# Elimination of the Negative Soret Cotton Effect of Cytochrome *c* by Replacement of the Invariant Phenylalanine Using Site-Directed Mutagenesis

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**Abstract:** The circular dichroism (CD) was determined for variants of *Saccharomyces cerevisiae* iso-1-cytochrome *c*, in which phenylalanine-87, an invariant residue in eukaryotic and many prokaryotic cytochromes *c*, is replaced with a serine, tyrosine, or glycine. The major differences between the CD spectra of the wild-type protein and the variants occur in the Soret (385–445 nm) region of the spectrum. Specifically, a negative Cotton effect that occurs between 415 and 418 nm, with an intensity of (5.3–5.7) × $10^4$ ° $cm^2$/dmol in the spectrum of the oxidized state of the wild-type protein, is absent from the spectra of the oxidized variants. Difference spectroscopy of the variants suggest that this same negative Cotton effect may also be present in the spectrum of the reduced wild-type protein. Two mechanisms by which phenylalanine-87 could contribute to the Cotton effect are considered. A direct mechanism involves the interaction of the $\pi$ to $\pi^*$ transition of the aromatic side chain of phenylalanine-87 with the delocalized $\pi$-electron system of the heme prosthetic group. In an indirect mechanism, phenylalanine-87 could contribute to this Cotton effect by defining a unique conformation for the polypeptide surrounding the heme. A review of published data suggests that the occurrence of this negative Cotton effect in different cytochromes *c* correlates with the presence and orientation, with respect to the plane of the heme, of a phenylalanine residue in a position equivalent to that of phenylalanine-87 in the *S. cerevisiae* protein.

The *c*-type cytochromes are a collection of structurally related heme-containing proteins found throughout nature whose biological function is to transfer electrons. While the circular dichroism (CD) and optical rotary dispersion of these proteins have been studied for 20 years,[1] little progress has been made in assigning specific Cotton effects to specific amino acid residues. In a theoretical study of the Soret CD of myoglobin and hemoglobin, Hsu and Woody concluded that the induced Cotton effects of these proteins are due to a coupled oscillator interaction between the heme-centered $\pi$ to $\pi^*$ transition and the allowed $\pi$ to $\pi^*$ transitions in nearby aromatic amino acids.[2] Furthermore, these authors suggest that the orientation of the particular aromatic amino acid relative to the heme determines the intensity of the Cotton effect. Therefore, it is expected that slight structural alterations in the polypeptide surrounding the heme would lead to alteration in the Soret CD. This notion is borne out by the observation of variations in the Soret CD of closely related cytochromes *c*.[3]

The invariant phenylalanine of eukaryotic cytochromes *c*, phenylalanine-87 in *Saccharomyces cerevisiae* iso-1-cytochrome *c*,[4] phenylalanine-82 in horse heart[5] and tuna[6] cytochromes *c*, and phenylalanine-90 in rice[7] cytochrome *c* has been shown by X-ray crystallographic studies to be a surface residue, the phenyl ring of which is parallel to and 5 Å distant from the methionine ligand side of the heme plane.[8-10]

We have previously reported the use of oligonucleotide-directed mutagenesis[11] to create three mutants of *S. cerevisiae* cytochrome *c* that possess a serine, glycine, or a tyrosine in place of phenylalanine at position 87 of the protein sequence.[12] The identity of each variant was confirmed by amino acid analysis.[12] The variants are able to drive the reaction of $H_2O_2$ with cytochrome *c* peroxidase.[12] Although the electronic spectra of the variants are essentially identical with those of the wild-type protein, the proposed influence of the heme environment on the CD of cytochrome $c$[1-3,13] prompted us to investigate the CD spectra of these variants. The results of this study establish the usefulness of this mutagenic approach in the examination of heme protein CD spectra.

