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A Specific Depression of the Reactivity of Peroxidase Complex II towards Donor Molecules by Substitutions in the Haem Group

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The substitution of the vinyl groups in the horse radish peroxidase protohaematin by acetyl groups has previously [1] been found to give decreased overall peroxidase activity. It is now demonstrated that the substitution affects the reaction between peroxidase-peroxide complex II and donor (k_4) whereas the rate of formation of complex I remains constant. The substitution has a greater effect upon the reactivity with uric acid than with ascorbic acid. The reason for the change in the complex II-donor reaction is briefly discussed. The experiment suggests a specific control of enzyme reactivity by side chain substitutions in the haematin.

For several reasons it has become of interest to determine the way in which the enzymic activity of horse radish peroxidase is affected by changes in the structure of its haem group. The initial studies of Theorell, Bergström and Åkeson [2] showed that successful recombinations with meso- or deutero-haematins and peroxidase apoprotein can be achieved. The activity with these haematins was found to be about half of that of the intact enzyme; a considerably higher activity with mesohaematin was, however, also reported [3]. Somewhat later monoazahaematin peroxidase was found to be one fifth as active as the intact enzyme [4]. Recently the activities of some of the previously studied artificial peroxidases were redetermined and in some cases also compared with the activities obtained with isomers of the haematins [1]. It is now well established that the propionic acid residues at the positions 6 and 7 are needed for the formation of an enzymatically active peroxidase [1,2,5]. Some 2,4-substitutions cause parallel shifts in peroxidase activity and pyridine haemochromogen spectrum [1]. Deuterohaematin is an exception, but its two free pyrrolic 2-positions may influence the activity. The homologue with three free 2-positions gave one tenth of the activity obtained with deuterohaematin itself [6].

Further studies of the artificial peroxidases are of interest also in view of the knowledge of haemoprotein structures now available through X-ray crystallography [7,8] and of the effect of haematin substitutions on the reactivity of myoglobin [9,10]. The fact that meso-, haemato-, and deuterohaematins but not diacetyldeuterohaematin can be utilized as growth factors by a haem-requiring *Staphylococcus* mutant [11] also will draw attention to the mechanism by which the artificial peroxidases differ.

In most determinations of the activity of reconstituted peroxidases, the purpurogallin test was used. A study of this reaction indicated the possibility of a rate-limiting step between the completion of the enzyme activity and the formation of coloured products [12]. Whereas such a slow step will not necessarily be of great consequence in determinations of protoperoxidase purity the situation may not be identical with substituted peroxidases. The mesidine test, used in some activity determinations, is at present being studied in the same way (B.C. *et al.*). A further complication is introduced by the fact that the over-all assays may measure either k_1 or k_4 according to the following equation [12]:

$$\frac{dx}{dt} = \frac{e}{(1/k_4 a) \times (1/k_1 x)} \qquad (1)$$

where k_1 and k_4 are defined below, a and x are the donor and peroxide concentrations and dx/dt is the rate of disappearance of x.

It is the purpose of this paper to study a reconstituted peroxidase of an activity sufficiently different from that of the protohaematin enzyme to permit the assigning of the decrease in activity to one or more steps in the following reaction mechanism (*cf.* Ref.[13]):

$$HRP + H_2O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} HRP - I + H_2O \qquad (2)$$

$$HRP - I + AH_2 \xrightarrow{k_7} HRP - II + AH^\circ \qquad (3)$$

$$HRP - II + AH_2 \xrightarrow{k_4} HRP + AH^\circ \qquad (4)$$

Diacetyldeuterohaematin peroxidase is such an enzyme [1].

MATERIAL AND METHODS

Horse radish peroxidase was prepared as previously described [14], and Fraction C was crystallized twice from ammonium sulfate solution. The preparation of the apoprotein and the coupling was performed as usual [5], ethanol being added to stabilize the haematin solutions in alkali [15]. After the chromatography on Dowex — 2 the concentrations of the reconstituted enzymes were determined by pyridine haemochromogen measurements [5].

The methyl peroxide solution was standardized by UV absorption as previously described [16]. Tenth molar acetate buffer of pH 4.6 and 26° were used in all experiments.

