Technical Papers

Intermediate Compounds Formed when Horseradish Peroxidase Reacts with Potassium Molybdicyanide and Potassium Chloriridate

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The titration of compound II of peroxidase with reducing agents[1(a)] showed it to be one equivalent above the ferric state of the enzyme and thus a compound containing iron with an effective oxidation number + 4. This leads to a new mechanism (*1*) for peroxidase action based on one-equivalent reduction steps:

$$\text{Per} + H_2O_2 \rightarrow \text{compound I} \xrightarrow[\text{reduction}]{\text{one equiv}}$$

$$\text{compound II} \xrightarrow[\text{reduction}]{\text{one equiv}} \text{Per} \quad \ldots \; a$$

The reduction of compound I to compound II has subsequently been confirmed in a direct titration by Chance (*2*). Accordingly, compound I could be a *complex* containing H_2O_2 or its anion O_2H^-, or a *compound* of iron with an effective oxidation number + 5. Other oxidizing agents, HOCl, HOBr, $NaClO_2$, ClO_2, etc., were found to replace H_2O_2 in a typical peroxidase reaction, the oxidation of guaiacol to tetraguaiacol.[1(b)] These oxidizing agents give intermediate compounds like H_2O_2: with cytochrome-*c* peroxidase an intermediate indistinguishable from compound II is formed exclusively as the stable intermediate, whereas with horseradish peroxidase the resulting absorption spectra are typical of a mixture of compounds I and II. The redox properties of compound II and the fact that the spectra obtained with the various oxidizing agents are identical within experimental error suggest a common structure in all systems. These oxidizing agents, however, possess two or more oxidizing equivalents, and the underlying chemical changes are thereby obscured. The recent discovery by George and Irvine (*3*) that simple single electron transfer oxidizing agents like potassium chloriridate and molybdicyanide can oxidize metmyoglobin to its effective quadrivalent iron compound (*4*), previously described as a hydrogen peroxide complex (*5*), thus called for similar experiments with peroxidase.

[1] P. George. *Nature*, **169**, 612 (1952), which contains a summary of four papers recently published: (*a*) The chemical nature of the secondary hydrogen peroxide compound formed by cytochrome-*c* peroxidase and horseradish peroxidase. (*b*) The formation of intermediate compounds when horseradish peroxidase and cytochrome-*c* peroxidase react with strong oxidizing agents. (*c*) Experiments on the third intermediate compound formed when hydrogen peroxide reacts with horseradish peroxidase. (*d*) A comparison of the effect of strong oxidizing agents on horseradish peroxidase, haemin, and tetrasulfonated copper phthalocyanine.

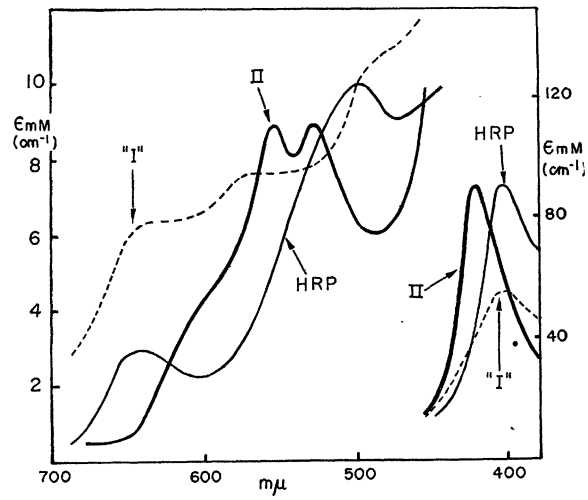

FIG. 1. Spectra of the first intermediate, "I," and compound II formed by the action of potassium chloriridate on horseradish peroxidase. Soret region: 1.5 μM-enzyme, 16.4 μM-K_2IrCl_6 at pH 10.2; visible region: 4.5 μM-enzyme, 34.5 μM-K_2IrCl_6 at pH 9.2. (Spectrum of peroxidase denoted by HRP.)

A sample of horseradish peroxidase prepared by Keilin and Hartree's method was used (*5*). With 1.5 μM enzyme and about 35 μM potassium molybdicyanide, full formation of an intermediate identical to the previous compound II was obtained at pH 11.0; reduction by potassium ferrocyanide regenerated the enzyme completely. At pH 9.2 only 40% conversion occurred. The presence of cyanide ions in some molybdicyanide solutions was occasionally observed by the production of the peroxidase-cyanide complex instead of compound II and its consequent resistance to reduction by ferrocyanide.

