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Kinetics and Energetics of Intramolecular Electron Transfer in Yeast Cytochrome *c* Peroxidase[†]

Pui S. Ho, Brian M. Hoffman,* Natalie Solomon, Chae Hee Kang, and Emanuel Margoliash

ABSTRACT: The oxidation of ferric cytochrome *c* peroxidase by hydrogen peroxide yields a product, compound ES [Yonetani, T., Schleyer, H., Chance, B., & Ehrenberg, A. (1967) in *Hemes and Hemoproteins* (Chance, B., Estabrook, R. W., & Yonetani, T., Eds.) p 293, Academic Press, New York], containing an oxyferryl heme and a protein free radical [Dolphin, D., Forman, A., Borg, D. C., Fajer, J., & Felton, R. H. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 614–618]. The same oxidant takes the ferrous form of the enzyme to a stable Fe(IV) peroxidase [Ho, P. S., Hoffman, B. M., Kang, C. H., & Margoliash, E. (1983) *J. Biol. Chem. 258*, 4356–4363]. It is 1 equiv more highly oxidized than the ferric protein, contains the oxyferryl heme, but leaves the radical site unoxidized. Addition of sodium fluoride to Fe(IV) peroxidase gives a product with an optical spectrum similar to that of the fluoride complex of the ferric enzyme. However, reductive titration and electron paramagnetic resonance (EPR) data demonstrate that the oxidizing equivalent has not been lost but rather transferred to the radical site. The EPR spectrum for the radical species in the presence of Fe(III) heme is identical with that of compound ES, indicating that the unusual characteristics of the radical EPR signal do not result from coupling to the heme site. By stopped-flow measurements, the oxidizing equivalent transfer process between heme and radical site is first order, with a rate constant of 0.115 s^{-1} at room temperature, which is independent of either ligand or protein concentration. This is slow relative to fluoride binding to the ferric enzyme, indicating that the rate-limiting step is the intramolecular transfer of oxidizing equivalents. Arrhenius plots of the observed rate constants for the Fe(IV) peroxidase and the Fe(III) enzyme yield some of the thermodynamic parameters that describe intermediates in the reaction pathway.

To fully understand the catalytic mechanism for cytochrome *c* peroxidase, one must be able to study both the inter- and the intramolecular electron-transfer reactions involved and their contributions to the overall mechanism. Recently (Ho et al., 1983), we developed a procedure for the preparation in nearly pure form of a cytochrome *c* peroxidase intermediate that is oxidized by 1 equiv above the resting ferric enzyme. This intermediate proved to be the oxyferryl peroxidase not containing the protein free radical normally associated with the fully oxidized hydrogen peroxide product, compound ES (Dolphin et al., 1971). The properties of the Fe(IV) enzyme (denoted as Fe^{IV} R), in conjunction with previous results from reductive titrations (Coulson et al., 1971) and transient-state kinetics for the reduction of compound ES (Jordi & Erman, 1974), allowed the development of a model of the oxidation and subsequent reduction pathways followed by cytochrome *c* peroxidase (Figure 1) (Ho et al., 1983). A novel aspect of this mechanism is the prediction that the conformation of the protein radical site determines its reduction potential. It was proposed that the stable form of the unoxidized site (the R conformer) has a high potential and can be oxidized only by a higher potential heme than that of Fe(IV) peroxidase, so that a significant flow of oxidizing equivalents between the heme and radical sites normally occurs only (1) during the hydrogen peroxide oxidation of the ferric enzyme and (2) within the initial transient intermediate formed upon the one-electron reduction of this oxidized product.

Earlier, Coulson et al. (1971) had reported that the addition of fluoride to a sample of compound ES previously reduced by 1 equiv caused an increase in the radical and the Fe(III) populations. This was interpreted as a shift in the equilibrium of the mixture of intermediates at 1 oxidizing equiv above native in favor of the ferric form, as a result of ligation of the ferric heme iron by the anion. In terms of our proposed mechanism, fluoride binding would serve to increase the reduction potential of the ferryl/ferric redox couple, allowing

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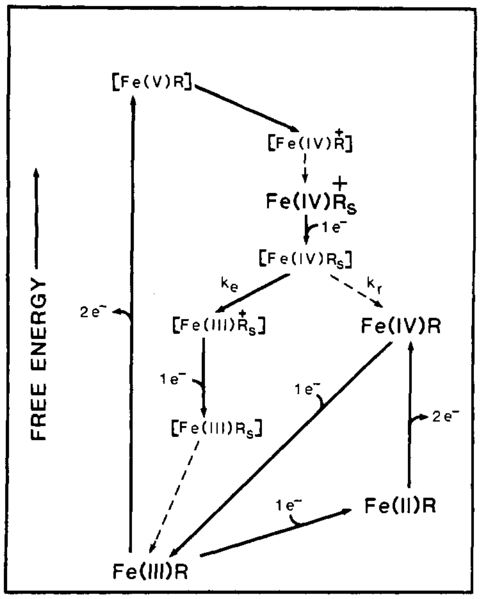

FIGURE 1: Schematic representation of the proposed catalytic mechanism of cytochrome *c* peroxidase (Ho et al., 1983). Transient intermediates or species not stably prepared are in square brackets, stably prepared species are in boldface, electron-transfer reactions are indicated by solid arrows, and conformational changes are indicated by broken arrows. The relative free energies of the $Fe^{III}R_s^{+}$ and $Fe^{IV}R$ intermediates need not be identical, as was addressed in the original discussion.

the oxidation of the R conformer of the radical site. The results reported by Coulson et al. were not entirely quantitative because attempts to reduce compound ES by 1 equiv with inorganic reductants yield solutions containing not only the intermediates described but also unreduced compound ES and fully reduced, native ferric enzyme.

