A Novel Reaction Catalyzed by Unadenylylated Glutamine Synthetase from *Escherichia coli*

AMP-DEPENDENT SYNTHESIS OF PYROPHOSPHATE AND L-GLUTAMATE FROM ORTHOPHOSPHATE AND L-GLUTAMINE*

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The unadenylylated, manganese form of glutamine synthetase (L-glutamate: ammonia ligase (ADP forming), EC 6.3.1.2 from *Escherichia coli* catalyzes a novel, AMP-dependent (reversible) synthesis of pyrophosphate and L-glutamate from orthophosphate and L-glutamine: Gln + 2 P → Glu + PP + NH₃. The hydrolysis of the L-glutamine amide bond is coupled to the stoichiometric synthesis of pyrophosphate, although as PP accumulates, additional hydrolysis of L-glutamine occurs in a secondary reaction catalyzed by the [manganese<enzyme>•AMP•PP] complex. The synthesis of PP, probably occurs at the subunit catalytic site in the positions normally occupied by the βγ-phosphates of ATP. To promote PP synthesis, AMP apparently binds to the subunit catalytic site rather than to the allosteric inhibitor site; equilibrium binding results suggest that P directs the binding of AMP to the active site. In this reaction, Mg will not substitute for Mn, and adenylylated glutamine synthetase is inactive. Pyrophosphate is synthesized by the unadenylylated, manganese enzyme at ~2% of the rate of that of ATP in the reverse biosynthetic reaction. If P, is replaced by arsenate, the enzymatic rate of the AMP-supported hydrolysis of L-glutamine is 100-fold faster than is PP, and synthesis is one-half the rate of the ADP-supported, irreversible arslenylation of L-glutamine. This latter activity also is supported by GMP and IMP, suggesting that the catalytic site of glutamine synthetase has a rather broad specificity for the nucleotide base. The reactions supported by AMP directly relate to the mechanism of glutamine synthetase catalysis.

Each subunit of the dodecameric glutamine synthetase (M, = 600,000) from *Escherichia coli* has a catalytic site (1, 2), separate binding sites for at least some of the feedback inhibitors (3-9), a specific tyrosyl residue that is the site of regulation by adenylylation-deadenylylation (3-5, 10), and two metal ion sites (n, and n) that must be saturated for activity expression (1). Mn at the n, site induces the nucleotide substrate at the subunit catalytic site, while Mn (Mg or Ca) binding to high affinity n, sites promotes an active conformation (1, 5). Distances from the phosphorus of covariantly bound 5'-adenylate to Mn at n, and n, subunit sites are ~10 and ~7 Å, respectively (11).

AMP is a feedback inhibitor of the biosynthetic reaction catalyzed by *E. coli* glutamine synthetase (3, 4):

\[
\text{L-Glutamate + ATP + NH}_3 \xrightarrow{\text{Mn}^{2+}} \text{L-glutamine + ADP + P}_i \quad (1)
\]

Unadenylylated glutamine synthetase requires Mg²⁺ in the forward reaction, but can catalyze the reverse reaction in the presence of either Mg²⁺ or Mn²⁺ (4, 12, 13). AMP binds independently to 12 sites of the dodecamer with \(K_A = 8 \times 10^7\) M⁻¹ at 4°C and pH 7.3 in the absence of other ligands (14). An allosteric subunit site for AMP (distinct from that binding the nucleotide substrate) has been inferred from equilibrium binding (6, 14), NMR (8), and calorimetric studies (6). Separate subunit sites for ADP and AMP also are shown by the measurements of the simultaneous binding of [³²P]ADP and [³²P]AMP to unadenylylated glutamine synthetase reported here. Nevertheless, AMP (substituted for the nonconsumable substrate ADP) supports the γ-glutamyl transfer reaction catalyzed by unadenylylated glutamine synthetase in the presence of Mn²⁺ or Cd²⁺ (1, 4):

\[
\text{L-Glutamine + NH}_3\text{OH} \xrightarrow{\text{ADP or AMP, Mn}^{2+}} \text{γ-glutamylhydroxamate + Ni,F}_3 \quad (2)
\]

Adenylylation of the subunit decreases the affinity >2000-fold for ADP-Mn in Reaction 2 (1). The binding of ADP-Mn and arsenate or of ADP-Mn and P; (\(K_a = 0.03\) to 0.08 μM for ADP-Mn) is strongly synergistic (1, 13). The unadenylylated, manganese enzyme has a >3000-fold greater affinity for ADP-Mn than for AMP in Reaction 2. This fact and the report of Rhee et al. (13) that the unadenylylated, manganese enzyme in the presence of L-glutamine and P, can phosphorylate AMP to yield ATP (without detecting intermediate ADP formation) suggested that commercial preparations of AMP might contain small amounts of ADP which were actually responsible for both reactions. We have found that chromatographically purified AMP does support the γ-glutamyl transfer Reaction 2 catalyzed by unadenylylated, manganese enzyme. However, the product of the reaction studied by Rhee et al. (13) has been discovered to be pyrophosphate rather than ATP. This novel, AMP-dependent reaction is described in the present paper and can be written:

\[
\text{L-Glutamine + 2 P,} \xrightarrow{\text{Mn}^{2+}, \text{AMP}} \text{L-glutamate + PP + NH}_3 \quad (3)
\]

Mg²⁺ but not Mg²⁺ supports this activity of the unadenylylated enzyme; fully adenylylated glutamine synthetase does not catalyze Reaction 3. Reaction 3 is analogous to the reverse...
biosynthetic Reaction 1 in which the β,γ-phosphate bond of ATP is synthesized, although Reaction 3 is catalyzed at only ~2% of the rate of ATP synthesis. Reaction 3 also is related to the irreversible arsenolysis of L-glutamine observed by Levintow and Meister (15) and Purich and Huang.

\[ \text{L-Glutamine} + \text{H}_2\text{O} \rightarrow \text{Me}^{2+}, \text{ADP} \text{or AMP} \rightarrow \text{L-glutamate} + \text{NH}_4^+ \]  

(4)

AMP may bind exclusively to the nucleotide substrate site of the enzyme subunit under the conditions of Reactions 2, 3, and 4.

