Variable-Temperature Spectroelectrochemical Study of Horseradish Peroxidase†

Z. Shadi Farhangrazi,§ Martin E. Fossett,‖ Linda S. Powers,*,§ and Walther R. Ellis, Jr.*,‖

National Center for the Design of Molecular Function, Utah State University, Logan, Utah 84322, and Department of Chemistry, University of Utah, Salt Lake City, Utah 84112

Received August 30, 1994; Revised Manuscript Received November 1, 1994®

ABSTRACT: The reduction potentials of the compound II/ferric and compound I/compound II couples have been studied, using potassium hexachloroiridate as a mediator titrant, by thin-layer spectroelectrochemistry. Compound I, which is 2 equiv more oxidized than the ferric (*i.e.*, resting) form of the enzyme, was reversibly formed via a compound II intermediate; no evidence for a ferric porphyrin π-cation radical intermediate was obtained. At 25 °C, $E°'$(compound I/compound II) = 897.9 ± 3 mV (NHE) and $E°'$(compound II/ferric) = 869.1 ± 2 mV. Redox thermodynamic parameters, obtained from the temperature dependences of the reduction potentials of both couples, are reported. The reaction entropies ($\Delta S°_{rc}$) for the compound II/ferric and compound I/compound II couples are 19.8 ± 3.9 and 12.1 ± 3.7 eu, respectively. This result indicates that the reorganization energy for the macrocycle-centered couple is lower than that for the metal-centered one. Together with our observation that $E°'$ for the former is *ca.* 30 mV greater than that for the latter, these results suggest that compound I is more reactive toward outer-sphere reductants than compound II. In particular, the electron self-exchange rates for the compound I/compound II and compound II/ferric couples are estimated to be 4.4×10^{-1} and 4.9×10^{-4} M^{-1} s^{-1}, respectively. Surprisingly, the formation of compound I from ferric HRP is accompanied by an almost zero standard entropy ($\Delta S°'$) change.

Horseradish peroxidase (HRP[1]) is one of the most intensively studied members of a family (Everse *et al.*, 1991) of heme enzymes that catalyze the oxidation of a variety of reductants by hydrogen peroxide. Due to its intense chromophore and ease of isolation, HRP has played a prominent role (Michaelis & Menten, 1913) in the progress of enzymology, including the early debate concerning the nature of enzymes as biological catalysts. This enzyme continues to attract attention because of widespread interest in the structures and reactivities of oxidized intermediates, containing ferryl iron, that also appear during the turnovers of other heme enzymes, including cytochrome P-450, prostaglandin H synthase, cytochrome *c* oxidase, and catalase (Naqui & Chance, 1986). HRP is additionally of biotechnological interest due to its common use (Tijssen & Kurstak, 1984; Frew *et al.*, 1986; Maidan & Heller, 1991; Garguilo *et al.*, 1993) in biosensors based on colorimetric or amperometric detection.

A variety of intermediates have been detected during the last 5 decades. Two of these, termed compounds I and II, have been the subjects of numerous spectroscopic and kinetic studies. When taken together, these experiments clearly indicate that steps involving single-electron transfers operate during the turnover of HRP. Compound I is a green intermediate that is formed from the reaction of the resting (*i.e.*, ferric) enzyme with hydrogen peroxide. Spectroscopic data, including electronic absorption (Dolphin *et al.*, 1971), Mössbauer (Schulz *et al.*, 1984), ENDOR (Roberts *et al.*, 1981a,b), and EXAFS (Chance *et al.*, 1984), indicate that compound I contains a ferryl (*i.e.*, tetravalent) iron weakly spin-coupled to a porphyrin π-cation radical. Thus, compound I is 2 equiv more oxidized than the resting enzyme. Compound I subsequently decays to a red intermediate, compound II, which contains a ferryl iron center. In both compounds I and II, the tetravalent oxidation state is stabilized by an axial oxo ligand that is absent in the resting enzyme. (The other axial ligand, histidine, is present in all forms of the enzyme.) During turnover, reducing substrates decompose compound I to compound II, which, in turn, decays to the ferric resting enzyme.

Compound II is less oxidized than compound I but more oxidized than the ferric enzyme, as indicated in Figure 1. A second potential intermediate having the same level of oxidation as compound II but containing a ferric porphyrin π-cation radical is not observed during enzyme turnover. However, this species has been identified (Kaneko *et al.*, 1980) as the product of the oxidation of Zn-substituted HRP. The issue of metal- vs ring-centered oxidations of heme enzymes (and metalloporphyrin complexes) is an important one. In particular, work (Calderwood *et al.*, 1985) on iron(III) porphyrin model complexes suggests that the presence of strongly basic axial ligands can lead to oxidation of the ferric center to a ferryl one prior to oxidation of the porphyrin ring.

