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Circular dichroism studies of the binding of mammalian and non-mammalian cytochromes *c* to cytochrome *c* oxidase, cytochrome *c* peroxidase, and polyanions

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Abstract

The effects of binding of *Candida krusei*, *Drosophila melanogaster*, horse, human, and rat cytochromes *c* to beef cytochrome *c* oxidase (ferrocytochrome *c*: oxygen oxidoreductase, EC 1.9.3.1) and yeast cytochrome *c* peroxidase (ferricytochrome *c*: hydrogen-peroxide oxidoreductase, EC 1.11.1.5) on their circular dichroism spectra were determined. The binding to cytochrome oxidase results in a positive increase in the ellipticities of the positive and negative Cotton effects at 404 nm and 417 nm of cytochrome *c*. The horse, human, and rat cytochromes *c* display less of an increase in the ellipticity of the positive Cotton effect at 404 nm, but more of a positive change in the negative Cotton effect at 417 nm than the *C. krusei* or *D. melanogaster* proteins. Interaction with yeast cytochrome *c* peroxidase elicits only a positive change in the ellipticity of the positive Cotton effect at 404 nm. No significant change is observed in the negative Cotton effect at 417 nm. Rat cytochrome *c* variants with a phenylalanine in place of tyrosine-67 and/or an alanine in place of proline-30 all display circular dichroism spectral changes upon binding to cytochrome *c* oxidase or cytochrome *c* peroxidase identical to those of the unaltered protein. The increase in ellipticity at 404 nm upon binding occurs even though replacement of tyrosine-67 results in the loss of the positive Cotton effect at this position. Polyglutamate and phosvitin complexes of cytochrome *c* show changes in the circular dichroism spectrum similar to those observed with cytochrome *c* peroxidase. However, the magnitudes of the spectral changes were considerably less. A model is proposed in which the main cause of the circular dichroism spectral changes observed upon complexation arise from the exclusion of solvent from the exposed front heme edge. According to this model, the exclusion of solvent changes the relative asymmetry of the environment of the electronic transitions of the heme prosthetic group of cytochrome *c*, resulting in observed circular dichroic effects.

Key words: Cytochrome *c*; Cytochrome oxidase; Cytochrome *c* peroxidase; Circular dichroism

1. Introduction

Eukaryotic cytochromes *c* are proteins of approx. 12400 Da that contain a single covalently attached heme prosthetic group. The axial ligands to the iron center of the heme are a histidine imidazole and a methionine sulfur. Cytochrome *c* functions in the electron transport chain of mitochondria as an electron shuttle between cytochrome *c* reductase (coenzyme-Q:cytochrome *c* oxidoreductase, EC 1.10.2.2) and cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) (COX). Cytochromes *c* in fungi also transfer electrons to cytochrome *c* peroxidase (ferricytochrome *c*:hydrogen-peroxide oxidoreductase, EC 1.11.1.5) (CCP) [1,2].

Many of the physio-chemical properties of the eukaryotic cytochromes *c* vary among the proteins from different phylogenetic groups or organisms. For exam-

Abbreviations: CD, circular dichroism; COX, cytochrome *c* oxidase; CCP, cytochrome *c* peroxidase; NMR, nuclear magnetic resonance; P30A, cytochrome *c* with an alanine substituted for proline at position 30; Y67F, cytochrome *c* with a phenylalanine substituted for tyrosine at position 67;

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ple, the kinetics of electron transfer between various cytochromes *c* and CCP in a preformed complex are different for fungal and non-primate mammalian proteins [3]. Furthermore, the lability of the coordinate bond between the methionine-80 and the heme iron center also displays phylogenetic differences. Typically, the coordination of methionine-80 in non-mammalian eukaryotic cytochromes *c* is more sensitive to changes in pH and to displacement by azide, imidazole, and cyanide [2].

The binding properties of cytochrome *c* with COX and yeast CCP also display such differences, the fungal and primate cytochromes *c* having tighter binding to beef COX than the non-primate mammalian proteins [4–6].

The electron transfer and binding properties of cytochrome *c* have been studied by chemical modification and site-directed mutagenesis [7–10]. The enzymatic interaction domain includes the solvent exposed edge of the heme. Electrostatic interactions act to stabilize the complex formed with the various physiological redox partners. It has been proposed that these electrostatic interactions involve the cationic patch on the cytochrome *c* and not any specific salt bridges [11], with the bound cytochrome *c* in a dynamic equilibrium of various orientations to the enzyme. Recent research on the complex formed by cytochrome *c* with cytochrome b_5 or yeast CCP supports such a proposal [12]. The orientation between the two proteins that results in electron transfer may just be a subpopulation.