## Experimental Section

Oligonucleotide-directed mutagenesis of the *CYC 1* gene was performed, and the resultant variant proteins were prepared as described in ref 12. The identity of the variants was confirmed as described in ref 12 with the exception that the constant-boiling HCl (Pierce) was made 1% (v/v) in freshly distilled phenol and 1 mM in ethylenediaminetetraacetic acid (EDTA), sodium salt. These measures improve the recovery of tyrosine.[14] *S. cerevisiae* cytochrome *c* was purchased from Sigma, the iso-1 component isolated, and its identity confirmed as described above.

Protein samples were prepared for spectroscopy in a potassium phosphate buffer (pH 7.3 at 25 °C) containing 0.1 M phosphate and 0.01 M EDTA. As *S. cerevisiae* iso-1-cytochrome *c* possesses a reactive sulfhydryl group (cysteine-107) that causes the protein to autoreduce, solutions of ferricytochrome *c* and ferrocytochrome *c* were made 2 mM in $NH_4[Co(dipicolinato)_2]$[15] or 1,4-dithiothreitol, respectively, to assure complete oxidation or reduction. Protein solutions were passed through a 0.45-mm filter (Millipore) prior to recording spectra. Protein con-

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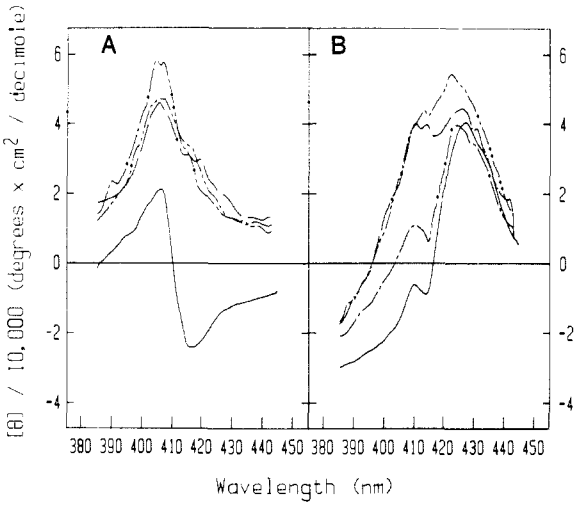


**Figure 1.** Soret CD of oxidized (A) and reduced (B) *S. cerevisiae* iso-1-cytochrome *c* (—), and the serine-87 (— - - —), tyrosine-87 (— - —), and glycine-87 (— · —) variants.

centrations used for calculation of 0.45-$\mu$m ellipticities were calculated from the absorbance at 410 nm for the oxidized proteins and 416 nm for the reduced proteins using molar absorptivities of $1.06 \times 10^5$ and $1.29 \times 10^5$ $M^{-1}$ $cm^{-1}$, respectively.[16] Absorbance measurements were made on a Cary 118 C or a Shimadzu UV-250 spectrophotometer. Absorbancies were kept below 1.5 for all samples.

CD measurements were carried out on a Jasco J-500 C recording polarimeter attached to a DP-500N data processor. A thermostated cell holder (Jasco) was used in conjunction with an external thermostated bath for temperature regulation. For the temperature variation experiments, bath temperature was corrected to cell temperature by using a calibration curve. Temperature was measured with a Fluka 8020A multimeter fitted with a 80T150C thermocouple. (+)-10-Camphorsulfonic acid (Eastman Organic Chemicals, recrystallized) and D-(−)-pantoyl lactone were used as calibrants. Unless otherwise stated, spectra were collected at 25 ± 1 °C. A 1-cm cylindrical cell (2.7-mL volume) was used for recording the temperature dependence of the spectra, while a specially designed 1-cm cell (0.6-mL volume) was used in the collection of isothermal data.

Molar ellipticities were calculated by using the equation $[\theta] = \theta_{obsd}/10lc$, where $\theta_{obsd}$ is the measured ellipticity in degrees, $l$ is the path length of the cell in cm, and $c$ is the protein concentration in mol/$cm^3$. Thus, the units of molar ellipticity are deg $cm^2$/dmol of protein.

