Biochimica et Biophysica Acta, 483 (1977) 90—99

BBA 68166

GLUTAMINASE ACTIVITY OF GLUTAMINE-DEPENDENT CARBAMOYL-PHOSPHATE SYNTHASE FROM RAT ASCITES HEPATOMA

REGULATION BY ADENOSINE TRIPHOSPHATE-MAGNESIUM AND MAGNESIUM ION

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(Received December 20th, 1976)

Summary

Glutamine-dependent carbamoyl-phosphate synthase (EC 2.7.2.9) from rat ascites hepatoma cells (AH 13), the first enzyme of de novo pyrimidine nucleotide biosynthesis, catalyzed the hydrolysis of L-glutamine in the absence of the other substrates at 5.9% the rate of glutamine hydrolysis in the complete system. The activity was stimulated 5.1-fold by 10 mM ATP plus 15 mM $MgCl_2$; 2.1-fold by 5 mM Mg^{2+}, an essential activator of the enzyme, and 2.4-fold by 10 mM $MgATP^{2-}$, a substrate. The apparent activation constants for Mg^{2+} and $MgATP^{2-}$ were 0.5 and 1.6 mM, respectively. $MgATP^{2-}$ plus Mg^{2+} had little effect on the apparent K_m for glutamine; the values in the absence and presence of 10 mM ATP plus 15 mM $MgCl_2$ were 21 and 27 μM, respectively. Experiments with [γ-^{32}P]ATP showed that $MgATP^{2-}$-stimulated glutamine hydrolysis was associated with little or no cleavage of ATP. Adenylyl imidodiphosphate-magnesium could not replace $MgATP^{2-}$. However, the addition of adenylyl imidodiphosphate-magnesium and bicarbonate stimulated the activity 1.8-fold; bicarbonate alone was not effective. Coordination of glutamine hydrolysis and carbamoyl-phosphate synthesis controlled by $MgATP^{2-}$, Mg^{2+} and probably by bicarbonate is suggested. Effects of the reaction products, enzyme modifiers and related compounds on the glutaminase activity are also described.

Introduction

Glutamine-dependent carbamoyl-phosphate synthase (EC 2.7.2.9) of higher animals catalyzes the first step of de novo pyrimidine biosynthesis and plays a

Abbreviations: carbamoyl-*P*, carbamoyl-phosphate; *PP*-ribose-*P*, 5-phosphoribosyl 1-pyrophosphate; ATPase, adenosine triphosphatase.

key role in the regulation of the pathway [1—10]. Previous studies in this laboratory [3] using a partially purified enzyme preparation from hematopoietic mouse spleen showed that it catalyzes the following reaction:

2 ATP + L-glutamine + HCO_3^- + H_2O
→ 2 ADP + carbamoyl-*P* + P_i + L-glutamate

The equation is the same as has been proposed for the enzyme of *Escherichia coli* [11]. In either systems, glutamine can be replaced by a high concentration of ammonia. Meister and coworkers [12,13] showed that the enzyme of *E. coli* catalyzed the hydrolysis of glutamine as a partial reaction of the overall process of carbamoyl-*P* synthesis. They also demonstrated that glutamine hydrolysis and ammonia-dependent carbamoyl-*P* synthesis occurred at distinct sites on the different subunits of the enzyme [13,14]. Although little information is available on the animal enzymes, probably much the same mechanism is involved in view of the similarity of the reactions catalyzed by the bacterial and mammalian enzymes.

It was found that the enzyme partially purified from mouse spleen catalyzed the hydrolysis of glutamine even in the obscure of exogenously added bicarbonate at about 50% of the rate in the complete system [3]. However, the significance of the observation was obscure because the enzyme used was not pure. Recently, we succeeded in purifying the synthetase of ascites hepatoma cells to a homogenous state as a multienzyme complex with the second and third enzymes of the pathway [15]; the existence of the synthetase as a multienzyme complex in animal tissues was first reported by Hoogenraad et al. [16] in mouse spleen and by Shoaf and Jones [17] in Ehrlich ascites carcinoma. This enabled a study of the mechanism of coupling between glutamine hydrolysis and carbamoyl-*P* synthesis.

