Aggregation States and Catalytic Properties of the Multienzyme Complex Catalyzing the Initial Steps of Pyrimidine Biosynthesis in Rat Liver†

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ABSTRACT: Glutamine-dependent carbamoyl-phosphate synthetase was purified about 2100-fold from the cytosol of rat liver using 30% (v/v) dimethyl sulfoxide and 5% (w/v) glycerol as stabilizers. Throughout the purification, aspartate transcarbamylase and dihydroorotase, the second and third enzymes of pyrimidine biosynthesis, were copurified with the synthetase. These three enzymes sedimented as a single peak with a sedimentation coefficient of 27 S in sucrose gradients containing the stabilizers, indicating their existence as a multienzyme complex. The aggregation states of the complex were analyzed by sucrose gradient centrifugation under conditions approximating those used for enzymatic assay and correlated with the kinetic properties of the synthetase. In the presence of 10% glycerol and 10 m*M* $MgATP^{2-}$ at 18°, the synthetase showed high activity and the three enzymes sedimented as a single peak with a coefficient of 25 S. The three enzymes also existed as a complex with the same coefficient when 50 μM PP-ribose-P was added in place of $MgATP^{2-}$. However, in the presence of 3 m*M* $MgUTP^{2-}$, the sedimentation coefficient of the complex shifted to 28 S, indicating alteration in its molecular shape, rather than size. With 10% glycerol alone, the complex partially dissociated and the synthetase activity appeared in three peaks with coefficients of 26, 19, and 9 S (carbamoyl-phosphate synthetases (CPSase) *a, b,* and *c,* respectively). CPSases *a, b,* and *c,* thus obtained, were all sensitive to regulation by UTP and PP-ribose-P, but they differed in their apparent K_m values for $MgATP^{2-}$ (5.1, 4.8, and 1.7 m*M* for CPSases *a* and *b,* and the enzyme within the original complex, respectively) and in their sensitivities to effectors. These results suggest that the aggregation may modify the catalytic and regulatory properties of the synthetase. Attempts to reassociate the components were unsuccessful.

Glutamine-dependent carbamoyl-phosphate synthetase (CPSase II[1]) has recently been demonstrated in a number of animal systems including various mammalian tissues (Tatibana and Ito, 1967, 1969; Hager and Jones, 1967b) and experimental tumors (Hager and Jones, 1967a; Yip and Knox, 1970; Ito et al., 1970). Studies in this laboratory (Tatibana and Ito, 1967, 1969; Ito et al., 1970; Inagaki and Tatibana, 1970) and others (Hager and Jones, 1967a,b; Lan et al., 1969; Yip and Knox, 1970) have established that the enzyme catalyzes the first step of de novo pyrimidine biosynthesis and plays a key role in the control of this pathway. The enzyme is subject to feedback inhibition by UTP and also to activation by PP-ribose-P (Tatibana and Shigesada, 1972a,c).

Adult rat liver also contains this type of CPSase in the cytosol (Nakanishi et al., 1968), in addition to the ammonia- and *N*-acetylglutamate-dependent enzyme (CPSase I) which is localized in the mitochondria and provides carbamoyl-P primarily for urea synthesis. Thus, there are two different pathways for carbamoyl phosphate metabolism in the liver. This unique feature prompted us to examine the properties of CPSase II of this tissue. During its purification from rat liver, we found that the enzyme exists as a complex with aspartate transcarbamylase (ATCase) and dihydroorotase, the second and third enzymes of the orotic acid pathway (Mori and Tatibana, 1973). Similar enzyme complexes were known to occur in other systems, including bakers' yeast (Lue and Kaplan, 1969, 1971), *Neurospora* (Williams et al., 1970), hematopoietic mouse spleen (Hoogenraad et al., 1971), Ehrlich ascites carcinoma (Shoaf and Jones, 1971), and human lymphocytes (Ito and Uchino, 1972). However, little is known about the relationship between the aggregation states and regulatory properties of the enzymes from mammals. To study this relationship the molecular and catalytic properties of the enzymes should be examined under identical or very similar conditions, since the activity of CPSase II varies greatly in response to changes in environmental conditions (Tatibana and Shigesada, 1972b).

In this study, we purified the hepatic enzyme, examined its general properties, and then simulated these conditions during sucrose gradient centrifugation. Centrifugal analyses performed under selected conditions revealed that the allosteric regulation of CPSase II activity by UTP and PP-ribose-P does not involve dissociation and association of the complex, but is associated with conformational changes of the complex as a whole. The complex dissociates when the concentration of cryoprotectants, such as dimethyl sulfoxide (Me_2SO) and glycerol, is reduced. The kinetic and regulatory properties of the dissociated CPSase, obtained in this way or by treatment with pancreatic elastase (Mori and Tatibana, 1973), were different from those of the enzyme in the complex. It is, therefore, suggested that the CPSase II molecule maintains its proper conformation when present as a component of the complex.

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[1] Abbreviations used are: CPSase II, glutamine-dependent carbamoyl-phosphate synthetase; CPSase I, ammonia- and *N*-acetylglutamate-dependent carbamoyl-phosphate synthetase; ATCase, aspartate transcarbamylase; Me_2SO, dimethyl sulfoxide; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Experimental Procedure

Materials. ATP, UTP, and PP-ribose-P were obtained as described previously (Tatibana and Shigesada, 1972c). Dihydro-L-orotic acid was obtained from Sigma, and dithiothreitol from Nakarai Chemicals, Kyoto, Japan. Me_2SO and glycerol were obtained and purified as described previously (Tatibana and Ito, 1969). To prepare hydroxylapatite calcium phosphate was prepared by the method of Siegelman et al. (1965) and treated with sodium hydroxide as described by Levin (1962). The preparation gave a sufficiently high flow rate. $Ba^{14}CO_3$ and [^{14}C]cyanate were obtained from the Radiochemical Centre, Amersham, England. $KH^{14}CO_3$ was prepared from $Ba^{14}CO_3$ as described previously (Tatibana and Ito, 1967). [^{14}C]Carbamoyl-P was prepared from [^{14}C]cyanate by a microscale modification of the method of Spector et al. (1957) for synthesis of nonradioactive carbamoyl-P. The preparation was recrystallized from ethanol–H_2O (2:1, by volume) to remove radioactive impurities. Ornithine transcarbamylase was purified from bovine liver essentially as described by Marshall and Cohen (1972). Protocatechuate 3,4-dioxygenase from *Pseudomonas aeruginosa* was a gift from Drs. M. Nozaki and O. Hayaishi, Kyoto University School of Medicine.