This paper presents a study of the consequences of the addition of fluoride to cleanly prepared Fe(IV) peroxidase. Since the effective oxidizing equivalents of this system center only on the heme iron, electron transfer induced by ligation of the anion can be readily identified as an oxidation of the radical site by the ferryl heme and quantitated. The kinetics and the energetics of intramolecular electron transfer in cytochrome *c* peroxidase are characterized and related to the previously proposed mechanism for the peroxidase catalytic cycle. Evidence is also reported in support of the conclusion (Hoffman et al., 1979, 1981) that the free radical site in cytochrome *c* peroxidase is not an aromatic amino acid side chain such as tryptophan.

Materials and Methods

All buffers, oxidants, reductants, sodium fluoride, and organic solvents were reagent grade and were used without further purification. Carbon monoxide and nitrogen gases from Matheson were dried and freed of oxygen by passage through columns of Drierite and Ridox. The oxidized and reduced cytochromes *c* were prepared by the procedure of Margoliash & Walasek (1967), as modified by Brautigan et al. (1978), and separated from any polymeric material as previously described (Brautigan et al., 1978).

Preparations of Yeast Cytochrome c Peroxidase. Cytochrome *c* peroxidase, in the native ferric oxidation state was prepared from pressed commercial yeast (Red Star) according to Nelson et al. (1977), with the modifications previously described (Ho et al., 1983). The concentration of peroxidase solutions was determined spectrophotometrically with an extinction coefficient at 408 nm of 100 mM^{-1} cm^{-1} at room temperature (Kang et al., 1977). Oxidation of the enzyme to compound ES, with both the heme and radical sites oxidized, was achieved by stoichiometric addition of a cold, fresh 1.0 mM solution of H_2O_2 in the appropriate buffer to the native ferric peroxidase.

Ferrous peroxidase was quantitatively prepared by the procedure developed by Ward & Chang (1982) for the photoreduction of myoglobin. A stock solution of the cytochrome *c* peroxidase was diluted into cold, degassed potassium phosphate buffer at pH 7.0 containing 2% 2-propanol and 0.008% acetophenone, in a rubber septum capped glass cuvette. The sample was irradiated for 2 min with unfiltered light from a 200-W xenon–mercury arc lamp (Oriel Corporation) to yield the stable Fe(II) enzyme. The carbonyl ferrous peroxidase was prepared by stoichiometric addition of 1 mM assayed sodium dithionite to a carbon monoxide flushed ferric enzyme sample.

Fe(IV) peroxidase was prepared by oxidation of the ferrous enzyme with H_2O_2 (Ho et al., 1983). For optical and stopped-flow measurements, oxidation of the photoreduced enzyme with stoichiometric hydrogen peroxide was sufficient to yield the desired product. For the more concentrated EPR samples, the preparation required using the CO–Fe(II) enzyme with a 10% excess of hydrogen peroxide added in the dark. The oxidation was effected by photodissociation of the carbonyl ligand with available room light.

Spectral Procedures. All ultraviolet and visible spectra were recorded on a Beckman ACTA III spectrophotometer. Liquid nitrogen temperature electron paramagnetic resonance (EPR) spectra were obtained on a modified Varian FRAT EPR spectrometer, while liquid helium temperature spectra at 4.2 K were recorded on a modified Varian E-4 spectrometer (Hoffman et al., 1981). Numerical integrations of the 4.2 K spectra were performed on a Fabritek Model 1074 Instrumental Computer. Electron-spin concentrations were calculated by the procedure of Aasa & Vänngård (1975). The magnetic fields of the spectrometers were checked against recrystallized diphenylpicrylhydrazyl.

Titrations of the cytochrome *c* peroxidase intermediates with reduced horse cytochrome *c* were performed by using aliquots of approximately 1 mM assayed reductant and monitored as the appearance of excess reduced cytochrome *c* at 560 nm. The manipulation of the absorbance data and the correction for the spontaneous decay of the peroxidase intermediates were as previously described (Ho et al., 1983).

Kinetic Procedures. Transient-state kinetics for fluoride binding to the various forms of the enzyme were measured on a Dionex stopped-flow instrument interfaced to an On-Line Instrument Systems (OLIS) Model 3820 data station, utilizing a Northstar Horizon minicomputer. Data were collected, stored, and analyzed by the OLIS STOPFLOW program. The temperatures for various kinetic runs were varied and controlled by circulation of thermostated water around the drive syringes and the observation cell, and the actual temperatures were measured at the drive syringes. Fluoride binding to the native ferric peroxidase was monitored as the change in absorbance at 406 nm and for the Fe(IV) peroxidase at 424 and 406 nm; identical rate constants were obtained at both wavelengths. The association and dissociation rate constants and the equilibrium constant for fluoride binding to the Fe(III) peroxidase was calculated as previously reported (Erman, 1974).

