Mechanistic Studies of Glutamine Synthetase from *Escherichia coli*. Fluorometric Identification of a Reactive Intermediate in the Biosynthetic Reaction†

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ABSTRACT: Conformational changes that occur in unadenylylated glutamine synthetase from *Escherichia coli* during the biosynthesis reaction have been investigated by a fluorometric method. The results disclose formation of a highly reactive enzyme-bound intermediate from ATP and L-glutamate. In the absence of NH_4^+, this intermediate will slowly decompose to ADP, P_i, and pyrrolidone carboxylate. The observed rates of the biosynthetic reaction indicate that the formation of this intermediate is a prerequisite for the reaction of NH_4^+ to form products and the turn over rate is limited by the release of products from the enzyme. Fluorescence changes in the presence of various inhibitors generally correlate exactly with their known inhibitory effects toward glutamine synthesis. These fluorescence changes can be used to determine the binding constants of various substrates and inhibitors to the enzyme. A number of binding constants, so determined, are reported.

In recent years a number of attempts have been made to elucidate the mechanistic details involved in the catalytic reactions of glutamine synthetase (Tate and Meister, 1973; Wedler and Boyer, 1972). The *Escherichia coli* enzyme is known to catalyze several different reactions (see recent review by Stadtman and Ginsburg, 1974; Meister, 1974). One of these reactions of physiological significance (usually referred to as the biosynthetic reaction) involves the synthesis of L-glutamine from L-glutamate.

$$NH_4^+ + ATP + \text{L-glutamate} \xrightarrow{Mg^{2+}} ADP + P_i + \text{L-glutamine}$$

Succinct outlines of the current state of knowledge on the above reaction along with detailed descriptions of the other chemical and physical properties of this enzyme have been presented in a number of recent reviews (Tate and Meister, 1973; Stadtman and Ginsburg, 1974; Ginsburg and Stadtman, 1973; Wohlhueter *et al.*, 1973; Ginsburg, 1972).

Despite numerous studies of the biosynthetic reaction, the exact reaction mechanism is uncertain. There are at least two schools of thought on this topic. Meister and coworkers (Weisbrod and Meister, 1973; Rowe *et al.*, 1970) have obtained considerable evidence in support of a two-step process in this reaction. The first step involves the formation of γ-glutamyl phosphate from ATP and L-glutamate as a distinct reaction intermediate. In the second step, addition of NH_4^+ results in amide formation coupled with displacement of the phosphoryl group. Evidence which was cited in support of the formation of a discrete γ-glutamyl phosphate intermediate is the observation of significant pyrrolidone carboxylate formation in the absence of added ammonia (Weisbrod and Meister, 1973). The pyrrolidone carboxylate formation was obtained with both adenylylated and unadenylylated enzyme. However, the formation of pyrrolidone carboxylate does not exclude the formation of an enzyme bound transition intermediate where chemical bonds are only partially formed. In addition, Tsuda *et al.* (1971) have suggested the formation of an acyl phosphate intermediate in studying reactions of cycloglutamate with sheep brain enzyme.

Employing kinetic isotope exchange techniques, Wedler and Boyer 1972) found that glutamine synthetase containing an average of ten adenylyl groups per mole does not catalyze a detectable ADP ⇌ ATP, P_i ⇌ ATP, glutamate ⇌ glutamine, or NH_3 ⇌ glutamine exchanges unless *all* substrates are present. These findings appear inconsistent with a discrete two-step mechanism and indicate rather that a random substrate addition mechanism is operative. From their results, Wedler and Boyer favor a concerted reaction mechanism in which all substrates must be present at the active site before any bond breaking can take place.

In this paper we report results of some fluorescent studies with unadenylylated glutamine synthetase from *E. coli*. Since the unadenylylated enzyme has an absolute requirement for magnesium ion, fluorescence changes associated with the addition of Mg^{2+} and the subsequent addition of various substrates to the enzyme were measured. The results provide further insights into the mechanistic details of this "biosynthetic" reaction.

Experimental Section

Materials. The preparation of glutamine synthetase ($E_{1.0}$) (the subscript indicates the average number of adenylylated subunits per dodecamer) was isolated using the procedure developed by Woolfolk *et al.* (1966) from *E. coli* grown in a medium containing 20 mM NH_4Cl and 0.67 M glycerol. The state of adenylylation of this enzyme preparation was determined both spectroscopically (Shapiro and Stadtman, 1970) and by γ-glutamyl transfer assay (Stadtman *et al.*, 1968) and found to be 1.0 ± 0.2. The specific activity of the purified enzyme was determined using the modified procedure of Ginsburg *et al.* (Ginsburg *et al.*, 1970) and the results obtained agree well with published values for the purified enzyme.