Base-line-corrected CD data were entered into an OLIS (Jefferson, GA) Model 3820 Data System with a digitizing pad and manipulated with the OLIS Graphics Operating System.

## Results

The CD spectra of the wild-type protein and the serine-87, tyrosine-87, and glycine-87 variants were measured between 700 and 190 nm for both ferri- and ferrocytochrome *c*. The spectra of the wild-type protein are typical of all eukaryotic cytochromes *c* thus far examined.[3] Although there are minor differences between the wild-type and the variants and among the variants throughout the region examined, by far the most significant changes occur in the Soret region (385–445 nm) (Figure 1). No changes in the spectra were observed upon dilution of the oxidant or reductant, suggesting that these reagents are not responsible for the differences observed.

To visualize better the differences between the wild-type and variant proteins, the spectrum of reduced or oxidized wild-type protein was subtracted from the appropriate spectrum of the reduced or oxidized variants. From examination of the difference spectra of the oxidized variants, shown in Figure 2A, it is evident that each variant lacks a Cotton effect with an ellipticity between 5.3 and 5.7 $\times 10^4$° $cm^2$/dmol at between 415 and 418 nm (width at half-height, 15 nm) that is present in the spectrum of the wild-type protein. Although the locations of the maximum differences for the reduced proteins (Figure 2B), 413 nm for the serine variant and 411 and 414 nm for the glycine variant, are

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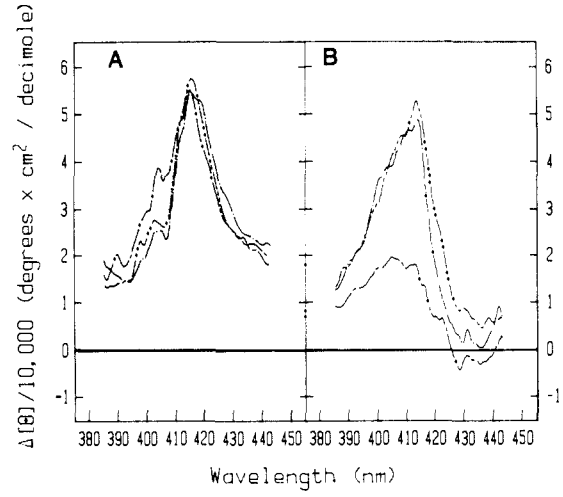


**Figure 2.** Difference Soret CD spectra (variants minus wild type) of the oxidized (A) and reduced (B) proteins. The line types are explained in the legend to Figure 1.

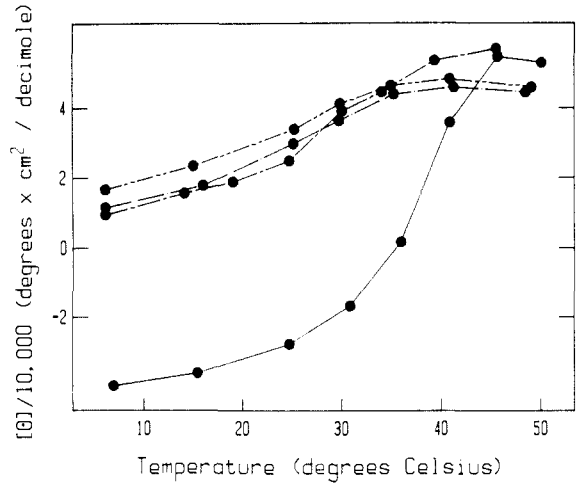


**Figure 3.** Temperature dependence of the Soret CD spectra of oxidized *S. cerevisiae* iso-1-cytochrome *c* and the serine-87, tyrosine-87, and glycine-87 variants. The analytical wavelengths are 415, 417.5, 418, and 418 nm for the wild-type protein and the serine-87, tyrosine-87, and glycine-87. The line types are explained in the legend to Figure 1.

very similar to those values obtained from difference spectra of the oxidized variants, the Cotton effects for these reduced variants are slightly less intense [$(4.6–5.3) \times 10^4$° $cm^2$/dmol] and broader (width at half-height, 25 nm). The difference spectrum of the reduced tyrosine variant, on the other hand, exhibits an attenuated difference spectrum with an ellipticity of $1.9 \times 10^4$° $cm^2$/dmol at 405 nm with possible fine-structure maxima at 400 and 413 nm.

