Conformational Changes in Glutamine Synthetase from *Escherichia coli*. II. Some Characteristics of the Equilibrium Binding of Feedback Inhibitors to the Enzyme*

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ABSTRACT: The equilibrium binding of each of the feedback inhibitors, adenosine monophosphate and L-tryptophan, to glutamine synthetase from *Escherichia coli* has been studied. Both inhibitors bind to the extent of 12 moles/600,000 g of enzyme or 1/subunit and the binding of each appears to be independent of the state of adenylylation of native glutamine synthetase. The binding sites for adenosine monophosphate are roughly equivalent (independent) with an apparent intrinsic association constant of ~8000 and appear to involve hydrophobic rather than electrostatic forces in the specific binding mechanism. The binding of adenosine monophosphate is not influenced by the presence of or absence of protein-bound Mn^{2+} or Mg^{2+}, even though Mn^{2+} or Mg^{2+} is required for stabilization of the active taut enzyme form. In contrast to the binding characteristics of adenosine monophosphate, the L-tryptophan binding to glutamine synthetase (with either Mn^{2+} or Mg^{2+}) is cooperative. A Hill coefficient and an interaction free energy have been estimated at ~2.5 and ~700 cal, respectively, with half-saturation occurring at 0.91 mM L-tryptophan. Equilibrium binding measurements of [^{32}P]adenosine monophosphate in the presence of high and low concentrations of L-[^{14}C-methylene]tryptophan show that the binding of L-tryptophan is not significantly influenced by a low level of adenosine monophosphate. However, the binding of adenosine monophosphate, for which the enzyme has the higher affinity in the absence of other effectors, is markedly decreased by L-tryptophan. This effect is enhanced with Mg^{2+} rather than Mn^{2+} present. Glutamate at a concentration approximately equal to the K_m for this substrate with Mg^{2+}, but not Mn^{2+}, also decreased the affinity of one enzyme preparation for adenosine monophosphate. Probably the number of adenosine monophosphate binding sites is not decreased by glutamate and Mg^{2+} since the affinity decrease alone accounts for the discrepancy between the binding and kinetic data. Possibly, the substrate ammonia has an analogous effect on the affinity of glutamine synthetase for adenosine monophosphate in the presence of Mn^{2+}. Kinetic responses to adenosine monophosphate have been shown to vary with the extent of adenylylation of the enzyme as a function of substrate concentrations and other assay conditions. Substrates or L-tryptophan appears to induce a negative interaction between the enzyme and adenosine monophosphate. The complexity of the response of glutamine synthetase to inhibitors and the interrelationship of substrate to inhibitor binding is further illustrated by the fact that the binding of another feedback inhibitor, L-alanine (with Mn^{2+} or Mg^{2+}), could be detected only in the presence of the substrate glutamine.

The glutamine synthetase of *Escherichia coli* has been shown by Woolfolk and Stadtman (1964, 1967a) to be susceptible to multiple feedback inhibition by products of glutamine metabolism in the cell. Inhibitors of glutamine synthetase were found to include L-tryptophan, AMP, CTP, glucosamine 6-phosphate, carbamyl phosphate, and L-histidine, the biosynthesis of which utilize the amide nitrogen of glutamine. Other inhibitors include glycine, serine, and alanine, which may arise through a transamination reaction involving glutamine (Meister, 1962; Woolfolk *et al.*, 1966; Stadtman *et al.*, 1968a). The cumulative inhibition pattern observed with the addition of combinations of these inhibitors at saturating levels suggested that each inhibitor acts independently in partially inhibiting the enzyme (Woolfolk and Stadtman, 1964, 1967a; Stadtman *et al.*, 1968a). Inhibition that is partially competitive with different substrates was observed with some of the inhibitors (Woolfolk and Stadtman, 1967a), and this fact complicates the analysis of the inhibitor–protein interactions (Stadtman *et al.*, 1968a).

Another mechanism for the regulation of glutamine synthetase from *E. coli* involves adenylylation and deadenylylation of this enzyme under certain conditions of growth (Kingdon and Stadtman, 1967b; Heilmeyer *et al.*, 1967; Holzer *et al.*, 1968). Enzymes catalyzing the adenylylation (Kingdon *et al.*, 1967; Wulff *et al.*, 1967; Stadtman *et al.*, 1968b) and deadenylylation (Shapiro and Stadtman, 1968a; Battig *et al.*, 1968; Shapiro, 1969) have been studied *in vitro*. Glutamine synthetase may be adenylylated to the extent of 12 5′-adenylyl groups/enzyme molecule of 600,000 molecular weight or 1 equiv/subunit (Kingdon *et al.*, 1967). Shapiro and Stadtman (1968b) have shown more recently that the adenylyl group is covalently bound to

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the polypeptide structure of glutamine synthetase through a stable phosphodiester bond to a specific tyrosyl hydroxyl group.

The adenylylation of glutamine synthetase dramatically changes the catalytic properties of the enzyme. The divalent cation specificity is changed from Mg^{2+} to Mn^{2+}, with the adenylylated enzyme form being intrinsically less active with Mn^{2+} than the unadenylylated enzyme with Mg^{2+} (Kingdon *et al.*, 1967; Kingdon and Stadtman, 1967b). The subunits appear to act independently in catalysis, with the divalent cation requirement dictated by the presence of or absence of covalently bound AMP (Stadtman *et al.*, 1968b). These aspects of the catalytic changes induced by adenylylation are discussed in greater detail in the accompanying paper which is concerned with the binding of divalent cations (Denton and Ginsburg, 1969) to glutamine synthetase. The studies of Kingdon and Stadtman (1967a,b), Kingdon *et al.* (1967), and Shapiro *et al.* (1967) concurrently demonstrated that adenylylation of glutamine synthetase changes the feedback inhibition pattern of this enzyme. When preparations of relatively nonadenylylated and adenylylated enzymes were compared, it was found that the γ-glutamyl transfer activity of the former was not inhibited by histidine, AMP, CTP, or tryptophan whereas the latter enzyme is markedly inhibited by these compounds (Kingdon and Stadtman, 1967a; Shapiro *et al.*, 1967). The responses of enzymes containing 1.2 and 12 equiv of AMP to effectors in the biosynthetic assay were observed by Kingdon *et al.* (1967) to be more complex and not necessarily predictable from the γ-glutamyl transfer assay experiments. It has become obvious that the superimposed control mechanisms for glutamine synthetase further complicate the interpretation of the previously observed feedback inhibition patterns.

Physical and chemical studies (Woolfolk *et al.*, 1966; Shapiro and Stadtman, 1967; Valentine *et al.*, 1968; Shapiro and Ginsburg, 1968) have revealed that glutamine synthetase is composed of 12 like, if not identical, subunits of about 50,000 molecular weight which are molecularly arranged in two superimposed hexagonal arrays. In solution, the native (*taut*) protein containing bound Mn^{2+} behaves as an essentially spherical particle. The particle density was found to be a function of the adenylylation of the enzyme, as was possibly the sedimentation and viscosity behavior (Shapiro and Ginsburg, 1968). Removal of specific divalent cations converts the taut into a *relaxed* enzyme which is catalytically inactive (Kingdon *et al.*, 1968), is more susceptible to dissociation (Woolfolk and Stadtman, 1967b) or reaction and inactivation with organic mercurials (Shapiro and Stadtman, 1967), and has 12–24 exposed tyrosyl and tryptophanyl residues (Shapiro and Ginsburg, 1968). The extent of adenylylation did not appear to influence the taut–relaxed enzyme interconversion in the physical–chemical studies (Shapiro and Ginsburg, 1968); however, subtle effects of adenylylation on this interconversion are implicated from the Mn^{2+} binding studies (Denton and Ginsburg, 1969).

This paper describes some of the characteristics of the binding of the inhibitors AMP, L-tryptophan, and L-alanine to preparations of glutamine synthetase which only vary in primary structure according to the extent of adenylation. Some effects of activating divalent cations on the inhibitor binding have been studied in these cases. A later paper will present studies on the binding of substrates to this enzyme. The very intimate interrelationship between inhibitor and substrate binding will be evident.

Materials and Methods

Reagents. The sodium salts of ATP and AMP, *p*-mercuriphenylsulfonate, and γ-glutamyl hydroxamate were obtained from the Sigma Chemical Corp. and of phosphoenolpyruvate from Boehringer Mannheim Corp. The disodium salt of DPNH was supplied by the California Corp. for Biochemical Research. Other nucleosides and nucleotides were obtained as the sodium salt from P-L Biochemicals Inc.; chromatographically pure L-alanine or other amino acids were obtained from Mann Research Laboratory Inc. or Nutritional Biochemicals Corp., respectively. Uniformly labeled [^{14}C]AMP and L-[^{14}C]alanine were obtained from New England Nuclear Corp.; [^{32}P]AMP and L-[methylene-^{14}C]tryptophan were supplied by Nuclear-Chicago. Compounds for equilibrium dialysis studies were determined to be >95% pure in non- and isotopically labeled compounds. Amino acids were analyzed by the technique of Dreyer (1960), using high-voltage electrophoresis in 4% formic acid and development with the cadmium ninhydrin reagent (Blackburn, 1965) after locating radioactivity with Vanguard Model 880 Autoscanner. Nucleotides were analyzed by descending paper chromatography in isobutyric acid–1 N NH_4OH (10:6) and radioactivity located as above or by exposure on X-ray film.

Stock solutions of imidazole (Eastman Organic Chemicals) were treated lightly with charcoal and filtered to remove any yellow color. Deionized water with a conductivity $\leq 1.7 \times 10^{-6}$ ohm^{-1} was obtained from a water deionizing unit (Model DJ-128) of the Crystal Research Laboratory, Inc., and this water was used exclusively for the preparation of solutions.

Glutamine synthetase as isolated from *E. coli* by the procedure of Woolfolk *et al.* (1966) contains varying equivalents of covalently bound AMP to the protein. The enzymes designated as $E_{\overline{9}}$ and $\overline{1.2}$[1] have an average of 9 and 1.2 equiv, respectively, of AMP covalently bound to the enzyme molecule, and these have been described by Shapiro *et al.* (1967) and were generous gifts of Drs. Bennett M. Shapiro and Henry S. Kingdon, respectively. The preparation $E_{\overline{2.3}}$, with an average of 2.3 AMP groups/molecule, was a gift of Dr. M. D. Denton of this laboratory, and it is described in the accompanying manuscript (Denton and Ginsburg,

[1] $E_{\overline{n}}$, glutamine synthetase preparations with an average of $\overline{n}$ equivalents of covalently bound 5′-adenylyl groups; PMPS, *p*-mercuriphenylsulfonate.

