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Mechanism of Cytochrome *c* Peroxidase. *O*-Benzoylhydroxylamine as an Analog of Hydrogen Peroxide†

Andrew F. W. Coulson* and Takashi Yonetani

ABSTRACT: A number of reagents, some of which are electronic analogs of hydrogen peroxide, will replace it in the reactions of cytochrome *c* peroxidase. These compounds include *N*-bromosuccinimide, sodium hypochlorite, and the novel oxidizing agent *O*-benzoylhydroxylamine. If fragments of the oxidant played a functional role in the structure of the oxidized form of the enzyme, it would be expected that the product formed from *O*-benzoylhydroxylamine would differ from that formed from hydrogen peroxide. The products formed on reaction of the two oxidizing agents with cytochrome *c* peroxidase are indistinguishable. This result carries implications for the structure of the so-called ES compound. The extension in the range of specific substrates for cytochrome *c* peroxidase allows identification of the structure which compounds must possess to be oxidizing substrates for the enzyme. A mechanism for the first step of the reaction is suggested. *O*-Benzoylhydroxylamine is also a reducing agent, and its reaction with the enzyme is analogous to that of hydrogen peroxide with catalase. The final product of the reaction is the inert nitric oxide complex of ferrous cytochrome *c* peroxidase.

Cytochrome *c* peroxidase (EC 1.11.1.5, ccp[1]) is a hemoprotein which catalyses the oxidation of cytochrome *c* by hydrogen peroxide (Yonetani, 1970). The enzyme has an obligatory order mechanism in which ferric ccp first reacts with peroxide. The so-called "ES compound" of the enzyme which is formed then oxidizes 2 mol of ferrous cytochrome *c* and is itself reduced to the native state.

$$\text{ccp} + H_2O_2 \longrightarrow \text{ES}$$

$$\text{ES} + 2\text{ferrocytochrome } c \longrightarrow \text{ccp} + 2\text{ferricytochrome } c$$

Physical measurements provide clues to the nature of ES, but its precise structure remains unknown. A chemical approach to the problem is to see how the properties of ES depend on the nature of the oxidizing substrate. Schonbaum (Schonbaum, 1970) has shown that stoichiometric quantities of ethanol can be distilled out of frozen solutions of ES prepared from ccp and ethyl hydroperoxide while the protein remains in its oxidized form and this implies that at least half of the oxidant molecule is freed from the protein on formation of ES.

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[1] Abbreviations used are: ccp, cytochrome *c* peroxidase; ES, product of reaction of ccp with H_2O_2: $BzONH_2$, *O*-benzoylhydroxylamine.

In this paper we shall describe experiments to explore the properties of ES formed with unnatural oxidizing agents. Related earlier work (George, 1953; Fergusson, 1956) demonstrated the special difficulties that ccp makes for this kind of study. Since all the enzyme reactions are redox processes and may involve only the transfer of electrons, there is every chance that powerful oxidizing reagents will oxidize the protein directly to ES compound by routes which have no relation to the enzymic mechanism. Many of the oxidizing agents studied by these authors are not so inert as H_2O_2 and it was suggested by Fergusson that the reactions observed with most or all of the reagents studied by George and by Fergusson might proceed by the intermediacy of peroxides. These peroxides could arise either by the oxidation of water, or by reduction of oxygen by protein free radicals produced by the primary oxidant.

It is therefore necessary to establish carefully that the reactions studied are truly analogous at the molecular level to the normal reaction of hydrogen peroxide, and for this reason we have chosen to study reagents whose structure or specificity suggest that they may be electronic analogs of the specific substrate.

Materials and Methods

Instruments. Visible and ultraviolet spectra and absorbance measurements were made on a Perkin-Elmer Model 124, with scale expander and pen recorder, on a Cary Model 15, or on a Perkin-Elmer Model 124.

Electron paramagnetic resonance (EPR) spectra were recorded on a Varian Model V-4502.

High speed reactions were observed with an Aminco-Morrow stopped-flow apparatus.

Materials. Ccp was prepared as described previously (Yonetani and Ray, 1965). Concentrations of ccp solutions were measured spectrophotometrically, using the value ϵ 93 mM^{-1} cm^{-1} (Yonetani, 1970). Sperm whale myoglobin was obtained from Calbiochem and horseradish peroxidase from Sigma.

All other reagents were commercial samples of laboratory or analytical grade except for the sodium hypochlorite solution which was sold for use as a bleach. Aqueous bromine solutions were made by shaking bromine (5 ml) with water (25 ml) and separating the layers. One milliliter of the aqueous solution was diluted to 1 l. with water, to give a final concentration of about 190 μM. Aqueous sodium hypobromite solutions were made by slow addition of 9.7 ml of Br_2 with stirring to a solution of 21.5 g of NaOH in 270 ml of water at 0°. Concentrations of these solutions were estimated either from the weight of bromine used, or by titration via potassium iodide and iodine against sodium thiosulfate solution standardized against solid KIO_3. This method was used also to determine the concentration of the sodium hypochlorite solutions.

O-Benzoylhydroxylamine, in the form of its hydrochloride, was made by Jencks' method (Jencks, 1958) or by that of Carpino et al. (Carpino et al., 1959). The IR spectra of these two products were identical and so were the mobilities of the free base on thin-layer chromatography (TLC) (silica gel plate developed with benzene; R_f 0.3). The proton nuclear magnetic resonance (NMR) spectrum of a solution of the free base in $CDCl_3$ had the following peaks (shifts are given in ppm from TMS): (1) broad singlet, shift = 6.6, relative intensity 2, $-NH_2$; (2) triplet (J = 8 Hz), shift = 7.43, relative intensity 2, 3-phenyl protons; (3) triplet (J = 7 Hz), shift = 7.57, relative intensity 1, 4-phenyl protons; (4) doublet (J = 8 Hz), shift = 8.02, relative intensity 2, 2-phenyl protons.

