Binding of Adenosine Triphosphate and Adenosine Diphosphate by Glutamine Synthetase*

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ABSTRACT: The γ-glutamyl transfer activity of purified sheep brain glutamine synthetase is activated by low concentrations of either adenosine triphosphate (ATP) or adenosine diphosphate (ADP). Studies with ATP-γ-^{32}P indicate that cleavage of ATP to ADP is not responsible for the activating effect of ATP. Although a number of other nucleotides and related compounds do not activate the transfer reaction, deoxy-ADP, deoxy-ATP, and adenosine tetraphosphate produce activation. Both deoxy-ATP and adenosine tetraphosphate are also active in glutamine synthesis. The curves for activation of the γ-glutamyl transfer reaction by ADP and ATP are identical at low concentrations of nucleotide but diverge at higher concentrations of nucleotide, with ADP being a more effective activator. The concentrations of ATP and ADP required for maximal activation are proportional to enzyme concentration, suggesting that the nucleotides are tightly bound to the enzyme. Direct evidence for binding of ATP and ADP to glutamine synthetase was obtained in gel filtration experiments with columns of Sephadex. Binding of ATP to the enzyme is not associated with its cleavage to ADP, and the bound ATP is available for glutamine synthesis. Studies on activation of the γ-glutamyl transfer reaction by ATP and ADP and the gel filtration experiments indicate that about 16 moles of nucleotide are bound readily by the enzyme; the enzyme exhibits equal affinity for ATP and ADP at low concentrations of nucleotide. The enzyme binds about twice as much ADP (but not ATP) at higher concentrations of nucleotide. The data suggest that binding of the "readily bound" nucleotide to the enzyme decreases its affinity for additional nucleotide. A tentative model is proposed to explain these findings.

Glutamine synthetase catalyzes several reactions, all of which require adenine nucleotides either in stoichiometric or catalytic amounts (Meister, 1962). Earlier studies in this laboratory showed that adenosine triphosphate (ATP[1]) is required for the binding of glutamate by glutamine synthetase, and that adenosine diphosphate (ADP) is needed for binding of glutamine. These findings and other considerations led to the conclusion that the enzyme does not have a complete binding site for amino acids until either ATP or ADP combines with the enzyme (Krishnaswamy *et al.*, 1962). It therefore appears that the initial step in the reaction sequence leading to glutamine synthesis involves combination of the enzyme with ATP, and similarly that reaction of ADP with enzyme is the first step in the reversal of synthesis, the γ-glutamyl transfer reaction, and the arsenolysis of glutamine.

The γ-glutamyl transfer reaction

$$\text{L-glutamine} + NH_2OH \xrightarrow{P_i(As_i),\ Mn^{2+}(Mg^{2+}),\ ADP} \text{L-}\gamma\text{-glutamylhydroxamate} + NH_3$$

is remarkable in that exceedingly low concentrations of ADP suffice to produce maximal activation. The very low nucleotide requirement for the γ-glutamyl transfer reaction has made it possible to obtain direct evidence for the binding of nucleotide to the enzyme.

This report describes studies on the binding of ADP to the enzyme in the presence of Mn^{2+} and in the absence of amino acid substrate. In the course of this work, it was discovered that ATP also activates the γ-glutamyl transfer reaction and that at low concentrations of nucleotide activation by ATP is equivalent to that observed with ADP. Evidence was obtained that cleavage of ATP to ADP is not responsible for the observed activation by ATP, and that ATP is also bound to the enzyme in the presence of Mn^{2+}. The present findings support the previous conclusion (Krishnaswamy *et al.*, 1962; Meister *et al.*, 1962) that combination of the enzyme with nucleotide is associated with steric or electronic changes in the enzyme which permit subsequent attachment of amino acid substrate. These studies also permit an estimate of the number of nucleotide binding sites on the enzyme.

Experimental Section

Materials

Sheep brain glutamine synthetase[2] and the various

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[1] Abbreviations used in this work: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; As_i, inorganic arsenate; AMP, adenosine 5′-monophosphate; A4P, adenosine tetraphosphate.

unlabeled substrates were obtained as previously described (Pamiljans *et al.*, 1962). Adenosine tetraphosphate, deoxy-ATP, deoxy-ADP, and the other nucleotides were obtained from the Sigma Chemical Co.; the adenosine tetraphosphate contained approximately 12% ATP and 1.3% ADP, which were removed by chromatography as described (Lieberman, 1955). ATP-8-^{14}C, ADP-8-^{14}C, and AMP were obtained from Schwartz BioResearch, Inc. We are indebted to Dr. Terrell C. Myers and Dr. James W. Flesher for their kindness in supplying us with samples of nucleotide phosphonic acid derivatives possessing the following structures: adenine–ribose–5′-O-PO_2H–CH_2–PO_3H_2, adenine–ribose–5′-OPO_2H–O–PO_2H–CH_2–PO_3H_2, and adenine–ribose–5′-OPO_2H–CH_2–PO_2H–O–PO_3H_2.