The rate for the oxidation of the radical site subsequent to fluoride addition was directly examined by EPR. A sample of the EPR-silent Fe(IV) peroxidase was prepared at 0 °C as described above. A 20-fold excess of sodium fluoride was

Table I: Association (k_{ON}) and Dissociation (k_{OFF}) Rate Constants for Fluoride Binding to Ferric Cytochrome *c* Peroxidase[a]

T (°C)	k_{ON} (M^{-1} s^{-1})	k_{OFF} (s^{-1})
0	$(6.77 \pm 0.23) \times 10^3$	0.236 ± 0.029
5	$(9.75 \pm 0.24) \times 10^3$	0.266 ± 0.048
10	$(1.13 \pm 0.10) \times 10^4$	0.397 ± 0.022
15	$(1.65 \pm 0.12) \times 10^4$	0.469 ± 0.088
20	$(2.11 \pm 0.24) \times 10^4$	1.041 ± 0.150

[a] The enzyme and anion were both in 0.2 M potassium phosphate buffer, containing 2% 2-propanol and 0.008% acetophenone, at pH 7.0. Rate constants were calculated from linear least-squares fit of the observed rate constants (k_{obsd}) vs. concentration of sodium fluoride as described by Erman (1974). Five to ten measurements of k_{obsd} for fluoride concentrations of 10, 20, 35, and 50 μM were made at each temperature.

added to the sample, which was quickly transferred to quartz EPR tubes, and frozen in liquid nitrogen at 9 and 40 s after addition of the anion. The samples were completely frozen within 2 s after immersion into the liquid nitrogen. A sample to which was added a volume of buffer equal to the volume of fluoride was used as the $t = 0$ reference. The increase in the $g = 6$, high-spin ferric, and $g = 2$ radical signals was monitored at 77 and 4.2 K. The signal intensities of the 4.2 K spectra were doubly integrated and compared to the intensities of the native ferric enzyme and compound ES, both with fluoride added, and compared to the intensities predicted at $t = \infty$ from the optical equilibrium measurements.

Results

Fluoride Binding to the Native Ferric Peroxidase. Pseudo-first-order kinetics of fluoride binding were observed when 1–4 μM samples of the ferric enzyme were flowed against equal volumes of 10–100 μM sodium fluoride solutions at temperatures from 0 to 20 °C. Following the procedure previously discussed (Erman, 1974), the rate constants for fluoride binding to (k_{ON}) and dissociation from (k_{OFF}) the ferric enzyme at each temperature were calculated from the slope and intercept, respectively, for the linear relationship between the observed rate constants and the final fluoride concentrations used in the stopped-flow kinetic measurements (Table I). The temperature dependences of the calculated rate constants from Table I were analyzed with the Arrhenius equation, $\log k = -\Delta E^{+}/(kT)$, and gave activation energies of 8.9 ± 1.2 kcal/mol for the forward and 10.5 ± 1.5 kcal/mol for the reverse binding pathways. The dissociation constant (K_D) for the ferric–fluoride complex was calculated as the ratio of k_{OFF} to k_{ON}, and the free energies for dissociation were calculated by the standard equation $\Delta G = -RT \ln K_D$ (Table II).

Fluoride Binding to Compound ES. The UV–visible spectrum of compound ES does not change upon the addition of sodium fluoride up to 1 mM. Furthermore, EPR spectra of the radical site at 4.2 K were unaltered in shape and undiminished in intensity by addition of the anion. Thus, addition of sodium fluoride to ES does not reduce the ferryl heme or radical sites, nor does fluoride bind to the ferryl heme.[1]

Fluoride-Induced Intramolecular Electron Redistribution in Fe(IV) Peroxidase. The addition of sodium fluoride to Fe(IV) peroxidase causes an increase in absorbance at 406 nm and a concomitant decrease at 418 nm, indicative of the formation of a fluoride-bound ferric species at the expense of the ferryl enzyme; the observed difference spectrum corresponds to that obtained by subtracting the spectra of the Fe(IV) enzyme from that of the $Fe^{III}F^-$ protein. The extent of conversion of ferryl to ferric heme upon fluoride addition was calculated by employing a value for the difference in extinction coefficients between the Fe(IV) peroxidase and ferricfluoro peroxidase of $\Delta\epsilon$(406 nm) = 44.2 ± 2.6 mM^{-1} cm^{-1}. The relatively clean isobestic points for the spectra obtained during these titrations indicate that no long-lived intermediates are produced in observable quantities (Figure 2).

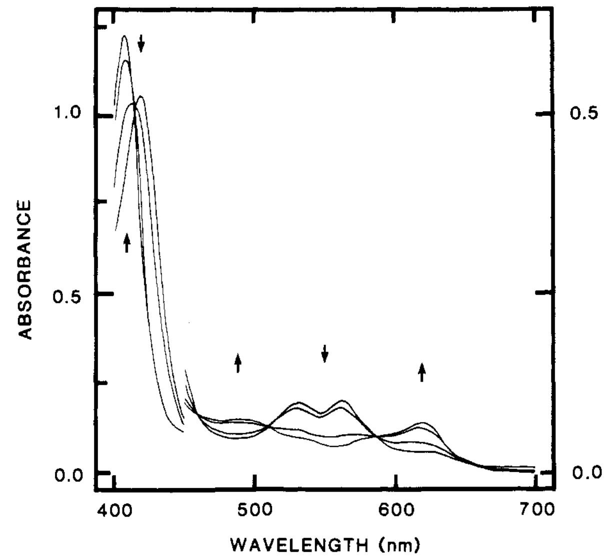

FIGURE 2: Optical spectra of Fe(IV) peroxidase titration with sodium fluoride. A 12 μM solution of Fe(IV) peroxidase prepared in 0.1 M potassium phosphate buffer at pH 7.0 containing 2% 2-propanol and 0.008% acetophonene was titrated with small volumes of 1 M sodium fluoride in 0.1 M potassium phosphate buffer at pH 7.0 to give final fluoride concentrations of 0, 1, and 15 mM at 25 °C. The final spectrum of the titrated solution after 48 h, presumed to be entirely the fluoride-bound ferric enzyme, is included for comparison.

