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Transamidinase of Hog Kidney

I. PURIFICATION AND PROPERTIES*

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A transamidinase activity was first reported independently by Borsook and Dubnoff (1) and by Bloch and Schoenheimer (2) who demonstrated the synthesis of guanidinoacetic acid from arginine and glycine in mammalian kidney. The enzyme catalyzes the transfer of the amidine group from donors such as L-arginine, guanidinoacetic acid, L-canavanine (3, 4), and homoarginine (5, 6) to acceptors such as glycine, L-ornithine, L-canaline, γ-aminobutyric acid, β-alanine (7), and hydroxylamine, (8). A method for the preparation of transamidinase has been described by Ratner and Rochovansky (5). Starting with hog kidney as the source material, they purified the enzyme 80-fold. With this preparation the influence of pH, substrate specificity, kinetics of the forward and reverse reactions, and the equilibrium constant were studied. For a further investigation of the properties of the enzyme and particularly of the nature of the transfer reaction, a more highly purified preparation was necessary. A method has now been developed which yields a 900-fold purified preparation from hog kidney. The purity of the enzyme preparation has been investigated by sucrose gradient centrifugation, gel filtration, and chromatography on phosphocellulose. Other properties of the enzyme such as substrate specificity and pH optimum have been studied and found to be similar to those of the less pure transamidinase preparation (5).

EXPERIMENTAL PROCEDURE

Materials and Methods

Materials—Fructose diphosphate aldolase and α-glycerophosphate dehydrogenase were purchased from Boehringer und Soehne and L-canavanine sulfate from Mann Research Laboratories. Diethylaminoethyl cellulose (DEAE-cellulose) DE-50 and phosphocellulose P-70 were purchased from Whatman. Sephadex G-100, particle size 40 to 120 μ, was supplied by Pharmacia, Uppsala, Sweden.

Analytical Methods—In the routine assay, transamidinase activity was measured in the test system described by Ratner and Rochovansky (5) by following ornithine formation estimated by the method of Chinard (9). A unit was defined as the amount of enzyme that catalyzes the formation of 1 μmole of ornithine or guanidinoacetic acid per hour at 37°. The enzymatic activity was also measured according to the following reactions.

$$\text{Ornithine} + \text{guanidinoacetate} \rightleftharpoons \text{arginine} + \text{glycine} \quad (1)$$

$$\text{Ornithine} + \text{canavanine} \rightleftharpoons \text{arginine} + \text{canaline} \quad (2)$$

$$\text{Arginine} + \text{hydroxylamine} \rightleftharpoons \text{ornithine} + \text{hydroxyguanidine} \quad (3)$$

The formation of arginine in Reaction 1 was estimated as urea, after reaction with arginase (5) by the method of Peters and Van Slyke (10). In Reaction 2 arginine formation was estimated by the Sakaguchi method (11) modified by Albanese and Frankston (12). In Reaction 3 ornithine formation was estimated as already described. Aldolase and glycerophosphate dehydrogenase activities were measured in the test system described by Racker (13).

Sucrose density gradient centrifugations were performed as described by Martin and Ames (14) for 15 hours at 37,000 rpm and 10°. Calculations of s_{20} value were based on several runs and were in good agreement with each other. All sucrose solutions used for such density gradients were prepared in 0.02 M phosphate buffer, pH 7.5. Protein concentration was determined from the ratio of absorption at 280 and 260 mμ (15); the results were calibrated against dry weight of dialyzed samples of the purified enzyme.

RESULTS

Purification of Enzyme

All operations were carried at 2° except when otherwise indicated.