ATP-γ-^{32}P was prepared by reversal of glutamine synthesis. Inorganic phosphate-^{32}P, obtained from the Oak Ridge National Laboratory, was neutralized with sodium hydroxide and its purity was checked by ascending paper chromatography on Whatman No. 3MM paper using a solvent consisting of isopropyl ether and formic acid (3:2); after development for 24 hr at 26°, the chromatogram was dried and a radioautogram was made. The labeled phosphate was eluted from the paper with water. A reaction mixture consisting of L-glutamine (50 μmoles), Tris–HCl buffer (pH 7.2; 100 μmoles), $MgCl_2$ (50 μmoles), sodium ADP (10 μmoles), potassium phosphate (pH 7.2; 10 μmoles), glutamine synthetase (300 units), and sodium phosphate-^{32}P (pH 7.2; 2 mcuries), in a final volume of 1.0 ml, was incubated at 37° for 3.5 hr. After addition of 0.2 ml of cold 18% perchloric acid, the denatured protein was removed by centrifugation at 4° and the perchloric acid was precipitated from the supernatant solution by addition of KOH to pH 7. After removal of the potassium perchlorate by centrifugation, the supernatant solution was added to the top of a column of Dowex 1-formate (1 × 3 cm) (Pressman, 1960). Inorganic phosphate and ADP were eluted by washing the column with 30 ml of ammonium formate (0.3 M; pH 5.0), followed by 80 ml of 0.05 M ammonium formate in 4 M formic acid. ATP-γ-^{32}P was eluted with 0.5 M ammonium formate in 4 M formic acid. The eluate was diluted fivefold with water and lyophilized. The residue was dissolved in water, and this solution was adjusted to pH 9.5 and then evaporated *in vacuo* at 30° to remove ammonia. The ammonia-free residue was dissolved in water and adjusted to pH 7 by addition of HCl.

Methods

Determination of Glutamine Synthetase Activity. Glutamine synthetase activity was determined in reaction mixtures consisting of imidazole–HCl buffer (pH 7.2; 50 μmoles), $MgCl_2$ (20 μmoles), 2-mercaptoethanol (25 μmoles), sodium L-glutamate (50 μmoles), hydroxylamine hydrochloride adjusted to pH 7.2 with NaOH (100 μmoles), sodium ATP (10 μmoles), and enzyme, in a final volume of 1.0 ml. After incubation at 37° for 15 min, 1.5 ml of a solution containing 0.37 M $FeCl_3$, 0.67 N HCl, and 0.20 M trichloroacetic acid was added. After removal of the precipitated protein by centrifugation, the colors were compared with that given by authentic γ-glutamylhydroxamic acid in a Weston colorimeter equipped with a 535 mμ filter. Suitable controls in which enzyme, ATP, and glutamate were separately omitted were employed. Under the conditions used, 1 μmole of γ-glutamylhydroxamic acid gave an absorbancy of 0.340. The reaction rate was linear with enzyme concentration over the range 0.2–1.2 μmoles of hydroxamate formed. A unit of glutamine synthetase activity is defined as that amount of enzyme which catalyzes the synthesis of 1 μmole of γ-glutamylhydroxamic acid under the conditions of assay.

Protein was determined by the procedure of Lowry *et al.* (1951) using crystalline bovine serum albumin as a standard. The values obtained for protein concentration by this method were divided by 1.35 to give the correct protein values. This factor was calculated from dry weight determinations of the enzyme (obtained by lyophilization of the enzyme followed by drying at 26° *in vacuo* over phosphorus pentoxide for 48 hr). A Kjeldahl nitrogen determination based on the protein concentration as determined by the procedure of Lowry *et al.* (1951) gave a value of 12.6%; a value of 16.9% was obtained on the basis of dry weight. Measurements of refractive index based on dry weight give a value close to the average value obtained with a number of globular proteins (Haschemeyer, 1965).

In this paper, the molarity of enzyme was calculated from the value obtained for protein concentration, corrected as described above, and a molecular weight of 525,000 (Haschemeyer, 1965). The specific activity of the purified enzyme is 200 units/mg.

Determination of γ-Glutamyl Transfer Activity. γ-Glutamyl transfer activity was determined in reaction mixtures consisting of imidazole–HCl buffer (pH 7.2; 50 μmoles), $MnCl_2$ (5 μmoles), L-glutamine (50 μmoles), potassium phosphate (pH 7.2; 25 μmoles), sodium ADP (0.1 μmole), hydroxylamine hydrochloride (100 μmoles) adjusted to pH 7.2 with NaOH, and enzyme, in a final volume of 1.0 ml. After incubation at 37° for 15 min the reaction mixture was treated with 1.5 ml of ferric chloride reagent, and the colors were compared as described above. Controls in which enzyme, $MnCl_2$, and phosphate were separately omitted were employed.