Circular dichroism (CD) is a powerful tool for studying the structure/functional properties of heme containing proteins [13–19]. This is because free heme is a symmetrical molecule and therefore displays no CD spectrum. The protein induces an asymmetry in the electronic absorption of the heme, resulting in the observed optical activity [20]. The CD spectra of eukaryotic cytochromes *c* are all identical and have been studied extensively [2]. Cytochrome *c* is unique in that exciton splitting in the Soret band occurs [21,22]. The electronic basis of this is not yet understood. The replacement of phenylalanine-82 with a non-aromatic amino acid results in the loss of the negative Cotton effect arising from the Soret absorption [23–25]. Bosshard and coworkers have reported the successful use of CD to study the binding of horse cytochrome *c* to cytochrome oxidase [24,26,27]. These studies have shown that the spectral changes associated with complexation are similar but nevertheless significantly different from those observed in titration of the coordination of methionine-80 to the heme iron at alkaline pH.

In this paper we report CD studies of the binding of various mammalian and non-mammalian cytochromes *c* with COX, CCP, and polyanions. We also report the effects of various site-directed mutations of cytochrome *c* that affect the stability of the coordination of methionine-80 to the heme iron on the individual spectra and those of the complexes. These results show a distinction between the spectral changes associated with binding of the mammalian and the non-mammalian cytochromes *c* to COX and CCP. This distinction is independent of the site-directed changes in the amino acid sequence of cytochrome *c* studied that alter the stability of the coordination of methionine-80.

2. Materials and methods

2.1. Proteins

All natural and recombinant cytochromes *c* were prepared and handled as previously described [7,28]. The proteins were prepared as previously described [29–31]. Beef COX was the generous gift of Dr. Anton O. Muijsers (University of Amsterdam).

CCP was isolated from Red Star pressed baker's yeast by a modified version of the procedure developed by Nelson et al. [32]. The cells (1 kg) were lysed by incubation at 4°C for 14 h in 1 l ethyl acetate with 2 mM phenylmethylsulfonyl fluoride/5 kg yeast. The lysate was diluted with 10 l of 15 mM sodium acetate (pH 5). The insoluble debris was removed by centrifugation at $5000 \times g$ for 20 min at 4°C, followed by three filtrations of the supernatant with 3 g/l Hyflo Supercell (Fisher Scientific) added each time. The CCP was absorbed onto DEAE-Sepharose FF (Pharmacia), eluted with 500 mM sodium acetate (pH 5.0) and chromatographed on G-75 Sephadex (Pharmacia) in 60 mM sodium acetate (pH 5.0). Only those fractions containing CCP with an absorbance ratio of 408 nm/280 nm exceeding 1.2 were pooled, absorbed on DEAE-Sepharose FF and eluted with the 500 mM sodium acetate, yielding solutions of 0.5–2.0 mM CCP. The CCP was slowly crystallized by dialysis at 4°C for 48 h against each of 30 mM sodium acetate, 15 mM sodium acetate, both at pH 5.0, and distilled water. The crystals were washed with cold distilled water, dissolved in 500 mM sodium acetate, and stored in liquid nitrogen. The CCP was recrystallized, dissolved in 500 mM sodium acetate, and dialyzed at pH 7 for 48 h each against 100 mM potassium phosphate, 40 mM potassium phosphate/60 mM potassium chloride, 10 mM potassium phosphate/40 mM potassium chloride, and finally twice against 2 mM potassium phosphate/25 mM potassium chloride. The CCP displays an EPR signal at $g = 5.98$. Only slight changes in the UV-visible absorption spectrum of the CCP above 600 nm were observed between pH 5 and pH 8 ($\lambda_{max} = 645$ nm).

Polyanions, polycations, and copolymers were purchased from Sigma.

2.2. Instrumentation

Ultraviolet-visible absorbance spectra were collected on a Hitachi 557 spectrophotometer. CD spectra were obtained using a Jasco-500 spectropolarimeter with a time constant of 1 s and a slit width of 2 nm employing a 1 cm cell. The CD data presented are the average of between 5 and 10 scans collected at 20 nm/min. Samples in which the concentration of total cytochrome exceeded 10 μM were examined in a 1 mm path length cuvette.

