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Elementary Steps in the Formation of Horseradish Peroxidase Compound I: Direct Observation of Compound 0, a New Intermediate with a Hyperporphyrin Spectrum†

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ABSTRACT: The reaction of horseradish peroxidase (HRP) with H_2O_2 has been studied in 50% v/v methanol/water over the 25.0 to −36.0 °C temperature range by using the low-temperature stopped-flow technique. All reactions were carried out under pseudo-first-order conditions with $[H_2O_2] \gg [HRP]$. Arrhenius plots for the pseudo-first-order rate constant k_{obs} were linear over the 17.6 to −36.0 °C temperature range studied with an activation energy of 4.8 ± 0.5 kcal/mol. Above 0 °C, k_{obs} varies linearly with peroxide concentration. However, saturation kinetics are observed below −16.0 °C, indicating that there is at least one reversible elementary step in this reaction. Double-reciprocal plots at −26.0 °C at pH* 7.3 for the reaction give k_{obs}^{max} = 163 s^{-1} and K_M = 0.190 mM. Rapid-scan optical studies carried out at −35.0 °C with $[H_2O_2] \gg K_M$ reveal the presence of a transient intermediate referred to as compound 0 whose conversion to compound I is rate limiting. The Soret region of the optical spectrum of compound 0 resembles that of a "hyperporphyrin" with prominent bands near 330 and 410 nm. The temperature dependencies of k_{obs}^{max} and K_M have been measured over the −16.0 to −26.0 °C range and give an activation energy for k_{obs}^{max} of 1.6 ± 0.7 kcal/mol and an enthalpy of formation for compound 0 of 4.0 ± 0.7 kcal/mol.

One of the most intensively studied reactions in enzymology is that between the hydroperoxidases and H_2O_2. Early pioneering studies indicated that horseradish peroxidase (HRP)[1] (Theorell, 1941) and catalase (Chance, 1947) each react with H_2O_2 to give a discrete, catalytically active reaction intermediate that is referred to as compound I. Subsequent studies have shown that this reaction is common to all of the hydroperoxidases and is characterized by the two-electron oxidation of these heme enzymes (Frew & Jones, 1984). An important goal in the elucidation of the mechanism of action of any enzyme is to identify and characterize both thermodynamically and kinetically all possible elementary steps in the catalytic pathway (Hammes & Schimmel, 1970; Gutfreund, 1975; Auld, 1977). In spite of intensive study, the elementary steps that lead to the formation of compound I have remained largely undefined.

Jones and Dunford (1977) have summarized the kinetic evidence which indicates that the reaction of HRP with H_2O_2 to form compound I is not a diffusion-controlled elementary bimolecular reaction

$$HRP + H_2O_2 \xrightarrow{k} I \qquad (1)$$

but rather a chemically controlled reaction in which there is a preequilibrium of reactants to form at least one precursor complex, HRP–H_2O_2 (where this designation implies that no electron transfer has occurred between the reactants, but is unspecific with regard to its structure), followed by a rate-limiting redox step. The simplest preequilibrium mechanism is

$$HRP + H_2O_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} HRP\text{–}H_2O_2 \xrightarrow{k_2} I \qquad (2)$$

The first piece of evidence in favor of mechanism 2 is the observation that the second-order rate constant of $\sim 10^7$ M^{-1} s^{-1} is lower than expected for an elementary diffusion-controlled bimolecular reaction (Dunford & Hewson, 1977). Second, the rate constants for compound I formation from some of the larger peroxybenzoic acids (e.g., *m*-chloroperoxybenzoic acid) are *larger* than for H_2O_2 (Davies et al., 1976). Third, the second-order rate constant for compound I formation from H_2O_2 is independent of viscosity, while that for *m*-chloroperoxybenzoic acid is viscosity dependent and diffusion controlled (Dunford & Hewson, 1977).

In principle, mechanisms 1 and 2 can be distinguished kinetically. If the first step in mechanism 2 equilibrates rapidly ($k_1[H_2O_2] + k_{-1} \gg k_2$) and $[HRP] \ll [H_2O_2]$, the pseudo-first-order rate constants, k_{obs}, for mechanisms 1 and 2 are

$$k_{obs} = k[H_2O_2] \qquad (3)$$

$$k_{obs} = k_{obs}^{max}[H_2O_2]/([H_2O_2] + K_M) \qquad (4)$$

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[1] Abbreviations: HRP, horseradish peroxidase; pH*, apparent protonic activity; OEP, octaethylporphyrin.