Gel Filtration. Columns containing Sephadex G-100 were prepared in 1-ml pipets (height 16 cm; volume 0.75 ml); the columns were equilibrated with Tris–HCl buffer (0.02 M; pH 7.2) containing 0.15 M NaCl and 0.005 M $MnCl_2$ at 22°. The reaction mixture to be added to the column was mixed in a drop on the surface of a piece of parafilm, and immediately transferred quantitatively to the top of the column. Elution was carried out with the same buffer used for equilibration with a

[2] The early steps in the isolation of the enzyme were carried out at the New England Enzyme Center at Tufts University School of Medicine, Boston, Mass. We are indebted to Dr. Stanley E. Charm, Director of the Center, for his valuable contribution to this preparation.

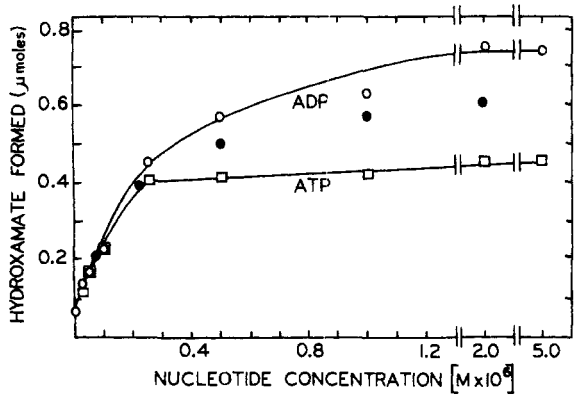

FIGURE 1: The effect of ADP and ATP concentration on γ-glutamyl transfer activity. The reaction mixtures contained enzyme (0.013 mμmole), imidazole–HCl buffer (pH 7.2; 100 μmoles), L-glutamine (50 μmoles), potassium phosphate (pH 7.2; 25 μmoles), $MnCl_2$ (5 μmoles), and ATP or ADP (as indicated), in a final volume of 1 ml; incubated at 37° for 15 min. The solid symbols represent experiments with equimolar concentrations of ATP and ADP.

flow rate of 0.1 ml/3 min. The eluate was collected in 0.05–0.1-ml fractions, which were immediately cooled in ice and then diluted with cold buffer to a final volume of 0.45 ml. The enzyme emerged from the column in the 0.15–0.35-ml volume; free ATP and ADP appeared in the 0.5–0.9-ml volume fractions. The recovery of enzyme was determined by measurements of glutamine synthetase activity. Samples of the fractions were plated on planchets and the radioactivity was determined in a gas flow counter.

In some experiments the Sephadex columns were equilibrated with Tris–HCl buffer (0.02 M; pH 7.2) containing 0.15 M NaCl, 0.005 M $MnCl_2$, and 0.0001 M ATP or ADP. Reaction mixtures containing enzyme and the same concentrations of $MnCl_2$ and nucleotide as used for equilibration were placed on the column. The recovery of enzyme from the column was determined by measurements of synthetase activity. Samples of the eluted fractions were also treated at 0° with perchloric acid (final concentration, 3.3%); after removal of the precipitated protein by centrifugation, the concentration of nucleotide was determined from the absorbancy at 257 mμ. This procedure is similar to that used by Hummel and Dreyer (1962) for studies on the binding of cytidylic acid to ribonuclease.

Results

Activation of γ-Glutamyl Transfer Activity by Adenine Nucleotides. As indicated in Figure 1, both ADP and ATP activated γ-glutamyl transfer activity. Under these conditions ATP and ADP produced equivalent activation at concentrations of nucleotide less than about 2.5×10^{-7} M. At concentrations of ATP higher than 2.5×10^{-7} M little further increase in the reaction rate was observed, while with ADP the reaction rate continued to increase, reaching a maximum at a concentration of ADP of 2×10^{-6} M. When equimolar concentrations of ATP and ADP were used, the activation observed was equivalent to the average of the effects observed with either nucleotide alone. Some transfer activity was observed in the absence of added nucleotide; this "blank" reaction proceeded at 2–10% of the maximal rate observed with added ADP and varied in extent with different preparations of the enzyme. For a given enzyme preparation, the rate of the "blank" reaction was proportional to the amount of the enzyme. Attempts were made to reduce the "blank" reaction by dialysis of the enzyme at pH 7.2 in 0.02 M Tris buffer containing 0.05 M 2-mercaptoethanol, treatment of the enzyme with charcoal, adsorption of the enzyme on a column of DEAE-cellulose followed by elution with 0.25 M potassium phosphate (pH 6.6), and passage of the enzyme through columns of Sephadex. However, these procedures failed to affect the extent of the "blank." When solutions of the enzyme (previously heated at 70° for 10 min) were added to reaction mixtures containing active enzyme and other components as indicated in Figure 1 (except added nucleotide), an increase in transfer activity was observed. Assuming that such activation is produced by ATP (or ADP) liberated from the enzyme by heating, it may be calculated that from 0.26 to 2.4 moles of nucleotide is bound per mole of enzyme. Presumably such tightly bound nucleotide accounts for the "blank" reaction observed in these and earlier studies on the γ-glutamyl transfer reaction catalyzed by preparations of glutamine synthetase.