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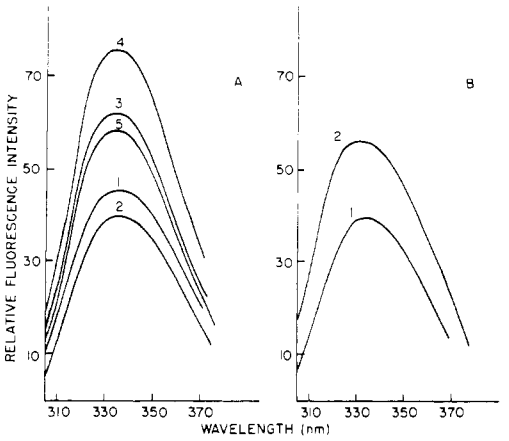


FIGURE 1: Comparison of the fluorescence changes obtained from the addition of reactants (A) and products (B) to glutamine synthetase. The reaction was carried out in 50 mM Hepes buffer (pH 7.20) containing 0.1 M KCl at 25°. Excitation wavelength was 300 nm. (A) Curve 1, fluorescent intensity of 12 μM (subunit) relaxed glutamine synthetase ($E_{1,0}$); curve 2, like curve 1, + 15 mM Mg^{2+}; curve 3, like curve 2, + 1.5 mM ATP; curve 4, like curve 3, + 20 mM L-glutamate; curve 5, like curve 4, + 20 mM NH_4^+. (B) Curve 1, fluorescent intensity of 12 μM (subunit) relaxed glutamine synthetase ($E_{1,0}$) + 15 mM Mg^{2+}; curve 2, like curve 1, + 1.5 mM ADP, 1.5 mM P_i, and with or without 1.5 mM glutamine.

The analog of ATP, 5′-adenylylimide diphosphate (AMP-P-N-P) and [^{14}C]-L-glutamic acid were purchased from International Chemical Nuclear. [^{3}H]Ethanol was obtained from New England Nuclear Corp. The other reagents namely, L-glutamic acid, D-glutamic acid, NaATP, NaADP, L-methionine (*SR*)-sulfoximine, L-methionine sulfone, L-methionine *dl*-sulfoxide, and L-glutamic acid γ-methyl ester, were obtained from the Sigma Chemical Co.

Methods. Fluorescence measurements were made using a Hitachi-Perkin-Elmer MPF-2A instrument equipped with a Hewlett-Packard 7004B X-Y recorder. Constant temperature was maintained using thermostated cell holders and constant temperature circulating baths. A Cary 17 spectrophotometer equipped with thermostated cells was used for absorption spectra measurements and for the biosynthetic enzyme assays.

Unless it is specified, all fluorescent experiments were carried out at 25° and a pH of 7.2. The solutions were buffered with 50 mM Hepes and contained 0.1 M KCl. In addition, special care was taken to remove trace amounts of ammonia in the enzyme (Pamiljans *et al.*, 1962). Redistilled deionized water was used. The amount of NH_3 present in the system is negligible as indicated by the fact that only a negligible amount of [^{14}C]glutamine is formed when enzyme is added to [^{14}C]glutamate, ATP, and Mg^{2+}.

Equilibrium dialysis experiments were carried out in homemade Lucite cells similar to those described previously (Englund *et al.*, 1969). The membrane used was Union Carbide dialysis tubing which had been boiled in 5% Na_2CO_3–50 mM EDTA, pressure stretched to increase its porosity and stored in 50% ethanol at 4°. The samples were withdrawn from the cells with Hamilton syringe for counting in Bray's solution. In all solutions, 20 μM of ^{3}H-labeled ethanol was added and the counts due to ^{3}H were used to correct for the error from dilution. The enzyme concentrations used for these experiments are in the range of 3–5 mg/ml. The experiments were performed at 4° and a pH of 7.2 Hepes solution containing 0.1 M KCl. A Beckman LS-250 liquid scintillation system was used for counting.

Results

Fluorescence Changes Induced by Reaction Substrates. Fluorescence emission spectra were recorded using an excitation wavelength of 300 nm with the emission spectra showing a maximum at 336 nm. At this excitation wavelength, the emission noted can be ascribed solely to the tryptophan residues in the enzyme of which there are three or four per subunit (Woolfolk *et al.*, 1966). Figure 1A shows the fluorescence changes due to successive addition of Mg^{2+}, ATP, L-glutamate, and NH_4^+ to the relaxed enzyme (divalent metal ion free). The magnitude of the changes provoked by each of these substrates is dependent on the concentration until saturation is achieved. (At saturation level, one enzyme subunit bound with one molecule of added ATP and L-glutamate.) For the curves shown in Figure 1A, each of the substrates was present in an amount sufficient to saturate the enzyme. The addition of Mg^{2+} results in a fluorescence decrease, followed by large increases upon the additions of ATP and L-glutamate. Subsequent addition of NH_4^+ produces a sharp decrease in fluorescence. Although the total fluorescence enhancement by ATP plus L-glutamate is always constant for saturating amounts of each substrate, the relative enhancement of each is somewhat dependent on the Mg^{2+} concentration with the relative contribution of ATP to the total increase being more pronounced at lower Mg^{2+} concentrations.

