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Intermediates in the Reaction Between Hydrogen Peroxide and Horseradish Peroxidase

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When excess H_2O_2 is added to horseradish peroxidase, a compound with an intense absorption band in the near infrared is observed. It is unstable and transforms to the green hemoprotein P-670. The relationship between this new compound, P-940, and other intermediates in the reaction of peroxidase with H_2O_2 is examined and discussed.

Peroxidases, ubiquitous in the plant world, catalyze the oxidation of certain compounds by H_2O_2. One of the best known of these enzymes is horseradish peroxidase (HRP). It has a molecular weight of about 40 000 and contains, like myoglobin, one iron protoporphyrin IX group per molecule.

Several peroxidase-H_2O_2 species with characteristic absorption spectra are known.[1] A slight excess of H_2O_2 converts ferric HRP in aqueous solution to "compound II" *via* "compound I".

$$\text{HRP} \xrightarrow{+H_2O_2} \text{comp. I} \xrightarrow{e^-} \text{comp. II}$$

The reduction in the last step of the above equation is effected even without addition of an extra hydrogen donor; apparently an endogenous donor is always present in preparations of HRP, and possibly this is a part of the protein. At higher concentrations of H_2O_2, comp. II is reversibly transformed to "compound III".

$$\text{comp. II} \underset{-H_2O_2}{\overset{+H_2O_2}{\rightleftharpoons}} \text{comp. III}$$

In a solution of comp. III, the green "compound IV", or P-670, as we shall call it, slowly develops on standing. The formation of P-670 is irreversible, and the porphyrin ring is probably attacked (see below).

In studying these compounds and attempting to deduce their nature, the absorption spectra in the 280 – 700 nm range only have been examined. In this

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paper we report results of an investigation of the peroxidase-H_2O_2 reactions in which we have taken special interest in the absorption spectra of the above HRP-compounds in the near infrared region; this reveals that at least one additional compound is present in the reaction system, and also permits a comparison with other known hemoprotein complexes. The myoglobin-H_2O_2 system was also examined experimentally with special emphasis on the long-wavelength spectra.

EXPERIMENTAL

Materials. The hemoproteins used were obtained from Koch-Light Laboratories, Colnbrook, England.

Horseradish peroxidase: salt free, lyophilyzed (Batch No. 41414). A chromatographic test, as described by Paul,[2] showed that the isoenzymes B, C, D, and E were present in approximately the same ratio as previously found.[2,3] The batch contained no isoenzyme A.

Sperm whale myoglobin: crystalline, salt free, lyophilyzed (Batch No. 41152). Analytical grade chemicals were used throughout.

Methods. Spectra were recorded on a Beckman DK2A spectrophotometer, equipped with a thermostatted cell holder. Concentrations of hemoproteins were always determined photometrically. The "time-drive" attachment of the spectrophotometer was used in the kinetic experiments at constant wavelength.

H_2O_2 was injected into standard 1 cm-3 ml cells by means of a syringe; its plastic needle was used as a stirrer to get rapid mixing.

Whatman CM32 carboxymethyl cellulose was used for the chromatography.

RESULTS

Spectrophotometric observations on the reaction between H_2O_2 and sperm whale myoglobin are given in Fig. 1; the myoglobin-H_2O_2 compound has no absorption bands in the 750–1250 nm region and is therefore different from oxymyoglobin (Fig. 1) as well as high-spin Fe(III)-myoglobins.[4]

