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The Oxidation of Aminotriazole by Horse Radish Peroxidase (HRP)

K. G. PAUL and E. MARGOLIASH*

Medicinska Nobelinstitutet, Biokemiska avdelningen, Stockholm, Sweden

The mutual effects of horse radish peroxidase and 3-amino-1,2,4-triazole have been studied spectrophotometrically. With the present technique no evidence was found for the formation of a compound between aminotriazole and peroxidase or any of the peroxidase-peroxide complexes. The complexes I and II reacted with aminotriazole at the rates of 460 and 200 $min^{-1}M^{-1}$ at pH 7.4 and room temperature. Aminotriazole did not inhibit the activity of horse radish peroxidase in the presence of continuously supplied hydrogen peroxide, but rather protected the peroxidase against destruction by hydrogen peroxide.

The observation that plants which had been exposed to 3-amino-1,2,4-triazole (AT) contained less chlorophyll than normal plants led to the discovery that extracts from such plants showed a subnormal catalase activity [1,2]. In livers and kidneys from rats, which have been given AT perorally, intraperitoneally, or intravenously, the catalase activity was decreased to such an extent that only 10 %, occasionally less, remained of the normal value per unit weight or nitrogen. The liver catalase activity dropped remarkably rapidly, maximal reduction being reached already after 3 h. Normal values were found on the seventh day. Blood catalase, haemoglobin, and liver cytochrome c remained normal. In experiments *in vitro* with AT and homogenized livers from normal animals or crystallized catalase considerably higher concentrations of AT were needed to bring about any decrease [2]. The *in vitro* effect could be abolished by dialysis, in contrast to the *in vivo* effect, and was attributed to some unspecific action of AT in high concentration. "Liver peroxidase" was slightly decreased after 3 h but normal again after 24 h. In another investigation [3] the same result with liver homogenate was obtained, but no inhibition of pure catalase occurred unless liver homogenate was added. It was also noticed that molecular oxygen was necessary for the effect. The δ-aminolaevulinic acid dehydrase activity in the AT-rats

* On leave from the Hebrew University—Hadassah Medical School, Jerusalem.

was found to be somewhat reduced although less than the catalase. Minimum activity, 75 % of the normal, was found 8 h after the injection of AT, and the activity had returned to normal after 36—48 h. Quite recently it was demonstrated [5,6] *in vitro* with pure catalase that AT reacts not with catalase itself but with complex I between catalase and hydrogen peroxide, resulting in a complete inhibition of catalatic activity. This reaction was irreversible and one mole of AT was incorporated into the enzyme protein for each mole of catalase haem reacted. These results explain the rapid decrease in catalase activity of liver and kidney observed *in vivo* after the administration of AT [1,2], making it unnecessary to invoke an effect of AT on the formation of the enzyme. The time for restoration of full liver catalase activity agrees well with the life span of liver catalase.

Although several factors can account for the lack of effect of AT on erythrocyte catalase *in vivo* the presence of hydrogen donors in the red cells may be the most important one [6]. Such donors would prevent the irreversible inhibition of catalase by AT by reacting rapidly with catalase-hydrogen peroxide complex I, thus competing with the slow, irreversible reaction of AT with the complex. The possibility that the effects of AT administration could be caused by some metabolite of AT rather than by AT itself was made less likely, although not excluded, by the failure to find any evidence, direct or indirect, for the participation of such a metabolite in the inhibitory reaction [5].

The less pronounced effect on δ-aminolaevulinic acid dehydrase may be caused by inhibition of action or synthesis or both, and may also be quite unspecific.

In the present investigation some of the mutual effects of another hydroperoxidase, horse radish peroxidase, and AT have been studied in the presence of hydrogen peroxide.

METHODS AND MATERIAL

AT from Light & Co Ltd was used without pretreatment. HRP (component C) had been prepared as previously described [7]. Spectra were taken with Beckman spectrophotometers. Dr. R. Ordell, Serafimerlasarettet, Stockholm, kindly put a useful DK 2 instrument at our disposal.

RESULTS AND DISCUSSION

The titration of AT (117 mg in 20 ml of water, room temperature) with molar hydrochloric acid and sodium hydroxide revealed an ionization at pH 4.35. Another group was traced at pH < 2 after correction for the acid, and a third one was seen at pH 10. Although a comparison with pyridine and aniline shows that the three groups are not immediately identifiable the titration made it possible to choose pH-values where only one form of AT is present.