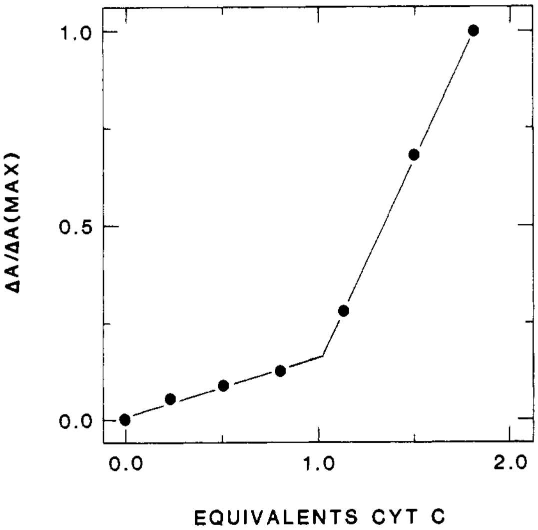

FIGURE 3: Titration of Fe(IV) peroxidase + F^- sample with reduced cytochrome *c*. A 7.5 μM Fe(IV) peroxidase sample in the presence of 2.0 mM sodium fluoride was prepared in 0.1 M potassium phosphate buffer at pH 7.0 containing 2% 2-propanol and 0.008% acetophenone. Titration of this sample with 5-μL increments of a 0.77 mM initial concentration of reduced cytochrome *c* was monitored at 550 nm at room temperature. The equivalents of reductant added were corrected for decay of the Fe(IV) peroxidase as discussed previously (Ho et al., 1983).

The optical changes resulting from fluoride addition to the Fe(IV) peroxidase are not associated with reduction of the enzyme to the ferric resting state. To establish this, a solution of 7.5 μM enzyme plus 2.0 mM F^-, and having an optical

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[1] The 77 K EPR spectra for compound ES were slightly broadened in the presence of fluoride.

Table II: Equilibrium Constants for Various Pathways

	Fe(III) + F⁻		Fe(IV) + F⁻	$Fe^{IV}R \rightleftarrows Fe^{III}r^+$	
T (°C)	K_D ($\times 10^5$ M)	ΔG (kcal/mol)	K_F (M^{-1})	K_E ($\times 10^3$)	$\Delta E°$ (mV)
0	3.49 ± 0.21	5.58	195[a]	6.81	117
5	2.73 ± 0.15	5.71	237 ± 23	6.47	121
10	3.51 ± 0.30	5.78	255 ± 10	8.95	115
15	2.84 ± 0.49	6.00	299 ± 40	8.49	119
20	4.72 ± 0.20	5.81	345 ± 22	16.3	104
25	4.58[a]	5.93	415 ± 66	19.0	102

[a] Extrapolated values from Arrhenius plots of the fluoride association and dissociation rate constants.

Table III: EPR Quantitation for Fe(IV) Peroxidase plus F⁻ at 4.2 K

		percentage increase	
sample	t (s)[a]	Fe(III)[b]	protein radical[c]
Fe(IV) enzyme	0	3.5	5.2
Fe(IV) enzyme + F⁻	9	24.9	29.5
Fe(IV) enzyme + F⁻	30	45.5	43.4

[a] Time of incubation at 0 °C of the Fe(IV) peroxidase plus fluoride. [b] Change in $g = 6$ high-spin ferric signal relative to the difference between the Fe(IV) peroxidase and native ferric peroxidase at this field. [c] Change in the $g = 2$ radical signal relative to the difference between the Fe(IV) peroxidase and compound ES at this field.

spectrum corresponding to an $Fe^{III}F^-$ to Fe(IV) ratio of 0.64, was titrated with reduced horse cytochrome *c*. It was found that 0.99 ± 0.10 equiv was required to reduce the sample to the ferric state (Figure 3). Thus, the protein in the presence of the anion has an optical spectrum corresponding to a mixture of Fe(III) and Fe(IV) states but, nevertheless, remains fully at the oxidation level of the Fe(IV) enzyme. This observation strongly suggests that addition of F⁻ causes a shift in the distribution of oxidizing equivalents according to the overall equilibrium expressed in eq 1.

$$Fe^{IV}R + F^- \xrightleftharpoons{K_F} Fe^{III}F^-R_s^+ \qquad (1)$$

EPR spectra were recorded subsequent to the addition of a 20-fold excess of fluoride to the Fe(IV) enzyme. Aliquots were taken from the 0 °C mixtures at intervals, and the reaction was quenched by rapid freezing in liquid nitrogen as described above. The 77 and 4.2 K spectra show parallel increases in both the high-spin ferric signal at $g = 6$ and the radical signal at $g = 2$ with time, confirming that the optical changes indeed result from oxidation of the radical site by the ferryl heme (Table III). The resulting 4.2 K radical signal, $g_\parallel = 2.05$ and $g_\perp = 2.01$, was indistinguishable in line width and saturation behavior from that of the compound ES (Figure 4). Thus, the EPR results confirm that F⁻ addition causes a redistribution of oxidizing equivalents, as indicated in eq 1. Assuming first-order kinetics, both EPR signals appear with approximate first-order rate constants at 0 °C of ca. 0.1–0.2 s^{-1}.

