Electrochemical, Kinetic, and Circular Dichroic Consequences of Mutations at Position 82 of Yeast Iso-1-cytochrome *c*†

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ABSTRACT: Replacement of Phe-82 in yeast iso-1-cytochrome *c* with Tyr, Leu, Ile, Ser, Ala, and Gly produces a gradation of effects on (1) the reduction potential of the protein, (2) the rate of reaction with $Fe(EDTA)^{2-}$, and (3) the CD spectra of the ferricytochromes in the Soret region under conditions where contributions from the alkaline forms of these proteins are absent. The reduction potential of cytochrome *c* is lowered by as little as 10 mV (Tyr-82) or by as much as 43 mV (Gly-82; pH 6.0) as the result of these substitutions. The second-order rate constants for reduction of these cytochromes range from a low of 6.20 (2) × 10^4 for the Tyr-82 variant to a high of 14.8 × 10^4 M^{-1} s^{-1} for the Ser-82 variant [pH 6.0, 25 °C, μ = 0.1 M (sodium phosphate)]. Analysis of these rates by use of relative Marcus theory produces values of k_{11}^{corr} that range from 10.9 M^{-1} s^{-1} for the wild-type protein to 190 M^{-1} s^{-1} for the Gly-82 mutant [25 °C, μ = 0.1 M, pH 6.0 (sodium phosphate)]. Reinvestigation of the effect of substituting Phe-82 by a Tyr residue on the CD spectrum of the protein now reveals little alteration of the intense, negative Cotton effect in the Soret CD spectrum of ferricytochrome *c*. On the other hand, substitution of nonaromatic residues of various sizes at this position results in loss of this spectroscopic feature, consistent with previous findings. The results of these functional and spectroscopic studies are correlated with the crystallographically determined three-dimensional structures of the wild-type protein and of the Gly-82 and Ser-82 variants.

Phenylalanine-82 is a phylogenetically conserved residue of mitochondrial cytochrome *c* and was the first residue of this protein to be modified by site-directed mutagenesis (Pielak et al., 1985). Mutations at this position have been of considerable interest as the result of modeling studies that predict critical roles for this residue in electron transfer reactions between cytochrome *c* and both cytochrome *c* peroxidase (Poulos & Kraut, 1980) and cytochrome b_5 (Wendoloski et al., 1987). Experimental studies with position-82 mutants have revealed crucial roles for this residue in regulating the rate of electron transfer from iso-1-ferrocytochrome *c* to the Zn–cytochrome *c* peroxidase π-cation radical (Liang et al., 1987, 1988) and in stabilizing the native structure of the ferricytochrome *c* heme environment (Pielak et al., 1985; Pearce et al., 1989). Structural analyses of the wild-type yeast iso-1-cytochrome *c* and several position-82 variants have recently been performed with both X-ray crystallography (Louie et al., 1988a; Louie & Brayer, 1989) and 2-D NMR spectroscopy (Pielak et al., 1988a,b).

Further characterization of the electrochemical and electron transfer properties of these variants is required to allow the correlation of mutation-induced structural changes with their functional consequences. We therefore report the electrochemical analyses of six position-82 mutants, kinetic studies of their reduction by $Fe(EDTA)^{2-}$, and their Soret CD spectra. The results are interpreted in terms of available structural information for these proteins.

Experimental Procedures

Protein Preparation. Wild-type mutant forms of *Saccharomyces cerevisiae* iso-1-cytochrome *c* were prepared as described previously (Pielak et al., 1985; Cutler et al., 1987). In the present study, however, final purification was achieved by ion exchange chromatography with an FPLC system fitted with a Pharmacia Mono-S HR 10/10 cation exchange column. The yeast cytochromes were loaded onto the column in 20 mM MES (pH 6.0) buffer and eluted with 1.0 M NaCl in the same buffer with a linear salt gradient. Yeast cytochromes *c* eluted between 0.3 and 0.4 M NaCl. Horse heart cytochrome *c* was purified by being loaded onto the Mono-S column in pH 7.0 sodium phosphate buffer (20 mM) and eluted with 1.0 M NaCl in the same buffer with a linear salt gradient. The horse heart cytochrome eluted between 0.1 and 0.2 M NaCl.

