Catalytic Cooperativity and Subunit Interactions in *Escherichia coli* Glutamine Synthetase: Binding and Kinetics with Methionine Sulfoximine and Related Inhibitors†

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ABSTRACT: Glutamine synthetase (GS) from *Escherichia coli*, a critical regulatory enzyme of nitrogen metabolism, is composed of 12 identical subunits. Several separate lines of evidence suggest homotropic subunit interactions. Steady-state kinetics with the unadenylylated enzyme at pH 7.5 indicate that L-methionine (*S*)-sulfoximine, Met(O)(NH)-*S*, a transition state analogue, is a linear competitive inhibitor vs. L-glutamate with K_{is} = 0.96 μM. Met(O)(NH)-*S* also irreversibly inhibits GS in the presence of MgATP. The rate of this reaction shows a sigmoidal dependence on [Met(O)(NH)-*S*] with a Hill n_H = 2.2 and half-saturation $I_{0.5}$ = 25 μM. The only kinetic models that successfully rationalize K_{is} = 1 μM vs. $I_{0.5}$ = 25 μM and the observed sigmoidal inactivation kinetics involve multiple interacting sites for Met(O)(NH): either more than one site per subunit or site–site interactions between subunits. Equilibrium binding studies of [Me-^{3}H]Met(O)(NH) to GS in the presence of magnesium adenosine 5′-(β,γ-imidotriphosphate) (MgAMP-PNP) yielded a biphasic Scatchard plot that extrapolated to a maximum of 1.0 Met(O)(NH) bound/subunit, thus indicating that the subunit interaction model is valid. The enzyme was irreversibly but partially inhibited to various extents (50%, 75%, 90%) with Met(O)(NH)-*S* and MgATP and was then characterized in terms of the properties of the remaining (active) subunits: K_m's for substrates, K_{is} for Met(O)(NH)-*S* vs. L-Glu, and k_{max}, $I_{0.5}$, and Hill n_H for the Met(O)(NH)–ATP inactivation reaction. The most dramatic effects were that K_m (NH_3) increased and K_m (ATP) decreased as the enzyme became increasingly inhibited. These data, along with fluorescence titrations [Rhee, S. G., Chock, P. B., Wedler, F. C., & Sugiyama, Y. (1981) *J. Biol. Chem. 256*, 644–648] and UV difference spectra [Shrake, A., Whitley, E. J., & Ginsburg, A. (1980) *J. Biol. Chem. 255*, 581–589], indicate negatively cooperative binding of Met(O)(NH)-*S* to Mg unadenylylated GS, n_H = 0.50–0.70. Subunit interactions are therefore proposed to be involved in conformational changes about bound Met(O)(NH) that necessarily precede the reaction with ATP that causes inactivation. Such changes are also proposed to provide the driving force for product dissociation during net turnover (catalytic cooperativity). These hypotheses are further supported by experiments with several newly synthesized analogues of Met(O)(NH) that included ethionine sulfoximine and a rotationally restricted "cyclo-Met(O)(NH)", namely, (cis, trans) 3-amino-3-carboxytetrahydrothiopyran sulfoximine.

The pioneering studies by Meister and co-workers demonstrated that Met(O)(NH)[1] inhibits the *Escherichia coli* and ovine brain enzymes, specifically the 2*S*,5*S* stereoisomer. Met(O)(NH) binds sufficiently more tightly to *E. coli* glutamine synthetase than the substrate glutamate to be considered a transition state analogue (Gass & Meister, 1970; Villafranca et al., 1976; Wedler & Horn, 1976). In addition to being a competitive inhibitor vs. L-glutamate, Met(O)(NH) causes irreversible inhibition in the presence of ATP, apparently due to formation of a very tight enzyme–methionine sulfoximine phosphate–ADP complex (Ronzio et al., 1969).

Glutamine synthetase from *E. coli* consists of 12 apparently identical subunits arranged in the form of two eclipsed hexagons. Each subunit can be covalently modified by enzymatic adenylylation at a specific tyrosine residue or can exist in an unadenylylated form (Stadtman & Ginsburg, 1974). Until recently, there was little evidence to indicate homologous subunit interactions, although heterologous interactions between adenylylated and unadenylylated subunits had been deduced in the binding of glutamate to the catalytic site (Ginsburg et al., 1970).

Indeed, it is not altogether surprising or unforeseen that intrasite conformational changes must be of importance during catalysis, when one considers the dramatic fluorescence changes elicited by Met(O)(NH) or by sequential addition of substrates (Rhee et al., 1981; Shrake et al., 1980; Timmons et al., 1974) that apparently are responses to the changes in geometry (trigonal to tetrahedral) that necessarily occur about the C-5 of L-glutamate during catalysis.