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respectively, where $k_{obs}^{max} = k_2$ and $K_M = (k_{-1} + k_2)/k_1$.[2] Thus, if the mechanism is of the type shown in eq 2, k_{obs} should show saturation behavior as $[H_2O_2]$ is increased above K_M. Saturation kinetics at ambient temperature for the formation of compound I in the pH range of 5–9 have not been observed for any hydroperoxidase[3] (Frew & Jones, 1984). While this can be considered to constitute evidence in favor of mechanism 1, this behavior is also predicted if the highest values of k_{obs} that can be measured in stopped-flow experiments at ambient temperature ($\sim$500 s^{-1}) correspond to $[H_2O_2] \ll K_M$.

Stopped-flow cryoenzymology has been developed to greatly expand the range of study of reactions such as that described above by exploiting the rate reductions brought about by lowering the temperature (Hui Bon Hoa & Douzou, 1973; Hanahan & Auld, 1980; Van Wart & Zimmer, 1981). It has been demonstrated that this technique can resolve elementary steps and detect labile catalytic intermediates not observable by other methods (Van Wart & Lin, 1983; Lin et al., 1988). Recently, this technique has been used to show that the reaction of HRP with H_2O_2 and EtO_2H to form compound I *does* exhibit saturation kinetics at subzero temperatures in methanol- and DMSO-based cryosolvents (Balny et al., 1987). The present study uses the low-temperature stopped-flow technique to carry out a much more detailed study of the reaction of HRP with H_2O_2 in 50% v/v methanol/10 mM phosphate in the 25.0 to −36.0 °C range. It is shown that below −16.0 °C saturation kinetics are observed, but with considerably different rate constants than reported earlier (Balny et al., 1987). Most importantly, our parameters predict conditions that have been used in rapid-scan studies to directly observe the optical spectrum of the intermediate whose conversion to compound I is rate limiting. These studies reveal that the new intermediate, which is called compound 0 since it precedes compound I on the catalytic pathway, has a hyperporphyrin optical spectrum. This unexpected result raises new questions with regard to the interactions of H_2O_2 with the heme group of hydroperoxidases.

Materials and Methods

Materials. HRP (EC 1.11.1.7) isozyme C was purchased from Sigma Chemical Co. (type VI) and used without further purification. The ratio of absorbance at 403/280 nm was above 3.0. Reagent grade H_2O_2 (30% v/v) was obtained from J. T. Baker Chemical Co. and spectranalyzed methanol was purchased from Fisher Scientific. All buffer solutions were prepared in reagent grade water with a resistivity of 18 MΩ/cm prepared with a Millipore Milli-Q system. The concentrations of HRP and H_2O_2 were determined spectrophotometrically by using $\epsilon_{403} = 1.02 \times 10^5$ M^{-1} cm^{-1} (Schonbaum & Lo, 1972) and $\epsilon_{240} = 39.4$ M^{-1} cm^{-1} (Nelson & Kiesow, 1972), respectively.

Low-Temperature Stopped-Flow Experiments. The low-temperature stopped-flow experiments were carried out with a modified form of an instrument described elsewhere (Van Wart & Zimmer, 1981). For single-wavelength measurements, monochromatic light is carried to the observation cell (path length, 2 cm) and the transmitted light taken to a Hamamatsu Model R136 photomultiplier tube with Oriel liquid light guides. The current from the phototube is amplified and converted to a voltage signal that is transmitted to an IBM-XT computer using a Data Translation Model 2801a A/D converter and stored on a floppy disk. Graphical display of the data, the conversion of transmittance to absorbance, and subsequent quantitative analysis were accomplished with standard programs. More than 200 data points were used in each rate constant determination. The reported rate constants are the average of at least three trials. For the rapid-scan stopped-flow experiments, white light from an Oriel 75-W xenon lamp was attenuated with a neutral density filter and focused onto the observation cell and the transmitted light focused onto a Princeton Applied Research Model 1226 spectrograph equipped with a Model 1420 intensified diode array detector. Data from the diode array were transferred to an IBM-XT computer with a Model 1461 detector interface and stored on a floppy disk. Data acquisition, storage, and retrieval were performed by using the OMA III program. The wavelength calibration of the diode array was achieved by using the lines from a mercury lamp.