The addition of ADP and P_i to Mg-glutamine synthetase also produces fluorescence increases as shown by curve 2, Figure 1B. The amounts of ADP and P_i added to obtain curve 2 are comparable to those produced by the reaction taken place in Figure 1A, with the assumption that all the ATP was being converted to ADP and P_i. The similarity between curve 2 in Figure 1B and curve 5 in Figure 1A suggests that the sharp fluorescence decrease noted upon the addition of NH_4^+ to the reaction mixture is due to the formation of ADP and P_i from ATP. The P_i level used in Figure 1B is in subsaturating quantity (K_d is 5 mM). Under this condition, addition of 1.5 mM glutamine to either the Mg-E-ATP or Mg-E-ADP-P_i complex, an amount which is equivalent to the total glutamine formed from the above NH_4^+ addition (but significantly less than the K_m value which is 7 mM), produces essentially no change in enzyme fluorescence.

When the sequence of substrate addition to the enzyme is varied, some interesting changes are noted. No enzyme fluorescence change was observed when glutamate was added to the Mg^{2+}-enzyme complex. However, the subsequent addition of ATP produces a total fluorescence increase identical with that shown in curve 4 of Figure 1A resulting from the sum of ATP and glutamate fluorescence increases. Thus it is clear that either glutamate does not bind (or binds very weakly) to the enzyme-metal complex in the absence of ATP or the binding of glutamate to the enzyme in the absence of ATP does not produce a conformational change that effects the fluorescence intensity. Utilizing the flourescence enhancement produced due to glutamate binding to Mg-E-ATP, titration was carried out to determine the binding constant. It was evaluated from the modified Scatchard type plot

$$\frac{[\text{glutamate}]_0}{\bar{X}} = [E]_0 + K_{diss}\left(\frac{1}{1-\bar{X}}\right) \qquad (1)$$

where $\bar{X}$ is defined as $\bar{X} = \Delta F/\Delta F_T$, ΔF_T is the total fluorescence change at saturation and ΔF is the fluorescence

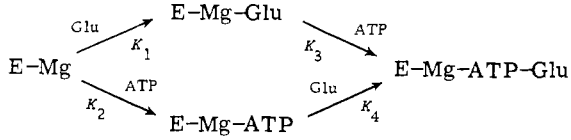
TABLE I: Results of the Fluorometric Titrations Used to Determine Binding Constants for Various Substrates and Inhibitors.[a]

	K_{diss} (mM)
ATP + relaxed enzyme	0.56
ATP + enzyme-$(Mg^{2+})_n$	0.26
ATP + enzyme-$(Mg^{2+})_n$-glutamate	0.04
L-Glutamate + enzyme-$(Mg^{2+})_n$-ATP	3
L-Methionine (*SR*)-sulfoximine + enzyme-$(Mg^{2+})_n$	2.6[b]
L-Methionine (*SR*)-sulfoximine + enzyme-$(Mg^{2+})_n$-ATP	0.05[b]
L-Methionine *dl*-sulfoxide + enzyme-$(Mg^{2+})_n$-ATP	0.6
P_i + enzyme-$(Mg^{2+})_n$-ADP	5.0

[a] All measurements are at pH 7.2, 25°, 50 mM Hepes buffer, and 0.1 M KCl. [b] These values refer to binding constants using unknown mixtures of the *SR* isomers of L-methionine sulfoximine. Since it has been shown that only one isomer is effective in inhibiting the reaction (Weisbrod and Meiser, 1973), the K_{diss} obtained in the present studies will be larger than that for the pure active inhibitor. However, the differences in dissociation constants in the presence and absence of ATP is real and the ratio of these values should be the same if only pure active isomer (L-methionine (*S*)-sulfoximine) were employed.

change obtained for a specific L-glutamate concentration. $[E]_0$ is the total enzyme concentration, and K_{diss} is the dissociation constant for the complex. A plot of $[\text{L-glutamate}]_0/\bar{X}$ *vs.* $1/(1-\bar{X})$ gives a straight line with K_{diss} as slope and $[E]_0$ as intercept. The binding constants for glutamate and a number of other substrates determined by this method are given in Table I.

The K_{diss} for L-glutamate from Mg-E-ATP is 3 mM. In the presence of 10 mM Mg^{2+} and absence of glutamate a K_{diss} of 0.26 mM is obtained for ATP which is in agreement with that derived from equilibrium dialysis data (Ginsburg, 1972). However, the presence of glutamate decreases K_{diss} for ATP by about sixfold. This suggests a mutual enhancement of ATP and glutamate binding to the E-Mg complex as shown below. If this scheme is correct, $K_1K_3 = K_2K_4$;



from which it can be calculated that glutamate will bind to the E-Mg complex, with a dissociation constant of ~20 mM. In addition, with glutamate dissociation constants of 3 mM (Table I) and 20 mM for the E-Mg-ATP-Glu and E-Mg-Glu complex, respectively, explains our failure to obtain these constants by equilibrium dialysis method. Other attempts to evaluate the binding constant of L-glutamate to glutamine synthetase in the absence of ATP have failed (Ginsburg *et al.*, 1970; Meister, 1974). It is worth pointing out that E-Mg-ATP-Glu decomposes slowly to ADP, P_i, and pyrrolidone carboxylate. This observation is in agreement with the finding of Weisbrod and Meister (1973).

