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Abstract

In the previous papers, it has been shown that the substrate inhibition of xanthine oxidase (xanthine: O2 oxidoreductase, EC 1. 2. 3. 2) induced by exce purines requires a small amount of exogenous metallic ions. Among these ions, Cu^2+ was the most typical one. At any stage of enzyme reaction, the inhibition began immediately on addition of a small amount of Cu^2+ such as 6.6 X 10-7 M. Since the depreed activity was not restored by the addition of chelating agents such as histamine and EDTA, it was suggested that the substrate, Cu^2+ and enzyme form a stable inactive enzyme complex, from which chelating agent can no longer remove Cu. The present communication describes the further investigations concerned with the formation of the substrate-enzyme complex in the presence of Cu^2+ and with the catalytic nature of this complex on other substrate and acceptor systems.

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FORMATION OF AN INACTIVE SUBSTRATE-Cu-ENZYME COMPLEX OF XANTHINE OXIDASE

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In the previous papers[1-3], it has been shown that the substrate inhibition of xanthine oxidase (xanthine : 0_2 oxidoreductase, EC 1. 2. 3. 2) induced by excess purines requires a small amount of exogenous metallic ions. Among these ions, Cu^{2+} was the most typical one. At any stage of enzyme reaction, the inhibition began immediately on addition of a small amount of Cu^{2+} such as 6.6×10^{-7} M. Since the depressed activity was not restored by the addition of chelating agents such as histamine and EDTA, it was suggested that the substrate, Cu^{2+} and enzyme form a stable inactive enzyme complex, from which chelating agent can no longer remove Cu. The present communication describes the further investigations concerned with the formation of the substrate-enzyme complex in the presence of Cu^{2+} and with the catalytic nature of this complex on other substrate and acceptor systems.

Together with hypoxanthine and Cu^{2+}, the purified xanthine oxidase[4] (A_{280}/A_{450}, 5.9) was left standing in ice water for 20 min. A fraction was isolated from the mixture by precipitating with $(NH_4)_2SO_4$ at 0.6 saturation (pH 5.6) and washed twice with $(NH_4)_2SO_4$ solution of the same concentration. This fraction showed stronger ultraviolet absorbancy in 0.1 M potassium phosphate buffer (pH 7.4) than the original enzyme solution or the fraction similarly obtained from the mixture of the substrate and enzyme, not containing Cu^{2+}. Substances which showed higher absorbancy in the ultraviolet region were released in the $(NH_4)_2SO_4$ solution by acidifying to pH 2.8. Fig. 1 indicates that the substances combined to enzyme are hypoxanthine and xanthine, since after the effect of xanthine oxidase they are converted to uric acid showing a maximum absorption at 290 mμ. Subsequent experiments showed that Cu^{2+} was indispensable for the formation of the complex, and that in the presence of 1.7×10^{-6} M of enzyme the maximal yield of the complex was demonstrated at 5×10^{-4} M of Cu^{2+} and at 2×10^{-4} M of hypoxanthine, respectively. Using C^{14}-labelled hypoxanthine, similar results were obtained by counting the radioactivity of the filtrate of a Sephadex G 25 column (bufferized with 0.005 M potassium phosphate, pH 7.4) or the acid soluble fraction prepared from the acetone precipitate.

