Salt-Dependent Switch in the Pathway of Electron Transfer from Cytochrome *c* to Cytochrome *c* Peroxidase Compound I

Marites R. Nuevo, Hua-Hsien Chu, Lidia B. Vitello, and James E. Erman*

Department of Chemistry
Nothern Illinois University
DeKalb, Illinois 60115

Received March 8, 1993

Cytochrome *c* peroxidase (CcP) catalyzes the reduction of hydrogen peroxide to water using two reducing equivalents from ferrocytochrome *c*.[1] The catalytic mechanism involves the 2-equiv oxidation of the native Fe(III) enzyme to a higher oxidation state called CcP compound I.[2,3] The oxidized sites in CcP compound I (CcPI), an Fe(IV) heme and a free radical localized on Trp-191, are reduced back to their native states by sequential electron transfer from ferrocytochrome *c*. Transient-state studies of the reduction of CcPI by ferrocytochrome *c* show intriguing results. Flash photolysis studies (using flash-generated flavin semiquinone to reduce cytochrome *c*) show that the reduction of CcPI is strongly dependent upon ionic strength and that the Fe(IV) site is preferentially reduced at low ionic strength.[4] However, there appears to be plasticity in the mode of electron transfer to the oxidized sites in CcPI. Millett and co-workers, using ruthenium-modified cytochrome *c*s to generate the ferrocytochrome *c* state by flash photolysis, find that the radical site in CcPI is reduced prior to the Fe(IV) site.[5] Millett and co-workers have also reported that native horse ferrocytochrome *c* reduces the radical site prior to the Fe(IV) site in CcPI at 104 mM ionic strength (μ).[5c] These findings prompted us to measure the ionic strength dependence of the reduction of the Fe(IV) and radical sites in CcPI.

Our laboratory has been using stopped-flow studies to investigate the reduction of CcPI by ferrocytochrome *c*.[6] These studies differ from the flash photolysis work in that CcPI is fully reduced to the native state by excess ferrocytochrome *c*. We observe two pseudo-first-order rates, attributed to the sequential reduction of CcPI to CcP compound II (CcPII), followed by reduction of CcPII to CcP, eq 1. We have shown previously that at μ = 10

$$\text{CcPI} \xrightarrow{k_A} \text{CcPII} \xrightarrow{k_B} \text{CcP} \qquad (1)$$

mM the Fe(IV) site is preferentially reduced, resulting in CcPII with an Fe(III) heme and the oxidized Trp-191 radical.[6] The spectrum of this species, which we call CcP compound II_R (CcPIIR), is essentially identical to that of the native enzyme, Figure 1, since the radical site has little absorbance in the near-UV and visible region of the spectrum. We now report that at μ = 200 mM, the radical site is preferentially reduced, giving a compound II that retains the Fe(IV) site (CcPIIF), confirming the results of Hahm et al.[5c]

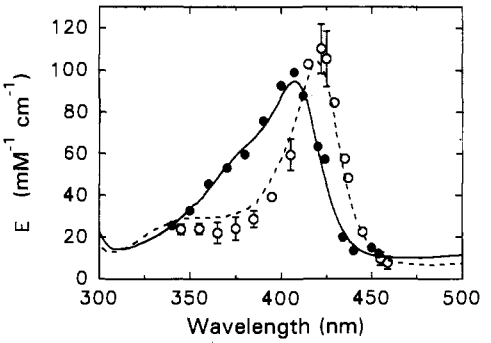

Figure 1. Spectrum of CcP compound II generated during the transient reduction of CcP compound I by horse ferrocytochrome *c*. ●, 4.2 mM phosphate, pH 7.5, 10 mM ionic strength (from ref 6a). ○, 10 mM phosphate, pH 7.5, 200 mM ionic strength with added KNO_3. —, spectrum of native Fe(III) CcP. - -, spectrum of CcP compound I.

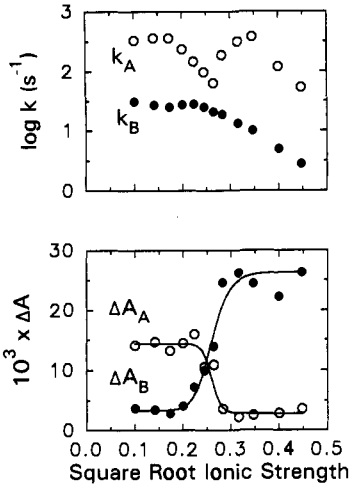

Figure 2. (Bottom panel) Absorbance change at 426 ± 2 nm for the conversion of CcP compound I to compound II (ΔA_A, ○) and the conversion of CcP compound II to CcP (ΔA_B, ●) as a function of the square root of ionic strength. (Top panel) The observed rate constants for the fast phase (k_A) and slow phase (k_B) of the reduction of 0.26 μM CcP compound I by 2.6 μM horse ferrocytochrome *c*. The buffer was 4.2–10 mM phosphate, pH 7.5, with added KNO_3 to adjust ionic strength.

The spectrum of CcPIIF is essentially identical to that of CcPI, Figure 1. The spectrum of CcPII at μ = 200 mM, pH 7.5, was determined from two independent experiments. In the first experiment, 0.50 μM CcPI was mixed with 5.0 μM horse ferrocytochrome *c*, and the absorbance changes were monitored at 16 different wavelengths between 345 and 459 nm. Two exponential phases were observed with values of k_A and k_B averaging 122 ± 15 and 5.14 ± 0.30 s^{-1}, respectively. The absorptivity of CcPII was calculated from the amplitude of the *slow* reaction phase, ΔA_B. The slow reaction phase was slow enough that 100% of the expected amplitude could be measured. In the second experiment, 0.50 μM ferrocytochrome *c* was mixed with excess CcPI (5.0 μM). A single exponential phase was observed at each of the 16 wavelengths monitored, with an average rate of 97 ± 13 s^{-1}. The absorptivity of CcPII was calculated from the amplitude of the single kinetic phase. The average values of the absorptivity determined from the two experiments are shown in Figure 1.