1969). All enzymes preparations show >95% homogeneity by disc gel electrophoresis and ultracentrifugation (Shapiro and Ginsburg, 1968).

The glutamine synthetase preparations are stored at 4° as crystals or precipitates suspended in ~50% saturated ammonium sulfate which are collected by centrifugation as needed. Prior to equilibrium dialysis studies, enzyme solutions were dialyzed exhaustively three 1000× volumes of imidazole buffer (pH 7.4–7.5) containing 1 mM Mn^{2+} or Mg^{2+}, with or without 0.1 M KCl, over a 3-day period at 4°) and were clarified by centrifugation. Dialyzed enzyme solutions (>0.2%) are stable at 4° for many months.

Protein concentrations were obtained from absorbancy measurements at 280 mμ as described elsewhere (Shapiro and Ginsburg, 1968). For the determination of protein concentrations in the presence of [^{32}P]AMP, [^{14}C]AMP, or L-[methylene-^{14}C]tryptophan, radioactivity measurements, and the molar extinction coefficients of the isotopically labeled compound were used to correct the spectra. The spectral constants for AMP were obtained from Morell and Bock (1954), and those for L-tryptophan were from Beaven and Holiday (1952) and Wetlaufer (1962). These were used also for the determination of the specific activity of radioactive standards.

Glutamine Synthetase Assays. For biosynthetic activity, the spectrophotometric coupled assay system of Kingdon *et al.* (1968), which measures initial catalytic rates of ADP formation in a Mg^{2+} system, was used at ~24°. The components of this assay system in 1 ml at pH 7.1 ± 0.1 include: 50 mM imidazole chloride, 90 mM KCl, 50 mM NH_4Cl, ATP, $MgCl_2$, or $MnCl_2$, 30–100 mM sodium glutamate, 1 mM potassium phosphoenolpyruvate, 100 μg of lactic dehydrogenase, and 25 μg of pyruvate kinase dissolved in 0.1 M KCl (obtained from the Boehringer Mannheim Corp. essentially free of myokinase activity). Early studies utilized 100 mM glutamate before an inhibitory effect was found with the $E\overline{9}$ preparation.[2] Routinely, 50 mM $MgCl_2$ and 3.75 mM ATP or 0.375 mM ATP in AMP inhibition studies were used in this assay. At the lower ATP level (0.2 and 0.4 mM) the AMP kinetics are noncompetitive with respect to this substrate. Lower inhibitions with AMP are effected by raising the ATP to saturating concentrations (≥3.75 mM). Then, AMP is partially competitive with ATP, but only at high ATP levels. Therefore, the investigations of pH, different enzyme preparations, and temperature (see Results and Figure 2) effects on the AMP inhibition were studied at the low "noncompetitive" ATP level. Saturating ATP levels in this assay system were used to test for reversible inactivation of glutamine synthetase by the chelating of divalent cations with ETDA (relaxation), and the irreversible inactivation of the relaxed enzyme form produced by reaction with PMPS (Shapiro and Ginsburg, 1968).

When $MnCl_2$ was substituted for $MgCl_2$, 1 mM excess over the amount of ATP present (assuming a 1:1 ATP-Mn^{2+} complex; Perrin and Sharma, 1966) was used for the pyruvate kinase requirement (Lohmann and Meyerhof, 1934).

The γ-glutamyl transfer assay was used almost according to a procedure of Stadtman (see Stadtman *et al.*, 1968b) for differentiating between adenylylated and nonadenylylated subunits in the absence of inhibitors. This assay system utilizes limiting glutamine (20 mM) and hydroxylamine (20 mM), 40 mM imidazole buffer (instead of the mixed buffer system of E. R. Stadtman containing 50 mM of each 2,4-dimethylimidazole, 2-methylimidazole, and imidazole[3]), 0.4 mM ADP, 20 mM potassium arsenate, and 0.3 mM Mn^{2+} ± 60 mM Mg^{2+}, with 2 ml of the "stop" ferric chloride reagent of Shapiro and Stadtman (1968a) added after the timed incubation at 37°. The pH is adjusted so that after the addition of divalent cations (and in the studies reported here, inhibitor additions) the final pH is pH 7.15 (24°). Under the original conditions of Stadtman *et al.* (1968b),[3] it was shown that 60 mM Mg^{2+} completely inhibits the γ-glutamyltransferase activity of adenylylated subunits which are otherwise active with only 0.3 mM Mn^{2+} present. Nonadenylylated subunits are active under both conditions. This assay system, which requires Mn^{2+} (Woolfolk *et al.*, 1966), was used to test the different enzyme preparations ($E\overline{2.3}$ and $E\overline{9}$) for L-tryptophan and AMP inhibitions.

Equilibrium dialysis techniques were as previously described (Ginsburg and Mehler, 1966). Suitable aliquots were removed from the protein and solvent sides simultaneously for radioactivity, protein, enzyme activity, and pH determinations. A Model PHM 25 Radiometer equipped with a scale expander and Leeds and Northrup No. 124138 microelectrode assembly was used for pH determinations. Temperature corrections were made according to that determined for the imidazole buffer (+0.02 pH unit/°C temperature decrease) by a procedure described previously (Ginsburg and Carroll, 1965). If necessary, solutions were scanned on a Cary 15 recording spectrophotometer before counting. At protein concentrations of >2 mg/ml, enzyme activities of the protein side were not significantly changed by repeated equilibrations for 72-hr periods. However, only 48-hr equilibration times were used for protein solutions at <2 mg/ml. The Mark I Nuclear-Chicago Scintillation system with the solvent of Bray (1960) was used for radioactivity

[2] Unpublished data of M. D. Denton and A. Ginsburg.

[3] It was subsequently learned that this change in buffer composition and ionic strength introduced here shifts the isoactivity pH and the empirical relationship between the adenylylation state of the enzyme determined by E. R. Stadtman in the two assay systems (±Mg^{2+}). The reader is referred to Figure 3 in Stadtman *et al.* (1968b) which also shows the inverse relationship between the extent of adenylylation and activity in the Mg^{2+} assay system. For this reason, the results with the $E\overline{2.3}$ (but not $E\overline{9}$) preparation at pH 7.15 show a slightly greater specific activity in the Mg^{2+} assay system, and under these conditions the results in the 0.3 mM Mn^{2+} ± Mg^{2+} assays cannot be used to describe the extent of adenylylation of the $E\overline{2.3}$ preparation. It appears, however, that under these conditions adenylylated subunits are completely inhibited with 60 mM Mg^{2+} present in the transfer assay.

determinations. No significant quenching by protein was observed, and since standard solutions were adjusted to the same volume as aliquots removed from the equilibrium dialysis cells for each determination, no correction was necessary for quenching produced by the aqueous phase. In the experiments in which [^{32}P]AMP was added to [^{14}C]amino acids, two channels were used to resolve the counts from each compound; appropriate standards were used to determing the corrections, which were minimized by utilizine 8–10-fold more ^{14}C than ^{32}P counts. ^{32}P counts were first corrected for ^{14}C in that channel (<2% of the ^{14}C channel counts) and then ^{14}C counts were corrected for ^{32}P counts in that channel (23% of the ^{32}P channel counts). Sampling was repeated in these cases for duplicate values.

In all cases reported, the total counts from protein and solvent sides accounted for the amount of the radioactive compound added initially within 1–2%. As is usual, the radioactivity of the protein and solvent sides of the membrane are used to calculate total and free concentrations of the radioactive compound, respectively. Throughout these studies a molecular weight of 600,000 for the enzyme has been used to calculate molar concentrations of protein from absorbancy measurements at 280 mμ (Shapiro and Ginsburg, 1968).

In studies of L-[^{14}C]alanine binding to glutamine synthetase in which Mg^{2+} and L-glutamate were present, an anomalous situation was found in which a large number of counts were associated with the membrane. Although the protein appeared to be competing for the membrane complex (divalent cation-glutamate–alanine), there was no way to quantitate this data. If the divalent cation supplied was Mn^{2+}, this did not occur, but then the enzyme did not bind L-[^{14}C]alanine at ~5 mM concentration. It was found that L-glutamine (with Mn^{2+} or Mg^{2+}) could substitute for L-glutamate in producing binding of L-alanine, with the total counts now accounted for by protein and solvent sides. L-Glutamine was effective at 1.5 mM but lower concentrations of this compound were not tried. To avoid membrane artifacts in a Mg^{2+}–substrate–inhibitor system, a different procedure was used for the investigation of the effect of L-glutamate on AMP binding in the presence of Mg^{2+}. Instead of equilibrium dialysis, the centrifugation technique of Velick *et al.* (1953) as modified by Friedman and Colman (1967) was used. After centrifugation of 3 mg of enzyme with [^{32}P]AMP in a Spinco Model L ultracentrifuge at 39,000 rpm (4°) for 16 hr, the nonsedimented [^{32}P]AMP was determined. Appropriate corrections were made for the [^{32}P]AMP gradient produced by tubes run without protein. The siphoned off [^{32}P]AMP was counted and assayed for any nonsedimented protein (<1% correction). Since the affinity of the Mg–enzyme for AMP is markedly decreased by the presence of L-glutamate, a quantitative AMP binding curve could not be obtained by this procedure.

Analysis of the Data. The AMP binding data (see Results and Figure 1) could be expressed simply by the mass law binding expression (see Klotz, 1953)

$$\bar{\nu} = \frac{n'k_A'c}{1 + k_A'c} \quad (1)$$

where $\bar{\nu}$ is the average moles of inhibitor bound to 1 mole of protein (600,000 g), k_A' is the apparent intrinsic association constant for the binding of inhibitor at the molar concentration, c, of free inhibitor (with activity coefficients assumed to be unity) and n' is the apparent number of equivalent binding sites per mole of protein. As in the accompanying paper (Denton and Ginsburg, 1969), electrostatic corrections to the data do not appear to be applicable (see Results).