**MATERIALS AND METHODS**

Equine muscle AMP, yeast AMP, CMP, ADP, and ATP were purchased from Sigma Chemical Co. Also, AMP, GMP, and UMP (P-L Biochemical Inc.), AMP (Calbiochem), and IMP (Boehringer Mannheim Biochemical) were used. Commercial AMP was purified on DEAE-Sephadex A-25 employing a gradient elution with 0.05 to 0.16 M (NH₄)₂CO₃, followed by 8 to 10 repeated lyophilizations of the isolated AMP to remove (NH₄)₂CO₃. [³²P]AMP (1.4 μCi/μmol, Amersham Radiochemical Centre) was diluted to about 1000 cpm/μmol with the purified AMP and relyophilized before use. For the experiments in Figs. 1, 2A, and 3 to 5, to 10X lyophilized muscle or yeast AMP-glutamine synthetase, commercial AMP was used without further purification. Carrier-free [³²P]orthophosphate at pH 2 to 3 (Amersham Radiochemical Centre) was purified before use by chromatography on Dowex 1-X2 (Cl⁻ form, Bio-Rad Laboratories) using the conditions of Fig. 3 (below). Similarly, sodium [³²P]pyrophosphate (5.5 mCi/μmol, New England Nuclear) was purified on Dowex 1-X2 (Cl⁻ form) in 1 M NH₄Cl. The [¹⁴C]ADP (stored frozen at pH 6.8) was a previously studied sample. Chromatography on PEI-cellulose® thin layers by the procedure of Cashel et al. (16) indicated that radioactively labeled nucleotides, P₃, and PP, were pure (Figs. 1 and 2 below). L-[¹⁴C]Glutamine was prepared as needed from L-[¹⁴C]glutamate (200 mCi/mmol, ICN Pharmaceuticals) by treatment with excess glutamine synthetase from Escherichia coli. The [¹⁴C]glutamate was dissolved in water at a concentration of 0.25 mg/ml (-162 units/ml). Hepes and Trizma base were from Sigma Chemical Co. All other reagents were as described previously (1).

Thin layer chromatography was on precoated plastic sheets of PEI-cellulose (Polygram gel 300 PEl from Brinkmann Instruments) in an Eastman chromatogram (No. 13259) developing apparatus. Development was ascending with 0.25 to 1.0 M ammonium carbonate (KMe₅EDTA) as was EM Laboratories. Enzymatic activity versus AMP concentration curve was affected by volatile contaminant(s) present in commercial preparations of AMP, one of which could be (NH₄)₂CO₃ (an inhibitor of Reaction 2).

**RESULTS**

**AMP-supported γ-Glutamyl Transfer Activity**—With AMP obtained from different commercial sources (P-L Biochemicals, Calbiochem, and Sigma Chemical Co.), the maximum activity of the unadenylylated, manganese enzyme in Reaction 2 with arsenate present varied from 7 to 43 units/mg at 37°C. Therefore, a contamination of commercial preparations of AMP with small amounts of ADP was suspected. Two preparations of AMP obtained from Sigma Chemical Co. (yeast AMP, Lot 44C-7290, and muscle AMP, Lot 87C-7090), which varied 6-fold in their ability to support Reaction 2, were purified by chromatography on a DEAE-Sephadex A-25 column using a gradient elution with 0.04 to 0.5 M (NH₄)₂CO₃. With either lot of AMP, a minor component (~0.05% of the AMP) eluted at the position of ADP, absorbed at 259 nm, and supported the γ-glutamyl transfer activity of glutamine synthetase. However, the chromatographed AMP from both lots (after lyophilization to remove ammonium carbonate) supported the activity of the unadenylylated, manganese enzyme in Reaction 2 to the same extent (~50 units/mg at 37°C, pH 7.2, with Kₗ = 1 mM for AMP). Subsequently, it was found that apparent differences between commercial batches of AMP could be removed by repeated lyophilizations.

Thus, AMP supports the γ-glutamyl transfer reaction catalyzed by the unadenylylated, manganese enzyme as previously reported (1, 4). However, the amplitude and shape of the enzyme activity versus AMP concentration curve is affected by volatile contaminant(s) present in commercial preparations of AMP, one of which could be (NH₄)₂CO₃ (an inhibitor of Reaction 2).

**AMP- and ADP-Supported Reactions with L-Glutamine and Orthophosphate**—Fig. 1 shows an autoradiogram of PEI-cellulose thin layers of AMP-supported and ADP-supported reactions catalyzed by unadenylylated glutamine synthetase in the presence of Mn²⁺, L-glutamine, and [³²P]ATP with AMP as the only nucleotide present. [³²P]ATP is synthesized in the reverse biosynthetic Reaction 1 (Fig. 1, lane 1). With AMP as the only nucleotide present, [³²P]ATP is synthesized in the reverse biosynthetic Reaction 1 (Fig. 1, lane 1). With AMP as the only nucleotide present, [³²P]ATP synthesis decreases with decreasing AMP, while that of a slower migrating [³²P]-labeled compound increases. With 5 and 7 μM AMP added to 12 mM AMP, radioactivity at both positions is visible (lanes 5 and 6, Fig. 1). With 1 μM AMP added to 12 mM AMP (lanes 7 and 2, Fig. 1), [³²P]ATP synthesis decreases with decreasing AMP, while that of a slower migrating [³²P]-labeled compound increases. With 5 and 7 μM AMP added to 12 mM AMP, radioactivity at both positions is visible (lanes 5 and 6, Fig. 1). With 1 μM AMP added to 12 mM AMP (lanes 7 and 2, Fig. 1), only the new [³²P]-labeled product is synthesized. Fig. 1 (lane 9) also shows that 80 mM NH₄Cl completely blocks the AMP-supported reaction. A control sample in lane 10 without nucleotide (AMP or ADP) shows only [³²P]; at the solvent front.