Methods. The reactions of oxidizing agents with ccp were observed by the addition of small aliquots of aqueous oxidizing agents to buffered (0.1 M phosphate (pH 6) except as noted) solutions of ccp (either 10 or 100 μM) in 1-cm or 1-mm path-length cells. Changes in absorbance at 420 nm were observed as functions of time and of the volume of reagent added.

Reduction reactions were observed in a similar way by addition of small aliquots of aqueous potassium ferrocyanide solution to oxidized protein solutions. When necessary, ferrocyanide solution concentrations were determined by titration against ccp oxidized with hydrogen peroxide.

Samples for EPR spectroscopy were prepared by mixing suitable volumes of aqueous oxidant solutions and, when necessary, aqueous ferrocyanide solutions with 200-μl aliquots of 1 mM ccp solutions in pH 6 0.1 M phosphate buffer; 20-μl portions of the reaction mixture were removed and diluted with 2 ml of the same buffer for observation of the uv and visible spectra. The remainder of each sample was rapidly frozen in liquid nitrogen and the EPR spectrum examined.

Results

Reaction of ccp with N-Bromosuccinimide. Addition of *N*-bromosuccinimide to ccp solutions produced an increase in absorbance of the protein at 420 nm corresponding to oxidation of ccp to its ES compound. The reaction was complete within 30 sec when 10 μM ccp was allowed to react with similar concentrations of *N*-bromosuccinimide at pH 6. A titration curve for the reaction is shown in Figure 1. The overall change in absorbance at 420 nm corresponds to 96% of that expected for complete formation of ES compound (Yonetani, 1970), and 3.5 mol of *N*-bromosuccinimide were required to oxidize each mole of ccp. At the end of the titration, the spectrum was scanned. Peaks were observed at 419, 530, and 560 nm and the ϵ values were 96 mM^{-1} cm^{-1}, 15 mM^{-1} cm^{-1}, and 17 mM^{-1} cm^{-1}, respectively (cf. for ES (Yonetani 1970) 419 nm, 100 mM^{-1} cm^{-1}; 561 nm, 14 mM^{-1} cm^{-1}). The initial rate of decay of the product (from the decrease in absorbance at 420 nm) was 0.5% min^{-1}.

Addition of a 5.3-fold excess of *N*-bromosuccinimide to ccp produced apparently complete oxidation to ES. Figure 1 shows a reductive titration of the product with ferrocyanide. The end point corresponds to recovery of 80% of the oxidizing equivalents involved in the formation of the ES compound. The curvature of the reductive titrations of ES compound has been considered before (Coulson et al., 1971).

Reaction of ccp with N-Chlorosuccinimide. This reaction was examined under similar conditions to those used for the bromo compound. The reaction was slow, and the yield of the oxidized product much reduced. No attempt was made to take the reaction to conclusion, but from the absorbance changes in the early part of the reaction it was calculated that a 30-fold excess of oxidant would have been required for formation of ES.

Stability of N-Bromosuccinimide in Water. Since *N*-bromosuccinimide may be purified by crystallization from hot water, it is evidently reasonably stable at its own pH (about 5 for a 10 mM solution). Acidification of aqueous *N*-bromosuccinimide with acetic acid produced no extensive rapid formation of bromine, but addition of bromide

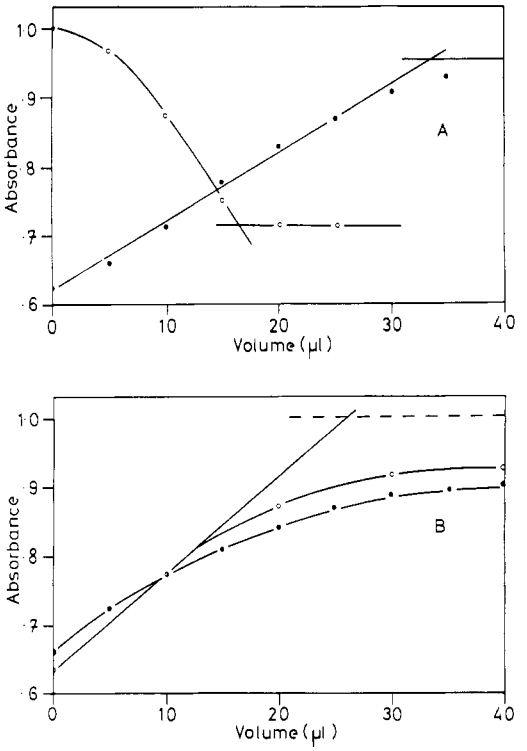

FIGURE 1: (a) (●) Titration of cytochrome *c* peroxidase (2 ml; 9.5 μM) with *N*-bromosuccinimide (2.0 mM). (○) Titration of ES compound formed from cytochrome *c* peroxidase (2 ml, 10.4 μM) and *N*-bromosuccinimide (55 μM) with potassium ferrocyanide (2.0 mM). (b) (●) Titration of cytochrome *c* peroxidase (2 ml; 9.1 μM) with sodium hypochlorite (10 mM). (○) Titration of cytochrome *c* peroxidase (0.22 ml, 90 μM) with aqueous *O*-benzoylhydroxylamine (1.13 mM). In each case, the absorbance at 420 nm (1-cm path-length cuvets; 1-mm path length for the *O*-benzoylhydroxylamine reaction), corrected for dilution, is plotted against the volume of reagent added. The broken line indicates the expected value for complete formation of ES compound.

