Reaction of Cytochrome c with the Radical in Cytochrome c Peroxidase Compound I

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The reaction between cytochrome *c* and cytochrome *c* peroxidase has become a paradigm for biological electron transfer because of the extensive structural and biophysical characterization of the system.[1,2] However, certain aspects of the kinetic mechanism remain poorly understood.[3] It is generally agreed that the resting ferric form of cytochrome *c* peroxidase (C*c*P) is oxidized by hydrogen peroxide to Compound I (CMPI), which contains an oxyferryl heme Fe(IV) and a radical, probably located at Trp-191.[4] CMPI is then sequentially reduced to CMPII and C*c*P by two molecules of ferrocytochrome *c*. Two forms of the singly oxidized state CMPII have been identified, CMPII(IV,R) containing the oxyferryl heme Fe(IV) and CMPII(III,R$^{\bullet}$) containing the radical.[5] An important question is whether cytochrome *c* reacts initially with Fe(IV) in CMPI to form CMPII(III,R$^{\bullet}$) or instead with the radical to form CMPII-(IV,R).[6-11]

In the present paper we report conditions under which the reaction between native horse cytochrome *c* and the radical in CMPI can be measured by stopped-flow spectroscopy.[12] Excess CMPI (0.6 μM) was mixed with ferrocytochrome *c* (0.3 μM) in order to isolate the first step in the reaction, and high ionic strength buffer (2 mM sodium phosphate, pH 7, 100 mM NaCl) was used to decrease the rate enough to resolve the entire reaction in the stopped-flow instrument (2-ms deadtime). The 416-nm absorbance transient indicated complete oxidation of ferrocytochrome *c*, but no reduction of CMPI Fe(IV) was observed at 434 nm (a cytochrome *c* isobestic[14]) (Figure 1). These results are consistent with electron transfer from ferrocytochrome *c* to the radical in CMPI according to reaction 1, since the $\Delta\epsilon$ due to reduction of the radical is very small at both wavelengths.[14]

$$\text{cyt } c^{2+} + \text{CMPI(IV,R}^{\bullet}) \rightarrow \text{cyt } c^{3+} + \text{CMPII(IV,R)} \quad (1)$$

$$\text{cyt } c^{2+} + \text{CMPII(IV,R)} \rightarrow \text{cyt } c^{3+} + \text{CcP(III,R)} \quad (2)$$

Experiments with a range of CMPI concentrations in excess of the cyt c^{2+} concentration confirmed that reaction 1 was second-order, with a rate constant of $(1.3 \pm 0.2) \times 10^8$ M^{-1} s^{-1} (Figure 1, Table I). No transient was observed at 434 nm, even at a CMPI concentration of 2 μM, where the pseudo-first-order rate constant of the 416-nm transient was 300 s^{-1}. This rules out initial reduction of Fe(IV) in CMPI followed by conversion of CMPII(III,R$^{\bullet}$) to CMPII(IV,R), since the later reaction has a rate constant of 6 s^{-1}.[6,15]

Mixing an excess of ferrocytochrome *c* with CMPI resulted in a biphasic transient at 416 nm and a slow monophasic transient at 434 nm (Figure 2). This is consistent with the mechanism given by reactions 1 and 2 above. Both reactions are detected at 416 nm, while only reaction 2 is detected at 434 nm. Experiments with a range of cyt c^{2+} concentrations in excess of CMPI confirmed that reaction 2 was second-order with a rate constant of $(9 \pm 1) \times 10^6$ M^{-1} s^{-1} (Figure 2, Table I). Both second-order rate constants increased with decreasing ionic strength (Table I). However, at ionic strengths below 50 mM, the amplitude of the 416-nm transient upon mixing 0.3 μM cyt *c* with 0.5 μM CMPI was less than 40% of the expected absorbance change, indicating that a substantial portion of the reaction was too fast to be resolved in the stopped-flow.

Pelletier and Kraut have recently determined the crystal structure of the complex between yeast iso-1-cytochrome *c* and cytochrome *c* peroxidase at high ionic strength and have proposed an electron-transfer pathway that extends from the exposed heme methyl group of cytochrome *c* through peroxidase residues Ala-194, Ala-193, and Gly-192 to the indole group of Trp-191, which is in contact with the heme group.[2] The present results suggest that horse cytochrome *c* uses this pathway to reduce the Trp-191 indole radical at high ionic strength, since it provides a short, direct route for rapid electron transfer. The smaller rate constant for reduction of the oxyferryl heme Fe(IV) could be due to a larger reorganization energy as well as a longer distance. The mechanism given by reactions 1 and 2 should also apply under steady-state conditions at high ionic strength. There is no need to postulate the involvement of a more reactive precursor of CMPI[3d,6] or a more highly oxidized species[16] since the radical is so reactive.

