a spectrum that compares very well to that of the native cytochrome [12,13]. The most notable difference between the spectrum of the native protein and those of cyt *c*-His80 and cyt *c*-Imid is the absence of the 695 nm band in the latter two spectra. The 695 nm band is a methionine sulfur to the ferric heme ion charge transfer transition [6,18,19]. While the absence of the 695 nm band in the spectrum of cyt *c*-His80 is consistent with absence of methionine as a ligand, it does not establish the identity of the new axial ligand.

The electronic absorption spectrum of cyt *c*-Cys80 is substantially different from that of native cytochrome *c* (Fig. 1). The red shift and hypochromicity of the Soret band in the spectrum of cyt *c*-Cys80 relative to that of the native protein is an indication of increased ligand field strength about the heme iron [20] consistent with Cys80 thiolate ligation. Peaks of low intensity at 635 and 734 nm are presumed to originate from sulfur to ferric iron charge transfer transitions [2].

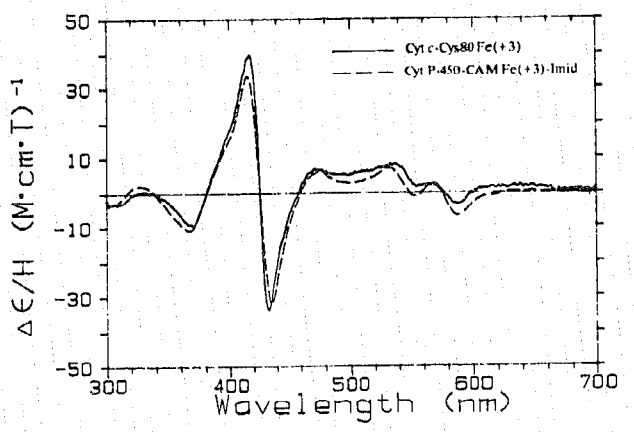

Fig. 2. Magnetic circular dichroism spectra at 4°C, pH 7.0, of ferric cyt *c*-Cys80 and the imidazole complex of ferric cytochrome P-450-CAM. Cyt *c*-Cys80 (32 μM) (——) was examined in 50 mM sodium phosphate buffer, 0.2 M NaCl, and the P-450-CAM imidazole complex (25 μM P-450-CAM, 22 μM camphor, 88 mM imidazole) (— — —) in 100 mM potassium phosphate buffer. The spectrum of cyt *c*-Cys80 has been red-shifted by 8 nm, see text. The spectrum of the P-450 adduct is reproduced from Sono et al. [21].

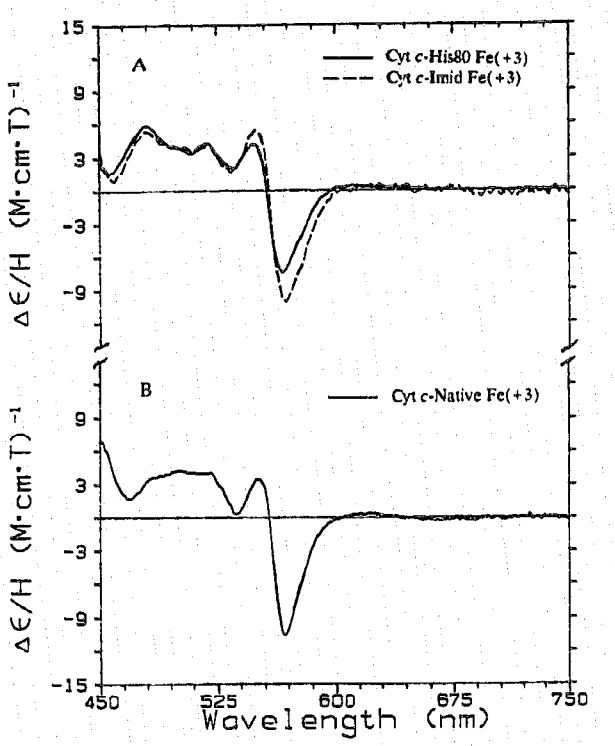

Fig. 3. Magnetic circular dichroism spectra in the visible region at 4°C, pH 7.0, of (A) ferric cyt *c*-His80 and the imidazole complex of native ferric cytochrome *c* and of (B) native ferric horse heart cytochrome *c*. Cyt *c* His80 (152 μM) (——) was examined in 50 mM sodium phosphate buffer, 0.2 M NaCl; cyt *c*-Imid (51 μM protein, 2.5 M imidazole) (— — —) in 50 mM potassium phosphate buffer; and cytochrome *c* (95 μM) (——) in 50 mM potassium phosphate buffer.

The MCD spectrum of ferric cyt *c*-Cys80 in the UV-visible region is compared to that of the imidazole complex of ferric cytochrome P-450-CAM [21] in Fig. 2. It is well established that cytochrome P-450 has a proximal cysteine thiolate ligand [9,22,23] and that imidazole can bind to form a six coordinate complex [21]. The consistent match of every feature in the MCD spectra of the imidazole complex of P-450-CAM [21] and cyt *c*-Cys80 (Fig. 2) provides strong evidence that the axial ligands in the latter are a thiolate from cysteine and imidazole from histidine.

The similarity of the electronic absorption spectra of cyt *c*-His80 and native cytochrome *c* (Fig. 1) illustrates the difficulty in spectroscopically distinguishing between bis-histidine and histidine-methionine ligation in ferric, low-spin heme proteins. The MCD spectra of the two proteins in the Soret region are also closely matched [11]. The visible region MCD features of ferric low-spin heme proteins, on the other hand, are more sensitive to axial ligand identity than the Soret region [17]. Fig. 3A shows a comparison of the MCD spectra of cyt *c*-His80 and cyt *c*-Imid in this region. The two MCD spectra have essentially identical band shapes and intensities throughout the region. In contrast, the spectrum of native cytochrome *c* (Fig. 3B), while broadly similar to the above spectra (Fig. 3A), lacks the fine structural features found between 470 and 530 nm in the MCD spectra of cyt *c*-His80 and cyt *c*-Imid. Taken together, these data are most consistent with coordination of His80 in cyt *c*-His80 although the data are not as compelling as for cyt *c*-Cys80.

Thomson and co-workers have shown that near-IR MCD spectroscopy can better distinguish between bis-histidine- and histidine/methionine-ligated ferric heme proteins [4,24]. While the amount of cyt *c*-Cys80 sample was insufficient for near-IR MCD analysis, a near-IR absorption spectrum of the sample was measured and the lowest energy (and most intense) band in the near-IR was observed at approximately 1350 nm. This is much closer to the position of the lowest-energy near-IR transition observed for cyt *c*-Imid at 1480 nm than to that seen at 1720 nm for native cytochrome *c* [11]. These data provide additional support for the conclusion that histidine is an axial ligand to the ferric heme iron in the cyt *c*-His80 mutant.

The proper interpretation of electron transfer studies done with these cytochrome *c* mutants [2] is dependent on accurate axial ligand assignment. The process utilized to produce these axial ligand replacement mutants involves the denaturation and subsequent refolding the protein polypeptide [25]. Not all attempts at axial ligand replacement have been successful in high yield [2]. Thus, careful analysis of the resultant proteins is required to be certain that axial ligation by the 'expected' amino acid has taken place. The utility of MCD spectroscopy in the analysis of native heme proteins of unknown ligation state is well developed [3,4,9,10,21,26]. The present study illustrates the usefulness of the method to establish the identities of the replacement axial ligands in mutant heme proteins.

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