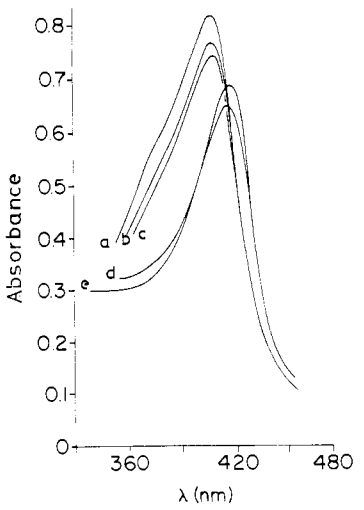

FIGURE 2: Soret band spectra of cytochrome *c* peroxidase (2.5 ml; 8.7 μM in pH 7 0.1 M phosphate buffer) after addition of bromine. The molar excesses of oxidant added at each stage were: (a) 0; (b) 0.08; (c) 0.94; (d) 9.5; (e) 95. Spectrum d has a greater absorbance at the peak than spectrum e.

ion at pH 5 produced a rapid formation of bromine and the reaction was faster still in acid. Thus, there is no rapid irreversible formation of HOBr or Br_2 in neutral solutions of *N*-bromosuccinimide, and either of these could only be the true oxidizing agent if it were present at a low equilibrium concentration, and if its reaction rate were correspondingly faster than that observed with *N*-bromosuccinimide.

Reaction of Aqueous Bromine with ccp. No significant change was observed in the spectrum of ccp when aqueous bromine was added to it under the usual conditions until the bromine was present in a large excess (Figure 2). With 95-fold excess of oxidant, slow progressive changes occurred in the Soret region which apparently represent loss of heme and destruction of the enzyme.

Reaction of Sodium Hypobromite with ccp. Preliminary experiments showed that addition of sodium hypobromite solutions to aqueous ccp produces spectral changes expected for the formation of ES, but that even a moderate excess leads to rapid destruction of the enzyme. The pH dependence of these effects was examined by addition of small (two-fold) and large (20-fold) excesses of NaOBr solution to ccp in 0.1 M phosphate buffers of pH between 5 and 8. The apparent yield of ES after the first addition was greatest at pH 6 (58%) and fell to 15–20% at pH 7 and 8. The initial rate of decay after the second addition was not strongly pH dependent, except that it became very slow at pH 8. Further experiments were carried out at pH 5 or 6. A titration at pH 6 ended with a spectral change corresponding to 88% formation of ES with a 3.5-fold molar excess of oxidant. At pH 5 the reaction was slower and a final absorbance change of 82% of that expected, required a 12-fold excess of oxidant. The yield was larger when larger aliquots of oxidant were used. The peak positions and absorbance values for the spectrum of the NaOBr product corresponded to those found in the spectrum of ES compound formed from H_2O_2. The ES compound formed on addition of NaOBr was titrated with ferrocyanide. All the oxidizing equivalents represented by the ES compound formed were recovered on reduction.

Reaction of ccp with Sodium Hypochlorite. Preliminary experiments showed that the reaction of NaOCl resembled that of NaOBr, but that both the initial oxidation and the subsequent decay reactions were faster than in the first case. The effect of pH was examined also in this case, and it was found that the apparent yield of ES was greatest at pH 6–7; the decay reaction was fastest at pH 8, while at pH 5 the larger excess of oxidant precipitated the protein within a few seconds of mixing. Figure 1 shows the course of a titration of ccp with NaOCl. The end point corresponds to the absorbance change expected for 82% formation of ES, and extrapolation from the first part of the curve showed that an 11-fold excess of oxidant would have been required for complete reaction. The spectrum of the product had peaks at 419, 530, and 560 nm. Back titration with ferrocyanide gave a 94% recovery of oxidizing equivalents.

Absence of Catalatic Reaction with NaOCl. The convex titration curve of Figure 1 might have been due to a catalatic reaction of ccp with NaOCl, and an oxygen electrode was used to see whether an evolution of oxygen could be detected. No evolution of oxygen was observed when 1 or 10 mol/mol of NaOCl was added to 10 μM enzyme solution. The reaction, under similar conditions, of equimolar mixtures of NaOCl and H_2O_2 gave electrode responses at least 20 times larger than the minimum detectable.

Reaction of O-Benzoylhydroxylamine with ccp. Preliminary experiments showed that *O*-benzoylhydroxylamine oxidized ccp to its ES compound slowly but at low molar ratios. The reaction was faster in alkaline solution, but the yield was highest at pH 6, and this pH was used for subsequent titrations.

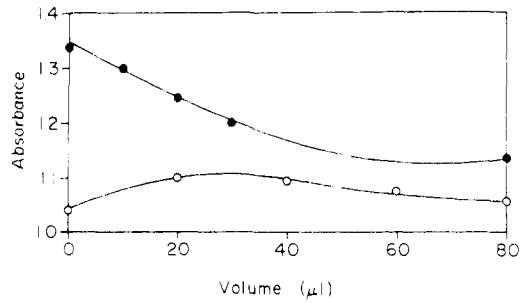

FIGURE 3: Complementary titrations of cytochrome *c* peroxidase (134 μM, O) and of its ES compound (●)—formed by reaction with hydrogen peroxide—with *O*-benzoylhydroxylamine at pH 8. The absorbance at 420 nm (1-mm path length) is plotted against the volume of *O*-benzoylhydroxylamine solution added (2.1 mM).

