COPURIFICATION OF CARBAMOYL PHOSPHATE SYNTHETASE AND ASPARTATE TRANSCARBAMOYLASE FROM MOUSE SPLEEN[1]

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Summary. A purification technique developed for the glutamine dependent carbamoyl phosphate synthetase from mouse spleen resulted in the copurification of aspartate transcarbamoylase. The two activities remained associated through $(NH_4)_2SO_4$ precipitation, hydroxylapatite adsorption and sucrose density gradient centrifugation. The pH curve of the aspartate transcarbamoylase exhibited two optima, one at pH 9.4 and a much higher one at pH 10.2. At pH 7.4 and 37°, aspartate transcarbamoylase had a K_m for aspartate of 8.4 mM and V_{max} of 0.35 μmoles product formed per min. per mg. protein. The K_m for carbamoyl phosphate was 1.8×10^{-3} mM. Activity of aspartate transcarbamoylase was not affected by any one of a number of pyrimidine nucleotides.

In bacteria, a single enzyme, carbamoyl phosphate synthetase (CPS), supplies carbamoyl phosphate for the synthesis of both arginine and orotic acid (1). In contrast, eukaryotic organisms, such as fungi (2,3), amphibia (4) and mammals (5,6,7) use one CPS for arginine biosynthesis and another CPS for pyrimidine synthesis. A bifunctional enzyme complex of the pyrimidine-specific CPS and aspartate transcarbamoylase (ATC, EC 2.1.3.2), the second enzyme of the pyrimidine biosynthetic pathway, was reported to exist in yeast (8,9) and in Neurospora (10).

The present paper reports the copurification of CPS and ATC from hematopoietic mouse spleen and presents some kinetic properties of ATC.

METHODS

Materials.[2] Uniformly labeled ^{14}C-L-aspartic acid, specific activity 10 mCi/mole (Amersham/Searle), was absorbed on a 1.2 x 1.5 cm column of Dowex 50W-X8 (H^+ form) to remove the small amount of radioactive impurity and eluted with 2 M HCl. The

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[2] L-aspartic acid, D-L-carbamoyl aspartic acid, L-glutamine, dithiothreitol, glycylglycine, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) were obtained from Calbiochem. Ammonium sulphate was special enzyme grade from Mann. Hydroxylapatite was a Bio-Rad product (Biogel HTP). All other reagents were analytical grade.

eluate was evaporated to dryness over NaOH pellets and the ^{14}C-aspartic acid adjusted to 20μCi/ml, pH 7.4. $Na_2{}^{14}CO_3$ was prepared by addition of 2 M HCl to $Ba^{14}CO_3$ (61.1 mCi/mmole, Amersham/Searle). The $^{14}CO_2$ evolved was absorbed on filter paper saturated with 2 M NaOH and $Na_2{}^{14}CO_3$ was eluted with deionized, boiled water. Commercial preparations of carbamoyl phosphate, ATP and UTP were assayed (11) and corrected for inorganic phosphate.

Enzyme Preparation. Male mice of Swiss strain, approximately 20 g, were given four daily subcutaneous injections of 0.1 ml of 1.0% (w/v) phenylhydrazine, pH 7.4. The mice were killed on the 5th day, when each spleen weighed 0.5 to 1.0 g. CPS was prepared using the method of Tatibana and Ito (12). After each step of the purification the product was assayed for activity of CPS and ATC. The enzyme preparation was frozen and stored in liquid nitrogen.

Enzyme Assays. ATC was assayed by isolation of ^{14}C-carbamoylaspartate formed from ^{14}C-aspartate as outlined by Porter et al (13). The standard ATC reaction mixture (0.3 ml) contained carbamoyl phosphate (3 mM), ^{14}C-aspartic acid (5×10^5 cpm), unlabelled sodium aspartate to make the final aspartate concentration 10 mM and sodium HEPES, pH 7.4,[1] (50 mM).