All stopped-flow measurements were performed under pseudo-first-order conditions with $[H_2O_2] \geq 10[HRP]$, and values of the pseudo-first-order rate constant, k_{obs}, were obtained from fits to the exponential change in absorbance at 397 nm. The apparent second-order rate constants for the reactions, k'_2, were calculated by dividing k_{obs} by $[H_2O_2]$. The cryosolvent used in all of the low-temperature stopped-flow experiments was 50% v/v methanol/10 mM phosphate. The apparent protonic activity in this solvent, pH*, at subzero temperatures was estimated from tables of the temperature dependence of pH* for this solvent (Douzou, 1977), as described earlier (Lin et al., 1988). A minimum of 1 h was allowed for temperature equilibrium to occur before kinetic runs were initiated. The temperature of the stopped-flow system was monitored throughout all experiments with an Omega copper/constantan grounded thermocouple and an Omega Model 2176 A-T digital thermometer to an accuracy of ±0.2 °C.

Results and Discussion

Choice of Cryosolvent. In order to use the low-temperature stopped-flow technique to study the compound I formation reaction at subzero temperatures, an appropriate cryosolvent is needed. Methanol has been chosen as an organic cosolvent for a variety of reasons, including its low viscosity, high dielectric constant, and low freezing point (Douzou, 1977). Specifically, a 50% v/v methanol/water solution has a freezing point of −55 °C, and over the 20 to −40 °C temperature range, the dielectric constant varies from 60.3 to 83.9 and the viscosity from 1.61 to 38 cP, respectively (Douzou, 1977). Even at −40 °C, the viscosity is below the value of 50 cP found to be the limit for correct mixing in the stopped-flow instrument (Travers et al., 1975). Phosphate was chosen as the buffer because of its small change in pK with temperature and high solubility at low temperatures. Methanol/water-based cryosolvents have been used for HRP in the past without difficulties (Douzou, 1977; Van Wart & Zimmer, 1981, 1985; Balny et al., 1987).

Effect of Methanol on HRP and Rate of Formation of Compound I. The effect of methanol on the pseudo-first-order rate constant, k_{obs}, for the formation of compound I from HRP and H_2O_2 at 0 °C at pH* values of 5.5, 7.5, and 9.0 is shown in Figure 1. The value of k'_2 calculated from similar data at 23 °C in the absence of methanol is 1.4×10^7 M^{-1} s^{-1} and is in good agreement with values reported in the literature (Hewson & Dunford, 1975; Davies et al., 1976; Dunford &

[2] For more complex versions of mechanism 2 in which there are other intermediates between the precursor complex and compound I, k_{obs} will be the same form as given in eq 4, except that k_{obs}^{max} and K_M will be comprised of other combinations of elementary rate constants.

[3] Note that saturation kinetics have been observed at ambient temperature for the reaction of HRP with H_2O_2 between pH 10 and 11.5 (Job & Dunford, 1978).

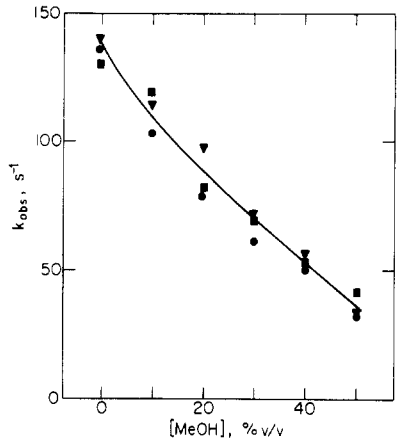

FIGURE 1: Effect of methanol on k_{obs}, the pseudo-first-order rate constant for the reaction of 1 μM HRP and 15 μM H_2O_2 to form compound I, at pH* values of (●) 5.5, (▼) 7.5, and (■) 9.0 at 0 °C.