The data obtained for the binding of [^{14}C]AMP in the presence of adenine, adenosine, or GMP could be treated by the expression of Lewis and Saroff (1957) for direct competition

$$\bar{\nu}_{AMP} = \frac{n'k_1'c_1}{1 + k_1'c_1 + k_2''c_2} \quad (2)$$

If an added compound (eq 2) competes with AMP (eq 1) for identical equivalent sites (n') on the glutamine synthetase molecule, the observed value of $\bar{\nu}_{AMP}$ for the equivalents of AMP bound per protein molecule will be a function of the free concentration of the competing compound, c_2, which has an apparent association constant of k_2'', and of c_1, and k_1' for AMP. Since the binding parameters for AMP were established first in the absence of added compounds, $\Delta\bar{\nu}_{AMP}$ is used to describe the displacement or enhancement of AMP binding under different conditions

$$\Delta\bar{\nu} = \bar{\nu}_{obsd} - \bar{\nu}_{theory} \quad (3)$$

where $\bar{\nu}_{obsd}$ is the observed value of $\bar{\nu}$ and $\bar{\nu}_{theory}$ is the value of $\bar{\nu}$ calculated from eq 1 and n' and k' values with $c_2 = 0$.

The binding of L-tryptophan to glutamine synthetase was cooperative, and these data were treated in the manner suggested by Wyman (1964). L-Alanine binding to the enzyme (with L-glutamine present) also appeared to be cooperative, but in this case the affinity of the enzyme for the inhibitor was so low, and accuracy accordingly low, that no attempt was made to quantitate these data.

Results

Figure 1 shows three sets of AMP binding data for different preparations of glutamine synthetase ($E_{\overline{1\cdot2}}$, $E_{\overline{2\cdot3}}$, and $E_{\overline{9}}$ shown right to left). The abscissa is displaced with each set to resolve the data obtained with each enzyme preparation. Within experimental error, the binding data for all three enzyme preparations fit a single theoretical curve constructed from eq 1 with $n' = 12$ and $k_A' = 8000$. Thus, glutamine synthetase appears to have 12 independent and equivalent binding sites for AMP.

It is important to note especially that under the conditions of the equilibrium dialysis experiments, the extent of adenylylation does not influence the AMP binding. Initial AMP binding measurements were made on $E_{\overline{9}}$.

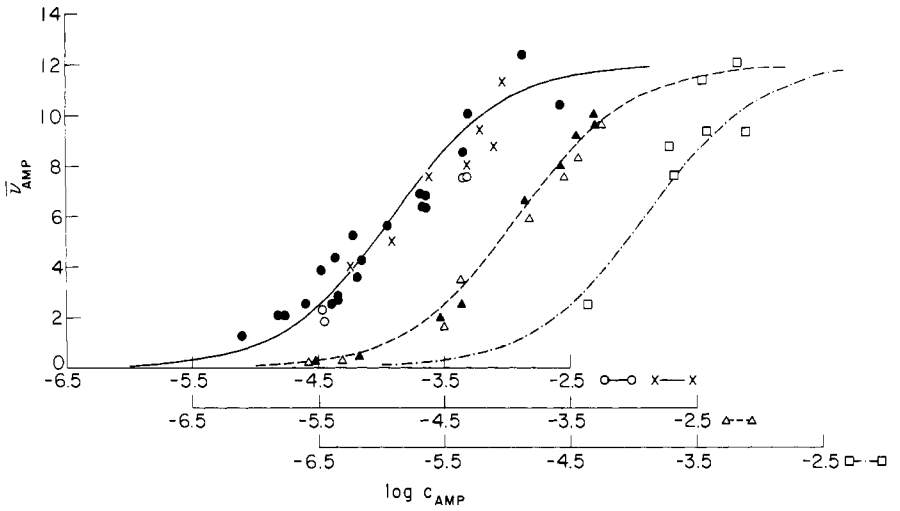

FIGURE 1: Equilibrium binding of [^{14}C]- or [^{32}P]AMP to three different preparations of glutamine synthetase at 4° and pH 7.3–7.5. The curves which are theoretical for $k_A' = 8000$ and $n = 12$ (eq 1) are displaced; left to right are points associated with measurements on $E_{\bar{9}}$ (●—●, ○—○, X—X), $E_{\overline{2.3}}$ (▲—▲, △—△), and $E_{\overline{1.2}}$ (□—□). Protein concentrations ranged from 1 to 9 mg per ml. Dialysate compositions: (a) 1 mM $MnCl_2$, 0.02 M imidazole chloride, and 0.10 M KCl (●—●, □—□); (b) same as part a but at low ionic strength without KCl (○—○); (c) relaxed $E_{\bar{9}}$ with 0.01 M EDTA in buffer a (X—X); (d) 5 mM $MnCl_2$ (▲—▲) or 50 mM $MgCl_2$ (△—△) in 0.02 M imidazole chloride–0.10 M KCl.

Subsequently, the $E_{\overline{1.2}}$ enzyme was isolated and shown by Kingdon and Stadtman (1967a,b) to markedly differ from $E_{\bar{9}}$ in many catalytic properties including a much decreased sensitivity to inhibition by AMP. It was of interest therefore to examine the AMP binding characteristics of a relatively unadenylylated enzyme preparation. Since only a small amount of the $E_{\overline{1.2}}$ enzyme was available for binding studies, more accurate and detailed measurements were made with the $E_{\overline{2.3}}$ preparation. It is apparent from the results shown in Figure 1 that the site of the covalent attachment of 5′-adenylyl groups (recently identified as a tyrosyl residue by Shapiro and Stadtman, 1968b) is distinct from the inhibitory site for AMP. This was suggested already by the inhibition studies which showed that adenylylation increases the sensitivity of the enzyme toward AMP inhibition (Kingdon and Stadtman, 1967a,b; Shapiro *et al.*, 1967). The fact that $E_{\overline{1.2}}$, $E_{\overline{2.3}}$, and $E_{\bar{9}}$ all bind 1 equiv of AMP/subunit with approximately the same affinity is more difficult to reconcile with the kinetic data. However, this problem is examined below.

The data of Figure 1 also show that the binding of AMP by glutamine synthetase is the same with either Mn^{2+} or Mg^{2+} present. Further, the relaxed, inactive enzyme (illustrated with $E_{\bar{9}}$) with 0.01 M EDTA present does not significantly differ from the native taut form in the binding of AMP. The enzyme with EDTA was shown to be in the relaxed form by being fully susceptible to organic mercurial inactivation by reaction with PMPS at pH 8 (Shapiro and Stadtman, 1967) and by being initially inactive in the time-dependent reactivation process induced by substrates in the spectrophometric assay at 24° (Kingdon *et al.*, 1968; Shapiro and Ginsburg, 1968). Then, the binding of AMP is independent of the binding of specific divalent cations. Further, the conformational change induced by specific divalent cations does not appear to influence the AMP binding site.

Early studies with the $E_{\bar{9}}$ preparation showed that increasing the ionic strength by adding 0.1 M KCl (Figure 1) did not decrease the AMP binding. A decrease in the AMP binding would be expected if the chloride anion competed with AMP at a phosphate group binding site. In fact, the binding of AMP appeared to be slightly enhanced at the higher salt concentration. Possibly, the addition of 0.1 M KCl prevents some aggregation from occurring during the dialysis which might otherwise lead to a blockage of potential AMP binding sites. Indeed, there was no effect of varying the protein concentrations from 1 to 7 mg per ml on the AMP binding with the higher salt concentration. The use of higher salt concentrations had the added advantage of stabilizing the relaxed enzyme form as a dodecameric aggregate (Shapiro and Ginsburg, 1968). Routinely then all subsequent studies were performed with 0.1 M KCl added to the 20 mM imidazole buffer.

The reduced viscosity and sedimentation coefficient of the enzyme with 0.01 M AMP present were the same as those found for the taut enzyme (see Shapiro and Ginsburg, 1968) in the absence of AMP. Also, the ultraviolet spectrum of the protein–AMP complex was simply the summation of the spectra of the protein and AMP components. This would indicate that neither component is spectrally perturbed by binding.

Table I shows the results obtained by adding various nonisotopically labeled compounds to the $E_{\bar{9}}$ preparation in the [^{14}C]AMP binding studies. The largest decreases in AMP binding, $\Delta\bar{\nu}$, were obtained when the analogs adenine, adenosine, or GMP were present. These compounds appear to be competitive for the same protein sites that bind AMP, and this result is consistent with the kinetics determined by Woolfolk and Stadtman (1967a) for inhibition by AMP plus

AMP analogs. Treatment of these data with eq 2 gave fairly good agreement in k_2'' values calculated at different concentrations of the added compound. The calculated apparent intrinsic association constants, k_2'', for adenine and adenosine were as large as that found for AMP itself, whereas k_2'' for GMP is about one order of magnitude less. Other nucleotide monophosphates appeared to have little consistent effect on the AMP binding. The effectiveness of adenine or adenosine in competing with AMP would suggest that the charged phosphate group is not important in the binding mechanism. Indeed, the fact that 0.1 M KCl did not decrease the AMP binding (see above) already suggested that the affinity for AMP was not primarily due to the charged phosphate group. The amino groups on the purine bases do appear to have a function in the binding of AMP since IMP did not bind to these protein sites.

When a mixture of feedback inhibitors, consisting of histidine, tryptophan, serine, and glycine, was added, AMP binding was only decreased by an amount observed with tryptophan alone (see below). Thus, the binding of histidine, serine, and glycine appears to be completely independent of the AMP binding which supports the previous kinetic observations of Woolfolk and Stadtman (1964, 1967a). L-Alanine alone also did not influence the AMP binding, but alanine appears to be a unique case (see below), such that it probably was not bound to the enzyme except when the substrates glutamate and ammonia were also present (see Discussion). L-Alanine with the substrates glutamate and/or NH_4Cl (with Mn^{2+}) may slightly enhance the binding of AMP by the $E_{\bar{9}}$ preparation, but this effect was not further pursued.

Another feature of the AMP binding characteristics of glutamine synthetase is illustrated in Figure 2. There is about one order of magnitude difference between the affinity of the enzyme estimated by binding and kinetic measurements. The assay conditions used to obtain the data of Figure 2 were those that gave noncompetitive inhibition kinetics with respect to ATP (see Methods). The presence of the substrates in this Mg^{2+} biosynthetic assay appear to lower the affinity of the enzyme for AMP. Further, with ATP limiting, the $E_{\overline{1.2}}$ and $E_{\bar{9}}$ preparations have identical AMP inhibition curves, which is not the case under different assay conditions (Kingdon and Stadtman, 1967a,b; Kingdon *et al.*, 1967). Lowering the temperature to 12° did not change the inhibitory properties of AMP, so it seems unlikely that the difference in affinity illustrated by curves 1 and 2 is due to the difference in temperature between the binding studies (4°) and kinetic measurements (24°).