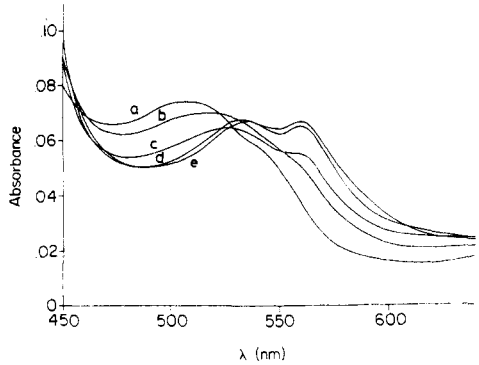

FIGURE 4: Visible spectra of cytochrome *c* peroxidase (90 μM) on reaction with *O*-benzoylhydroxylamine. The samples contained (a) 0; (b) 0.6; (c) 1.2; (d) 3.5; (e) 7, mol of oxidant/mol of cytochrome *c* peroxidase.

Figure 1B shows the result of spectrophotometric titrations of ccp with *O*-benzoylhydroxylamine at pH 6. Further addition of a large excess of oxidizing agent did not cause oxidation beyond the plateau shown. Extrapolation from the first part of the curve to the expected end point showed that complete reaction would have required a 1.4-fold molar excess of oxidant. In other experiments this value ranged between 1.2 and 2.0.

It was shown that solutions with excess *O*-benzoylhydroxylamine in which reaction was apparently complete nevertheless still contained oxidant. Addition of 0.5 mol/mol of aliquots of ferrocyanide to the product of reaction of ccp with a fivefold excess of oxidant produced a rapid partial reduction followed by a slow reoxidation to the same plateau.

Experiments to demonstrate a converse effect by addition of hydrogen peroxide were more difficult, since at pH 6 the reaction with *O*-benzoylhydroxylamine goes too nearly to completion for further oxidation to be easily observed, while at pH 8, although the oxidation is less complete, the usual decay of ES compound is comparable in rate to the oxidation by *O*-benzoylhydroxylamine. Nevertheless, Figure 3 shows the result of complementary titrations of ccp and of ES with *O*-benzoylhydroxylamine at pH 8.

Effect of Prior Incubation of O-Benzoylhydroxylamine on Titration with ccp. No change in the titration curves was found when the oxidant solution used had undergone prior incubation for 10 min either in pH 6, 0.1 *M* phosphate buffer, or in concentrated bovine serum albumin solution. No change in the titration behavior was observed when the aqueous oxidant solution used had been stored for 1–2 days at 0°.

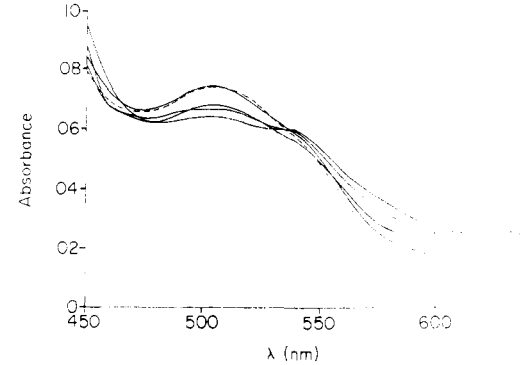

FIGURE 5: Visible spectra of cytochrome *c* peroxidase after oxidation with *O*-benzoylhydroxylamine and reduction with excess ferrocyanide. Same samples as in Figure 4; 3.2 mol of ferrocyanide/mol of enzyme was added to each sample. The dashed line is the spectrum of native ccp; the concentrations of oxidant increases with the absorbance at 600 nm.

Recovery of Oxidizing Equivalents from ES Formed by O-Benzoylhydroxylamine. Two molar equivalents of oxidant was added in a small volume to 100 μM ccp, and the resulting ES compound titrated at 420 nm with ferrocyanide. The end point corresponded to 98% recovery of the oxidizing equivalents represented by the ES formed.

Inactivation of ccp by O-Benzoylhydroxylamine. The enzymic activity of ccp was assayed by measuring the rate of oxidation of 0.5 mM potassium ferrocyanide (observing the absorbance change at 420 nm) by 0.05 mM H_2O_2 in the presence of 0.5 μM ccp. Incubation of the enzyme with 5 mM *O*-benzoylhydroxylamine produced 98% inhibition in 20 min.

Products of Reaction of $BzONH_2$ with ccp. A series of samples of ccp were allowed to react with 0.6–3.5 molar equiv of oxidant. The visible spectrum was recorded when the reaction appeared to be complete. A small excess of ferrocyanide was added to each sample, and the spectrum scanned again after complete reduction. Figures 4 and 5 show the spectra of the oxidized and rereduced materials. Evidently the first aliquots of oxidizing agent give rise to a product having absorption peaks at 530 and 560 nm which is fully reducible to native ccp, but with a greater excess of BzNOH another material, stable to ferrocyanide, and with an absorption peak near 540 nm appears. Part of the excess absorption below 460 nm is due to ferricyanide. When a fivefold excess of *O*-benzoylhydroxylamine was added to ccp, the first product had peaks at 530 and 560 nm. After 10 min this material was partially converted to a mixture of native ccp and the new irreducible component. Table I gives the wavelengths of absorption peaks and isosbestic points for these three materials.

Figures 6 and 7 show the EPR spectra of ccp and of mixtures of the protein with various ratios of *O*-benzoylhydroxylamine.

