EVIDENCE FOR THE FORMATION OF AN INACTIVE UREASE-HYDROXAMIC ACID COMPLEX

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Hydroxamic acid has been found to be a potent and specific inhibitor of urease activity (urea amidohydrolase, ED 3.5.1.4) (Kobashi, Hase and Uehara, 1962; Fishbein et al, 1965). A previous report (1966) showed that inhibitory function to be the -CONHOH group and described the relation between the chemical structure of various hydroxamic acids and their inhibitory effects on the activity of urease. In this communication we present conclusive evidence for the formation of a urease-hydroxamic acid complex. ^{3}H-labeled caprylohydroxamic acid, one of the most potent inhibitors of urease, was used in these studies, allowing determination of the molar ratio in the enzyme-inhibitor complex.

^{3}H-labeled caprylohydroxamic acid was prepared by the method of Wiltzbach (1957). Radioactivity was measured with a liquid scintillation spectrometer (Packard Co. Tri-Carb). The labeled hydroxamic acid (1,620 counts/min/mμmole) was purified by repeated crystallization. Further purification was achieved by paper chromatography with butanol: acetic acid:water = 4:1:2 as the developing solvent, following which the radioactive spot of caprylohydroxamic acid was eluted with water. During these purification procedures, the specific radioactivity decreased to the constant value, 470 counts/min/mμmole, from which the

number of moles of caprylohydroxamic acid were calculated, on the assumption that the interaction of the hydroxamic acid with urease did not affect its specific radioactivity. Crystalline urease (126,000 units/g) was prepared by the method of Uehara and Kobashi (1959). Ureolytic activity was estimated by the colorimetric method of van Slyke and Archibald (1944). Moles of urease was calculated from Sumner units or mg of protein and its molecular weight, 473,000 (Sumner et al, 1938). The number of moles of inactive complex formed was calculated by comparing the specific activity of the inhibited sample with that of the starting material.

In the experiment shown in Table I, 29.2 mμmoles of crystalline urease dissolved in 0.1 M phosphate buffer (pH 7.8) was allowed to react with 348 mμmoles of ^{3}H-labeled caprylohydroxamic acid in a total volume of 14.0 ml. Inhibition was measured after incubation at 23°C for 90 min. Urease activity was reduced to 10.6% of the initial activity and hence the amount of active enzyme remaining was 3.1 mμmoles. The difference between the initial and final amounts of active enzyme in-

TABLE I

Molar Ratio of Hydroxamic Acid to Urease-Hydroxamic Acid Complex

Procedure	Active urease (mμmoles)	Urease-HXA complex (mμmoles) Urease	HXA	HXA/urease
After incubation	3.1	26.1*	---	---
Crystallization	3.4	25.8*	133	5.15
Washed twice with acetone	3.6	25.6*	58.5	2.29
Recrystallization at pH 7.8	0.62	5.82	13.2	2.28

Experimental conditions and procedure were described in the text.
HXA:Hydroxamic acid
* Calculated by the difference between the initial amount of urease and that of active enzyme after incubation.

dicates that 26.1 mμmoles of urease-hydroxamic acid complex was formed. Cold acetone was added dropwise to the reaction mixture and at a concentration of approximately 20% acetone, active urease and inactive complex crystallized as octahedrons about 5μ in diameter. In these crystals, the amounts of active enzyme, inactive complex and hydroxamic acid were 3.4 mμmoles, 25.8 mμmoles and 133 mμmoles respectively. Hence the molar ratio of hydroxamic acid to protein in the complex was calculated to be 5.15. To remove free hydroxamic acid adsorbed to the crystals, they were washed twice with 10 ml of 80% cold acetone, which resulted in a decrease of the ratio of 2.29. The crystals were centrifuged, dissolved in 0.1 M phosphate buffer (pH 7.8), and recrystallized by the dropwise addition of cold acetone. Mixed crystals of active and inactive complex were obtained, in which the molar ratio was 2.28. After recrystallization the amount of complex was calculated by determination of protein and specific ureolytic activity. During these procedures, traces of cysteine and EDTA were added to the reaction mixture in order to avoid inactivation by denaturation. In a control experiment, we could not observe any decrease of specific activity of urease under the same conditions.

Table II shows the results of experiments conducted under conditions similar to those in Table I. Using a urease preparation of low specific activity, 10,300 units/g, the ratio of hydroxamic acid to inactive urease was determined in Exp. No.4 shown in Table II. Regardless of the purity of the urease preparation, the ratio was found to be the same as in the case of crystalline urease.

From the results shown in Table I and II, it is quite clear that two moles of hydroxamic acid bind per mole of urease to form and inactive enzyme-inhibitor complex, which suggests that the number of active sites per molecule of urease is probably two. According to the work of Hellerman et al (1943) and Desnuelle et al (1949), there are 23 sulfhydryl groups

TABLE II

Ratio of Hydroxamic Acid to Urease-Hydroxamic Acid Complex

Exp.No.	Initial reaction components (mμmoles)		Molar ratio of HXA to complex				
			Crystallization	Washed with acetone			Recrystallization
	Urease	HXA		1st	2nd	3rd	
1	45	250	3.26	2.23	2.08	---	1.49**
2	64	314	2.62	2.22	2.12	1.90	----
3	24	343	---	---	5.39	---	2.36
4	33*	238	---	2.58	2.79	2.18	---

Experimental conditions and procedures were the same as in Table I except as described in this table.
* This urease was a crude preparation (10,300 units/g), and thus an amorphous precipitate was obtained in these procedures. Amount of enzyme was calculated from the ureolytic activity.
** The complex was recrystallized at pH 5.5.

per mole of urease, the oxidation of which results in complete enzymic inactivation. It has been suggested that this represents one -SH group per active site of urease, which corresponding to the data of Hand (1939) from diffusion rate studies. However, our results suggest that most of the sulfhydryl groups are involved in the maintenance of the enzyme's configuration rather than in a direct function at the active site.

Hydroxamic acid inhibits urease activity with strict specificity, because the inhibition is not affected by various amino acids and other proteins added, and in addition hydroxamic acid has no inhibitory action on a variety of enzymes except urease (Kobashi, Hase and Uehara, 1962). In both crystalline and crude urease preparations, the number of hydroxamic acid groups bound per mole of enzyme was the same, supporting the notion of strict specificity of inhibition by hydroxamic acid.

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REFERENCES

Desnuelle, P., and M. Rovery, Biochim. Biophys. Acta, 3, 26 (1949).
Fishbein, W. N., T. S. Winter, and J. D. Davidson, J. Biol. Chem., 240, 2402 (1965).
Fishbein, W. N., and P. P.Carbone, J. Biol. Chem., 240, 2407 (1965).
Hand, D. B., J. Am. Chem. Soc., 61, 3180 (1939).
Hellerman, L., F. B. Chinard and V. R. Deitz, J. Biol. Chem., 147, 443 (1943).
Kobashi, K., J. Hase and K. Uehara, Biochim. Biophys. Acta, 65, 380 (1962).
Kobashi, K., K. Kumaki and J. Hase, Biochim. Biophys. Acta, In press.(1966)
Sumner, J. B., N. Gralen and Eriksson-Quensel, J. Biol. Chem., 125, 37 (1938), Science, 87, 395 (1938).
Uehara, K. and K. Kobashi, Seikagaku, 31, 342,715 (1959).
van Slyke, D. D. and R. M. Archibald, J. Biol. Chem., 154, 632 (1944).
Wilzbach, K. E., J. Am. Chem. Soc., 79, 1013 (1957).