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Participation of horseradish oxyperoxidase (compound III) in interenzymic reaction steps

When horseradish peroxidase (donor:H_2O_2 oxidoreductase, EC 1.11.1.7) acts as an oxidase, O_2 is bound to ferroperoxidase to yield oxyperoxidase (also called oxyferroperoxidase, compound III or complex III) (refs. 1–3):

$$\text{ferroperoxidase} + O_2 \rightarrow \text{oxyperoxidase} \quad (1)$$

When hoseradish peroxidase acts as a peroxidase, there is also a route leading to the formation of oxyperoxidase. The terminal step is the addition of H_2O_2 to compound II (ref. 4):

$$\text{compound II} + H_2O_2 \rightarrow \text{oxyperoxidase} \quad (2)$$

Several types of steps for the further conversion of oxyperoxidase in reacting peroxidase systems have been proposed (*cf.* refs. 1, 3, 5). One such step involves a reaction between oxyperoxidase and compound II (Eqn. 3) (refs. 3, 5) and another step, a reaction with ferroperoxidase (Eqn. 4) (refs. 1, 3, 5):

$$\text{oxyperoxidase} + \text{compound II} \rightarrow 2\ \text{ferriperoxidase} + O_2 \quad (3)$$

$$\text{oxyperoxidase} + \text{ferroperoxidase} \rightarrow 2\ \text{ferriperoxidase} + H_2O_2 \quad (4)$$

These two steps can be described as interenzymic, since they imply an electron transfer reaction between two peroxidase molecule species. Whether interenzymic steps indeed occur, to what extent oxyperoxidase is converted *via* such steps, and the species with which oxyperoxidase reacts have not been firmly established and are the subjects of this note.

A highly purified horseradish peroxidase preparation (Worthington, electrophoretically purified grade) with an absorbance ratio $A_{403\ nm}/A_{275\ nm}$ of 3.05 was used. The initial rate of the peroxidase-catalyzed oxidation of *o*-dianisidine with H_2O_2 as the H-acceptor was obtained spectrophotometrically by recording the rate of ab-

TABLE I

REACTION ORDER WITH RESPECT TO THE ENZYME AND INITIAL RATE OF THE PEROXIDASE-CATALYZED OXIDATION OF *o*-DIANISIDINE AT DIFFERENT H_2O_2 AND *o*-DIANISIDINE CONCENTRATIONS

The initial rate of absorbance change (dA/dt) was measured at several enzyme concentrations to obtain the reaction order (*cf.* Fig. 1). The rates given in the table were calculated through intrapolation to an enzyme concentration of $5 \cdot 10^{-10}$ M.

o-Dianisidine concn. (mM)	*H_2O_2 concn. (mM)*	*dA/dt (min^{-1})*	*Reaction order with respect to peroxidase*
0.03	0.03	0.072	1.00
0.03	0.3	0.390	1.16
0.03	3	0.280	1.27
0.03	31	0.013	1.63
0.1	31	0.062	1.12
0.3	31	0.130	1.06

sorbance change at 500 nm. Measurements were performed in 30 mM potassium phosphate (pH 6.0) at a temperature of 25°.

In the presence of sufficiently high H_2O_2 concentrations, oxyperoxidase is formed[4]. Since oxyperoxidase undergoes further reaction at a comparatively low rate[5], inhibition by H_2O_2, also acting as a substrate, can be observed[6] (see also Table I).

Normal enzymic reactions are strictly first order with respect to the enzyme[7]. If, however, the reaction mechanism includes an interenzymic step of the type described by Eqns. 3 and 4, any order up to 2 can be expected. When the logarithm of the rate is plotted against the logarithm of the enzyme concentration, linear curves are obtained in the case of the reaction examined here (see Fig. 1). In such a plot the slope

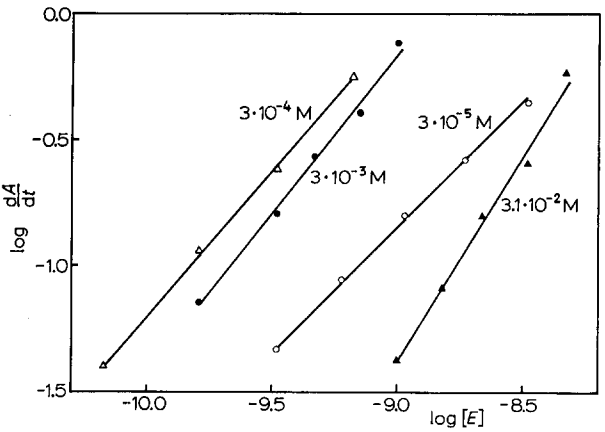

Fig. 1. The logarithm of the initial rate of absorbance change is plotted against the logarithm of the enzyme concentration to determine the order of the reaction with respect to the enzyme. The measurements were performed with $3 \cdot 10^{-5}$ M *o*-dianisidine as oxidizable substrate. The H_2O_2 concentrations used are given in the figure. The slopes of the lines are equal to the orders, and these are given in Table I.

is equal to the order with respect to the enzyme. At a low H_2O_2 concentration an order of 1 is found, but when the H_2O_2 concentration is increased to an inhibitory or nearly inhibitory level, at which oxyperoxidase may be expected to be present, orders ranging up to 1.63 can be observed (see Table I).

Compound II and probably also oxyperoxidase react with substrates oxidized in peroxidase reactions[5]. If the concentration of the oxidizable substrate is increased, both the reaction steps mentioned should contribute to a reduction of the oxyperoxidase concentration. An increase in the *o*-dianisidine concentration would therefore be expected to counteract the inhibition and the anomalous reaction order apparent at high H_2O_2 concentrations. Such a phenomenon can also be observed (see Table I).

Previously reaction orders higher than 1 with respect to the enzyme have been observed apparently only when the enzyme preparation contained a dissociable activator or coenzyme[7]. In such cases the anomalous order is due to the coupling of the activator or coenzyme concentration with the enzyme concentration, and the reaction can not be considered as truely higher than first order with respect to the enzyme. Dialysis of the peroxidase used in this study for a total of 72 h against 6 changes of 20 mM potassium phosphate (pH 7.0) had no effect on the reaction orders

observed. Thus anomalous orders could not have been caused by a dissociable low molecular weight activator.

Horseradish peroxidase oxidizes substrates to free radicals, which undergo further reactions[8]. These reactions may be complex, and it could perhaps be suggested that they might explain the unusual kinetics. We have obtained results closely similar to those described above when I^- or pyrogallol was used as oxidizable substrate instead of *o*-dianisidine. The primary product formed from I^- is presumably atomic iodine. Iodine atoms dimerize very rapidly to I_2 (ref. 9), which should eliminate complex reaction chains and the possibility that the order with respect to the enzyme is affected.

The oxidizable substrates used are not capable of reducing the enzyme to ferroperoxidase[10], which rules out ferroperoxidase as a possible participant in interenzymic reaction steps. In the presence of an oxidizable substrate, compound I is a short-lived intermediate present only in very low concentration[11] and its participation is therefore quite improbable. The concentration of ferriperoxidase, which reacts with H_2O_2, will also be low at the high H_2O_2 concentrations at which high orders with respect to the enzyme are best observed. This leaves reaction between compound II and oxyperoxidase as the most probable explanation for the present data. Since a reaction order as high as 1.63 has been observed, a high proportion of the oxyperoxidase can apparently be transformed *via* the interenzymic step under suitable conditions.

Participation by oxyperoxidase in an interenzymic reaction step is, in our opinion, by far the most plausible explanation for the anomalously high reaction orders with respect to the enzyme, which are reported here.

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