The wild-type phenylalanine at position 82 was replaced by tyrosine, serine, glycine, isoleucine, leucine, and alanine. In addition, all cytochromes possessed a second mutation in which the cysteine at position 102 was replaced by threonine. This mutation eliminates rapid autoreduction and dimerization of the cytochrome (Cutler et al., 1987; Narita & Titani, 1969).

Direct Electrochemistry. The direct electrochemistry of the wild-type and mutant cytochromes was achieved at a gold surface modified with 4,4′-dithiodipyridine (Allen et al., 1984; Taniguchi et al., 1982a,b). The gold disc electrode (0.16 cm^2) was cleaned by being polished with an alumina (0.3 μm)/water slurry on a Mastertex polishing cloth (Beuhler), modified by

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being dipped into a saturated solution of the modifier (Aldrithiol-4, Aldrich), and washed extensively with water. Cyclic voltammetry was carried out in a three-electrode, two-compartment glass cell. The calomel reference electrode (Radiometer K401) was maintained at 25 °C and connected to the sample compartment by a Luggin capillary (0.1 mm). A platinum wire counter electrode was inserted around the capillary in the sample compartment. The sample temperature was controlled by inserting the cell into a small, jacketed beaker filled with water and thermostated by a refrigerated circulating water bath. Experiments were performed on 0.5 mL of 0.4 mM protein solutions in μ = 0.1 M buffer, pH 6.0 (50 mM KCl with the remaining ionic strength provided by sodium phosphate).

The potential was controlled by an Ursar Electronics potentiostat, and the current output was recorded on a Kipp and Zonen BD 90 X-Y recorder. A potential range of −10 to 530 mV (vs SHE) was studied at sweep rates of 5–200 mV s^{-1}. The midpoint potentials were measured from steady-state voltammograms recorded at a sweep rate of 20 mV s^{-1}. The temperature dependence of the midpoint potentials was measured between 10 and 40 °C. Identical results were obtained between experiments in which the electrode was cleaned and remodified between measurements at each temperature and experiments in which the electrode remained in the solution over the course of the experiment.

Kinetic Studies. $Fe(EDTA)^{2-}$ solutions were prepared in phosphate buffer (Wherland et al., 1975; Wherland, 1977). Kinetic measurements were made with a computer-interfaced stopped-flow spectrophotometer as described previously (Reid & Mauk, 1982) with reaction temperature regulated to ±0.2 °C. Buffered ferricytochrome *c* solutions (5 μM) were prepared on the day of use. The reduction of ferricytochrome *c* by $Fe(EDTA)^{2-}$ was monitored at 412.5 nm under pseudo-first-order conditions with reductant concentration in at least 20-fold excess (μ = 0.1 M, sodium phosphate buffer, pH 6.0, 25 °C). Second-order rate constants were obtained from weighted linear least-squares analysis of the dependence of k_{obs} on reductant concentration. Activation parameters were calculated from Eyring plots fitted by weighted linear least-squares analysis.

Circular Dichroism Spectroscopy. Proteins were prepared for CD measurements in 20 mM MES buffer (pH 6.0, 25 °C). Conversion to ferricytochrome *c* was achieved by addition of $NH_4[Co(dipicolinate)_2]$ to a final concentration of 2 mM. The oxidant was removed by passing the samples over a column (2.5 × 10 cm) of Sephadex G-25 resin. Protein solutions were diluted to a concentration of less than 0.014 mM after filtration through a 0.22-μm filter. The visible spectrum of each sample was recorded with a Shimadzu UV-250 spectrophotometer to verify complete oxidation.