Early suggestions (George, 1953; Fergusson, 1956) that the *in vivo* oxidant H_2O_2 could potentially be replaced by outer-sphere inorganic oxidants were amplified in a report (Hayashi & Yamazaki, 1979) that potassium hexachloroiridate(IV) turns over HRP, producing compound I and compound II intermediates. More importantly, two revers-

† This work was supported by NIH Research Resource Grant 5P41 RR06030 (L.S.P.) and NIH Grant GM43507 (W.R.E.).

* To whom correspondence should be addressed.

§ Utah State University.

‖ University of Utah.

® Abstract published in *Advance ACS Abstracts,* February 15, 1995.

[1] Abbreviations: HRP, horseradish peroxidase; ENDOR, electron nuclear double resonance; EXAFS, extended X-ray absorption fine structure; NHE, normal hydrogen electrode; OTTLE, optically transparent thin-layer electrode; $E°'$, reduction potential at 25 °C and pH 7.0.

0006-2960/95/0434-2866$09.00/0

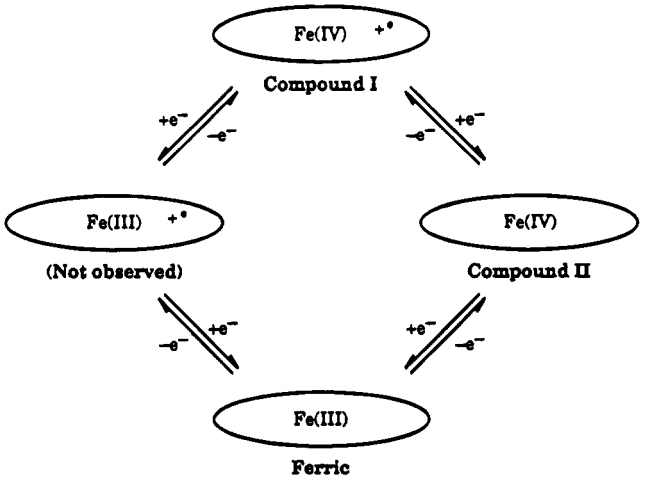

FIGURE 1: Reaction scheme illustrating the interconversion between the ferric "resting" and compound I forms of HRP via pathways involving one-electron redox reactions. Only one species possessing an intermediate level of oxidation, compound II, was observed in these experiments. For simplicity, only the oxidation states of the iron and porphyrin ring are indicated.

ible interconversions were identified, compound II/ferric and compound I/compound II. The $K_2IrCl_6-K_3IrCl_6$ system has also been used to obtain reduction potentials for these couples in *Arthromyces ramosus* peroxidase (Farhangrazi *et al.*, 1994).

In this report, we present the results of controlled potential electrolyses of HRP using an OTTLE cell for detection of intermediates by electronic absorption spectroscopy. Redox thermodynamic parameters for the compound II/ferric and compound I/compound II couples are determined from measurements of the reduction potentials as a function of temperature. A surprising conclusion, in view of the available physical data for these forms of the enzyme, is that the formation of compound I from resting HRP is accompanied by an almost zero standard entropy change.

MATERIALS AND METHODS

Materials. HRP isoenzyme C was obtained from Toyobo Co. (Osaka, Japan) and used without further purification. This preparation exhibited an A_{403}/A_{280} ratio (typically referred to as the RZ value) of 3.0 and was greater than 95% pure as determined by isoelectric focusing and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Potassium hexachloroiridate(III), a redox mediator titrant ($E^{\circ\prime}$ = 877 mV vs NHE in the solution described below) in these experiments, was used as received from Aldrich. All other reagents were of the best analytical grade available. Enzyme/mediator solutions in 0.1 M potassium phosphate buffer, pH 7.0, were freshly prepared for each titration. An equimolar ratio of mediator/HRP was used with a final concentration of each being *ca.* 100 μM. This mediator concentration was chosen to circumvent problems with correction for spectral interference; $IrCl_6^{3-}$ is colorless in the 340–575 nm range examined in this study, and $IrCl_6^{2-}$ contributes less than 2% to the absorbance in the Soret region. Solutions were degassed by repeated cycles of gentle evacuation, followed by flushing with purified Ar, on a vacuum manifold.