In order to determine whether the activating effect of ATP on transfer activity requires its conversion to ADP, experiments were carried out with ATP-γ-^{32}P under the conditions described in Figure 1. It was found that with 0.2×10^{-6} M ATP-γ-^{32}P the formation of inorganic phosphate was linear with time for at least 30 min, reaching only 30% of completion after 15 min of incubation. The ADP thus formed cannot be responsible for the observed activation by ATP because the activating effect of ATP is equal to that of ADP at concentrations less than 0.2×10^{-6} M. In addition, the transfer reaction activated by ATP is linear with time for at least 30 min; if activation by ATP required its conversion to ADP, an exponential curve would be expected.

The following nucleotides did not activate γ-glutamyl transfer activity under the conditions described in Figure 1: AMP, guanosine 5′-diphosphate, uridine 5′-diphosphate, cytidine 5′-diphosphate, guanosine 5′-triphosphate, uridine 5′-triphosphate, and cytidine 5′-triphosphate. Adenosine diphosphoribose, reduced diphosphopyridine nucleotide, inosine 5′-diphosphate, and inosine 5′-triphosphate were only slightly active when these were studied at concentrations a thousandfold greater than those of ADP or ATP; such activation could be due to the presence of as little as 0.1% of ADP or ATP in the preparations of these products employed. The three nucleotide phosphonic acid derivatives whose structures are given under Materials

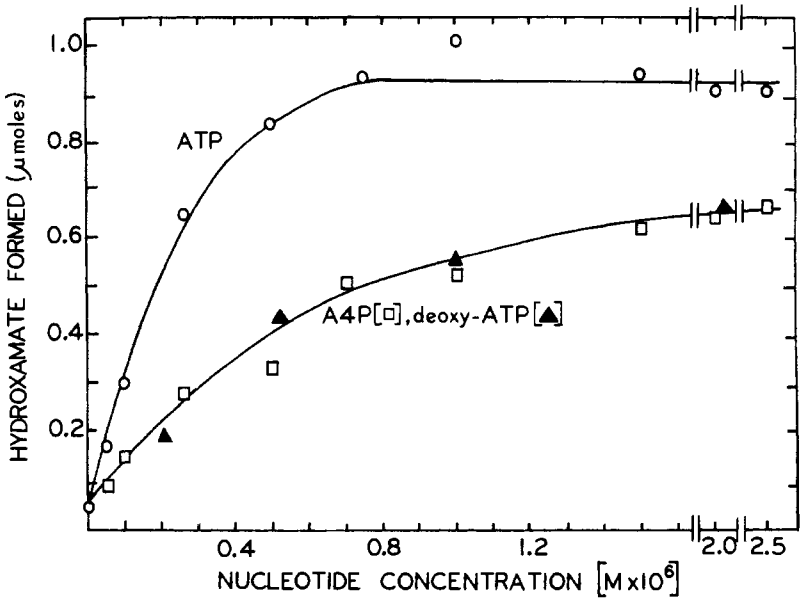

FIGURE 2: The effect of adenosine tetraphosphate and deoxy-ATP on γ-glutamyl transfer activity. The reaction mixtures contained enzyme (0.028 mμmole), imidazole–HCl buffer (pH 7.2; 100 μmoles), L-glutamine (50 μmoles), potassium phosphate (pH 7.2; 25 μmoles), $MnCl_2$ (5 μmoles), and adenosine tetraphosphate (A4P), deoxy-ATP, or ATP (as indicated), in a final volume of 1 ml; incubated at 37° for 15 min.

(see above) did not activate the γ-glutamyl transfer reaction when tested in concentrations ranging from 2.5×10^{-7} to 2.0×10^{-6} M, nor did any of these compounds replace ATP in the synthesis reaction. The nucleotide phosphonic acid derivatives did not inhibit the transfer or synthesis reactions when tested at concentrations of 5×10^{-3} and 0.5×10^{-3} M, respectively.

Substantial activation of γ-glutamyl transfer activity was observed with adenosine tetraphosphate (Figure 2). The adenosine tetraphosphate preparation employed was shown to be free of ATP and ADP by column chromatography (Lieberman, 1955). Less activation was observed with adenosine tetraphosphate than with either ATP or ADP. The maximal activity obtained with adenosine tetraphosphate was the same as that with ATP at a nucleotide concentration of 10^{-4} M. Attempts were made to demonstrate enzymatic formation of adenosine tetraphosphate by carrying out the reversal of glutamine synthesis in the presence of ATP (in place of ADP) and ^{32}P-phosphate; however, no adenosine tetraphosphate formation was detected. On the other hand, adenosine tetraphosphate was active (in place of ATP) in the synthesis reaction; the reaction with adenosine tetraphosphate proceeded at about 10% of the rate observed with ATP, and the formation of inorganic phosphate and γ-glutamylhydroxamate was equimolar. Presumably adenosine tetraphosphate is initially converted to ATP, which can then be used in an additional synthesis reaction. However, the reaction with adenosine tetraphosphate did not proceed to completion and generally stopped when approximately 10% of the total nucleotide phosphorus was liberated as inorganic phosphate. Addition of ATP to such reaction mixtures led to further formation of γ-glutamylhydroxamate, indicating that the adenosine tetraphosphate preparation did not significantly inhibit the enzyme. Further studies on the utilization of adenosine tetraphosphate are needed.