Purification of CPSase II. Wistar strain rats, weighing 250 to 350 g, were killed by exsanguination from the abdominal aorta under ether anesthesia and the livers (57 g) were excised. Homogenization, the first ammonium sulfate fractionation, and high speed centrifugation at 105,000*g* were performed as described for purification of the mouse spleen enzyme (Tatibana and Ito, 1967). The supernatant solution thus obtained was subjected to the second ammonium sulfate fractionation by adding 0.65 vol of the same saturated ammonium sulfate solution as used in the preceding step. After standing for 5 min at 0° the precipitate was collected by centrifugation at 20,000*g* for 15 min and dissolved in 17.1 ml (0.3 vol of the original tissue) of 0.15 *M* potassium phosphate (pH 7.8) containing 30% Me_2SO, 5% glycerol, and 1 m*M* dithiothreitol (second ammonium sulfate fraction). The solution was mixed with 11.4 ml of hydroxylapatite, previously equilibrated with the same buffer, and centrifuged at 1000*g* for 5 min. The hydroxylapatite was washed twice with 5.7 ml of the same buffer. The supernatant and washings were combined (first hydroxylapatite fraction), diluted with 1.5 vol of 2 m*M* potassium phosphate (pH 7.0) containing 30% Me_2SO, 5% glycerol, and 1 m*M* dithiothreitol, and adjusted to pH 6.7 with 1 *M* acetic acid. Then the solution was applied to a hydroxylapatite column (3.0 × 4.2 cm) previously equilibrated with the same phosphate buffer (2 m*M*) as used for enzyme dilution. Elution was performed with a linear gradient of 0.03–0.21 *M* potassium phosphate buffer (pH 7.8) (240 ml) containing 30% Me_2SO, 5% glycerol, and 1 m*M* dithiothreitol at a flow rate of 80 ml/hr. Active fractions were combined, concentrated to about 2 ml by ultrafiltration using Amicon XM-300 membrane at room temperature, and dialyzed for 4 hr at 4° against 0.03 *M* Hepes[1] (pH 7.2) containing 30% Me_2SO, 5% glycerol, and 1 m*M* dithiothreitol (second hydroxylapatite fraction).

The enzyme (0.5 ml) was then layered on a sucrose gradient (4.5 ml) containing 5 to 20% sucrose, 0.05 *M* Hepes (pH 7.2), 30% Me_2SO, 5% glycerol, and 1 m*M* dithiothreitol, and centrifuged at 65,000 rpm for 6 hr at 18°. After centrifugation fractions of 22 drops were collected. The fractions with the highest activities were pooled and dialyzed as above (sucrose gradient fraction).

Enzyme Assays. CPSase II activity was assayed in two systems. In system A the reaction mixture contained 7.5% Me_2SO and 2.5% glycerol, and in system B it contained 10% glycerol. The incubation time was 15 min in both systems. Other conditions were as described previously (Tatibana and Shigesada, 1972b), unless otherwise indicated. In studies on enzyme effectors, the concentrations of ATP and $MgCl_2$ were reduced to 3 and 8 m*M*, respectively. The concentrations of $MgCl_2$, ATP, UTP, and PP-ribose-P were determined as described previously (Tatibana and Shigesada, 1972b,c).

ATCase activity was assayed with [^{14}C]carbamoyl-P as substrate by following the formation of [^{14}C]carbamoyl aspartate. The reaction mixture contained 40 μmol of Tris-HCl (pH 8.5), 1.0 μmol of L-aspartic acid, 40 nmol of [^{14}C]carbamoyl-P (150 cpm/nmol), and enzyme in a total volume of 0.2 ml. After incubation for 15 min at 37°, the reaction was terminated by adding 0.1 ml of 3 *M* formic acid and the mixture was kept at 80° for 5 min. Then it was evaporated to dryness in vacuo over KOH and dissolved in 0.5 ml of water and its radioactivity was determined with 10 ml of Bray's solution (Bray, 1960) in a Beckman scintillation spectrometer. To determine the K_m value for carbamoyl-P, assay was carried out using [^{14}C]carbamoyl-P (1000 cpm/nmol) in a total volume of 1.0 ml.

Dihydroorotase activity was assayed by measuring the carbamoyl aspartate formed from dihydroorotate in a 0.3-ml system containing 30 μmol of potassium phosphate buffer (pH 7.0), 0.6 μmol of dihydroorotate, and enzyme on incubation for 2 hr at 37°. Carbamoyl aspartate was determined by the method of Prescott and Jones (1969).

One unit of CPSase II, ATCase, or dihydroorotase activity is defined as that amount of enzyme which produces 1 nmol of product per minute under the above conditions (measuring CPSase II in system A). The specific activity is expressed as units of enzyme per milligram of protein. Protein was determined as described previously (Tatibana and Ito, 1967). Protocatechuate 3,4-dioxygenase was assayed as described by Fujisawa and Hayaishi (1968).

Sucrose Gradient Centrifugation. Sucrose gradient centrifugation was performed by the method of Martin and Ames (1961). Linear 5 to 20% sucrose gradients (4.5–4.9 ml) containing 0.05 *M* Hepes (pH 7.2), 1 m*M* dithiothreitol, Me_2SO, and glycerol, and substrates and effectors, when indicated, were prepared and equilibrated overnight at 18°. The enzyme solution (0.04–0.5 ml), which was brought to the same concentrations of cryoprotectants as those in the gradient, was layered on the gradient and centrifuged at 65,000 rpm at 18° for 2.5 or 6.0 hr in a Hitachi 65P ultracentrifuge using an RPS 65TA rotor. Gradients containing PP-ribose-P, which is labile, were used soon after their preparation. After centrifugation, fractions of 22 drops were collected from the bottom of the tube. Sedimentation coefficients were calculated by the formula (eq 4) of Martin and Ames (1961) using protocatechuate 3,4-dioxygenase ($s_{20,w}$ = 19.4 S) (Fujisawa and Hayaishi, 1968; Fujisawa et al., 1972) as an internal standard. In a strict sense, this method is not suitable for the present work, because the gradient contained high concentrations of cryoprotectants. However, this does not change any of the basic conclusions of this report. In addition, the sedimentation coefficients obtained by this method were in good agreement with the values calculated by eq 1 and 2 of Martin and Ames (1961) based on the viscosity and density of the medium.