In the reactions of Fig. 1, 1 μM ADP-Mn is in the absence of AMP is sufficient to saturate 58% of the nucleotide substrate sites of the enzyme with ADP-Mn (1, 13). With 12 mM ADP present, there are decreasing amounts of [³²P]ATP synthesized as the ratio of ADP/AMP is decreased from about 0.88 to 0.05% in Fig. 1. Since the rate of [³²P] synthesis in Reaction 3 is only about 2% of that of ATP synthesis in the reverse biosynthetic Reaction 1 (Table III below), AMP predominately competes with ADP binding in the experiments of Fig. 1. The apparent inhibition of pyrophosphatase by NH₄Cl in Fig. 1 could be an effect on Kₗ of Reaction 3 (see below).
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Fig. 1. Autoradiogram of PEI-cellulose thin layers of reaction mixtures with unadenylylated, manganese glutamine synthetase, L-glutamine, ADP, AMP, and [32P]orthophosphate. Reactions were for 2 h at room temperature (pH 7.2) in 1.0 ml and contained 50 mM Hepes/KOH, 12 mM L-glutamine, 100 mM KCl, 11 mM 32P, (~1700 cpm/nmol), 1.0 mM MnCl2, 0.70 mg of GSi/ml, and varying concentrations of nucleotide. Lane I, 1 mM ADP (without AMP); lanes 2 to 7, 12 mM AMP and 100, 50, 10, 7, 5, and 1 mM ADP, respectively (arrow indicates direction of increasing ADP concentration); lanes 8 and 9, 12 mM AMP (without ADP) with 80 mM NH4Cl also present in the reaction of lane 9; lane 10, without ADP or AMP present. After terminating reactions by the addition of EDTA (4 mM), aliquots (~2 pl) were spotted on PEI-cellulose (origin); chromatograms were developed (ascending) with 0.85 M KH2PO4 at pH 3.4 (16) and then exposed for 6 days to x-ray film. The mobilities of standard PPi, ATP, and P, are given on the right.

The chromatograms of Fig. 1 were developed with 0.85 M KPO4 buffer (pH 3.4). Under these conditions, ADP, ATP, PPi, and adenosine tetraphosphate (in order of decreasing mobility) are well separated (16). Lower concentrations of this developing buffer gave poorer resolutions of ATP and PPi, which may account for the error of Rhee et al. (13) in identifying the 32P-labeled product of Reaction 3 as [32P]ATP, rather than correctly as 32PP.

Identification of Reaction 3 Products—Inorganic pyrophosphate was identified as a product of Reaction 3 by chromatography, hydrolysis by inorganic pyrophosphatase, and charcoal adsorption (Figs. 1 to 3). The product, L-glutamate, was identified chromatographically (Fig. 3). The supporting nucleotide in Reaction 3 was shown to be unaltered chemically by using [32P]AMP (Fig. 2B).

After the synthesis of the 32P-labeled product of the AMP-supported reaction (Fig. 2A, lane 1) a portion of the reaction mixture was treated with yeast inorganic pyrophosphatase with Mg2+ present. Subsequent chromatography (lane 2, Fig. 2A) revealed that the 32P-labeled product was hydrolyzed by the yeast inorganic pyrophosphatase which is absolutely specific for PPi, in the presence of Mg2+ (22). In a control experiment, authentic 32PPi was added to a reaction mixture without glutamine synthetase present (lane 3, Fig. 2A) and then was treated with inorganic pyrophosphatase (lane 4, Fig. 2A) in the same way as was the enzyme reaction product. After inorganic pyrophosphatase treatment (lanes 2 and 4, Fig. 2A), all of the radioactive appears at the solvent front as 32P.

Further identification of products of the AMP-supported reaction catalyzed by unadenylylated glutamine synthetase was from ion exchange chromatography on Dowex 1-Cl- (Fig. 3). Elution positions are shown by arrows in Fig. 3. Starting with L-[14C]glutamate and 32P, in the AMP-supported reaction, L-[14C]glutamate and 32PPi are identified as the only radioactively labeled reaction products.

In AMP-supported reactions with L-glutamine and 32P, radioactivity was not adsorbed to any significant extent by charcoal (Table I). Under the same conditions, [32P]ATP formed from L-glutamine, ADP, and 32P, (Table I) in the reverse biosynthetic Reaction 1 is adsorbed by charcoal (12). The isolated 32P-labeled product of Fig. 3 represented by the hatched area also was not adsorbed to charcoal; 97 ± 2% of the radioactivity was recovered in the eluate after charcoal treatment. Therefore, a 32P-labeled nucleotide is not synthesized in Reaction 3; the reaction product 32PPi is not adsorbed...
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**Fig. 3.** Separation of the reactants and products of Reaction 3 on Dowex 1-X2 (Cl- form). Unadenylylated glutamine synthetase (0.85 mg of GS1) was incubated at 30°C (pH 7.2) in 1.0 ml of a reaction mixture containing 50 mM Hepes/KOH, 0.5 mM MnCl2, 12 mM AMP, 100 mM KCl, 15 mM L-[14C]glutamine (95 cpm/nmol), and 13 mM 32P (1000 cpm/nmol). After 2 h, EDTA (4 mM) was added; the mixture was placed on a column (0.9 X 15 cm) of Dowex 1-Cl at 4°C and the column washed with 10 ml imidazole/HCl at pH 7.3 for 20 fractions (5 ml/fraction). At Fraction 21, 10 mM potassium citrate (adjusted to pH 4.5 with HCl) was connected to the imidazole buffer reservoir (500 ml) to generate a gradient of decreasing pH and increasing ionic strength (-- --). Radioactivity in the fractions was determined by scintillation counting and is expressed in counts per minute (cpm) per ml of 14C-labeled hatched area were treated with charcoal as in Table I and 5 ± 2% of the radioactivity was recovered in the filtrate.