This communication deals with the glutaminase activity of homogeneous carbamoyl-phosphate synthase II from ascites hepatoma cells and its enhancement by $MgATP^{2-}$ and Mg^{2+}, and in the presence of a $MgATP^{2-}$ analogue, by bicarbonate. Effects of reaction products, enzyme modifiers and related compounds on the activity are also described.

Materials and Methods

Materials. ATP and *PP*-ribose-*P* were obtained from Kyowa Hakko Kogyo, Tokyo, UTP from Boehringer Mannheim and adenylyl imidodiphosphate from Sigma Chemical Co. Concentrations of ATP and $MgCl_2$ were determined as described previously [18]. *N*-(Phosphonacetyl)-L-aspartate was synthesized according to the method of Swyryd et al. [19]. [^{14}C]Glutamine (40 Ci/mol) was obtained from the Radiochemical Centre, Amersham, and purified by paper electrophoresis at pH 6.0 in 0.1 M pyridine acetate buffer to remove [^{14}C]glutamate and [^{14}C]pyrrolidone carboxylic acid which interfere with the glutaminase assay. [γ-^{32}P]ATP, which was prepared according to the method of Glynn and Chappel [20], was a gift from Dr. T. Hisata of this laboratory. Ornithine carbamoyltransferase (EC 2.1.3.3) was purified from buvine liver according to the method of Marshall and Cohen [21].

Enzyme purification. Carbamoyl-phosphate synthase II was purified to an essentially homogeneous state from rat ascites hepatome cells (AH 13) as a complex with aspartate carbamoyltransferase (EC 2.1.3.2) and dihydro-orotase (EC 3.5.2.3) as described previously [15]. The purified enzyme had a specific activity of about 0.5 unit (μmol citrulline formed in 1 min) per mg of protein under the standard conditions [18].

Enzyme assays. Glutaminase activity was assayed in a system (final volume, 50 μl) containing 50 mM potassium *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonate (pH 7.0), 20 μM L-[^{14}C]glutamine (40 000 cpm/nmol), 10% (w/v) glycerol, 1 mM dithiothreitol and enzyme. The test tubes containing the reaction mixture were flushed with nitrogen, stoppered and incubated for 15 min at 37°C. The reaction was stopped by the addition of 100 μl of 50% (v/v) ethanol containing 10 mM EDTA, 0.2 mM L-glutamine and 0.2 mM L-glutamate. The [^{14}C]glutamate formed was separated from [^{14}C]glutamine by paper electrophoresis in 0.1 M pyridine acetate buffer (pH 6.0) on Whatman 3MM paper at 15 V/cm for 45 min. The areas of the paper containing glutamine and glutamate were identified by lightly spraying the dried paper with a 0.05% solution of ninhydrin in acetone; the paper was cut into sections and the radioactivity was determined in a Beckman scintillation spectrometer.

ATPase activity was assayed with [γ-^{32}P]ATP as a substrate by following the release of $^{32}P_i$. After the reaction, unreacted ATP was removed by treatment with activated charcoal, and the unadsorbed radioactivity that represents $^{32}P_i$ was counted. Details are included in the legend to Table II. The overall reaction of carbamoyl-*P* synthesis was measured with [^{14}C]bicarbonate as a substrate by following the formation of [^{14}C]citrulline in the presence of excess amounts of L-ornithine and ornithine carbamoyltransferase as described previously [18].

Results

Enhancement of glutaminase activity by Mg^{2+}, $MgATP^{2-}$ and adenylyl imidodiphosphate-Mg plus bicarbonate

Carbamoyl-phosphate synthase II purified to homogeneity from rat ascites hepatoma cells catalyzed the hydrolysis of L-glutamine in the absence of the other substrates at 5.9% of the rate of glutamine hydrolysis in the complete system for carbamoyl-*P* synthesis (Table I). The hydrolysis proceeded linearly with time up to at least 30 min. In order to elucidate the mechanism underlying the coupling of glutamine hydrolysis and carbamoyl-*P* synthesis, effects of the substrates on the glutamine hydrolysis were examined. $KHCO_3$ at 20 mM affected only slightly the basal glutaminase activity. However, the activity was stimulated 2.1-fold by the addition of 5 mM $MgCl_2$. Free Mg^{2+} is required for carbamoyl-*P* synthesis as an essential activator in addition to a substrate $MgATP^{2-}$ [18]. The activity was stimulated 5.1-fold by 10 mM ATP plus 15 mM $MgCl_2$ *; the extent of activation by 10 mM $MgATP^{2-}$ was calculated to be 2.4-fold. ATP alone slightly inhibited the activity. The stimulation of the glu-

