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# Yeast cytochrome *c* with phenylalanine or tyrosine at position 87 transfers electrons to (zinc cytochrome *c* peroxidase)$^+$ at a rate ten thousand times that of the serine-87 or glycine-87 variants

**(site-directed mutagenesis/electron transfer/*Saccharomyces cerevisiae*)**

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ABSTRACT **Of the many factors known to influence the rate of electron transfer between two metalloproteins, it is particularly difficult to assess the role of the polypeptide matrix intervening between the donor and acceptor sites. To determine whether the phylogenetically conserved Phe-87 of yeast iso-1-cytochrome *c* helps to mediate electron transfer between cytochrome *c* and cytochrome *c* peroxidase, we have constructed mutants of cytochrome *c* that are altered at this position and now have studied the kinetics of long-range electron transfer within their complexes with zinc-substituted cytochrome *c* peroxidase. We find that the rate of electron transfer from reduced cytochrome *c* to the zinc cytochrome *c* peroxidase $\pi$-cation radical is four orders of magnitude greater when phenylalanine or tyrosine is present at position 87 than when serine or glycine is present.**

Phenylalanine-87 of yeast iso-1-cytochrome *c* is phylogenetically conserved, and the possibility that this residue might be involved directly in electron transfer between cytochrome *c* (Cyt*c*) and cytochrome *c* peroxidase (Cyt*c*P; ref. 1) is raised by computer modeling studies (1, 2). Our recent preparation of three mutants of cytochrome *c* in which this residue is replaced by serine, glycine, or tyrosine (3, 4) provides a unique opportunity to test this hypothesis. Steady-state kinetic studies showed only modest effects of substitution at this position (3), but the enzymic reaction is complex (5), and such studies are imperfect windows on the electron-transfer step because they inevitably convolve bimolecular processes and the intracomplex transfer event. Recently, we have shown that it is possible to study long-range electron transfer (6) within the preformed Cyt*c*P–Cyt*c* protein complex by substituting zinc protoporphyrin IX (ZnPor) for heme in cytochrome *c* peroxidase (7, 8). Electron transfer from ZnCyt*c*P to Fe(III)Cyt*c* is initiated by flash photoproduction of the zinc protoporphyrin triplet state ($^3$ZnPor), which can reduce a ferriheme partner with rate constant $k_t$ (Fig. 1, *Inset*). The resulting intermediate, B, returns to the ground state by electron transfer from Fe(II)Por to the $\pi$-cation radical, ZnPor$^+$, in a thermal reaction that is analogous to the physiological oxidation of Fe(II)Cyt*c* by $H_2O_2$-oxidized Cyt*c*P (5). The present report demonstrates that substitution of Phe-87 by serine and tyrosine has no effect on the photostimulated forward reaction; glycine substitution appears to perturb the complex and reduces $k_t$. However, the thermal return process is more than $10^4$ times faster for the wild-type protein and the Tyr-87 variant than for the serine or glycine variants.

## MATERIALS AND METHODS

Preparation of the three mutants of yeast (*Saccharomyces cerevisiae*) iso-1 cytochrome *c* and of the wild-type protein have been described (3, 4). ZnCyt*c*P was prepared by a procedure modified from that reported for the preparation and reconstitution of the apoperoxidase (9). To avoid autoreduction of the yeast iso-1 Fe(III)Cyt*c*, all experiments were done at 0°C, using anaerobic solutions in 1 mM potassium phosphate buffer, pH 7.0; the ZnCyt*c*P was present at *ca*. 4–9 $\mu$M, and the [Fe(III)Cyt*c*]/[ZnCyt*c*P] molar ratio was varied between 1.1 and 2. Note that our recently published experiments (8) employed yeast iso-2 cytochrome *c*, which is not susceptible to autoreduction, and the results reported there were obtained at 25°C.