Both deoxy-ADP and deoxy-ATP activated the γ-glutamyl transfer reaction. Deoxy-ATP activated to the same extent as adenosine tetraphosphate (Figure 2). When tested at equivalent concentrations between 0.1 $\times 10^{-6}$ and 10^{-6} M, deoxy-ADP gave about 75% of the effect observed with ADP; at concentrations higher than 10^{-5} M these nucleotides exhibited equivalent activation. Deoxy-ATP was as effective as ATP in the synthesis reaction; the K_m for deoxy-ATP was 2.6×10^{-3} M or, within experimental error, the same as that observed previously for ATP (2.3×10^{-3} M; Pamiljans *et al.*, 1962).

Although activation of γ-glutamyl transfer activity by ATP was observed earlier, it had been concluded that such activation was due to cleavage of ATP to ADP and that ADP was specifically required for activation (Meister, 1962). Previous findings also indicated that ATP inhibited the transfer reaction when this nucleotide was added in high concentrations. We have confirmed this observation, but find that the concentration of ATP required for inhibition is 5×10^{-3} M or about 20,000 times the concentration of ATP required for maximal activation under the conditions described in Figure 1. On the other hand, ADP did not inhibit γ-glutamyl transfer activity even when added in concentrations as high as 2×10^{-2} M.

The shape of the curve for activation of γ-glutamyl transfer activity by ATP (and to a lesser extent that for

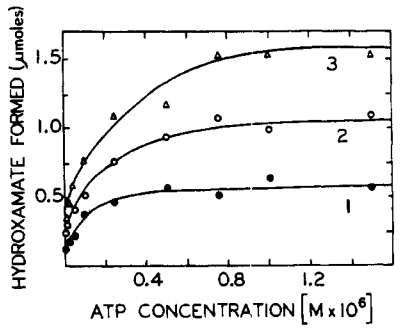

FIGURE 3: The effect of enzyme concentration on the ATP-stimulated γ-glutamyl transfer reaction. The reaction mixtures contained enzyme (curve 1, 0.017 mμmole; curve 2, 0.034 mμmole; curve 3, 0.051 mμmole), imidazole–HCl buffer (pH 7.2; 100 μmoles), L-glutamine (50 μmoles), potassium phosphate (pH 7.2; 25 μmoles), $MnCl_2$ (5 μmoles), and ATP as indicated, in a final volume of 1 ml; incubated at 37° for 15 min.

ADP) suggests that the enzyme is being titrated by the nucleotides. Furthermore, the concentration of nucleotide required to achieve saturation of the enzyme is proportional to the concentration of enzyme; this is indicated by the data given in Figure 3 which shows the activation by ATP obtained with three concentrations of enzyme. The molar concentration of ATP required for maximal activation is about 18 times that of the enzyme. Analogous results were obtained in similar experiments with ADP.

Binding of ATP and ADP to Glutamine Synthetase. When reaction mixtures containing the enzyme, ATP, or ADP, and manganese ions were prepared and immediately added to the top of columns of Sephadex G-100 and then eluted from the column with buffer containing manganese chloride as described under Methods, substantial quantities of nucleotide emerged from the column with the enzyme. A typical experiment is described in Figure 4. When manganese chloride was omitted from the reaction mixture and eluting buffer, no significant binding of nucleotide was observed. No evidence for binding of nucleotides was observed when magnesium ions were substituted for manganese ions.

In another type of experiment, illustrated in Figure 5, much larger amounts of radioactive nucleotide and enzyme were used. In these experiments there was no sharp break in the curve for the elution of nucleotide from the column, but substantially the same nucleotide to enzyme ratio was obtained in the early effluent fractions.

Table I summarizes the results of a number of experiments in which reaction mixtures containing enzyme and nucleotide were placed on Sephadex columns. Experiments 3 and 13 were carried out under the conditions described in Figure 5; experiment 2 is described in Figure 4. The results obtained with ATP-γ-^{32}P are in essential agreement with those carried out with ^{14}C-ATP. The finding of slightly lower values in the studies with ATP-γ-^{32}P as compared to those with ATP-^{14}C may be due to slight nonenzymatic cleavage of

