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DECARBAMOYLATING ACTIVITY OF ORNITHINE TRANSCARBAMOYLASE

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SUMMARY. We have purified from beef liver an enzyme which decarbamoylates carbamoyl-hemoglobin and to a much lesser extent carbamoyl histones. Carbamoyl casein was a poor substrate while carbamoyl trypsin, fibrinogen and ovoalbumin were not affected. The optimal pH is 7.4. Addition of Mg^{++}, Mn^{++} or Ca^{++} was without effect. On testing citrulline as a substrate we found high activity leading us to suspect that the activity of the decarbamoylase preparation was due to contaminating ornithine transcarbamoylase activity. Evidence for this is the similar ratio of transcarbamoylase to decarbamoylase activities of both ornithine transcarbamoylase and of the purified preparation of decarbamoylase from beef liver. Also, δ-PALO, the specific inhibitor of ornithine transcarbamoylase inhibited both preparations to the same extent. Interestingly, ornithine transcarbamoylase from bacteria also has decarbamoylase activity while aspartic transcarbamoylase does not.

We reported briefly the existence in liver extracts and purified preparations thereof, of a "decarbamoylase" which had activity with a number of carbamoylated proteins (1). Of carbamoylated proteins thus far tested carbamoyl-hemoglobin was by far the best substrate. This finding appears potentially of practical interest because carbamoyl-hemoglobin has been identified in a number of abnormal conditions, particularly uremia (2).

We have purified the decarbamoylase activity from rat liver and more extensively from beef liver. On testing various potential substrates we found that a number of carbamoyl amino acids were slightly active and surprisingly, that citrulline was a good substrate. Although ornithine transcarbamoylase is known to

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be highly specific, we wondered whether it might possibly have decarbamoylase activity. On testing highly purified ornithine transcarbamoylase (OTC) from beef liver, we found that it did in deed have decarbamoylase activity. Moreover, the transition state analog and highly specific ornithine transcarbamoylase inhibitor δ-N (phosphonacetyl)-L-ornithine (δ-PALO), (3), inhibited both the transcarbamoylase and decarbamoylase activities of our purified preparations, as well as those of beef liver OTC.

The findings presented in this paper suggest that the decarbamoylase activity of the extensively purified preparations reflects contaminant OTC activity. In other words, OTC co-purified with a protein(s) having similar physical properties and therefore our suggestion of a decarbamoylase (1) "per se" appears to be an artefact. However, the hitherto unexpected decarbamoylase activity of OTC is of interest. These findings are illustrated in this paper.

MATERIALS AND METHODS

Proteins, including human hemoglobin, were incubated overnight at 37 °C with (^{14}C) KCNO (specific radioactivity: 0.25 mCi/mmol) at a concentration of 5 µCi per 30 mg of protein. Incubations were done in 50 mM Tris-HCl pH 7.2. Residual cyanate was removed by dialysis against water, or by desalting by the method of Penefsky (4). The preparation of carbamoylated intermediates of human hemoglobin was by the method of Williams et al. (5). Hemoglobin was methylated by the method of Rice and Means (6). Histones, Type II-S from Sigma, were acetylated by the method of Inoue and Fujimoto (7). Beef liver OTC was purified as described by Marshall and Cohen (8), δ-PALO was purified as described by Martinis et al. (3). Carbamoyl aminoacids were prepared by incubating overnight at 37°C 90 µmoles of (^{14}C) KCNO (specific radioactivity 0.07 mCi/mmol) with one mg of the aminoacid in 0.5 ml of 0.1 M Tris-HCl buffer pH 7.2. Residual cyanate was decomposed by the addition of acetic acid 10% (w:v). The mixture was kept for 2 h at 40 °C and subsequently neutralized with 1 M Tris Base.

Assay of decarbamoylase activity. The incubation medium contained 110 µmoles of sodium phosphate pH 7.0, 140 µg of carbamoylated hemoglobin (containing 0.7 µmoles of (^{14}C)carbamoyl group/mg hemoglobin) and 125-250 µg of purified enzyme in a volume of 1.1 ml. The assay mixture was placed in test tubes which were then sealed with rubber stoppers pierced by a syringe needle holding two penicillin paper disks (5 mm diameter) impregnated

with 50 µl of toluene/NCS solubilizer for liquid scintillation counting 1:1 solution and a syringe containing trichloroacetic acid. After incubation for 60 min at 37°C, the reaction was stopped by injection of 200 µl of 10% trichloroacetic acid into the tubes which were kept shaking for 30 min. at 40 °C. To determine the $(^{14}C)CO_2$ trapped in the disks, they were placed in 3 ml of scintillation fluid and counted in a liquid scintillation Spectrometer (Intertechnique PG4000). The content of (^{14}C) CO_2 as product of the reaction was estimated by the difference between tubes with and without enzyme.