Spectroelectrochemistry. Controlled potential titrations were carried out in an OTTLE cell as described in Taniguchi *et al.* (1982), except that the cell was maintained in an isothermal configuration. The three-electrode configuration consisted of a gold mesh working electrode (Buckbee-Mears Co., Minneapolis, MN), a Bioanalytical Systems Model RE-4 Ag/AgCl(3 M NaCl) miniature reference electrode, and a Pt wire counter electrode. The Ag/AgCl reference electrode was calibrated against a saturated calomel electrode (used for standardization purposes only) prior to each experiment. All potentials reported herein are referenced versus the NHE and were corrected for the temperature dependence of the Ag/AgCl reference electrode potential. Potentials were applied across the OTTLE cell with a Hi-Tek Instruments (Southampton, U.K.) Model DT2101 potentiostat and measured (±0.1 mV) using a Keithley Model 177 digital multimeter. Constant temperature was maintained by a Forma Scientific Model 2006 circulating water bath, and the OTTLE cell temperature was monitored with a Beckman Model 461 digital thermometer connected to a Cu-constan microthermocouple (Omega Engineering, Inc., Los Angeles, CA). UV/visible spectra were recorded using a computer-interfaced Cary 2215 spectrophotometer. Data were col-

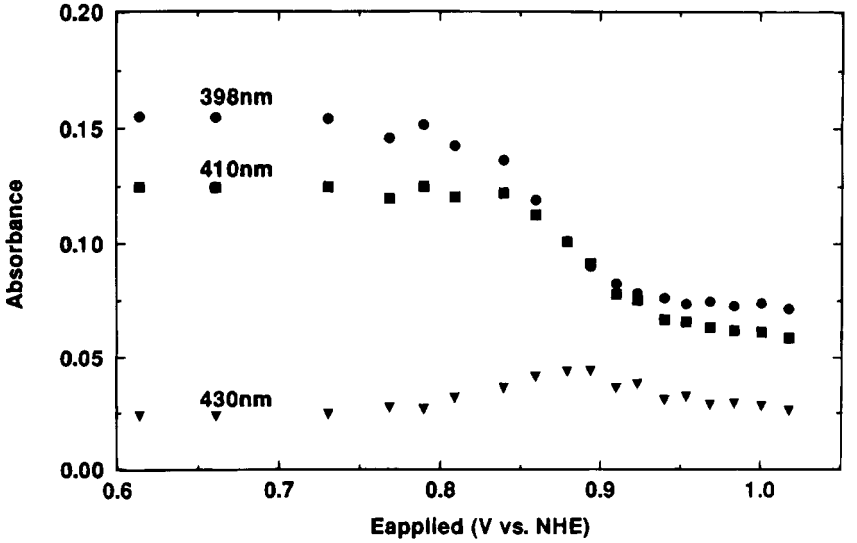

FIGURE 2: Absorbance changes obtained during a controlled potential titration of HRP at 24.9 °C. At 398 nm, the resting enzyme is observed to decay at increasing potentials. The formation of compound I at increasing potentials is indicated by a decrease in absorbance at 410 nm. The 430 nm points indicate the formation and decay of compound II.

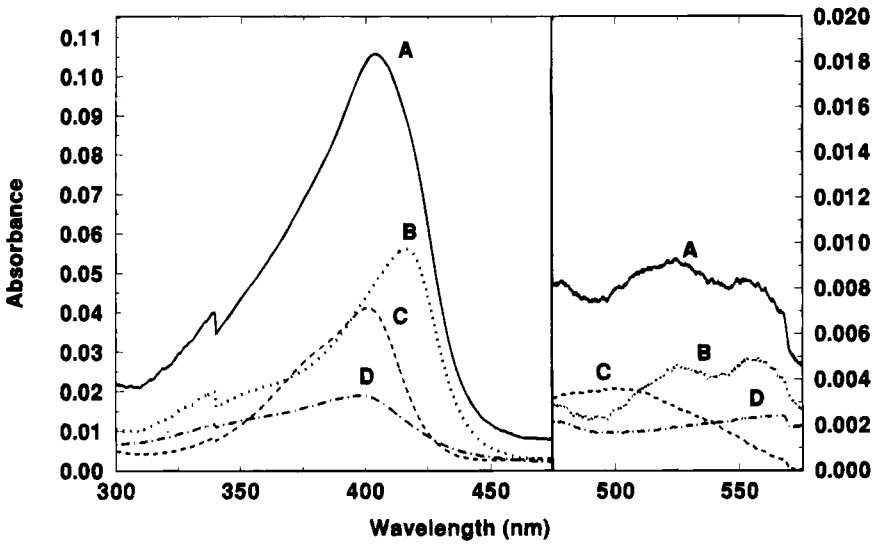

FIGURE 3: Deconvolution of the 24.9 °C absorbance spectrum (A) obtained at $E_{applied}$ = 879.2 mV. Three components are present, B, compound II; C, ferric HRP; and D, compound I. The discontinuity at 340 nm is due to a lamp change.