**Table II**

**Requirements for pyrophosphate synthesis**

A complete reaction mixture (1.0 ml) at pH 7.2 contained 40 mM Hepes/KOH, 28 to 50 mM KCl, 0.5 mM MnCl2, 12 mM AMP (lyophilized Lot 57C-7090 from Sigma Chemical Co.), 10 mM L-[14C]glutamine (freshly prepared, 95 cpm/nmol), 13 mM 32P (52 cpm/nmol), and 0.43 mg unadenylylated glutamine synthetase (GST). After 150 min at 30°C, reaction products were separated at 4°C on a Dowex 1-Cl column as in Fig. 3 and counted. The micromolar quantity of each product is an average; the number of independent determinations is given in parentheses. Results were the same using 3.8 mg of GST for 10 min at 30°C. Different components of the reaction were omitted or replaced as indicated.

| Reaction mixture | Product recovery |
|------------------|------------------|
|                  | 32PP, L-[14C]Glutamate* |
| Complete         | 38 (3) 57 (4)     |
| Minus GS1        | 0 0               |
| Minus [32P]      | 0 0               |
| Minus L-[14C]glutamine | 0 0         |
| Minus AMP        | 0 0               |
| Mg2+ (50 mM)     | 0 0               |
| GST instead of GS1 | 0 0          |

* Values are corrected for nonenzymatic hydrolysis of L-[14C]glutamine (<5%, see text).

**Fig. 4.** Time course of the AMP-dependent Reaction 3 at 30°C, measuring the synthesis of L-[14C]glutamate (Curve 1) and 32PP (Curve 2) from L-[14C]glutamine and 32P, (open symbols). The rate of L-[14C]glutamine hydrolysis (independent of PPi) synthesis at 30°C that is supported by AMP and PPi in Reaction 5 (see text) is given by Curve 3 (O). Reaction 3 mixtures at pH 7.2 contained 50 mM Hepes/KOH, 100 mM KCl, 12 mM AMP, 0.5 mM MnCl2, 15 mM L-glutamine, 13 mM P, and unadenylylated glutamine synthetase (1.75 mg of GST/ml in Curve 1; 0.087 or 0.87 mg of GST/ml in Curve 2). Reaction 5 (Curve 3) was run as in Reaction 3 with 0.125 mg P, substituted for orthophosphate. The amount of product is expressed as nanomoles of L-glutamate or formed/mg of GS; The formation of L-[14C]glutamate in Curves 1 and 3 was measured by passing a 1.0-ml portion of each reaction mixture (containing L-[14C]glutamine, 35 cpm/nmol) at the indicated time through a column (0.5 X 5 cm) of Dowex 1-Cl (in a Pasteur pipette) which had been equilibrated with 10 mM imidazole/HCl buffer at pH 7.3. L-[14C]Glutamine passed through the column and the bound L-[14C]glutamate subsequently was released into a scintillation vial by washing the column with 50 ml HCl buffer at pH 7.3. L-[14C]Glutamate values were corrected for the nonenzymatic hydrolysis of L-[14C]glutamine which was 0.2% of the total L-glutamine present under these conditions. The synthesis of 32PP in Curve 2 was measured after separation of products and reactants on PEI-cellulose thin layers (Fig. 1). At the times indicated, a 50-ul aliquot of the reaction mixture (containing 32P, 2000 cpm/nmol) was taken and the reaction stopped by EDTA addition (5 ml 40 mM EDTA) at 0°C; 20 ml then was placed in a 5 cm band on PEI-cellulose which was developed as in Fig. 1. The product migrating at the position of standard PPi was cut out and counted in Aquasol. The dashed sections of Curves 1 and 2 show extrapolations to the corresponding values of Table II.

by charcoal and accounts completely for the synthesized 32P radioactivity in the elution profile of Fig. 3. Furthermore, Fig. 2B shows that when 32PAMP and P, are substituted for AMP and 32P, in Reaction 3, 32P AMP is unchanged. (In the experiments of Fig. 2B, the addition of NH4OH produces Reaction 2 in which AMP and P, are not consumed.)

**Reaction 3 Requirements and Apparent Stoichiometry**—For the data of Table II, L-[14C]glutamine was used as soon as possible after preparation (see "Materials and Methods") and reactions were stopped by immediate chromatography on Dowex 1-Cl at 4°C (Fig. 3). Nevertheless, the nonenzymatic hydrolysis of L-[14C]glutamine (estimated from controls without enzymes or P,) was 3% of the total glutamine present in these experiments. When a smaller bed volume of Dowex 1-Cl was used (Figs. 4 and 5, below), the nonenzymatic hydrolysis of L-[14C]glutamine was reduced to 0.2%. Even storage of L-[14C]glutamine frozen (without intermediate thawing) produced ~0.2% hydrolysis/24 h. Prusiner and Milner (17) also observed an instability of L-glutamine. All L-[14C]glutamate quantities in Table II are corrected for the nonenzymatic hydrolysis of L-[14C]glutamine.

Table II shows that the AMP-supported synthesis of PP, and L-glutamate from P, and L-glutamine catalyzed by unadenylylated glutamine synthetase has absolute requirements for P, L-glutamine, AMP, and Mn2+ (50 mM MgCl2 substituted...
for Mn<sup>2+</sup> produced no reaction). The reaction also is specific for the unadenylated form of glutamine synthetase, since the substitution of the fully adenylylated enzyme at an even higher concentration did not produce detectable 32PPi synthesis (Table II). The concentration of KCl (28 to 130 mM) in Reaction 3 appeared to be unimportant.