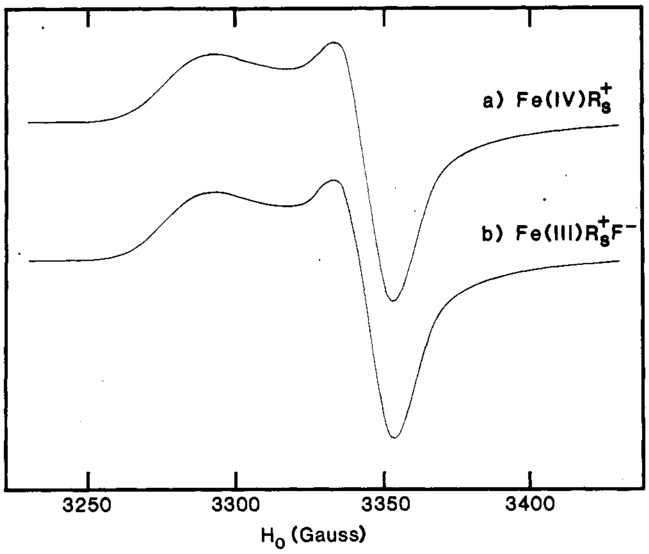

FIGURE 4: EPR spectra of cytochrome *c* peroxidase free-radical signal at 4.2 K. Compound ES (a) and Fe(IV) peroxidase in the presence of 1000-fold excess sodium fluoride were both prepared in 0.1 M potassium phosphate buffer at pH 7.0 containing 2% 2-propanol and 0.008% acetophenone. Spectra for these samples were recorded at 9.62 GHz, 20-μW power, and 10-G modulation amplitude.

Stopped-Flow Measurements on the Electron Redistribution Process. The reaction of Fe(IV) peroxidase with fluoride (eq 1) was monitored by following the absorbance changes in the stopped-flow apparatus when 5–20 μM enzyme solutions were flowed together with equal volumes of solution containing 15–100-fold excess sodium fluoride. The resulting final ratios of $Fe^{III}F^-$ to Fe(IV) heme were calculated from the change ($t \rightarrow \infty$) in absorbance at 424 nm for these kinetic traces, with a value of $\Delta\epsilon(424\ nm) = 40\ mM^{-1}\ cm^{-1}$ for the (resting ferric fluoride − ferryl) peroxidase difference spectrum. The equilibrium constant (K_F) for eq 1 calculated in this way correlated well with those determined from equilibrium titrations. Linear-regression analysis of ln K_F vs. reciprocal temperature, for temperatures between 5 and 25 °C (Table II), yielded a value, $\Delta H = 4.7 \pm 1.4$ kcal/mol, for the equilibrium of eq 1.

The loss of the 424-nm ferryl heme absorbance in the stopped flow and the increase of the 406-nm fluoroferric heme absorbance each is first order and slow relative to fluoride binding to the native ferric peroxidase under the same conditions. The room temperature rates obtained at the two wavelengths [$k_{obsd}(20\ °C) = 0.11\ s^{-1}$] agree and are independent of the concentration of sodium fluoride (Figure 5) and of the initial concentration of the Fe(IV) peroxidase. From this we conclude that the rate-limiting step for the process is the intramolecular oxidation of the radical site by the ferryl heme and that this step is rapidly followed by binding of fluoride to the ferric heme thus formed:

$$Fe^{IV}R \xrightarrow[slow]{k_e} Fe^{III}r^+ \xrightarrow[rapid]{k_F} Fe^{III}F^-r^+ \qquad (2)$$

Since the spectrophotometric techniques only monitor changes in the heme site and do not distinguish between conformers of the oxidized radical site, the designations r and r^+ have been used where appropriate to represent the unoxidized and oxidized radical sites, respectively, without reference to conformation. Electron-transfer rate constants for the reaction were obtained as a function of temperature (Table IV), and an Arrhenius plot corresponds to an activation energy of $\Delta E^+ = 13 \pm 1.5$ kcal/mol for this process.

Thermodynamic Calculations for Electron Redistribution in Fe(IV) Peroxidase. The overall equilibrium constant (eq 1) for binding fluoride ion to ferryl peroxidase, $K_F(20\ °C) =$

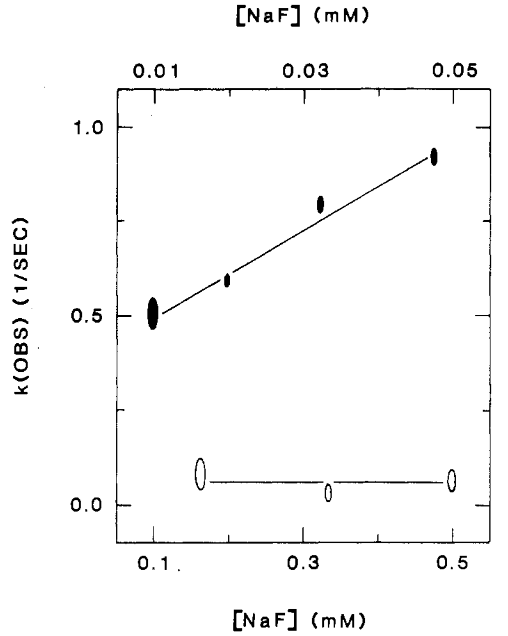

FIGURE 5: Observed rate constants (k_{obsd}) for fluoride reaction with cytochrome *c* peroxidase and Fe(IV) peroxidase at 10 °C. The closed symbols represent reaction with the native ferric enzyme (upper abscissa) and the open symbols with the Fe(IV) peroxidase (lower abscissa). The size of the ellipses are proportional to the error in measurement. Both samples were in 0.1 M potassium phosphate buffer at pH 7.0 containing 2% 2-propanol and 0.008% acetophenone.

