Direct Measurement of the Reduction Potential of Catalytically Active Cytochrome *c* Peroxidase Compound I: Voltammetric Detection of a Reversible, Cooperative Two-Electron Transfer Reaction

Madhu S. Mondal, Helen A. Fuller, and Fraser A. Armstrong*

Inorganic Chemistry Laboratory, Oxford University South Parks Road, Oxford OX1 3QR, U.K.

Received July 25, 1995

Yeast cytochrome *c* peroxidase (C*c*P)[1] catalyzes the reduction of hydrogen peroxide to water by cytochrome *c*(II) in a cycle of processes that have attracted intense and varied interest directed toward developing an understanding of biological electron transfer.[2] As depicted in Scheme 1, the mechanism involves two-electron oxidation of the Fe(III) "resting" form of the enzyme to a form known as C*c*P compound I (or "ES"), which contains oxyferryl heme (Fe^{IV}═O) and a radical located on the indole ring of Trp-191.[3] The resting form is regenerated by two sequential one-electron reactions via a form known as C*c*P compound II. Compound II can exist in at least two forms, [Fe(IV): Trp^0] and [Fe(III): Trp^+] depending upon pH, ionic strength, and the presence of ligands that favor Fe(III).[4,5] However, only [Fe(IV): Trp^0] has been isolated and characterized in detail.

In view of the importance of understanding the mechanism and associated energetics of this system, it is surprising that little information has emerged on the reduction potentials of the catalytically relevant states shown in Scheme 1, and then only via indirect methods.[6] From kinetic data and Marcus theory, the reduction potential of compound II was calculated to be +1.09 V.[7] For the couple $Trp^{+/0}$, a value of 0.65 V was estimated by electrostatic calculations of the degree by which the radical is stabilized in the protein compared to being free in solution.[8]

Cytochrome *c* peroxidase adsorbs at a pyrolytic graphite "edge" (PGE) electrode to produce an extremely active surface for reduction of hydrogen peroxide.[9] It was found that, for low concentrations of peroxide, a peak at +750 mV was obtained for reduction at stationary electrodes.[9a] Using a microelectrode array model for electrocatalysis by adsorbed enzymes, it was concluded that a dense layer of active catalysts must be present to account for the sharp, peak-like catalytic waveform.[9d] Since such a high coverage should be detectable by voltammetry without amplification from turnover, we searched for conditions under which a rapid and reproducible response could be elicited.

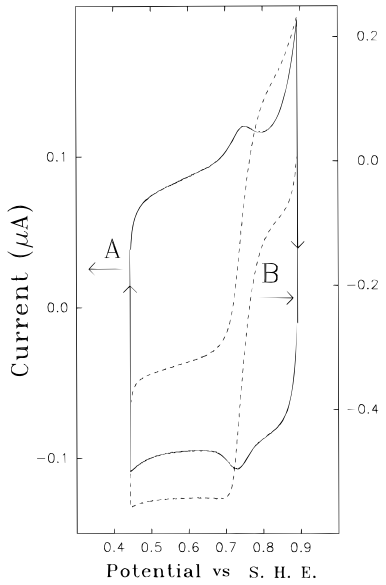

Figure 1. (a) Cyclic voltammogram obtained several cycles after introducing a freshly polished PGE electrode to a 0.7 μM solution of C*c*P in 20 mM phosphate buffer, pH 6.1 at 4 °C: electrode surface area, 0.03 cm^2; scan rate, 20 mV s^{-1}. The voltammogram appears over the course of several scans after a potential hold for 30 s at +600 mV. The signal remains unchanged upon rotation of the electrode at 500 rpm. The broken line (B) represents the voltammetric response at the same C*c*P-adsorbed rotating-disk PGE electrode upon addition of 20 μM H_2O_2 at 400 rpm. Scan rate was 20 mV s^{-1}. The noticeable ridge in the catalytic reduction current at ca. 710 mV stems from the very high rate of turnover of the adsorbed enzyme, which for low substrate concentrations prevents the steady state from being reached, even at high rotation rates.

