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Transamidinase of Hog Kidney

II. ISOLATION OF A STABLE ENZYME-AMIDINE COMPLEX*

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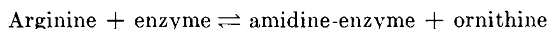
Two hypotheses have been formulated to explain the reaction mechanism of the transamidinase. Walker (1) postulates the formation of an amidine-enzyme compound as shown in the following reactions.


Arginine + enzyme ⇌ amidine-enzyme + ornithine

Amidine-enzyme + glycine ⇌ guanidinoacetate + enzyme

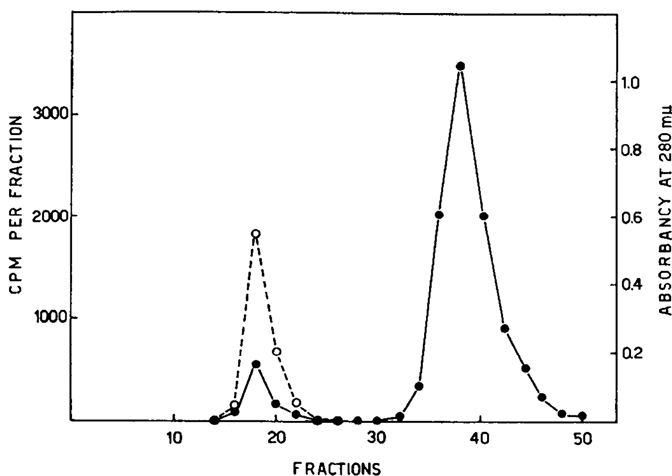


Fig. 1. Formation of the transamidinase-amidine complex. The incubation mixture (0.4 ml) contained 0.5 mg of transamidinase (specific activity, 70 units per mg of protein), 5×10^{-5} M DL-arginine guanido-^{14}C (specific activity, 0.68 mC per mM), and 0.015 M phosphate buffer, pH 7.5. After incubation at 20° for 30 min, the incubation mixture was applied to a Sephadex G25 column (1.2 × 25 cm) equilibrated with 0.015 M phosphate buffer, pH 7.5. Elution was performed with the same buffer. Fractions (0.7 ml) were collected every 2 min. Temperature was 4°. ○- - -○, protein concentration expressed as absorbance at 280 mμ; ●——●, radioactivity in counts per minute.

Ratner and Rochovansky (2) proposed the possibility of a single displacement reaction with both substrates being present simultaneously on the enzyme surface, the new bond being formed as the old bond is broken. The attempts to isolate the enzyme-amidine compound have so far been unsuccessful (2). The studies on the inhibitory relationship between the various substrates (2, 3), although very interesting, have not yet provided a way to distinguish between the two proposed mechanisms. This paper reports the isolation of the transamidinase-amidine compound obtained after incubation of the enzyme with arginine-^{14}C. The radioactive amidine group easily dissociates from the enzyme on warming to 100°. The compound released has been identified as urea. The transfer of the amidine group from the isolated transamidinase complex to ornithine and glycine has also been studied.

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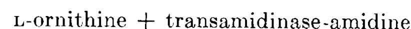
EXPERIMENTAL PROCEDURE

Sephadex G25 was supplied by Pharmacia, Uppsala, Sweden. DL-Arginine guanido-^{14}C (specific activity, 0.68 mC per mM) was purchased from the New England Nuclear Corporation and uniformly labeled L-arginine-^{14}C (specific activity, 7.7 mC per mM) from the Radiochemical Centre, Amersham, England. Transamidinase was prepared as described in the preceding paper (4). The enzymatic activity was measured in the test system described by Ratner and Rochovansky (5). Protein concentration was determined spectrophotometrically at 280 mμ. The method was calibrated by dry weight determinations on the dialyzed enzyme. Radioactivity measurements were made with solid samples on a low background gas flow Geiger-

Table I

Equilibrium constant of reaction

L-Arginine + transamidinase ⇌


L-ornithine + transamidinase-amidine

The incubation mixtures (1 ml) contained transamidinase (specific activity, 70 units per mg of protein), and DL-arginine guanido-^{14}C (specific activity, 0.68 mC per mM) as indicated in the table, and 0.015 M phosphate buffer, pH 7.5. After 15 min of incubation at 2°, the mixtures were submitted to Sephadex G25 chromatography as described in Fig. 1. The fractions containing the enzyme-amidine complex were pooled and analyzed for the radioactivity and for the protein content.