Acetone Powder Extraction—Hog kidneys were removed from animals immediately after slaughter and chilled; they were then divested of fat and medullary tissue and cut into small pieces. The cortex was homogenized in a Waring Blendor for 3 min with 5 volumes of acetone chilled to −15°. The resulting suspension was filtered in a large Buchner funnel. The residue was re-extracted in the blender for 3 min with equal amounts of acetone as before. The defatted tissue was spread out and allowed to dry at room temperature and was finally stored in a vacuum desiccator. The dried acetone powder (150 g) was suspended in 1050 ml of potassium 0.14 M phosphate buffer, pH 7.3, and extracted for 30 min with stirring. The suspension was centrifuged for 15 min at 14,000 × g and the precipitate was discarded (acetone powder extract, 950 ml; see Table I).

Acid Ammonium Sulfate Fractionation—The supernatant solution was treated with 108 g of solid ammonium sulfate and the resulting precipitate was centrifuged and discarded. The pH was brought to 3.5 by addition of a 20% saturated ammonium sulfate solution containing 1 N H_2SO_4. The precipitate was collected by centrifugation for 10 min at 14,000 × g and sus-

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TABLE I

Purification of hog kidney transamidinase

Step	Total units	Specific activity	Recovery
			%
Acetone powder extract	5200	0.08	
Acid ammonium sulfate	4800	0.7	92
Heat treatment	4700	1.26	90
Calcium phosphate gel	3900	3.24	74
Phosphocellulose	2450	12	46
DEAE-cellulose	1500	34	28
Sephadex G-100	950	75	18

pended with 200 ml of 0.08 M potassium phosphate buffer, pH 7.3. A second centrifugation yielded a clear supernatant fluid (acid ammonium sulfate fraction, 360 ml).

Heat Step—The acid ammonium sulfate fraction was diluted with 0.08 M potassium phosphate buffer, pH 7.3, to bring the protein concentration to 17 mg per ml. The protein solution was allowed to equilibrate at room temperature, and was divided in 80-ml aliquots, which were heated in a water bath at 49° for 15 min and then chilled to 0°. The precipitate was centrifuged at 14,000 × *g* for 5 min and discarded. The supernatant solution was diluted with 0.08 M phosphate buffer, pH 7.3, to a protein concentration of 7 mg per ml (heat fraction, 560 ml).

Calcium Phosphate Gel Adsorption—Calcium phosphate gel (1100 ml, dry weight, 12 mg per ml) was sedimented by centrifugation. The sediment was resuspended with the heat fraction. After 10 minutes at 0° the suspension was centrifuged at 5,000 × *g* and the precipitate was extracted for 30 min with 560 ml of 5% saturated ammonium sulfate solution. After centrifugation, the supernatant solution was discarded and the precipitate was re-extracted for 30 min with 560 ml of 20% saturated ammonium sulfate solution. After centrifugation the supernatant solution was collected and brought to 70% saturation by the addition of solid ammonium sulfate. After centrifugation for 15 min at 14,000 × *g* the protein precipitate was dissolved with 30 ml of 0.02 M phosphate buffer, pH 7.5, and dialyzed for 4 hours against the same buffer (calcium phosphate gel fraction, 48 ml).

Phosphocellulose Chromatography—The calcium phosphate fraction was placed on a phosphocellulose column (2 × 30 cm) that had been equilibrated with 0.02 M acetate buffer, pH 5.5. The column was washed with 100 ml of the same buffer. Elution was begun with a linear gradient from 0.02 M acetate buffer, pH 5.5 (500 ml), to 0.075 M phosphate buffer plus 0.1 M sodium acetate, pH 7.1 (500 ml). Elution of 77% of the activity was obtained with the last fractions. The fractions with higher specific activity were pooled (180 ml), the protein was precipitated by addition of 85 g of solid ammonium sulfate and was collected by centrifugation. The precipitate was dissolved with 10 ml of 0.02 M phosphate buffer, pH 7.5, and dialyzed for 4 hours against the same buffer (phosphocellulose fraction, 15 ml).