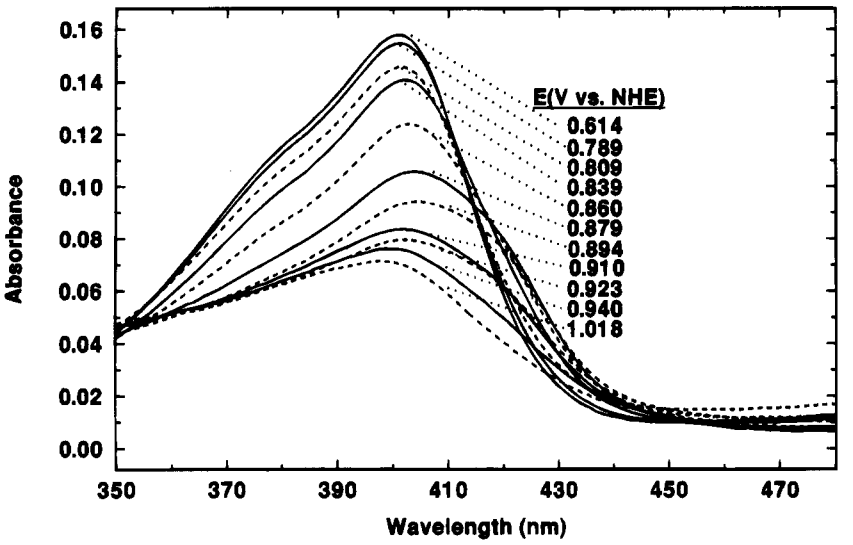

FIGURE 4: Selected overlay UV/visible spectra obtained during the titration of Figure 2. Solid lines: spectra obtained during oxidation of resting HRP to compound I. Dashed lines: spectra obtained during rereduction of compound I.

lected and processed using SpectraCalc (Galactic Industries, Salem, NH) software. Typically, 1−1.5 h was required for equilibration after each potential step; an entire spectroelectrochemical titration therefore required 20−24 h.

Data Analysis. The redox states of ferric, compound I, and compound II HRP were monitored at 398, 410, and 430 nm, respectively; these wavelengths are isosbestic points for the other two species not measured (Hayashi & Yamazaki, 1979). All Nernst plots contain a minimum of six data points. Weighted linear fits to plots of reduction potentials for the compound II/ferric and compound I/compound II couples vs temperature yielded $dE°'/dT$ values, from which $\Delta G°'$, $\Delta H°'$, and $\Delta S°'$ parameters were calculated. Errors in fits to the data were calculated as outlined in Barlow (1989).

RESULTS

Absorbance changes at three isosbestic wavelengths, obtained during a spectroelectrochemical titration at 24.9 °C, are shown in Figure 2. Both oxidative and reductive data points are shown, demonstrating good reversibility for the entire titration. The visible spectrum (spectrum A in Figure 3) obtained at $E_{applied}$ = 879.2 mV contains a component (spectrum B) that displays the α- and β-bands (λ = 525 and 557 nm) expected for compound II. Deconvolution of spectrum A in Figure 3 reveals a total of three components: B, compound II; C, ferric HRP; and D, compound I. Upon inspection of spectra obtained at other applied potentials, it is clear that compound II builds up and then decays as the applied solution potential is increased. No evidence was obtained for an additional species possessing a porphyrin π-cation radical (other than compound I). Figure 4 displays overlay UV/visible absorbance spectra for a subset of the potentials applied. As the potential is increased, the Soret maximum decreases markedly, as expected for the generation of compound I. Nernst plots of the absorbance changes at 398 and 410 nm, set out in Figure 5, were used to compute the reduction potentials of the compound II/ferric (869.1 ± 1.5 mV vs NHE; n = 1.1) and compound I/compound II (897.9 ± 2.8 mV vs NHE; n = 1.1) couples, respectively. Attempts to obtain pH dependences of the potentials were