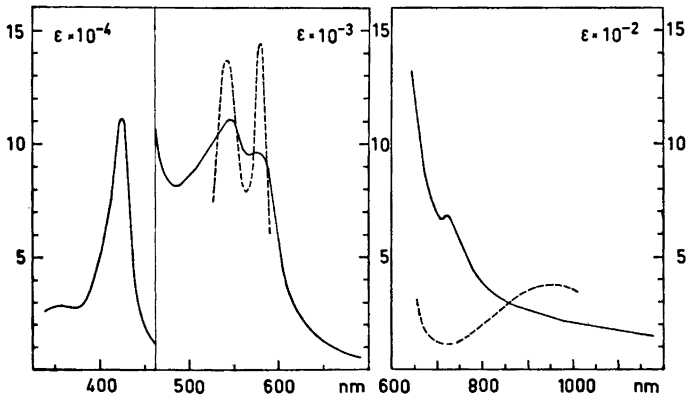


Fig. 1. ———, Spectrum of metmyoglobin plus excess H_2O_2; R ≃ 110, 0.05 M phosphate buffer, pH = 7.00, T = 3°C; – – –, part of the spectrum of oxymyoglobin.[4,14] R is the mole ratio of H_2O_2 to hemoprotein.

Comp. I, II, and III of peroxidase, prepared at 3°C by adding the appropriate amounts of H_2O_2 and identified by their visible spectra, have no absorption bands in the near infrared, in marked contrast with high-spin Fe(III)-peroxidase (Fig. 2).

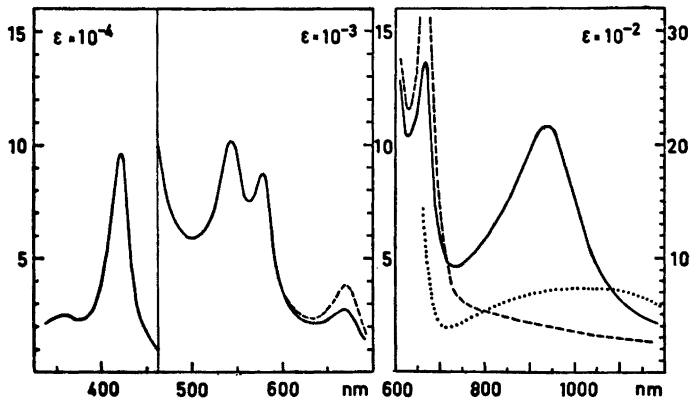


Fig. 2. ———, Spectrum of a ferric HRP solution (3 ml) 3 min after addition of excess H_2O_2 (0.1 ml), R≃300; 0.05 M phosphate buffer, pH=7.00, T=3°C; – – –, 60 min after H_2O_2-addition, now a mixture of ~85 % comp. III and ~15 % P-670; ·····, near infrared spectrum of ferric HRP.

However, while measuring the spectrum of comp. III it was observed, that following the addition of excess H_2O_2 to an aqueous solution of ferric HRP, a strong absorption band emerges at 940 nm (Fig. 2); a feature which was not seen in the corresponding experiment with metmyoglobin (Fig. 1). On standing, the band gradually dies away again, and this change is accompanied by the appearance of the 670 nm peak due to P-670.

The rise of the 940 nm band was found to take place at all of the pH-values 4.88, 6.03, 7.00, 7.96, 8.86, and 11.10. In acidic and basic solution, the recorded maximum absorption was less intense than at neutral, and consequently 7.00 was chosen as the most convenient pH for a closer examination of this phenomenon.

Kinetic experiments revealed that the decrease of the 940 nm band and the increase in concentration of P-670 are directly coupled. In Fig. 3 it is shown that recordings of the optical densities at 940 nm and 670 nm become linear at approximately the same time at three different temperatures. Our interpretation is that the near infrared band is due to an intermediate, hereinafter called P-940, in the formation of P-670.

A further injection of H_2O_2 at a time just after the absorption at 940 nm had reached the constant value (Fig. 3) did not cause another jump in this absorption tracing. Therefore, P-940 is not disappearing because the H_2O_2 is being used up, and comp. III is not directly transformed to P-940 by action of H_2O_2.