Figure 8 shows an EPR difference spectrum obtained by subtracting point-by-point the EPR signal of a rereduced (with ferrocyanide) sample of ccp from that of the reaction mixture of ccp and *O*-benzoylhydroxylamine. Within the limitations of the experiment, the *g* value, the line width, and shape of this difference signal were indistinguishable from the EPR spectrum of a sample of ES produced by reaction of H_2O_2 with ccp (Coulson et al., 1971).

Reaction of O-Benzoylhydroxylamine Reaction Product with Reducing Agent. The inhibition of ccp with *O*-benzoylhydroxylamine described above prevented the observa-

Table I: Absorption Maxima and Isosbestic Points in Optical Spectra of Compounds of Cytochrome *c* Peroxidase.

Compound	λ_{max} (nm)				Isosbestic Point (nm)	Ref
ccp	507			647		*a*
ES	530	560			518	*a*
$BzONH_2$ (first product)	530	560			520	*b*
ccp Fe^{2+}–NO	544		572		528	*c*
$BzONH_2$ (second product)	540		570		530	*b*

[a] Yonetani and Ray (1965). [b] Present results. [c] Yonetani et al. (1972).

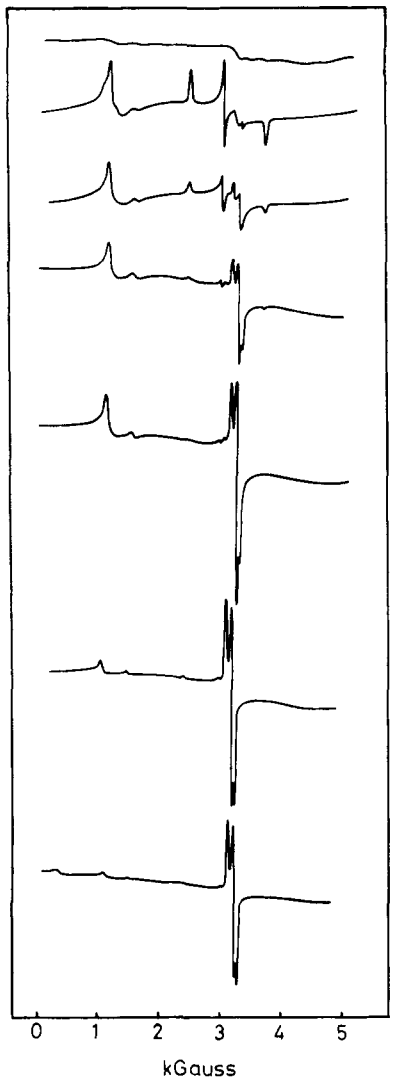

FIGURE 6: EPR spectra of mixtures of ccp and *O*-benzoylhydroxylamine, taken under the conditions described in the text. From the top, the samples contained: oxidant alone; ccp alone; 0.2 mol of oxidant/mol of ccp; 0.6 mol/mol; 1.1 mol/mol; 2.2 mol/mol; 4.4 mol/mol. The last two spectra were recorded with gain settings one-half and one-fourth that used for the first five spectra.

tion of the ccp-catalyzed oxidation of ccp substrates by this oxidant. However, the time courses of the reaction of ferrocyanide with the ES compounds formed from H_2O_2 and from *O*-benzoylhydroxylamine were identical.

Interaction of Other Hemoproteins with O-Benzoylhydroxylamine. No reaction was observed when a 100-fold excess of *O*-benzoylhydroxylamine was added to myoglobin at pH 6. Under similar conditions, the oxidant appeared to oxidize horseradish peroxidase to compound I. A similar observation has been reported recently by Schonbaum (Schonbaum, 1973). The reaction was not examined in detail, but it was observed that a 20-fold excess of the reagent gave rise to about 50% formation of compound I.

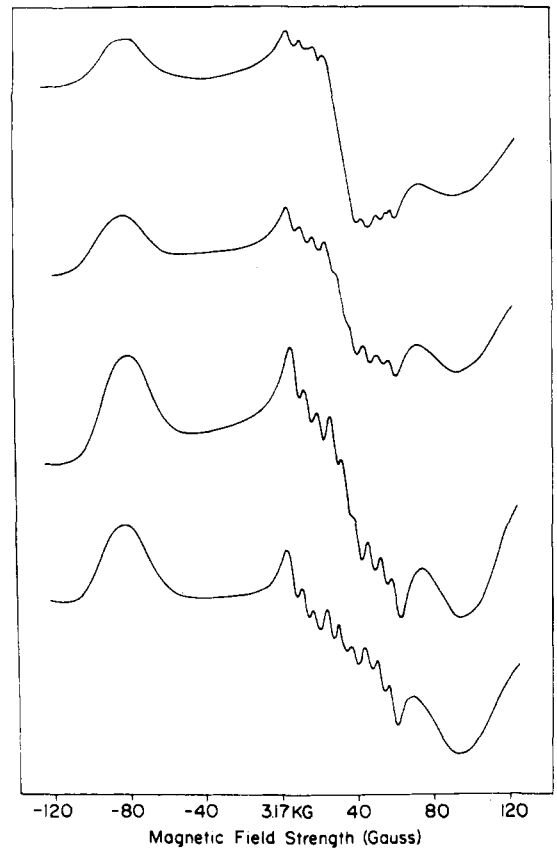

FIGURE 7: EPR spectra (g = 2 region) of mixtures of ccp and *O*-benzoylhydroxylamine, observed under the conditions described in the text. From the top, the samples contained: 1.1 mol of oxidant/mol of ccp; 2.2 mol/mol; 4.4 mol/mol; 2.2 mol/mol, and excess ferrocyanide. The second and third spectra were recorded with a gain setting of one-half that used for the first and fourth.