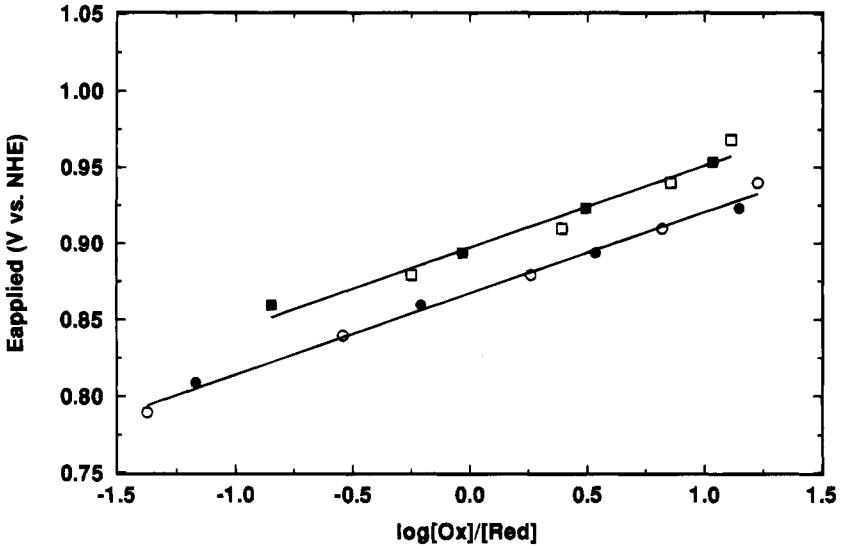

FIGURE 5: Nernst plots of the absorbance changes measured in Figure 4. Upper plot: compound I/compound II couple; lower plot, compound II/ferric couple. Open symbols: oxidative points. Filled symbols: points obtained during rereduction.

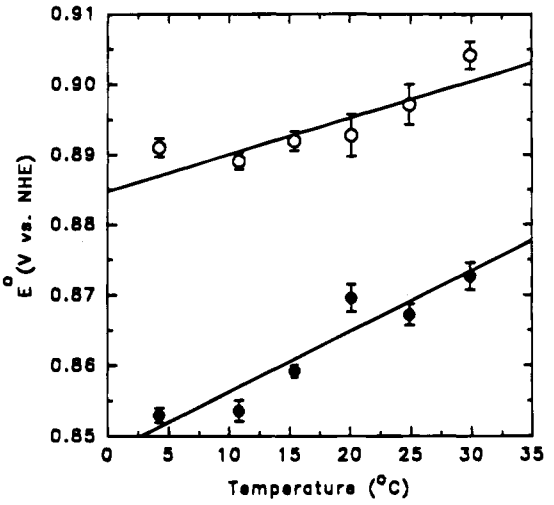

FIGURE 6: Temperature dependences of reduction potentials. Upper plot: compound I/compound II couple, $dE°/dT = 5.24 \pm 1.59 \times 10^{-4}$ V/°C. Lower plot: compound II/ferric couple, $dE°/dT = 8.57 \pm 1.67 \times 10^{-4}$ V/°C.

frustrated by the instability of spectra of either compound I (at pH > 8) or compound II (at pH < 6).

Figure 6 graphically summarizes the results of experiments carried out over the 4.2−29.9 °C range. Nernst plots for the indicated temperatures were linear. However the n values deviate from unity at the extremes of the temperature range studied; in particular, $n = 0.9$ at 29.9 °C and progressively increases to $n = 1.3$ at 4.2 °C. Careful inspection of the temperature dependence of the electronic absorption spectrum (not shown) of the resting enzyme indicates that there is a distribution of high-spin species that gradually changes in this temperature interval.

Weighted linear fits to the data in Figure 6 were used to calculate reaction entropies ($\Delta S°_{rc} = nF(dE°/dT)$) for the two redox couples. The reaction entropy for the metal-centered oxidation is clearly more positive (Table 1) than that of the porphyrin-centered oxidation. Standard entropies of reduction, obtained by referencing the reaction entropies to the NHE, together with the enthalpies of reduction, are also set out in Table 1.