The inhibition data fit a curve constructed from eq 1 for $n' = 12$ (zero activity) and $1/K_i' = 667$. The apparent inhibition constant, $K_i' = 1.5$ mM, was estimated from plots of reciprocal velocity *vs.* AMP concentration at low ATP levels, under which condition classical noncompetitive inhibition behavior with respect to ATP is observed (see Dixon and Webb, 1964). Complex kinetic responses were observed with the analogs of AMP shown in Table I since adenine, adenosine, ADP, and possibly GMP all are competitive with ATP too (see Woolfolk and Stadtman, 1967a).

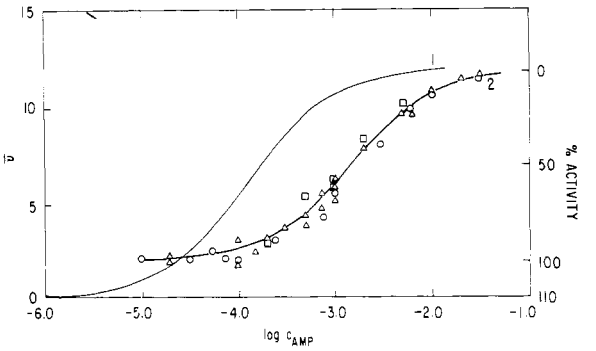

FIGURE 2: A comparison of the kinetically determined "noncompetitive" (with respect to ATP) AMP inhibition of glutamine synthetase with the AMP binding curve. Curve 1 is reproduced from Figure 1 for $\bar{v}$ and is theoretical for $k' = 8000$; $n' = 12$; curve 2 is theoretical for $k' = 667$ ($K_i = 1.5$ mM); $n' = 12$, where % activity = $100(1 - \bar{v})/12$). The per cent activity of the enzyme without added AMP was measured from initial rates by the spectrometric coupled assay at pH 7.1 (see Methods) with varying concentrations of AMP and a subsaturating concentration of 0.375 mM ATP, 50 mM $MgCl_2$, 50 mM NH_4Cl, and 100 mM glutamate. $E_{\bar{9}}$ (3–9 μg) assayed at 12.2° (△) or at 24° (○). $E_{\overline{1.2}}$ (0.5–2 μg) assayed at 24° (□). Note that the $\bar{v}$ scale from 0 to 12 is compared with the per cent activity from 110 to 0%.

The effect of pH in the range of pH 6.8–8.2 on the kinetics of the AMP inhibition of the $E_{\bar{9}}$ enzyme preparation was investigated with the same assay system also. At 0.375 mM ATP, a constant specific activity of E_9 was observed from pH 7 to 8.2. Also, the inhibition observed with 2 mM AMP present (~60%) was not a function of pH in this pH range. However, with 3.75 mM ATP the pH–activity profile of $E_{\bar{9}}$ showed a peak at pH 7.7 with a broad shoulder at pH 7.1–7.3.

Figure 3 illustrates the equilibrium binding of L-tryptophan to two preparations of glutamine synthetase ($E_{\overline{2.3}}$ and $E_{\bar{9}}$).[4] The L-tryptophan saturation curve (Figure 3a) is sigmoidal in shape with half-saturation at 0.91 mM L-tryptophan. A Hill plot of the same data (see Wyman, 1964, 1967) is shown in Figure 3b and it further illustrates the cooperativity of L-tryptophan binding to this enzyme. An interaction energy of ~700 cal with a Hill coefficient of ~2.5 can be estimated from this plot by the graphical method of Wyman (1964). In Figure 3b, the data fit assymptotes of unit slope indicating that the interaction between tryptophan binding sites is finite; the positive displacement of the assymptotes shows the cooperative or stabilizing nature of the binding with increasing tryptophan concentrations.

[4] Preliminary data for the equilibrium binding of L-[^{14}C-methylene]tryptophan to the $E_{\bar{9}}$ preparation was obtained at low L-tryptophan concentrations by Dr. Bennett M. Shapiro. It is quoted here in order to illustrate the correspondence of these data to those obtained here with the $E_{\overline{2.3}}$ preparation.

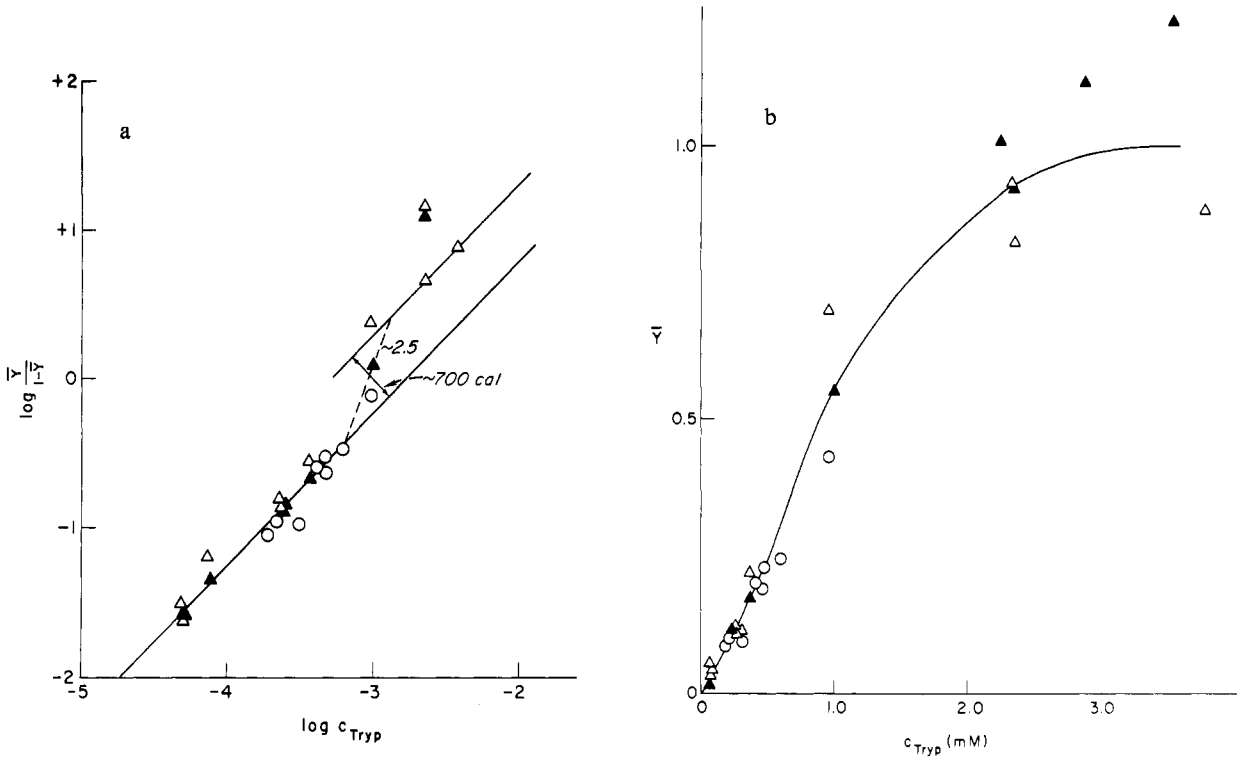

FIGURE 3: Equilibrium binding of L-[^{14}C-methylene]tryptophan to glutamine synthetase at 4° and pH 7.4–7.5. (a) Saturation curve for the binding of L-tryptophan to glutamine synthetase where $\bar{Y} = \bar{\nu}/12$ and c is the free concentration of L-tryptophan (see Methods). The data for the $E_{\bar{9}}$ preparation were obtained by Dr. B. M. Shapiro;[3] (○—○) $E_{\bar{9}}$ (10–11 mg/ml) in 0.05 M imidazole chloride, 5 mM $MnCl_2$, and 0.10 M KCl; $E_{\overline{2,3}}$ (10–12 mg/ml) in 1 mM $MnCl_2$ (▲) or 1 mM $MgCl_2$ (△), 0.02 M imidazole chloride, and 0.10 M KCl. (b) Hill plot of the data from a where $\bar{Y}/(1 - \bar{Y})$ is the fractional saturation. $N_{max} \simeq 2.5$ with the energy of interaction estimated to be 700 cal by the graphical method of Wyman (1964).

No significant differences in the L-tryptophan binding data were obtained with the two enzyme preparations. Then, the binding characteristics of L-tryptophan alone do not appear to be a function of the adenylylation state of the glutamine synthetase. At saturating L-tryptophan concentrations, the $E_{\overline{2,3}}$ preparation binds this inhibitor to the extent of 12 moles/mole of enzyme or 1 equiv of L-tryptophan/subunit. The L-tryptophan binding to the enzyme was the same with either Mn^{2+} or Mg^{2+} present.

The cooperative nature of L-tryptophan binding to glutamine synthetase is in contrast to the results obtained in the binding of AMP (Figure 1). Cooperativity in binding suggests that a conformational change of the protein is involved. However, the transition induced by L-tryptophan is not influenced by the presence of either Mn^{2+} or Mg^{2+} or by the degree of adenylylation of the enzyme. Then, AMP and L-tryptophan each bind to the extent of 12 equiv, and the binding of each inhibitor is independent of the state of adenylylation of the enzyme or of the divalent cation present.

Kinetic studies have suggested that glutamine synthetase is sensitive to the independent action of at least eight feedback inhibitors (Woolfolk and Stadtman, 1964, 1967a; Stadtman *et al.*, 1968a). Therefore, it was desirable to measure the simultaneous binding of more than one inhibitor. For experimental reasons, [^{32}P]-AMP and L-[^{14}C]tryptophan were selected as the combined inhibitors to equilibrate with glutamine synthetase ($E_{\overline{2,3}}$). The results of these studies are shown in the upper portion of Table II. Again, effects of specific divalent cations present, if any, were tested. In some other equilibrium dialysis experiments with the $E_{\overline{2,3}}$ preparation, L-[^{14}C]alanine was combined with [^{32}P]-AMP and these results are shown in the lower part of Table II.

Particular attention should be focused on the $\Delta\bar{\nu}$ values of Table II since these values represent positive or negative displacement of the number of equivalents of a particular inhibitor bound in the absence of the other as shown in Figures 1 and 3a. The observed $\bar{\nu}$ values for each inhibitor are included to indicate the extent of saturation of the enzyme with inhibitor at the specified free concentration of AMP, L-tryptophan, or L-alanine. In considering the upper date of Table II first, it is immediately apparent that whereas L-tryptophan decreases the binding of AMP, the latter effector has little or no significant influence on the binding of L-tryptophan by the enzyme. These results are even more significant if the higher affinity of the enzyme for AMP is recalled. (Half-saturation with AMP or L-tryptophan occurred at 0.125 or 0.91 mM, respectively.) In these experiments, the concentration of AMP was kept fairly constant (for $\bar{\nu} = 4.1$–4.5 in the absence of L-tryptophan), while the L-tryptophan was varied from very low to saturating concentrations. The deviations in the L-tryptophan binding data of Table II,

TABLE I: [^{14}C]AMP Binding to Glutamine Synthetase[a] in the Presence of Substrates, Inhibitors, and/or Structural Analogs.