In contrast to the fact that no change in fluorescence is caused by addition of L-glutamate to the enzyme-Mg^{2+}

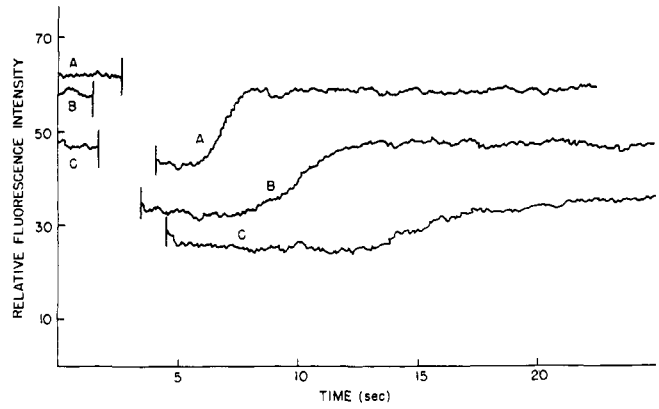


FIGURE 2: Demonstration of the effect of successive additions of limiting amounts of NH_4^+ to a reaction mixture containing glutamine synthetase (12 μM subunit), Mg^{2+} (20 mM), ATP (2.3 mM), L-glutamate (20 mM), and KCl (0.1 M) in 50 mM Hepes buffer (pH 7.20) at 25°. Excitation wavelength was 300 nm. The reaction was followed by the change in fluorescence at 336 nm. Curve A, addition of 0.67 mM NH_4^+; curve B, addition of another 0.67 mM NH_4^+; curve C, addition of a third 0.67 mM NH_4^+. The breaks in each of the curves above represents the time required to add each aliquot of NH_4^+ and to stir the mixture.

complex, addition of high concentrations (with a K_{diss} of about 30 mM) of L-glutamate to the relaxed enzyme results in a very slow fluorescence increase. This slow increase in fluorescence is also observed when L-glutamate is added to relaxed enzyme-ATP complex. The formation of this divalent metal ions free enzyme-ATP complex is also accompanied by an increase in fluorescent intensity of the protein. A qualitative scheme reflecting the protein fluorescence changes when ATP and L-glutamate are added to the relaxed enzyme is shown in Figure 5.

It is of interest to note that the titration of relaxed enzyme with Mg^{2+} shows that there are at least two distinct binding constants of Mg^{2+} on glutamine synthetase. The saturation of the first binding site produces a small quenching of the tryptophan fluorescence intensity. The Mg^{2+} concentration required to saturate the first binding site of a 12 μM enzyme solution is about 35 μM. No further fluorescence change is observed until Mg^{2+} concentration exceeded 100 μM. However, the changes in the ultraviolet absorption spectrum associated with the binding of Mg^{2+} to the enzyme under these experimental conditions shows that 100 μM of Mg^{2+} is enough to saturate at least the first binding site (Hunt and Ginsburg, 1972). It appears that second (and, possibly additional) Mg^{2+} bindings are considerably weaker and it is not possible to determine these binding constants from fluorescence changes since the fluorescence decrease appears to continue indefinitely with the added Mg^{2+}. Nevertheless, these fluorescence experiments clearly demonstrate that there is a small but significant fluorescence decrease associated with the binding of Mg^{2+} to the highest affinity sites on the enzyme.

Time-Dependent Fluorescence Changes during Reaction. Utilizing the sharp fluorescence decrease due to addition of NH_4^+ to E-Mg-ATP-Glu complex, one can follow the rate of NH_4^+ reaction. When limiting quantities of NH_4^+ was added to a solution containing large excesses of Mg^{2+}, ATP, and L-glutamate, it produces a fast initial decrease in fluorescence which is then followed by a slow increase in fluorescence intensity toward the pre-NH_4^+ addition level. Continued additions of small aliquots of NH_4^+ produces a repetition of the above effect except the rate for returning

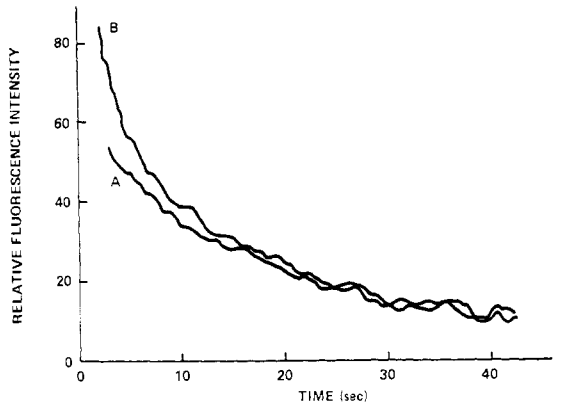


FIGURE 3: Demonstration of the effect of varying the last substrate added on the rate of fluorescence decrease under conditions of limited amounts of ATP. The final reaction mixture containing enzyme (3.6 μM subunit); Mg^{2+} (12 mM), ATP (0.6 mM), L-glutamate (13 mM), NH_4^+ (3.3 mM), and KCl (0.1 M) in 50 mM Hepes buffer (pH 7.20) at 25°. The reaction was followed by the change in fluorescence at 336 nm. Curve A represents fluorescence decay when either ATP or L-glutamate is the last reactant added. Curve B is the decay curve obtained when NH_4^+ is added last.