Table 1: Thermodynamic Parameters for HRP Couples

parameter	compd II/ferric	compd I/compd II
$\Delta S°_{rc}$ (eu)	19.8 ± 3.9	12.1 ± 3.7
$\Delta G°'$ (kcal/mol)[a]	−(20.04 ± 0.05)	−(20.71 ± 0.05)
$\Delta S°'$ (eu)[b]	4.2 ± 3.9	−(3.5 ± 3.7)
$\Delta H°'$ (kcal/mol)[a]	−(18.8 ± 1.2)	−(21.2 ± 1.2)

[a] At 25.0 °C. [b] $\Delta S°' = \Delta S°_{rc} - 15.6$ eu. This correction references the reaction entropies to the NHE.

DISCUSSION

While a ferric porphyrin π-cation radical-containing HRP intermediate does not build up to a sufficiently high concentration to be observed in these experiments, it has been demonstrated (Kaneko *et al.*, 1980) that such a species can be produced by one-electron oxidation of Zn-substituted HRP. The reported reduction potential for this couple is 740 mV vs NHE, suggesting that the presence of a more positively charged iron center in native HRP should increase the potential for the porphyrin-centered couple. Our results indicate that the presence of a ferryl center raises the ring-centered reduction potential by 160 mV. One approach to the problem of a possible ferric porphyrin π-cation radical species produced from resting native HRP involves the study of anionic adducts, using cyanide or halides, in an effort to stabilize the trivalent oxidation state of the iron center. This issue is of potential relevance to the nature of the turnover of the native enzyme with hydrogen peroxide; in this inner-sphere electron-transfer process, the reduction potential for the compound II/ferric couple may be significantly greater than that reported here. If this is the case, it is possible that a heretofore uncharacterized ferric porphyrin π-cation radical intermediate (instead of compound II) is the predominant precursor to compound I.

A study (Hayashi & Yamazaki, 1979) of the HRP couples concluded that the reduction potential of the compound II/ferric couple is *ca.* 20−25 mV greater (in the pH 6.06−6.53 range) than that of the compound I/compound II couple, in contrast to an earlier report by George (1953) who tentatively concluded that the reduction potentials of the compound I/compound II and compound II/ferric HRP couples are approximately 1.3−1.6 and 1.0 V (NHE),

respectively. Our results indicate that compound I is a somewhat (30 mV) stronger oxidant than compound II. The observation that the ferryl center forms prior to the oxidation of the porphyhrin ring is in agreement with the work of Calderwood and co-workers (1985) on porphyrin model complexes. We believe that our results are more reliable than the previous ones for the following reasons: (1) we show (Figure 5) that our titrations are reversible, which was not demonstrated in either of the earlier studies; (2) Hayashi and Yamazaki (1979) indirectly determined HRP reduction potentials using oxidative and reductive *kinetic* measurements, which commonly lead to errors in equilibrium constants; (3) our experiments employed equimolar concentrations of HRP and potassium hexachloroiridate mediator, in contrast to the large excesses of either K_2IrCl_6 or K_3IrCl_6 (which could weakly bind to HRP and thereby shift the reduction potentials of interest here) used by Hayashi and Yamazaki (1979); and (4) the experiments of George (1953) only provide very approximate estimates of the potentials, due to the natures of the oxidants and reductants used.

Unlike situations involving the generation of compound I using chemical oxidants, such as H_2O_2 or K_2IrCl_6, this intermediate can be maintained for many hours using controlled potential electrolysis. Thus, variants (Heineman, 1978) of this experiment could be of value in characterizing other compound I-like intermediates, produced from various heme enzymes (including cytochrome P-450), that have eluded structural study. One interesting option involves EXAFS spectroelectrochemistry (Smith *et al.*, 1985; Igo *et al.*, 1991) to study structures of enzyme intermediates.

Our Nernstian n values typically displayed statistically significant deviations from unity. In the 4.2−29.9 °C temperature range, the electronic absorption spectrum of ferric HRP was observed to consist of a temperature-dependent distribution of Gaussian components. Evangelista-Kirkup *et al.* (1985) have noted that the resonance Raman spectrum of ferric HRP at 290 K contains porphyrin skeletal modes indicative of five-coordinate high-spin heme together with a six-coordinate high-spin component (due to addition of a distal water molecule). The microheterogeneous nature of the resting enzyme is, in our view, responsible for the deviation from ideal Nernstian behavior in our experiments.

Our thermodynamic results, presented in Table 1, indicate that conversion of resting HRP to compound I using outer-sphere oxidants is accompanied by a nearly zero change in the standard entropy associated with the enzyme. Stated alternatively, the partial molar entropies of these two species are nearly identical. In view of the available spectroscopic, particularly EXAFS, data for these forms of HRP, this finding is quite unexpected because of structural changes such as the addition of an oxo ligand. Water is a leaving group when compound II is reduced to ferric HRP, yielding a marginally positive entropy change that is much smaller than that expected for transfer of coordinated water (for which $S° \sim 0$) to bulk water ($S° = 16.72$ eu at 25 °C). Protonation of the oxo group most likely is responsible for the observed compound II → ferric HRP standard entropy change.