CD measurements were conducted with a Jasco J-500 C recording polarimeter interfaced to a DP-500N data processor. *d*-(+)-10-Camphorsulfonic acid (Eastman Organic Chemicals, recrystallized) and *d*-(−)-pantoic acid lactone were used as calibrants. Spectra were collected on samples placed in a 1-cm cell (0.6-mL volume) at 25 °C, and the average of four scans (370–450 nm) was collected for each.

Results

Electrochemistry. The electrochemistry at a 4,4′-dithiodipyridine-modified gold electrode of all the cytochromes investigated here was judged to be quasi-reversible at all temperatures. The peak to peak separations at 20 mV s^{-1} were 57 ± 2 mV at 25 °C. At pH 6.0 no difference in the current response among any of the mutants and the wild-type protein could be detected. The midpoint potential of horse heart cytochrome *c* was determined to be 270 mV vs SHE (25 °C, pH 7.0, μ = 0.1 M), in good agreement with reported values (Taniguchi et al., 1980).

Table I: Electrochemical Properties of Position-82 Iso-1-cytochrome *c* Mutants[a]

protein	E_m (mV vs SHE)	$\Delta H°$ (kcal/mol)	$\Delta S°_{rc}$ (eu)	$\Delta G°$ (kcal/mol)
F82	290 (2)	−14.0 (2)	−9.1 (4)	−6.7 (5)
Y82	280 (2)	−13.6 (1)	−8.3 (3)	−6.5 (5)
L82	286 (2)	−14.6 (2)	−11.6 (7)	−6.5 (4)
I82	273 (2)	−14.6 (1)	−12.3 (3)	−6.3 (5)
A82	260 (2)	−13.0 (2)	−8.1 (5)	−6.0 (5)
S82	255 (2)	−13.8 (2)	−11.0 (5)	−5.9 (5)
G82	247 (2)	−12.7 (2)	−8.0 (4)	−5.7 (5)

[a] 25 °C, pH 6.0, μ = 0.1 M, and SHE reference.

The thermodynamic parameters derived from the temperature dependence of the midpoint potentials are listed in Table I. At 25 °C (pH 6.0), the midpoint potentials of the cytochromes examined span a range of 43 mV, which corresponds to differences in free energy of reduction that vary at most by 1 kcal/mol. However, some differences between mutants are apparent through examination of their standard enthalpies and entropies. Specifically, the Ser-82, Leu-82, and Ile-82 variants exhibit relatively large standard entropy values, though this effect is compensated by enthalpic considerations for the latter two proteins. On the other hand, replacement of Phe-82 with Ala and Gly influences the reduction potentials of these mutants largely through enthalpic effects.

$Fe(EDTA)^{2-}$ Reduction Kinetics. Those mutant cytochromes with an alkaline p*K* below 8 (Ala, Ser, Ile, Leu, Gly) exhibited biphasic kinetics even at pH 6. For these proteins, the reduction of the oxidized protein is the principal rate process at this pH and was several orders of magnitude faster than the rate of the slower phase. Consequently, the second-order rate constants for reduction of the native protein were readily determined. The slower phase exhibits the same rate constant as observed for the conversion of the alkaline forms of these proteins to the native conformation in pH-jump experiments (Pearce et al., 1989) and is consequently ascribed to this conformational change. The wild-type and Tyr-82 proteins [alkaline pK_a = 8.5 and 8.3, respectively (Pearce et al., 1989)] demonstrated monophasic kinetics. For all position-82 variants, the observed rate constants for cytochrome reduction varied linearly with reductant concentration over the range examined, in agreement with similar measurements with horse heart cytochrome *c* (Hodges et al., 1974). The uncertainties in rate constants derived from weighted linear least-squares fits to the dependence of k_{obs} on $[Fe(EDTA)^{2-}]$ are probably best regarded as a lower limit of error that may arise from limitations of reproducibility. As a result, small differences (5–10%) in rate constants or other fitted parameters should not be emphasized.