To determine where the transition between preferential reduction of the radical site and preferential reduction of the Fe(IV) site occurs, we have measured the amplitudes of the two kinetic phases as a function of ionic strength. Data from a series of experiments using 0.26 μM CcPI and 2.6 μM horse ferrocytochrome *c* are shown in Figure 2. The amplitudes of the two kinetic phases show a sharp transition between μ = 50 and 80

(1) Bosshard, H. R.; Anni, H.; Yonetani, T. In *Peroxidases in Chemistry and Biology*, Vol. *II*; Everse, J., Everse, K. E., Grisham, M. B., Eds.; CRC Press: Boca Raton, FL, 1990; pp 51–84.
(2) Jordi, H. C.; Erman, J. E. *Biochemistry* **1974**, *13*, 3734–3741.
(3) Coulson, A. F. W.; Erman, J. E.; Yonetani, T. *J. Biol. Chem.* **1971**, *246*, 917–924.
(4) (a) Hazzard, J. T.; Poulos, T. L.; Tollin, G. *Biochemistry* **1987**, *26*, 2836–2848. (b) Hazzard, J. T.; Moench, S. J.; Erman, J. E.; Satterlee, J. D.; Tollin, G. *Biochemistry*, **1988**, *27*, 2002–2008. (c) Hazzard, J. T.; McLendon, G.; Cusanovich, M. A.; Tollin, G. *Biochem. Biophys. Res. Commun.* **1988**, *151*, 429–431.
(5) (a) Geren, L.; Hahm, S.; Durham, B.; Millett, F. *Biochemistry* **1991**, *30*, 9450–9457. (b) Hahm, S.; Durham, B.; Millett, F. *Biochemistry* **1992**, *31*, 3472–3477. (c) Hahm, S.; Geren, L.; Durham, B.; Millett, F. *J. Am. Chem. Soc.* **1993**, *115*, 3372–3373.
(6) (a) Summers, F. E.; Erman, J. E. *J. Biol. Chem.* **1988**, *263*, 14267–14275. (b) Erman, J. E.; Kang, D. S.; Kim, K. L.; Summers, F. E.; Matthis, A. L.; Vitello, L. B. *Mol. Cryst. Liq. Cryst.* **1991**, *194*, 253–258.

0002-7863/93/1515-5873$04.00/0

mM. Above μ = 80 mM, the small ΔA_A indicates that only absorbance changes related to oxidation of ferrocytochrome *c* contribute to the amplitude of the fast phase, while the reduction of the Fe(IV) site in CcPI and the cytochrome *c* absorbance changes contribute to ΔA_B. We observe essentially 100% of the expected absorbance change for the reaction above μ = 80 mM.

Below μ = 50 mM, the Fe(IV) site of CcPI is preferentially reduced, followed by reduction of the radical site in the slow phase of the reaction. We observe only about 65% of the expected amplitude for ΔA_A due to partial reaction of the fast phase during the deadtime of the stopped-flow instrument. The value of k_A is 350 ± 35 s^{-1} between 10 and 30 mM ionic strength. The observed rate constants are shown in Figure 2. In the transition region between μ = 40 and 80 mM, k_A shows anomalous behavior. The values of k_A decrease from 350 s^{-1} at low ionic strength to a value of 64 ± 7 s^{-1} at μ = 70 mM, followed by an increase back to 320 ± 30 s^{-1} at μ = 100 mM. Above μ = 100 mM, k_A decreases with increasing ionic strength, reaching a value of 54 ± 5 s^{-1} at μ = 200 mM.

Correlation of the data presented here with the crystallographic structures of the yeast cytochrome *c*–CcP complex[7] obtained at high ionic strength and of the horse cytochrome *c*–CcP complex[7] obtained at low ionic strength is interesting. In the yeast cytochrome *c*–CcP complex crystallized from high ionic strength buffer, heme methyl-3 of cytochrome *c* is nestled between the two methyl groups of Ala-193 and Ala-194 of CcP. This interaction gives a short electron-transfer pathway from heme methyl-3 of cytochrome *c* to Trp-191, the radical site of CcP compound I, via residues Gly-192 and Ala-193. If the horse cytochrome *c*–CcP complex at high ionic strength resembles that of the high ionic strength yeast cytochrome *c*–CcP complex, reduction of Trp-191 prior to the Fe(IV) site in CcP would be expected. The horse cytochrome *c*–CcP complex crystallized at low ionic strength has a slightly different orientation of the two redox proteins. Heme methyl-3 of cytochrome *c* is rotated away from the Ala-193, Ala-194 interface of CcP, and there is more intimate contact between Glu-35, Asn-38, and Glu-290 of CcP with Lys-87, Lys-8, and Lys-72 of horse cytochrome *c*, respectively. The electron-transfer pathway in this complex may proceed via the distal side of the heme rather than the proximal side, resulting in preferential reduction of the Fe(IV) site at low ionic strength. The switch between preferential reduction of the Fe(IV) site to the radical site is probably controlled by the orientation of the two proteins in the complex. At low ionic strength the orientation is dominated by the electrostatic forces, and at high ionic strength the orientation is dominated by the hydrophobic interactions.

Acknowledgment. This investigation was supported in part by research grant NSF MCB-9121414.

(7) Pelletier, H.; Kraut, J. *Science* **1992**, *258*, 1748–1755.