CPS activity was determined using the radiochemical assay of Levine and Kretchmer (16). In the standard assay for CPS, glutamine was 1.25 mM, ATP (sodium salt) 25 mM, magnesium (as sulfate or chloride) 37.5 mM, $Na_2{}^{14}CO_3$ 5 mM (1.4×10^7 cpm) and sodium HEPES 50 mM (pH 7.4) in a total volume of 0.8 ml. Optimal CPS activity was obtained by adding 7.5% (v/v) dimethyl sulfoxide and 2.5% (w/v) glycerol (12). Both assays, carried out at 37°, were started by addition of enzyme. Samples were counted in 10 ml of scintillation solution (17) in a Packard Model 2003 scintillation spectrometer.

Protein Determinations. Protein was determined using the Oyama and Eagle (18) modification of the method of Lowry et al (19) with human serum albumin as standard. Since HEPES and dimethyl sulfoxide (20) interfere with this method, samples were precipitated and washed with trichloracetic acid (12).

Sucrose Gradients. Linear sucrose density gradients (20%-30% sucrose) were prepared as described by Oliver et al (21). To stabilize the CPS, Mg-ATP was added to the gradients to a final concentration of 15 mM Mg^{++} and 10 mM ATP. Samples (0.2 ml) of crude homogenate or purified enzyme (0.55 mg protein) were layered on the gradient, and then centrifuged at 300,000 x g_{av} for 4 hours in the SW 65 Ti rotor of the Spinco L2-65 ultracentrifuge. The gradient was collected through a puncture, as 0.3 ml fractions.

[1] Carbamoyl phosphate undergoes a base catalyzed decomposition of 9.3% per minute at 37° at pH 10.2 (14). Therefore kinetic analysis were carried out at pH 7.4, the optimum for CPS (15) rather than 10.2, the pH optimum for ATC.

Calculations and Data-Processing. Kinetic determinations were based on initial rates obtained from time-progress studies. Data were analyzed using an IBM 360/50 time-sharing system with on-line communication and programs written in the PL/ACME language.

RESULTS

Copurification of CPS and ATC. Although the procedure for purification used in this study was developed expressly for CPS (12), considerable purification of ATC was also obtained (Table I). The recovery of both enzymes was similar. The decreased activity of CPS compared to ATC after ammonium sulphate fractionation may be due to presence of ammonium ions and glutamine in the CPS assay medium (5,6). In six separate purifications, the increase in specific activity ranged from 15- to 99- fold for CPS and 26- to 65- fold for ATC. The yields varied from 15 to 41% for CPS and 13 to 60% for ATC.

TABLE I

PURIFICATION OF CPS AND ATC [1]

Fraction	Activity (Units[2])		Protein (mg)	Specific Activity (units/mg protein)		Purification	
	CPS	ATC		CPS	ATC	CPS	ATC
Homogenate	32.85	15,600	1343.0	0.02	11.6	-	-
20,000g supernatant	43.22	15,680	948.0	0.04	11.6	1.0	1.0
Ammonium sulfate	8.65	9,300	78.5	0.11	118.0	2.5	7.2
1st hydroxylapatite	4.82	*	6.0	1.57		34.0	
2nd hydroxylapatite	8.20	2,500	4.7	1.75	530.0	38.0	32.0
Dialysate	3.17	1,000	2.3	1.38	439.0	30.0	27.0

[1] Details of the purification procedure are given by Tatibana and Ito (12). The starting material was 11.0 g wet weight of mouse spleen.

[2] One unit of enzyme activity is equal to 1mμmole product formed/min. at 37°.

* Not determined.

Elution from Hydroxylapatite. Elution of CPS, ATC, and protein from the hydroxylapatite column is shown in Fig. 1. Similar elution patterns for CPS and ATC indicate close association of these two enzymes.