For oxidized eukaryotic cytochrome *c*, Myer[13] has shown that as the temperature is increased, the sign of the Cotton effect at 418 nm changes from negative to positive and has suggested that this change is a measure of the integrity of the heme environment. Thus, to examine the possibility that the absence of the Cotton effect in the variants is due to the same transition taking place at a lower temperature, the spectra were rerun at 6.1 °C. Although there is a decrease in the ellipticity under these conditions, only a positive Cotton effect is observed for the variants (Figure 3). Even spectra recorded at −4.3 °C (in 10% ethylene glycol) retain a positive Cotton effect at 418 nm. As spectra of mixtures of wild-type and variant proteins are strictly additive, it is unlikely that there is some agent in the solutions of the variants that destabilizes the proteins. Thus, if a change to a negative Cotton effect is possible for the variants, it must take place at a temperature below −4.3 °C.

The temperature dependence of the ellipticities at 417.5, 415, or 418 nm for solutions of oxidized wild-type protein and the serine,

**Table I.** Correlation of the Low-Energy Soret CD Band of Oxidized *c*-Type Cytochromes with Heme Microenvironment

| cytochrome | wavelength, nm | $[\theta] \times 10^{-4}$, deg cm$^2$/dmol | amino acid at pos equiv to F-87 | relationship between phenylalanine and heme |
|---|---|---|---|---|
| *S. cerevisiae* iso-1-cytochrome *c* | 417 (min) | −2.4[a] | phenylalanine-87[g] | unknown |
| serine-87 *S. cerevisiae* iso-1-cytochrome *c* | 418 | +3.1[a] | serine-87[h] | |
| tyrosine-87 *S. cerevisiae* iso-1-cytochrome *c* | 418 | +2.5[a] | tyrosine-87[h] | |
| glycine-87 *S. cerevisiae* iso-1-cytochrome *c* | 418 | +2.9[a] | glycine-87[h] | |
| beef heart cytochrome *c* | 418 (min) | −5.1[b] | phenylalanine-82[i] | parallel[n] |
| tuna cytochrome *c* | 418 (min) | −3.0[b] | phenylalanine-82[j] | parallel[o] |
| *R. rubrum* cytochrome $c_3$ | 411 (max) | +8.2[c] | phenylalanine-93[k] | parallel[p] |
| *Pseudomonas aeruginosa* cytochrome $c_{551}$ | 418 (max) | +7.2[d] | asparagine-64[l] | none[q] |
| *R. molischianum* iso-2-cytochrome $c_2$ | 418 (min) | −3.2[e] | phenylalanine-73[k] | unknown |
| *E. colid* $b_{562}$ | 421 (min) | −7.3[f] | phenylalanine-68[m] | parallel[r] cytochrome |

[a] This work. [b] Myer, Y. P. *Biochim. Biophys. Acta* **1970**, *214*, 94–106. [c] Reference 19. [d] Vinogradov, S.; Zand, R. *Arch. Biochem. Biophys.* **1968**, *125*, 902–910. [e] Flatmark, T.; Dus, H.; Deklerk, H.; Kamen, M. D. *Biochemistry* **1970**, *9*, 1991–1996. [f] Reference 24. [g] Reference 4. [h] Reference 12. [i] Nakashima, T.; Higa, H.; Matsubara, H.; Benson, A.; Yasundbu, K. T. *J. Biol. Chem.* **1966**, *241*, 1166–1177. [j] Reference 6. [k] Reference 18. [l] Ambler, R. P.; Wynn, M. *Biochem. J.* **1973**, *131*, 485–498. [m] Reference 22. [n] Reference 9. [o] Reference 8. [p] Reference 20. [q] Matsura, Y.; Takano, T.; Dickerson, R. E. *J. Mol. Biol.* **1982**, *156*, 389–409. [r] Reference 26.