* In the presence of 10 mM ATP and 15 mM $MgCl_2$, the amount of free ATP would be insignificant because of the high stability constant for $MgATP^{2-}$ complex and the respective concentrations of $MgATP^{2-}$ and free Mg^{2+} would be approx. 10 and 5 mM (see ref. 18).

TABLE I

EFFECT OF CARBAMOYL-PHOSPHATE SYNTHASE II SUBSTRATES ON GLUTAMINE HYDROLYSIS

The reaction mixture (50 μl) contained 50 mM potassium *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonate (pH 7.0), 20 μM [^{14}C]glutamine (40 000 cpm/nmol), 10% (w/v) glycerol, 1 mM dithiothreitol, 0.01 (A) and 0.02 (B) munit of enzyme (0.02 and 0.04 μg of protein, respectively) and the additions indicated. Incubation and the following procedure are described in the text. AMP-PNP, adenylyl imidodiphosphate.

Expt.	Additions	Glutamate formed	
		pmol	percent *
A	None	3.7	5.9
	$KHCO_3$ (20 mM)	4.3	6.8
	ATP (10 mM)	3.1	4.9
	$MgCl_2$ (5 mM)	7.9	12.5
	ATP (10 mM), $MgCl_2$ (15 mM)	18.9	30.0
	AMP-PNP (10 mM), $MgCl_2$ (15 mM)	7.3	11.6
	ATP (10 mM), $MgCl_2$ (15 mM), $KHCO_3$ (20 mM)	63.0	100
	AMP-PNP (10 mM), $MgCl_2$ (15 mM), $KHCO_3$ (20 mM)	12.9	20.5
B	None	8.4	
	$KHCO_3$ (20 mM)	9.0	
	$MgCl_2$ (5 mM)	17.7	
	$MgCl_2$ (5 mM), $KHCO_3$ (20 mM)	18.2	
	AMP-PNP (10 mM), $MgCl_2$ (15 mM), $KHCO_3$ (20 mM)	26.6	

* Expressed as percentage of the value in the presence of 10 mM ATP, 15 mM $MgCl_2$ and 20 mM $KHCO_3$.

taminase activity by $MgATP^{2-}$ was observed even when the reaction was conducted in a system at pH 6.5, previously "degassed" by evacuation. Therefore, it is unlikely that $MgATP^{2-}$-stimulated glutamine hydrolysis took place by the formation of carbamoyl-*P* with the endogenous bicarbonate remaining in the reaction mixture. This was unequivocally demonstrated by the lack of stimulation of ATP hydrolysis by glutamine (see below).

Adenylyl imidodiphosphate-Mg, which is an analogue of $MgATP^{2-}$ [22] and was shown to be a competitive inhibitor of the AH 13 enzyme (Mori, M. and Tatibana, M., unpublished) could not replace $MgATP^{2-}$. However, the addition of bicarbonate (20 mM) caused a 1.8-fold increase in the activity over that in the presence of adenylyl imidodiphosphate and $MgCl_2$; bicarbonate plus Mg^{2+} had little effect (Table I).

The effect of varying concentrations of Mg^{2+} on the glutaminase activity is shown in Fig. 1. The maximal activation was observed at 5 mM Mg^{2+} and the concentration required to give a half maximal activation (apparent K_a value) was calculated to be 0.5 mM. Mg^{2+} at concentrations higher than 10 mM, to some extent, inhibited the activity. The profile in Fig. 1 is similar to that of the activation by free Mg^{2+} of carbamoyl-*P* synthesis by the enzymes from mouse spleen [18] and rat ascites hepatoma [9]. Mn^{2+} was also effective in stimulating the glutaminase activity; $MnCl_2$ at 1 and 5 mM stimulated the activity 1.8- and 1.6-fold, respectively. The velocity of glutamine hydrolysis plotted against varying concentrations of $MgATP^{2-}$ gave a kinetics which was slightly sigmoidal