DEAE-cellulose Chromatography—To a DEAE-cellulose column (1.2 × 27 cm) that had been equilibrated with 0.02 M phosphate buffer, pH 7.5, were added 15 ml of the phosphocellulose fraction. The column was washed with the same buffer, the enzymatic activity was not retained and was found in the first fractions of the effluent solution. The increase of the specific activity was 3-fold. The active fractions were pooled (18 ml), the protein was precipitated by addition of 8.5 g of solid ammonium sulfate, the precipitate was collected by centrifugation and dissolved in 3 ml of 0.015 M phosphate buffer, pH 7.5. The protein solution was dialyzed for 4 hours against the same buffer (DEAE-cellulose fraction, 4 ml).

Sephadex G-100 Filtration—To a Sephadex G-100 column (2.2 × 140 cm) that had been equilibrated with 0.015 M phosphate buffer, pH 7.5, were added 4 ml of the DEAE-cellulose fraction. Elution was begun with the same buffer at the rate of 6 ml per hour. Fractions of 2 ml were collected. Two distinct protein peaks were eluted. The enzymatic activity was recovered with the second one which appeared after 24 to 30 hours of elution. The fractions with higher specific activity were pooled (20 ml) and the protein was precipitated by addition of 9.4 g of solid ammonium sulfate. The precipitate was collected by centrifugation, dissolved in 1 ml of 0.015 M phosphate buffer, pH 7.5, and dialyzed for 4 hours against the same buffer (Sephadex G-100 fraction, 2 ml).

Properties of Enzyme

Homogeneity of Transamidinase Preparation—The following tests have been used in order to check the purity of the transamidinase preparation: phosphocellulose chromatography, Sephadex G-100 filtration, and sucrose density gradient centrifugation. As shown in Figs. 1 to 3, with the three different tests, a single symmetrical peak is obtained for both the protein and the enzymatic activity; furthermore the specific activity of the transamidinase preparation is constant in all the fractions across the protein peak.

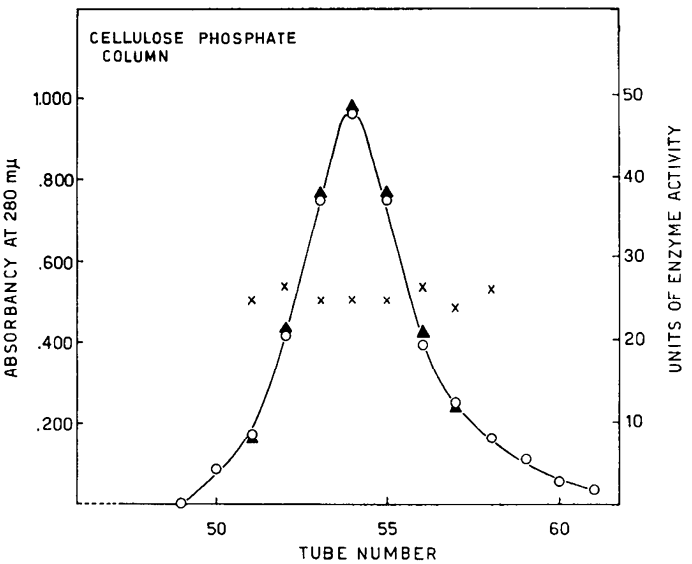

FIG. 1. Elution pattern of transamidinase from phosphocellulose column. A column (1 × 20 cm) was equilibrated with 0.02 M acetate buffer, pH 5.5, the flow rate was 30 ml per hour, and fractions of 1.5 ml per tube were collected at 4°. Transamidinase, 4 mg (specific activity, 75 units per mg of protein) dissolved in 0.5 ml of 0.015 M phosphate buffer, pH 7.5, was applied to the column and the elution was carried out with a linear gradient between 0.02 M acetate buffer, pH 5.5, and 0.075 M phosphate buffer plus 0.1 M sodium acetate, pH 7.1 (50 ml in each of the two chambers). ○——○, protein concentration expressed as absorbance at 280 mμ (A_{280} = 1.5 for 1 g per liter); ▲——▲, units of transamidinase per ml (see text); ×——×, specific activity (average value, 70 units per mg of protein).