Free AMP Concn, c_1 (mM)	Compd, c_2 Added to [^{14}C]AMP	mM[b]	$\bar{\nu}_{AMP}$[c] (obsd)	$\Delta\bar{\nu}_{AMP}$ ($\bar{\nu}_{obsd} - \bar{\nu}_{theory}$[d])	k_2''[e]
0.575	Adenine	0.515	5.4	−4.5	10,000
0.302		0.258	4.5	−4.0	12,000
0.510	Adenosine	0.503	4.4	−5.2	13,000
0.265		0.251	3.9	−4.3	14,000
0.581	GMP	1.95	6.5	−3.4	1,500
0.295		0.97	6.05	−2.4	1,400
0.549	CMP	2.83	9.7	−0.1	
0.266		1.41	9.3	+1.1	
0.555	IMP	2.67	9.1	−0.7	
0.283		1.33	8.0	−0.3	
0.548	UMP	2.03	11.1	+1.3	
0.278		1.01	8.7	+0.4	
0.381 (0.210)	Glutamate	2.5 (1.25)			
	NH_4Cl	2.5 (1.25)	9.7 (7.2)	+0.6 (−0.3)	
0.121 (0.291)	L-Histidine	5 (2.5)			
	L-Tryptophan	2.5 (1.25)			
	L-Serine	5 (2.5)	4.5 (7.4)	−1.4 (−1.0)	
	Glycine	2.5 (1.25)			
0.114	L-Alanine	2.5	5.6	−0.1	
0.0558	L-Glutamate	5	5.6	+1.9	
	NH_4Cl	5			
	L-Alanine	1.25			

[a] Enzyme preparation $E_{\bar{9}}$. Equilibrium dialysis at 4° and pH 7.4 for 60–200-hr periods in the presence of 0.02 M imidazole chloride, 0.1 M KCl, 1 mM $MnCl_2$, [^{14}C]AMP (c_1), and added compounds (c_2). Protein concentrations were 0.7–1.6 × 10^{-5} M and the specific activity of the [^{14}C]AMP was 6–10 × 10^5 cpm/μmole. Values given in parentheses represent reequilibrations at the given c_1 and c_2 concentrations. [b] Total concentration of added compound. [c] $\bar{\nu}_{AMP}$ is the moles of [^{14}C]AMP bound per mole (600,000 g) of protein. [d] $\bar{\nu}_{theory}$ is calculated from eq 1 for $n' = 12$, $k_A' = 8000$ (see Figure 1). [e] k_2'' values are calculated from eq 2 for the competitive case (see Methods) with $n = 12$, $\bar{\nu}_{obsd}$ for AMP, $k_1' = 8000$, c_1 and c_2 where the free concentration of c_2 is approximated from the total concentration of c_2, $\Delta\bar{\nu}_{AMP}$, and the protein concentration.

$\Delta\bar{\nu}_{Trp}$, are within the experimental accuracy of the experiments. The binding data are least accurate at the high concentrations of inhibitor where the difference in radioactivity between the protein and solvent sides of the membrane is decreased from that obtainable at low inhibitor concentrations.

The effect of L-tryptophan on the binding of AMP by the enzyme is enchanced in the experiments in which Mg^{2+} instead of Mb^{2+} was present. These results are in contrast to the experiments in which only one of these inhibitors was present where no effect of divalent cation was observed. Somehow, then, the divalent cation is specifically linked to the effectiveness of L-tryptophan in decreasing the enzyme's affinity for AMP.

The binding of L-alanine to glutamine synthetase appears to be a special case. No binding of this inhibitor at about 10 mM concentrations to the enzyme could be detected in equilibrium dialysis experiments. This is despite the fact that L-alanine is one of the more potent inhibitors in a kinetic assay (Woolfolk and Stadtman, 1964, 1967a). When L-glutamate was added to the equilibrium dialysis cells with Mg^{2+} present, a complex appeared to be formed with L-[^{14}C]alanine that was adsorbed onto the membrane (see Methods) for which the protein appeared to be competing. With Mn^{2+} present, the addition of L-glutamate had no effect. However, binding of L-[^{14}C]alanine could be observed when L-glutamine was added (only 1.5–3 mM L-glutamine concentrations were tried), without any discernable experimental artifacts being introduced. Representative results of equilibrium dialysis experiments with L-[^{14}C]alanine and 1.5 mM L-glutamine present are shown in the lower part of Table II. The affinity of the enzyme for L-alanine under these experimental conditions is so low that only approximate results could be obtained. The binding of L-alanine with L-glutamine present appeared to be cooperative from these and other data. L-Alanine did not have an apparent effect on the binding of [^{32}P]AMP to the Mg–enzyme. In the presence of Mn^{2+}, L-alanine did appear to slightly decrease the affinity of glutamine synthetase for AMP. However, these values are tentative, and as shown in Table I, enhanced AMP binding to the $E_{\bar{9}}$ preparation was observed with L-alanine, L-glutamate, ammonia, and Mn^{2+} (under which conditions L-alanine would be expected to bind

TABLE II: Combined Inhibitor Binding to Glutamine Synthetase.[a]

Divalent Cation Present	Free Concn of Inhibitor					
	C_{AMP}[b] (mM)	C_{Trp}[c] (mM)	$\bar{\nu}_{AMP}$[d]	$\Delta\bar{\nu}_{AMP}$[e]	$\bar{\nu}_{Trp}$[d]	$\Delta\bar{\nu}_{Trp}$[f]
	Equilibrium Binding of [^{32}P]AMP and L-[^{14}C]Tryptophan					
Mn^{2+}	0.079	2.29	2.5	−2.0	11.0	0
Mg^{2+}	0.093	2.22	1.4	−3.8	12.9	+2
Mn^{2+}	0.066	0.29	3.6	−0.6	1.4	−0.3
*Mg^{2+}	0.066	0.26	2.8	−1.3	1.8	+0.3
Mn^{2+}	0.065	0.061	3.4	−0.7	0.4	−0.4
*Mg^{2+}	0.073	0.054	2.5	−1.9	0.7	+0.1
		C_{Ala}(mM)			$\bar{\nu}_{Ala}$[h]	
	Equilibrium Binding of [^{32}P]AMP and/or L-[^{14}C]Alanine[g]					
Mn^{2+}	0	7.21			10.2	
	0	3.69			4.8	
	0.069	2.91	2.4	−1.9	4.0	
*Mg^{2+}	0	3.11			17	
	0.048	1.89	3.7	+0.4	29	

[a] Enzyme preparation $E_{\overline{2.3}}$. Equilibrium dialysis at 4° in the presence of 0.02 M imidazole chloride, 0.1 M KCl, and either 1 mM $MnCl_2$ or $MgCl_2$ (pH 7.4) after prolonged dialysis of the enzyme against the same buffer (see Methods). It was found by the γ-glutamyl transferase assay without added Mn^{2+} (see Methods) that the enzyme dialyzed against Mg^{2+} still contained catalytic amounts of Mn^{2+} (yielding ~25% of the activity in the 0.3 mM $MnCl_2$ assay system). However, increasing the Mg^{2+} concentration to 34–50 mM Mg^{2+} (asterisk) cases did not alter the results even though this is sufficient Mg^{2+} to completely displace the bound Mn^{2+}. Results are from two 72-hr dialysis periods with a duplicate agreement in $\Delta\bar{\nu}$ (see footnotes *e*, *f*, and *i*) of ±0.1 for AMP and L-tryptophan at $C_{Trp} < 1$ mM and $\Delta\bar{\nu}$ of ±2 at $C_{Trp} > 1$ mM. [b] 4.6–3.6 × 10^5 cpm/μmole of [^{32}P]AMP. [c] 1.8 × 10^5 or 1.6–3.8 × 10^6 cpm per μmole of [^{14}C]tryptophan at high and low concentrations, respectively, of tryptophan. [d] $\bar{\nu}$ is the moles of inhibitor bound per mole (600,000 g) of enzyme. Protein concentrations were 1.7–1.9 × 10^{-5} M. [e] $\Delta\bar{\nu}_{AMP} = \bar{\nu}_{obsd} - \bar{\nu}_{theory}$, where $\bar{\nu}_{theory}$ is calculated from eq 1 for $n' = 12$; $k_A' = 8000$. [f] $\Delta\bar{\nu}_{Trp} = \bar{\nu}_{obsd} - \bar{\nu}_0$, where $\bar{\nu}_0$ is observed $\bar{\nu}_{Trp}$ in the absence of AMP (Figure 3a). [g] 1.53 mM L-glutamine present also for L-[^{14}C]alanine (1.6 × 10^5 cpm/μmole) binding (see text). [h] Results are approximate since the protein at 1% concentration binds only 1.5–5% of the total counts.

also; see Discussion). Lower extents of L-alanine binding to the enzyme were observed with Mn^{2+} than with Mg^{2+}. It must be stressed that the binding data for L-alanine are extremely inaccurate and only should be accepted with reservation. Nevertheless, the data are presented because of the uniqueness of the L-alanine binding which was observed only in the presence of the substrate, L-glutamine.

Tryptophan is partially competitive with glutamate under assay conditions (Woolfolk and Stadtman, 1967a). For this reason, and since L-tryptophan has an effect on the binding of AMP by the enzyme (Table II), the binding of AMP with L-glutamate present were determined. These results are shown in Table III. For these experiments, the glutamate concentration was maintained at 5 mM, which is the approximate K_m' for this substrate.[2] The upper portion of Table III shows that with the magnesium–enzyme, glutamate lowers the affinity, and possibly also the maximum equivalents of AMP bound, *n*. If *n* is assumed to not change, the affinity of the enzyme for AMP approached $1/K_i$ values determined kinetically (Figure 2). Unfortunately, the lower affinity of the enzyme for AMP with glutamate and Mg^{2+} present made it impossible to obtain an accurate binding curve by the method employed. It is certain, however, that glutamate and Mg^{2+} affect the binding of AMP by glutamine synthetase. With Mn^{2+} present, glutamate did not appear to have any effect on the binding of AMP by the $E_{\overline{2.3}}$ enzyme preparation (lower part of Table III). Since glutamate and/or ammonia with L-alanine appeared to have an effect on the binding of AMP by $E_{\overline{9}}$ (Table I), 5 mM NH_4Cl was added in these equilibrium dialysis experiments. Glutamate and Mn^{2+} with ammonia at the lower AMP levels did appear to decrease the affinity of $E_{\overline{2.3}}$ for AMP. However, these effects of substrates on the inhibitor binding require further investigation. It will be recalled that glutamate, ammonia, Mn^{2+}, and L-alanine appeared to enhance AMP binding to $E_{\overline{9}}$ (Table I). Some of the complexities of inhibitor and substrate relationships are apparent under different assay conditions (Figures 2, 4, and 5).