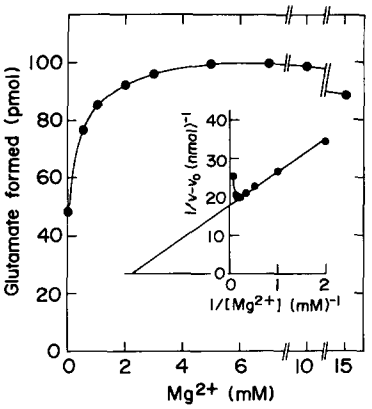

Fig. 1. Effect of varying concentrations of free Mg^{2+} on glutaminase activity. The enzyme (0.12 munit, 0.24 μg of protein) was assayed as described in Table I except for the addition of varying concentrations of $MgCl_2$. The inset shows the data plotted in the double reciprocal form; v_0 is the velocity in the absence of $MgCl_2$.

at low concentrations of $MgATP^{2-}$ (Fig. 2). However, the double reciprocal plots were essentially linear at higher $MgATP^{2-}$ concentrations and the apparent K_a value calculated from the linear portion was 1.6 mM.

The apparent K_m values for L-glutamine in the absence and presence of 10 mM ATP plus 15 mM $MgCl_2$ were 21 and 27 μM, respectively; these values were close to the apparent K_m value of the enzyme for glutamine in carbamoyl-*P* synthesis assay (21 μM). The result indicates that $MgATP^{2-}$ and Mg^{2+} stimulate the glutaminase activity by increasing the maximal velocity of the enzyme without significant effect on the affinity for glutamine.

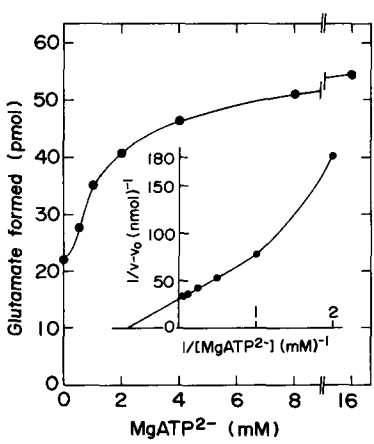

Fig. 2. Effect of varying concentrations of $MgATP^{2-}$ on glutaminase activity. The enzyme (0.03 munit, 0.06 μg of protein) was assayed as described in Table I except for the addition of varying concentrations of $MgATP^{2-}$. $MgCl_2$ was added in 5 mM excess over ATP; the control test tube without $MgATP^{2-}$ contained 5 mM $MgCl_2$. The inset shows the data plotted in the double reciprocal form; v_0 is the velocity in the absence of $MgATP^{2-}$.

Hydrolysis of ATP

We also attempted to determine whether $MgATP^{2-}$-stimulated glutamine hydrolysis is associated with the hydrolysis of ATP and the release of $^{32}P_i$ from [γ-^{32}P]ATP under the conditions for glutaminase assay was measured. The enzyme catalyzed the hydrolysis of ATP in the absence of the other substrates at 23% of the rate of ATP hydrolysis in the complete system for carbamoyl-*P* synthesis (Table II). The ATPase activity was enhanced by about 30% on addition of 20 mM bicarbonate. To be noted is that the apparent K_m for $MgATP^{2-}$ in bicarbonate-stimulated ATP hydrolysis was 0.21 mM, which was eight times lower than that in the overall carbamoyl-*P* synthesis reaction (1.7 mM). It has also been reported that the apparent K_m for $MgATP^{2-}$ in bicarbonate-dependent ATPase activity on the *E. coli* enzyme was an order of magnitude lower than that in the carbamoyl-*P* synthesis reaction [23]. The bicarbonate-dependent ATPase activity might be considered as a partial reaction of carbamoyl-*P* synthesis, by analogy with carbamoyl-phosphate synthase II of *E. coli* [23] and ammonia-dependent carbamoyl-phosphate synthase (EC 2.7.2.5) from log liver [24] and rat liver [25].