Table I: Purification of CPSase II, ATCase, and Dihydroorotase from Rat Liver.[a]

Fraction	Total Act. (Units)			Total Protein (mg)	Sp Act. (Units/mg of Protein)		
	CPSase II	ATCase	Dihydro-orotase		CPSase II	ATCase	Dihydro-orotase
Homogenate supernatant	240[b]	21,600	1680	5200	0.046[b]	4.2	0.32
First ammonium sulfate		12,200		324		37.3	
Second ammonium sulfate	145[c]	10,100	564	128	1.13[c]	79.0	4.4
First hydroxylapatite	129			33	3.99		
Second hydroxylapatite	78	5,290	86	6.2	12.7	854	13.9
Sucrose gradient	15.5	2,460	45	0.16	97.0	15,400	281

[a] The starting material was 57 g wet weight of rat liver. [b] Approximate values measured using the 105,000*g* supernatant of rat liver homogenate after treatment with hydroxylapatite to remove CPSase I. [c] The preparation at this step still contained CPSase I, but its activity was less than 10% of that presented.

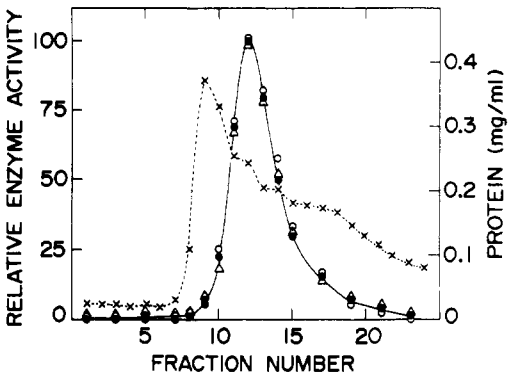

FIGURE 1: Chromatography on a hydroxylapatite column. The first hydroxylapatite fraction containing 48.2 mg of protein was applied on a column of hydroxylapatite (3.0 × 4.2 cm). Elution was carried out as described in the text. Fractions of 10 ml were collected: (●-●) CPSase II; (○-○) ATCase; (△-△) dihydroorotase; (x- - -x) protein concentration. The ordinate represents the activity of each enzyme as a percentage of that at the top of the peak.

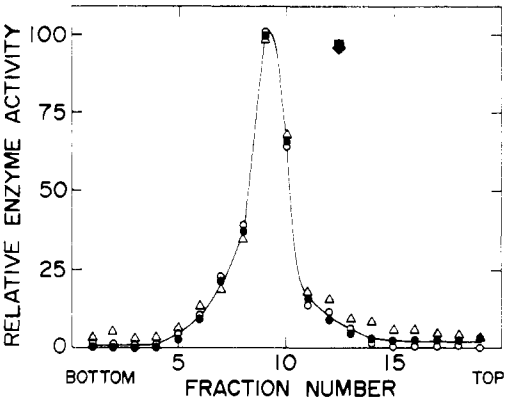

FIGURE 2: Sucrose gradient centrifugation in the presence of 30% Me_2SO plus 5% glycerol. Enzyme solution (0.04 ml) containing 0.81 unit of CPSase II activity (68 μg of protein) and 135 μg of protocatechuate 3,4-dioxygenase was centrifuged at 65,000 rpm for 6 hr at 18° in a 65TA rotor: (●-●) CPSase II; (○-○) ATCase; (△-△) dihydroorotase. CPSase II was assayed in system A except that both 3.3 m*M* glutamine and 30 m*M* NH_4Cl were used as nitrogen donors. The recoveries of CPSase II, ATCase, and dihydroorotase activities were 68, 68, and 76%, respectively. The position of the peak of protocatechuate 3,4-dioxygenase ($s_{20,w}$ = 19.4 S) is indicated by an arrow.

Results

Copurification of CPSase II, ATCase, and Dihydroorotase. CPSase II was purified about 2100-fold from rat liver cytosol as described under Experimental Procedure. As shown in Table I, the purification of CPSase II resulted in concomitant purification of ATCase and dihydroorotase, the second and third enzymes of pyrimidine biosynthesis, and, furthermore, in all the steps the behaviors of these two enzymes coincided with that of CPSase II. For example, upon chromatography on a hydroxylapatite column, all three activities were eluted in the same, single peak (Figure 1), and upon sucrose gradient centrifugation in the presence of 30% Me_2SO plus 5% glycerol, these three activities sedimented together as a single peak with a sedimentation coefficient of 27 S (Figure 2).

The ratio of the activities of CPSase II and ATCase was approximately constant throughout the purification procedure, except on sucrose gradient centrifugation (Table I). The lower recovery of CPSase II observed in the last step (20%) compared to that of ATCase (47%) is probably due to the preferential inactivation of the glutamine site (Trotta et al., 1971) of CPSase II at this step. When assayed with 40 m*M* NH_4Cl instead of 3.3 m*M* glutamine as nitrogen donor, the recovery of CPSase II activity was 44% and the activity ratio of CPSase II to ATCase was approximately the same as those in preceding steps. A similar preferential decrease of the glutamine-dependent activity upon ultracentrifugation was reported previously for CPSase II from a hepatoma cell line (Ito et al., 1970). With respect to dihydroorotase, the poor yield throughout the whole purification procedure is ascribable to enzyme inactivation, because dihydroorotase is more labile than the other two enzymes under these purification conditions.

The following enzymes were not detected in our most purified preparations: (1) dihydroorotate dehydrogenase (the fourth enzyme of pyrimidine synthesis), assayed with molecular oxygen, NAD^+, $NADP^+$, FAD, Methylene Blue, phenazine methosulfate, dichlorophenol indophenol, potassium ferricyanide, and *o*-quinone as oxidants; (2) orotidylate phosphoribosyltransferase (the fifth enzyme of the pathway), (3) glutamine phosphoribosylpyrophosphate amidotransferase (the first enzyme of purine synthesis), and (4) 5′-nucleotidase (a marker enzyme of microsomes), assayed with AMP as a substrate. All activities were assayed by radioactive methods.