Enzyme Kinetics. Figures 4 and 5, together with Figure 2 and Table IV, illustrate some of the variations observed in the response of glutamine synthetase preparations to AMP under different assay conditions.

A. γ-GLUTAMYL TRANSFER ACTIVITY. The results of Mecke *et al.* (1966) (see also Stadtman *et al.*, 1968b) have indicated that the γ-glutamyl transfer activity is not changed by adenylylation of glutamine synthetase.

TABLE III: [^{32}P]AMP Binding to Glutamine Synthetase ($E_{\overline{2.3}}$) in the Presence of 5 mM Sodium Glutamate.

50 mM Mg^{2+}[a] C_{AMP} (mM)	$\bar{\nu}_{obsd}$	$\Delta\bar{\nu}$[b]	Extrapolated[c] K_D' (mM)	Kinetic[d] K_i' (mM)
0.0322	0.6	−1.8	0.62	
0.361	2.0	−6.9	1.5	1.5
5 mM Mn^{2+}				
0.00406	0.6	+0.3		
0.0610	4.1	+0.3		
0.527	10.1	+0.4		
+5 mM NH_4^+				
0.0442	2.1	−1.0		
0.584	8.0	−2.0		
0.740	11.1	+0.8		

[a] Ultracentrifuge method of Velick *et al.* (1953) used (see Methods) for the binding measurements in the presence of Mg^{2+}. [b] $\Delta\bar{\nu} = \bar{\nu}_{obsd} - \bar{\nu}_{theory}$, where $\bar{\nu}_{theory}$ calculated from eq 1 with $k_A' = 8000$; $n' = 12$. [c] Assuming n' is not changed ($n' = 12$). [d] At a low, noncompetitive level of ATP (see Methods, Figure 2).

TABLE IV: The Response of $E_{\overline{2.3}}$ to Various Metabolic Effectors in a Biosynthetic Assay.[a]

Divalent Cation Present	Sp Act.	Rel Sp Act. with Added Effector: L-Histidine* 20 mM	L-Histidine* 40 mM	Glycine** 20 mM	Glycine** 40 mM	L-Alanine 20 mM	L-Alanine 40 mM	L-Tryptophan** 5 mM	L-Tryptophan** 15 mM	AMP 20 mM	AMP 40 mM
Mg^{2+}	65	108	109	29	19	22	14	92	81	46	30
Mn^{2+}	5	78	66	49	40	42	31	83	59	65	45

[a] Assay procedure of Woolfolk *et al.* (1966) measuring P_i release after 10 min at 37° was used. The assay mixtures without added effector contained 50 mM imidazole buffer (pH 7.1), 5 mM ATP, 50 mM $MgCl_2$ or 5 mM $MnCl_2$, 50 mM NH_4Cl (* reduced to 10 mM), 100 mM solidm L-glutamate (**reduced to 7.5 mM), and 0.5–5 μg of glutamine synthetase ($E_{\overline{2.3}}$). For each condition, the activity (micromoles of P_i formed per minute per milligram) in the absence of effectors is set at 100.

However, it was shown by Kingdon and Stadtman (1967a) that enzyme preparations of different AMP content (Shapiro *et al.*, 1967) have markedly different sensitivities toward the various metabolic effectors of glutamine synthetase in the γ-glutamyl transfer assay. Figure 4 corroborates the latter published results in showing that the γ-glutamyl transfer activity of $E_{\overline{9}}$ is much more inhibited by AMP or L-tryptophan than that of $E_{\overline{2.3}}$.

The studies of Stadtman (see Stadtman *et al.*, 1968b) have shown the following: (1) in the γ-glutamyl transfer assay with 0.3 mM $MnCl_2$ present, both adenylylated and unadenylylated subunits are active with each having a different pH profile and an isoactivity pH ≃ pH 7.15;[3] (2) the γ-glutamyl transfer activity appears to be expressed independently in hybrid molecules containing both types of subunits; (3) the addition of 60 mM $MgCl_2$ to the assay completely inhibits the γ-glutamyl transfer activity of adenylylated subunits and alters the pH–activity profile of unadenylylated subunits. These assay conditions were approximated in the studies shown in Figure 4. In the 0.3 mM $MnCl_2$ assay at pH 7.15 the $E_{\overline{2.3}}$ and $E_{\overline{9}}$ preparations have different inhibition curves, with $E_{\overline{2.3}}$ being less sensitive to AMP or L-tryptophan inhibition and also appearing to be saturated with these inhibitors at the higher AMP or tryptophan concentrations. The addition of 60 mM $MgCl_2$ to these assays with the inhibitors present normalized the per cent inhibition of $E_{\overline{2.3}}$ or $E_{\overline{9}}$ to the same value with each inhibitor (results are shown only for the $E_{\overline{9}}$ preparation at one concentration of AMP or tryptophan in Figure 4). This would be the anticipated result if only unadenylylated subunits were active under these conditions. However, other considerations suggest that the effect of Mg^{2+} on the γ-glutamyl transfer activity in the presence of inhibitors is complex. In view of the fact that Mg^{2+} affects the γ-glutamyl transfer activity of unadenylylated subunits (see above), it is possible that Mg^{2+} also sensitizes these subunits to inhibition by AMP or L-tryptophan. Alternatively, some interaction between subunits of hybrid molecules occurs in the presence of inhibitors

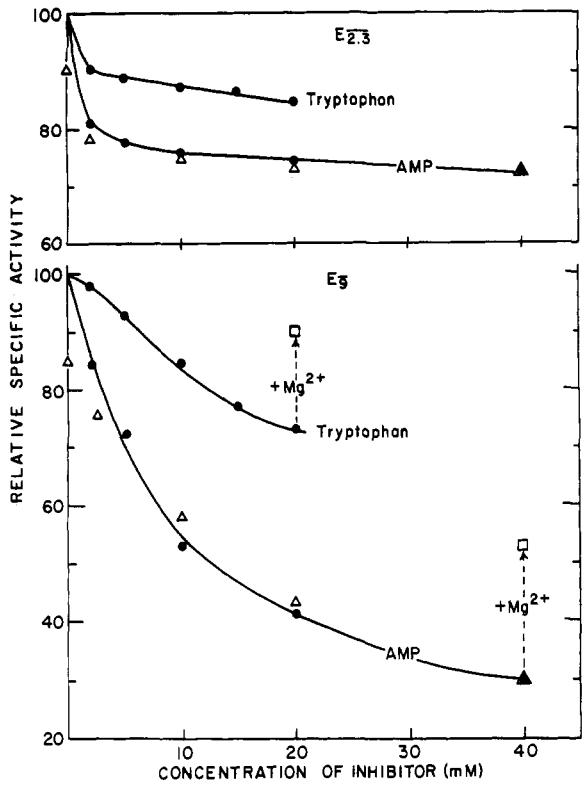

FIGURE 4: L-Tryptophan and/or AMP inhibition of the γ-glutamyl transfer activities at pH 7.15 of two glutamine synthetase preparations ($E_{\overline{2.3}}$ and $E_{\overline{9}}$). Relative specific activities at varying L-tryptophan or AMP concentrations (●) are shown by the curves, whereas the points (△) show the activity relative to that with no added inhibitor at varying AMP concentrations in the presence of 10 mM L-tryptophan. The addition of 60 mM $MgCl_2$ (see Methods) inhibits the γ-glutamyl transfer activity of the adenylylated enzyme in the absence of other metabolic effectors,[3] and the specific activities of $E_{\overline{2.3}}$ and $E_{\overline{9}}$ under these conditions are 54 and 13 μmoles of γ-glutamylhydroximate formed per min at 37° per mg, respectively. The decreased inhibition of the $E_{\overline{9}}$ preparation by 20 mM L-tryptophan or 40 mM AMP with 60 mM $MgCl_2$ present are shown by □ and are representative of the results obtained with $E_{\overline{2.3}}$ also.

and Mg^{2+}. Either or both of these possibilities must be considered in reconciling the Mg^{2+} results of Figure 4 with the fact that adenylylation correspondingly increases the sensitivity of the γ-glutamyl transfer activity of glutamine synthetase preparations to inhibition by various effectors (Kingdon and Stadtman, 1967a; Shapiro *et al.*, 1967). In these studies, more inhibition of $E_{\overline{2.3}}$ by AMP was produced by adding Mg^{2+} to the Mn^{2+} assay system that potentially measures both unadenylylated and adenylylated subunits. However, the inhibition of $E_{\overline{2.3}}$ by AMP with Mg^{2+} was the same as that observed with $E_{\overline{9}}$ under the same conditions and this suggests that Mg^{2+} does sensitize unadenylylated subunits to inhibition by AMP.

The AMP inhibition of the γ-glutamyl transfer activity of either $E_{\overline{2.3}}$ or $E_{\overline{9}}$ (Figure 4) was essentially the same with or without 10 mM L-tryptophan present. This result is compatible with those from the binding studies which show that L-tryptophan decreases the affinity of the enzyme for AMP (Table II). However, tryptophan is competitive with glutamine (Kingdon and Stadtman, 1967a) so that AMP and glutamine under assay conditions may even reverse the conformational change induced by tryptophan. The nonhyperbolic inhibition curves with L-tryptophan also suggest the cooperative nature of the observed binding of this inhibitor (Figure 3a). The shapes of the tryptophan inhibition curves of Figure 4 are similar to those previously observed in the biosynthetic assay by Woolfolk and Stadtman (1967a). The results from the mixed inhibitor studies in the γ-glutamyl transfer assay (shown by the open triangles in Figure 4) do not show the cumulative inhibition previously observed in the biosynthetic assay (Woolfolk and Stadtman, 1964, 1967a; Stadtman *et al.*, 1968a). Further studies will be required to learn whether or not the results obtained with mixtures of inhibitors is also a function of the assay procedure used.