Sample preparation and flash photolysis experiments were performed as described recently (10). Observations of the intermediate B involved measurements of very weak absorbance changes ($\Delta A_{max} \approx 10^{-3}$) and signal-to-noise ratios were enhanced by signal averaging. However, excitation by the flash lamp-pumped dye laser (R6G; ≈0.5 J) causes a small, net photoreduction. Thus, data collection was restricted to $\lesssim$ 30 transients, which gave negligible ($\lesssim$ 5%) net reduction.

## RESULTS

As embodied in the kinetic scheme (Fig. 1, *Inset*), decay of the $^3$ZnCyt*c*P excited state within the [ZnCyt*c*P–Fe(II)Cyt*c*] complex is first order, with decay rate, $k_d$. This rate constant is the same whether ZnCyt*c*P is free in solution or in the complex and, in particular, is independent of the identity of the complexed Fe(II)Cyt*c*. Fig. 1 presents $^3$ZnPor decay traces at 0°C, 1 mM buffer, for ZnCyt*c*P and for the 1:1 [ZnCyt*c*P–Fe(III)Cyt*c*] complexes prepared from the wild-type cytochrome *c* and the three cytochrome *c* variants. The traces for the complexes were invariant as the concentration ratio was varied over the range, [Fe(III)Cyt*c*]/[ZnCyt*c*P] ≈ 1.1–2. Our original work (7) showed that the wild-type protein forms a 1:1 complex stoichiometrically, even under the less favorable conditions of 10 mM buffer and room temperature. Although the range of concentrations employed here is limited, these observations strongly imply that the cytochrome *c* mutants, as well as the wild-type protein, form a stoichiometric complex with ZnCyt*c*P under the conditions used here.

Excited-state electron transfer to Fe(III)Cyt*c* sharply increases the $^3$ZnCyt*c*P decay rate, to $k_p = k_d + k_t$, for the wild-type protein and the serine and tyrosine variants. In each of these cases, $k_t \approx 200$ sec$^{-1}$ (Table 1). In contrast, the substitution of glycine for phenylalanine at position 87

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Abbreviations: Cyt*c*, cytochrome *c*; Cyt*c*P, cytochrome *c* peroxidase; Por, protoporphyrin IX.