Properties of Rat Liver CPSase II. The catalytic and regulatory properties of the rat liver enzyme were examined using a preparation of the second hydroxylapatite fraction. The properties were qualitatively the same as those reported for the mouse spleen enzyme (Tatibana and Ito, 1967; Tatibana and Shigesada, 1972c). The rat liver enzyme is active with either glutamine or ammonia as nitrogen donor. Its apparent K_m values for glutamine and NH_4Cl were 17 μ*M*

Table II: Effects of UTP and PP-Ribose-P on the Maximal Velocity and Apparent K_m for ATP of CPSase II.[a]

Enzyme Source	Temp of Assay (°C)	Addition	V_{max}[b] (nmol/min per mg of Protein)	Apparent K_m for ATP (mM)
Rat liver	37	None	10.5	1.7
		UTP, 1,0 mM	10.5	15
		PP-Ribose-P, 50 μM	11.9	0.50
	18	None	2.7	1.0
		UTP, 0.3 mM	2.7	5.0
		UTP, 1.0 mM	2.7	>20
		PP-Ribose-P, 50 μM	3.2	0.10
Mouse spleen[c]	37	None	22.8	5.2
		UTP, 0.5 mM	22.8	9.0
		UTP, 2.0 mM	22.8	20
		PP-Ribose-P, 50 μM	22.8	0.72

[a] CPSase II activity was assayed under the standard conditions in system B except for variation in ATP concentration. The concentration of free Mg^{2+} was fixed at 5.0 mM. The maximal velocity (V_{max}) was obtained by extrapolation of double reciprocal plots to infinite substrate concentration. The apparent K_m was obtained from a standard Hill plot of log $[v/(V_{max} - v)]$ vs. log [ATP]; enzyme: 0.10 unit, 9.5 μg of protein at 37°; 0.20 unit, 19 μg of protein at 18°. [b] Values for a partially purified enzyme preparation (second hydroxylapatite fraction). [c] Previously reported (Tatibana and Shigesada, 1972c) or calculated from the data reported (Tatibana and Shigesada, 1972c).

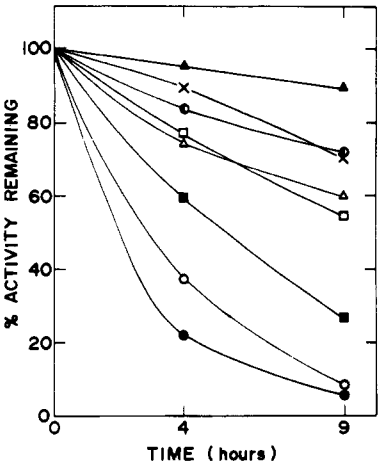

FIGURE 3: Effects of ATP, UTP, and PP-ribose-P on the stability of CPSase II in 10% glycerol at 18°. Enzyme (0.12 unit, 11 μg of protein) was kept at 18° in solution (0.20 ml) containing 0.05 M Hepes (pH 7.2), 10% glycerol, 1 mM dithiothreitol, and the following additional compounds: none (●-●); 0.3 mM ATP (○-○); 1 mM ATP (■-■); 3 mM ATP (□-□); 10 mM ATP (◐-◐); 0.3 mM UTP (x-x); 3 mM UTP (▲-▲); 50 μM PP-ribose-P (△-△). At the indicated times activity in 0.05-ml aliquots was assayed in system B.

and 10 mM, respectively, at pH 7.0 in system B. The ratio of enzyme activity with 0.2 mM glutamine to that with 40 mM NH_4Cl was 1.32.

As observed with the mouse spleen enzyme, the rat liver enzyme was inhibited by UTP and activated by PP-ribose-P; UTP increased and PP-ribose-P decreased the apparent K_m for ATP with little change in the maximal velocity. (Hereafter ATP and UTP represent $MgATP^{2-}$ and $MgUTP^{2-}$, respectively, in this paper; 5 mM free Mg^{2+} was added in excess of the concentration of $MgATP^{2-}$ or $MgUTP^{2-}$ in all experiments including enzymatic assays, enzyme stability tests, and sucrose gradient centrifugation.) The data are shown in Table II. The properties of the mouse spleen enzyme, reported previously (Tatibana and Shigesada, 1972c), are also shown for comparison. Kinetic analyses were carried out at both 37 and 18°; the data at 18° are described in the next section. As shown, at 37° the rat enzyme has a lower apparent K_m for ATP (1.7 mM) than that of the mouse enzyme (5.2 mM), and is more sensitive to UTP inhibition and slightly less sensitive to PP-ribose-P activation than the mouse enzyme.

CPSase II from mammalian sources is extremely labile so cryoprotectants, such as Me_2SO and glycerol, were added during purification, storage, and enzyme assay. These agents not only increased the stability of the enzyme, but changed its catalytic properties significantly (Tatibana and Shigesada, 1972b). The activity of the liver enzyme increased with an increase in the concentration of glycerol to 10 to 15% glycerol, but activity was strongly inhibited by higher concentrations of glycerol. In the presence of 30% Me_2SO plus 5% glycerol, which gave maximal stability, no catalytic activity was detected. Further kinetic studies showed that these cryoprotectants increased the affinity of the enzyme for ATP, but depressed its maximal velocity (H. Ishida, M. Mori, and M. Tatibana, unpublished observations).

When the concentrations of the solvents were decreased, the enzyme became markedly less stable. In 10% glycerol at 18° half the activity was lost in about 2 hr (Figure 3). The stability increased on addition of substrates and effectors, as shown in Figure 3; ATP, UTP, and PP-ribose-P all stabilized the enzyme but UTP was much more effective than ATP and the effects of ATP and PP-ribose-P were additive. ATP and UTP have been reported to stabilize the enzyme from other sources (Hager and Jones, 1967a; Levine et al., 1971; Tatibana and Shigesada, 1972b).

Approximation of Conditions for Sucrose Gradient Centrifugation and Enzyme Assay. The conformation of CPSase II seems to change significantly in response to changes in environmental conditions, as described above. Thus, to correlate the kinetic and molecular properties of the enzyme, these should be measured under identical or very similar conditions. However, the enzyme is unstable under the standard conditions used for its assay and, on the other hand, when the enzyme is maximally stabilized, i.e. in the presence of high concentrations of cryoprotectants, its catalytic activity and sensitivity to allosteric effectors are considerably decreased. Thus, as a compromise between the conditions giving high activity and high stability of the enzyme, we usually carried out the centrifugation at 18° using 10% glycerol as the basic medium. Although the enzyme is not very stable under these conditions (Figure 3), the presence of the substrate, ATP, as well as the effectors, UTP and PP-ribose-P, which were present in the gradient in most experiments, had a considerable stabilizing effect, as described above.