Specificity—The following compounds have been tested: amidine donors, L-canavanine, L-arginine, guanidinoacetic acid; amidine acceptors, L-ornithine, glycine, and hydroxylamine; and all have been found active as substrates of the transamidinase.

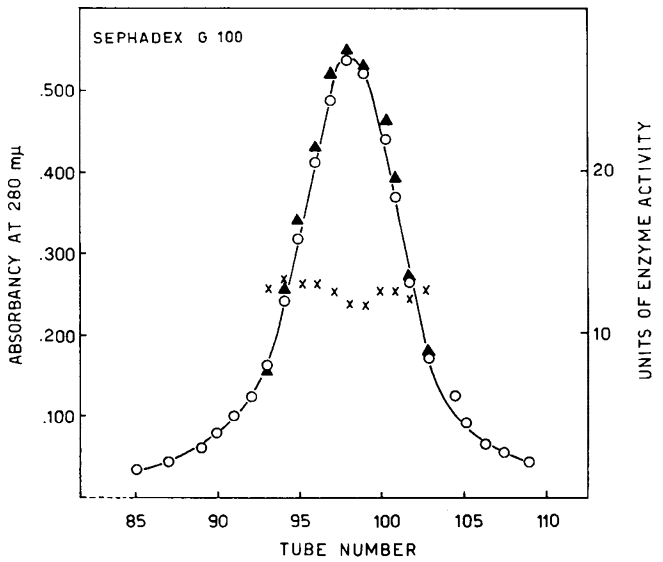

FIG. 2. Elution pattern of transamidinase from Sephadex G-100 column. A column (2.2 × 140 cm) was equilibrated with 0.015 M phosphate buffer, pH 7.5, the flow rate was 6 ml per hour, and fractions of 2 ml per tube were collected at 4°. Transamidinase, 5.5 mg (specific activity, 75 units per mg of protein) dissolved in 1 ml of 0.015 M phosphate buffer, pH 7.5, was applied to the column and the elution was carried out with the same buffer. ○——○, protein concentration expressed as absorbance at 280 mμ (A_{280} = 1.5 for 1 g per liter); ▲——▲, units of transamidinase per ml (see text); ×——×, specific activity (average value, 70 units per mg of protein).

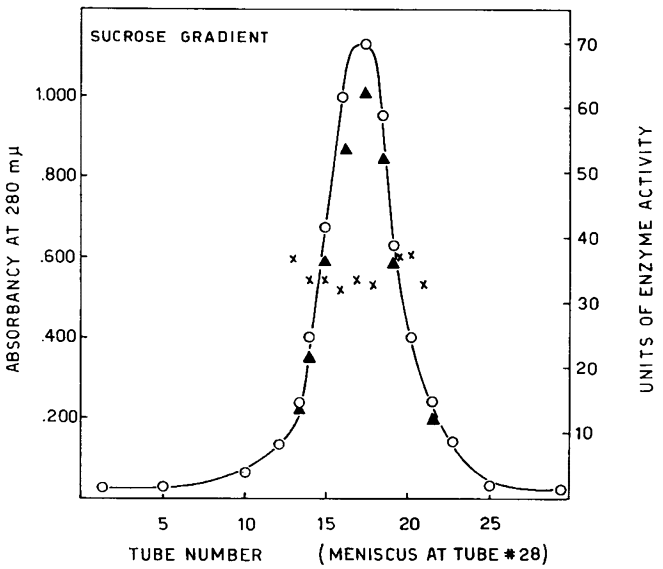

FIG. 3. Sedimentation pattern for transamidinase in sucrose density gradient centrifugation. Transamidinase, 0.8 mg (specific activity, 75 units per mg of protein) in 0.1 ml of 0.02 M phosphate buffer, pH 7.5, was layered on a sucrose gradient. After 15 hours of centrifugation at 37,000 rpm at 10°, the gradient was fractionated and analyzed. ○——○, protein concentration expressed as absorbance at 280 mμ (A_{280} = 1.5 for 1 g per liter); ▲——▲, units of transamidinase per ml (see text); ×——×, specific activity (average value, 75 units per mg of protein).