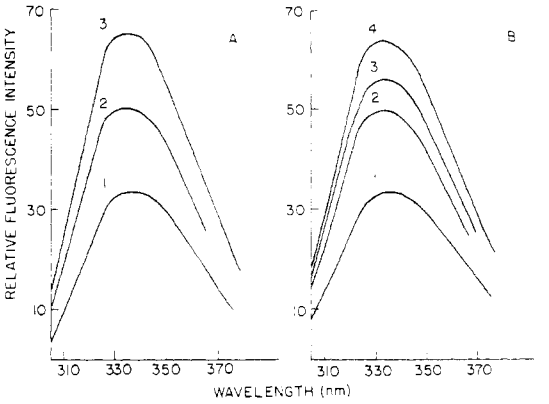


FIGURE 4: Fluorescence changes obtained with L-methionine (*SR*)-sulfoximine in the absence (A) and presence of L-glutamate (B) when added to enzyme-Mg^{2+}-ATP complex. The reaction was carried out in 50 mM Hepes (pH 7.20) containing 0.1 M KCl at 25°. Excitation wavelength was 300 nm. (A) Curve 1, 12 μM (subunit) of glutamine synthetase + 20 mM Mg^{2+}; curve 2, like curve 1, + 1.5 mM ATP; curve 3, like curve 2, + 1.5 mM L-methionine (*SR*)-sulfoximine. (B) Curve 1, 12 μM (subunit) of glutamine synthetase + 20 mM Mg^{2+}; curve 2, like curve 1, + 1.5 mM ATP; curve 3, like curve 2, + 8 mM L-glutamate; curve 4, like curve 3, + 1.5 mM L-methionine (*SR*)-sulfoximine.

to the high fluorescence state becomes slower as the amount of products being cumulated. Furthermore, the amplitude of this increase becomes progressively less as the extent of reaction increases. This effect is demonstrated in Figure 2. Eventually, a point is reached where the further addition of NH_4^+ has no effect on the fluorescence level. This point is achieved when either of the substrates ATP or L-glutamate have been consumed.

When stoichiometric limiting quantities of NH_4^+ were added to a reaction mixture containing glutamine synthetase (12 μM subunit), Mg^{2+} (20 mM), ADP (0.5 mM, 1 mM), ATP (2.3 mM), and L-glutamate (20 mM), it was found that the time required to rebuild the high fluorescence complex is longer and the amplitude of the fluorescence increase is less than that obtained in the absence of ADP. The rate and amplitude of fluorescence increase after the NH_4^+ addition is inversely proportional to the concentration of ADP added. This effect observed with ADP was found to be more pronounced when both ADP and P_i were present in the reaction mixture.

Finally, a series of experiments were carried out to determine if there are significant differences on the rate of reaction if the order of addition of substrate is varied. Three consecutive experiments were carried out in which the final component added was either ATP, glutamate, or NH_4^+. In each case, the reaction mixture, minus the last component, was allowed to incubate for 5 min and all reaction concentrations were the same in each experiment. The concentrations employed were enzyme (3.6 μM subunits), Mg^{2+} (12 mM), ATP (0.6 mM), L-glutamate (13 mM), and NH_4^+ (3.3 mM). Thus the ATP was the limiting reactant. It was found (Figure 3) that when NH_4^+ is added last, a fast initial rate is observed followed by a relatively slower rate. This slower rate is essentially the same as that obtained when either L-glutamate or ATP is added last. These results suggest that a highly reactive intermediate which consists of enzyme, Mg^{2+}, ATP, and L-glutamate is formed, and the formation of this intermediate has to be completed before NH_4^+ can react to give products. However, the turn over rate probably is limited by the products release from the enzyme.

Effect of the ATP:Mg^{2+} Ratio on the Fluorescence Level. At glutamine synthetase concentrations of 50 μM (subunit), [ATP] = 6.5 mM and [Mg^{2+}] = 2.5 mM, the concentration of free Mg^{2+} is $\leq$13.5 μM, based on an Mg^{2+}-ATP dissociation constant of 5×10^{-5} M. Under these conditions, addition of NH_4^+ had no effect on the fluorescence level. However, when the concentration of total Mg^{2+} was increased to 2.9 mM so that the free Mg^{2+} was 16 μM, the biosynthetic reaction was found to occur, as indicated by the decrease in fluorescence intensity. Thus a relatively small increase in the free Mg^{2+} exerts a very marked effect on the fluorescence changes. The concentration of free Mg^{2+} required for reaction agrees qualitatively with the results of the binding of the first Mg^{2+} to the enzyme measured by fluorescence changes as mentioned above.

In contrast, there appears to be no specific requirement for *free* ATP. At ratios of Mg^{2+}:ATP as high as 23 (10 mM Mg^{2+}, 0.43 mM ATP), the fluorescence decrease with the addition of NH_4^+ is still observed, even though the free ATP concentration was only 2.2 μM. Therefore ATP is probably bound to the taut enzyme surface as MgATP complex. This complex could be derived from a random addition of Mg^{2+} and ATP or direct MgATP binding to the enzyme.