Experiment	Total transamidinase	Initial DL-arginine		Transamidinase-amidine complex*		L-Ornithine	Keq†
	mμmoles	*cpm*	*mμmoles*	*cpm*	*mμmoles*	*mμmoles*	$\times 10^{-2}$
1	9.7	11,100	26	1,054	2.47	2.47	8
2	9.7	22,200	52	871	2.04	2.04	2.3
3	3.23	60,600	142	854	2	2	4.7
4	9.7	60,600	142	1,861	4.36	4.36	5.3
5	2.5	111,000	260	811	1.9	1.9	4.7

* Calculated from the specific activity of the arginine assuming 1 active site per molecule of enzyme; molecular weight 100,000 (4).

† Average value = 5×10^{-2}.

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Müller counter purchased from the Società Elettronica Lombarda, Milano, Italy.

RESULTS

Formation of Enzyme-Amidine Complex—In the absence of acceptor, transamidinase reacts with arginine guanido-^{14}C to form a stable transamidinase-amidine complex. The complex can be isolated by gel filtration. The elution pattern of the reaction mixture from a Sephadex G25 column is shown in Fig. 1. A radioactive protein peak is eluted first, well separated from the radioactive substrate, arginine guanido-^{14}C. The equilibrium constant of the reaction of formation of the enzyme-amidine complex has been determined. For these experiments the transamidinase-amidine complex was isolated after incubation of the enzyme preparation with DL-arginine guanido-^{14}C for 10 min at 2°. Total transamidinase was estimated from the quantity of protein. The concentration of complex was calculated from the radioactivity, compared with the specific activity of the arginine-^{14}C used for labeling. Ornithine formation was assumed to be equimolecular with the enzyme complex formation. The following equilibrium constant was obtained (Table I).

$$\frac{\text{Transamidinase-amidine} \times \text{L-ornithine}}{\text{Transamidinase} \times \text{L-arginine}} = 5 \times 10^{-2}$$