B. Mg^{2+}-DEPENDENT BIOSYNTHETIC ACTIVITY. Adenylylation of glutamine synthetase inactivates the enzyme in a Mg^{2+} biosynthetic assay (Kingdon *et al.*, 1967; Wulff *et al.*, 1967) while reciprocally activating the Mn^{2+} biosynthetic activity (Kingdon *et al.*, 1967). The results of Figures 2 and 5 indicate that the Mg^{2+} biosynthetic activities of different enzyme preparations ($E_{\overline{1.2}}$, $E_{\overline{2.3}}$, or $E_{\overline{9}}$), which are as much as three- to fourfold different (see legend to Figure 5), have the same sensitivity toward AMP at different ATP levels. The partial competitive behavior of ATP with AMP in the inhibition studies suggests that the ATP substrate induces a conformational change in the enzyme (Dixon and Webb, 1964). Then, unadenylylated, Mg^{2+} specific subunits in a hybrid molecule (assuming their existence) would appear to be equally influenced by the high ATP levels. As shown by the results in Table III, glutamate with Mg^{2+} decreases the affinity and possibly the number of binding sites for AMP. Since the AMP inhibition curves in Mg^{2+} assay systems are the same for enzyme preparations with markedly different Mg^{2+} specific activities (Figures 2 and 5a), the number of equivalents of AMP bound under assay conditions might be expected to equal that found in the binding experiments (Figure 1). Then, the glutamate effect (Table III) can be related also to a conformational change which decreases the affinity of the enzyme for AMP. Kinetically, the substrate ATP appears to similarly influence the enzyme affinity for AMP. Under the conditions of the Mg^{2+} assay, however, limiting the glutamate concentration did not appear to have any effect on the observed AMP inhibition. It is quite possible that there are more than one ATP or glutamate sites per enzyme subunit (Denton and Ginsburg, 1969b), and this should be taken into account in correlating the effects of substrates on the enzyme–inhibitor interactions.

C. Mn^{2+}-DEPENDENT BIOSYNTHETIC ACTIVITY. The results obtained in the Mn^{2+} biosynthetic assay were quite different from those observed with the Mg^{2+}-activated enzymes. As illustrated by Figure 5b–d, enzymes adenylylated to different extents vary in their response to AMP in a Mn^{2+} biosynthetic assay as a

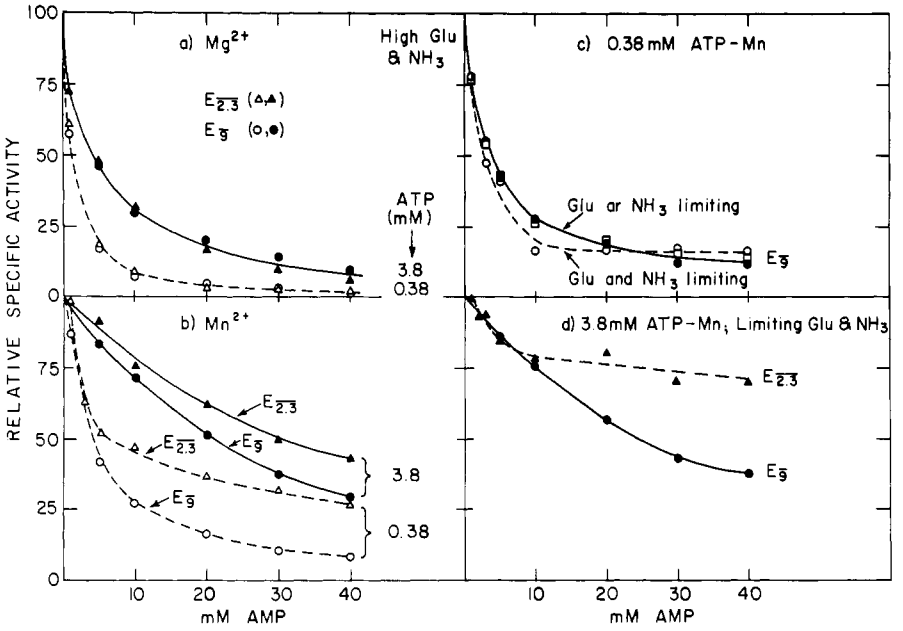

FIGURE 5: Kinetic responses of two glutamine synthetase preparations ($E_{\overline{2\cdot3}}$ and $E_{\overline{9}}$) to varying concentrations of AMP under different conditions in the spectrophotometric coupled assay at pH 7.1 and 24°. Specific activities (in the units of micromoles of ADP formed per minute at 24° per milligram of enzyme) relative to those with no inhibitor are plotted. With zero AMP, the Mg^{2+} specific activities in (a) of $E_{\overline{2\cdot3}}$ and $E_{\overline{9}}$, respectively, were 42 and 16 at 3.8 mM ATP (▲, ●) and 23 and 9 at 0.38 mM ATP (△, ○). In b, with no AMP present, the Mn^{2+} specific activities of $E_{\overline{2\cdot3}}$ and $E_{\overline{9}}$, respectively, were 2 and 5.2 at 3.8 mM ATP and 100 mM NH_4Cl, and 1.3 and 3.6 at 0.38 mM ATP. In the studies of a–c, 30 mM glutamate and 50 mM NH_4Cl were present unless otherwise noted; in c and d, limiting concentrations of glutamate and NH_4Cl are 7.5 and 5–10 mM, respectively. In part a 50 mM $MgCl_2$ was used. In the Mn^{2+} assay systems of b–d, 1 mM $MnCl_2$ in excess to the concentration of ATP present was added.

function of the substrate concentrations (ATP, glutamate, and ammonia).

In the Mn^{2+} assay, the adenylylated subunits are activated so that the $E_{\overline{9}}$ preparation is about three times more active than $E_{\overline{2\cdot3}}$ (see legend to Figure 5). This is consistent with the conclusion that in Mn^{2+} essentially only adenylylated subunits are active (Stadtman *et al.*, 1968b). If so, it is evident that subunit interaction influences the activity of the adenylylated subunits since, in the presence of Mn^{2+}, the $E_{\overline{9}}$ preparation was more inhibited by AMP than was $E_{\overline{2\cdot3}}$. These differences in the AMP inhibition curves became more marked at the low ATP or high ATP with limiting glutamate and ammonia concentrations (Figure 5b–d, respectively). From the studies with $E_{\overline{9}}$ at 0.38 mM ATP, it appears that the AMP inhibition curve is changed only when both glutamate and ammonia are limiting (Figure 5b,c). It should be noted that glutamine synthetase has a K_m' for ATP which is about tenfold lower with the ATP–Mn complex than with that of ATP–Mg; however, adenosine triphosphate differentially activates the more adenylylated enzyme forms in the Mn^{2+} assay.[2] It would appear that an interaction between adenylylated and unadenylylated subunits in hybrid enzyme molecules in the Mn^{2+} assay can occur, and that this interaction is enhanced at high ATP and limiting glutamate and ammonia concentrations. The interaction between the subunits has a marked effect on the sensitivity of the Mn^{2+}-activated, adenylylated subunits toward AMP inhibition.

Table IV summarizes the response of the $E_{\overline{2\cdot3}}$ preparation to various effectors in a Mg^{2+} or Mn^{2+} biosynthetic assay at 37°. Complete inhibition curves for the metabolic effectors of glutamine synthetase in a Mg^{2+} or Mn^{2+} biosynthetic assay have been published for other enzyme preparations (Woolfolk and Stadtman, 1967a; Kingdon *et al.*, 1967; Stadtman *et al.*, 1968a). L-Histidine and L-tryptophan are more effective inhibitors in the Mn^{2+} assay whereas the Mg^{2+}-activated $E_{\overline{2\cdot3}}$ is more sensitive to glycine, L-alanine, or AMP inhibition.

Discussion

The studies of Woolfolk and Stadtman (1964, 1967a) have suggested that the glutamine synthetase from *E. coli* is regulated by the independent action of at least eight different feedback inhibitors (Stadtman *et al.*, 1968a). Indirect evidence supporting the view that the glutamine synthetase molecule has separate binding sites for the metabolic effectors has been obtained from studies on the sulfhydryl reactivity of the enzyme (Shapiro and Stadtman, 1967). Another regulatory mechanism for this enzyme has been discovered more recently which involves *in vivo* adenylylation of the glutamine synthetase molecule (Kingdon *et al.*, 1967; Shapiro *et al.*, 1967; Wulff *et al.*, 1967). Adenylylation has been shown to enhance the susceptibility of the chemically modified enzyme to multiple feedback inhibition (Kingdon and Stadtman, 1967a,b; Kingdon *et al.*, 1967; Stadtman *et al.*, 1968b). The direct binding

studies presented here have only partially verified these conclusions from kinetic analyses. It does appear that the glutamine synthetase molecule has different binding sites for inhibitors, but that interaction between these sites may occur with more than one inhibitor present. Substrates (*i.e.*, glutamate, ammonia, and ATP, or glutamine, hydroxylamine, and ADP) appear to be able to couple the state of adenylylation of the enzyme to the action of inhibitors, and therefore, interactions between substrate and inhibitor binding sites can occur also. Some of the effects of substrates on the glutamine synthetase, which are a function of the adenylylation state of the enzyme, are considered in greater detail in another paper.[2] Specific divalent cations, the binding properties of which have been presented (Denton and Ginsburg, 1969), only appear to be linked to the inhibitor binding when there is an interaction between sites. In general, the binding studies have indicated that whereas the binding of individual effectors to the enzyme may be rather straightforward, the binding of mixtures of inhibitors, or inhibitor with substrate, can be complex. Unfortunately, then, it is impossible to correlate the binding data for individual effectors directly with the kinetically observed feedback inhibition patterns (Woolfolk and Stadtman, 1964, 1967a; Stadtman *et al.*, 1968a,b). However, the binding studies reported here have been of value in indicating some of complexities of inhibitor binding to this allosteric enzyme.

The studies reported here on the equilibrium binding of two feedback inhibitors, AMP and L-tryptophan, to glutamine synthetase have shown that each of these inhibitors are bound to the extent of 12 equiv at different sites of the enzyme molecule. Since the glutamine synthetase molecule is composed of 12 subunits of apparently identical amino acid composition, molecularly arranged in two superimposed hexagons (Valentine *et al.*, 1968; Woolfolk *et al.*, 1966; Shapiro and Stadtman, 1967), it is reasonable to conclude that each subunit of the enzyme has distinct binding sites for each inhibitor. The binding characteristics of AMP or L-tryptophan were not influenced by the extent of adenylylation of the enzyme preparation examined.