Isolation of the products of the AMP-supported reaction with both substrates radioactively labeled (Table II) consistently gave a higher value for L-[14C]glutamate than for 32PPi formation. This deviation from the anticipated exact coupling (as indicated in Reaction 3) is real. An explanation arose from the experiments of Fig 4 and does not involve an uncertainty in the value of L-[14C]glutamate synthesized due to appreciable nonenzymatic hydrolysis of L-[14C]glutamine.

The time course for the formation of each L-glutamate and PPi in Reaction 3 is shown in Fig. 4 (Curves 1 and 2, respectively). At early times (<20 min), there is an exact correspondence between the amounts of L-glutamate and PPi formed. Initially, therefore, there is a coupling between hydrolysis of the amide bond of L-glutamine and synthesis of the pyrophosphate bond. After about 20 min at 30°C, Curves 1 and 2 of Fig. 4 show different time extrapolations. Extrapolations from about 90 min to the data in Table II for 150 min are shown by the dashed portions of Curves 1 and 2 (Fig. 4). The data are internally consistent and indicate that more L-glutamate than PPi is formed at ~30 to 150 min. These data suggested that in addition to Reaction 3, a secondary reaction occurred at the later times which involved L-glutamate hydrolysis independent of PPi synthesis. Such a secondary reaction could be initiated by an accumulation of synthesized PPi if PPi binds to the unadenylated subunit. Hydrolysis of L-glutamate (with K<sub>a</sub> > 4 x 10<sup>3</sup> M<sup>-1</sup> in the presence of 15 mM Pi) to form an [enzyme-AMP-PP]<sub>i</sub> complex that can catalyze the hydrolysis of L-glutamine in a reaction related to Reaction 4. This latter possibility was tested (Curve 3, Fig. 4) by measuring the rate of L-glutamine hydrolysis in a reaction mixture containing the unadenylated enzyme, Mn<sup>2+</sup>, AMP, PPi, and L-[14C]glutamine, with the amount of PPi being about 4-fold the concentration of unadenylated subunits. Hydrolysis of L-glutamine was observed. The rate of this secondary reaction (Curve 3, Fig. 4) and the accompanying inhibition of PPi synthesis in Reaction 3 approximately account for the difference between Curves 1 and 2 at the later times in Fig. 4. This new reaction may be written:

L-glutamate + H<sub>2</sub>O<sup>-</sup>[Mn<sub>2+</sub>-GS-AMP-PP]<sub>i</sub>→ L-glutamate + NH<sub>4</sub><sup>+</sup> (5)

where the unadenylated subunit (GS) complex [Mn<sub>2+</sub>-GS-AMP-PP]<sub>i</sub> is involved in catalyzing L-glutamine hydrolysis. The reaction rate in Curve 3 (Fig. 4) is 15% of that of the initial L-glutamate or PPi synthesis in Curves 1 and 2 of Fig. 4.

Reversal of Pyrophosphate Synthesis in Reaction 3—The reversal of Reaction 3 at pH 7.2 could be demonstrated when the unadenylated enzyme was incubated with high concentrations of L-glutamate (80 to 200 mM) and NH<sub>4</sub>Cl (200 mM) in the presence of 1.0 mM 32PPi, 12 mM AMP, and 0.5 mM MnCl<sub>2</sub>. Under these conditions, hydrolysis of 32PPi to 32P was observed (Table III). The reverse Reaction 3 required AMP and Mn<sup>2+</sup>; no hydrolysis of 32PPi was observed in this reaction when 10 mM MgCl<sub>2</sub> was substituted for 0.5 mM MnCl<sub>2</sub> (Table III).

Nucleotide-supported Arsenolysis Reaction 4—Fig. 5 shows the rates of ADP- and AMP-supported arsenolysis reactions catalyzed by the unadenylated, manganese enzyme. Note that L-glutamine hydrolysis is linear with time in Reaction 4, which is not the case for the reverse biosynthetic Reaction 1 (13) or for PPi synthesis in Reaction 3 (Fig. 4). The relative rate of the AMP- to the ADP-supported Reaction 4 is ~0.5 which is about the same ratio as observed in Reaction 2 (Table III). GMP and IMP (at 12 mM concentration) are ~30% as effective as AMP in supporting Reaction 4 (Fig. 5). GMP and IMP also support the γ-glutamyl transfer Reaction 2, although K<sub>m</sub> values are higher for GMP and IMP than that for AMP. In addition, GDP and IDP are substrates in the reverse biosynthetic reaction (13).

AMP, ADP, and Divalent Cation Support in Reactions 1 to 4—Table III summarizes activity data for unadenylated glutamine synthetase in Reactions 1 to 4 so that the activities in AMP- and ADP-supported reactions with either Mn<sup>2+</sup> or Mg<sup>2+</sup> present may be compared. Note that the AMP-supported PPi synthesis in Reaction 3 with Mn<sup>2+</sup> present is only 1.5% of the rate of ATP synthesis in the reverse biosynthetic Reaction 1. The hydrolysis of PPi in the reverse Reaction 3 at pH 7.2 occurs at ~36% of the rate of the forward direction, with no attempt being made here to optimize the concentrations of reactants in either direction of Reaction 3. When P<sub>i</sub> in Reaction 3 is replaced by arsenate, the AMP-supported, irreversible arsenolysis Reaction 4 is ~100-fold faster than PPi synthesis in Reaction 3 and is one-half the rate of the ADP-supported arsenolysis of L-glutamine. In forming the product γ-glutamylhydroxamate in Reaction 2 with arsenate present, NH<sub>4</sub>OH increases the rates (relative to rates in Reaction 4) of both the AMP- and ADP-supported reactions 100-fold.