To the best of our knowledge, this work represents the first determination of redox thermodynamic parameters for oxidizing couples (*i.e.*, those with oxidation states higher than the Fe(III)/Fe(II) couple) in heme enzymes. It has been noted (Marcus & Sutin, 1975; Sutin *et al.*, 1980) that there is a close connection between redox thermodynamic parameters and kinetic activation parameters for outer-sphere electron-transfer reactions. In this context, our reaction entropies suggest that the reorganizational energy for the porphyrin-centered couple is much less than that for the iron-centered one. This conclusion is in keeping with the observation (Sitter & Terner, 1985; Hashimoto & Kitagawa, 1986; Oertling & Babcock, 1988) that the resonance Raman spectrum of compound I displays porphyrin skeletal modes (frequencies > 1000 cm^{-1}) similar to those of compound II produced at pH 7 and higher. (In other words, ring-centered oxidation does not result in a large change in macrocycle geometry.) Thus, considerations of thermodynamic driving forces, reorganizational energy barriers, and kinetic accessibility suggest that HRP compound I is intrinsically a much more reactive oxidant than compound II in one-electron outer-sphere redox reactions.

Hasinoff and Dunford (1970) reported a study of the oxidation of ferrocyanide by compounds I and II; their reported values for the second-order rate constants (pH 7) are 8×10^5 M^{-1} s^{-1} for compound I reduction and 2×10^4 M^{-1} s^{-1} for compound II reduction. Using $E^{o\prime} = 407$ mV (NHE) and $k_{11} = 7 \times 10^4$ M^{-1} s^{-1} (Shporer *et al.*, 1965) for the $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ couple, we estimate, using the Marcus cross relation without any work corrections, 4.4×10^{-1} and 4.9×10^{-4} M^{-1} s^{-1} for the electron self-exchange rates for the compound I/compound II and compound II/ferric HRP couples, respectively.

Like HRP compound I, yeast cytochrome *c* peroxidase compound I (also designated compound ES) is 2 equiv more oxidized than the ferric resting enzyme. However, the yeast enzyme intermediate is a red species that contains a ferryl center and an amino acid free radical; crystallographic (Edwards *et al.*, 1987) and spectroscopic (Sivaraja *et al.*, 1989) characterization of this intermediate point to Trp-191 as the site of the free radical. Interestingly, H_2O_2 oxidation (Erman *et al.*, 1989) of a Trp-191→Phe-191 mutant of cytochrome *c* peroxidase produced a ferryl porphyrin π-cation radical intermediate, reminiscent of HRP compound I, that rapidly decayed to compound II. Purcell and Erman (1976) have calculated, using Marcus theory, a value of 1.087 V vs NHE for the reduction potential of the cytochrome *c* peroxidase compound II/ferric couple (*ca.* 200 mV more positive than that for the analogous HRP couple). Thus, the yeast cytochrome *c* peroxidase ferryl porphyrin π-cation radical/compound II reduction potential must also be at least 200 mV greater than that for the HRP compound I/compound II couple. In contrast, Wikström (1988) measured a value of 801 mV for the ferrylcytochrome a_3/ferricytochrome a_3 reduction potential of beef heart mitochondrial cytochrome *c* oxidase, approximately 70 mV less than the analogous HRP compound II/ferric couple. It is therefore evident that environmental effects play a significant role in tuning the potentials of oxidizing (radical and ferryl/ferric) couples in heme enzymes, as has been observed for a variety of other metalloprotein couples.

ACKNOWLEDGMENT

We thank Professors Isao Yamazaki and Gregory Schonbaum for helpful discussions.

REFERENCES

Barlow, R. J. (1989) *Statistics: A Guide to the Use of Statistical Methods in the Physical Sciences*, Wiley, Chichester, U.K.

