Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 1330–1333, March 1986
Biophysics

Direct measurements of intramolecular electron transfer rates between cytochrome *c* and cytochrome *c* peroxidase: Effects of exothermicity and primary sequence on rate

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Communicated by Gerhard L. Closs, October 21, 1985

ABSTRACT **Rapid mixing of ferrocytochrome *c* peroxidase [cyt *c* peroxidase(II)] and ferricytochrome *c* [cyt *c*(III)] results in the reduction of cyt *c*(III) by cyt *c* peroxidase(II). In 10 mM phosphate, pH 7.0, the rate of decay of cyt *c* peroxidase(II) and the rate of accumulation of cyt *c*(II) give equal first-order rate constants: $k = 0.23 \pm 0.02\ s^{-1}$. Equivalent results are obtained by pulse radiolysis using isopropanol radical as the reducing agent. This rate is independent of the initial cyt *c*(III):cyt *c* peroxidase(II) ratios. These results are consistent with unimolecular electron transfer occurring within a cyt *c*(III)–cyt *c* peroxidase(II) complex. When cyt *c* is replaced by porphyrin cyt *c* (iron-free cyt *c*), a complex still forms with cyt *c* peroxidase. On radiolysis, using e^-_{aq} as the reducing agent, intracomplex electron transfer occurs from the porphyrin cyt *c* anion radical to cyt *c* peroxidase(III) with $k = 150\ s^{-1}$. This large rate increase with increasing $\Delta G°$ suggests that the barrier for intracomplex electron transfer is large. Finally, we have briefly investigated how the cyt *c* peroxidase(II) → cyt *c*(III) rate depends on the primary structure of cyt *c*(III). We find the reactivity order to be as follows: yeast ($k = 3.4\ s^{-1}$) > horse ($k = 0.3\ s^{-1}$) > tuna ($k = 0.2\ s^{-1}$). These results mirror a report [Ho, P. S., Sutoris, C., Liang, N., Margoliash, E. & Hoffman, B. M. (1985) *J. Am. Chem. Soc.* 107, 1070–1071] on excited state reactions of the cyt *c*/cyt *c* peroxidase couple.**

The interaction of cytochrome *c* peroxidase (cyt *c* peroxidase; EC 1.11.1.5) with cytochrome *c* (cyt *c*) has been extensively studied as a model for understanding the control of redox rates and protein–protein recognition in biological systems (1–3). The crystal structures of both proteins (4, 5) and a computer model of the complex (6) have been published (Fig. 1). Furthermore, it is possible in this complex to employ metal substitution to systematically vary parameters such as the reaction exothermicity, which play an important role in controlling biological electron transfer rates. For example, theory predicts (7–11) that the electron transfer rate constant $k_t = k°\nu_{el}\nu_n$, where $k° \approx 10^{14}\ s^{-1}$ and the electronic (ν_{el}) and nuclear (ν_n) frequency factors are given by $\exp(-\alpha R)$ and $\exp-(\Delta G°+\lambda)^2/4\lambda kT$, respectively. Electron transfer within the 1:1 (cyt *c*:cyt *c* peroxidase-enzyme–substrate) complex [cyt *c* peroxidase-enzyme–substrate is formed on reaction of H_2O_2 with cyt *c* peroxidase(III)] is believed to be very rapid, based on the large turnover number observed for cyt *c* peroxidase (2). However, $\Delta E°$ for this reaction is very large (0.9 V) (12). A recent study also inferred fast electron transfer ($k_t = 2.7 \times 10^4\ s^{-1}$) from cyt *c* peroxidase(II) to cyt *c*(III) ($\Delta E° = 0.46$ V; refs. 13 and 14) based on the assignment of a weak near-IR absorption to an intervalence charge transfer transition (15). Corroboration of this rate by a more direct measurement is desirable in the context of understanding biological electron transfer rates.