The stopped-flow apparatus [17] (optical path length 1 cm) was used to record the cycle of formation and disappearance of the enzymesubstrate intermediates. With this, a double-beam spectrophotometer [18] was used to record the absorbancy changes of the reactants, the wavelengths being set to the peak and trough values for the difference between the free enzyme and the secondary peroxidase complex, II. For protoperoxidase the appro-

priate wavelengths were 427 and 395 mμ, and for diacetyldeuteroperoxidase 415 and 388 mμ. The initial rate of the absorbancy change was measured from a mirror oscillograph recording and the full cycle of appearance and disappearance of the intermediate was recorded on a slower time scale (Fig. 1).

The peroxide was added to a solution containing the enzyme and the donor in the stopped-flow apparatus. This procedure was desirable since any trace of peroxide formed by autoxidation of the donor would be removed prior to testing the activity. The final concentration of haematin was 0.5 μM.

The molecular extinction coefficient of the protohaematin peroxidase was $\varepsilon = 108$ cm^{-1} mM^{-1} and that of the diacetyldeuterohaematin peroxidase was 71 cm^{-1} mM^{-1} at 403 and 405 mμ, respectively. The reconstituted protohaematin peroxidase gave this value whereas the unsplit material [14] gave 98 cm^{-1} mM^{-1}, both on the basis of pyridine haemochromogen measurements. $\varepsilon = 108$ has been used for the present calculations.

According to determinations in the split-beam recording spectrophotometer [19] the extinction coefficient of the secondary complex as measured at the pairs of wavelengths 427—395 mμ and 415—388 for proto- and diacetyldeuterhaemin peroxidases, respectively, were 84 and 59 cm^{-1} mM^{-1}. In the flow apparatus, however, where the absorbancy change is measured immediately after the reaction of peroxidase with donor and peroxide, the values were higher, 106 and 74 cm^{-1} mM^{-1}. These were used in calculating k_4 from the cycles of the intermediate compounds.

RESULTS

Typical experimental results are shown on the superimposed tracings of Fig. 1 which represents the formation and disappearance of complex II of protohaematin peroxidase (upper trace) in the presence of 7 μM uric acid and 1.5 μM methyl peroxide. Time proceeds from left to right and illustrates the reaction kinetics which ensue upon mixing peroxide with enzyme plus donor. The upward deflections of the traces indicate increases of absorbancy at 427 and 415 mμ, measured with respect to 395 and 388 mμ, respectively. The intermediate forms abruptly in both cases. In the case of protohaemin peroxidase the concentration of the intermediate falls to zero in a few seconds, the value of $t_{\frac{1}{2}\ off}$ being 4.8 sec. In the case of diacetyldeuterohaemin peroxidase $t_{\frac{1}{2}\ off}$ is 8 times larger (40 sec.).

A quantitative determination of the values of k_4 can be obtained from the formula [20,21]:

$$k_4 = \frac{x_o}{p_{max}\ t_{\frac{1}{2}\ off}\ [AH_2]} \qquad (5)$$

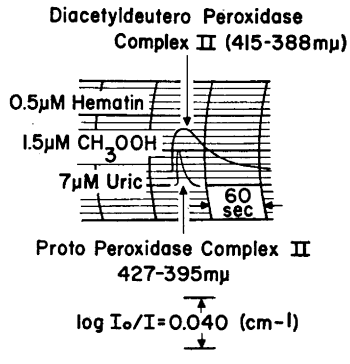

Fig. 1. Superimposed spectrophotometric records showing the kinetics of formation and disappearance of protohemin peroxidase and deuterohemin peroxidase, measured at the appropriate wavelengths indicated on the diagram. Time moves from left to right; an upward deflection of the trace corresponds to increased absorbancy at the longer wavelengths. 0.5 μM peroxidase, 0.1 M acetate buffer, pH 4.6, 26°C. Other conditions indicated on the diagram (Expts. 132 b—6,7).

Table 1. Comparison of the kinetics of proto- and diacetyldeuterohematin peroxidase (Expt. 132 b).