Table IV: Observed Rate Constants for Fluoride Binding to Fe(IV) Peroxidase[a]

T (°C)	k_{obsd} ($\times 10^2$ s^{-1})
5	4.0 ± 0.4
10	6.6 ± 0.7
15	9.2 ± 0.2
20	11.5 ± 0.1
25	21.3 ± 0.4

[a] Approximately 3.5 μM solutions of the Fe(IV) peroxidase were prepared as previously described (Ho et al., 1983) in 0.1 M potassium phosphate buffer, containing 2% 2-propanol and 0.008% acetophenone, at pH 7.0, and flowed against equal volumes of 1 mM solutions of sodium fluoride, in the same buffer system. The reaction was monitored at 424 nm, and 4–10 determinations of k_{obsd} were made at each temperature.

345 ± 2 M^{-1} (Table II), corresponds to a difference in free energy between the product and the reactant of $\Delta G = 3.4$ kcal/mol at room temperature. The stopped-flow measurements indicate that the net process of eq 1 can be written in terms of two individual equilibria, namely, partitioning of the oxidizing equivalent between heme and radical sites (eq 3a) and fluoride binding to the ferri heme in the presence of the protein cation radical (eq 3b). Thus, K_F ($=K_E/K_D$) is the ratio

$$\mathrm{Fe^{IV}R} \xrightleftharpoons{K_E} \mathrm{Fe^{III}r^+} \tag{3a}$$

$$\mathrm{Fe^{III}r^+ + F^-} \xrightleftharpoons{K_D^{-1}} \mathrm{Fe^{III}F^-r^+} \tag{3b}$$

$$\text{net:}\quad \mathrm{Fe^{IV}R + F^-} \xrightleftharpoons{K_F} \mathrm{Fe^{III}F^-R_s{}^+}$$

of two individual equilibrium constants. That of chief interest, K_E, describes the equilibrium between Fe^{IV}R and all contributing intermediate states of the enzyme with the oxidized radical site; it can be calculated from the measured K_F if the fluoride ion dissociation constant (K_D) is known.

The binding of fluoride to the native ferric peroxidase provides a model for anation of the Fe^{III}F$^-$R$_s{}^+$ species. Comparisons of the optical spectra of the Fe(IV) peroxidase to that of compound ES (Ho et al., 1983) and of the $g = 2$ radical signal induced by fluoride binding to the Fe(IV) peroxidase to that of compound ES above show that the radical site is not strongly coupled to either the ferric or ferryl heme sites. The presumed additional positive charge of the cationic radical could in principle alter fluoride association to and dissociation from the enzyme. However, it has been proposed (Erman, 1974) that at neutral pH values fluorine enters the heme pocket as the neutral species, hydrofluoric acid, and that the pH dependence for the dissociation constant is small; thus, we expect electrostatic contributions of the cationic, oxidized radical site to provide only minor contributions to anion binding to the ferric intermediate and consider the parameter describing the native ferric peroxidase, K_D given in Table II, to be valid as a first approximation for fluoride binding to this intermediate as well. This quantity was therefore used in conjunction with that for the overall process of eq 1 (K_F) to determine the temperature dependence of the electron-hole partition equilibrium constant (K_E); Table II presents the results and their formulation in terms of the difference in standard reduction potential for the Fe(IV) and radical species: $\Delta E_0 = [RT/(nF)] \ln K_E$. From the calculated value of K_E, it is expected that the enzyme oxidized by 1 equiv above the ferric resting state should exhibit ca. 98% of the Fe^{IV}R form at ambient temperature, consistent with the optical spectra. From the temperature variation of K_E (Table II), we obtain a value $\Delta H_E = 4.7$ kcal/mol for electron transfer from R to ferryl heme (eq 3a); the absence of signals from the oxidized radical in low-temperature EPR spectra of the Fe(IV) peroxidase confirms that this process is endothermic. This allows the proposal of a reaction pathway for the reaction of the Fe(IV) peroxidase with fluoride complete with relative enthalpies for intermediates and hypothetical transient species (see Figure 6).

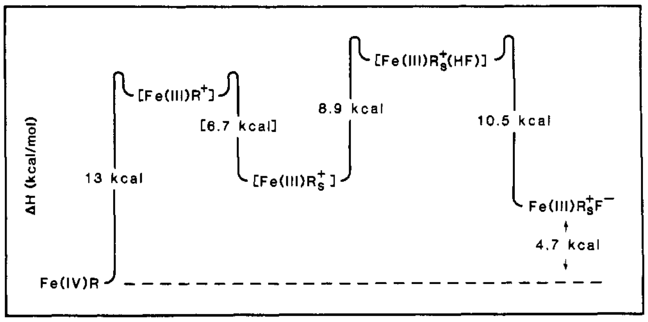

FIGURE 6: Reaction pathway and energetics for Fe(IV) peroxidase reaction with fluoride. All unstable species not isolated are in brackets. The Fe^{III}R$^+$ and Fe^{III}R$_s{}^+$ transient intermediates were inferred from the catalytic pathway proposed earlier for the enzyme (Ho et al., 1983), and initial binding of HF to form the Fe^{III}R$_s{}^+$(HF) intermediate was inferred from the pH dependence of fluoride binding to the native ferric peroxidase as reported by Erman (1974). The activation energy for destabilization of the R$_s$ radical conformer to the R‡ species (in brackets) was not directly measured but was calculated from the other measured quantities.