Storage of the Enzyme. Activity of ATC was stable at -20°, but CPS was completely inactivated within 48 hours at this temperature. Following storage of the preparation in a liquid nitrogen freezer (-196°) it was found that both enzyme activities were consistently increased. The basis for this increase in activity is unknown, but it was not due to freezing and thawing of the enzyme preparation.

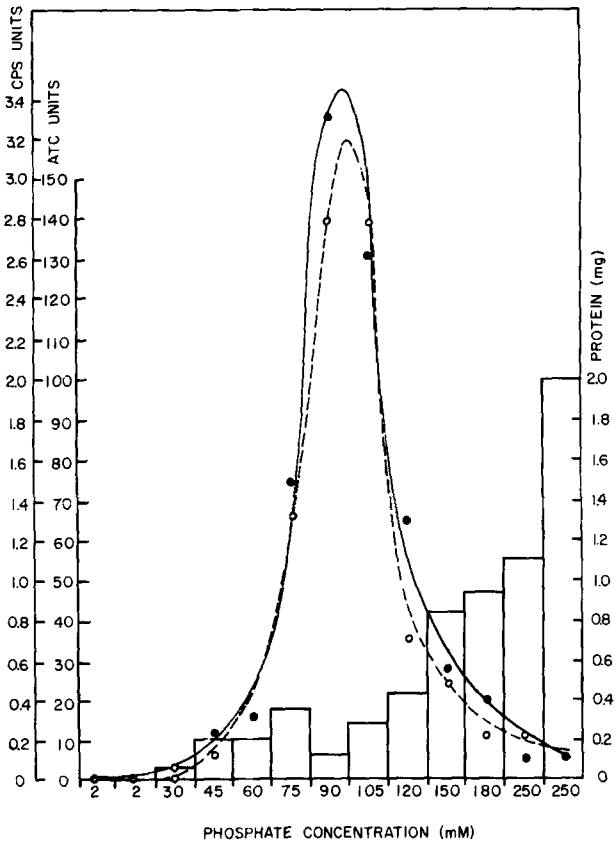

Figure 1. Profile of enzyme fractionation on a hydroxylapatite column. The enzyme preparation was purified through the ammonium sulphate step. The enzyme was eluted in 7 ml fractions from the 5 ml hydroxylapatite column with step-wise increases in concentration of phosphate buffer, pH 7.6. The CPS and ATC were determined by the standard procedure described in the experimental section. ATC activity --0--0--; CPS activity -●-●-. The histogram represents protein. One unit of activity equals 1000 mμmole of product/min. at 37°C. In the four fractions containing the most enzyme activity, 87.7% of the ATC activity, 82.7% of the CPS activity, and 16% of the protein were recovered.

When the preparation was re-assayed one month later, both enzyme activities were slightly decreased from that found after 20 hours of storage.

Sucrose Gradient Centrifugation. When homogenates were subjected to sucrose gradient centrifugation, both CPS and ATC were recovered in the same fraction of the gradient (Fig. 2A). This fraction corresponds to the 600,000 molecular weight ATC-peak obtained from rat liver (21), which is the main peak for ATC in a mouse spleen homogenate (22). After purification of the enzymes both activities were still closely associated, although there was a slight shift of the activities to a more slowly sedimenting fraction (Fig. 2B).