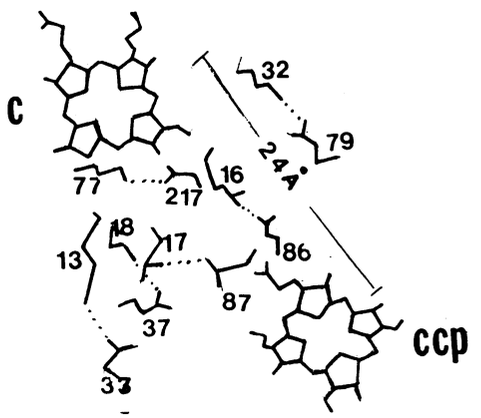

FIG. 1. Computer model of the cyt *c*–cyt *c* peroxidase complex. c, cyt c; ccp, cyt c peroxidase.

We, therefore, wish to report the direct measurement of electron transfer from cyt *c* peroxidase(II) to cyt *c*(III) within the cyt *c*(III)–cyt *c* peroxidase(II) complex. In addition, we report data for electron transfer in the derivative system, cyt *c* peroxidase–porphyrin (Por) cyt c^- ($\Delta E° \approx 1$ V), where Por-cyt c^- is the anion radical of Por-cyt *c*. Por-cyt *c* is iron-free cyt *c*. These systems together provide unique data on the dependence of protein electron transfer rates on $\Delta G°$.

Finally, Ho *et al.* (16) reported an interesting study on the rate of oxidative quenching of the Zn–cyt *c* peroxidase triplet by cyt *c*(III). They found that ^3k_q depends markedly on the primary structure of the cytochrome with ^3k_q (yeast C) > 10 ^3k_q (horse C). With the methods in hand to study a simple Fe(II)/Fe(III) redox reaction in the cyt *c*/cyt *c* peroxidase system, we report a comparative study of how the cyt *c* peroxidase(II) + cyt *c*(III) reaction depends on the primary structure of cyt *c*.

MATERIALS AND METHODS

cyt *c* peroxidase was isolated by the method of Nelson *et al.* (17) with the following modifications: (*i*) substituting cross-linked DEAE-agarose for DEAE-cellulose, (*ii*) extracting the enzyme in 0.05 M sodium acetate buffer (pH 5.0), thereby allowing the enzyme to be loaded directly onto the DEAE column, and (*iii*) concentrating the enzyme by ultrafiltration rather than on a second DEAE column. Por-cyt *c* was prepared from Sigma type VI cyt *c* by the method of Vanderkooi *et al.* (18). The product was purified by G-200

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Abbreviations: cyt *c*, cytochrome *c*; Por, porphyrin.

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and DEAE columns to a single electrofocusing component. The thermal reactions were carried out as follows: cyt *c* peroxidase(III) (3.3 μM) in 10 mM sodium phosphate, pH 7.0 containing 0.008% acetophenone and 2% (vol/vol) isopropyl alcohol, was sealed in a 1-cm cuvette and extensively degassed under Ar. cyt *c* peroxidase(II) was formed *in situ* by UV-irradiation (19). The photoreduction was monitored by absorbance changes in the Soret region (Fig. 2). Once reduction was complete, 100–200 μl of a degassed solution of cyt *c*(II) (100 μM) were immediately added to the cuvette with a gas-tight syringe. After shaking the reactants, absorbance changes were followed by using a rapid response (0.1 s^{-1}) spectrophotometer (HP model 8451A). The mixing procedure generally took 3 s to complete, which represents one half-life for the electron transfer reaction. Absorbance readings were taken every 0.1 s over the next 15 s, or five half-lives. Plots of $\ln(A_0 - A_\infty)/(A_t - A_\infty)$ vs. t(A_0, A_t, and A_∞ are the absorbance reading at t = 0, t, and 60 s, respectively) were linear with correlation coefficients of 0.994–0.999. Samples for pulse radiolysis were prepared by mixing equal concentrations (2–20 μM) of cyt *c* peroxidase(III) and cyt *c*(III) in phosphate buffer (1 mM, pH 7.0). The solutions were degassed under Ar and transferred by syringe to anaerobic quartz cells for pulse radiolysis. All measurements were conducted at ambient temperature (24 ± 1°C). The pulse radiolysis system has been described (20). Again, plots of absorbance changes were linear.