Reaction of Other Hydroxylamines with ccp. No reaction could be detected between ccp and hydroxylamine, hydroxylamine-*O*-sulfonic acid, or *N*-methylhydroxylamine. All these compounds reduced ES (formed from ccp and H_2O_2) principally to ferric ccp at similar rates. Examination of the EPR spectrum of ccp after oxidation with hydrogen peroxide and reduction with hydroxylamine showed a barely detectable signal similar to that observed when excess *O*-benzoylhydroxylamine was allowed to react with ccp.

Discussion

The peroxidases are of particular interest to molecular enzymology, since the structures of the intermediates have still not been firmly established despite many years of work, and because the mechanisms of the reactions are apparently unique with no obvious low-molecular weight analogs.

An effective technique for characterizing intermediates in enzyme reactions is to vary the structure of the substrate and measure the consequent changes in the enzyme kinetics and in the physical properties of the intermediate.

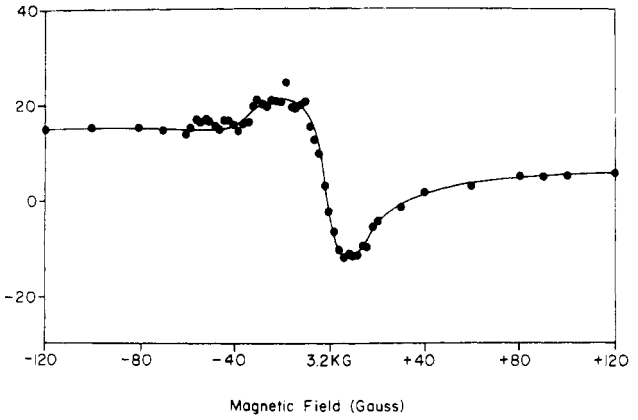

FIGURE 8: EPR difference spectrum of first product of reaction of cytochrome *c* peroxidase with *O*-benzoylhydroxylamine. The EPR spectra of samples of ccp treated with 1.5 mol of oxidant/mol of protein in the presence and absence of a small excess of ferrocyanide were measured, and subtracted for each other point by point.

Previous attempts (George, 1953; Fergusson, 1956) to use this approach with ccp largely failed because of the difficulty of showing that nonstoichiometric reaction with powerful oxidizing agents was related to the reaction of the enzyme with its usual substrates. Alkyl hydroperoxides (Yonetani and Ray, 1965) and peroxy acids (Coulson, unpublished results) have been shown to replace hydrogen peroxide in the enzyme reaction. Wittenberg et al. (Wittenberg et al., 1968) have reported that the four-electron oxidizing agent, chlorite, stoichiometrically oxidizes ccp to ES compound.

The experiments described here suggest that other oxidizing agents will act as true electronic analogs of hydrogen peroxide and allow observation of an enzyme intermediate formed from an oxidant having no structure in common with hydrogen peroxide itself.

The reaction of *O*-benzoylhydroxylamine with ccp, though complex, can be analyzed in some detail.

There are several reasons for believing that this material is acting as a genuine substrate analog for ccp. The fact that it will oxidize ccp and, apparently, horseradish peroxidase, but not the related hemoprotein myoglobin, implies that its reaction with the peroxide-requiring enzymes is specific. Furthermore, the reaction of ccp with *O*-benzoylhydroxylamine has close to a one-to-one stoichiometry, and the oxidized form of the enzyme retains both oxidizing equivalents of the oxidant and can be reduced back to the fully active state with ferrocyanide (these statements require some further justification which is given below). It does not seem likely that *O*-benzoylhydroxylamine is oxidizing water, or bringing about a reduction of oxygen by protein to a hydroperoxide, since aqueous solutions of the compound are stable for many hours and the course of the reaction with ccp is not affected by prior incubation either in the presence or in the absence of an added protein.

Nevertheless the reaction is complicated. The fact that the reaction does not go to completion, even if excess oxidizing agent and viable ferric ccp remain in solution, suggested that a steady state was established, and this was confirmed by the complementary pair of titrations (at pH 8) of ccp and ES to the same end point. Presumably *O*-benzoylhydroxylamine is capable of reducing ES back to the native enzyme and this seems reasonable in view of the fact that hydroxylamine and its methylated and sulfonylated derivatives bring about a rapid reduction of ES formed from hydrogen peroxide. The overall reaction is analogous to the catalase reaction, and this conforms to the accepted view that the only difference between catalase and peroxidase is the different pattern of specificity for electron donors. This behavior also explains the curvature of the oxidative titrations of ccp by *O*-benzoylhydroxylamine and legitimates the deduction of the stoichiometry of the reaction by extrapolation from the first part of the curve to the expected end point. Similar observations on catalase itself in its reaction with hydrogen peroxide were analyzed in detail by Chance et al. (Chance et al., 1952).