It is of interest that the unadenylated enzyme can not catalyze either direction of Reaction 3 with Mg<sup>2+</sup> substituted for Mn<sup>2+</sup> (Table III). Moreover, Mg<sup>2+</sup> is unable to support Reaction 2 (and insignificantly Reaction 4) when AMP is the

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*E. R. Stadtman and P. Z. Smyrniotis, unpublished data.*
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**TABLE III**

| Reaction (in text) | Products measured | Nucleotide and divalent cation support |
|-------------------|------------------|--------------------------------------|
|                   |                  | AMP | ADP |
| Glu, PPi          | Mn²⁺ Mg²⁺ Mn⁺⁺ Mg⁺⁺ |
| (Reverse 3)       | 1.6 0            |
| Glu, ATP          | 0.65 0          |
| (Reverse 1)       | 1.1 12          |
| Glu               | 200 0.7 410 46  |
| (Arsenolysis)     |                  |
| 2 (With P)        | γ-Glu-NH₂OH⁻     |
| 2 (With arsenate) | γ-Glu-NH₂OH⁻     |

| TABLE IV |

**Simultaneous binding of [³²P]AMP and [¹⁴C]ADP to unadenylated glutamine synthetase**

Equilibrium dialysis was performed as described previously (14). Equilibration of 2 to 6 mm of unadenylated glutamine synthetase (GS) per ml was for 29 to 50 h at ~25°C in buffers at pH 7.2 containing 20 to 40 mM Tris/HCl, 100 mM KCl, and 1.0 mM MnCl₂. Free concentrations of nucleotides are given; enzyme subunit M, = 50,000 (5).

| Unadenylated glutamine synthetase and effectors present | Equivalents of [³²P]AMP bound/subunit | Equivalents of [¹⁴C]ADP bound/subunit |
|--------------------------------------------------------|---------------------------------------|-------------------------------------|
| GS; +0.5 mM AMP³²⁺                                    | 0.7                                   | 0.8                                 |
| GS; +0.07 mM ADP¹⁴⁺                                    | 0.7                                   | 0.7                                 |
| GS; +0.5 mM AMP + 0.04 mM ADP                         | 0.7                                   | 0.7                                 |
| GS; with 10 mM L-Glutamine                           | +1 mM AMP                             | 0.7                                 |
| GS; with 10 mM L-Glutamine                           | +1 mM AMP + 0.15 mM ADP              | 1.1                                 |
| GS; with 10 mM P,                                    | +1 mM AMP                             | 0.6                                 |
| GS; with 10 mM P,                                    | +1 mM AMP + 0.15 mM ADP              | 0.1                                 |

*For AMP, Kᵦ = 4 x 10⁻³ M⁻¹; [³²P]AMP = 160 to 600 cpm/nmol.*

supporting nucleotide (see also Ref. 1). In contrast, unadenylated glutamine synthetase specifically requires Mg⁺² in the biosynthetic reaction 1 (3, 4, 13) and catalyzes the reverse biosynthetic reaction 4-fold faster with Mg⁺² than with Mn⁺² present (Table III).

**Nucleotide Binding Sites**—The equilibrium dialysis results given in Table IV show that there are separate binding sites for ADP and for AMP on each subunit of the unadenylated, manganese enzyme. In the absence of other substrates, ~0.7 equivalent/subunit of each [³²P]AMP and [¹⁴C]ADP can be simultaneously bound. In obtaining these data, it was observed that the binding of AMP was somewhat slower in the presence than in the absence of ADP; longer equilibration times were required in these cases. Nevertheless, the data of Table IV are consistent with each subunit binding ADP at the catalytic site and binding AMP at an allosteric inhibitor site. A site for AMP distinct from the catalytic site has been suggested previously from other data (6, 8, 14). In addition, AMP binds with equal affinity to the inactive, divalent cation-free enzyme and to the active, manganese or magnesium enzyme (14), whereas Mn⁺² (or Mg⁺²) is required for tight binding of ADP (1) or ATP (12) to the subunit catalytic site.

Since AMP supports Reactions 2 to 5, it is possible that AMP binds to the ADP site of the subunit under these reaction conditions in which L-glutamine and Pₐ, arsenate, or PP, are present. Alternatively, it is possible that AMP supports these reactions by binding to a noncatalytic site, since AMP is unchanged (Fig. 2B). When 10 mM L-glutamine was present in the binding experiments of Table IV, both AMP and ADP were bound simultaneously to the enzyme subunit. However, orthophosphate, which is synergistic to the binding of ADP (13), is antagonistic to the binding of AMP when ADP is bound also to the subunit catalytic site. These results indicate that AMP is almost totally excluded from binding in the allosteric site of the subunit when ADP and P, are bound to the catalytic site. In the absence of ADP, orthophosphate apparently has little effect on AMP binding (Table IV). In this case, however, Pₐ may direct the binding of AMP to the ADP site. Whatever binding sites are involved, the stoichiometry of AMP binding to glutamine synthetase does not exceed 1 equivalent of AMP bound/enzyme subunit (Table IV, Ref. 13) which could be due to mutually exclusive binding at two sites.

The AMP binding site at the subunit catalytic site has a rather broad specificity for the purine base, since ADP, GDP, and IDP are phosphorylated in the reverse biosynthetic Reaction 1 (13). Since AMP, GMP, and IMP (substituted for ADP with the unadenylated, manganese enzyme) support Reactions 2 and 4 (Fig. 5), it appears likely that these 5'-nucleotide monophosphates bind to the ADP site when L-glutamine and orthophosphate or L-glutamine and arsenate are present. Recall also (Fig. 1) that with varying amounts of ADP and AMP in mixtures, ATP synthesis in the reverse Reaction 1 was apparently competitive with PP, synthesis in Reaction 3. With low ratios of ADP to AMP, both products ([³²P]ATP and [³²P]PP, were seen (Fig. 1), whereas high proportions of ADP only produced [³²P]ATP.