The data obtained indicate that the 12 AMP binding sites are independent and equivalent. The free energy of binding can be estimated at about 5 kcal from the apparent intrinsic association constant. (This association constant is about one order of magnitude greater than that estimated from the $1/K_i$ value observed kinetically, and this discrepancy appears to be due to the absence of substrate(s) in the binding measurements (see below) rather than due to an external effect such as temperature.) The ionized phosphate group does not appear to participate in the binding mechanism, since increasing the ionic strength does not decrease AMP binding. In kinetic studies at pH 7–8, the inhibition of the enzyme by AMP was not changed by pH variations. Further, the enzyme appears to have approximately the same, if not slightly greater, affinity for adenine or adenosine at the AMP binding sites. GMP was less effective in competing with AMP for AMP binding sites, with the enzyme having an apparent association constant for GMP about one-fifth of that for AMP. CMP, IMP, or UMP did not compete with AMP in the binding studies. Thus, the purine, 6-amino group, and possibly also the ribosyl portions of the AMP molecule appear to be directly involved in the specific binding of AMP to the enzyme. Hydrophobic forces therefore must be predominantly responsible for the enzyme–AMP interaction. Kinetic studies indicate that the AMP site is distinct from the catalytic ATP site (Woolfolk and Stadtman, 1964, 1967a) at which adenine or adenosine (or ADP) also compete. The AMP binding site is different also from the site of attachment of AMP in the adenylylation reaction (Shapiro and Stadtman, 1968b), since enzyme preparations of different AMP content all bound AMP to the same extent.

It has been shown previously that the removal of Mn^{2+} from the native taut enzyme by EDTA treatment induces structural changes in the glutamine synthetase molecule resulting in a more labile and catalytically inactive enzyme form (Shapiro and Stadtman, 1967; Woolfolk and Stadtman, 1967b; Kingdon *et al.*, 1968; Shapiro and Ginsburg, 1968). This relaxed enzyme form is a slightly more asymmetric particle than the taut form with exposed sulfhydryl, tyrosyl, and tryptophanyl residues, and it is more susceptible than the native enzyme to dissociating agents. The behavior of the relaxed enzyme has been shown to be the same with or without the EDTA–Mn^{2+} complex removed (Shapiro and Ginsburg, 1968). The relaxed enzyme form has been shown here to have approximately the same affinity for AMP as does the taut enzyme. Then, the conformational changes induced by the removal of Mn^{2+} from the enzyme would not appear to alter the AMP binding sites. It is of interest that in the studies of Shapiro and Stadtman (1967), AMP and L-tryptophan each protected the relaxed enzyme from inactivation by organic mercurials; in contrast, ATP was found to enhance the rate of the mercurial-dependent inactivation of relaxed enzyme. The observations on the sulfhydryl reactivity in the presence of added compounds may be related to conformations stabilized and/or to a protection against or enhancement of the dissociating action of sulfhydryl reagents (Shapiro and Stadtman, 1967). However, an antagonistic action of ATP and AMP in binding might be implied from kinetic observations. At high ATP levels, AMP inhibition is less than that observed at low "noncompetitive" ATP concentrations. The addition of a saturating concentration of AMP to the taut enzyme produced no detectable changes in the sedimentation or viscosity properties of the enzyme (Shapiro and Ginsburg, 1968).

The equilibrium binding of L-tryptophan to glutamine synthetase is weakly cooperative with a Hill coefficient and an interaction energy estimated to be ~2.5 and ~700 cal, respectively. The midpoint of the transition occurs at 0.91 mM L-tryptophan, and the over-all transition appears to be the same with Mg^{2+} or Mn^{2+} present or with different glutamine synthetase preparations. Even though the enzyme has a greater affinity for AMP than for L-tryptophan, the latter effector decreases the affinity of the enzyme for AMP (Table II). Since AMP does not have a reciprocal influence on the

binding of L-tryptophan, the effect of L-tryptophan on the AMP binding has to occur through an interaction between separate AMP and L-tryptophan binding sites and not by competition at the same binding site. The divalent cation has some effect on this interaction that is not apparent from the binding of the individual effectors. The decrease in AMP binding produced by L-tryptophan was greater with Mg^{2+} than with Mn^{2+}. Although the L-tryptophan transition appears to be the same with either Mg^{2+} or Mn^{2+} present, different conformers must be stabilized by these divalent cations such that the Mg^{2+}–tryptophan conformers have the lower affinity for AMP. The addition of a mixture containing nearly saturating levels of L-histidine, L-tryptophan, L-serine, and glycine (Table I) to [^{14}C]-AMP, only produced a decrease in AMP binding by an amount equivalent to that produced by L-tryptophan alone. This suggests that L-tryptophan is the only one of these inhibitors that affects the binding of AMP. Also, L-alanine, in the presence of glutamine and Mg^{2+} (see below), did not appear to significantly change the AMP binding, but varying effects of L-alanine on AMP binding were observed with substrates and Mn^{2+} (Tables I and II). It is apparent that the other feedback inhibitors of glutamine synthetase do not necessarily all bind in an independent manner to the enzyme, since AMP in mixed inhibitor studies has been shown to be an exception. Also, the cooperativity of the L-tryptophan binding indicates that the binding of this effector by itself to the enzyme subunits involves some interaction so that these binding sites are not independent or equivalent.

In the studies of Woolfolk and Stadtman (1967a), L-tryptophan was shown to be partially competitive with the substrate, L-glutamate. Glutamate was found here to decrease the affinity of the enzyme for AMP in the presence of Mg^{2+}. The number of binding sites for AMP are probably not changed by glutamate because the observed decrease in the affinity alone can account for the comparatively low value of $1/K_i$ estimated kinetically. Indirectly, the evidence suggests that glutamate and tryptophan are antagonistic in binding, but that both compounds stabilize conformations of the Mg^{2+}–enzyme which have a decreased affinity for AMP.

With Mn^{2+}, glutamate alone had little or no effect on AMP binding. A decrease in the amount of AMP bound to the enzyme was observed when ammonia was added to this system (Table III). However, an increase in the amount of AMP bound to another enzyme preparation was observed with Mn^{2+}, L-glutamate, ammonia, and L-alanine present (Table I). The effects of the different substrates and inhibitors on inhibitor binding are difficult to quantitate since the affinities of the enzyme for substrates or inhibitors under assay conditions (Woolfolk and Stadtman, 1964) are so low that binding measurements by the techniques used here become extremely inaccurate. This was the case in the studies of L-alanine binding to glutamine synthetase considered below.

The binding of L-alanine to glutamine synthetase is of interest. This binding could be detected (at as high as 10 mM L-alanine concentrations) *only* in the presence of glutamine. However, under assay conditions, L-alanine behaves as a potent "noncompetitive" inhibitor of the enzyme (Woolfolk and Stadtman, 1964, 1967a; Table IV). Perhaps the unusual requirement of glutamine for the binding of L-alanine to the enzyme can be correlated to an observation of Kingdon *et al.* (1967) that inhibition of glutamine synthetase activity with L-alanine is less at subsaturating than at high concentrations of glutamate and ammonia. These observations suggest that the binding of L-alanine requires the presence of one or more substrates (*i.e.*, ammonia, glutamate, or glutamine). On this basis, a possible role for L-alanine in regulating glutamine synthetase activity in *E. coli* has been proposed by Stadtman *et al.* (1968b).

The kinetic responses of glutamine synthetase to the different inhibitors have been presented elsewhere (Woolfolk and Stadtman, 1967a; Stadtman *et al.*, 1968a; Kingdon and Stadtman, 1967a,b). Preliminary kinetic studies with AMP and L-tryptophan and two enzyme preparations ($E_{\overline{2.3}}$ and $E_{\overline{9}}$) have been presented here to illustrate that the responses to inhibitors are very much dependent upon the assay conditions employed and, in particular, upon the substrate concentrations. In this respect, the responses of glutamine synthetase to most of the inhibitors appear to be coupled to the action of one or more substrates. It is obvious that much more detailed kinetic analyses are required with enzyme preparations that are homogeneous with respect to the molecular distribution of covalently bound AMP.

The binding of the effectors AMP or L-tryptophan, individually, can be described by either of several models for allosteric enzymes. For example, the inhibitor AMP binds equally well to the inactive, relaxed enzyme or to the Mg^{2+}- or Mn^{2+}- activated enzyme forms, but with a lowered affinity to the enzyme in the presence of substrates. In the absence of AMP, the L-tryptophan-induced transition appears to be the same with either divalent cation. These observations would seemingly fit a two-state allosteric model involving nonexclusive inhibitor binding such as that proposed by Rubin and Changeux (1966) in an extension of the two-state allosteric model of Monod *et al.* (1965) or the alternate models of Koshland *et al.* (1966). However, the complexities observed here in the binding of two inhibitors of glutamine synthetase from *E. coli* require the interpretation that this enzyme is able to assume multiple conformations in solution. The specific divalent cation or the adenylylation state of the enzyme may or may not be coupled to the inhibitor binding under different conditions; that is, in at least one case examined thoroughly here, Mg^{2+} and Mn^{2+} can be shown to stabilize conformers of the enzyme–tryptophan complex that differ in their affinity for AMP. Under assay conditions, the analysis of the protein–inhibitor interactions is even more complex. Substrate-modulated interactions between adenylylated or non-adenylylated subunits in the dodecameric aggregate are an important aspect of the linkage between substrates, divalent cation (Mg^{2+} or Mn^{2+}), and inhibitor bind-

ing. Finally, the fact that the binding of L-alanine appears to require the presence of substrate (glutamine, or glutamate and ammonia) suggests that the behavior of this enzyme is not adequately described by the two-state allosteric models of Monod *et al.* (1965) or Rubin and Changeux (1966) or by the alternate models proposed by Koshland *et al.* (1966).

A mechanism by which the multiple feedback inhibitors of glutamine synthetase could exert their cumulative effects has been proposed by Stadtman *et al.* (1968a). Assuming that the enzyme had independent binding sites for each inhibitor, the model proposed postulated two mutually exclusive inhibitor sites per subunit, a noninhibitory site and an inhibitory site, which may or may not overlap. The binding data presented here could be compatible with such a model if it is stipulated further that the substrates and adenylylation state of the enzyme dictate the form assumed by the inhibitory sites. Since the inhibitors do not all bind independently to the different sites on the enzyme, inhibitors in some cases may also induce a change in the conformations of other inhibitory sites on the same subunit of similar sites present on other subunits of the macromolecule.

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