Several new lines of evidence, all involving the binding of the transition state analogue L-methionine sulfoximine, Met(O)(NH), have demonstrated homologous intersubunit interactions: (a) Rhee et al. (1981) have found that the first-order kinetic plots of the irreversible inhibition by Met(O)(NH) and ATP deviated from linearity, indicating that inactivation of one subunit could affect the properties of neighboring subunits, (b) fluorescence titrations of the enzyme with the *S* isomer of Met(O)(NH) showed nonhyperbolic saturation, with a Hill coefficient (n_H) equal to 0.65, indicating negative cooperativity (Rhee et al., 1981), and (c) Shrake et al. (1980) found that the binding of Met(O)(NH) induced UV difference spectral charges that gave n_H values of 0.5 and 0.7 for the Mg-unadenylylated and Mn-adenylylated enzymes, respectively.

In the present study, data from steady-state kinetics, the kinetics of irreversible inhibition, and the equilibrium binding of Met(O)(NH) are consistent with homologous subunit in-

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[1] Abbreviations: Met(O)(NH) or MSOX, L-methionine sulfoximine; GS, glutamine synthetase; AMP-PNP, adenosine 5′-(β,γ-imidotriphosphate); TNS, 2-(*p*-toluidinyl)naphthalene-6-sulfonate; HPLC, high-pressure liquid chromatography; TES, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid; TEA, triethanolamine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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teractions in unadenylylated *E. coli* glutamine synthetase. In particular, we have used enzyme partially inactivated by Met(O)(NH) and ATP to "freeze" various conformational states for detailed kinetic analysis. This approach has yielded new insights to the conformational changes transmitted from one active site to another, including an unexpected intersubunit synergism exerted by Met(O)(NH) upon ATP binding.

Experimental Procedures

Materials

L-Methionine (*SR*)-sulfoximine was synthesized from L-methionine (Sigma) by the procedures of Lavine (1947) and Christensen et al. (1969) by oxidation to the sulfoxide in acidic H_2O_2 and then reaction with hydrazoic acid. The synthesis of L-ethionine (*SR*)-sulfoximine from L-ethionine (Sigma) was carried out by similar procedures. Separation of the isomers of L-methionine sulfoximine was accomplished by two different methods: (a) that of Christensen et al. (1969), by fractional recrystallization as the (2:1) *d*-10-camphorsulfonic acid complexes, repeated 5 times, and (b) fractional recrystallization of *N*-phthaloyl-L-methionine sulfoximine that was cyclized between the imino group of the sulfoximine and the α-carboxylate by use of acetic anhydride at 92 °C or dicyclohexylcarbodiimide (DCC) at 20 °C. Deblocking was accomplished by treatment with mild acid followed by hydrazine. Either procedure gave isomers that were >99.5% pure, as determined by infrared spectral analysis, by HPLC and NMR analysis of the (cyclized) *N*-phthaloylmethionine sulfoximine, and by determination of the percent inactivation of glutamine synthetase in the presence of Met(O)(NH) and ATP (Rhee et al., 1981; Christensen et al., 1969).

AMP-PNP was from ICN Pharmaceuticals and was further purified by chromatography on Sephadex A-25, eluted with ammonium bicarbonate, and then lyophilized. L-Glutamate, L-glutamine, L-methionine, ATP, and ADP were from Sigma Chemical Co. All other chemicals and biochemicals were reagent grade.

L-[*methyl*-^{3}H]Methionine was from New England Nuclear and was converted to L-methionine (*SR*)-sulfoximine, after addition of 0.1 g of carrier L-methionine, by the above procedures. The specific radioactivity of the product was 0.01 Ci/mol.

The two (cis, trans) isomers of 3-amino-3-carboxytetrahydrothiopyran sulfoximine (cyclo-Met(O)(NH)), i.e.

were synthesized by the production of the hydantoin (12) from tetrahydrothiopyran-4-one and then conversion to the sulfoximide by the methods cited above, and fractional crystallization of the two isomers was followed by hydrolysis with strong acid (60% H_2SO_4–$CHCl_3$, 65 °C, 72 h). After removal of H_2SO_4 with $Ba(OH)_2$, recrystallization from acetone–water yielded two crystal forms, I and II. For I (granules), mp 252 °C dec (Anal. Found: C, 34.27; H, 6.71; N, 13.32). For II (needles), mp 278–283 °C (Anal. Calcd: C, 33.58; H, 6.20; N, 12.54. Found: C, 33.92; H, 6.50; N, 12.90).