The results in Table 1 show that the peroxide but not AT inactivates HRP under the given conditions and that AT to some extent protects HRP against inactivation by the peroxide. This protection may simply depend upon the continuous consumption of the peroxide passing the cellophane. During the dialysis a yellow colour appeared in the solutions inside and outside the sac.

Table 1. Activity changes in HRP during dialysis against peroxide and AT. 1.5 ml of 10 μM HRP and 20 mM AT in phosphate buffer of pH 7.4 were dialysed at 37° against 100 ml of 20 mM AT and 0.25 mM hydrogen peroxide in the same buffer. Controls were made without AT or peroxide. For the assay of the peroxidatic activity 50 μl of the solution inside the sac were diluted with 1.00 ml of water, 10 μl of the dilution being taken for the assay with 3 ml of guaiacol — buffer (pH 7) mixture. t = the time (in min after the addition of peroxide) during which the absorbancy at 470 mμ increased by 0.050 [8,9].

Hours of dialysis	HRP AT H_2O_2	HRP AT —	HRP — H_2O_2
	Peroxidase activity in min^{-1} $(1/t)$		
0	3.8	4.6	3.9
1	4.0	4.1	3.4
2	3.9	4.4	3.7
3	3.4	4.3	2.4
4	2.9	3.9	1.8
7	3.3	4.3	1.3

It faded only slowly at neutral pH but instantaneously upon acidification or alkalinization. Its formation and disappearance were followed spectrophotometrically at pH 4.9, where it seemed to have its intensity maximum (Fig. 1). As long as any peroxide remained the yellow colour increased in intensity.

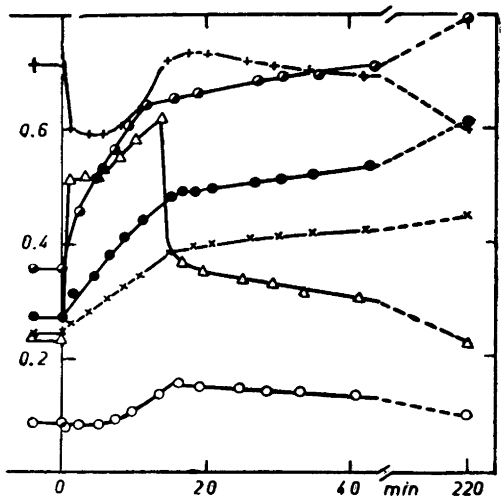

Fig. 1. Spectral changes during the reactions between HRP (10 μM), hydrogen peroxide (10 mM), and AT (5 mM) in acetate buffer of pH 4.9. The peroxide was added to the other reactants at time 0. The HRP-absorption at 460 mμ is independent of the peroxide concentration. Free HRP and complex I are isosbestic at 427 mμ, free HRP and complex II at 412 mμ.

○ 460 mμ + 412 mμ ● 268 mμ
△ 427 mμ × 285 mμ ◒ 250 mμ

When the peroxide had been consumed, as indicated by the sudden changes in the Soret band, the production of the yellow pigment ceased and the colour began to fade. The spectral changes after the termination at about 15 min of the enzymic reactions appeared as first order reactions. The changes must reflect several reactions since the specific rate constant varied from one wavelength to another and since the constant obtained at 427 mμ showed a sudden change at 55 min (Table 2).

Table 2. Specific velocity constants for the non-enzymic reactions of the primary oxidation products occurring during the time range 15—220 min in Fig. 1.

mμ	250	268	285	410	427	460
min^{-1}	1.0	1.3	1.9	−0.9	−1.2 −0.7	− 1.1

The spectral changes during the first minutes were followed by scanning the range 440—380 mμ at suitable time intervals. It was possible and advantageous to work with equimolar concentrations of HRP and peroxide. This

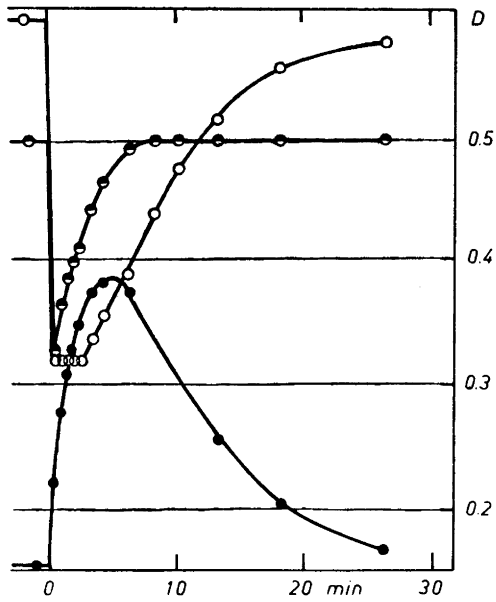

Fig. 2. Spectral changes in the Soret band region during the reactions between HRP (7 μM), hydrogen peroxide (7 μM), and AT (140 μM) in 0.1 M phosphate buffer of pH 7.4. Peroxide and AT were added simultaneously at time 0.