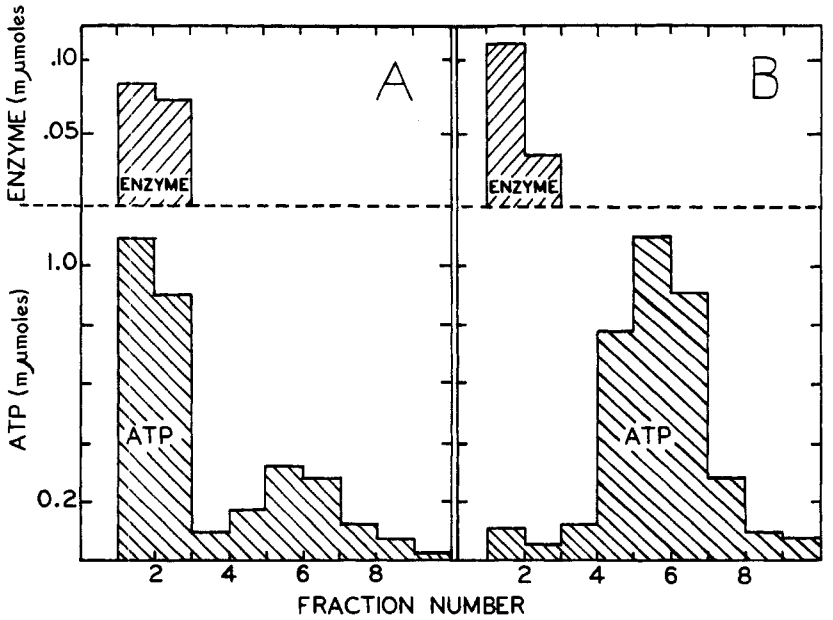

FIGURE 4: Binding of ATP to glutamine synthetase. (A) A reaction mixture containing Tris–HCl buffer (0.4 μmole; pH 7.2), NaCl (3 μmoles), $MnCl_2$ (0.5 μmole), enzyme (0.13 mμmole), and ATP-^{14}C (3 mμmoles; 13,700 cpm), in a final volume of 0.05 ml, was added to the top of a Sephadex G-100 column; elution and analyses were carried out as described under Methods. (B) $MnCl_2$ was omitted from the reaction mixture and from the eluting solution.

TABLE I: Binding of Nucleotides to Glutamine Synthetase.[a]

Expt No.	Enzyme Added (mμ-moles)	Nucleotide Added (mμmoles)	Enzyme Eluted (mμ-moles)	Nucleotide Found with Enzyme (mμ-moles)	Nucleotide/Enzyme[b]
1	0.55	30 (ATP-^{14}C)	0.50	6.74	14
1a	0.55	30 (ATP-^{14}C)	0.44	0.28	0.6[c]
2	0.20	3 (ATP-^{14}C)	0.18	2.19	12
3[d]	4.88	300 (ATP-^{14}C)	3.26	52.6	16
4	0.33	30 (ATP-^{14}C)	0.20	3.03	15
5	0.23	4.8 (ATP-^{32}P)	0.09	2.30	12
6	0.18	2.5 (ATP-^{32}P)	0.14	1.68	12
7	0.14	2.0 (ATP-^{32}P)	0.11	1.38	13
8	0.21	2.0 (ATP-^{32}P)	0.11	1.31	12
8a	0.21	2.0 (ATP-^{32}P)	0.17	0.21	1.2[c]
9	0.19	5.9 (ATP-^{32}P)	0.11	1.40	13
10	0.37	20 (ADP-^{14}C)	0.33	6.27	19
11	0.43	20 (ADP-^{14}C)	0.27	4.24	16
11a	0.43	20 (ADP-^{14}C)	0.26	0.18	0.7[c]
12	0.43	20 (ADP-^{14}C)	0.23	3.40	15
13[d]	4.88	200 (ADP-^{14}C)	3.53	55.6	16

[a] Reaction mixtures containing Tris–HCl buffer (0.4 μmole; pH 7.2), NaCl (3 μmoles), $MnCl_2$ (0.5 μmole), enzyme (in the amounts indicated), and labeled nucleotide (as indicated), in a final volume of 0.05 ml, were added to columns of Sephadex G-100; elution and other procedures were carried out as described under Methods. [b] This ratio was obtained by dividing the value for nucleotide found with the enzyme by the value for enzyme eluted. [c] $MnCl_2$ was omitted from the reaction mixture and the eluting solution. [d] Reaction mixtures containing Tris–HCl buffer (7 μmoles; pH 7.2), NaCl (52 μmoles), $MnCl_2$ (2.5 μmoles), enzyme (in the amounts indicated), and labeled nucleotide (as indicated), in a final volume of 0.5 ml, were added to columns of Sephadex G-100; elution and other procedures were carried out as described under Methods.

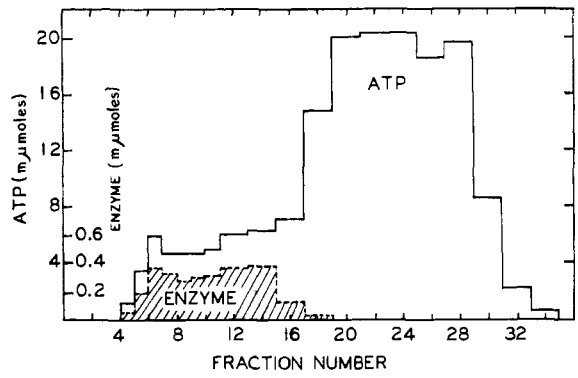

FIGURE 5: Binding of ATP to glutamine synthetase. A reaction mixture containing Tris–HCl buffer (7 μmoles; pH 7.2), NaCl (52 μmoles), $MnCl_2$ (2.5 μmoles), enzyme (4.88 mμmoles), and ATP-^{14}C (300 mμmoles; 1.37×10^6 cpm), in a final volume of 0.5 ml, was added to the top of a Sephadex G-100 column; elution and other procedures were carried out as described under Methods.