tyrosine, and glycine variants is shown in Figure 3. These observed changes were shown to be reversible by recording a spectrum at 25 °C, heating the sample to 55 °C, recording the spectrum, then quickly cooling to 25 °C, and re-recording the spectrum and observing that the initial and final spectra were the same within experimental error. The intensity of the Soret CD of the reduced, wild-type protein and the variants was found to be nearly temperature independent as has been observed for horse heart cytochrome *c*.[13]

## Discussion

The CD spectra of oxidized and reduced *S. cerevisiae* iso-1-cytochrome *c* are typical of those reported for other eukaryotic cytochromes *c*.[3] The ultraviolet (intrinsic) CD of the serine, tyrosine, and glycine variants are nearly identical with the spectra of the wild-type protein (data not shown). The near identity of the intrinsic CD suggests that substitution of phenylalanine with serine, tyrosine, or glycine does not dramatically affect the global secondary structure of the protein.

However, the Soret CD spectra of the variants differ significantly (Figure 1). The difference spectra (generated by subtracting the appropriate wild-type spectrum from each of the spectra of the variants) are shown in Figure 2. Comparison of the data in Figures 1A and 2A reveals that a negative Cotton effect at 417 nm with an ellipticity of approximately $6 \times 10^{4\circ}$ cm$^2$/dmol, demonstrated by wild-type ferricytochrome *c*, is absent in the spectrum of the oxidized variants. A band is also observed in the difference spectra of the reduced serine and glycine variants (Figure 2B), although the peaks are wider and less intense, and are shifted to slightly lower wavelength. The peak in the difference spectra of the reduced variants is very similar to that of the oxidized variants, suggesting that a negative Cotton effect may be present in the spectrum of both the reduced and oxidized wild-type protein. If this is the case, the negative Cotton effect in the spectrum of the reduced wild-type protein is masked by one of opposite sign (compare the 410–420-nm region in parts A and B of Figure 1).

Taken together, these observations demonstrate that the amino acid substitutions cause some conformational change in the variants. Since very little change is observed in the intrinsic CD spectrum of the variants with respect to the wild-type protein and a dramatic change is observed in the Soret region, the conformation change induced by the mutations is probably limited to the immediate surroundings of the heme.

One explanation for the elimination of the negative Cotton effect is that the substitutions might bring about the spectral change directly. If this is the case, the invariant phenylalanine residue serves to form a unique heme environment in oxidized cytochrome *c*. Support for this possibility is provided by the finding that the difference spectra for all the oxidized variants are identical—as are the difference spectra of the reduced serine and glycine variants. For the reduced protein, the difference spectrum for the tryosine-87 variant (Figure 2B) suggests that it may provide an environment more similar to that created by phenylalanine-87 than to the environment created by the presence of the serine or glycine in this position.

In their theoretical study of the origin of Soret Cotton effects in myoglobin and hemoglobin, Hsu and Woody[2] suggested that the dominant mechanism is a coupled oscillator interaction between the heme Soret transition and transitions in nearby aromatic amino acid residues. Thus, the source of the negative Cotton effect observed for oxidized and implied for reduced cytochrome *c* may be the direct interaction of the $\pi$ to $\pi^*$ transition of the aromatic side chain of phenylalanine-87 with the $\pi$ to $\pi^*$ transition of the heme. This explanation is consistent with the difference spectral data for the serine and glycine variants (Figure 2) since the side chains of these amino acids, which do not possess a delocalized $\pi$-electron system, do not give rise to large Cotton effects. However, this rationale is not consistent with the data for the oxidized tyrosine variant. If the aromatic ring of tyrosine-87 were in the same position as that of the phenylalanine residue, its $\pi$ to $\pi^*$ transition should interact with the $\pi$ to $\pi^*$ transition of the heme to produce a Soret Cotton effect similar to that observed for the wild-type protein. This partially may be the case for the reduced tyrosine variant (Figure 2B).