○ 397 mμ (complex I and complex II isosbestic)
× 412 mμ (complex II and free HRP isosbestic)
● 427 mμ (complex I and free HRP isosbestic)

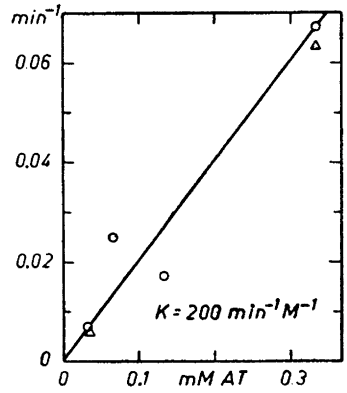

Fig. 3. Velocity constant for the appearance of free HRP (○) and disappearance of complex II (△) as a function of the concentration of AT. HRP, peroxide, and time as in Fig. 2. The values are corrected for the »spontaneous decay» of the complex.

actually converted the enzymatic system to a stoichiometric reaction. The specific rate constant for the spontaneous increase in density at 412 mμ for a solution 7 μM in HRP and 7 μM in hydrogen peroxide in 0.1 M phosphate buffer of pH 7.4 was 0.0035 min^{-1}. An example of the spectral changes during the reactions between HRP-hydrogen peroxide complexes I and II and AT is given in Fig. 2. In these experiments the contribution of the yellow pigment to the absorption in the Soret band region was negligible. With 7—350 μM AT isosbestic points were found at 397 mμ during the initial and at 412 mμ during the terminal part of the reaction. The isosbestic point for complex I/free HRP (427 mμ) could only occasionally be visualized with this technique because of the similarity in shape of the absorption curves of complex II and free HRP.

The specific velocity constant for the appearance of free HRP (Fig. 3) was obtained from the absorbancy changes occurring at 397 mμ after the isosbestic level at 412 mμ had been reached. At a few concentrations of AT it was possible to follow, with this technique, the decrease in absorption at 427 mμ sufficiently accurately to permit the calculation of the velocity constant for the disappearance of complex II (Fig. 3).

The specific velocity constant for the disappearance of complex I was calculated from the changes at 427 mμ during the first minutes when the absorbancy at 397 mμ remained constant (Fig. 4).

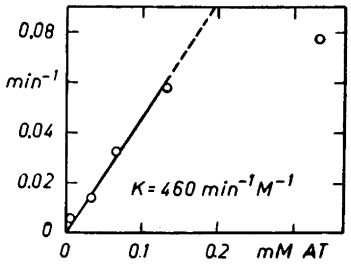

Fig. 4. Velocity constant for the disappearance of complex I. Same conditions as in Fig. 3.

Under the experimental conditions used above (Fig. 2—4) AT gave no spectrophotometrically operable compound with HRP or the HRP-peroxide complexes as long as the concentration of AT was lower than 350 uM. This is obvious from several facts. Two isosbestic points were found, and it is unlikely that both are threefold isosbestic. They were found at the wavelengths which have been reported for the isosbestic points between complex I and II (397 mμ) and complex II and free HRP (412 mμ)[10]. The velocity constants for the appearance of free HRP and the disappearance of complex II agreed fairly well (Fig. 3). At 350 μM AT, however, the disappearance of complex I was markedly hampered (Fig. 4), and the decrease in density at 397 mμ upon the addition of AT and peroxide was not instantaneous. It should be kept in mind, however, that in the case of catalase a compound between AT and the catalase-peroxide complexe was formed only at considerably higher concentrations of AT as well as of the complex. At such a high peroxide concentration as was used in the catalase experiments HRP would have been rapidly inactivated.

At the concentrations of AT used in the present investigation nothing has been found that would put AT in a class of its own as regards the reactions with HRP. The two HRP-peroxide complexes seem to react with AT at the rates of 460 and 200 min^{-1} M^{-1}, thus quite slowly. This fact, and the purity of the HRP preparation, makes the system a suitable one for studies of the peroxidative reactions.

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