The Use of Other Substrates and Inhibitors. Addition of the ATP analog 5'-adenylylimide diphosphate (AMP-P-N-P) to the enzyme-Mg^{2+} complex resulted in a fluorescence increase identical with that produced by ATP. However, in sharp contrast with the ATP system, the subsequent addition of L-glutamate produced no fluorescence enhancement. Furthermore, the final addition of NH_4^+ had no effect on the fluorescence intensity. Thus it is clear that the highly fluorescent and reactive complex obtained when ATP and L-glutamate are added to the enzyme-Mg^{2+} complex is not produced when AMP-P-N-P is employed, even though the AMP-P-N-P appears to provoke the same initial conformational change as ATP when added to the metal-enzyme system. The structural analog of glutamine, L-methionine (*SR*)-sulfoximine, was shown by Meister and coworkers (Ronzio *et al.*, 1969; Weisbrod and Meister,

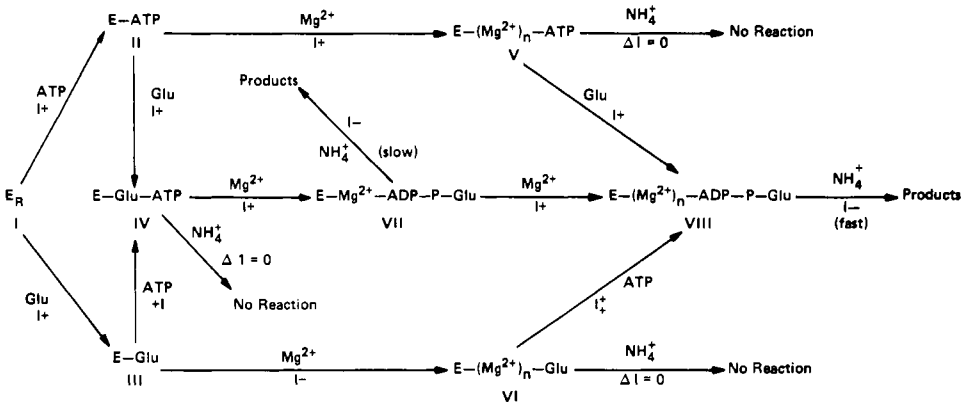


FIGURE 5: Schematic diagram showing the successive addition of the various substrates to relaxed glutamine synthetase. If the addition of a substrate resulted in a fluorescence change then the change in the conformational state of the enzyme is denoted by a Roman numeral. The notations I+ and I– are used to represent fluorescence intensity increases and decreases, respectively. Except for II and IV, all ATP represents MgATP complex.

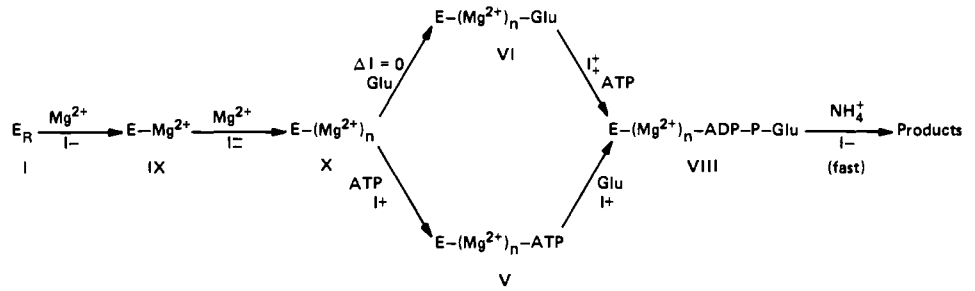


FIGURE 6: Schematic diagram of the fluorescence changes observed when substrates are added to the Mg^{2+} activated glutamine synthetase. (See Figure 5 for the meaning of notations.)

1973) to be a potent inhibitor of the glutamine synthetase from *E. coli* and mammalian tissue. We found that addition of L-methionine (*SR*)-sulfoximine to the E-Mg^{2+}-ATP complex results in a very marked fluorescence increase. Fluorescence titration measurements showed the binding of this molecule to be very tight with a K_{diss} of 0.05 mM which agrees with the findings of Weisbrod and Meister (1973). Addition of L-glutamate does not produce any further fluorescence increase and the addition of NH_4^+ has no immediate effect on the fluorescence intensity. If the order of addition of L-glutamate and L-methionine (*SR*)-sulfoximine is reversed, one observed the usual increase with glutamate addition to the E-Mg^{2+}-ATP complex, followed by an additional small increase when L-methionine (*SR*)-sulfoximine is added (Figure 4). The result is consistent with a displacement of L-glutamate by the L-methionine (*SR*)-sulfoximine and the small fluorescence increase upon sulfoximine addition corresponds to the fact that the fluorescence intensity of the L-methionine (*SR*)-sulfoximine-ATP-Mg^{2+}-enzyme complex is slightly higher (20%) than the L-glutamate-ATP-Mg^{2+}-enzyme complex. The fact that L-glutamate must be displaced by the L-methionine (*SR*)-sulfoximine is further demonstrated by the lack of any fluorescence changes with added NH_4^+ when the L-methionine (*SR*)-sulfoximine is present.