Although phenylalanine-87 is invariant in all eukaryotic[17] and many prokaryotic cytochromes *c*,[17c] substitutions at this position do occur in some prokaryotic *c*-type cytochromes. Structural and spectroscopic data for a number of cytochromes are compiled in Table I. Note that those proteins that possess a phenylalanine in the equivalent position to phenylalanine-87 also possess a negative band in the Soret CD spectrum.

Cytochrome $c_2$ from *Rhodospirillum rubrum*, however, is an exception to this generalization. This cytochrome possesses a phenylalanine at the position equivalent to phenylalanine-87,[18] yet it exhibits a CD maximum at 418 nm.[19] According to the refined crystallographic data,[20] this phenylalanine is nearly parallel to and within 6 Å of the heme plane. If, as suggested above, the negative Soret Cotton effect observed for all eukaryotic and most prokaryotic cytochromes *c* is due to the interaction of the phe-

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(20) In the original crystal structure, the aromatic ring of this phenylalanine was thought to be at a 45° angle with respect to the heme plane: Salemme, F. R.; Freeer, S. T.; Xuong, N. H.; Alden, R. A.; Kraut, J. *J. Biol. Chem.* **1973**, *248*, 3910–3921. However, upon further refinement this phenylalanine was found to be nearly parallel: Bhatla, G. E. Ph.D. Dissertation, University of Calfornia, San Diego, 1981.

nylalanine with the heme, we can only speculate as to the reason(s) for this exception. Perhaps, in solution, the aromatic side chain of this phenylalanine may rotate about its $C_\alpha$–$C_\beta$ bond, thus averaging the environment. Measurement of the temperature dependence of the proton magnetic resonance ($^1$H NMR) spectrum has shown that the aromatic ring of phenylalanine-82 of horse heart cytochrome *c* does not rotate.[21] Although the technique of $^1$H NMR has been applied to cytochrome $c_2$,[22] its temperature dependence has not been determined. Another related possibility is that this phenylalanine is not in the same orientation in the crystal as it is in solution. The chemical shift of the methyl protons belonging to the $\beta$-carbon of each of the two thio ether bridges of cytochrome *c* may be relevant to this point. For horse heart cytochrome *c*, one of these two resonances is dramatically shifted upfield. This upfield shift probably originates from the close proximity of these protons to phenylalanine-82.[23] The failure to observe this shift in the spectrum of cytochrome $c_2$[22] may indicate that phenylalanine-82 and the equivalent residue in cytochrome $c_2$ are not in the same environment in solution. It is also possible that indirect and compensatory effects involving other residues surrounding the heme in cytochrome $c_2$ may modify its Soret CD spectrum.

Cytochrome $b_{562}$ from *Escherichia coli* exemplifies the correlation between a negative Cotton effect at 420 nm[24] and the relative orientation of the phenylalanine[25] and the heme.[26] Although it possesses the same ligand complement to the iron, this bacterial cytochrome is not a member of the cytochrome *c* family as its heme is not covalently bound to the apoprotein. Furthermore, the tertiary structure of cytochrome $b_{562}$ is made up of four $\alpha$-helices rather than the typical cytochrome *c* fold.[26] However, examination of the three-dimensional structure of cytochrome $b_{562}$ reveals a phenylalanine in contact with and parallel to the methionine ligand side of the heme—a position strikingly similar to that of the invariant phenylalanine in cytochrome *c*.[8-10] As this phenylalanine is one of the few common features between the structure of cytochrome $b_{562}$ and the *c*-type cytochromes, the observation of this negative Soret Cotton effect supports the conclusion that the negative Soret Cotton effect observed in all eukaryotic ferricytochromes *c*,[3] some prokaryotic ferricytochromes *c*,[3] and *E. coli* ferricytochrome $b_{562}$[24] may arise from the interaction of a phenylalanine parallel to and within 5 Å of the methionine side of the heme plane with the delocalized $\pi$-electron system of the heme. This correlation may also hold for the reduced proteins,[3] but, as discussed above, the proposed negative and the positive bands of the Soret CD may tend to cancel each other in the reduced protein.