Scheme 1

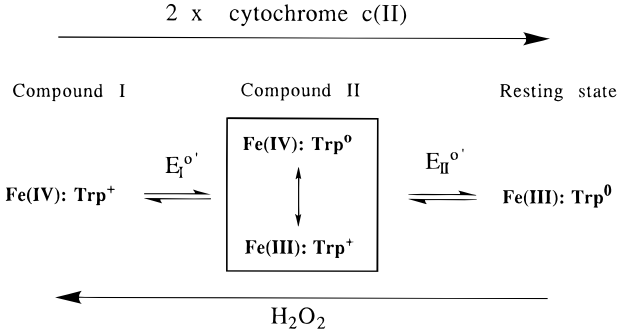

Figure 1 shows the result obtained when a freshly polished PGE electrode is introduced to a 0.7 μM solution of C*c*P in 20 mM phosphate buffer, at 4 °C.[10] Symmetrical peak-like signals, with reduction potential +740 mV, appear over the course of several cycles and are unchanged when the electrode is rotated

(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. Poulos, T.; Fenna, R. In *Metal Ions in Biological Systems, Vol. 30*, Sigel, H., Sigel, A., Eds.; Marcel Dekker Inc.: New York, 1994; pp 25−75.

(2) See, for example: Stemp, E. D.; Hoffman, B. *Biochemistry* **1993**, *32*, 10848−10865. Hahm, S.; Miller, M. A.; Geren, L.; Kraut, J.; Durham, B.; Millett, F. *Biochemistry* **1994**, *33*, 1473−1480. Wang, Y.; Margoliash, E. *Biochemistry* **1995**, *34*, 1948−1958.

(3) Sivaraja, M.; Goodin, D. B.; Smith, M.; Hoffman, B. M. *Science* **1989**, *245*, 738−740. Erman, J. E.; Vitello, L. B.; Mauro, J. M.; Kraut, J. *Biochemistry* **1989**, *28*, 7992−7995.

(4) Coulsen, A. F. W.; Erman, J. E.; Yonetani, T. *J. Biol. Chem.* **1971**, *246*, 917−924.

(5) Ho, P. S.; Hoffman, B. M.; Solomon, N.; Kang, C. H.; Margoliash, E. *Biochemistry* **1984**, *23*, 4122−4128.

(6) By contrast the reduction potentials of the noncatalytic redox couple Fe(III)/Fe(II) are well-established. See, for example: Goodin, D. B.; McRee, D. E. *Biochemistry* **1993**, *32*, 3313−3324.

(7) Purcell, W. L.; Erman, J. E. *J. Am. Chem. Soc.* **1976**, *98*, 7033−7037.

(8) Miller, M. A.; Han, G. W.; Kraut, J. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 11118−11122.

(9) (a) Armstrong, F. A.; Lannon, A. M. *J. Am. Chem. Soc.* **1987**, *107*, 7211−7212. (b) Paddock, R. M.; Bowden, E. F. *J. Electroanal. Chem.* **1989**, *260*, 487−494. (c) Scott, D. L.; Paddock, R. M.; Bowden, E. F. *J. Electroanal. Chem.* **1992**, *341*, 307−321. (d) Armstrong, F. A.; Bond, A. M.; Büchi, F. N.; Hamnett, A.; Hill, H. A. O.; Lannon, A. M.; Lettington, O. C.; Zoski, C. G. *Analyst* **1993**, *118*, 973−978. (e) Scott, D. L.; Bowden, E. F. *Anal. Chem.* **1994**, *66*, 1217−1223.