ATP-γ-^{32}P to unlabeled ADP. It is conceivable that the binding of ATP is associated with cleavage and separate binding to the enzyme of the ADP and terminal phosphate moieties of ATP. However, the following study renders this possibility unlikely. Fractions of enzyme–ATP-γ-^{32}P complex obtained from Sephadex columns were treated with 0.006 M EDTA for 10 sec and then mixed with 0.4 volume of 16% ethanol containing activated charcoal; about 80% of the total ^{32}P present was adsorbed on the charcoal. Under these conditions at least 90% of the enzyme protein was not adsorbed as measured by determinations of glutamine synthetase activity. Evidence that the ATP bound to the enzyme is available for glutamine synthesis was obtained in experiments in which the enzyme–ATP complex was incubated with NH_4Cl and ^{14}C-glutamate. Thus, in a representative experiment, a sample of enzyme containing 0.14 mμmole of enzyme and 1.7 mμmoles of ATP obtained from a Sephadex column was incubated with ^{14}C-L-glutamate (11.8 mμmoles; 1.3×10^6 cpm), Tris–HCl buffer (pH 7.2; 44 μmoles), NH_4Cl (100 μmoles), and $MnCl_2$ (0.1 μmole), in a final volume of 0.3 ml for 1 min at 37°. The reaction was terminated by addition of 0.05 ml of 18% perchloric acid and the precipitated protein was removed by centrifugation. The amount of ^{14}C-glutamine formed, determined by paper electrophoretic study of the supernatant solution (Krishnaswamy *et al.*, 1962), was found to be 1.47 mμmoles, indicating utilization of 86% of the ATP associated with the enzyme.

The data given in Table I indicate that close to 16 moles of ADP are bound per mole of enzyme; similar but slightly lower values were obtained with ATP. The data on activation of the transfer reaction by ATP and ADP (Figure 1) indicated that both nucleotides produced equivalent activation at low concentrations; with higher concentrations of ADP (but not ATP) additional activation was observed. The maximal activation found with ADP approached a value that was about twice that observed with ATP.[3] These observations suggested that more ADP than ATP was bound by the enzyme

[3] The ratio of maximal ADP activation to maximal ATP activation observed with different preparations of the enzyme varied from 1.3 to 2.0; ratios close to 2.0 were typically found with freshly prepared enzyme. Storage of the enzyme at 0° was often associated with a decrease in this ratio without appreciable change of synthetase activity.

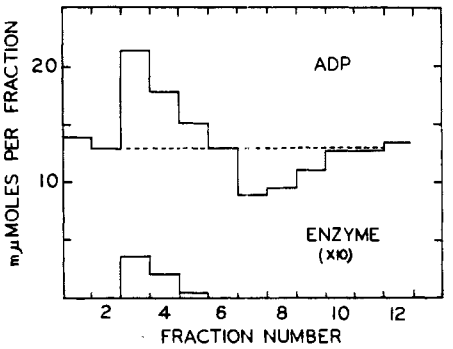

FIGURE 6: Binding of ADP to glutamine synthetase in the presence of a high concentration of nucleotide. A reaction mixture containing Tris-HCl buffer (2 μmoles, pH 7.2), NaCl (15 μmoles), $MnCl_2$ (0.5 μmoles), ADP (12.8 mμmoles), and enzyme (0.85 mμmole), in a final volume of 0.1 ml, was added to the top of a Sephadex G-100 column; elution and analysis for enzyme and ADP were carried out as described under Methods.

at higher nucleotide concentrations, but that the affinity for the additional increment of ADP was less than that for the initial amount of ADP bound. In order to examine this possibility, experiments with Sephadex columns, in which the eluting buffer contained the same concentration of nucleotide as the reaction mixture, were carried out as described under Methods. A representative experiment is described in Figure 6. Under these conditions, the enzyme was eluted with additional nucleotide followed by a trough of approximately equivalent amount in the elution curve for nucleotide. The values obtained in studies of this type for the binding of ADP and ATP to three different enzyme preparations are given in Table II. The data show that substantially more ADP than ATP is bound to the enzyme and that the ADP to ATP ratios for binding are, within experimental error, the same as the corresponding ratios for maximal γ-glutamyl transfer activation.

Discussion

The data provide conclusive evidence that glutamine synthetase can combine with ATP and ADP in the absence of glutamate or glutamine, and are therefore in accord with the conclusion that binding of glutamate and glutamine to the enzyme requires prior combination of the enzyme with nucleotide (Krishnaswamy *et al.*, 1962). The findings also provide further evidence that enzyme-bound ADP is a constituent of the enzyme-activated glutamate complex (Meister, 1962).