TABLE II

Rate of amidine transfers catalyzed by transamidinase

Incubations were carried out at 38° in a final volume of 1 ml in the presence of 0.1 M phosphate buffer, pH 7.5, and of 0.035 mg of transamidinase (specific activity, 75 units per mg of protein).

Substrates present		Amount of product formed	
Amidine donor	Amidine acceptor	15 min	30 min
μmoles/ml	*μmoles/ml*	*μmoles/ml*	
Arginine, 10	Glycine, 16	0.7	1.2
Arginine, 10	Hydroxylamine, 40	0.07	0.14
Guanidino acetate, 30	Ornithine, 3	0.13	0.2
Canavanine, 10	Ornithine, 3	0.45	0.93

TABLE III

Hydrolytic activity of transamidinase

Incubations were carried out at 37° for 20 min in a final volume of 1 ml in the presence of 0.1 M phosphate buffer, pH 7.5. The specific activity of the enzyme is 75 units per mg of protein.

Enzyme	Amidine donor	Amidine acceptor	Amount of product formed
μg/ml	*μmoles/ml*	*μmoles/ml*	*μmoles/ml*
5.3	Arginine, 10	Glycine, 16	Ornithine, 0.146
265	Arginine, 10		Ornithine, 0.074
265	Guanidino acetate, 25		Glycine, 0.09*

* Glycine determined by the ninhydrin method (17).

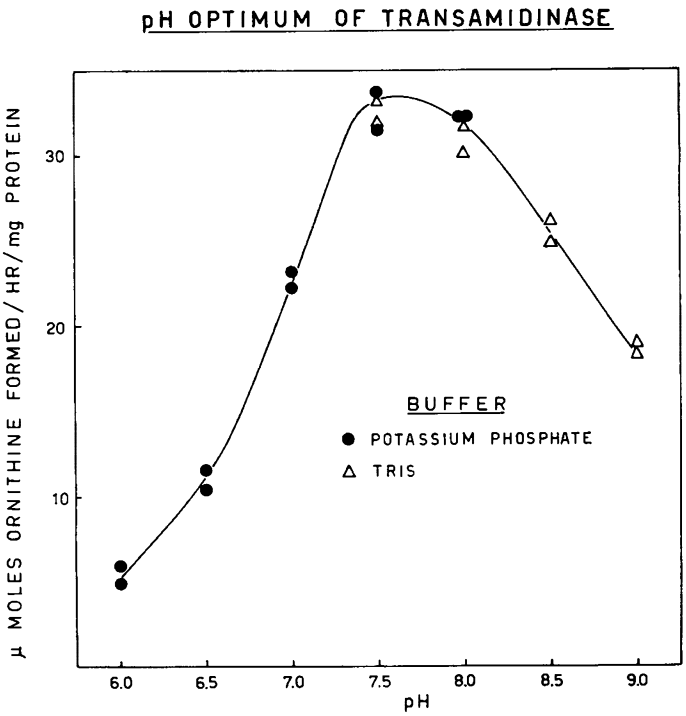

FIG. 4. Effect of pH on activity of transamidinase. Measurements of enzymatic activity were performed as described under "Experimental Procedure." ●——●, 0.1 M phosphate buffer; △——△, 0.1 M Tris buffer.