Like the enzymes from other sources (Williams et al., 1970; Tatibana and Shigesada, 1972b), CPSase II from rat liver is cold labile when the concentration of cryoprotectants is low. With 10% glycerol the stability was higher at 18° than at 0 or 37°. Furthermore, at 18°, the enzyme activity was 26% of that at 37° and the sensitivity to allosteric effectors was qualitatively the same as that at 37° (Figure 4 and Table II). On the other hand, the activity at 4° was only 1.5% of that at 37°. (Arrhenius plots for the reaction had a discontinuity of slope at about 20° with a downward bend. The activation energies at higher and lower temperatures were calculated to be 9160 and 31,000 cal, respective-

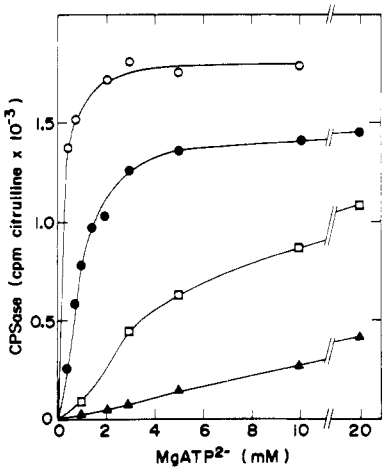

FIGURE 4: Effect of ATP concentration on inhibition by UTP and activation by PP-ribose-P of CPSase II activity at 18°. CPSase II activity was assayed in system B with the following additions: none (●-●); 0.3 m*M* UTP (□-□); 2.0 m*M* UTP (▲-▲); 50 μM PP-ribose-P (○-○); enzyme: 0.20 unit, 19 μg of protein.

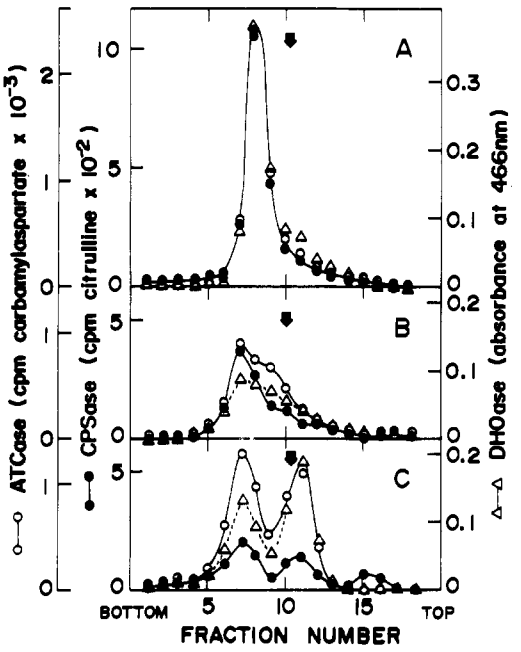

FIGURE 5: Effect of ATP on sedimentation of enzymes in 10% glycerol. Sucrose gradients (4.8 ml) contained 0.05 *M* Hepes (pH 7.2), 1 m*M* dithiothreitol, 10% glycerol, and (A) 10 m*M* ATP, (B) 1 m*M* ATP, or (C) no other compound. Then 0.12 ml of enzyme solution containing 1.2 units of CPSase II activity (102 μg of protein) and 135 μg of protocatechuate 3,4-dioxygenase was layered on top. Centrifugation was carried out for 2.5 hr. Fractions of 22 drops were collected and assayed for CPSase II (●-●), ATCase (○-○), and dihydroorotase (△- -△). CPSase II activity was assayed in system B except that both 3.3 m*M* glutamine and 30 m*M* NH_4Cl were used as nitrogen donors. Arrows indicate the position of the peak for protocatechuate 3,4-dioxygenase. The recoveries of CPSase II activity were 41, 28, and 18% in A, B, and C, respectively.

ly.) Based on these considerations, 10% glycerol and 10 m*M* ATP were included in a standard medium for centrifugation, and the operation was performed at 18°.

The enzyme sample used in centrifugation experiments contained 0.88 to 1.2 unit of CPSase II in a final volume of 0.04 to 0.20 ml per gradient. Assuming several-fold dilution during centrifugation, this enzyme concentration is comparable to that used in measurements of enzymatic activity (about 0.2 unit of CPSase II in 0.3 ml of assay mixture).

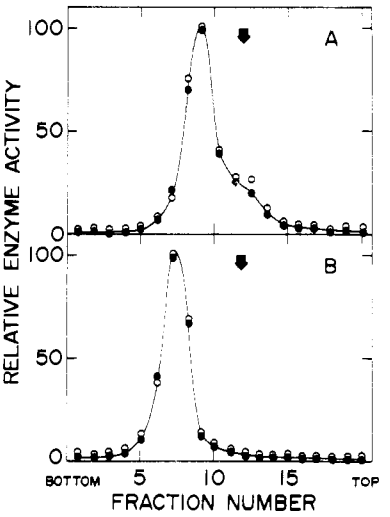

FIGURE 6: Effects of PP-ribose-P and UTP on sedimentation of the enzymes in 10% glycerol. Conditions were as for Figure 5, except that the gradient contained 50 μM PP-ribose-P (A) or 3 m*M* UTP (B): (●-●) CPSase II; (○-○) ATCase. CPSase II activity was assayed as for Figure 5. The recoveries of CPSase II activity were 54 and 72% in A and B, respectively.

Effects of ATP, UTP, and PP-Ribose-P on Sedimentation of the Enzymes. To test the possibility that allosteric inhibition and activation of CPSase II are associated with changes in the aggregation states of the enzyme complex, the effects of ATP, UTP, and PP-ribose-P on the sedimentation profile of the complex were studied. Upon sucrose gradient centrifugation in the presence of 10% glycerol and 10 m*M* ATP at 18°, the three enzymes sedimented in a single sharp peak with a sedimentation coefficient of 25 S (Figure 5A). When the concentration of ATP was reduced to 1 m*M*, which is close to the apparent K_m value for the CPSase II reaction, the peaks of the three activities became lower and less sharp with a shoulder of slower sedimenting enzymes (Figure 5B). Without ATP (Figure 5C) partial dissociation of the complex took place.