On the other hand, glutamine did not markedly increase the hydrolysis of ATP under the conditions where $MgATP^{2-}$-stimulated glutamine hydrolysis was expected to take place (Table II). The slight increase in the rate of ATP hydrolysis by glutamine (0.021 nmol/min, Table II), if significant at all, was far less than the increase in the rate of glutamine hydrolysis brought about by $MgATP^{2-}$ which was calculated to be about 0.10 nmol/min under the conditions. Thus, it can be concluded that the $MgATP^{2-}$-stimulated hydrolysis of glutamine was not coupled with cleavage of ATP.

TABLE II

EFFECTS OF CARBAMOYL-PHOSPHATE SYNTHETASE II SUBSTRATES ON ATP HYDROLYSIS

The reaction mixture (100 μl) contained 50 mM potassium *N*-hydroxyethylpiperazine-*N'*-2-ethane sulfonate (pH 7.0), 10 mM [γ-^{32}P]ATP (1290 cpm/nmol), 15 mM $MgCl_2$, 10% (w/v) glycerol, 1 mM dithiothreitol, 0.5 mM L-ornithine, 4 units (μmol/min at 37°C) of ornithine carbamoyltransferase free from ammonia, 0.6 munit of enzyme (1.2 μg of protein) and the additions indicated. After incubation for 10 or 20 min at 37°C, the reaction was stopped by the addition of 0.9 ml of 0.1 M HCl containing 10 μmol of inorganic phosphate. The mixture was applied on a column of charcoal (25 mg) which was then washed with 1 ml of water. The effluent and washings were combined, to it was added 10 ml of Bray's schintillation fluid and radioactivity was measured. The amounts of $^{32}P_i$ present in the test tube without enzyme at 10 and 20 min were 3660 and 3890 cpm, respectively. These values were subtracted from all of the values of P_i release.

Additions	Time (min)	P_i released (nmol)
None	10	2.64
	20	5.50
$KHCO_3$ (20 mM)	10	3.41
	20	7.50
Glutamine (0.2 mM)	10	2.84
	20	5.93
Glutamine (0.2 mM), $KHCO_3$ (20 mM)	10	11.2
	20	23.0

Effects of substrates, products, effectors and related compounds of the enzyme system on glutaminase activity

The effects of various compounds on the glutaminase activity were examined under the following three conditions: in the absence (A) and presence (B) of $MgATP^{2-}$, and in the presence of $MgATP^{2-}$ and bicarbonate (C), and the results are summarized in Table III together with the effects of these compounds on the overall reaction of carbamoyl-*P* synthesis. $MgUTP^{2-}$, which strongly inhibited carbamoyl-*P* synthesis [1,7,8,26], had little effect on the glutaminase activity either in System A or B. The extent of inhibition in System C was much less than that of carbamoyl-*P* synthesis, indicating the occurrence of some uncoupling of glutamine hydrolysis and carbamoyl-*P* synthesis in the presence of $MgUTP^{2-}$. *PP*-ribose-*P*, an allosteric activator of the enzyme [8,26], was also ineffective on the glutaminase activity in System A or B.

L-glutamate, a reaction product of glutamine hydrolysis, inhibited the glutaminase activity moderately in Systems A and B, while the inhibition was much less marked in System C. Ammonia, an alternative substrate whose binding site is thought to be different from the binding site of glutamine in analogy with the case of *E. coli* enzyme [13,14], was only slightly inhibitory. It should be noted that carbamoyl-*P* which does not appear to be directly related with the glutaminase reaction, inhibited the latter activity.

The enzyme of AH 13 cells [15] as well as the enzymes from other animal

TABLE III

EFFECT OF VARIOUS COMPOUNDS ON GLUTAMINE HYDROLYSIS AND CARBAMOYL-*P* SYNTHESIS

Glutamine hydrolysis was measured as described in Table I in the absence (A) and presence (B) of 10 mM ATP and 15 mM $MgCl_2$, and in the presence of 10 mM ATP, 15 mM $MgCl_2$ and 20 mM $KHCO_3$ (C). Carbamoyl-*P* synthesis was measured in a system (300 μl) which contained 50 mM potassium *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonate (pH 7.0), 10 mM ATP, 15 mM $MgCl_2$, 3.3 mM L-glutamine, 16.7 mM $KH^{14}CO_3$ (2000 cpm/nmol), 0.5 mM L-ornithine, 4 units of bovine liver ornithine carbamoyltransferase free from ammonia, 10% (w/v) glycerol, 1 mM dithiothreitol, enzyme and the additions indicated. Enzyme: A, 0.15 munit; B, 0.06 munit; C, 0.03 munit; carbamoyl-*P* synthesis assay, 0.05—0.2 unit. The values of glutamine hydrolysis and carbamoyl-*P* synthesis are expressed as percentage of the control values.