3. Results

3.1. CD spectra of various cytochromes c

Cytochromes *c* display major positive and negative Cotton effects in the Soret band region at 404 nm and 417 nm, respectively, and weak negative Cotton effects at 383 nm and 460 nm. These features are independent of pH between 6.8 and 8.0 and of ionic strength between 10 mM and 150 mM. They are also the same for proteins derived from mammals (primate or non-primate), fungi and insects.

Replacement of proline-30 by an alanine in the rat and Drosophila cytochromes *c* (P30A), has no significant effect on the CD spectrum. In contrast, the replacement of tyrosine-67 with a phenylalanine in rat cytochrome *c* (Y67F), results in a CD spectrum lacking the positive Cotton effect in the Soret band region (Fig. 1). The negative Cotton effect is now observed at 412 nm. This apparent shifting is the result of the loss of the spectral overlap with the positive Cotton effect. Likewise, the corrected position of the positive Cotton effect, 410 nm, is obtained from the difference spectrum between the wild type rat cytochrome *c* and Y67F mutant proteins. The Y67F protein also displays a decrease in the ellipticity of the small negative Cotton effect at 383 nm and an increase in the broad negative elliptical band observed around 460 nm. The simplest interpretation of these observations is that in native cytochromes *c* the overlap of the strong positive and negative Cotton effects at 410 nm and 412 nm, respectively, result in apparent maxima at 404 nm and 417 nm. The opposite polarities in the CD maxima enhance their separation. The double mutant rat cytochrome *c* Y67F/P30A shows a CD spectrum indistinguishable from that of the Y67F protein (data not shown).

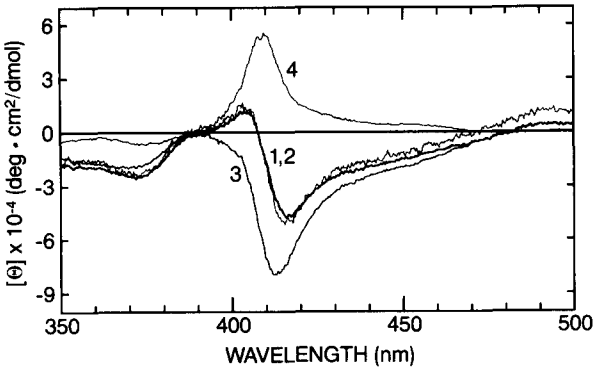

Fig. 1. Circular dichroism spectra of recombinant wild type and site-directed mutagenized rat cytochromes *c*. The circular dichroism spectra of (1) 2.1 μM recombinant rat cytochrome *c*, (2) 2.1 μM P30A rat cytochrome *c*, (3) 2.1 μM Y67F rat cytochrome *c*, and (4) the difference between the rat and the Y67F mutant protein spectra. The various proteins are in 25 mM Tris-acetate buffer (pH 7.9). Spectra were collected as described in Materials and methods.

3.2. CD spectra of cytochrome c complexes with cytochrome c oxidase or cytochrome c peroxidase

Even though the naturally occurring cytochromes *c* display identical CD spectra, the changes observed upon binding with either COX or CCP indicate two types of complexes (Fig. 2). The rat and human cytochromes *c* display changes in their CD spectra identical to those observed by Weber et al. [27] for the binding of horse cytochrome *c* with beef COX. These changes are an increase in ellipticity at 404 nm and 417 nm and a decrease at 432 nm. The change at 432 nm can be ascribed to the COX, because COX has an absorption band at 432 nm while cytochrome *c* does not. In contrast to these mammalian proteins, the Candida and Drosophila cytochromes *c* display a larger increase in ellipticity at 404 nm and less of a change at 417 nm (see Fig. 2A). At an ionic strength of 150 mM these spectral changes were not observed.

Differences in the CD spectral properties of the mammalian and non-mammalian cytochromes *c*-CCP complexes were also observed (Fig. 2B). The non-mammalian cytochromes *c* display a larger increase in ellipticity at 404 nm than the mammalian proteins. Though spectral changes at 417 nm were observed for the CCP complexes, spectral overlap between the Soret bands of cytochrome *c* and CCP (410 nm and 408 nm) makes interpretation difficult. Furthermore, the very broad negative Cotton effect centered around 400 nm of CCP may also serve to mask spectral changes in the cytochrome *c*. Therefore, it is hard to interpret the apparent differences between the COX and CCP difference spectra for the complexation of mammalian versus non-mammalian cytochromes *c* (Fig. 2A curve 3 versus Fig. 2B curve 5).