The second-order rate constants for the reduction of the mutant proteins were comparable to or greater than the rate constants obtained for the wild-type protein. On the basis of these results, the six cytochromes can be divided into three groups: Phe-82 and Tyr-82 with the lowest rate constants; Leu-82, Ile-82, and Ala-82 with intermediate values; and Ser-82 and Gly-82 with the highest rate constants, about 2-fold greater than that of wild type.

Eyring plots (Figure 1) for most position-82 variants were linear between 10 and 35 °C. For the Leu-82, Ile-82, and Ala-82 variants, however, these plots were linear only up to

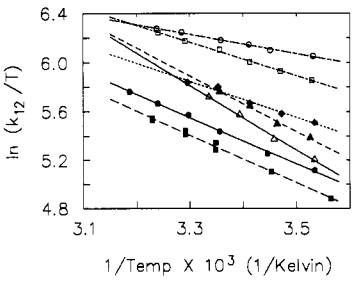

FIGURE 1: Eyring plots determined from the temperature dependence of the rate data for $Fe(EDTA)^{2-}$ reduction of position-82 variants of iso-1-cytochrome c (pH 6.0, μ = 0.1 M, $[Fe(EDTA)^{2-}]$ = 0.2 mM): (●) wild type; (■) Tyr-82; (△) Leu-82; (▲) Ile-82; (◆) Ala-82; (○) Ser-82; (□) Gly-82.

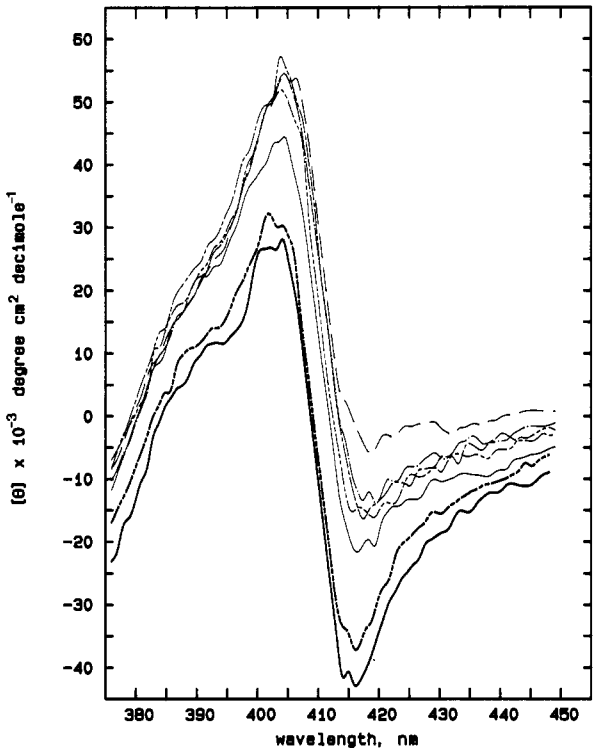

FIGURE 2: Soret circular dichroism spectra of wild-type and mutant forms of *S. cerevisiae* iso-1-cytochrome c (pH 6.0, 25 °C): (heavy line) wild type; (double-dashed heavy line) Tyr-82; (—) Gly-82; (- - -) Ser-82; (- - -) Ala-82; (-·-) Leu-82; (--) Ile-82.

25 °C. At higher temperatures, the Ile-82 variant exhibited an increase in the contribution of the slower phase, which suggests that the thermodynamics of the alkaline transition must be altered significantly for this mutant. The activation parameters obtained from the Eyring plots are shown in Table II. The variations in activation entropies and enthalpies are not large, and the values determined are similar to those reported for horse heart cytochrome c (Hodges et al., 1974). The activation parameters for the wild-type protein and the Tyr-82 mutant are identical within experimental error. The greater reduction rates observed for Ala-82, Ser-82, and Gly-82 appear to be due to lower activation enthalpies, which are offset partially by somewhat more negative activation entropies. The significance of the activation parameters determined for the Leu-82 and Ile-82 variants is uncertain in light of the limited temperature range over which data could be collected.