The addition of L-methionine (*SR*)-sulfoximine to a Mg^{2+}-enzyme complex in the absence of ATP also causes an increase in fluorescence. However, it was found that this binding is much weaker than in the presence of ATP. The K_{diss} obtained was 2.6 mM. Thus, in comparison with L-glutamate, L-methionine (*SR*)-sulfoximine binding to the Mg^{2+}-enzyme complex is much tighter than that of L-glutamate both in the presence and absence of ATP. The detailed studies by Weisbrod and Meister (1973) have shown that the effective isomer with respect to this inhibition is the L-methionine (*S*)-sulfoximine isomer.

Experiments were carried out with a number of other substrates and inhibitors and the fluorescence changes observed agree well with literature predictions based on assay experiments (Ronzio *et al.*, 1969). For example, L-methionine sulfone binds tightly to the enzyme-Mg^{2+}-ATP complex producing a large fluorescence increase. In the absence of ATP, a fluorescence increase is also obtained; however, the binding of the sulfone alone is much weaker than in the presence of ATP. Addition of up to 20 mM L-glutamate to the mixture containing saturating amounts of Mg^{2+}, ATP, and L-methionine sulfone produced no fluorescence change. Finally, the addition of NH_4^+ also produced no fluorescence changes. Thus, it is clear that the normal fluorescence sequences we observe with L-glutamate and NH_4^+ addition are inhibited by the presence of L-methionine sulfone.

The above observations can be contrasted with that of L-methionine addition (up to 2 mM) which produced no fluorescence changes when added to the saturated enzyme-Mg^{2+}-ATP complex. Furthermore, added L-glutamate and NH_4^+ now produce the normal fluorescence effects and one can conclude that L-methionine is not an inhibitor in this system.

The addition of L-methionine *dl*-sulfoxide to the enzyme-Mg^{2+}-ATP complex produced a fluorescence increase and we estimate a dissociation constant of 0.6 mM for this substrate addition to the enzyme complex. Subsequent addition of 20 mM L-glutamate produced a further fluorescence increase and the addition of NH_4^+ showed that reaction was taking place. Although this experiment was done only on a semi-quantitative basis, the results do

show that L-methionine *dl*-sulfoxide binding to the enzyme-Mg^{2+}-ATP complex is considerably weaker than that of L-methionine (*SR*)-sulfoximine. This work is consistent with the inhibition studies of Ronzio *et al.* (1969) who found that L-methionine *dl*-sulfoxide is only 10% as effective as L-methionine (*SR*)-sulfoximine in inhibiting the biosynthetic reaction.

The substitution of D-glutamate in place of L-glutamate produced no fluorescence change when added to the enzyme-Mg^{2+}-ATP complex. This result is consistent with the fact that in the Mg^{2+} activated unadenylylated enzyme system, D-glutamate exhibits less than 1 or 2% of the activity shown by L-glutamate (Ginsburg *et al.*, 1970). In addition, the substitution of γ-methyl monoglutamate ester in place of L-glutamate produced no fluorescence change nor reaction inhibition. Similarly, the addition of DL-methionine produced neither fluorescence change nor inhibition.

It is noteworthy that all of the above fluorescence changes induced by various substrates as well as the lack of effect noted with other molecules correlate exactly with classical inhibition studies of Meister and coworkers. As a result, we feel justified in concluding that the fluorescence changes observed in this work reflect accurately conformational changes of the enzyme associated with its catalytic activity.

Discussion

The results shown above can best be discussed in terms of the schematic diagrams shown in Figures 5 and 6. These figures summarize the various substrate-induced fluorescence changes noted in this work. With the assumption that protein fluorescent change is reflecting a different conformational state of the enzyme, a Roman numeral is used to represent a specific form of the enzyme indicated by its fluorescent change due to the addition of a substrate. In Figure 5 we start with relaxed enzyme (I) and show the conformational sequence for L-glutamate and ATP addition in the absence of Mg^{2+}. Addition of either L-glutamate or ATP induces a fluorescence increase as shown. Note that the fluorescence level reached by ATP and L-glutamate addition is independent of the order of addition. Furthermore, the fluorescence changes in the absence of Mg^{2+} are much slower processes than in the presence of this divalent cation. The addition of NH_4^+ to this mixture (IV) produced no fluorescence change. If, instead of NH_4^+, small amounts of Mg^{2+} are added to the enzyme-glutamate-ATP complex (IV) a small fluorescence increase is observed (IV $\rightarrow$ VII). When the concentration of Mg^{2+} added is sufficient to produce enough free Mg^{2+} to saturate the first Mg^{2+} site on the enzyme (but not enough free Mg^{2+} to saturate the second site), the addition of NH_4^+ produces a slow decrease in fluorescence indicative of the overall reaction. If instead of NH_4^+, more Mg^{2+} is added, there is a significant fluorescence increase and the half-life for this conformational change is 70 sec (VII $\rightarrow$ VIII). It is noteworthy that this half-life is identical with that obtained for the change in ultraviolet spectrum induced by the addition of Mg^{2+} to relaxed enzyme (Hunt and Ginsburg, 1972).