The CD spectral changes associated with the binding of the rat mutant proteins Y67F, P30A, the double mutant, Y67F/P30A, or by the Drosophila mutant, P30A, to either COX or CCP, were all identical to those observed for their respective, unaltered cytochromes *c* (data not shown). This is surprising, since even though the replacement of tyrosine-67 with a

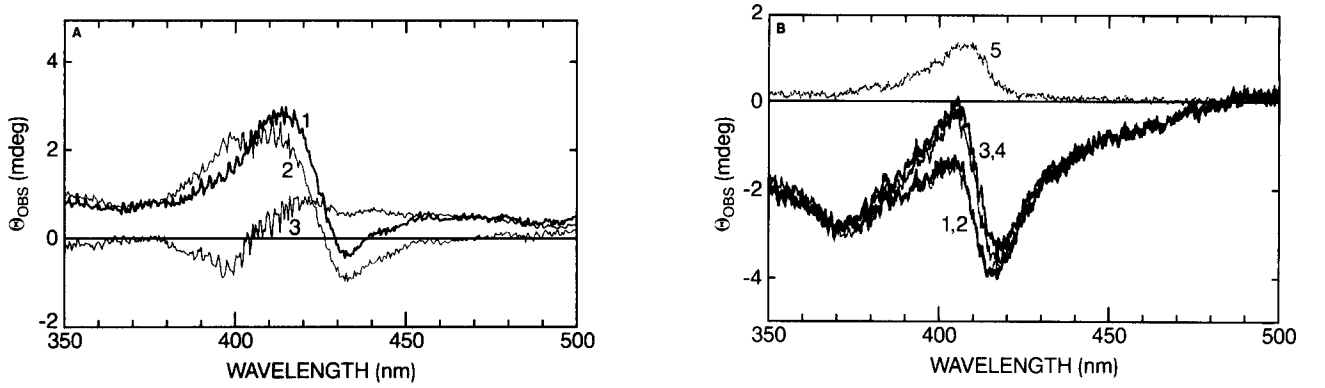

Fig. 2. Circular dichroism spectral studies of the binding of cytochrome *c* to cytochrome *c* oxidase and cytochrome *c* peroxidase. (A) Difference spectra of the binding of cytochrome *c* oxidase with (1) recombinant rat cytochrome *c* and (2) *Candida krusei* cytochrome *c*. Curve 3 is the difference between curves 1 and 2. All of the proteins were at 2.4 μM in 25 mM Tris-acetate (pH 7.9)/0.5% β-dodecyl maltoside. Spectra were collected as described in Materials and methods. (B) Circular dichroism spectra of the complexes formed by 4 μM cytochrome *c* with 4 μM cytochrome *c* peroxidase in 10 mM potassium phosphate (pH 6.8). (1, bold line) Rat cytochrome *c*-CCP; (2, thin line) human cytochrome *c*-CCP; (3, bold line) *C. krusei* cytochrome *c*-CCP; (4, thin line) Drosophila cytochrome *c*-CCP; (5) half the difference between the sum of curves 3 and 4 minus the sum of curves 1 and 2. The observed peak in curve 5 is at 408 nm.

phenylalanine results in the complete loss of the positive Cotton effect at 410 nm, complexation of that mutant protein induces a change in the ellipticity at this position.

3.3. CD spectra of cytochrome c complexes with polyglutamate and phosvitin

The influence of polyanions incapable of carrying out redox reactions on the CD spectra of cytochrome *c* was also examined. Fig. 3 illustrates the spectral changes observed upon adding polyglutamate to horse cytochrome *c* or phosvitin to rat cytochrome *c* in 10 mM potassium phosphate (pH 6.8). A slight increase in the ellipticity of the positive Cotton effect at 404 nm is observed. This result could not be mimicked by the amino acids glutamate or aspartate, or potassium chloride, or polymers of lysine. The spectral changes in cytochrome *c* induced by phosvitin or polyglutamate are qualitatively analogous to those resulting from the binding to CCP. However, the magnitudes of these changes are only 20% of those induced by the physiological redox partners examined. These decreases in the magnitude of the CD spectral changes may reflect looser binding between the polyanions and cytochrome *c*.