RESULTS AND DISCUSSION

By assuming $k_t \approx 10^4$ s^{-1} as predicted by Potasek (15), we attempted to measure this rate directly using the following scheme:

$$\text{cyt } c\text{(III)–cyt } c \text{ peroxidase(II)–CO} \underset{k_{CO}}{\overset{h\nu}{\rightleftharpoons}} \text{cyt } c\text{(III)–cyt } c \text{ peroxidase(II)// /CO} \xrightarrow{k_t} \text{cyt } c\text{(II)–cyt } c \text{ peroxidase(III)} + \text{CO}.$$

cyt *c*(III)–cyt *c* peroxidase(II)// /CO represents the species obtained on CO photodissociation where CO is apparently trapped within the cyt *c* peroxidase matrix (21).¶ Since the geminate recombination rate constant, k_{CO}, is $\approx$5 s^{-1} at pH 7.0 (21), we expected electron transfer to compete favorably with CO recombination. However, flash photolysis in the presence of cyt *c*(III) shows no electron transfer transients. This indicates that $k_t < k_{CO}$ for cyt *c*(III)–cyt *c* peroxidase(II); i.e., k_t < 5 s^{-1}. This result suggested that simple mixing experiments could be used to measure k_t.

Hence, to measure k_t, cyt *c*(III) and cyt *c* peroxidase(II) were directly mixed as described above. The decrease in cyt *c* peroxidase(II) is monitored mainly at 440 nm, and the reduction of cyt *c*(III) is monitored at 421 nm, which is an isosbestic point in the peroxidase spectrum (Fig. 2). The decay at 440 nm and the accumulation at 421 nm are both strictly first order (Fig. 3) and give essentially identical rate constants. Rates were measured for initial cyt *c*:cyt *c* peroxidase ratios of 1:1, 2:1, and 3:1 (mol/mol). The observed rate constants are 0.22 ± 0.2, 0.24 ± 0.01, and 0.21 ± 0.02 s^{-1}, respectively. To ensure that cyt *c*(III) was reduced by the peroxidase only, we examined the spectra of the products in the visible region. When the cyt *c* peroxidase(III) spectrum is subtracted from the product spectrum at different cyt *c*:cyt *c* peroxidase ratios, only cyt *c*(II) is present at a 1:1 ratio, but equal concentrations of cyt *c*(II) and cyt *c*(III) are present at 2:1, as expected if cyt *c* peroxidase(II) is the only reductant. The above results are consistent with the following scheme:

$$\text{cyt } c\text{(III)} + \text{cyt } c \text{ peroxidase(II)} \overset{K}{\rightleftharpoons} \text{cyt } c\text{(III)–cyt } c \text{ peroxidase(II)} \xrightarrow{k_t} \text{cyt } c\text{(II)–cyt } c \text{ peroxidase(III)},$$

where the overall average value of k_t, the intramolecular electron transfer rate constant, is 0.23 ± 0.02 s^{-1} at 25°C and pH 7.0.

A first order rate constant is also consistent with the rate-limiting step being dissociation of a dead-end complex followed by rapid bimolecular electron transfer. Since the observed kinetics are independent of the cyt *c* concentration, the formation of a dead-end complex would have to block the approach of a second cyt *c* molecule to the cyt *c* peroxidase heme in a bimolecular step. It seems unlikely that the dead-end site and the bimolecular closest-approach site should overlap on the protein surface. We, therefore, con-

¶In 0.2 M Tris·HCl buffer over a pH range from 6.5 to 8.5, Iizuka *et al.* (22) observed bimolecular kinetics for the recombination of photodissociated CO with the heme of cyt *c* peroxidase. The reason for the discrepancy between our results (21) and theirs is not understood at present.