However, this is not the end of the complexity. EPR spectra of the reaction mixtures of ccp and $BzONH_2$ show that a third component is formed whose concentration continues to increase at oxidant concentrations far greater than those at which the reaction observed at 420 nm appears to be complete. This material is easy to identify from its characteristic EPR spectrum, which is identical with that of the nitric oxide complex of ferrous ccp (Yonetani et al., 1972). A simple suggestion can be made for the stoichiometry of this reaction

$$ES + PhCOONH_2 \longrightarrow PhCO_2^- + ccp\text{–}Fe^{2+}\text{–}NO$$

and though the stoichiometry is not certain (the referee has pointed out that this complex may also arise by reduction of ferric ccp by NO^-), it is clear that the overall course of the reaction between ccp and *O*-benzoylhydroxylamine is:

$$ccp \xrightarrow[BzNOH]{1} ES \xrightarrow[BzNOH]{2} ccp$$
$$\downarrow{\scriptstyle 3}\ BzNOH$$
$$ccp\text{–}Fe^{2+}\text{–}NO$$

Since the nitric oxide ferrous complex is essentially inert, this scheme also provides an explanation for the rapid inactivation of low concentrations of ccp by excess oxidant. The time dependence of the optical spectrum of a mixture of ccp with *O*-benzoylhydroxylamine shows that a steady-state concentration of ES is reached fairly rapidly by reactions 1 and 2, and that reaction 3 then slowly bleeds off ccp into the dead-end nitric oxide complex. This complex was observed also in reaction mixtures in which ES formed from hydrogen peroxide had been reduced with hydroxylamine.

We can now proceed with the main purpose of this investigation, the examination of the properties of ES complex formed from an oxidizing agent having no structure in common with hydrogen peroxide. The two subsidiary reactions limit the precision with which the properties of this material can be compared to that formed from hydrogen peroxide. Within these limits, Figures 4 and 5 and Table I show that the optical spectra of the two materials cannot be distinguished. The kinetics of reduction of the two forms of ES by ferrocyanide were identical. The EPR spectra could not be compared directly, since that of the BzNOH reaction was swamped by the sharper spectrum of the nitric oxide ferrous ccp even at low concentrations of the latter. However, the nitric oxide complex is inert to ferrocyanide while ES is reduced to the ferric enzyme. Consequently, the difference spectrum for samples and after reduction gives the spectrum of the ES complex only. Figure 8 shows that, within the limits set by experimental difficulties, the EPR spectrum of the ES complex is not distinguishable from that formed from hydrogen peroxide and ccp.

The experiments with other oxidizing agents cannot be discussed individually in such detail, but the catalatic reac-

tion found with *O*-benzoylhydroxylamine does not seem to be shared with the other compounds. Only hypochlorite has a convex titration curve, and in this case no evolution of oxygen could be detected. Presumably the nonstoichiometry in these cases is due to destructive nonspecific oxidation of the protein. The rapid reaction with excess HOBr and HOCl is particularly striking; undoubtedly George's failure (George, 1953) to observe oxidation of ccp by these reagents was due to this secondary reaction. If a reagent were found which reacted faster with ccp than hydrogen peroxide does (e.g., peracetic acid, (Coulson, unpublished observation)) then hydroperoxides certainly could not be intermediates in its reaction. For this reason, and because George claimed that the reaction of HOCl with horseradish peroxidase was faster than the hydrogen peroxide reaction with this enzyme, an attempt was made to measure the reaction rate of ccp with HOCl, which was clearly the fastest reacting of the peroxide analogs examined here. Figure 9 shows that this reaction rate is really too fast to be studied by the stopped-flow technique, but that the hypochlorite reaction rate is only about half the peroxide reaction rate under similar conditions.

The titration results do indicate that all the compounds studied behave as electronic analogs of hydrogen peroxide. The *N*-bromosuccinimide reaction was evidently not due to HOBr or Br_2.

These results have implications both for the structure of the intermediate, and for the mechanism of the reaction in which it is formed.

Thus the known specific substrates of ccp are now hydrogen peroxide, *n*-alkyl hydroperoxides (up to octyl), *sec*- and *tert*-butyl hydroperoxides (Coulson, unpublished results), peracetic acid and perbenzoic acid, hypohalous acids, *N*-halosuccinimides, and *O*-benzoylhydroxylamine. Related substances which are not substrates include bromine, hydroxylamine, and its *N*-methyl and *O*-sulfonyl derivatives. The substrate structures have in common an electronegative atom linked to a moderately good leaving group which can be made into a much better leaving group by acid catalysis. A plausible mechanism therefore is that an early stage of the reaction involves acid-catalyzed heterolytic fission of the bond between these groups and that the electronegative group so formed is the effective oxidant.

The difference between hydroxylamine and *O*-benzoylhydroxylamine in terms of this mechanism is simply that in the latter compound the oxygen atom has been converted into a better leaving group. The sulfonyl group was expected to serve a similar purpose, but *O*-sulfonylhydroxylamine is not a substrate for the enzyme.

Experiments reported in a preliminary way by Schonbaum (Schonbaum, 1970) provide the only prior chemical evidence on the structure of the intermediate. He allowed ccp to react with ethyl hydroperoxide, and showed that at any rate half the acceptor molecule leaves the enzyme on formation of ES, since ethanol could be recovered unchanged from the solution without total destruction of the intermediate. An obvious question, therefore, is whether the second half of the molecule is on or off the enzyme at this stage. The work reported here does not answer this question directly. However, it does allow us to examine the spectral (optical and EPR) and kinetic properties of an intermediate formed from an oxidizing substrate having no structure in common with the hydroperoxides. The intermediates formed in the two reactions are indistinguishable, and this implies that if any fragments of substrate remain on the enzyme at the ES stage, then they play no further part in the reaction. In particular, if the stable free radical like EPR signal in ES represented an OH radical or related species derived from the substrate, then we should certainly have expected changes in the EPR spectrum on passing over to the corresponding nitrogen compound. There is now widespread agreement that the optical spectrum of ES (seen also in compound II of horseradish peroxidase) is due to a change in the oxidation state of the heme iron formally to Fe(IV). By the same token, further oxidation of the heme, with formation of a free radical, would have a profound effect on the spectrum, and it seems clear that this is not the site at which the radical is formed. But if the radical represents neither a fragment of substrate nor a heme structure, then it must be due either to a reversibly oxidized protein residue; or to oxidation of a heme ligand. Two specific suggestions have been made recently for the structure of ES complex; Peisach et al. (Peisach et al., 1968) propose that it is a peroxyl complex of ferrous ccp, and Williams (Williams, 1973) that the free radical is located on an amino acid side chain. It seems unlikely that $BzONH_2$ could give rise to peroxyl in a facile reaction on the enzyme, and the evidence presented here therefore favors the second proposal.