4. Discussion

4.1. Differences between cytochromes c

The differences in the CD spectral changes observed with horse, rat, and human (mammalian) cy-

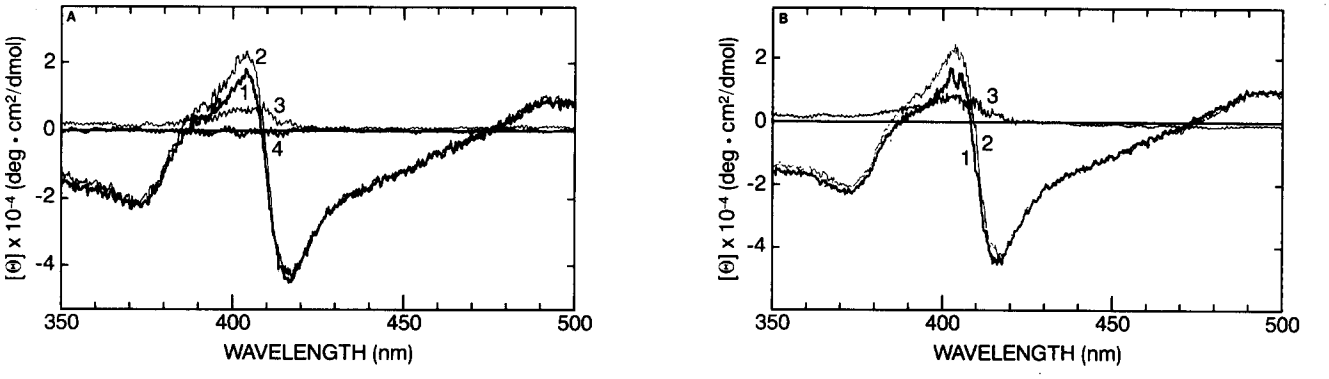

Fig. 3. Effects of polyanion binding on the CD spectrum of cytochrome *c*. (A) The binding of polyglutamate to horse cytochrome *c*. (1) 12 μM cytochrome *c* in 10 mM potassium phosphate (pH 6.8); (2) 12 μM cytochrome *c* with 15 μM polyglutamate (degree of polymerization = 95); (3) difference between curves 1 and 2. The difference spectrum plotted is the 5 point smoothing of the curve. (4) 12 μM horse cytochrome *c* with 1.5 mM sodium glutamate in 10 mM potassium phosphate (pH 6.8). (B) The binding of phosvitin to rat cytochrome *c*. (1) 5.3 μM rat cytochrome *c* in 10 mM potassium phosphate; (2) 5.3 μM rat cytochrome *c* with 4 μM phosvitin (molecular weight approx. 40000) in 10 mM potassium phosphate (pH 6.8); (3) difference between curves 1 and 2. The difference spectrum plotted is the 5 point smoothing of the curve.

tochromes *c* on the one hand, and the *Candida krusei* and *Drosophila melanogaster* (non-mammalian) cytochromes *c* on the other, upon binding with COX or CCP indicate a novel classification of the protein. A similar division occurs in the kinetics of electron transfer in the preformed complex of cytochrome *c* with CCP [3].

The CD spectral differences between the complexes formed by the two classes of cytochromes *c* with either COX or CCP cannot be ascribed to an artifact arising from the incomplete binding of the various proteins. Indeed, under similar conditions, the human cytochrome *c* has a K_d for COX of $< 1 \cdot 10^{-8}$ M and the horse cytochrome *c* has a K_d of $3 \cdot 10^{-8}$ M [4–6], so that identical spectral properties are most likely not due to partial binding.

A structural property that is consistent with the division of cytochrome *c* into mammalian and non-mammalian groups is the stability of the coordination of methionine-80 to the iron of the heme. The pK_a of the alkaline induced displacement of methionine-80 as the sixth ligand to the heme iron in the fungal cytochromes *c* is 8.3 and in the Drosophila protein is 8.8, while those of the mammalian proteins are equal to or greater than 9.2 [2,30]. Replacement of tyrosine-67 with a phenylalanine increases the stability of the methionine-80 heme iron bond, so that the pK_a is increased by more than one pH unit [29]. In contrast, replacement of proline-30 with an alanine lowers the pK_a by almost one pH unit [31]. As a result, the P30A mutant rat cytochrome *c* resembles the non-mammalian proteins in regard to the stability of the methionine-80 coordination. Our observation that these changes do not alter the spectroscopic properties of the complexes indicates that the stability of the heme crevice does not determine the CD spectral changes associated with complexation.