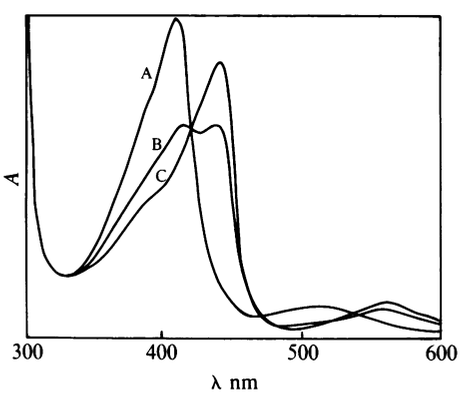


FIG. 2. Spectra of cyt *c* peroxidase(III) (curve A; λ_{max}, 408 nm), cyt *c* peroxidase(II) (curve C; λ_{max}, 436 nm), and the half-reduced mixture during photoreduction (curve B). Note the isosbestic point at 421 nm.

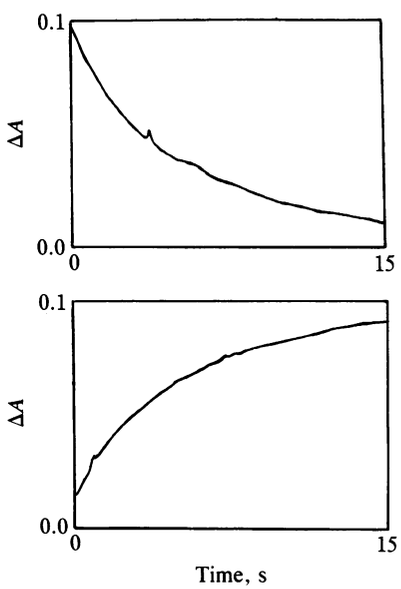
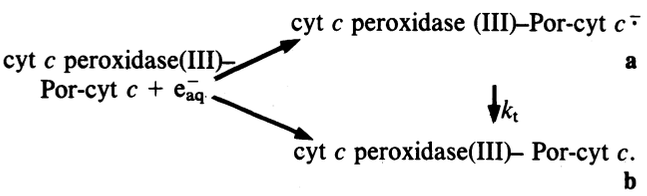


FIG. 3. Kinetics of the reaction cyt *c*(III)–cyt *c* peroxidase(II) → cyt *c*(II)–cyt *c* peroxidase(III) using horse cyt *c* following rapid mixing. The reaction is monitored by absorbance changes due to cyt *c* peroxidase(II) decay at 440 nm (*Upper*) and cyt *c*(II) accumulation at 421 nm (*Lower*) in 10 mM phosphate (pH 7.0) with 3 μM each of cyt *c* and cyt *c* peroxidase.

clude that the observed rate is, indeed, an intramolecular electron transfer rate within the protein–protein complex.

The results obtained in the thermal mixing experiments are generally confirmed by pulse radiolysis studies. When solutions containing 3×10^{-6} M cyt *c*(III)–cyt *c* peroxidase(III) complex are irradiated with a 10-ns pulse from a 20 MeV Linac linear accelerator, e^-_{aq} capture occurs at both the thermodynamically stable cyt *c*(III) site and the unstable cyt *c* peroxidase(III) site. The rate so measured for the equilibration reaction of cyt *c* peroxidase(II)–cyt *c*(III) by pulse radiolysis is $0.3 \pm 0.1\ s^{-1}$.

By contrast with the slow rate for cyt *c*(III)–cyt *c* peroxidase(II) → cyt *c*(II)–cyt *c* peroxidase(III) electron transfer, a far more rapid rate is observed (Fig. 4) for the more exothermic reaction of Por-cyt c^- in the following sequence:



The presence of the kinetic product **a** is demonstrated by an increased absorbance at 650 nm, which Dekok *et al.* (23) showed is characteristic of the Por-cyt c^- radical. In a pulse radiolysis study of Por-cyt *c*, Dekok *et al.* (23) found that electron capture by Por-cyt *c* was effectively blocked when Por-cyt *c* was bound to cytochrome oxidase. In the Por-cyt *c*–cyt *c* peroxidase system, complex formation significantly reduces electron capture, but fortunately does not eliminate it. The unstable Por-cyt c^-–cyt *c* peroxidase(III) intermediate decays with a first order rate constant of $150 \pm 10\ s^{-1}$. Concomitantly, an absorbance growth is observed at 436 nm, indicating the kinetically coupled formation of cyt *c* peroxidase(II) with $k_t = 150 \pm 20\ s^{-1}$. The observed rates are clearly first order over two half-lives and are independent of the total concentration over the range examined (2–20 μM).