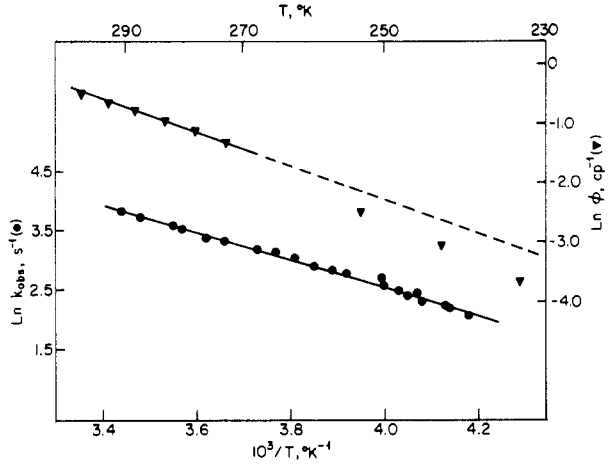

FIGURE 2: Arrhenius plots for (●) k_{obs} for the reaction of 1 μM HRP with 12.5 μM H_2O_2 to form compound I in 50% v/v methanol/10 mM phosphate, pH* 7.3, and (▼) the fluidity of 50% v/v methanol/water.

Hewson, 1977; Ohlsson et al., 1984; Balny et al., 1987). As the methanol concentration is increased, there is a monotonic and approximately equal decrease in k_{obs} at all three pH* values. In 50% v/v methanol, the rate of the reaction is lowered 3.5-fold.

Effect of Temperature on Rate of Formation of Compound I. Values of k_{obs} for the reaction of HRP with H_2O_2 to form compound I have been measured over the 17.6 to −36.0 °C temperature range in 50% v/v methanol/10 mM phosphate, pH* 7.3, hereafter referred to as cryosolvent, under pseudo-first-order conditions. An Arrhenius plot constructed from these data is shown in Figure 2 and is linear over the entire temperature range studied and gives E_a = 4.8 ± 0.5 kcal/mol. The linearity of this Arrhenius plot indicates that there is no change in the rate-determining step for the reaction or change in conformation of HRP that affects its catalytic efficiency over this temperature range. Thus, the overall reduction in rate is simply attributable to the lowered temperature.

Also shown in Figure 2 is an Arrhenius plot for the fluidity (φ, the inverse of the viscosity) of 50% v/v methanol/water obtained by using viscosity data from the literature for the 0–25 °C (Dunford, 1983) and −30 to −40 °C (Douzou et al., 1976) regions. Due to the large errors in the values in the −30 to −40 °C region, the line in the Arrhenius plot has been drawn by using only the more accurate data in the 0–25 °C range to give $E_a^{solvent}$ = 5.3 ± 0.2 kcal/mol. If the three points at lower temperatures are included, then $E_a^{solvent}$ = 6.9 ± 0.4 kcal/mol, an even larger value. Thus, the E_a value for the compound I formation reaction is lower than, but within experimental error of, the value for the fluidity of the solvent. This result is very similar to that obtained by Hewson and Dunford (1975) for this reaction in water over the 3.7–70.0 °C range.

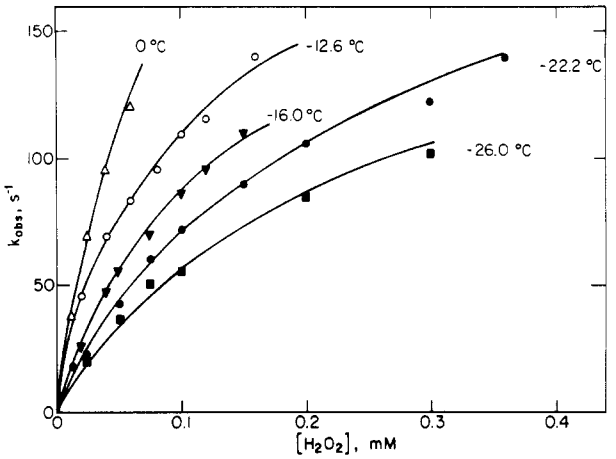

FIGURE 3: Variation in k_{obs} as a function of H_2O_2 concentration for the reaction of 0.8 μM HRP to form compound I in 50% v/v methanol/10 mM phosphate, pH* 7.3: (△) 0, (○) −12.6, (▼) −16.0, (●) −22.2, and (■) −26.0 °C.

Table I: Kinetic Parameters for Reaction of HRP with H_2O_2 To Form Compound I[a]

pH*	T (°C)	k_{obs}^{max} (s^{-1})	K_M (mM)	k_{obs}^{max}/K_M (mM^{-1} s^{-1})
7.3	−16.0	192	0.134	1430
	−19.0	186	0.151	1230
	−22.2	181	0.161	1120
	−25.0	179	0.174	1030
	−26.0	163	0.190	858
5.3	−26.0	181	0.264	686
9.0	−26.0	204	0.235	868

[a] All reactions were carried out in 50% v/v methanol/10 mM phosphate at the pH* and temperature indicated.