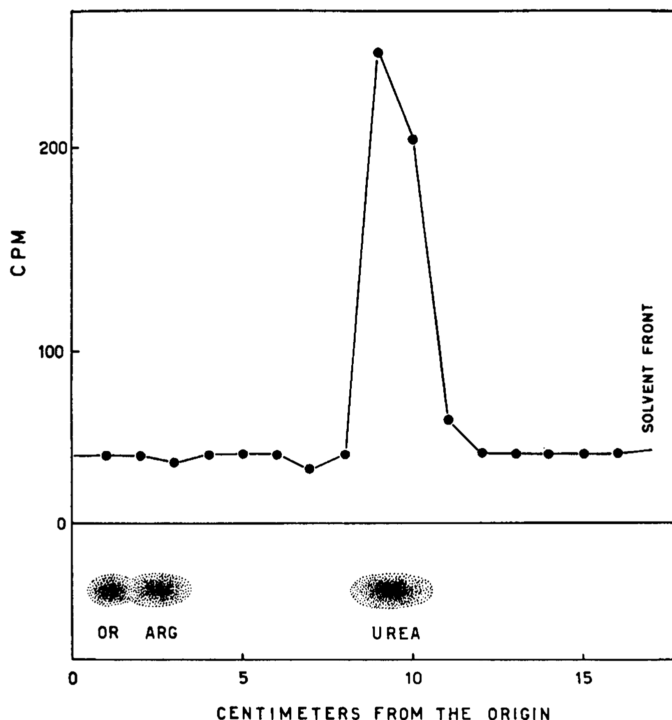


Fig. 2. Chromatography of the urea released by warming the transamidinase complex to 100°. The transamidinase complex (specific activity, 3400 cpm per mg of protein), 0.52 mg dissolved in 2 ml of 0.015 M phosphate buffer, pH 7.5, was heated for 5 min at 100°; the protein was precipitated by addition of 0.2 ml of 50% trichloroacetic acid. The radioactive supernatant was placed on a column of Dowex 50 (H^+) (0.5 × 6 cm). The column was washed with 3 ml of water, and the radioactivity eluted with 1 M NH_4OH. The radioactive fractions were pooled, desiccated under vacuum, and dissolved in 0.1 ml of water. To this solution 0.2 μmole of urea was added as internal standard. The sample was placed on Whatman No. 1 paper for ascending chromatography in 1-butanol-acetic acid-water, 4:1:1. The areas of radioactivity were located by cutting the paper into 1-cm wide pieces and assaying in the gas flow Geiger-Müller counter. The standards (urea, arginine, and ornithine) were detected with the nitroprusside-alkaline ferricianide reagent (6) or with 0.25% ninhydrin in acetone.

Table II

Identification with urease of urea released by warming transamidinase complex at 100°

The transamidinase complex (specific activity, 3400 cpm per mg of protein), 0.5 mg, dissolved in 0.5 ml of 0.015 M phosphate buffer, pH 7.5, was heated for 5 min at 100°; the protein was precipitated by addition of 0.05 ml of 50% trichloroacetic acid and the radioactive supernatant solution was neutralized. To the neutralized solution were added 0.2 ml of 0.2 M phosphate buffer, pH 7.5, and 0.01 ml of 1.5 M urea as carrier. The incubation mixture was transferred in the bottom of a Warburg flask containing in the first side arm 1 mg of urease in 0.5 ml of phosphate buffer, 0.04 M, pH 7.5; in the second side arm 0.3 ml of 0.6 N H_2SO_4, and in the center well 0.3 ml of 0.1 N NaOH. The Warburg flask was evacuated, and the reaction was started by mixing the urease with the incubation mixture. After 1 hour of incubation at 20°, the H_2SO_4 was added and the incubation continued. After 16 hours, the NaOH of the center well was transferred in a centrifuge tube, 0.1 ml of a saturated solution of $Ba(OH)_2$ was added, the precipitate was centrifuged, washed till neutrality with 50% water-ethanol solution, resuspended in the water-ethanol solution, and counted. In a control experiment performed exactly under the same conditions but without urease, no $BaCO_3$ was formed, and the radioactivity was still present in the incubation mixture.

Step	
	cpm
Enzyme-amidine complex added	1700
Supernatant solution after acid treatment	1600
$BaCO_3$ isolated after incubation with urease	1450

Characterization of Enzyme-Amidine Complex—The isolated enzyme-amidine complex when heated to 100° decomposes and the radioactivity dissociates from the protein. The radioactive material has been identified as urea by cochromatography with an authentic sample of urea (Fig. 2) and by isolation of labeled CO_2 after treatment with urease (Table II). The same results are obtained when the transamidinase complex is prepared with uniformly labeled arginine-^{14}C instead of that with arginine guanido-^{14}C. In both cases only radioactive urea dissociates from the enzyme; neither radioactive arginine nor ornithine can be detected.

Dissociation of Enzyme-Amidine Complex by Transamidinase Acceptors—When the enzyme-amidine complex is incubated with a suitable acceptor such as glycine or ornithine, the radioactivity is removed from the protein, and two new radioactive compounds are isolated by Sephadex G25 filtration. The radioactive products have been identified as guanidinoacetate and arginine, respectively, by cochromatography with the authentic samples by ascending chromatography in the solvent systems: 1-butanol-acetic acid-water (73:10:17) and phenol-ammonia-water (160:1:40) (Fig. 3).

DISCUSSION

We have described a stable ^{14}C-labeled transamidinase complex formed when the enzyme is incubated with arginine guanido-^{14}C.