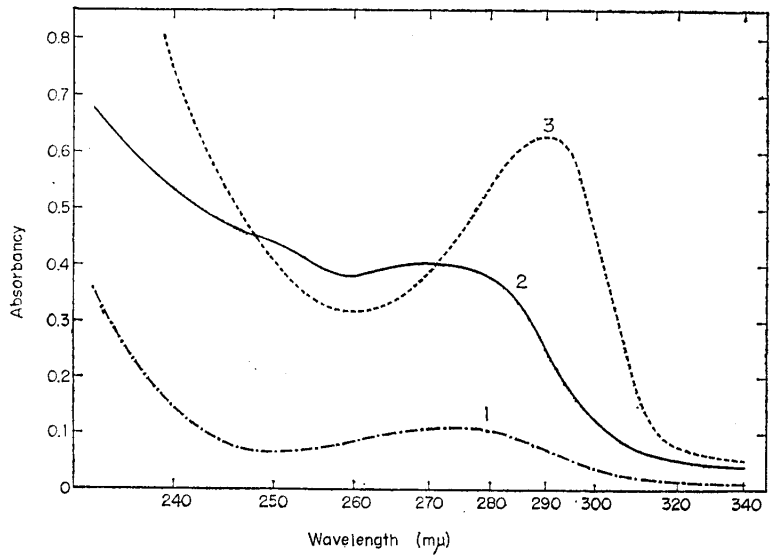

Fig. 1 Absorption spectrum of the acid soluble fraction from the inactive enzyme complex. 8 ml of the reaction mixture consisting of 1.4×10^{-6} M of enzyme, 5×10^{-4} M of hypoxanthine, 0 or 5×10^{-4} M of $CuSO_4$ and 3 ml of potassium phosphate buffer (pH 7.4) was left standing in ice water for 20 min. After addition of 3.1 g $(NH_4)_2SO_4$, the precipitate was washed twice with 2 ml of $(NH_4)_2SO_4$ solution of 0.6 saturation. The substrate was separately solubilized by addition of 0.1 ml of 1 N HCl to the precipitate suspended in 3 ml of $(NH_4)_2SO_4$ solution of 0.4 saturation. This solution served as the material for the spectrophotometric analysis after neutralizing to pH 7.4 with 1 N NaOH. Curve 1, the acid soluble fraction from enzyme and substrate; curve 2, the same fraction from the mixture of enzyme, Cu^{2+} and substrate (the inactive enzyme complex); curve 3, the same fraction as curve 2, but effected by xanthine oxidase.

The content of substrate and Cu of the inactive complex was determined. Table 1 suggests that one mole of the enzyme combines 24.9 moles of substrate and 46.0 moles of Cu. However, it is difficult to determine the accurate molar ratio of substrate to Cu, since this complex is converted to a more labile urate-enzyme complex in the room temperature and purines are more readily released than Cu from the complex.

Among the other purine derivatives, xanthine, uric acid, allopurinol (4-hydroxypyrazolo-3, 4-*d*-pyrimidine) and adenine formed a similar complex as hypoxanthine. However, nucleosides and nucleotides: inosine, adenosine, inosine monophosphate and adenosine monophosphate did not combine with the enzyme. Pb^{2+}, Fe^{2+}, Fe^{3+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Hg^{2+}, and *p*-chloromercuribenzoate were not able to bind hypoxanthine to the enzyme.

When the enzyme preparation heated at 60° for 5 min was used, the

Table 1 Substrate and Cu content of the inactive enzyme complex. The experimental conditions were the same as Fig. 1, except that the acid treatment was repeated once more. The substrate content was calculated from the absorbancy at 290 mμ as uric acid, which molar extinction coefficient is 11.3×10^3, after the treatment with xanthine oxidase of the acid extract. Cu content was determined colorimetrically with dithizone[5] after an $(NH_4)_2SO_4$ precipitate was hydrolysed with conc. HNO_3. The protein content of enzyme was estimated by absorption at 280 mμ and 260 mμ[6], and the molar concentration calculated on the basis of molecular weight 3.2×10^5.

Expt. No.	Bound substrate (A at 290 mμ)	Bound Cu (μg)	Substrate/enzyme (mole/mole)	Cu/enzyme (mole/mole)
1	1.27	31.9	30.6	40.5
	1.32	38.6	31.8	49.0
	1.29	41.3	31.0	52.3
2	0.75	26.6	18.0	33.9
	0.78	27.8	18.7	35.4
	0.82	30.9	19.6	39.4
3	0.89	41.3	24.0	52.3
	0.92	41.3	24.9	52.3
	0.94	46.3	25.7	58.7
Average value			24.9±1.8*	46.0±2.9*

* Standard error.

amount of the substrate being combined was decreased to 70 per cent that combined by the non-heated enzyme and when heated at 70° decreased to 8 per cent. This was in accord with the rate of oxidation of hypoxanthine by the enzyme preparations treated at respective temperature. The complex formation mediated by 5×10^{-4} M of Cu^{2+} was depressed to 14 per cent in the presence of the same molar concentration of *p*-chloromercuribenzoate. These results show that the formation of the complex depends directly on the activity of the enzyme.

In the reaction system containing histamine, the rate of oxidation of hypoxanthine by the substrate-Cu-enzyme complex (the $(NH_4)_2SO_4$ fraction from the mixture of substrate, Cu^{2+} and enzyme) was 5.5 per cent that by the original enzyme, while the fraction from the substrate and enzyme, was 80.5 per cent and the fraction from Cu^{2+} and enzyme, 65.0 per cent, respectively. The substrate-Cu-enzyme complex also lost the ability of the oxidation of hypoxanthine or acetaldehyde by methylene blue. However, for the oxidation of dihydronicotinamide adenine dinucleotide by oxygen this complex still retained the activity, which is not effected by histamine[1].

From these results, it is concluded that there is little difference between the properties of substrate-Cu-enzyme complex which contains the surprising amount of substrate and Cu and that of a stable inactive complex postulated in the experiments on the substrate inhibition in the previous report[3].

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