Effect of Hydrogen Peroxide Concentration on Rate of Formation of Compound I. Values of k_{obs} for the reaction of HRP with H_2O_2 to form compound I were measured as a function of [H_2O_2] at various subzero temperatures in the cryosolvent. The mixing time of the instrument is viscosity (and, thus, temperature) dependent, and the maximum values of k_{obs} that could be measured accurately at subzero temperatures are approximately 100–150 s^{-1}. At 0 °C, k_{obs} varies approximately linearly with [H_2O_2] up to the maximum measurable value (Figure 3). However, as the temperature is progressively lowered, the plots of k_{obs} vs [H_2O_2] start to exhibit the saturation behavior predicted by eq 4. Double-reciprocal plots of $1/k_{obs}$ vs $1/[H_2O_2]$ have been constructed, from which values of k_{obs}^{max} and K_M have been evaluated. These parameters will not be accurate if $[H_2O_2]_{max}$ is less than K_M. Thus, only the data at −16.0, −19.0, −22.2, −25.0, and −26.0 °C, which satisfy this criterion, have been used to evaluate k_{obs}^{max} and K_M. Saturation behavior is also observed for the reactions with H_2O_2 at pH* 5.3 and 9.0 at −26.0 °C (data not shown), and the values of k_{obs}^{max} and K_M are very similar to those at pH* 7.3. Values of k_{obs}^{max} and K_M for all of these reactions are summarized in Table I. Our values of k_{obs}^{max} = 204 s^{-1} and K_M = 0.235 mM at pH* 9.0 and −26 °C in 50% methanol/10 mM phosphate do not agree well with those of k_{obs}^{max} = 330 s^{-1} and K_M = 1.1 mM reported by Balny et al. (1987) at pH* 8.7 and −29.5 °C in 50% methanol/40 mM Tris. The results of similar studies using EtO_2H as the peroxide also disagree

with those of Balny et al. (1987). These differences will be discussed in a subsequent paper (Baek and Van Wart, in preparation).

The saturation kinetic behavior observed in Figure 3 is a reflection of the change in reaction order with respect to H_2O_2 predicted from eq 4 when $[H_2O_2]$ approaches and exceeds K_M. Control experiments have been performed in which HRP was recovered from reactions carried out at H_2O_2 concentrations in the saturation region at these temperatures. The enzyme was found to retain full activity in conventional assays, indicating that the leveling off of k_{obs} at high $[H_2O_2]$ is not due to damage to the enzyme brought about by the H_2O_2. Moreover, in all of these reactions, rapid scan studies (see below) showed that compound I was formed with the correct spectral properties and was ultimately converted completely to a normal compound II. The ability to observe saturation kinetics at subzero temperatures is the consequence of the overall lowering of k_{obs} for this reaction, thus enabling the use of $[H_2O_2]$ that are greater than K_M. For example, for the reaction with H_2O_2, the value of K_M extrapolated to 25 °C is 46 μM, while the maximum $[H_2O_2]$ usable at this temperature is 10 μM. Thus, saturation kinetics are not observable. However, the reduction in temperature to −26.0 °C lowers the overall rate 7.6-fold, and even though K_M is increased 4.1-fold, the maximum usuable $[H_2O_2]$ is increased 30-fold to 0.30 mM, a value that exceeds K_M. The observation of saturation kinetics constitutes direct evidence for a reversible step in the catalytic pathway and points to a mechanism of the type shown in eq 2 with one or more intermediates.

Direct Observation of a New Intermediate. The saturation kinetic behavior observed at subzero temperatures indicates that, at high $[H_2O_2]$, a reaction intermediate is formed whose conversion to compound I is rate limiting. If it is assumed that a single intermediate accumulates and that it is in a steady state with the reactants, then k_{obs} for the formation of compound I and decay of the intermediate are both given by eq 4. Moreover, the fraction of HRP present as the intermediate is given by

$$[\text{intermediate}]/[\text{HRP}] = [H_2O_2]/([H_2O_2] + K_M) \quad (5)$$

and its half-life will be $t_{1/2} \geq (k_{obs}^{max})^{-1}$. Thus, the intermediate should be directly observable under conditions where $[H_2O_2] > K_M$, provided that $t_{1/2}$ is long enough.