Effect of pH on ATC Activity. The pH profile for ATC has two optima, one at

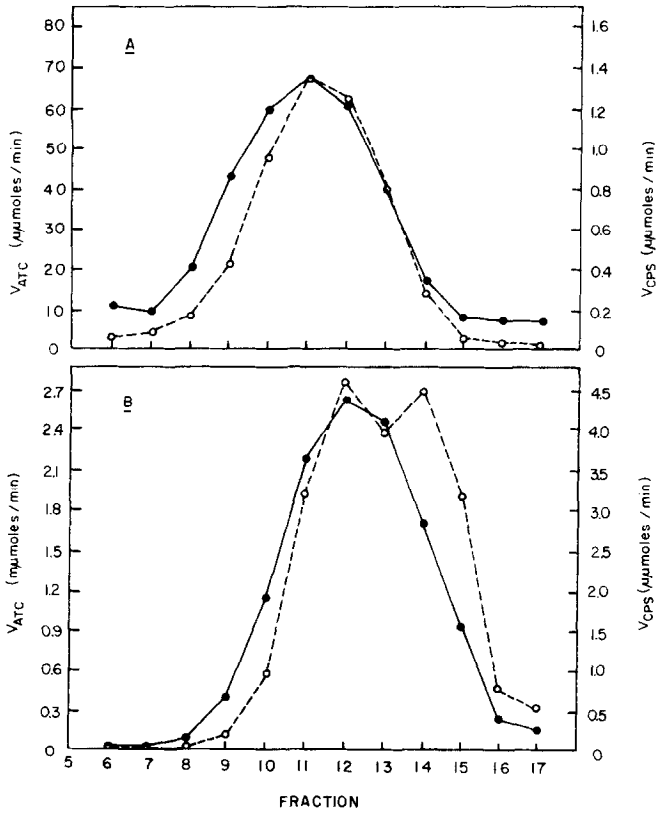

Figure 2. Distribution of CPS and ATC activity on 20-30% sucrose gradients (density: 1.074 - 1.113). The highest density shown in the figure is 1.102 (fraction 5). A: crude homogenate; B: purified enzyme. The experiments were carried out as described in the experimental section. ATC activity, -●-●-; CPS activity, --○--○--.

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pH 9.4 and one with a much higher velocity at pH 10.2. Above pH 10.2 the velocity of the reaction rapidly decreases.

Determination of Kinetic Parameters. The kinetics of ATC were investigated at pH 7.4, the optimum for CPS (15). Trial experiments showed that there was no variation in the velocity of ATC when the concentration of the carbamoyl phosphate was reduced to 0.2 mM. This finding results from the large difference between the K_m values for carbamoyl phosphate (about 0.01 mM) and aspartate (10 mM). Consequently, the usual technique of varying the concentration of each substrate in the range of its K_m resulted in formation of too little radioactive product for accurate determinations of true K_m. Reasonable approximations of the true K_m for mouse spleen ATC are shown in Table II and were obtained by using the method of Porter et al (13), who found similar large

TABLE II

ESTIMATES OF K_m AND MAXIMAL VELOCITY FOR ATC

SUBSTRATE	K_m (mM)	MAXIMAL VELOCITY (mμmoles/min/mg)
Aspartate	8.4	0.35
Carbamoyl phosphate	1.8×10^{-3}	--

Initial velocity data were obtained as described in the text. The K_m and V_{max} for aspartate were determined using saturating amounts of carbamoyl phosphate (1mM) with aspartate concentrations ranging from 0.50 to 7.5 mM. The K_m for carbamoyl phosphate was determined in the presence of limiting aspartate (4.5×10^{-2} mM) with carbamoyl phosphate concentrations ranging from 1×10^{-3} to 5×10^{-3} mM.

differences in the K_m values for the catalytic sub-unit of E. coli ATC. For both substrates the data gave an excellent fit to the rate equation describing simple Michaelis-Menten kinetics.

Effect of Pyrimidine Nucleotides on ATC Activity. A number of pyrimidine nucleotides, UTP, UMP, dUTP, dUMP, CTP, CMP, dCTP, dCMP, ITP and IMP, were tested for their effect on ATC activity at pH 7.4. They were added to the assay medium at concentrations of 1 mM and 0.5 mM. No inhibition of the ATC by any of these nucleotides was detected when the incubations were carried out in the presence of an excess of carbamoyl phosphate (0.5 mM) and with concentration of aspartate well below the K_m (1 mM).