(10) Bakers' yeast cytochrome *c* peroxidase was isolated according to the method of English et al. (English, A. M.; Laberge, M.; Walsh, M. *Inorg. Chim. Acta* **1986**, *123*, 113−116) and then crystallized and stored under liquid N_2. The enzyme gave a turnover number of 2810 s^{-1} (average of five assays) at 25 °C, and pH 7.0, and 15 μM cytochrome *c* (II) and saturating levels of H_2O_2. For comparison, at pH 6.0 and 23 °C, Yonetani and Ray obtained a turnover number of 2500 s^{-1} (Yonetani, T.; Ray, G. S. *J. Biol. Chem.* **1965**, *240*, 4503−4508). The electrochemical cell and electrodes have been described previously (see ref 16). Voltammetry was performed using an Autolab Electrochemical Analyzer (EcoChemie, Utrecht, Netherlands).

0002-7863/96/1518-0263$12.00/0

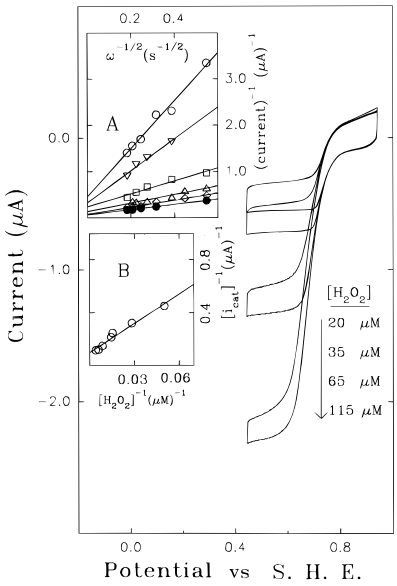

Figure 2. Variation of the catalytic current−potential response at the C*c*P-adsorbed rotating-disk PGE electrode as a function of H_2O_2 concentration: rotation rate, 400 rpm; scan rate, 20 mV s^{-1}; other conditions as for Figure 1. Inset A shows the Koutecky−Levich plot (see ref 16). The lines shown are the best fits to data obtained at various rotation rates and represent experiments at 20, 35, 65, 115, 170, and 242 μM H_2O_2. The currents due to intrinsic catalytic properties of the enzyme are determined from the inverse intercepts of these lines. Inset B shows the double reciprocal plot of the catalytic currents (obtained from the intercepts) at various H_2O_2 concentrations. The line drawn is the best fit to the data points giving $K_M = 92$ μM and $k_{cat} = 268$ s^{-1}.

at 500 rpm, thus showing that the species responsible is bound to the electrode surface.[11] The small peak separation (<20 mV at 20 mV s^{-1}) shows that interfacial electron transfer is facile. Continuing rotation and introducing even just a low level of H_2O_2 causes the peaks to convert to sigmoidal waveforms characteristic of steady-state catalysis. Thus effectively all of the redox species contributing to the reversible signals are catalytically active.

That the peak-like signals arise from a *cooperative two-electron* couple is clear from the half-height widths δ, which lie between 50 and 60 mV. Theory predicts that a one-electron reaction of a surface-confined population of non-interacting molecules should have $\delta = 85$ mV at 4 °C.[12] Nonidealities such as intermolecular Coulombic interactions or dispersion of species over a spectrum of reduction potentials would cause δ to increase, not decrease.[13] Thus, ruling out intermolecular cooperativity between the redox centers in these large molecules (MW 34 000), the decrease in δ (toward the two-electron value of 42 mV) for both peaks must be associated with cooperative transfer of more than one electron. We conclude that the reaction observed is the reversible two-electron interconversion between resting C*c*P and compound I *without the need for* H_2O_2, and (as indeed suggested by others) that this system behaves as a *cooperative* redox couple, in which oxidations of Trp-191 and the heme group are mutually supportive.[14,15]