Rapid-scan stopped-flow experiments of the reaction of HRP with 1 mM H_2O_2 have been carried out in the cryosolvent, pH* 7.3 at −35.0 °C, in order to observe the optical spectrum in the Soret band region of the intermediate. The results are shown in Figure 4, where curve a is HRP (λ_{max} = 406 nm) and curve b is compound II (λ_{max} = 420 nm) that is rapidly formed from compound I at this pH* and $[H_2O_2]$. When the time between stopped-flow experiments is long, compound II is present in the observation cell prior to a new mixing experiment. Curve c is a 21-ms scan initiated at the start of mixing. It shows a band due to some residual compound II at 420 nm and bands near 330 and 400 nm due to the new intermediate. Curve d is the next 21-ms scan which shows a trace of residual compound II (since the mixing time is 20–30 ms) and bands with decreased intensity near 330 and 400 nm due to the reaction intermediate. The next scan (not shown) indicates complete formation of compound I with a band at 405 nm. These spectral changes are not observed in control experiments involving the mixing of solvent with solvent, solvent with H_2O_2, or solvent with HRP. Since these spectra show that the species that exhibits the 330- and 400-nm bands precedes compound I, it is attributed to a reaction intermediate that will be referred to as compound 0.

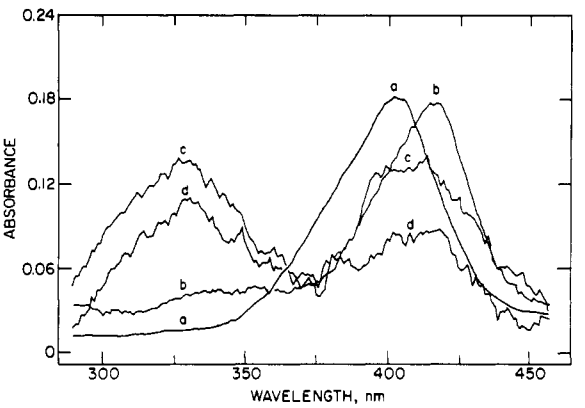

FIGURE 4: Rapid-scan optical spectra of the reaction of 1 μM HRP with 1 mM H_2O_2 in 50% v/v methanol/10 mM phosphate, pH* 7.3 at −35.0 °C. Curves a and b are spectra of HRP and compound II, respectively. Curves c and d are consecutive 21-ms scans initiated at the start of mixing, which show the formation of compound 0 and its conversion to compound I.

The optical spectrum of compound 0 resembles that of a "hyperporphyrin" with an extra band in the Soret region. A compound 0 in the reaction of microperoxidase 8 with H_2O_2 at −35.0 °C has also been observed that exhibits very similar absorption bands near 330 and 410 nm (Baek, Wang, and Van Wart, unpublished data). It is also noteworthy that an intermediate with a similar optical spectrum has been reported in the reaction of deuteroferriheme with *m*-chloroperoxybenzoic acid at 25 °C in water (Portsmouth & Beal, 1971; Kelly & Yasui, 1984). Hyperspectra can arise when metalloporphyrin–ligand complexes have charge-transfer transitions with the same symmetry and energy as the Soret π–π^* transition, in which case the transitions mix and split into high- and low-energy components (Gouterman, 1978). These criteria are met in Mn(III)–porphyrin–oxygen complexes in which there is a charge-transfer band from the porphyrin to a Mn(III)–O_2 hybrid orbital that mixes with the Soret transition (Hanson & Hoffman, 1980) and in the cytochrome P-450–CO complex which has an appropriate charge-transfer transition from the electron-rich mercaptide ligand to the porphyrin (Hanson et al., 1976).