The experiments reported here show clearly that ATP can activate the transfer reaction. Although ATP is split under the conditions employed for study of the transfer reaction, the rate of its cleavage is insufficient to explain the observed activation in terms of ADP formation. The relatively slow cleavage of ATP found

TABLE II: Binding of ATP and ADP to Glutamine Synthetase in the Presence of a High Concentration of Nucleotide.[a]

Enzyme Preparation	Nucleotide	Enzyme Eluted (mμmole)	Nucleotide Found with Enzyme (mμmoles)	Ratio Nucleotide Enzyme	Ratio of Binding ADP/ATP	Ratio of Transfer Activities[b]
A	ADP	0.40	9.2	23	1.5	1.6
	ATP	0.81	12.4	15		
B	ADP	0.75	15.5	21	1.2	1.3
	ATP	0.77	12.9	17		
C	ADP	0.55	14.8	27	1.8	2.0
	ATP	0.45	6.7	15		

[a] The reaction mixtures contained 0.02 M Tris-HCl buffer, 0.15 M NaCl, 0.005 M $MnCl_2$, enzyme, and nucleotide (10^{-4} M), in a final volume of 0.1 ml. These were added to equilibrated Sephadex G-100 columns; elution and analysis were carried out as described under Methods. A, B, and C represent different preparations of the enzyme exhibiting the same glutamine synthesis activity. [b] Ratio of the maximal transfer activity observed with ADP to that observed with ATP.

may probably be ascribed to the presence of some glutamate in the reaction mixture, either present as a contaminant in the glutamine preparation added or formed by reversal of glutamine synthesis. Even traces of ADP on the enzyme or in the ATP preparation added would be expected to be sufficient to promote some reversal of synthesis with consequent formation of glutamate. Thus, the release of labeled inorganic phosphate observed could readily be explained in terms of an exchange of the terminal phosphate of ATP with unlabeled inorganic phosphate of the medium by virtue of the reversible synthesis of glutamine.

It is noteworthy that the curves for the activation of the transfer reaction by ADP and ATP are identical at low concentrations of nucleotide and that they diverge at higher concentrations of nucleotide, with ADP being a more effective activator (Figure 1). However, a proportionately much higher concentration of ADP is required for maximal activation than for half-maximal activation. Thus, in the experiment described in Figure 1, half-maximal activation was achieved with an ADP concentration of about 2×10^{-7} M, while maximal activation required about 2×10^{-6} M ADP. These findings can evidently be correlated with the studies on the binding of nucleotides. Thus, in the experiments in which mixtures containing enzyme, nucleotide, and manganese ions were passed through Sephadex columns eluted with nucleotide-free buffers, about 14–16 moles of ^{14}C-nucleotide were bound per mole of enzyme (Table I). When nucleotide was present in the

buffer, substantially more ADP was bound than ATP; furthermore, the ratios of bound ADP to ATP were equivalent to those for maximal transfer activity (Table II). The findings indicate that, once bound to the enzyme, ATP and ADP are about equally effective in promoting the transfer reaction. However, the data also indicate that the enzyme binds approximately half of the total maximally bound ADP readily; the remainder of the ADP is bound with considerably less affinity.

We offer the following tentative explanation for these findings. The binding of some ADP (or ATP) to the enzyme lowers the affinity of the enzyme for additional nucleotide. Thus, about 16 moles of ADP is readily bound per mole of enzyme; further ADP is less readily bound. Our best present quantitative estimate (based on transfer activity studies with freshly prepared enzyme) indicates that about 32 moles of ADP is bound per mole of enzyme. The inability of the enzyme to bind more than about 16 moles of ATP/mole of enzyme suggests that the affinity for additional ATP is even less than for ADP. It is tempting to consider the possibility that the active sites on the enzyme occur in pairs and that the acceptance of a nucleotide molecule by one site of a pair reduces the affinity of the neighboring site for nucleotide. Such a model would appear to explain the equal affinity of the enzyme for ATP and ADP at low nucleotide concentrations. The greater affinity of the enzyme for ADP than ATP at higher nucleotide concentrations might then be ascribed to the greater effect of the somewhat bulkier ATP molecule in reducing the affinity of the second site for nucleotide. Further studies are needed to test the validity of this model. It seems probable that the enzyme contains a number of subunits; if each molecule of ADP is bound to a site on a single peptide chain, it would be plausible to postulate the existence of 32 such subunits. Haschemeyer (1965) has been able to dissociate the enzyme into eight apparently identical subunits by treatment with sodium dodecyl sulfate, urea, or guanidine hydrochloride. Further studies are needed to determine whether the subunits obtained in this manner can be dissociated to units of lower molecular weight.

In the present studies binding of nucleotides to the enzyme was observed only in the presence of manganese ions. No binding could be detected when magnesium ions were substituted for manganese ions. Similarly, under the conditions employed here for study of the γ-glutamyl transfer reaction, no activity was observed when magnesium ions were used in place of manganese ions, even when the concentrations of metal ions and nucleotides were increased, respectively, by 6- and 1000-fold. However, γ-glutamyl transferase activity has been observed with magnesium ions when much higher concentrations of enzyme are used. It seems apparent that the affinity of the enzyme for nucleotide is much greater with manganese than with magnesium ions, at least under the conditions employed. Additional work is needed to elucidate other aspects of the binding of nucleotides, metal ions, and amino acids to the enzyme.

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