The significance of this result is reinforced by a kinetic analysis of the electrocatalytic reduction of H_2O_2, which shows the species involved to be extremely active even at this temperature. After generation of the peak-type signals, varying concentrations of H_2O_2 were added, and the limiting catalytic currents were measured at different rotation rates. Results are shown in Figure 2. Data were analyzed according to the model used by Sucheta et al.[16] The coverage of enzyme molecules was determined as 6.2×10^{-12} mol cm^{-2} by base-line-corrected integration of peaks, assuming a two-electron reaction. This value equates to an area equivalent to a square 51 Å × 51 Å for each enzyme molecule, consistent with a coverage that is probably close to an idealized monolayer. From the turnover data and coverage, the kinetic constants $K_M = 92$ μM and $k_{cat} = 268$ s^{-1} were obtained. Therefore $k_{cat}/K_M = 2.9 \times 10^6$ M^{-1} s^{-1}.[17]

The catalytically relevant, macroscopic reduction potentials at pH 6.1 and 4 °C are thus partially quantified as $E_I^{\circ\prime} \leq 740$ mV $\leq E_{II}^{\circ\prime}$, with $E_{I/II}^{\circ\prime}$ for the overall two-electron reaction being 740 mV. It should be noted that the equal potential situation $E_I^{\circ\prime} \sim E_{II}^{\circ\prime}$ also yields a δ value below 83 mV if the two electron transfers are tightly coupled (an extreme example being electrons adding to a single center).[18] However, the individual $E^{\circ\prime}$ values cannot be determined reliably from the data, since the levels of nonideality (dispersion or interactions) which might increase δ from the limiting $2e^-$ value of 42 mV have not been assessed,[19] nor can the partitioning of compound II between its two alternative forms[4] be defined from the voltammetric experiment.

The experiments that we have conducted are rapid, economical, and sensitive, and have the capability of bridging the complicated divisions existing between thermodynamics, transient kinetics, and steady-state kinetics. Detailed studies of the reversible active-site transformations and the correlation with catalytic activity are now underway.

Acknowledgment. This work was supported by the UK Engineering and Physical Sciences Research Council. We thank Judy Hirst and Dirk Heering for experimental assistance and helpful discussions.

JA952489F

(11) No signals were observed in the absence of cytochrome *c* peroxidase. As the peaks developed, there was a small but noticeable decrease in the background capacitance, consistent with adsorption of the enzyme. The coated electrode could be transferred to a cell containing buffer solution devoid of enzyme, whereupon the peaks diminished in intensity but remained visible for at least 20 min. Peak currents were proportional to scan rate up to at least 250 mV s^{-1}.

(12) Laviron, E. In *Electroanalytical Chemistry, Vol. 12*. Bard, A. J., Ed.; Marcel Dekker: New York, 1982; pp 53−157. Laviron, E. *J. Electroanal. Chem.* **1979**, *97*, 135−149.

(13) Laviron, E. *J. Electroanal. Chem.* **1974**, *52*, 395−402. Matsuda, H.; Aoki, K.; Tokuda, K. *J. Electroanal. Chem.* **1987**, *217*, 1−13.

(14) Electron-transfer cooperativity accounts for the prominence of the voltammetric waves, since current is proportional to n^2 (see refs 12 and 13).

(15) Liu, R.-Q.; Miller, M. A.; Han, G. W.; Hahm, S.; Geren, L.; Hibdon, S.; Kraut, J.; Durham, B.; Millett, F. *Biochemistry* **1994**, *33*, 8678−8685.

(16) Sucheta, A.; Cammack, R.; Weiner, J.; Armstrong, F. A. *Biochemistry* **1993**, *32*, 5455−5465.

(17) Experiments conducted at pH 5 showed that the non-catalytic reduction potential increases to approximately +780 mV and the wave remains sharp, while at pH 7, the potential decreases to approximately +690 mV and the waves appear slightly broader. Introduction of H_2O_2 in either case induced turnover with potentials shifted in respective directions but with slightly lower currents than observed with the same concentration of H_2O_2 at pH 6.1.

(18) Plichon, V.; Laviron, E. *J. Electroanal. Chem.* **1976**, *71*, 143−156.

(19) There is substantial evidence against disproportionation of compound II. See refs 5 and 15: Thus it is unlikely that $E_{II}^{\circ\prime}$ is very much higher than $E_I^{\circ\prime}$.