It is possible that compound 0 exhibits a hyperporphyrin spectrum as the result of a similar charge-transfer transition involving its constituents. For example, if compound 0 is an Fe(V) porphyrin, it could emulate the situation in Mn(III)–porphyrin complexes. Alternatively, compound 0 could be a ferric porphyrin complex of HO_2^-, or O_2^{2-} in which there is a charge-transfer transition from these electron-rich species to the porphyrin, in a manner similar to that in cytochrome P-450. Alternatively, since the OEP dication radical exhibits well-defined absorption bands in the 300–360-nm region (Fajer et al., 1970), compound 0 could be an Fe(III) dication radical produced by selective oxidation of the porphyrin ligand. One other possibility that merits consideration is that compound 0 could be an Fe(III) isoporphyrin produced by attack or migration of HO_2^- to a methine carbon. A similar species exhibits two broad bands in the Soret region of the type observed here for compound 0 (Gold et al., 1984). Additional studies are under way to investigate these possibilities.

Mechanism of Compound I Formation. The mechanism for compound I formation must explain the saturation kinetics observed at high $[H_2O_2]$ and be consistent with the formation of compound 0 with its unusual optical spectrum. It was anticipated that the species whose optical spectrum was observed above would be the precursor complex shown in eq 2. HRP is known to formally bind the neutral form of acid

ligands, but the resultant heme complexes are believed to be between Fe(III) and the anionic form of the ligand with its proton stored on a basic amino acid residue (Dunford & Stillman, 1976). For example, Thanabal and associates (1988) have shown from NMR data that the HCN complex of HRP is an Fe(III)CN complex with the proton transferred to the distal His-42 to form an imidazolium side chain. Thus, the initial precursor complex in the compound I formation reaction has been thought to be the corresponding ferric hydroperoxyl anion Fe(III)(^-O_2H), as has been proposed for cytochrome *c* peroxidase (Poulos & Kraut, 1980). Since there is no precedent for heme species of this type having the unusual optical spectrum found here for compound 0, the possibility must be considered that compound 0 is formed from the precursor complex *after* an oxidation step involving electron transfer between HO_2^- and the heme group.

Mechanism 1 can be discarded because it does not account for the saturation kinetics. The two simpliest mechanisms that are consistent with the data are variations of mechanism 2. The first is mechanism 2 as shown in which k_2 is rate limiting and compound 0 *is* the precursor complex and has an unusual optical spectrum for reasons not presently understood. The second mechanism is

$$\mathrm{HRP} + \mathrm{H_2O_2} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{HRP\text{–}H_2O_2} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} 0 \xrightarrow{k_3} \mathrm{I} \qquad (6)$$

where HRP–H_2O_2 is a precursor complex, compound 0 is an oxidized form of HRP, and step 3 is the rate-determining step. If either of the first two steps equilibrates more rapidly than step 3 in this mechanism, then the rate of compound I formation will be given by eq 4 with

$$k_{obs}^{max} = k_2k_3/(k_2 + k_{-2}) \qquad (7)$$

$$K_M = [k_{-2}/(k_2 + k_{-2})]K_s \qquad (8)$$

where $K_s = k_{-1}/k_1$ (Bernasconi, 1976).

Other versions of mechanism 6 can be eliminated on the basis of the observed data. Mechanism 6 with $k_{-2} = 0$ fails to predict saturation kinetics, since eq 8 shows that this corresponds to $K_M = 0$. A combination of mechanisms 2 and 6 in which there is no precursor complex, but in which compound 0 is an oxidized form of HRP that is in direct equilibrium with reactants, has also been discarded. While this mechanism is formally consistent with the data, it seems unlikely that such an initial oxidation step would be reversible. Other mechanisms are possible, but mechanisms 2 and 6 are the least complex that are consistent with the current data.

Temperature Dependence of k_{obs}^{max} and K_M. Plots of ln k_{obs}^{max} and ln K_M^{-1} vs T^{-1} covering the −16.0 to −26.0 °C range (Table I) are shown in Figure 5. The plot for k_{obs}^{max} gives E_a = 1.6 ± 0.7 kcal/mol, and extrapolation of the data shows that lowering the temperature from 25.0 to −26.0 °C reduces k_{obs}^{max} less than 2-fold. K_M^{-1} is the apparent association constant for compound 0 in both mechanisms 2 and 6, which can for the present purposes be collectively written as

$$\mathrm{HRP} + \mathrm{H_2O_2} \underset{}{\overset{K_M^{-1}}{\rightleftharpoons}} 0 \xrightarrow{k_{obs}^{max}} \mathrm{I} \qquad (9)$$

where K_M^{-1} equals the steady-state concentration of compound 0 divided by the free concentrations of HRP and H_2O_2. The slope of the plot shown in Figure 5 for K_M^{-1} can then be used to calculate ΔH_0, the enthalpy change associated with the *formation* of compound 0 from HRP and H_2O_2 in its steady state. The value obtained from these data is ΔH_0 = 4.0 ± 0.7 kcal/mol. The positive sign of ΔH_0 means that the steady-state concentration of compound 0 is greater at higher temperatures and that its formation is an *uphill process* in the catalytic pathway. The activation energy, $E_a^{overall}$, calculated from the