Additions	Glutaminase activity			Carbamoyl-*P* synthesis
	A	B	C (percent of control)	
None	100	100	100	100
$MgUTP^{2-}$ (6 mM)	102	112	49	8
PP-ribose-*P* (1 mM)	95	90	130	131
L-Glutamate (10 mM)	53	46	93	
NH_4Cl (10 mM)	72	76	78	
Carbamoyl-*P* (4 mM)	58			
L-Aspartate (10 mM)	29			80 *
N-(Phosphonacetyl)-L-aspartate (0.2 mM)	106			106
DL-Carbamoyl-aspartate (4 mM)	66			
Dihydro-orotate (4 mM)	93			
Glycine (10 mM)	23	23	25	32 *
Alanine (10 mM)	59			61 *

* Glutamine concentration was reduced to 20 μM.

systems [16,17,26—28] exists as a multienzyme complex with aspartate carbamoyltransferase and dihydro-orotase. To determine whether or not there is an interaction between the glutamine site of the synthetase and the second and third enzymes, the effects of substrates and products of the latter two enzymes were examined. Of the compounds tested aspartate strongly inhibited the glutaminase activity. However, it is unlikely that the binding of aspartate to aspartate carbamoyltransferase caused the inhibition of the glutaminase activity, because aspartate is known to bind the enzyme only in the presence of carbamoyl-*P*, another substrate of the enzyme [29]. Furthermore, *N*-(phosphoacetyl)-L-aspartate, a potent transition state analogue inhibitor of the enzyme [19], was not effective. Carbamoyl aspartate was also moderately inhibitory, but the mechanism is not known.

Glycine at 10 mM was found to inhibit to glutaminase activity by 75—77% in all the systems (Table III). Glycine inhibited glutamine-dependent carbamoyl-*P* synthesis to almost the same extent (68%). The inhibition was competitive with respect to glutamine; the apparent K_i was 3.5 mM in the glutamine-dependent carbamoyl-*P* synthesis reaction. On the other hand, this amino acid stimulated carbamoyl-*P* synthesis from ammonia. The extent of stimulation by 10 mM glycine was 1.37-fold with 5 mM NH_4Cl as a substrate and 1.16-fold with 40 mM NH_4Cl. Alanine also inhibited both glutamine hydrolysis and glutamine-dependent carbamoyl-*P* synthesis, though to an extent much less than that by glycine. These results strongly suggest that glutamine and ammonia bind to distinct sites on the enzyme and that glutaminase activity of the enzyme is a reflection of the normal mechanism for utilization of glutamine in overall carbamoyl-*P* synthesis.

Discussion

Most glutamine amidotransferase have the capacity to utilize ammonia in addition to glutamine as the nitrogen donor. The essential feature is that glutamine and ammonia bind to distinct sites which may be on the same polypeptide chain [30,31] or on non-identical chains of the oligomeric enzymes [13,14, 32—35]. Carbamoyl-*P* synthetase of *E. coli* was shown to be composed of a light subunit (molecular weight, about 42 000) and a heavy subunit (molecular weight, about 130 000); the light subunit has the binding site for glutamine and catalyzes the hydrolysis of glutamine, and the heavy subunit catalyzes carbamoyl-*P* synthesis from ammonia but not from glutamine [13,14]. In contrast, the enzyme from AH 13 cells exists as a complex with the second and third enzymes of the pyrimidine pathway and the complex (molecular weight, about 870 000) is composed of 4—5 subunits of similar size (molecular weight, about 210 000) [15]. Our preliminary experiments suggested that both glutamine site and the sites responsible for carbamoyl-*P* synthesis from ammonia exist on the same polypeptide chain (Mori, M. and Tatibana, M., unpublished), and the present data indicate that the interaction exists between the glutamine binding site and the Mg^{2+} and $MgATP^{2-}$-binding sites on the enzyme. The hydrolysis of glutamine was almost completely suppressed in the absence of the other substrates and was enhanced by the addition of Mg^{2+} and $MgATP^{2-}$ (Table I). The binding of Mg^{2+} and $MgATP^{2-}$ to their binding sites may produce a conforma-