The sequence of changes starting with relaxed enzyme and initial addition of Mg^{2+} is shown in Figure 6. A small fluorescence decrease is observed for initial low Mg^{2+} addition corresponding to saturation of the first Mg^{2+} site (I $\rightarrow$ IX). Continued addition of Mg^{2+} at much higher concentrations reduces the fluorescence to still lower levels (IX $\rightarrow$ X); however, it was not possible to determine the saturation point for these subsequent Mg^{2+} binding sites. Since the stoichiometry of Mg^{2+} bound at this state is not known, $(Mg^{2+})_n$ is used in the figure. The order of addition of L-glutamate and ATP is not required in reaching the final fluorescence level of the saturated complex (VIII). The addition of L-glutamate to the enzyme-$(Mg^{2+})_n$ complex produces no fluorescence change (X $\rightarrow$ VI). As shown, the addition of ATP to the solution containing enzyme, Mg^{2+}, and glutamate produces a large increase in fluorescence which is exactly equal to the sum of the increases obtained by subsequent addition of ATP and L-glutamate to the enzyme-$(Mg^{2+})_n$ complex. The enzyme-$(Mg^{2+})_n$-ADP-P-glutamate complex (VIII) thus formed is extremely reactive toward NH_4^+ addition as noted in the previous section.

On the basis of the experiments reported here, it is clear that a reactive intermediate is formed when Mg^{2+}, ATP, and L-glutamate are present with glutamine synthetase. Meister *et al.* have suggested that this intermediate is a γ-glutamyl phosphate. The lack of any observed fluorescence increase upon L-glutamate addition when AMP-P-N-P is substituted for ATP can be cited as support for Meister's suggestion. Due to enzyme specificity, it may not be able to break the N-P bond in AMP-P-N-P. Therefore, it prevents phosphate transfer precluding the formation of a γ-glutamyl phosphate intermediate and thus a lack of fluorescence change when L-glutamate is added. However, the finding of Wedler and Boyer (1972), who observed isotopic exchange between ATP $\rightleftharpoons$ ADP, etc., only when a complete reaction mixture is present, is certainly consistent with the formation of a transition intermediate which involves only partial bond breakage of the O-P bond. In fact, their results can be rationalized even in terms of a γ-glutamyl phosphate intermediate if one assumes that both ADP and γ-glutamyl phosphate formed are enzyme bound. Such an explanation had been used to rationalize the results obtained in the synthesis of arginosuccinate (Rochovansky and Ratner, 1961, 1967) in which ATP $\rightleftharpoons$ PP_i exchange is obtained only when all substrates are present. In addition, Krishnaswamy *et al.* (Krishnaswamy *et al.*, 1962) also suggested an enzyme-bound activated glutamate derivative is formed by reaction of sheep brain glutamine synthetase with ATP and glutamate in the presence of Mg^{2+}.

Actually, we would like to point out explicitly, that the results from all three laboratories are consistent with the formation of a highly reactive intermediate from enzyme-Mg^{2+}-ATP and L-glutamate but it is not necessary that this intermediate involve the complete transfer of a phosphate group to the glutamate. In the absence of added NH_4^+, this intermediate cyclizes slowly leading to pyrrolidone carboxylate (Weisbrod and Meister, 1973). However, if NH_4^+ is added, the intermediate is destroyed producing discrete molecules of ADP. When AMP-P-N-P is employed in place of ATP an unfavorable geometry may obtain due to enzyme specificity and this condition prevents the formation of either γ-glutamyl phosphate or a reactive transition intermediate.

Obviously we cannot distinguish between the two possibilities of complete phosphate transfer as opposed to the formation of a transition intermediate involving partial bond formation between L-glutamate and ATP with the fluorometric method used. And, in reality, this distinction is really not all that important. However, the formation of a reaction intermediate clearly indicates that a sequential mechanism is operative in this reaction.

Finally, we feel that the fluorescence studies are consis-

tent with the requirement for the formation of an intermediate, and the turn over rate is limited by the release of products. This conclusion is based on the identical long term (*i.e.*, >5 sec) fluorescence decay regardless of which substrate is added last (Figure 3). The most likely product to be released last is ADP since it exhibits the highest affinity for the enzyme among the products. Unfortunately, the initial fluorescence changes are, in many cases, too fast to be measured by the techniques employed in this work. In order to obtain accurate specific rate constants for various steps, a faster technique such as stopped-flow methods is required. Such studies are currently in progress.

When L-methionine (*SR*)-sulfoximine, L-methionine sulfone, or L-methionine *dl*-sulfoxide is added to the E-$(Mg^{2+})_n$-ATP complex, a fluorescent enhancement similar to that produced by L-glutamate addition is observed. These results are consistent with the findings reported by Rowe *et al.* (1969) on sheep brain enzyme where they suggested the formation of the phosphate compound of methionine derivatives.

In summary, the use of fluorescence methods provides an unusually detailed picture of conformational changes in an enzymatic system in the case of glutamine synthetase and the biosynthetic reaction. The fluorescence changes observed can be related to conformational changes involving the active center of the enzyme. Many of the fluorescence changes discovered in this study can be used to determine the substrates or inhibitors binding constants. The binding constants for various substrates have been measured and reported in Table I. The presence of ATP has been found to exert a marked influence on the binding of L-glutamate and inhibitors to the enzyme, in each case the presence of ATP results in the formation of a much tighter complex.

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