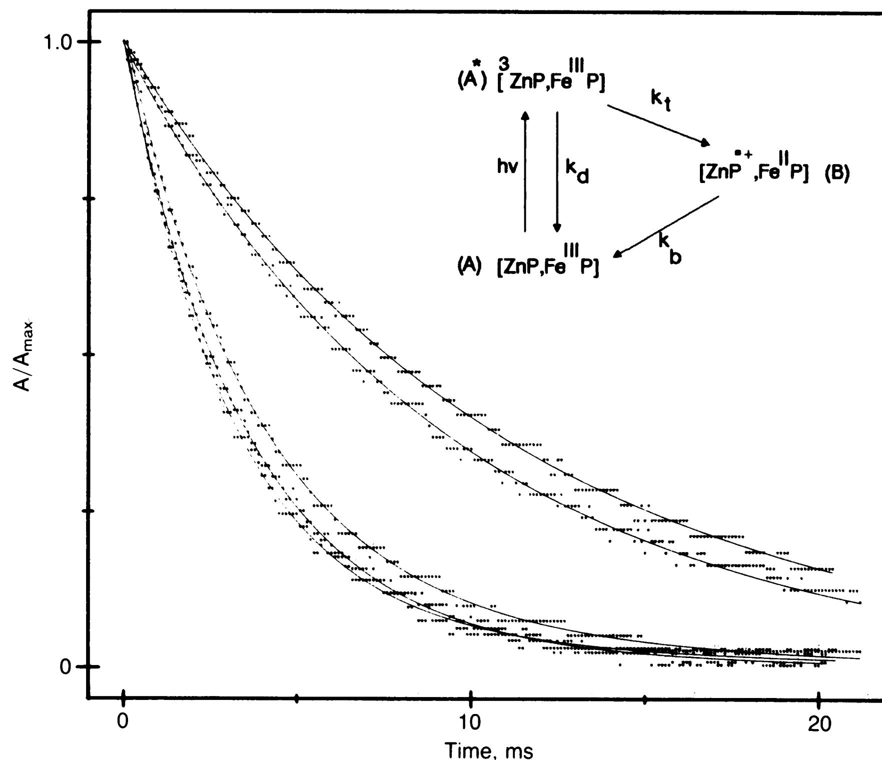


FIG. 1. Absorbance (A) decay of ³ZnCytcP alone and in [ZnCytcP–Fe(III)Cytc] complexes. From top to bottom: ZnCytcP; [ZnCytcP–(Gly-87)Fe(III)Cytc]; [ZnCytcP–(Tyr-87)Fe(III)Cytc]; [ZnCytcP–(Phe-87)Fe(III)Cytc]; [ZnCytcP–(Ser-87)Fe(III)Cytc]. Conditions: 1.0 mM potassium phosphate buffer, pH 7.0 at 0°C. *Inset*, kinetic scheme that defines the rate constants, resting state (A), excited state (A*), and electron-transfer intermediate (B) of the complex. P, protoporphyrin IX.

reduces $k_t$ by *ca.* 20-fold. The small 50-mV lowering of the reduction potential for the free glycine and serine mutants (11) might have been expected to lower $k_t$ several-fold in these cases. The absence of such an effect for the serine variant may reflect an opposing change in potential upon complex formation, or even a small change in the complex that is caused by the replacement of phenylalanine and that acts to slightly increase $k_t$. The unique behavior of the glycine variant is provisionally taken to indicate that elimination of the side-chain at position-87 of cytochrome *c* modifies the structure of the complex.

The rate constant, $k_b$, of the thermal return reaction can be determined if the kinetic difference spectrum of electron-transfer intermediate B (Fig. 1, *Inset*) can be detected and monitored. As the maximal concentration of B always is much less than the initial A* concentration, B is most readily detected by monitoring the Fe(III)Cytc/Fe(II)Cytc difference spectrum at the ³ZnCytcP/ZnCytcP isosbestic point (444.5 nm) (8). Flash excitation of the [ZnCytcP–(Ser-87)Fe(III)Cytc] complex gives a small but well-defined absorbance change at 444.5 nm, whereas the ZnCytcP

Table 1. Rate constants (0°C) for electron transfer within the complexes of yeast iso-1-cytochrome *c* variants and zinc cytochrome *c* peroxidase ($sec^{-1}$)*

| Residue at position 87 | $k_t \cdot sec^{-1}$ (³ZnPor → Fe(III)Por) | $k_b \cdot sec^{-1}$ ($ZnPor^+$ ← Fe(II)Por) |
|---|---|---|
| Phe | 201 (11) | 1.9 (6) × $10^4$ |
| Tyr | 173 (11) | 1.5 (6) × $10^4$ |
| Ser | 231 (22) | 2 (1) |
| Gly | 13 (2) | 1.4 (3) |

*Rate constants are defined in the kinetic scheme of the Fig. 1 *Inset*. Measurement gives $k_d$ = 92 (2) $sec^{-1}$. Conditions: 1.0 mM potassium phosphate buffer, pH 7.0, at 0°C. Error limits are indicated in parentheses as uncertainties in last digit(s).

isosbestic is preserved in [ZnCytcP–(Ser-87)Fe(II)Cytc], which exhibits no signal. The transient absorbance in the Fe(III)Cytc complex grows with a rate constant equal to $k_p$ (Fig. 2, *Inset*), precisely as predicted by the kinetic equations for the electron-transfer intermediate B under the condition $k_b < k_p$ (8). In this case, B rises during the lifetime of the ³ZnPor and then decreases slowly, with rate constant $k_b$, after ³ZnPor has disappeared.