These results clearly demonstrate that the rate of electron transfer in the cyt *c*–cyt *c* peroxidase complex is quite sensitive to the nature of the donor, or, more likely, to ΔG° for the electron transfer step. In this context, it is noteworthy that a similar trend seems to be emerging in other studies of electron transfer reactions within protein–protein complexes. For example, in the cyt *c*–cyt b_5 complex, k_t increases from $1800\ s^{-1}$ for $\Delta E^\circ = 0.2$ V to $10^5\ s^{-1}$ when $\Delta E^\circ = 0.8$ V (20).

These results have been interpreted as suggesting that a large reorganization energy, λ, is associated with protein repolarization around the developing charge. From basic Marcus theory (7–11), this energy can be compensated by the reaction exothermicity, i.e., $k_t = A[\exp-(\Delta G^\circ + \lambda)^2/4\lambda kT]$. If a similar interpretation is applied to the dependence of rate on ΔG° for the cyt *c*–cyt *c* peroxidase reaction, then for this complex λ is ≈2 eV. An equally large λ value (λ ≈ 2.3 eV) has been inferred from the temperature dependence of the electron transfer rate from the excited Zn triplet to Fe(III) within the Zn/Fe hybrid of hemoglobin (24). It is tempting to speculate that the large apparent λ values associated with the Fe(II)/(III) couple in both cyt *c* peroxidase and hemoglobin reflect the large internal reorganization that occurs in reducing the six coordinate Fe(III) species to produce a five coordinate Fe(II) species. In fact, a model is provided by the conversion of Fe(III) myoglobin to deoxy Fe(II) myoglobin where the heme reorganizational energy is of the order of 1 eV (25).

Finally, we have used pulse radiolysis to examine the dependence of electron transfer rate on the primary structure of cyt *c*. Ho *et al.* (16) reported the rate constant, ^3k_q, for the quenching of the triplet excited state of Zn-substituted cyt *c* peroxidase when bound to cyt *c*(III). This quenching was assigned to an electron transfer process, and the key finding was that ^3k_q (yeast C)/^3k_q (horse C) ≈ 10. We thought it desirable to examine whether a similar relationship exists in the ground state redox reaction. Using pulse radiolysis (Fig. 5), we also observed a dependence of rate on the primary structure of the cytochrome as follows: k_t (yeast) $= 3.4 \pm 0.2 > k_t$ (horse) $= 0.3 \pm 0.1 > k_t$ (tuna) $= 0.2 \pm 0.1\ s^{-1}$. These reports support and extend the observations by Ho *et al.* (16) but do not address whether such rate differences reflect activation energy or electronic coupling associated with different primary structure.

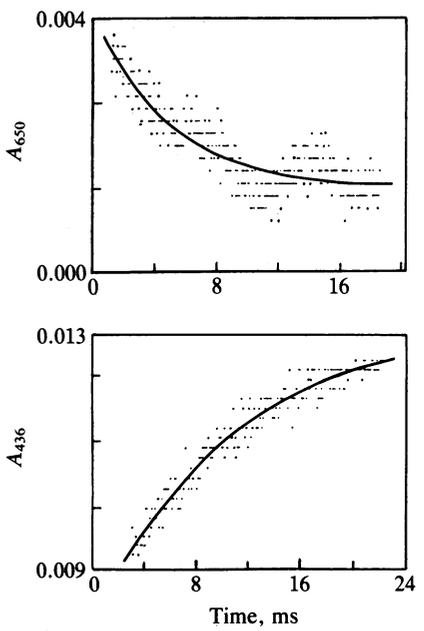


FIG. 4. Kinetics of the reaction cyt *c* peroxidase(III)–Por-cyt c^- → cyt *c* peroxidase(II)–Por-cyt *c* initiated by pulse radiolysis and followed by observing Por-cyt c^- decay at 650 nm (*Upper*, $k_t = 164\ s^{-1}$) or cyt *c* peroxidase(II) growth at 436 nm (*Lower*, $k_t = 147\ s^{-1}$).