A second possible explanation of the results described here is that the substitutions that we have introduced into yeast cytochrome *c* might bring about the observed spectral changes indirectly by causing reorganization of the polypeptide surrounding the heme at locations other than position 87. Support for this possibility is provided by the data for oxidized tyrosine variant (vide supra) and by the observation that the variants exhibit slight changes near 280 nm in their absorption spectra.[12] Absorbance in this region is dominated by the sole tryptophan residue of cytochrome *c*, tryptophan-64. While this residue is not adjacent to phenylalanine-87, it is on the same side of the heme. Insofar as the difference spectra for the oxidized variants and for the reduced glycine and serine variants are very similar, it follows that these substitutions bring about a nearly identical conformation. The attenuated difference spectrum observed for the reduced tyrosine variant suggests that this substitution causes a less profound conformational change, perhaps attributable to the similarities of tyrosine and phenylalanine.

With increasing temperature, the sign of the Cotton effect at 418 nm for horse heart cytochrome *c* changes from negative to positive. Myer[13] concluded that this alteration in sign reflects disruption of the heme environment at high temperatures.

In the case of iso-1-cytochrome *c*, the maximum ellipticity at higher temperatures is the same for the oxidized wild-type protein and the variants at position 87. Thus, the substitution of phenylalanine with serine, glycine, or tyrosine appears to result in a similar conformation at temperatures above 35 °C. It is possible, therefore, that the mutations at position 87 exert their effect on the sign of the Cotton effect by decreasing the stability of the polypeptide surrounding the heme. If this is true, the data in Figure 3 indicate that the transition temperature that would give rise to the negative Cotton effect is shifted to below 6.1 °C, and the data from the experiment employing 10% ethylene glycol suggest that the transition temperature is below −4.3 °C. However, the observation of a positive Cotton effect for both the wild-type and the variant proteins at higher temperatures may simply be coincidental.

## Conclusions

Substitution of phenylalanine-87 with serine, tyrosine, or glycine results in an altered Soret but essentially unaltered ultraviolet CD spectra. Therefore, any conformational changes in cytochrome *c* produced by these mutations are restricted to the vicinity of the heme. The spectral changes observed for the variants may arise both directly or indirectly from the substitution of the invariant phenylalanine. The direct effect would result from the disruption of the interaction between the $\pi$-electron systems of the heme and phenylalanine-87, which, in turn, results in the loss of the negative Soret Cotton effect observed in the wild-type protein. This idea is in agreement with theoretical considerations.[2] It is also in accord with the possibility that the negative Soret Cotton effect observed for the reduced and oxidized forms of eukaryotic cytochromes *c*,[3] many prokaryotic cytochromes *c*,[3c] and cytochrome $b_{562}$[24] is partially determined by the direct interaction of the $\pi$ to $\pi^*$ transition of a phenylalanine residue, the phenyl group of which is parallel to and within 5 Å of the methionine side of the heme, with the $\pi$ to $\pi^*$ transition of the heme. The possible indirect effect is one in which the phenylalanine determines the Soret CD by defining a unique conformation of the polypeptide surrounding the heme.

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**Registry No.** Cytochrome *c*, 9007-43-6; L-phenylalanine, 63-91-2.

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