Discussion

The ability of fluoride to shift the equilibrium concentrations of the cytochrome *c* peroxidase intermediates that are one oxidation level above the native ferric species has been previously reported by Coulson et al. (1971). They found that addition of fluoride to compound ES previously reduced by 1 molar equiv caused parallel increases of both the ferric and protein radical EPR signals at 77 K. Our studies with clean preparations of Fe(IV) peroxidase allow a more detailed examination of (1) electron transfer between the Fe(IV) heme and the radical site, (2) the initial stages of H_2O_2 oxidation of the enzyme, and (3) the identity of the radical site.

Fluoride binding to the heme of Fe(IV) peroxidase is a reaction for which the rate-determining step is not ligation but the slower intramolecular oxidation of the protein radical site by the ferryl heme [$k_e(20\ ^\circ C) = 0.11\ s^{-1}$; $\Delta E^{+} = 13$ kcal/mol]. This observation represents the first direct measurement of electron transfer between these two oxidizable sites. If the electron-transfer reaction corresponded to a single microscopic process with equilibrium constant K_e, as written in eq 2 and 3a, then from the forward rate constant (k_e) and the equilibrium constant (Table II) one would obtain as the rate constant for the reverse process, namely, the oxidation of the heme iron by the protein radical, $k_{-e}(20\ ^\circ C) = 11\ s^{-1}$.

However, such a relatively large rate constant is not consistent with earlier results. Indeed, the Fe(IV) peroxidase is the stable form of the enzyme oxidized 1 equiv above the ferri heme resting state. This, together with the large value calculated for k_{-e}, would require that reduction of compound ES by 2 equiv occur in two discrete steps regardless of whether the site of reduction is at the heme or the radical and that 1-equiv reduction of compound ES would quantitatively yield the Fe(IV) enzyme without free radical. However, addition of 1 reducing equiv to compound ES, at or below neutral pH, forms roughly 0.5 equiv of Fe(IV) peroxidase and half of a ferri heme radical form (Coulson et al., 1971), despite the fact that stopped-flow reduction of compound ES by ferrocyanide appears to proceed by a pathway in which the radical site is preferentially reduced over the oxyferryl heme (Jordi & Erman, 1974). Our proposed mechanism for cytochrome *c* peroxidase function reconciles these observations (Figure 1). The electron-transfer process (eq 3a) is taken to proceed via an intermediate, $Fe^{III}R^+$, in which the oxidized radical site has not relaxed to its stable conformation:

$$Fe^{IV}R \underset{k'_{-e}}{\overset{k'_e}{\rightleftharpoons}} Fe^{III}R^+ \underset{k_{-c}}{\overset{k_c}{\rightleftharpoons}} Fe^{III}R_s^+ \qquad (4)$$

The measured equilibrium constant for the overall reaction would then become $K_E = K'_E K_c = (k'_e/k'_{-e})(k_c/k_{-c})$. The species R^+ is considered to involve the radical site in an energetically unfavorable conformation, and thus, $K_c > 1$. Oxidation of the ferriheme by the radical proceeds by a pathway that involves conversion to the unfavorable conformer, $R_s^+ \rightarrow R^+$, making the process slow and thus rate limiting. Therefore, the $Fe^{III}R_s^+$ species formed during the reductive titration of compound ES does not convert to the more stable $Fe^{IV}R$ form. Oxidation of the radical site by the oxyferryl heme, as measured in the stopped-flow mixing of Fe(IV) peroxidase and fluoride, also reflects conformational reorganization, and the transfer rate constant according to eq 4 becomes

$$k_e = k'_e k_c/(k'_{-e} + k_c) \qquad (5)$$

However, in order for the $Fe^{III}R_s^+$ species to be kinetically stable, it is further necessary that $k_c \gg k'_{-e}$, and thus, the overall forward transfer rate simplifies to that for electron transfer: $k_e \doteq k'_e$.

The first stage in the proposed mechanism for H_2O_2 oxidation of ferric cytochrome *c* peroxidase (Figure 1) (Ho et al., 1983) involves a short-lived, strongly oxidizing intermediate that is presumed to be an oxyferryl heme with a π-cation radical centered on the porphyrin macrocycle, denoted as heme(V), and presumed to be analogous to the prosthetic group in compound I of horseradish peroxidase. The present experimental results allow us to set a lower limit to the reduction potential of the heme(V) intermediate relative to other species. The equilibrium constant (K_E) and the free-energy difference for the partitioning of oxidizing equivalents between the oxyferryl heme and protein radical sites correspond to a reduction potential for the radical site that is 104 mV more positive than that for the oxyferryl heme (Table II). Since hydrogen peroxide oxidation of the resting-state enzyme results in oxidation of over 95% of the protein radical site, the heme(V) must have a reduction potential that is at least 80 mV more positive than the R_s conformer of this redox center and thus at least 180 mV above the oxyferryl heme. The R conformer of the radical site would likely be at an even higher potential than R_s. This is generally consistent with the proposed identity of the heme(V), since the reduction potential of compound I of horseradish peroxidase is over 300 mV more positive than that of compound II (Hayashi & Yamazaki, 1979).