The observation that mammalian and non-mammalian cytochromes *c* form slightly different complexes is consistent with the X-ray structures of the horse cytochrome *c*- and the yeast cytochrome *c*-CCP complexes obtained by Pelletier and Kraut [33]. Both complexes were basically similar including pyrrole ring II of cytochrome *c* in the docking site. Pelletier and Kraut observed with the horse cytochrome *c* complex indications of three intermolecular hydrogen bonds, while the yeast chytochrome *c* complex indicated only one such interaction. These similarities and differences are consistent with our results which show both types of protein undergoing basically similar, yet distinctive changes in the electronic transitions of the Soret.

4.2. Differences between polyanions

The differences between the complexes formed by COX and by CCP, or polyglutamate, or phosvitin are also unique. The occurrence of spectral overlap between the Soret bands of cytochrome *c* and CCP make definitive interpretations of the spectral differences difficult. It is proposed that the COX and the CCP are interacting differently with the cytochrome *c*. This is based upon the observation that polyglutamate and phosvitin mimic CCP, but do not absorb in the Soret region and cannot produce any compensatory changes in the CD spectrum. Hence, if one concludes that polyglutamate and phosvitin interact with cytochrome *c* in a manner analogous to CCP, the data indicate differences in the binding of cytochrome *c* with COX as compared to CCP.

The affinities of CCP, polyglutamate, and phosvitin towards various cytochromes *c* are considerably less than that of COX [4,34, unpublished data]. This cannot be the sole basis for the distinction in the spectral changes, since the change in ellipticity at 417 nm with COX is greater for rat and horse cytochromes *c* than for the tighter binding fungal protein. The K_d for *C. krusei*, horse, and rat cytochromes *c* with COX are estimated as $< 6 \cdot 10^{-9}$ M, $2 \cdot 10^{-8}$ M, and $1 \cdot 10^{-8}$ M, respectively [34].

4.3. Comparison of polarizing factors

The lack of a positive Cotton effect at 404 nm with the Y67F and Y67F/P30A cytochromes *c* means that these proteins lack the polarizing factor(s) that induce this band. However, upon binding with COX these proteins behave identically to the unaltered cytochromes *c*, namely, they display a positive increase in ellipticity at 404 nm. Therefore, the change observed at 404 nm upon binding must be ascribed to a separate influence whose net effect on the spectrum is analogous to that of having a tyrosine at position 67.

The increase in the positive ellipticity at 404 nm with all polyanions, and the increase at 417 nm with COX can be envisioned as making the magnitude of the opposing positive and negative Cotton effects more comparable. Namely, the opposing polarizing environmental factors that cause the negative and positive Cotton effects are becoming more balanced.

Pielak et al. [23] and Rafferty et al. [25] have demonstrated that the replacement of phenylalanine-82 with a non-aromatic amino acid results in the loss of the negative Cotton effect at 417 nm. This result is consistent with CD studies of *Pseudomonas aeruginosa* cytochrome *c*-551 which also lacks a negative Cotton effect and has a proline in place of a phenylalanine at position 82 [36]. We have observed that the replacement of tyrosine-67 results in the loss of the positive Cotton effect. It is proposed that the negative Cotton effect arises from a polarization from the anterior edge of the heme backwards, possibly aligning with the positive end of the dipole moment of the protein at

phenylalanine-82. Similarly, one could consider that the positive Cotton effect may represent a polarization perpendicular or at an angle to the heme.

Of interest is the observation by Raman et al. [37] that the complex formed by antibody binding near residue 44 on the lower right side of cytochrome *c* induces a loss of the positive Cotton effect and an increase in the magnitude of the negative Cotton effect. How this occurred is not understood. It is also unclear how this phenomenon relates to the observation of Mayne et al. [38], that antibody binding near the w loop between residues 38 and 50, on the lower part of the heme crevice, induced a destabilization of the protein.

To explain the effects observed above, one may envision that the tyrosine at position 67 and its hydrogen bonded water molecule [38] increase the hydrophilic environment of the lower left side of the heme. This creates a polarizing influence angular to the two axial ligands to the heme iron, the sulfur of methionine-80 and the imidazole of histidine-18. Replacement of tyrosine-67 with a phenylalanine, resulting in the removal of an internal hydroxyl, makes the environment of the left side of the heme more hydrophobic and thereby more analogous to that of the right side. This would lead to the loss of elliptical character and the disappearance of the positive Cotton effect in the cytochromes *c* that lack tyrosine-67. By contrast the complexation of cytochromes *c*, studied above, acts to reduce the polarization in the plane of the prosthetic group by excluding solvent and making the anterior more hydrophobic and similar to the posterior of the heme. This increases the relative influence of the internal polarizing factors and enhances the positive Cotton effect.

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