In contrast to the present results at high ionic strength, Summers and Erman reported that in stopped-flow experiments at low ionic strength horse ferrocytochrome *c* reduced the Fe(IV) site in CMPI to form CMPII(III,R$^{\bullet}$), followed by conversion to CMPII(IV,R) before reaction with a second molecule of cytochrome *c* could

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(9) Hazzard and Tollin[8] reported a transient decrease in absorbance at 445 nm which they interpreted as electron transfer to heme Fe(IV). However, the extinction coefficient change is nearly the same for reaction with the radical as with Fe(IV) at this wavelength,[5] making it difficult to distinguish between the two reactions.

(10) Geren, L. M.; Hahm, S.; Durham, B.; Millett, F. *Biochemistry* **1991**, *30*, 9450-9457.

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(12) Cytochrome *c* peroxidase was purified as described by Geren et al.[10] and had the following absorbance ratios: $A_{408}/A_{280} = 1.17 \pm 0.03$; $A_{408}/A_{508} = 8.8 \pm 0.2$; $A_{408}/A_{380} = 1.52 \pm 0.03$; $A_{620}/A_{646} = 0.80 \pm 0.05$. These values are the same as those reported by Vitello et al.,[13] within experimental error.

(13) Vitello, L. B.; Huang, M.; Erman, J. E. *Biochemistry* **1990**, *29*, 4283-4288.

(14) The extinction coefficient changes are[5] as follow: cyt $c^{2+} \rightarrow$ cyt c^{3+}, $\Delta\epsilon_{416} = -40$ mM^{-1} cm^{-1}, $\Delta\epsilon_{434} = 0$; CMPI $\rightarrow$ CMPII(III,R$^{\bullet}$), $\Delta\epsilon_{416} = -13$, $\Delta\epsilon_{434} = -27$ mM^{-1} cm^{-1}; and CMPI $\rightarrow$ CMPII(IV,R), $\Delta\epsilon < 3$ mM^{-1} cm^{-1} at both wavelengths.

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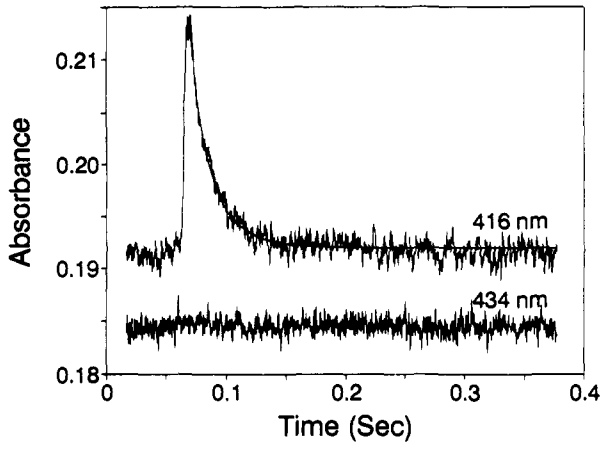

Figure 1. Ferrocytochrome *c* (0.3 μM) was mixed with CMPI (0.6 μM) in 2 mM sodium phosphate, pH 7, 100 mM NaCl at 25 °C in a Durrum stopped-flow spectrophotometer and detected at 416 and 434 nm. The solid line is the best fit to the second-order equation:

$$\Delta A = 2\Delta\epsilon\left(b_0 - \frac{\exp[(b_0 - a_0)kt] - 1}{\exp[(b_0 - a_0)kt]/a_0 - 1/b_0}\right)$$

where b_0 = initial [cyt c^{2+}], a_0 = initial [CMPI], and $k = 1.3 \times 10^8$ M^{-1} s^{-1}.