**DISCUSSION**

Unadenylated glutamine synthetase from *E. coli* has been discovered to catalyze a reversible, AMP-dependent synthesis of pyrophosphate and L-glutamate from orthophosphate and L-glutamine in the presence of Mn⁺². In this reaction, Mg⁺² cannot substitute for Mn⁺² and the fully adenylylated enzyme is inactive. Reactions 2 to 5 relate directly to the mechanism of enzyme-catalyzed synthesis of L-glutamine in Reaction 1 and will be discussed from this viewpoint.

If we label potential phosphate binding sites at the catalytic site of each subunit according to the α,β, and γ-phosphate positions occupied by ATP, pyrophosphate synthesis presumably occurs at the β,γ-phosphate positions. The Mn⁺² ion at the n₀ site which is involved in chelating ADP + P₀, or ATP at the catalytic site (1, 9, 23) would complex phosphates at the β,γ positions (24). We conclude that the nucleotide site of the enzyme subunit must be occupied either by ADP (GDP or IDP) or by AMP (GMP or IMP) for synthesis of the β,γ-phosphate bond to occur. The energy of pyrophosphate bond synthesis is coupled to that released by the hydrolysis of L-glutamine. However, Reaction 3 is not energetically favored since the free energy of formation of the PP, bound is ~6.4 kcal at pH 7.0, 0.5 mM Mg²⁺ (25) and the hydrolysis of L-glutamine only releases 3.4 kcal (26). The reversal of L-glutamine synthesis in Reaction 1 is even less favorable (15) with the comparable free energy of formation of the β,γ-phosphate bond of ATP = 7.9 kcal (25).

In pyrophosphate synthesis with AMP bound to the nucleotide site, there is no α,β-phosphate bond. If ADP occupies this site (with an intact α,β-phosphate bond), the synthesis of
the $\beta,\gamma$ phosphate bond to form ATP in the reversal of L-glutamine synthesis in Reaction 1 is ~70-fold faster than when the $\alpha,\beta$-phosphate bond is missing. Apparently, ADP + P, are better able than are AMP + 2 P, to promote the correct conformation for coupling $\beta,\gamma$-phosphate bond synthesis to L-glutamine hydrolysis. The structure of the nucleotide binding site of the enzyme subunit would be expected to be more flexible when AMP and P fill this site, recalling also that an additional oxygen and accompanying negative charges must be accommodated in this case.

The $\alpha,\beta$-phosphate bond of ADP also is not required for the arsenolysis Reaction 4 or for the $\gamma$-glutamyl transfer Reaction 2 (1), since AMP can replace ADP in either Mn$^{2+}$-supported reaction catalyzed by unadenylylated glutamine synthetase. When arsenate is substituted for P, in the AMP-supported Reaction 3, the reaction rate is increased 100-fold (Reaction 4) and then is approximately one-half of the reaction rate of the ADP-supported irreversible arsenolysis of L-glutamine (Table III). In studies with the pea seedling enzyme, Levintow and Meister (15) noted the close relationship of the arsenolysis reaction to $\gamma$-glutamyl transfer and reversal of L-glutamine synthesis. They suggested that when P, is replaced by arsenate in the reversal of L-glutamine synthesis, the reaction is displaced toward L-glutamate formation as a result of forming the unstable arsenate intermediate, which undergoes spontaneous hydrolysis. The rate of the transfer Reaction 2 with either AMP or ADP is 100-fold faster than that of arsenolysis (Reaction 4) because hydroxyxalamine is a much better nucleophilic attacking agent than is water (Table III).

The $^{18}$O studies of Varner et al. (27) and Rhee et al. (13) show that the oxygen of either orthophosphate (in the reversal of Reaction 1) or arsenate (in the ADP-supported arsenolysis Reaction 4) is transferred to L-glutamate. (This oxygen transfer from P, agrees with that determined earlier (28, 29) for the enzymatic synthesis of L-glutamine in which the $\gamma$-carboxyl oxygen of L-glutamate is transferred to P,.) Thus, P, and arsenate apparently have identical roles in the $\gamma$-glutamyl transfer Reaction 2 (as suggested earlier (15)) with both anions binding to the same site and forming enzyme-bound intermediates with L-glutamate (13, 30). It is reasonable to assume that a similar transfer of oxygen from P, or arsenate to L-glutamate occurs in the AMP-supported Reactions 2 through 4.

In Reactions 2 and 4, the nucleotides AMP and ADP probably occupy the same site on the enzyme subunit. This site has a low specificity for the purine base (13) which is true also for the brain enzyme (31). Substrates bind randomly to the E. coli enzyme (1, 13). However, a marked synergism in binding has been observed between Mn$^{2+}$ and substrates (1, 19, 23, 32, 33), between ATP and L-glutamate (34), between ADP and arsenate (1), and between ADP and P, (13). In contrast, the binding of AMP appears not to be influenced by the presence of P, (Table IV), although it is likely that P, directs the binding of AMP to the ADP site while blocking the binding of AMP to the subunit allosteric site.