For the serine variant the relatively rapid rate of production of B and its slow rate of loss render B observable at wavelengths other than 444.5 nm. As an example, Fig. 2 displays a kinetic progress curve taken at the Fe(III)Cytc/Fe(II)Cytc isosbestic (434 nm). The strong, early portion of the trace represents the decay of ³ZnCytcP. The weak, later portion, which primarily reflects the $ZnCytcP^+$/ZnCytcP absorbance difference, again represents the return of B to the initial state A. A fit of the long-time decay of B gives the rate constant $k_b$ = 2±1 $sec^{-1}$ for the complex with Ser-Cytc, slower by a factor of ≈100 than the forward rate. As confirmation of this result, we note: (*i*) there is no slowly decaying signal at any wavelength in the [ZnCytcP–Fe(II)Cytc] complex (also see ref. 8); (*ii*) the rate constant is invariant with λ; and (*iii*) the extrapolated zero-time absorbance changes for B agree in sign and magnitude with those expected from the expected B/A difference spectrum. Thus, the transient is indeed associated with intermediate B and not with an oxidized or reduced amino acid residue. The rate constant $k_b$ is unchanged by two-fold variation in the concentrations of Cytc and ZnCytcP, which suggests that it is not significantly affected by dissociation and reassociation of intermediate B. The thermal electron transfer rate for the glycine variant also is slow (Table 1).

Although the photostimulated reaction is indistinguishable for wild-type Phe-, Tyr-, and Ser-87 cytochrome *c* variants, the thermal electron transfer process responds very sharply to the identity of residue 87. The complexes of ZnCytcP with

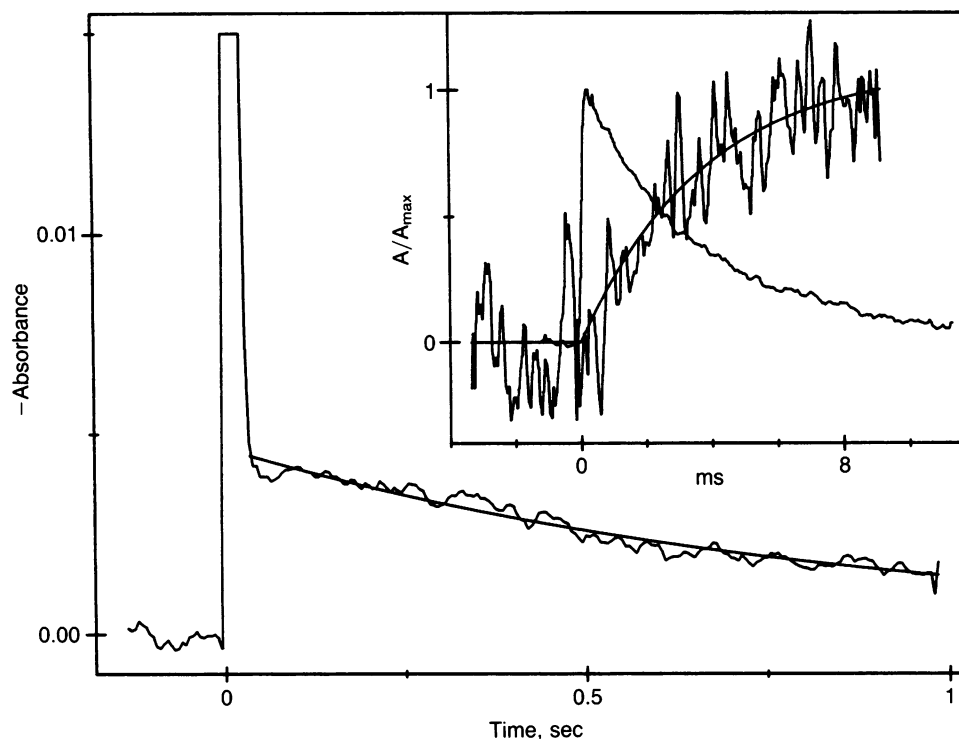


FIG. 2. Transient absorbance (A) after photolysis of [ZnCyt*c*P–(Ser-87)Fe(III)Cyt*c*]. The large, rapidly decaying component represents $^3$ZnPor and has been expanded off-scale in order to emphasize the slowly decaying component whose exponential fall, rate constant $k_b$, represents the electron transfer intermediate B. *Inset*, as predicted by the kinetic scheme (8), decay of $^3$ZnCyt*c*P (434 nm) and concomitant exponential rise of B (444.5 nm) with the same rate constant, $k_p = k_d + k_t$. Experimental conditions were as in Fig. 1.