Calderwood, T. S., Lee, W. A., & Bruice, T. C. (1985) *J. Am. Chem. Soc. 107,* 8272−8273.
Chance, B., Powers, L., Ching, Y., Poulos, T., Schonbaum, G. R., Yamazaki, I., & Paul, K. G. (1984) *Arch. Biochem. Biophys. 235,* 596−611.
Dolphin, D., Forman, A., Borg, D. C., Fajer, J., & Felton, R. H. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68,* 614−618.
Edwards, S. L., Xuong, N. H., Hamlin, R. C., & Kraut, J. (1987) *Biochemistry 26,* 1503−1511.
Erman, J. E., Vitello, L. B., Mauro, J. M., & Kraut, J. (1989) *Biochemistry 28,* 7992−7995.
Evangelista-Kirkup, R., Crisanti, M., Poulos, T. L., & Spiro, T. G. (1985) *FEBS Lett. 190,* 221−226.
Everse, J., Everse, K. E., & Grisham, M. B., Eds. (1991) *Peroxidases in Chemistry and Biology, Vols. I & II,* CRC Press, Boca Raton, FL.
Farhangrazi, Z. S., Copeland, B. R., Nakayama, T., Amachi, T., Yamazaki, I., & Powers, L. S. (1994) *Biochemistry 33,* 5647−5652.
Fergusson, R. R. (1956) *J. Am. Chem. Soc. 78,* 741−745.
Frew, J. E., Harmer, M. A., Hill, H. A. O., & Libor, S. I. (1986) *J. Electroanal. Chem. 201,* 1−10.
Garguilo, M. G., Huynh, N., Proctor, A., & Michael, C. A. (1993) *Anal. Chem. 65,* 523−528.
George, P. (1953) *Science 117,* 220−221.
Hashimoto, T., & Kitagawa, T. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83,* 2427−2421.
Hasinoff, B. B., & Dunford, H. B. (1970) *Biochemistry 9,* 4930−4939.
Hayashi, Y., & Yamazaki, I. (1979) *J. Biol. Chem. 254,* 9101−9108.
Heineman, W. R. (1978) *Anal. Chem. 50,* 390−402.
Igo, D. H., Elder, R. C., Heineman, W. R., & Dewald, H. D. (1991) *Anal. Chem. 63,* 2535−2539.
Kaneko, Y., Tamura, M., & Yamazaki, I. (1980) *Biochemistry 19,* 5795−5799.
Maidan, R., & Heller, A. (1991) *J. Am. Chem. Soc. 113,* 9003−9004.
Marcus, R. A., & Sutin, N. (1975) *Inorg. Chem. 14,* 213−216.
Michaelis, L., & Menten, N. (1913) *Biochem. Z. 49,* 433.
Naqui, A., & Chance, B. (1986) *Annu. Rev. Biochem. 55,* 137−166.
Nakatani, H., & Takahashi, K. (1986) *Biochemistry 25,* 3515−3518.
Oertling, W. A., & Babcock, G. T. (1988) *Biochemistry 27,* 3331−3338.
Purcell, W. L., & Erman, J. E. (1976) *J. Am. Chem. Soc. 98,* 7033−7037.
Roberts, J. E., Hoffman, B. M., Rutter, R., & Hager, L. P. (1981a) *J. Am. Chem. Soc. 103,* 7654−7656.
Roberts, J. E., Hoffman, B. M., Rutter, R., & Hager, L. P. (1981b) *J. Biol. Chem. 256,* 2118−2121.
Schulz, C. E., Rutter, R., Sage, J. T., Debrunner, P. G., & Hager, L. P. (1984) *Biochemistry 23,* 4743−4754.
Shporer, M., Ron, G., Loewenstein, A., & Navon, G. (1965) *Inorg. Chem. 4,* 361−364.
Sitter, R., & Terner, J. (1985) *J. Biol. Chem. 260,* 7515−7522.
Sivaraja, M., Goodin, D. B., Smith, M., & Hoffman, B. M. (1989) *Science 245,* 738−740.
Smith, D. A., Elder, R. C., & Heineman, W. R. (1985) *Anal. Chem. 57,* 2361−2365.
Sutin, N., Weaver, M. J., & Yee, E. L. (1980) *Inorg. Chem. 19,* 1098−1100.
Taniguchi, V. T., Ellis, W. R., Jr., Cammarata, V., Webb, J., Anson, F. C., & Gray, H. B. (1982) in *Electrochemical and Spectrochemical Studies of Biological Redox Components* (Kadish, K. M., Ed.) Advances in Chemistry Series No. 201, pp 51−68, American Chemical Society, Washington, DC.
Tijssen, P., & Kurstak, E. (1984) *Anal. Biochem. 136,* 451−457.
Wikström, M. (1988) *Chem. Scr. 28A,* 71−74.

BI942039K