An experiment where excess H_2O_2 was added to a solution of a mixture of comp. I and comp. II caused the 940 nm band to rise in exactly the same

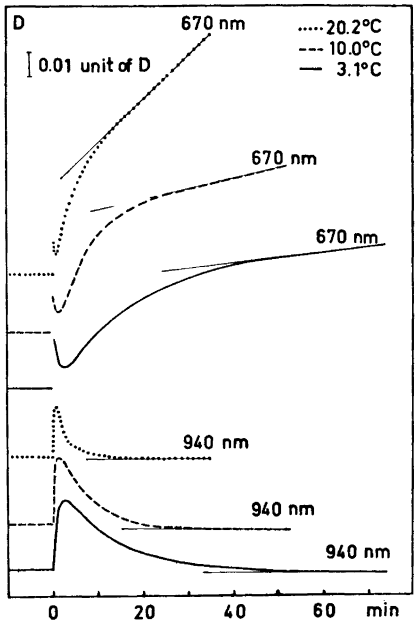


Fig. 3: Kinetics of the effect of excess H_2O_2 on ferric HRP. The optical densities at 670 nm and 940 nm recorded as a function of time at three different temperatures. 2.00×10^{-5} M HRP, 0.05 M phosphate puffer, pH = 7.00, R ≃ 300.

way as shown in Fig. 2. Apparently the amount of comp. I and comp. II present initially did not affect the height of the 940 nm band, *i.e.* the amount of P-940 formed. This indicates that P-940 does not originate directly from comp. I, but that it must arise from comp. II.

After standing overnight, most of the comp. III, in a solution used for the experiments referred to in Fig. 3, reverts to ferric HRP, and P-940 can again be developed by addition of a second portion of H_2O_2. In contrast, the myoglobin solutions became colourless after prolonged standing exposed to the first portion of H_2O_2. It seems that the porphyrin ring in comp. III is protected against oxidative attack by excess H_2O_2, but that this is not so in the corresponding myoglobin-H_2O_2 compound.

An analysis of the kinetic information from the experiments referred to in Fig. 3 shows that the decay of P-940 is first order in [P-940]; the half life times, $\tau_{\frac{1}{2}}$, and the rate constants, k, at the three different temperatures are given in Table 1.

Table 1.

T °C	$\tau_{\frac{1}{2}}$ min	k min^{-1}
3.1	8.9	0.0779
10.0	4.4	0.158
20.2	1.5	0.462

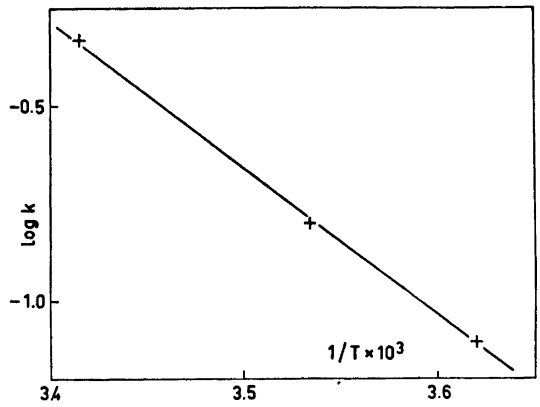


Fig. 4. Arrhenius plot for the decay of P-940.

From Fig. 4 one gets the Arrhenius equation for the rate constant

$$k = 2.57 \times 10^{12} \exp(-17\,100/RT) \text{ min}^{-1}$$

This yields an activation energy of 17.1 kcal mol^{-1} and an activation entropy of -11.9 e.u.

The spectrum of isolated P-670 has been published.[5] Using it, we can estimate that under our conditions (Fig. 2) about 15 % of the ferric HRP is initially converted to P-940, and the rest to comp. III. The ε_{940} for P-940 is actually $\sim 1.4 \times 10^4$ and must be at least 1.0×10^4.

An attempt to examine P-940 by EPR technique was hampered by O_2-evolution, when the concentrations were scaled up.

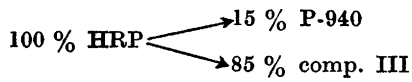
We have tried to get the long-wavelength spectrum of oxyperoxidase, but found some difficulty in preparing a solution of oxyperoxidase free from ferric HRP, which has a broad band in the near infrared (Fig. 2).

The B+C and D+E mixtures of HRP-isoenzymes, separated by chromatography, both yielded P-670 on exposure to H_2O_2.