When 50 μM PP-ribose-P was added to the gradient in place of ATP, the three enzymes cosedimented in a single peak with a small shoulder (Figure 6A). Here, the sedimentation coefficient was 25–26 S, which is close to that of the peak with 10 m*M* ATP. With 3 m*M* UTP, an allosteric inhibitor, these enzymes also sedimented as a single sharp peak (Figure 6B). However, there was a slight increase in the sedimentation velocity: the coefficient in the presence of UTP was 28 S, which is slightly but definitely larger than that in the presence of ATP. The shift by UTP was visualized more clearly by the following experiments. As shown in Figure 7A, the three activities cosedimented in a single sharp peak with a coefficient of 25 S when 10 m*M* ATP was present (see also Figure 5A). On further addition of 0.3 m*M* UTP, which inhibits CPSase II activity by 40% when assayed in a similar system, all the enzymes sedimented in a broader peak extending from the 25S form to the 28S form, suggesting that interconversion of the two forms was in rapid equilibrium (Cann and Goad, 1968). On the other hand, in the presence of 10 m*M* ATP and 3 m*M* UTP, which inhibits CPSase II activity by over 90%, the activities again appeared in a sharp peak of 28 S, and the profile was similar to that in the presence of 3 m*M* UTP alone (Figure 6B). It is noteworthy that the concentration dependency of the UTP-induced transition was comparable to that of the inhibitory effects of UTP on catalytic activity. The small

Table III: Properties of CPSases *a*, *b*, and *c*.[a]

	Assay System	CPSase *a*	CPSase *b*	CPSase *c*	Second Hydroxylapatite Fraction
Sedimentation coefficient		26 S	19 S	9 S	
Association with ATCase and dihydroorotase		Yes	Probably no	No	
Ratio of activity with 0.2 m*M* glutamine to that with 40 m*M* NH_4Cl at pH 7.0	A	0.42	0.64	0.76	1.18
Apparent K_m for glutamine	A	17 μM	9 μM	n.d.	20 μM
Apparent K_m for ATP	B	5.1 m*M*	4.8 m*M*	n.d.	1.7 m*M*
Inhibition by 1 m*M* UTP[b]	B	67%	74%	65%	86% (95%[c])
Activation by 50 μM PP-ribose-P[b]	B	38%	124%	67%	53% (122%[c])

[a] CPSases *a*, *b*, and *c* were obtained by sucrose gradient centrifugation under the same conditions as for Figure 5C. Kinetic analyses were performed immediately after preparation of the enzymes. [b] Assayed with 3 m*M* ATP. [c] Assayed with 1 m*M* ATP; n.d., not determined.

difference in the sedimentation rate may reflect some conformational change and not association-dissociation of the enzyme complex; the inhibited form may have a more compact structure than the catalytically active one. In all the above experiments the peak of the marker enzyme, protocatechuate 3,4-dioxygenase, was sharp and localized at essentially the same position.

The results obtained indicate that the allosteric regulation of CPSase II activity is not accompanied by association and dissociation of the complex but by a conformational change of the complex as a whole under the conditions used. The finding of identical variations of the sedimentation rates of all three enzymes depending on the presence of ATP and UTP (Figures 5, 6, and 7) provides additional support for the specific association of these three enzymes.

When other CPSase II substrates were further added to the gradient there was no change in the sedimentation behavior of the complex; in the presence of all the substrates, i.e. ATP, $MgCl_2$, $KHCO_3$, and glutamine in addition to Hepes, dithiothreitol, and glycerol, at the same final concentrations as in the assay medium (system B), the sedimentation pattern was exactly the same as that with 10 m*M* ATP alone. It was calculated that only minor parts of the added substrates were converted to products during centrifugal analysis in this experiment. These observations indicate that the reactive form of CPSase II remains in the undissociated complex with the sedimentation coefficient of 25 S.

Dissociation of the Complex. As shown above, in the absence of ATP and with a low concentration of cryoprotectant (10% glycerol), partial dissociation of the complex occurred. CPSase II activity appeared in three different peaks with sedimentation coefficients of 26, 19, and 9 S, respectively (Figure 5C, see also Figure 10A). The fastest sedimenting form (CPSase *a*) always coincided exactly with ATCase and dihydroorotase, in sedimentation. However, this was not the case with the intermediate form (CPSase *b*): in 4 of 14 runs, the CPSase was recovered one fraction before the other two enzymes. Thus, this form of CPSase II is probably not associated with the other two enzymes. The amount of the slowest form (CPSase *c*) was always less than those of CPSases *a* and *b* and it was not accompanied by the other activities. The existence of these three distinct forms indicates that these forms are not in rapid equilibrium during centrifugation. ATCase and dihydroorotase sedimented in two peaks with coefficients of 26 and 18 to 19 S,

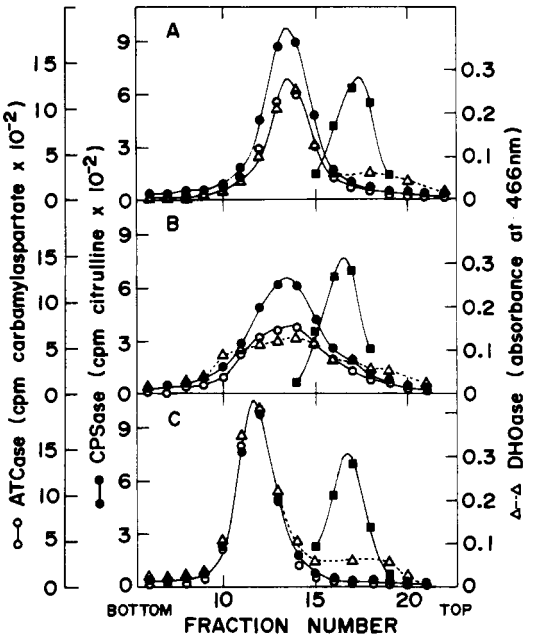

FIGURE 7: Effect of ATP plus UTP on the sedimentation velocity of the enzymes in 10% glycerol. Conditions were as for Figure 5, except that the gradient contained 10 m*M* ATP (A), 10 m*M* ATP plus 0.3 m*M* UTP (B), or 10 m*M* ATP plus 3 m*M* UTP (C). Fractions of 15 drops were collected: (●-●) CPSase II; (○-○) ATCase; (Δ- - -Δ) dihydroorotase; (■-■) protocatechuate 3,4-dioxygenase. CPSase II activity was assayed as for Figure 5. The recoveries of CPSase II activity were 40, 37, and 44% in A, B, and C, respectively.

respectively, but remained associated, suggesting that the association between ATCase and dihydroorotase may be tighter than that between CPSase II and ATCase and/or dihydroorotase.