Determination of protease or demethylase activity. The assay contained 120 µg of (^{14}C) methyl-hemoglobin (0.046 µmoles of (^{14}C) formaldehyde per mg of hemoglobin) and 25-30 µg of decarbamoylase in 200 µl of sodium phosphate buffer 90 mM pH 7.0, and was incubated for 1 h at 37 °C. The reaction was stopped by the addition of 100 µl of 20% trichloroacetic acid and 100 µl of 5% BSA. The tubes were centrifuged and the radioactivity determined in the supernatant.

Determination of deacetylase activity. The assay procedure was as follows: 250 µg of (^{14}C) acetyl-labeled histone (16.8 nmoles (^{14}C) acetyl/mg of histone) was incubated with the enzyme preparation at 37 °C in a final volume of 0.6 ml in 25 mM sodium phosphate buffer pH 7.0. After the incubation, the reaction mixture was acidified by the addition of 0.1 ml of 1 N HCl containing 5 µmoles of carrier acetic acid. Acetic acid was extracted by shaking with 3 ml of ethylacetate. 2 ml of the organic layer was taken, mixed with the scintillation solution and the radioactivity measured.

Determination of ornithine transcarbamoylase activity. The assay contained 2.5 µmoles of carbamoyl phosphate, 2.5 µmoles of L-ornithine and an appropriate amount of enzyme in 0.5 ml of 50 mM acetate-diethanolamine buffer pH 7.0. The incubation was for 10 min at 37 °C and the citrulline formed was assayed (9).

RESULTS AND DISCUSSION

Enzyme purification from beef liver. Since the activity is low, rat liver is not a convenient source from which to isolate sufficient enzyme to study its properties. Fresh bovine liver was therefore used routinely as the source of enzyme. The results of a typical purification are shown in Table I. Livers were washed in ice cold 0.15 M KCl and minced with scissors. They were homogenized in 4 volumes of cold 0.15 M KCl in a Waring blender. The homogenate was filtered through glass wool and centrifuged. Centrifugations were for 15 min at 17,000 x g at 4°C. The resulting supernatant is the crude fraction, from which the enzyme was obtained by precipitation with acetone. To each 100 ml of crude

TABLE I - Purification of the enzyme from 400 g of liver

Fraction	Protein (mg)	Total activity (pmoles)	Specific activity (pmoles/ mg prot)	Recovery %
Crude	26,600	21,100	0.8	100
Acetone	880	8,550	10	41
65°C Heated	220	6,670	30	32
Sephadex eluate	1.4	680	485	3

fraction were added 50 ml of cold acetone in a bath kept -10°C; the precipitate was discarded. The supernatant was mixed with more acetone to reach 60% (v/v). The resulting precipitate was dissolved in double distilled water to a volume ca. 0.1 that of the crude fraction. It was then heated for 5 min at 65°C, cooled, centrifuged for 15 min and the supernatant lyophilized.

The lyophilized material was dissolved in 1 ml of 0.1 M sodium phosphate buffer pH 7.0 (ca. 20 mg of protein/ml) and then applied to a Pharmacia column, 30 x 1.5 cm., packed with Sephadex G-100 Fine (bed volume of 45 ml). The column was equilibrated and eluted with 0.1 M sodium phosphate buffer pH 7.0. The elution was carried out at a flow rate of about 14 ml per hour. The fractions containing the decarbamoylase activity, eluted between 30-35 ml, were combined and lyophilized. The isolated enzyme was stable for many months when stored in a desiccator at 4°C.

Effect of pH. The effect of pH on the activity of the decarbamoylase was studied in 0.1 M citrate phosphate buffer between pH 3.4-7.0 and in 0.1 M sodium phosphate between 5.7-7.9. As can be seen in Fig. 1 maximal activity was obtained at pH 7.4.

Effect of cations. Between 0.01-5.00 mM, Mg^{++}, Mn^{++} and Ca^{++} had no effect on the actitivity.

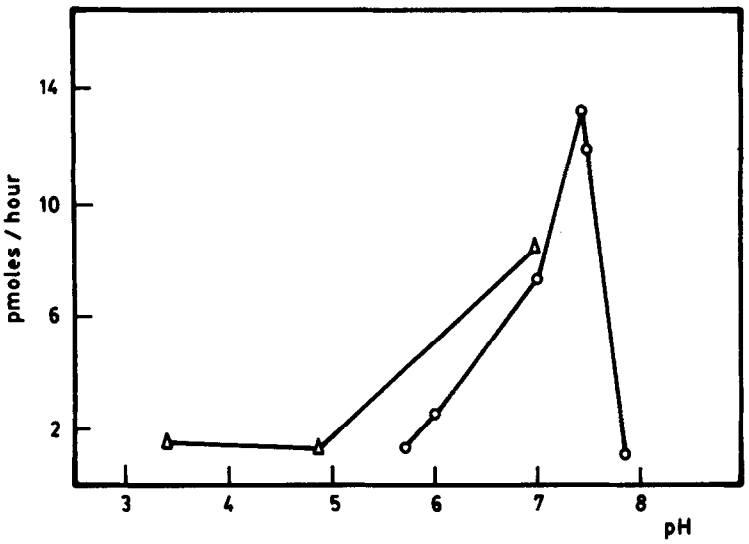

FIGURE 1.- Effect of pH on activity of hemoglobin decarbamoylase.