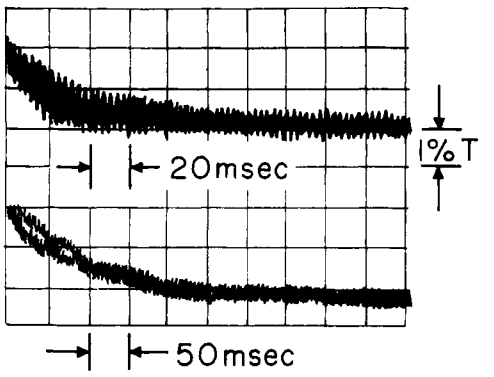

FIGURE 9: Time course of the reactions of cytochrome *c* peroxidase (0.5 μM) with hydrogen peroxide (0.5 μM, lower trace) and sodium hypochlorite (5 μM, upper trace). The reaction was followed at 420 nm; ten replicates of each run were superimposed.

We have shown (Coulson and Yonetani, 1972) that the loss of activity that occurs when ES is repeatedly allowed to decay to the ferric state is associated with destruction of tyrosyl residues, and it is tempting to suggest that the site of the free radical is a tyrosyl residue. Certainly this seems to be compatible with the *g* value of 2.004, but there is as yet little direct evidence.

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Essential and Nonessential Thiols of Yeast Hexokinase. Reactions with Iodoacetate and Iodoacetamide†

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ABSTRACT: The reaction of yeast hexokinase with iodoacetate or iodoacetamide has been investigated in detail, using pure hexokinase B. Of the four thiols in each subunit of the molecule, two (the "apparently essential thiols") are alkylated rapidly at 35°, and the enzymic activity is lost in parallel with their reaction. The other two thiols react subsequently to completion, but at a very much slower rate. In the conditions used, no other uptake of the reagent occurs elsewhere during these thiol alkylations. Electrophoretically homogeneous dialkylated and tetraalkylated protein species are formed, in the two stages of the reaction. The inactivating reaction at 35° with the apparently essential thiols is second order. The rate constant increases with increasing pH, in the range pH 7.0–8.5, in a manner consistent with control of the reaction by a group with pK_a of approximately 10. The absolute (pH independent) rate constant is of the same order as that for a normal thiol in model compounds. The availability of the apparently essential thiols appears to be associated with some conformational change in the molecule in the monomer form: it declines at high ionic strengths, is maximal at intermediate values where the dimer first dissociates, but is lowered in the dimer at very low ionic strengths. The reaction also shows a sharp temperature dependence: the dimer at 30° (in contrast to 35°) shows no availability of the apparently essential thiols. A similar transition to a state permitting fast inactivation is found with pH, above pH 8.5. The reaction of the two apparently essential thiols is strongly inhibited by glucose. ATP and ADP, and their Mg complexes, protect significantly, but less effectively than does glucose. The affinities of these substrates at the active site of the enzyme are measured in this protection system. These various reactions appear to be of value for identifying the cysteine-containing regions that are involved in the active center or in its maintenance in the structure.

There have long been indications that yeast hexokinase is a thiol-dependent enzyme. Variations in the findings of the earlier studies on this question are understandable in view of the recognition (Lazarus et al., 1966; Gazith et al., 1968; Schulze and Colowick, 1969) of the heterogeneity of previous preparations of this enzyme, which relied upon extensive autolysis of the yeast cells for its release, introducing proteolytic damage. In this laboratory, a method (Lazarus et al., 1966; Ramel et al., 1971; Rustum et al., 1971) is used which avoids such proteolysis, for the preparation of two isoenzymic forms of yeast hexokinase, A and B, in homogeneous (Rustum et al., 1971) form. Hexokinase B is the major and more active isoenzyme. (Similar material has been prepared by Colowick and coworkers: their preparation, which is by a different method, and in which they term the corresponding isoenzyme P-II, has recently been reported in detail (Womack et al., 1973).) Analysis of hexokinases A and B has established that there are eight SH groups in each molecule (Lazarus et al., 1968). The molecule has 104,000 molecular weight and consists of two identical subunits in each case (Rustum et al., 1971; Derechin et al., 1972; Schmidt and Colowick, 1973). On the basis of the inactivation of both enzymes by methylmercuric iodide, it was concluded (Lazarus et al., 1968) that four of these SH groups are not essential for catalytic activity. However, inactivation of hexokinase B with iodoacetate was, in certain conditions, associated with the carboxymethylation of all eight thiols. Earlier, Barnard and Ramel (1962), using a stable and homogeneous yeast hexokinase (but made by an earlier method, and suffering minor proteolytic damage), showed that inactivation by bromoacetate was associated with the carboxymethylation of at least one and at the most four SH groups per unit of about 50,000 molecular weight. In the present report we describe more precisely the reac-

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