Several other attempts to dissociate the complex into active components were unsuccessful. These included treatments with digitonin, Triton X-100, deoxycholate, urea, guanidine hydrochloride, and potassium thiocyanate. CPSase II was rapidly inactivated by all these treatments.

Properties of the Dissociated Forms of CPSase II. CPSases *a*, *b*, and *c* all utilized glutamine as well as ammonia as nitrogen donor, but the glutamine-dependent activities of CPSases *a* and *b*, assayed with 0.2 m*M* glutamine, were lower than their ammonia-dependent activities assayed with 40 m*M* NH_4Cl at pH 7.0 in system A (Table III). However, the apparent K_m values for glutamine of

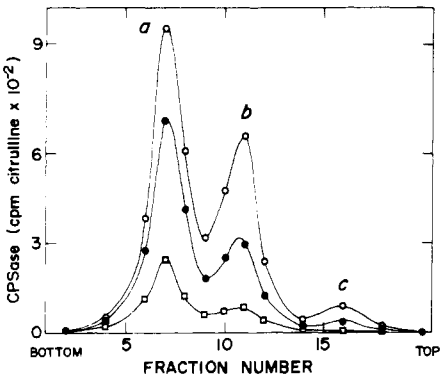

FIGURE 8: Inhibition by UTP and activation by PP-ribose-P of different forms of CPSase II. Centrifugation was performed as described for Figure 5C. Immediately after fractionation, CPSase II activity was assayed in system B, except for the use of 3 m*M* ATP and the following additions: none (●–●); 1 m*M* UTP (□–□); 50 μM PP-ribose-P (○–○).

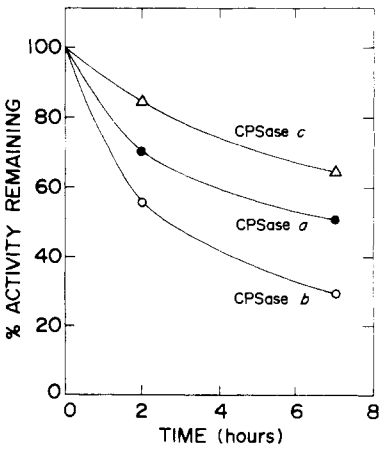

FIGURE 9: Stabilities of different forms of CPSase II at 18° in 10% glycerol. CPSases *a*, *b*, and *c* were prepared as described for Figure 5C and kept at 18°. Activity was assayed in system B at the indicated times after fractionation.

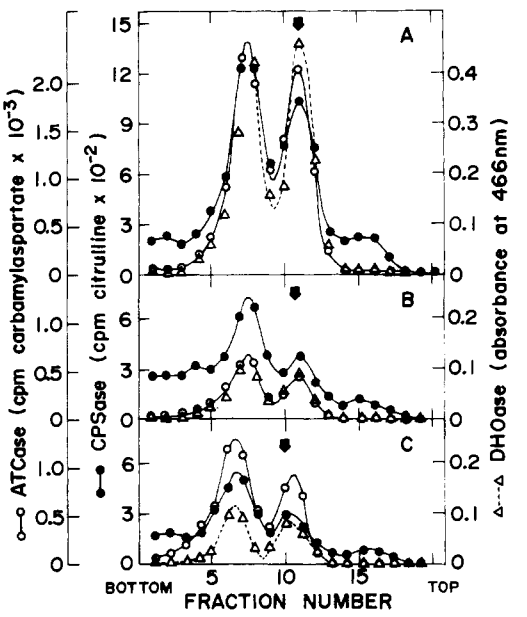

FIGURE 10: Recentrifugation of dissociated enzymes in the presence of ATP. Three portions of enzyme solution, each containing 2.02 units of CPSase II activity and 170 μg of protein, were centrifuged under identical conditions on three gradients as described for Figure 5C. One gradient was fractionated and assayed for CPSase II (●–●), ATCase (○–○), and dihydroorotase (△- - -△), as shown in the upper panel (A). The second gradient (B) was mixed, ATP was added to 10 m*M* concentration, and the whole mixture was concentrated to 0.2 ml at 18° in 2.5 hr in a collodion bag. The concentrated enzyme was recentrifuged for 2.5 hr on a sucrose gradient (4.8 ml) containing 10% glycerol and 10 m*M* ATP. The third gradient (C) was concentrated and recentrifuged in the same manner as for B except for omission of ATP. Fractions of 22 drops were collected and their activity was assayed. CPSase II activity was measured as described for Figure 5. The arrow indicates the position of the peak of protocatechuate 3,4-dioxygenase. The recovery of CPSase II activity in the first centrifugation was 19%, while those in B and C were 51 and 42%, respectively, of the activity subjected to the second analysis.

CPSases *a* and *b* (17 and 9 μM, respectively) were similar or even lower than that of the original enzyme (20 μM). In contrast, the apparent K_m values for ATP of CPSases *a* and *b* (5.1 and 4.8 m*M*, respectively) were about 3 times higher than that of the original enzyme (1.7 m*M*).

CPSases *a*, *b*, and *c* were all inhibited by UTP, but to a lesser extent than the original enzyme (Figure 8, Table III). PP-ribose-P activated CPSase *b* the most and CPSase *a* the least under the conditions used. It is noteworthy that CPSase *c*, which is not associated with the other enzymes and may be the protomeric form of the enzyme, was sensitive to both effectors.

The stabilities of CPSases *a*, *b*, and *c* in 10% glycerol at 18° are shown in Figure 9, although the exact stabilities of each form are uncertain because of the possible interconversion of the forms during the experiments. As shown, CPSase *c* was the most stable, while CPSase *b* was the least stable. However, the difference in stability was not great, so the sedimentation profiles obtained were probably not significantly modified by the different rates of inactivation of each form. As with the original enzyme 30% Me_2SO plus 5% glycerol were effective in stabilizing CPSases *a*, *b*, and *c*, but ATP and UTP were much less effective than with the original enzyme.

To analyze the effects of dissociation of the complex on the kinetic properties of ATCase, the apparent K_m values for carbamoyl-P of the different forms were studied. Those of the 26S form, the 18–19S form (Figure 5C), and the enzyme in the original complex were 6.9, 6.7, and 5.6 μM, respectively, showing that the forms were not discrete judging from their affinities for carbamoyl-P.