tional change in the protein molecule to activate the glutamine site in such a way that glutamine hydrolysis is catalyzed more efficiently. $MgATP^{2-}$-induced conformational change was suggested by our previous observation [26] that $MgATP^{2-}$ stabilized the enzyme and protected the enzyme complex from partial dissociation.

The overall reaction of carbamoyl-*P* synthesis requires 2 mol of ATP; 1 mol is required for the activation of CO_2 and the other for the formation of carbamoyl-*P* from carbamate. Presumably these two partial reactions occur at two distinct $MgATP^{2-}$-binding sites on the enzyme (Sites I and II) [23]. The apparent K_m values for $MgATP^{2-}$ of Sites I and II are 0.21 and 1.7 mM, respectively. The apparent K_a for $MgATP^{2-}$ in $MgATP^{2-}$-stimulated glutamine hydrolysis (1.6 mM) is close to the K_m value of Site II. However, it is unlikely that $MgATP^{2-}$ stimulates the glutaminase activity by binding to Site II, since $MgUTP^{2-}$ and *PP*-ribose-*P* which affect the carbamoyl-*P* synthetase activity by altering the affinity for $MgATP^{2-}$ of Site II [8,26], had little effect on $MgATP^{2-}$ stimulation of glutamine hydrolysis. Therefore, there seems to be a "regulatory" site for $MgATP^{2-}$ which is distinct from the two substrate sites for $MgATP^{2-}$. Adenylyl imidodiphosphate-Mg may have little affinity for this "regulatory" site. The experiments with [γ-^{32}P]ATP showed that $MgATP^{2-}$-stimulated glutamine hydrolysis was associated with little or no cleavage of ATP, suggesting that the binding of $MgATP^{2-}$ to its site may be sufficient for the stimulation of glutamine hydrolysis. However, Anderson et al. [12] showed that the hydrolysis of γ-glutamyl hydroxamate, a reaction related to glutamine hydrolysis, by the *E. coli* enzyme was activated by ATP, Mg^{2+} and bicarbonate, and that during the course of this reaction some ATP was cleaved. In view of this fact it is also possible that some, but not necessarily stoichiometric, cleavage of ATP is required for the stimulation of glutamine hydrolysis by $MgATP^{2-}$.

Glutamine hydrolysis and carbamoyl-*P* synthesis thus appear to be regulated in a coordinate manner by Mg^{2+}, $MgATP^{2-}$ and probably also by bicarbonate. Assuming that bicarbonate has the ability to stimulate the glutaminase activity 1.8-fold as suggested by the experiment with adenylyl imidodiphosphate-Mg (Table I), the effect, together with those by Mg^{2+} and $MgATP^{2-}$, can account for 53% of the rate of glutamine hydrolysis in the complete system for carbamoyl-*P* synthesis. In this respect it is noteworthy that the glutaminase activity of the *E. coli* enzyme is enhanced by alteration in subunit interactions [36,37]. It is also notable that the glutaminase activity of anthranilate synthetase (EC 4.1.3.27) from *Salmonella typhimurium* is stimulated by the substrate chorismate [32], that of CTP synthetase (EC 6.3.4.2) from *E. coli* by ATP analogues [30] and that of glutamate synthetase (EC 2.6.1.53) from *E. coli* by the substrates α-ketoglutarate and NADPH [35]. It appears that the glutaminase activity of glutamine amidotransferases may normally be present in a "latent" form in the absence of the other substrates and that the enhancement of the activity coupled with the synthetic reaction may occur through the interaction between glutamine site and the site or sites responsible for ammonia-dependent biosynthetic reactions.

Acknowledgements

Thanks are due to Miss M. Ohara for assistance with preparation of the manuscript. This work was supported in part by the Scientific Research Fund (D067020) from the Ministry of Education, Science and Culture, of Japan.

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