Table I. Second-Order Rate Constants for Reaction Between Ferrocytochrome *c* and Cytochrome *c* Peroxidase[a]

reductant	ionic strength (mM)	*k* (reaction 1)	*k* (reaction 2)
native cyt *c*	54	4.0×10^8	3×10^7
native cyt *c*	104	1.3×10^8	9×10^6
native cyt *c*	154	3.3×10^7	3.5×10^6
Ru-72-cyt *c*	104	3.5×10^7	4.0×10^6

[a] Rate constants in units of M^{-1} s^{-1} were measured at 25 °C in 2 mM sodium phosphate, pH 7 containing NaCl. The error limits are ±15%.

occur.[6] Hazzard et al. also reported that cytochrome *c* reacted with the heme Fe(IV) in CMPI at low ionic strength using a flavin flash photolysis technique,[7] but no conversion of CMPII-(III,R•) to CMPII(IV,R) was observed.[8,9] The apparent difference in mechanism at low ionic strength could arise from a different binding orientation for ferrocytochrome *c* that would favor electron transfer to the oxyferryl heme Fe(IV). The great sensitivity of the binding orientation to ionic strength and redox state is illustrated by recent binding[17] and crystallographic[2] studies.

Geren et al.[10] and Hahm et al.,[11] however, found that five different ruthenium-labeled horse and yeast cytochrome *c* derivatives obeyed reactions 1 and 2 under both low and high ionic strength conditions. For example, the reaction kinetics for (dicarboxybipyridine)(bisbipyridine)ruthenium-Lys-72-cytochrome *c* (Ru-72-cyt *c*[11]) are the same using the stopped-flow technique (Table I) as previously obtained using the ruthenium photoexcitation technique at this ionic strength.[11] The rate

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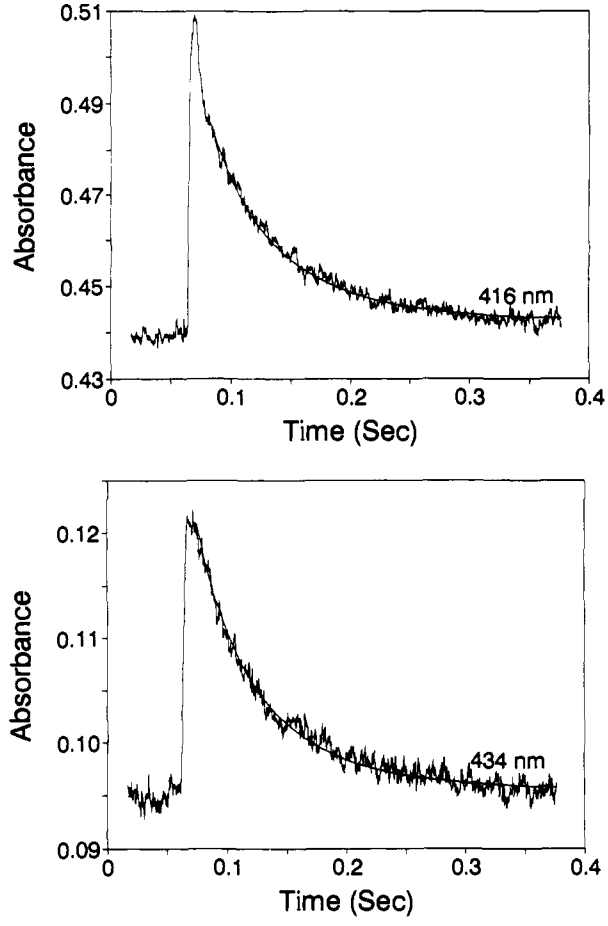

Figure 2. Ferrocytochrome *c* (2.2 μM) was mixed with CMPI (0.5 μM) under the same conditions as described for Figure 1. The solid line is the best fit of the second-order kinetic equation to the slow phase of the 416-nm transient (top) and the single phase of the 434-nm transient (bottom) with $k = 9 \times 10^6$ M^{-1} s^{-1}.

constants for both reactions are somewhat smaller than those of native cytochrome *c*, probably due to steric effects of the bulky ruthenium complex on the left side of the heme crevice. The photoexcitation technique was used to measure the reaction of Ru-72-cyt *c* at low ionic strength, where intracomplex electron transfer to the radical in CMPI is biphasic, with rate constants of 14 300 and 350 s^{-1}, respectively.[11]

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Supplementary Material Available: Experimental procedures and results; Table II, showing second-order rate constants for the reaction between ferrocytochrome *c* and cytochrome *c* peroxidase; Figure 3, showing the wavelength dependence of the reaction between ferrocytochrome *c* and excess CMPI (7 pages). Ordering information is given on any current masthead page.