Using the same enzyme concentration and a five- to tenfold molar excess of potassium chloriridate, the reaction follows a different path. At pH 9.2–11.6 an intermediate is formed first characterized by low absorption in the Soret region and strong absorption in the visible extending to 650 mμ. This then changes spontaneously into a second intermediate having the spectroscopic characteristics of compound II. Both spectra are shown in Fig. 1. The first intermediate is apparently stable while excess potassium chloriridate is still present, for its lifetime is longer the higher the initial iridate/enzyme ratio. The addition of one equivalent of potassium ferrocyanide converts the first intermediate very rapidly into compound II before the reduction of compound II to the ferric state of the enzyme, which is recovered fully on the addition of more ferrocyanide. When potassium chloriridate is added to compound II, formed either from the spontaneous reaction of the first intermediate or from hydrogen peroxide itself, the first intermediate again appears and then reverts to compound II again. Excess hydrogen peroxide converts the first intermediate

into compound II or compound III if the concentration is high enough—i.e., greater than 1.0 mM, as was found in the hydrogen peroxide system alone (*5*).[1(c)]

These results cannot be due to the initial formation of peroxides through the attack of the oxidizing agents on reducible groups present, for peroxides themselves do not react in an identical way. They reveal further the similarity that exists between compound II of peroxidase and the metmyoglobin intermediate, for both can be formed using single electron transfer oxidizing agents.

Compared with metmyoglobin, the appearance of the first intermediate when potassium chloriridate is used presents an additional feature of great importance, because its chemical properties parallel those of compound I in the H_2O_2 or alkyl hydroperoxide systems (*6*). It is reduced to compound II by ferrocyanide in accord with the above reaction scheme *a*, and appears again when compound II is oxidized by potassium chloriridate. It differs from compound I in the peroxide systems by its far greater stability and certain details of its absorption spectrum (*7*). Although it has a Soret band of similar low intensity, the absorption is somewhat greater below 405 mμ. There can be little doubt that it is a compound of the same chemical type, and in conjunction with the spectroscopic indications that an intermediate of low Soret band intensity is also formed with the other oxidizing agents, an enzyme-substrate-complex structure for compound I, Per-H_2O_2 or Per-OOH, appears less satisfactory compared with more general structures in which the iron has the effective oxidation number +5.

Further experiments showed that intermediates are only produced using potassium chloriridate in solutions of pH greater than 7.0, and that at pH 11.6, where the peroxidase is present in its alkaline form, the very rapid production of compound II via the first intermediate still occurs. This behavior is in contrast to the action of peroxides where reaction with the acid form of peroxidase is pH-independent and the alkaline form does not react (*6*). This suggests that the intermediates can be produced by electron transfer from the acid or alkaline form of peroxidase with chloriridate, whereas the peroxide reactions involve hydrogen atom transfer which is unfavored with the alkaline form. Since the redox potential of the chloriridate ion is independent of pH, these observations also confirm the participation of H in the redox reactions of the intermediates for which there are clear indications in the previous experiments.[1(a)]

No evidence could be found for the reduction of the first intermediate by the enzyme itself in the reaction

$$\text{First intermediate} + \text{enzyme} \rightarrow 2 \text{ compound II} \quad \ldots b$$
$$(\text{formally: } Fe^{5+} + Fe^{3+} \rightarrow 2\ Fe^{4+}).$$

An over-all reaction of this type is undoubtedly favored in the peroxide systems,[1(a)] for at low values of the H_2O_2/enzyme ratio the formation of compound II approximates to the stoichiometry

$$1 \text{ mol } H_2O_2 \equiv 2 \text{ mol compound II.}$$

The absence of reaction *b* can be regarded as a reason for the appearance of the first intermediate as a stable state. In terms of free energy, reaction *b* will be favored if the redox potential $E^{\circ}_{5+/4+} > E^{\circ}_{4+/3+}$. Compound formation by the +5 state in the chloriridate system would lower $E^{\circ}_{5+/4+}$, and the stability of the first intermediate would follow on the reversal of this relationship when $E^{\circ}_{5+/4+} < E^{\circ}_{4+/3+}$.

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Centrifugation in Field-aligning Capsules: Preparative Microcentrifugation[1]

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Preparative high-speed centrifugation is commonly employed for fractionating and concentrating many biological entities of small size. Some of these materials, notably viruses, may be obtained as highly active agents even in extremely small quantities. This circumstance makes it appear that microcentrifugation methods can be advantageously applied to those cases where only fractional milliliter samples of source material are available—quantities too small to be handled with the usual preparative centrifugation methods. A microtechnique for improving the specific activity of minute amounts of sedimentable materials serves also as a useful adjunct to microchemical and microphysical assay methods. Severe difficulties arise in standard centrifugation procedures when attempts are made to secure successive depositions of a small amount of material in round-bottom plastic tubes of the size commonly employed in angle rotors, since excessive losses of recoverable material occur owing to "shelving" and back-diffusion from the thinly spread deposits. Capillary centrifugation in swinging-cup rotors has previously been employed (*1–3*), but the largest values of centrifugal acceleration obtainable with such rotors are too small to pellet most viruses and macromolecules.

A method that might be described as "field-aligning capsule centrifugation" has been employed in this laboratory for the subfractionation of very small pellets obtained from 10-ml volumes of clarified supernatant fluid of virus-infected tissue cultures and controls. Pellets obtained initially by use of conventional equipment are resuspended in 0.01–0.1 ml of diluent

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