Finally, we consider the EPR signals obtained following the addition of fluoride to Fe(IV) peroxidase. The high-spin signal is normal for a fluoroferri hemoprotein, and the $g = 2$ signal is indistinguishable from that of compound ES. Thus, the signals could arise by disproportionation of the Fe(IV) enzyme into the fluoroferric peroxidase and compound ES. However, the H_2O_2 titrations of ferrous peroxidase (Ho et al., 1983) indicated that hole transfer between peroxidase forms is unobservably slow and that such disproportionation is unimportant. Therefore, it seems appropriate to assign the EPR signals to the $Fe^{III}R_s^+F^{-1}$ species, as indicated in eq 1. The $g = 2$ signal from this species is associated with a radical within a protein molecule containing $S = {}^5/_2$ fluoroferric heme, not a $S = 1$ oxyferryl heme as in compound ES. Nevertheless, it is indistinguishable in all respects from the EPR signal of compound ES in the presence of fluoride (Figure 4). Therefore, the EPR signal of the radical site is not influenced by the spin state of the iron, and the observed *g* values of compound ES must be intrinsic properties of the free-radical site, not the result of spin coupling between the ferryl heme and a tryptophanyl radical. Thus, these measurements support the earlier arguments against assigning tryptophan as the radical site, since the *g* values of $g_{\parallel} = 2.05$ and $g_{\perp} = 2.01$ are not consistent with the properties of such a radical (Hoffman et al., 1979, 1981). In addition, were the radical signal associated with tryptophan-51, as suggested by its proximity to the heme iron (Poulos & Kraut, 1980), the line broadening to be expected from dipolar splitting by a neighboring fluoroferri heme can be calculated to be ca. 150 G for either the neutral or cation radicals. Thus, the absence of observed broadening provides a strong argument that an aromatic amino acid, in particular tryptophan-51, is not directly involved in the protein radical site.

It may be noted that the absence of broadening does not appear to speak directly to the proposal that the radical is associated with a sulfur center, in particular with the sulfur closest to the heme, that of methionine-171. If the radical were localized on this sulfur alone, its EPR signal would be broadened by a maximal splitting of only ca. 19 G. Distributing the unpaired electron spin to a nucleophilic amino acid side chain and/or allowing a minor conformational rearrangement to increase the methionine sulfur distance from the heme iron by approximately 2 Å would decrease the dipolar interaction such that the effect becomes unobservable. Such processes could be viewed as an R to R_s conformational rearrangement of the radical site as originally described by the mechanism in Figure 1.

Registry No. Cytochrome *c* peroxidase, 9029-53-2; fluoride, 16984-48-8.

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Modification of the F_0 Portion of the H^+-Translocating Adenosinetriphosphatase Complex of *Escherichia coli* by the Water-Soluble Carbodiimide 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide and Effect on the Proton Channeling Function[†]

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ABSTRACT: 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), a water-soluble carbodiimide, inhibited ECF_1–F_0 ATPase activity and proton translocation through F_0 when reacted with *Escherichia coli* membrane vesicles. The site of modification was found to be in subunit c of the F_0 portion of the enzyme but did not involve Asp-61, the site labeled by the hydrophobic carbodiimide dicyclohexylcarbodiimide (DCCD). EDC was not covalently incorporated into subunit c in contrast to DCCD. Instead, EDC promoted a cross-link between the C-terminal carboxyl group (Ala-79) and a near-neighbor phosphatidylethanolamine as evidenced by fragmentation of subunit c with cyanogen bromide followed by high-pressure liquid chromatography and thin-layer chromatography.

The ATP synthase of animals, bacteria, and plants is a multisubunit enzyme often called F_1F_0, which is able both to catalyze the synthesis of ATP in response to a proton gradient and to generate a transmembrane proton gradient driven by ATP hydrolysis (Pederson et al., 1981; Fillingame, 1980; Senior & Wise, 1983; Futai & Kanazawa, 1983).

The F_1F_0 complex can be divided into a water-soluble part, F_1, which is extrinsic to the lipid bilayer and made up of five different subunits, generally called α, β, γ, δ, and ϵ. These subunits are most likely present in a stoichiometry of 3:3:1:1:1, respectively. It is the F_1 portion which contains the catalytic sites of ATP hydrolysis and ATP synthesis. The F_0 portion is intrinsic to the lipid bilayer and provides the transmembrane part, being composed of three different subunits in *Escherichia coli* called a, b, and c that are present in a stoichiometry of 1:2:(6–10), respectively (Foster & Fillingame, 1982).

Studies with the hydrophobic carbodiimide dicyclohexylcarbodiimide (DCCD)[1] have established that the functionality of F_1 is critically dependent on its binding to F_0. At low concentrations, this reagent binds specifically to a carboxyl in subunit c and inhibits both ATP synthesis and ATP hydrolysis by the intact complex [for a review, see Sebald & Hoppe (1981)].

The F_0 portion of the ATP synthase has been shown to act as a proton channel. DCCD blocks proton movement through this channel (Sebald & Hoppe, 1981).

In the ATP synthase of *E. coli*, called here ECF_1–F_0, the effect of DCCD is due to modification of Asp-61 of subunit c (Wachter et al., 1980a). Recently, there has appeared evidence that carboxyls of subunit c in addition to Asp-61 are important for proton channeling through ECF_1–F_0. Altendorf and colleagues have claimed that water-soluble carbodiimides react with a carboxyl near the N terminus of subunit c to inhibit proton translocation through F_0 (Wachter et al., 1980b; Altendorf et al., 1980). Patel & Kaback (1976) have also

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[1] Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; ECF_1–F_0, H^+-translocating ATPase complex of *E. coli*; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; [^{14}C]ETC, 1-ethyl-3-[3-[^{14}C](trimethylammonio)propyl]carbodiimide; DCCD, dicyclohexylcarbodiimide; FCCP, carbonyl cyanide *p*-(trifluoromethyl)phenylhydrazone; HPLC, high-pressure liquid chromatography; LDAO, lauryldimethylamine oxide; Mops, 3-(*N*-morpholino)propanesulfonic acid; PE, phosphatidylethanolamine; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTE, dithioerythritol; $NaDodSO_4$, sodium dodecyl sulfate.

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