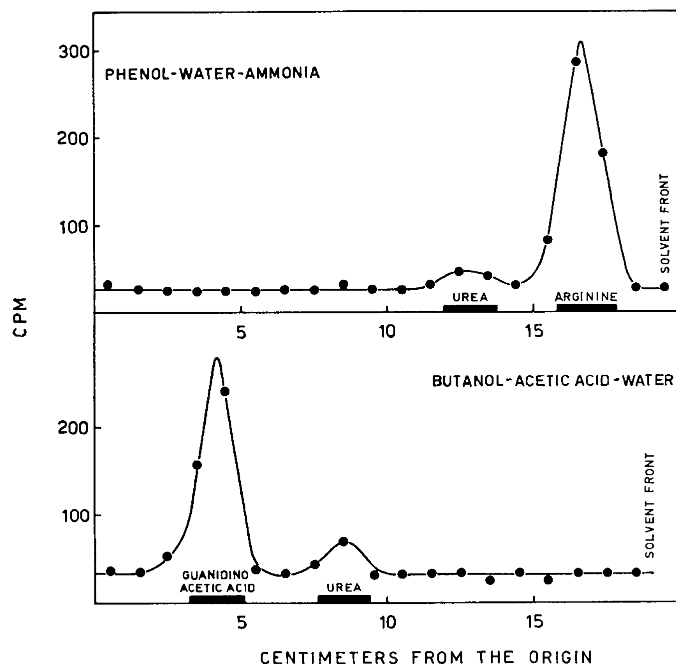
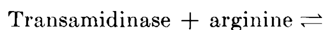
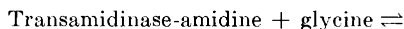


FIG. 3. Dissociation of the enzyme-amidine complex by transamidinase acceptors. The incubation mixtures (0.9 ml) contained 0.6 mg of enzyme-amidine complex (specific activity, 3400 cpm per mg of protein), 0.015 M phosphate buffer, pH 7.5, 0.005 ml of 0.1 M ornithine (*upper part of the figure*), or 0.005 ml of 0.1 M glycine (*lower part of the figure*). The mixtures were incubated for 20 min at 20° and chromatographed on Sephadex G25 columns (1.2 × 25 cm) equilibrated with water. Temperature was 4°. Fractions (0.7 ml) were collected every 2 min. A nonradioactive protein peak was eluted first followed by a peak of radioactivity. The radioactive fractions were pooled, concentrated, and applied to Whatman No. 1 paper. This was developed by ascending chromatography with the butanol-acetic acid-water, 73:10:17, or with the phenol-water-ammonia mixtures, 160:40:1. The areas of radioactivity and the standards (urea, arginine, guanidinoacetate) were located as described in Fig. 2.

The labeled group can be transferred to ornithine or glycine to form arginine or guanidinoacetate, respectively. These results confirm the hypothesis of Walker (1) and prove that the transamidinase reaction occurs through two separate steps according to the following mechanism.


Transamidinase + arginine ⇌

transamidinase-amidine + ornithine

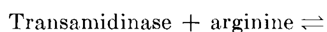

Transamidinase-amidine + glycine ⇌

transamidinase + guanidinoacetate

Some of the properties of the enzyme-amidine complex have been studied. It can be separated by filtration through Sephadex G25, it can be hydrolyzed by warming at 100°, and it slowly dissociates with liberation of urea.

SUMMARY

This paper reports the isolation of a stable transamidinase-amidine complex obtained by incubation of the enzyme with labeled arginine-^{14}C. The equilibrium constant of the reaction


Transamidinase + arginine ⇌

transamidinase-amidine + ornithine

has been calculated. The radioactive amidine group slowly dissociates from the enzyme at room temperature or rapidly hydrolyzes as urea on warming to 100°. The labeled amidine group is transferred from the enzyme-amidine complex to suitable acceptors such as glycine or ornithine with formation of labeled guanidinoacetate or arginine.

REFERENCES

1. WALKER, J. B., *J. Biol. Chem.*, **231,** 1 (1958).
2. RATNER, S., AND ROCHOVANSKY, O., *Arch. Biochem. Biophys.*, **63,** 296 (1956).
3. WALKER, J. B., *J. Biol. Chem.*, **224,** 57 (1957).
4. CONCONI, F., AND GRAZI, E., *J. Biol. Chem.*, **240**, 2461 (1965).
5. RATNER, S., AND ROCHOVANSKY, O., *Arch. Biochem. Biophys.*, **63,** 277 (1956).
6. ROCHE, J., THOAI, N. V., AND HATT, L., *Biochim. et Biophys. Acta*, **14,** 71 (1954).