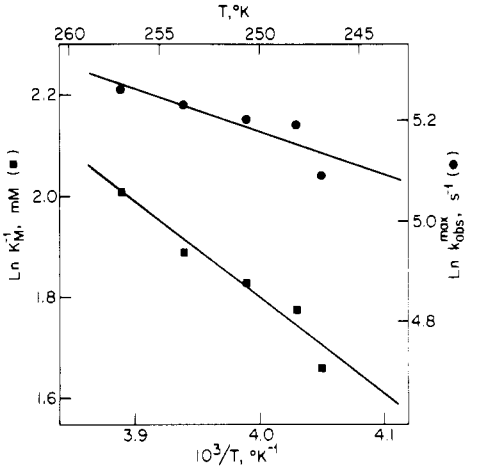

FIGURE 5: Arrhenius plots for (●) k_{obs}^{max} and (■) K_M^{-1} for the reaction of HRP with H_2O_2 to form compound I in 50% v/v methanol/10 mM phosphate, pH* 7.3.

Arrhenius plot shown in Figure 2 pertains to k_{obs}^{max}/K_M and reflects the two separate terms:

$$E_a^{overall} = E_a(k_{obs}^{max}) + \Delta H_0 \qquad (10)$$

The value of E_a of 5.3 ± 0.5 kcal/mol calculated from the −16 to −26 °C region of this plot agrees well with the value calculated from eq 10 by substitution of the values of 4.0 ± 0.7 and 1.6 ± 0.7 kcal/mol measured independently over the same temperature range for ΔH_0 and $E_a(k_{obs}^{max})$, respectively. Note that for mechanism 2 with $k_{-1} \gg k_2$ and mechanism 6 with $k_{-2} \gg k_2$, $K_M = K_s$ and ΔH_0 is the standard enthalpy of formation of compound 0.

Relationship between $E_a^{overall}$ and $E_a^{solvent}$. One of the interesting features of the compound I formation reaction is the relative value of $E_a^{overall}$ and the activation energy for the fluidity of the solvent, $E_a^{solvent}$. In aqueous solution over the 3.7–70.0 °C temperature range, $E_a^{overall}$ is 3.5 ± 1.0 kcal/mol, while E_a^{water} is 3.89 ± 0.04 kcal/mol (Hewson & Dunford, 1975). In the cryosolvent used here, $E_a^{overall}$ = 4.8 ± 0.5 kcal/mol, while $E_a^{solvent}$ = 5.3 ± 0.2 kcal/mol. Although $E_a^{overall}$ and $E_a^{solvent}$ are within experimental error for both solvents, the most probable value of $E_a^{overall}$ is actually *less than* $E_a^{solvent}$ in both. For an irreversible bimolecular elementary reaction, this should only be true if the reaction possesses a means of overcoming the normal barriers to diffusion. However, for the preequilibrium mechanism shown in eq 9, $E_a^{overall}$ is given by eq 10 and can attain values that are less than $E_a^{solvent}$, or even negative, depending on the value of $E_a(k_{obs}^{max})$ and the sign and magnitude of ΔH_0. Values of $E_a^{overall}$ less than $E_a^{solvent}$ have been observed for electron-transfer reactions between certain metal ion complexes that associate in solution prior to the redox step because the values of ΔH_0 were negative (Sutin, 1973). Jones and Dunford (1977) have made the interesting suggestion that the low value of $E_a^{overall}$ for the compound I formation reaction in water might be explained by similar circumstances. While it has not been possible to examine the individual values of $E_a(k_{obs}^{max})$ and ΔH_0 for the reaction in water, these parameters have been determined for the reaction carried out here in the cryosolvent. The results show that ΔH_0 is, in fact, positive and indicate that the basis for the low value of $E_a^{overall}$ for the compound I formation reaction must lie elsewhere.

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