The requirements for Mn$^{2+}$ and for the unadenylylated form of glutamine synthetase in Reactions 2 through 4 are interesting. In the biosynthetic Reaction 1, the unadenylylated enzyme is very active with Mg$^{2+}$ (but inactive with Mn$^{2+}$) as the supporting divalent cation (3). Adenylation of the protein converts glutamine synthetase to a form that requires Mn$^{2+}$ in Reactions 1 and 2 (3) and that has a lowered affinity for ADP and arsenate in Reaction 2 (1). Rhee et al. (13) proposed that the inability of Mn$^{2+}$ to support the biosynthetic activity of the unadenylylated enzyme in Reaction 1 is, in fact, due to the very high affinity (with corresponding slow off rates) that this enzyme form has for ADP ($K_D = 20$ nm) and P, ($K_D = 80$ nm). The unadenylylated, manganese enzyme does catalyze the reversal of L-glutamine synthesis in Reaction 1, although this reaction rate at pH 7 is 4-fold slower than with Mg$^{2+}$ present (Table III, Ref. 13). Since Mn$^{2+}$ supports ATP synthesis with the unadenylylated enzyme in the reverse Reaction 1 and since Mg$^{2+}$ binds P, and PP, as well as does Mn$^{2+}$, it is peculiar that Mn$^{2+}$ can not substitute for Mn$^{2+}$ in the AMP-supported Reactions 2 through 4 catalyzed by the unadenylylated enzyme. This suggests that in the presence of substrates, the conformation of the unadenylylated enzyme with Mn$^{2+}$ or with Mg$^{2+}$ differs in such a way that only the Mn$^{2+}$ form is able to catalyze the AMP-dependent synthesis of PP, in Reaction 3. The divalent cation requirement is linked to the nucleotide support since Mg$^{2+}$ can be substituted for Mn$^{2+}$ in the AMP-supported transfer and arsenolysis Reactions 2 and 4 catalyzed by the unadenylylated enzyme (4, 13).

The adenylated, manganese enzyme catalyzes Reaction 1 in both directions and also the ADP-supported $\gamma$-glutamyl transfer Reaction 2 (3). However, this enzyme form does not catalyze the AMP-dependent synthesis of PP, in Reaction 3 or the AMP-supported transfer Reaction 2 (1, 4). Thus, the Mn$^{2+}$-promoted conformations of the unadenylylated and adenylylated enzymes must differ when substrates are present. Recent evidence indicates that this is the case (35).

The role of divalent cations in the catalytic mechanism of glutamine synthetase from E. coli has been the subject of much study (1, 3, 5, 19, 23, 32, 33, 36-38). It has been shown that two Mn$^{2+}$ sites per enzyme subunit must be filled for activity expression in the $\gamma$-glutamyl transfer Reaction 2 (1). As already mentioned, Mn$^{2+}$ at the lower affinity ($n_1$) site complexes with the nucleotide at the catalytic site (1). The role of Mn$^{2+}$ at the high affinity ($n_2$) site is structural as well as serving a possible catalytic function. The binding of Mn$^{2+}$ to $n_2$ sites of the dodecamer promotes a conformational change from an inactive (relaxed) to an active (tightened) form (3-5). NMR (23, 36), EPR (33, 36-38), and equilibrium binding (32) data are consistent with the Mn$^{2+}$ ion at the subunit $n_2$ site also binding the $\gamma$-carboxyl group of L-glutamate, possibly with a highly immobilized water molecule intervening between Mn$^{2+}$ and the $\gamma$-carboxyl group. A Mn$^{2+}$ interaction with L-glutamate could serve to orient and polarize the $\gamma$-carboxyl group in Reaction 1 for its attack on the $\gamma$-phosphorus of ATP (28, 29), which is followed by nucleophilic attack by ammonia on the $\gamma$-glutamyl phosphate intermediate (30). It is noteworthy that Mn$^{2+}$ at the subunit $n_1$ site does not interact with L-glutamate (1, 19, 32). With the transition state analog L-methionine-S-sulfoximine phosphate and ADP irreversibly bound to the enzyme subunit (2, 30), both the $n_1$ and $n_2$ metal ions are locked tightly into the subunit structure (32).

The major reaction pathway of L-glutamine synthesis in Reaction 1 (reviewed by Meister (30) catalyzed by glutamine synthetase from E. coli) seems analogous to that catalyzed by the brain enzyme (30). This pathway involves the formation and utilization of enzyme-bound $\gamma$-glutamyl phosphate intermediate whose structure is analogous to that of L-methionine-S-sulfoximine phosphate (2, 30). Meister (30) has summarized the evidence from his laboratory for formation of the $\gamma$-glutamyl phosphate intermediate in L-glutamine synthesis. In addition, Todhunter and Purich (40) have obtained chemical evidence that $\gamma$-glutamyl phosphate is formed during the synthesis of L-glutamine catalyzed by glutamine synthetase from E. coli. The similarity in the partial reactions catalyzed by glutamine synthetases from different sources (2, 3, 13, 30) and the interaction of the different glutamine synthetases with the transition state analog L-methionine-S-sulfoximine (2, 30, 32, 35-38) suggests that the catalytic sites of
AMP-supported Reactions Catalyzed by Glutamine Synthetase

these enzymes are quite similar.5

Despite the similarities in catalytic mechanism between glutamine synthetase from E. coli and glutamine synthetases from other sources, there are distinct differences. The enzyme from E. coli is a dodecamer (3, 5) rather than an octamer (30) and is regulated by adenylylation-deadenylylation (3, 4, 10) which alters the divalent cation requirement and the affinity for nucleotide in Reaction 1. Nevertheless, the AMP-supported reactions catalyzed by the unadenylylated, manganese sources for nucleotide in Reaction 1. Nevertheless, the AMP-supported reactions catalyzed by glutamine synthetases from otheramp rather than ADP occupies the substrate nucleotide site.

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5 A related enzyme, γ-glutamylcysteine synthetase from rat kidney, also generates PPi, but in this case, the reactants are ATP and P1 in a partial reaction in which L-glutamate is inhibitory (41). Although the synthesis of PP is not of physiological significance in the reactions catalyzed by glutamine synthetase and γ-glutamylcysteine synthetase, Wood et al. (42) have reviewed other instances in which the bond energy of PP, is utilized.