Circular Dichroism. The CD spectrum of wild-type ferricytochrome c at pH 6.0 exhibits an intense, negative Cotton effect in the Soret region as expected on the basis of previous reports [see the following reviews: Myer (1978, 1983) and Myer and Pande (1979)] (Figure 2). Replacement of Phe-82 with a tyrosyl residue decreases the intensity of this Cotton effect somewhat, but the principal features remain unchanged. Replacement of Phe-82 with nonaromatic residues, however, has a much more pronounced influence on this spectrum. With Gly at position 82, the negative Cotton effect is largely eliminated, while substitution with Ser, Leu, and Ile results in progressive reduction in intensity to the point where no trace of the Cotton effect can be seen for the Ile mutant. This differential effect of mutation on the Soret Cotton effect does not arise from differences in thermal stability of the mutant proteins as identical results were obtained when the spectra were obtained at 4 °C (data not shown).

Table II: Kinetic Parameters for Reduction of Iso-1-cytochromes by $Fe(EDTA)^{2-}$ [a]

protein	$k_{12} \times 10^{-1}$ (M^{-1} s^{-1})	k_{11}^{corr} (M^{-1} s^{-1})	$\Delta S^{\ddagger}$ (eu)	$\Delta H^{\ddagger}$ (kcal/mol)
F82	7.20 (2)	10.9	−23.6 (3)	3.8 (1)
Y82	6.20 (2)	11.8	−23.5 (5)	3.9 (1)
L82	8.83 (3)	19	−18.3 (9)	5.3 (3)
I82	9.4 (1)	35.1	−19.2 (6)	5.0 (2)
A82	9.9 (2)	62.5	−25 (1)	3.2 (4)
S82	14.80 (3)	165	−29.7 (2)	1.6 (1)
G82	13.7 (3)	190	−26.1 (3)	2.7 (1)

[a] 25 °C, pH 6.0, and μ = 0.1 M.

Discussion

The studies outlined here provide fundamental functional information concerning several position-82 mutants of cytochrome c that is required for understanding the role played by this residue in stabilizing the structure and regulating the functional properties of cytochrome c. As we have previously found that mutations at this position of the protein reduce the pK_a for formation of the alkaline form of the protein, our studies have been performed at pH 6.0 to minimize complications arising from this conformational transition.

The reduction potentials recorded at pH 6.0 show a qualitative correlation with the size of the residue at position 82. As seen in Table I, the potential of the wild-type protein is the highest of the proteins studied in this series, with the mutants having progressively lower potentials in the order Leu > Tyr > Ile > Ala > Ser > Gly. In our preliminary report of the potentials of the Tyr, Ser, and Gly mutants (Pielak et al., 1985), the values reported for the Ser and Gly mutants were lower than those reported here by about 25 mV. This apparent discrepancy results from differences in the electrochemical techniques used in the two studies. At the pH used in the earlier study (7.2), a substantial fraction of the Ser and Gly mutants occurs in the alkaline form [the pK_a for the alkaline transition of these two mutants is 7.7 (Pearce et al., 1989)], which is known to have a low and interdeterminant reduction potential (Moore et al., 1984; Rodrigues et al., 1987). The spectroelectrochemical technique used for these experiments controls the position of equilibrium between all species in the bulk solution. As a result, the equilibrium among native and alkaline forms lowers the apparent reduction potential. Direct electrochemistry of cytochrome c allows the use of cyclic voltammetry, a dynamic technique, in the study of these proteins. In a cyclic voltammetry measurement, individual electroactive species can be observed independently within a mixture of interconvertible species as long as the rates of the interconversions in the homogeneous solution are below a certain limit.[1] The kinetics of interconversion of the native

and alkaline forms of ferricytochrome *c* is sufficiently slow that the alkaline equilibrium has no effect on the reduction potential of the native protein as measured by cyclic voltammetry.[1]