Failure to Reassociate the Components. The enzyme components dissociated by the first centrifugation (Figure 10A) were combined, concentrated by ultrafiltration, and recentrifuged in the presence of 10 m*M* ATP. The medium used for recentrifugation was the most favorable for keeping the complex intact. No tendency for reassociation of the components was observed (Figure 10B) and the profile was essentially the same as that before recentrifugation (Figure 10A). Figure 10C shows that the activity profile when the second centrifugation was performed in the absence of added ATP was very similar to that in Figure 10B. Reassociation was also not observed when recentrifugation was performed in a gradient containing 30% Me_2SO and 5% glycerol.

Discussion

CPSase II of rat liver exists as a multienzyme complex with the second and third enzymes of pyrimidine biosynthesis. The evidence for this presented here was: (1) simultaneous purification of the three enzymes through all the steps leading to 2100-fold purification of CPSase II (Table I); (2) cosedimentation of these enzymes in a sucrose gradient (Figure 2); (3) UTP, an allosteric inhibitor of CPSase

II, caused a shift in the sedimentation velocity and again the behaviors of the other two enzymes exactly coincided with that of CPSase II (Figures 6 and 7). Additional evidence reported previously (Mori and Tatibana, 1973) was that upon digestion of the preparation with elastase, the complex was dissociated into individual enzymes without significant inactivation of CPSase II or dihydroorotase. The existence of similar complexes of the initial enzymes of the pyrimidine pathway in a number of biological systems has been reported (Lue and Kaplan, 1969; Williams et al., 1970; Hoogenraad et al., 1971; Shoaf and Jones, 1971; Ito and Uchino, 1972).

Sedimentation analyses performed under carefully controlled conditions showed that allosteric regulation of CPSase II activity does not involve association and dissociation of the complex, but is related to changes in the conformation of CPSase II and possibly of other enzymes remaining in the aggregated state. It is remarkable that UTP caused a shift in the sedimentation velocity of the complex from 25 to 28 S. This difference of 3 S could be due to alteration in molecular shape, rather than in size. The CPSases in yeast (Lue and Kaplan, 1969) and *Neurospora* (Williams et al., 1970) are both present as complexes with ATCase and are also subject to feedback inhibition by UTP, but the nucleotide exerts different effects on the aggregation states of these two complexes. UTP promotes aggregation of the yeast complex (Lue and Kaplan, 1969), whereas it favors dissociation of the *Neurospora* complex (Williams et al., 1970). Thus, in this respect, the mammalian complex, reported here, is different from either of these two microbial systems. It is uncertain whether these different behaviors are due to different molecular properties of the enzymes or to different experimental conditions. In view of the labile and changeable nature of the complex, analyses should be carried out under standardized conditions.

When the concentration of cryoprotectant is reduced, the enzyme complex undergoes partial dissociation with formation of CPSases *a, b,* and *c*. Assuming that all these forms are spherical in shape and have partial specific volumes similar to that of protocatechuate 3,4-dioxygenase (mol wt 700,000), their molecular weights are calculated to be 1,090,000, 680,000, and 220,000, respectively. Of these three forms, CPSase *a* may have an altered conformation of the original complex, since it apparently differs from the original enzyme with respect to its apparent K_m for ATP and stability. On the other hand, CPSase *c* (9 S) may represent the protomeric form of CPSase II, since its sedimentation coefficient is close to those of CPSase of *Escherichia coli* (Anderson and Marvin, 1970) and the CPSases I of frog liver (Marshall et al., 1961) and rat liver (Guthöhrlein and Knappe, 1968), which were reported to be 8.5, 11.2, and 10.9 S, respectively.

Previous reports from this (Inagaki and Tatibana, 1970) and other laboratories (Oliver et al., 1969) showed that mammalian ATCase could be fractionated into several forms. From the data presented here and by Shoaf and Jones (1973), it is conceivable that dissociation of the enzyme complex during the fractionation procedure might be responsible, in part at least, for the observed multiple forms of the ATCase.

Generally, a multienzyme complex may have special significance in (a) keeping the component enzymes in a suitable conformation for proper catalytic and regulatory functions, (b) facilitating a concerted response of the enzymes to a regulatory signal directed to an enzyme (Lue and Kaplan, 1969), (c) stabilizing the enzymes involved, or (d) "channeling" a product of an enzyme to the next enzyme in a pathway (Lue and Kaplan, 1970). From our studies (this paper and Mori and Tatibana, 1973), it seems that hepatic CPSase II in the complex exhibits properties compatible with possibility a. The dissociated enzyme, obtained by elastase treatment, had a lower affinity for ATP than the associated enzyme. Furthermore, the dissociated enzyme showed sensitivities to effectors different from those of the enzyme within the complex, and did not have the cooperativity with regard to ATP of the "complex" enzyme. We studied possibility b, using the enzyme from mouse spleen, but were not able to show that substrates and effectors for CPSase II, i.e. ATP, Mg^{2+}, glutamine, bicarbonate, and UTP, significantly affected the ATCase activity (M. Tatibana and K. Shigesada, unpublished observation). With regard to possibility c, as far as stability in the presence of 30% Me_2SO and 5% glycerol is concerned, there is no significant difference between the dissociated and the "complex" enzymes.

The liver is unique among mammalian tissues in that it contains two different enzymes responsible for the synthesis of carbamoyl-P, i.e. CPSase I and CPSase II. Our preliminary experiments showed that the two enzymes are present in a single type of cells, hepatocytes (M. Oguchi and M. Tatibana, unpublished observation). Although the subcellular localizations of these two enzymes are different, this does not necessarily mean that the two carbamoyl-P pools, one in the mitochondria and the other in the cytosol, are completely segregated. It is possible that CPSase I, which has nearly 1000 times more total activity than CPSase II in the liver, may "interfere" with the pyrimidine pathway due to "leakage" from the mitochondria of carbamoyl-P produced by CPSase I. Thus, it is possible that the initial enzymes of the pyrimidine pathway of the liver have some organ-specific properties which assure control of the pathway. Although so far we have failed to show "channeling" of carbamoyl-P within the complex (possibility d discussed above) in studies in vitro, the complex does show organ specificity: the CPSases II of rat liver and mouse spleen show significant differences in their affinities for ATP and quantitative sensitivities to allosteric effectors (Table II). These differences appear to represent tissue specificity rather than species specificity, since the apparent K_m values for ATP of the rat spleen enzyme and the mouse spleen enzyme are the same (M. Mori and M. Tatibana, unpublished observation). It remains to be elucidated whether these differences are due to differences in the structures of the CPSases II or in the interactions between the components of the complexes.

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