Incubations were for one hour in citrate-phosphate (△) and sodium phosphate (●) buffers, as indicated in Methods, with carbamoyl-hemoglobin and 250 µg of the enzyme with a specific activity of 30 pmoles/mg protein.

Decarbamoylation of several proteins and aminoacids. A number of carbamoylated proteins were tested as substrates for the decarbamoylase (Table II). At the concentrations tested, and other than with hemoglobin, only carbamoylated histones showed activity and carbamoyl casein traces. The decarbamoylating effect was not due to protease or demethylase activities because there was negligible liberation of acid-soluble radioactivity in the presence of (^{14}C) methylated hemoglobin. We tested (^{14}C) acetyl-

TABLE II - Decarbamoylation of several proteins with the decarbamylase

Substrate used	Activity pmols/hour
Carbamoyl-hemoglobin	60.75
Carbamoyl-histones	0.51
Carbamoyl-casein	0.07
Carbamoyl-trypsin	0.00
Carbamoyl-fibrinogen	0.00
Carbamoyl-ovoalbumin	0.00

The assay was done under the conditions described in the paper, using 125 µg of enzyme.

labeled histones without effect. We also tested the decarbamoylase with hemoglobin specifically carbamoylated at either the ε-NH_2 groups of lysine or the NH_2-terminal valines. The enzyme showed the same activity with both types of carbamoylated hemoglobins.

The following carbamoyl aminoacids were tested, carbamoyl β-alanine, carbamoyl glutamic acid, carbamoyl-valine and carbamoyl-lysine; only carbamoyl-valine and carbamoyl-lysine were slightly active. Citrulline, however, appeared to be a good substrate. It appeared to us unlikely that the decarbamoylase liberated CO_2 from the ureid group of citrulline via the reversal of the reaction catalyzed by OTC. This reaction is very difficult to measure and generally ignored because of the inhibitory effect of ornithine (10).

We carried out a comparative study of the activities of the decarbamoylase and of highly purified OTC from beef liver. As shown in TableIII, both OTC and the decarbamoylase were able to decarbamoylate carbamoyl-hemoglobin and citrulline, and the decarbamoylase could also synthesize citrulline from carbamoyl phosphate and ornithine, i.e. it had OTC activity. Moreover, the ratios of activity were similar. Also, the specific inhibitor of

TABLE III- Relative velocities of ornithine transcarbamoylase and the purified "decarbamoylase" for citrulline and hemoglobin decarbamoylation

Reaction measured	Preparation	
	OTC (nmoles/min. mg)	Decarbamoylase (pmoles/min.mg)
Citrulline synthesis	500,000	80,000
Citrulline decarbamoylation	2.6	0.18
Hemoglobin decarbamoylation	109.0	8.1

Decarbamoylation of citrulline and carbamoyl-hemoglobin was carried out under the same conditions using 2.0 and 2.2 nmoles of citrulline and hemoglobin, respectively.

OTC, the transition analog δ-PALO (δ-N-(phosphonacetyl)-L-ornithine), inhibited both the decarbamoylase and the transcarbamoylase activities of the two preparations. OTC from Streptococcus foecalis also showed decarbamoylase activity with carbamoyl hemoglobin. It had an activity of 9.17 nmols/min/mg protein with carbamoyl-hemoglobin, under the same conditions used with beef liver OTC.

Interestingly, when the sodium phosphate buffer was replaced by acetate-diethanolamine buffer, both OTC and the decarbamoylase preparations failed to show activity with citrulline and there was only a slight activity (0.8 pmoles/min/mg prot.) when carbamoylated hemoglobin was used as substrate. Since it is difficult to obtain phosphate-free hemoglobin, it appears likely that phosphate is necessary for the decarbamoylation reaction. Aspartate transcarbamoylase from Streptococcus foecalis did not show decarbamoylase activity with carbamoyl hemoglobin when tested under conditions similar to those used with OTC.

The findings presented above strongly suggest that the decarbamoylase activity is due to ornithine transcarbamoylase. This was indeed unexpected since it was believed up to now that OTC had absolute specificity. Finally, the decarbamoylase activity may be an example of protective enzymes. Certainly urea, KCNO and carbamoyl phosphate are known to modify and carbamoylate proteins. Indeed, other modified proteins exist in tissues where there is a great excess of substrate, e.g. glucose and glycoproteins; possibly glycoxidases as well as OTC and other enzymes may be of importance in preventing protein modifications or restoring modified proteins. The apparent involvement of phosphate in the decarbamoylase activity of OTC, as well as in the back reaction, suggest a possible intermediate function for carbamoyl phosphate or such related products as carbamate.

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