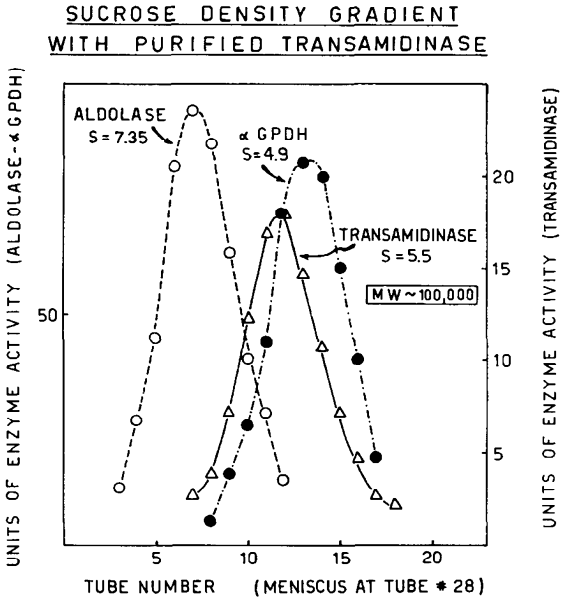

FIG. 5. Determination of sedimentation coefficient for transamidinase. Transamidinase, 0.35 mg (specific activity, 75 units per mg of protein), and 0.1 mg each of glycerophosphate dehydrogenase and aldolase in 0.1 ml of 0.02 M phosphate buffer, pH 7.5, were layered on a sucrose gradient. After 15 hours of centrifugation at 37,000 rpm at 10°, the gradient was fractionated and analyzed for enzymatic activity. △——△, transamidinase; ●-·-●, glycerophosphate dehydrogenase; ○- - -○, aldolase.

The relative rates for the different donor-acceptor pairs are reported in Table II and are in agreement with the data of Ratner and Rochovansky (16).

Hydrolytic Activity—The purified transamidinase preparation hydrolyzes both arginine and guanidinoacetic acid to ornithine and glycine, respectively, with liberation of stoichiometric amounts of urea. The assay conditions for the determination of the hydrolytic activity are reported in Table III. The rates of hydrolysis of arginine or guanidinoacetic acid were 1% and 1.2%, respectively, of the transamidinase activity between arginine and glycine.

Influence of pH—The pH optimum for the reaction

$$\text{Arginine} + \text{glycine} \rightleftharpoons \text{guanidinoacetate} + \text{ornithine}$$

carried out in phosphate and in Tris buffer is 7.5; the activity falls off rather sharply on either side of the optimal range (Fig. 4).

Sedimentation Analysis in Sucrose Density Gradient—The purified transamidinase submitted to centrifugation in a sucrose density gradient with rabbit muscle aldolase and glycerophosphate dehydrogenase as standards shows an s_{20} value of 5.5 (Fig. 5) which, assuming the sphericity of the protein, corresponds to an approximate molecular weight of 100,000.

DISCUSSION

A method is described for the purification of transamidinase from hog kidney that yields a preparation with a specific activity 10 times higher than that reported by Ratner and Rochovansky. The over-all purification is approximately 900-fold. The protein is homogeneous on chromatography and on centrifugation in sucrose density gradient, from which an approximate molecular weight of 100,000 has been calculated. The transamidinase preparation is free of contaminating enzymes; however a hydrolytic activity, corresponding to 1% of the transfer activity, is present, confirming similar findings obtained by Ratner and Rochovansky. Both arginine and guanidinoacetic acid are hydrolyzed; the latter is not a substrate of the arginase. This fact, together with the data that the ratio of the hydrolytic to the transfer activity is constant through a 10-fold increase in the purification, supports the idea that the hydrolytic activity is not dependent on arginase contamination but represents an intrinsic property of the enzyme.

SUMMARY

Transamidinase has been purified about 900-fold from hog kidney. The enzyme is homogeneous on chromatography and on centrifugation in sucrose density gradient. With this technique an s_{20} value of 5.5 has been obtained.

Other properties, including pH optimum and substrate specificity, have been studied.

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