The second-order rate constant for cytochrome *c* reduction by $Fe(EDTA)^{2-}$ varies from 6.2×10^4 to 14.8×10^4 M^{-1} s^{-1} depending on the identity of the residue at position 82, a 2.4-fold range. The relative insensitivity of this rate constant to the replacement of Phe-82 contrasts strongly with the behavior previously reported for the reaction between these proteins and Zn–cytochrome *c* peroxidase (Liang et al., 1986, 1987). This result demonstrates that mutations at this position of cytochrome *c* do not affect the electron transfer reactivity of the protein with (nonphysiological) inorganic reactants in the same manner that they affect the reaction of the Fe(II) mutant cytochromes with [Zn–cytochrome *c* peroxidase]$^{\bullet+}$. Evidently, the observations of Liang et al. result from the effect of such mutations on a critical (unidentified) mechanistic feature of the reaction between these two proteins that is not relevant to reaction of the cytochrome with $Fe(EDTA)^{2-}$ and presumably other small-molecule reactants. On the other hand, the results of the Marcus calculations (Table II) establish that the mechanism of cytochrome *c* reduction by $Fe(EDTA)^{2-}$ is affected to a detectable degree by replacement of Phe-82 with other residues. On the basis of the work of Gray and co-workers (Wherland & Gray, 1976; Cummins & Gray, 1979; Holwerda et al., 1980), the effects of mutations at position 82 on the apparent self-exchange rate for this family of cytochrome *c* mutants in the reaction with $Fe(EDTA)^{2-}$ (Table II) suggest that mutations at this position influence the kinetic accessibility of the heme prosthetic group in a manner consistent with the size of the amino acid side chain at that position. In other words, smaller residues at position 82 (Gly and Ser) give rise to greater values of k_{11}^{corr} than do larger residues (Phe and Tyr), with residues of intermediate size producing intermediate values for this parameter.

The availability of three-dimensional structures for the wild-type protein (Louie et al., 1988a) and for the Gly-82 (Louie & Brayer, 1989) and Ser-82 (Louie et al., 1988b) mutants in the reduced state permits an initial, qualitative assessment of the validity of this line of reasoning for both the electrochemical and kinetic data presented here. As analyzed by Louie and Brayer (1989), the surface area of the heme group that is exposed to solvent increases from 48.8 Å^2 (9.6% of the total heme surface) in the reduced wild-type structure to 70.0 Å^2 (13.7% of the total heme surface) in the reduced Ser-82 mutant. This increase in heme solvent accessibility correlates well with the increase in electron transfer reactivity observed in comparison of the Marcus calculations for the two proteins and with the decrease in reduction potential observed for the Ser-82 mutant. On the other hand, the exposure of the heme group to solvent in the Gly-82 variant is 44.4 Å^2 (8.7% of the total heme surface), a value effectively identical with that observed for the wild-type protein. Louie and Brayer have suggested that the origin of the lower reduction potential of the Gly-82 mutant is attributable to a conformational change produced by the mutation that results in an increase in the polarity of the protein groups that contribute to the heme binding environment. The present results are clearly consistent with this view.

At least two origins for the increased electron transfer reactivity of the Gly-82 mutant can be suggested at present. An increase in the hydrophilic character of the heme binding environment may simply facilitate close approximation of the hydrophilic reagent $Fe(EDTA)^{2-}$ to the heme edge and thereby facilitate greater orbital overlap with the prosthetic group. An alternative possibility is based on the observation by Louie and Brayer that the temperature factors for the peptide backbone in the region of Gly-82 suggest that this segment of the protein exhibits greater mobility than the corresponding region in either the Ser-82 mutant or the wild-type protein. If this mutation induces a fluxional motion of the protein structure in the vicinity of the heme binding site, it is conceivable that this motion might permit closer approach of the reducing agent to the heme group. However, if this explanation were valid, then it is surprising that the thermodynamic parameters for $Fe(EDTA)^{2-}$ reduction of the Gly variant do not exhibit a substantially greater enthalpic component than observed for reduction of the wild-type protein. In fact, the activation parameters for all of the proteins studied here are remarkably similar to each other, with the exceptions of the Leu-82 and Ile-82 mutants. Detailed structural origin of the relatively anomalous behavior of these mutants is not discernible from the information available, but presumably the stereochemical attributes of these relatively large residues result in detectable perturbation of the conformational folding of these forms of the protein.