Reaction Mechanism of ATC. Preliminary product inhibition studies carried out with the partially purified enzyme preparation gave results consistent with an ordered Bi-Bi mechanism (23). The experiments, conducted at pH 7.4, showed that when the concentration of carbamoyl phosphate was varied at a limiting concentration of aspartate (4.5×10^{-2} mM) reciprocal plots were linear. Only inorganic phosphate gave competitive inhibition with carbamoyl phosphate; no other combinations of product and substrate gave competitive inhibition.

DISCUSSION

The first two enzymes of the pyrimidine biosynthetic pathway copurify using the procedure devised for CPS purification by Tatibana and Ito (12). The activities of CPS and ATC from mouse spleen were still associated following ammonium sulfate fractionation, hydroxylapatite chromatography or sucrose density gradient centrifugation. More selective procedures for separating the two activities such as gel filtration chromatography or DEAE-cellulose chromatography have not been successful because it has not been possible to construct columns

with the DMSO required to stabilize CPS. It should be noted that the CPS activities at all steps in the purification procedure were 10 percent of the values obtained by Tatibana and Ito (12). This difference cannot be accounted for, but was consistently obtained.

Similar copurification of activities of CPS and ATC from baker's yeast was reported by Lue and Kaplan (8,9) and from Neurospora by Williams et al (10). Genetic evidence that the CPS and ATC of yeast are present as a single complex was given by Lacroute (24) who showed that both enzymes are coded by the same genetic region (ura-2) and that mutation of the ura-2 locus renders both enzyme activities insensitive to feedback inhibition by UTP. Likewise, support for a single CPS-ATC complex in Neurospora derives from genetic data which show that both enzyme activities are controlled by the pyr-3 locus (25,26).

There were a number of similarities in the kinetic properties of ATC from mouse spleen and E. coli. Enzyme from both sources have a pH optimum of 10.2 with a peak of less activity at a lower pH (27). Michaelis constants for the mammalian ATC at pH 7.4 were very close to those obtained at pH 7.8 for the catalytic subunit of E. coli ATC (13). In both studies the K_m for carbamoyl phosphate was very low (10^{-3} mM for mammalian ATC; 10^{-2} mM for E. coli ATC) and the K_m for aspartate was of the same order of magnitude (10 mM for mammalian ATC; 20 mM for E. coli ATC).

The existence in mammals of two distinct enzymes producing carbamoyl phosphate suggests that the products may be channeled through two separate pathways (6). Channeling has been demonstrated in vivo in Neurospora where the carbamoyl phosphate produced by one reaction is not freely available to the other pathway (3,28). Lue and Kaplan (29) have presented data which suggest that carbamoyl phosphate is channeled by the ATC-CPS complex from yeast. The very low K_m of ATC for carbamoyl phosphate (about 10^{-3} mM) coupled with an activity of ATC 300 times greater than CPS should lead to efficient use by ATC of proximally produced carbamoyl phosphate. These kinetic data suggest that efficient physiologic channeling probably occurs in vivo.

From the product inhibition pattern obtained, a tentative reaction mechanism can be proposed. Linear reciprocal plots with carbamoyl phosphate varied at limiting concentrations of aspartate suggests that the mechanism is Ordered. The pattern of produced inhibition is consistent with an Ordered-Bi-Bi mechanism (23) which predicts that only the last product to dissociate from the enzyme will be competitive with the first substrate to bind. This suggests that carbamoyl phosphate binds first, followed by aspartate and carbamoyl aspartate is released first, followed by ortho-phosphate. This mechanism is the same as that found by Porter at al (13) for the catalytic subunit of E. coli ATC.

Unlike native ATC from E. coli the mammalian enzyme was not subject to feed-

back regulation by pyrimidine nucleotides. Similar results were reported by Curci and Donachie (30) for ATC from red blood cells and by Koskimies et al (22) for liver. In contrast, the associated CPS is inhibited by UTP (7,15).

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