wild-type cytochrome *c* (8) or the tyrosine variant do not exhibit the slowly decaying absorbance by B that occurs when $k_b < k_p$ ($\approx$ 300 sec$^{-1}$). Instead, for each complex the absorbance of B, seen at 444.5 nm, rises with a high, but finite, rate (Fig. 3*B*), and then follows the time course of the $^3$ZnPor decay (Fig. 3*A*, *Inset*). This may be contrasted with the $^3$ZnPor signal monitored at 434 nm, which appears with the instrumental time constant (Fig. 3*A*), and with the reference compounds, which give no signal at 444.5 nm. According to the kinetic scheme (Fig. 1, *Inset*), these observations require that $k_b >> k_p$, in which case $k_b$ characterizes the exponential rise of the absorbance of B at 444.5 nm (8). For both these cytochrome *c* variants, analysis of the rise gives $k_b \approx 10^4$ sec$^{-1}$ at 0°C (Table 1), similar to the complex with yeast iso-2-C*c* at 25°C.

Interestingly, the 50-mV lower reduction potential for the glycine and serine variants corresponds to decreased stability of the reduced heme in these proteins. This would tend to increase $k_b$ for Gly- and Ser-cytochromes *c* totally at variance with observation. Thus, the change in potential not only fails to account for the dramatic difference in rates, but any contribution of the alteration in driving force would oppose, and thus diminish, the observed differences.

## DISCUSSION

The experiments reported here have produced a remarkable result: the thermal electron transfer from Fe(II)Cyt*c* to (ZnCyt*c*P)$^+$ within the protein–protein complex is *ca.* $10^4$ faster for the wild-type protein and the tyrosine variant than for the Ser-87 variant, whereas the rates of the photostimulated reaction for the three proteins are comparable. Because $k_t$ is largely unaffected by these mutations, it seems reasonable to infer that the complex of each with ZnCyt*c*P has essentially the same structure: Differences in the heme-heme distance of as little as $\approx$1 Å would have produced significant changes in the electron-transfer rate (6). For the glycine mutation, $k_t$ is reduced as well as $k_b$. The change in $k_t$ suggests that this mutation has in fact perturbed the complex of the Fe(III)Cyt*c* with ZnCyt*c*P; hence, we consider it to belong in a separate category and do not discuss it further.

The present observations indicate that the rate constant for the thermal, Fe(II)Por→ZnPor$^+$, reaction within the protein complex is controlled by interactions involving Phe-87, and that these are preserved upon substitution by the aromatic residue, tyrosine, but abolished when serine is introduced. These results make it tempting to infer that Fe(II)Por→ ZnPor$^+$ electron transfer is sharply enhanced by "hole" superexchange interactions (12) when an aromatic phenylalanine or tyrosine residue is incorporated at position 87 of cytochrome *c*. The insensitivity of the $^3$ZnPor→Fe(III)Por transfer rate, and indeed of the steady-state rate (3), to the identity of residue 87 could then mean that these processes are not facilitated by this form of electronic coupling. After all, the forward and reverse reactions within the ZnCyt*c*P complex are *different* chemical processes (Fig. 1, *Inset*), and the ZnPor$^+$ oxidant in the thermal reaction is a quite different species from the oxy-ferryl heme or amino acid free radical (5) that serve as oxidants in $H_2O_2$-oxidized Cyt*c*P.

Alternatively, it could be that the superexchange interactions are not effective in the initial, [ZnCyt*c*P–Fe(III)Cyt*c*], complex and are established by a conformation rearrangement following the photostimulated reaction. Indeed, we have noted that this system might undergo some such rearrangement (8), which in fact is predicted (13) on the basis of solution (14, 15) and x-ray (16, 17) structural studies that show cytochrome *c* undergoes a conformational change upon reduction.