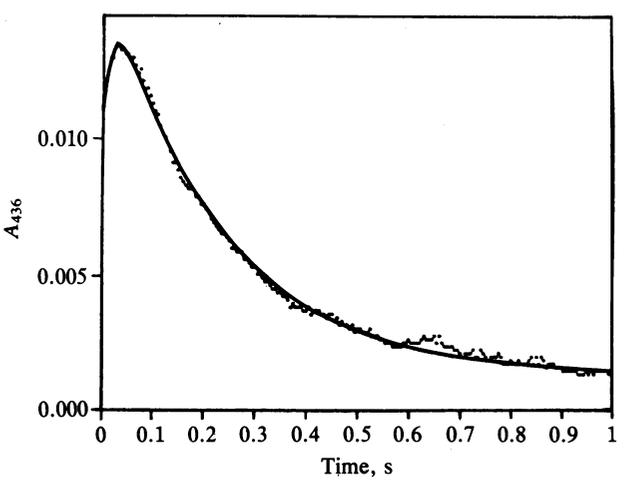


FIG. 5. Kinetics of the pulse-radiolysis-initiated electron transfer in the cyt *c*(III)–cyt *c* peroxidase(II) complex using yeast cyt *c* and monitored by cyt *c* peroxidase(II) absorbance at 436 nm. Because radical reduction to form cyt *c* peroxidase(II) occurs in competition with electron transfer to cyt *c*, the data were fit to a scheme having a fast and slow formation of the reactants followed by electron transfer to obtain $k_t = 3.4\ s^{-1}$.

SUMMARY

1. The present results provide the first direct measurements of electron transfer within the physiological cyt *c*–cyt *c* peroxidase complex. For the cyt *c*(III)–cyt *c* peroxidase(II) reaction ($\Delta E^\circ = 0.46$ V), the rate of intramolecular electron transfer is quite slow ($k_t = 0.2\ s^{-1}$).

2. This result appears to discount the previously reported rate, $k_t \approx 10^4\ s^{-1}$, which was based on the assignment of a weak band ($\varepsilon \approx 3\ M^{-1}\ cm^{-1}$) in the spectrum of the cyt *c*–cyt *c* peroxidase complex to an intervalence band (15).

3. In the Por-cyt c^+–cyt *c* peroxidase(III) reaction ($\Delta E^\circ \approx 1$ V), k_t increases to $\approx 150\ s^{-1}$, approaching the value inferred by the steady state measurements for the cyt *c*(II):cyt *c* peroxidase-enzyme–substrate system ($\Delta E^\circ = 0.9$ V, $k_t = 10^3\ s^{-1}$) (1).

4. These results suggest that a strong dependence of k_t on ΔG°, as inferred for the cyt *c*:cyt B_5 couple, may, in fact, be a more general property of biological electron transfer. Such a dependence of k_t on ΔG° is consistent with relatively large reorganization energies (≥ 0.8 eV) for protein electron transfer reactions.

5. Variations in the primary structure of cyt *c* cause variations in the rate of the cyt *c* peroxidase(II) + cyt *c*(III) reaction, suggesting that species specific rate processes may be common in the reactions of both cyt *c* and cyt *c* peroxidase.

Work at the Argonne National Laboratory was performed under the auspices of the Office of Basic Energy Sciences, Division of Chemical Science, U.S. Department of Energy under contract number W-31-109-ENG-38. Work at the University of Rochester was supported by the National Institutes of Health (GM 33881) and National Science Foundation (Chemical Dynamics Division); and work at Concordia University was supported by National Sciences and Engineering Research Council (Canada) and Fonds pour la Formation de Chercheurs et L'Aide à la Recherche (Quebec).

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