The spectropolarimetric properties of this family of mutants as observed in the Soret region of the oxidized proteins (Figure 2) establish that an aromatic residue at positions 82 is required for the intense, negative Cotton effect in this region of the spectrum. Large, aliphatic residues (e.g., Leu and Ile) are not sufficient to maintain this spectroscopic characteristic. We previously reported (Pielak et al., 1986) that replacement of Phe-82 with Tyr can also eliminate this negative Cotton effect, an observation that we now find to be incorrect. We attribute our previous observations to (1) the relative instability of position-82 mutants in which Cys-102 has not been converted to Thr, (2) greater instability of position-82 mutants at pH 7.3 owing to the lowered alkaline pK_a of these mutants, and (3) limitations in our previous method of cytochrome purification that did not use fast liquid protein chromatography. Repetition of the CD measurements reported previously (at pH 7.3) with cytochromes that possess Cys-102 and that have been purified by FPLC immediately prior to use reproduced the data shown in Figure 2 (data not shown). We conclude, therefore, that the differences in CD behavior that we not observe are not attributable to the presence of Thr at position 102 rather than Cys. Nonetheless, our conclusion that an aromatic residue at position 82 is required for the negative

[1] The electrochemistry of native cytochrome *c* in the presence of the alkaline form can be adequately described as a first-order, reversible, homogeneous reaction preceding reversible, heterogeneous electron transfer:

$$\text{cytochrome } c^{III}_{\text{alkaline}} \underset{k_b}{\overset{k_f}{\rightleftharpoons}} \text{cytochrome } c^{III}_{\text{native}}$$

$$\text{cytochrome } c^{III}_{\text{native}} + e^- \rightleftharpoons \text{cytochrome } c^{II}_{\text{native}}$$

The theoretical consequences of this kinetic scheme (referred to as C_rE_r) for voltammetric experiments have been described elsewhere (Saveant & Vianello, 1963; Nicholson & Shain, 1964; Bard & Faulkner, 1980). The effect of the conformational equilibrium on the electrochemical behavior of this system depends on the absolute and relative magnitudes of the kinetic parameter λ [$=(RT/nF\nu)(k_f + k_b)$ for cyclic voltammetry] and the equilibrium constant K ($=k_f/k_b$). If $\log (K\lambda^{1/2})^{-1} \geq 1$, then the electrochemistry is diffusion controlled and unaffected by the homogeneous kinetics. As formation of alkaline cytochrome *c* occurs with a rate constant of 1–15 s^{-1} and conversion of alkaline to native cytochrome *c* occurs with a rate constant of 0.02–0.06 s^{-1} (Pearce et al., 1989), the potential of cytochrome *c* as determined by cyclic voltammetry is unaffected by the formation of the alkaline protein at sweep rates (ν) of 20 mV s^{-1} or greater.

Cotton effect observed in the Soret region for ferricytochrome *c* remains unchanged. The observation of a relatively intense negative Cotton effect in this region of the spectrum for the Tyr mutant is consistent with crystallographic (G. D. Brayer, personal communication) findings that this substitution does not produce any notable distortion in the protein conformation.

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Registry No. L-Phe, 63-91-2; L-Tyr, 60-18-4; L-Leu, 61-90-5; L-Ile, 73-32-5; L-Ser, 56-45-1; L-Ala, 56-41-7; Gly, 56-40-6; $Fe(EDTA)^{2-}$, 15651-72-6; cytochrome *c*, 9007-43-6.

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