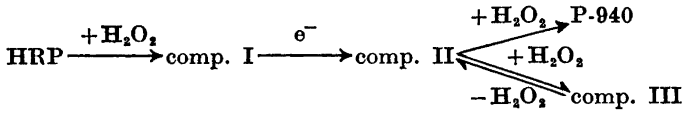
DISCUSSION

As a basis of the discussion, we propose the following reaction schemes, which are consistent with previous work and our present findings.

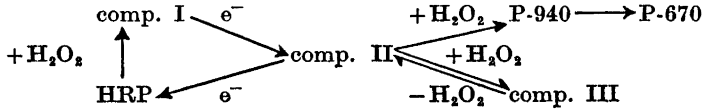
A. Addition of excess H_2O_2 gives:



the path being the following:



B. Steady state situation at a time just after the curves in Fig. 3 have become linear, *i.e.* [P-940] very low, and H_2O_2 still in excess:



As long as $[H_2O_2]$ is high, [comp. III] decreases, and [P-670] increases; all other concentrations are very low. When the excess of H_2O_2 is used up, [P-670] becomes constant, and comp. III reverts to HRP. In the end, the solution contains only ferric HRP and P-670. The conversion of comp. II to P-940 is negligible, when $[H_2O_2]$ is low.

We now turn to a discussion of the nature of the species dealt with above.

Comp. I is two oxidizing equivalents, and comp. II one oxidizing equivalent above ferric HRP. The absence of the long-wavelength absorption typical of high-spin Fe(III)-heme complexes shows that neither compound is high-spin Fe(III). Low-spin Fe(III) is of course unlikely with an oxygen-donor ligand. This confirms the Mössbauer data [6] which indicate that both comp. I and II are probably Fe(IV) compounds. The extra oxidizing equivalent of comp. I could be in the protein.

Comp. III cannot be a high-spin Fe(III)-complex either, for it has no near infrared absorption band. At present, the relationship of comp. III to the others in the series is uncertain, in fact its spectrum only is known.

From EPR measurements it was concluded that P-670 is a low-spin Fe (III)-compound,[7] and its similarity to choleglobin, an intermediate in the oxidative degradation of hemoglobin, was pointed out;[7] the resemblance of its absorption spectrum to that of free biliverdin,[8] especially the considerably reduced Soret band, is notable. Thus P-670 is possibly a Fe(III)-bile pigment-protein complex, *i.e.* a cleavage of the porphyrin ring has been accomplished.

P-940 is then to be regarded as an intermediate hemoprotein in an oxidation reaction, that results in the rupture of one of the methine bridges in the protoporphyrin IX ring. The extinction coefficient of the 940 nm band is a factor of ten too high to be assigned to a high-spin Fe(III)-heme or an O_2-heme species. A possible clue to its nature is given by the recent finding [9] that the one-electron oxidation of zinc tetraphenylporphyrin yields a π-cation radical, which exhibits an absorption band, $\varepsilon \sim 0.3 \times 10^4$, in the near infrared region.

On the basis of the visible spectra it has been suggested that metmyoglobin exposed to excess H_2O_2 yields oxymyoglobin;[10] this was used by Wittenberg *et al.*[11] to substantiate their suggestion that comp. III of peroxidase and oxyperoxidase may be identical. Our studies of the near infrared spectra show that a compound different from oxymyoglobin is formed with H_2O_2 (Fig. 1). As we failed to get a clean spectrum of oxyperoxidase in the long-wavelength region, we cannot say if the similarity in its ultraviolet-visible spectrum to that of comp. III extends to the near infrared. At present, the problem of equivalence or nonequivalence between oxyperoxidase and comp. III must still be regarded as unsolved. The discussion of these compounds by Noble and Gibson,[13] in a

paper published after the completion of this work, also seems to point in this direction.

An investigation of the horseradish peroxidase isoenzyme A, which responds differently from the rest of the isoenzymes, when exposed to H_2O_2,[12] is planned.

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