The intervention of a conformational rearrangement following the photostimulated reduction of Fe(III)Cyt*c*, of course, admits of other interpretations for the influence of residue 87 on the subsequent Fe(II)Por→ZnPor$^+$ reaction. For example, the volumes of the phenylalanine and tyrosine residues are greater (190, 194 Å$^3$) than that of serine (89 Å$^3$),

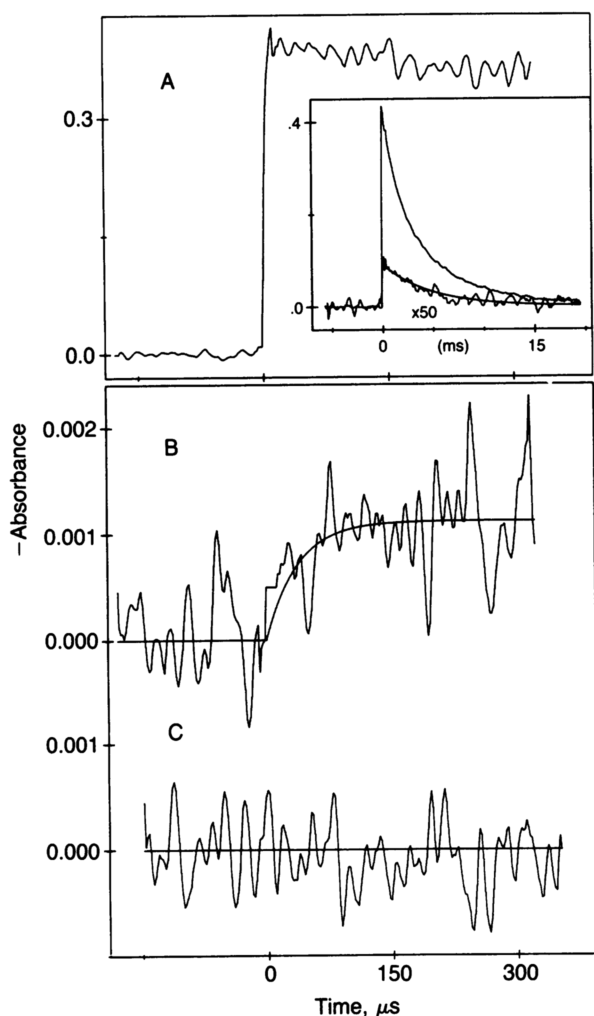


FIG. 3. Transient absorbance after photolysis of [ZnCyt*c*P–(Tyr-87)Fe(III)Cyt*c*]. (*A*) Short-time absorbance change for $^3$ZnPor in [ZnCyt*c*P–(Tyr-87)Fe(III)Cyt*c*], monitored at 434 nm. *Inset*, long-time decay of absorbance of $^3$ZnPor and of intermediate B (X50) occur with the same rate constant, $k_p$ (8). (*B*) Growth of intermediate B after photolysis of [ZnCyt*c*P–(Tyr-87)Fe(III)Cyt*c*]; $\lambda$ = 444.5 nm. Solid line is a fit to an exponential rise, with rate constant $k_b$ (8). Because of a signal from scattered light, six channels after t = 0 have been suppressed; these were not included in the fitting procedure. (*C*) Control, [ZnCyt*c*P–Fe(II)Cyt*c*] flashed under the same experimental condition as in (*B*). Conditions were as in Fig. 1. The signal in panel *A* represents a single transient; the *inset* to *A* involved a single transient for $^3$ZnPor and 23 for B; signals in *B* and *C* represent 33 and 32 transients, respectively.

which is greater than that of glycine (60 Å$^3$) (18). We suggested above that the structure of the Fe(III)Cyt*c* complex is perturbed for the glycine variant. It could be that the structure of the Fe(II)Cyt*c* complex is perturbed for both glycine *and* serine variants, or that the rate of a conformational switch is reduced in these cases.

In conclusion, two points are clear: (*i*) modifying a single, uncharged cytochrome *c* surface residue can have a remarkable effect on interprotein electron transfer. (*ii*) The same procedures that uncovered this effect can identify its origin by studying mutants in which Phe-87 is replaced by comparably sized aliphatic residues, such as isoleucine (167 Å$^3$).

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