Hematin	Proto-	Diacetyl-deutero-	Proto-	Diacetyl-deutero-
λ(mμ)	427–395	415–388	427–395	415–388
(x_o) CH_3OOH (μM) Donor	1.5 Ascorbic acid	1.5 Ascorbic acid	1.5 Uric acid	1.5 Uric acid
(a_o) Donor concentration (μM)	10	10	7	7
p_{max} (μM)	0.31	0.28	0.26	0.43
$t_{\frac{1}{2}\ off}$ (sec)	5.0	20	5.0	40
$k_4 \times 10^4$ ($M^{-1} \times sec^{-1}$)	0.97	0.26	2.0	0.13
Rate of formation (μM/sec)	0.49	0.42	0.63	0.51

The values of k_4 calculated in Table 1 by eqn. 5 show that the ratio of the activities for proto- and diacetyldeuteroperoxidases is 15 with uric acid as the donor. Experiments carried out with ascorbic acid as the donor give a ratio of 3.7. It should be noted that the values of k_4 for protoperoxidase obtained in the presence of ascorbic acid are significantly less than those obtained with previous preparations [22]. No explanation is put forward for this difference at the present time.

The data of Fig. 1 show qualitatively the cause of the low reactivity of diacetyldeuteroperoxidase, namely that there is a higher steady-state concentration of complex II in this substituted peroxidase than in the natural one. This suggests that the formation of the intermediate is not a ratelimiting factor and that its decomposition is the rate-limiting factor.

Support for the idea that the rate of formation of the intermediates is not greatly affected by the substitution of the haematin is afforded by kinetic data obtained with the stopped flow method. The last row of Table 1 gives the rates of formation of the intermediates in μmoles per liter per second (μM/sec) in the four experiments. It can be seen that the values for diacetyldeuterohaematin peroxidase are between 80 and 85 % of those for the protohaematin enzyme.

Table 2. Values of k_4 as determined in open cuvettes.

	Ascorbic acid	Uric acid
	$k_4 \times 10^{-4}$ M^{-1} s^{-1} (S.D.,n)	
Protohaematin-HRP	0.62 ± 0.04 (6)	1.88 ± 0.52 (5)
Diacetyldeutero-haematin-HRP	0.12 ± 0.02 (3)	0.21 ± 0.01 (7)

In another series of experiments varying amounts of peroxide were added to a solution of peroxidase and donor in an open cuvette (Table 2). In this series the ratio of activities in the presence of ascorbic acid was 5 and in the presence of uric acid 9. Measurements of the overall reaction in terms of the formation of "tetraguaiacol" from guaiacol were also studied. When performed as previously described [23] the guaiacol test gave the values 2.86 and 2.56×10^4 M^{-1} sec^{-1}. Under these conditions the test gives mainly k_1 with some contribution from k_4.

DISCUSSION

In these experiments it has for the first time been possible to assign to a substitution of groups in the haematin of the peroxidase a depression of one of the three reaction velocity constants for the action of this enzyme (see eqns. 2—4). That this is a specific effect is clear from a comparison of the lifetime of complex II of the proto- and diacetyldeuterohaematin enzymes in the presence of equal concentrations of donor and peroxide, as shown by Fig. 1. When suitable corrections have been made for the actual amount of complex II present, these curves show a 15-fold decrease in the rate of reaction with uric acid and an approximately fourfold decrease in the reaction with ascorbic acid.

Several causes for the reduction in reactivity of complex II can be discussed. The possibility that the splitting procedure somehow altered the apoprotein is ruled out by previous investigations [1,2,4] as well as by the present experiments, in which both peroxidases were recombined from the same batch of apoprotein. An alternative explanation is presented by a comparison with kinetic studies on catalase [24] which suggested a limited accessability of complex I to donor molecules of increasing chain length. At that time related effects had not yet been observed with horse radish peroxidase. A consideration of the crystallographic data on metmyoglobin [8] indicates that its haematin might be of limited accessability and, of most importance, that the vinyl groups of the haematin could be a factor in determining the accessibility of the iron porphyrin group to large molecules [10]. Although no noticeable limitation to the accessibility of peroxidase haematin is observed with the native enzyme, the haematin configuration in the diacetyldeuteroperoxidase may represent a less favourable steric situation. However, dipropionyldeuterohaematin peroxidase showed approximately the same activity as the diacetyl enzyme in the quaiacol test. If steric effects account for the decrease in the rate of reaction one would expect an inhibition of the reaction of complex I with the donor molecule to form complex II, provided that the same reactive site is involved in this transformation. Further, an inhibition of the reactivity of the peroxidase to alkyl hydrogen peroxides of increasing chain length to form complex I would also be expected. Experiments on this point will be taken up.

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