Unadenylylated glutamine synthetase, $E_{(0.7)}$ (the subscript indicates the average number of adenylylated subunits per dodecamer), was prepared as described previously (Stadtman et al., 1979). The specific activity of the purified enzyme was 114 units/mg, as measured by the pH 7.57 Mn(II)-dependent γ-glutamyl transferase assay method of Stadtman et al. (1979). This enzyme was judged to be homogeneous by pH 6.8 TES–TEA buffer native gels (Orr et al., 1972) and sodium dodecyl sulfate gel electrophoresis (Weber & Osborn, 1969).

Methods

Steady-State Kinetics. The enzyme activity was assayed by either the biosynthetic method of Shapiro & Stadtman (1970) or the γ-glutamyltransferase method of Stadtman et al. (1979). Unless otherwise stated, all assays contained 50 mM Hepes (pH 7.5), 100 mM KCl, and 50 mM $MgCl_2$ and were carried out at 37 °C. The concentration of substrates was varied by preparation of two assay mixtures, each at twice the desired final concentration of components, that were mixed in different proportions to the same final volume (0.1 mL), so as to produce a series of assay mixtures. Inhibitor, enzyme, and water were then added to a final volume of 0.2 mL. Enzyme used in assays was generally 1 μg or less. Fitting of lines to the data in Lineweaver–Burk plots was carried out by a weighted least-squares program.

Quenching Procedure. The irreversible inhibition of glutamine synthetase by L-methionine (*S*)-sulfoximine in the presence of ATP was arrested and monitored by diluting a small aliquot (10 μL) of the reaction mixture into a large volume (0.5 mL) of a quenching mixture containing 50 mM Hepes (pH 7.5), 100 mM KCl, 1 mM $MnCl_2$, 10 mM ADP, 20 mM K_2HAsO_4, and 150 mM L-glutamine, as described previously (Rhee et al., 1981). The enzyme thus prepared was subsequently assayed by the transferase method.

Preparation of Partially Inactivated Enzyme. Glutamine synthetase ($E_{(0.7)}$) was allowed to react with L-Met(O)(NH)-*S* (40 μM), ATP (10 mM), and $MgCl_2$ (50 mM) at pH 7.5, 20 °C, for varying lengths of time and then quenched by addition of $MnCl_2$–ADP–arsenate, so as to produce enzyme that was irreversibly inhibited to different extents. After quenching, the small molecules were removed by gel chromatography on Sephadex G-25 (fine), equilibrated with pH 7.5 Hepes, KCl, and $MgCl_2$ buffer. The activity remaining was assayed by the biosynthetic method, pH 7.5 with $MgCl_2$ present. The percent activity remaining has been compared to the number of subunits to which MSOX-P· was irreversibly bound (Figures 1 and 2 of Rhee et al., 1981). Thus, from these data one knows the enzyme that had 23–25% of the original activity had ca. 6.5 out of the 12 subunits occupied by MSOX-P·ADP and was designated $EX_{6.5}$. The enzyme that was 10% as active as native enzyme was designated EX_8 and so forth.

Equilibrium Binding. The reversible binding of L-[*methyl*-^{3}H]methionine (*SR*)-sulfoximine to glutamine synthetase ($E_{(0.7)}$) in the presence of 5 mM AMP-PNP, with 50 mM $MgCl_2$, 50 mM Hepes (pH 7.5), and 100 mM KCl, at ca. 30 °C was studied by use of the ultracentrifugation technique of Howlett et al. (1978). Dextran T40 (Pharmacia) was added at 2.0 mg/mL to stabilize the enzyme–ligand pellet. The K_{is} of AMP-PNP (vs. ATP) was determined by biosynthetic reaction kinetics to be 0.165 mM. Each enzyme–buffer–ligand mixture was made up to a final volume of 0.2 mL, 25 μL removed and counted by liquid scintillation with NEN Omnifluor–toluene solution in a Beckman LS-330 instrument. The mixtures were then centrifuged at top speed (122000*g*) in the 18° A-100 rotor in the Beckman airfuge TM for 45 min. Activity assays revealed that less than 0.1% enzyme activity remained in any part of the supernatant at the end of this time. Estimated rotor temperature was 25–30 °C. At the low range of binding, [^{3}H]MSOX was held at 10 μM, and the enzyme was varied from 5 to 25 μM, or both enzyme and MSOX were varied together up to 40 μM. At the high range of binding, the enzyme was held constant at 100 μM, and [^{3}H]MSOX was varied from 25 to 1000 μM. After the enzyme was

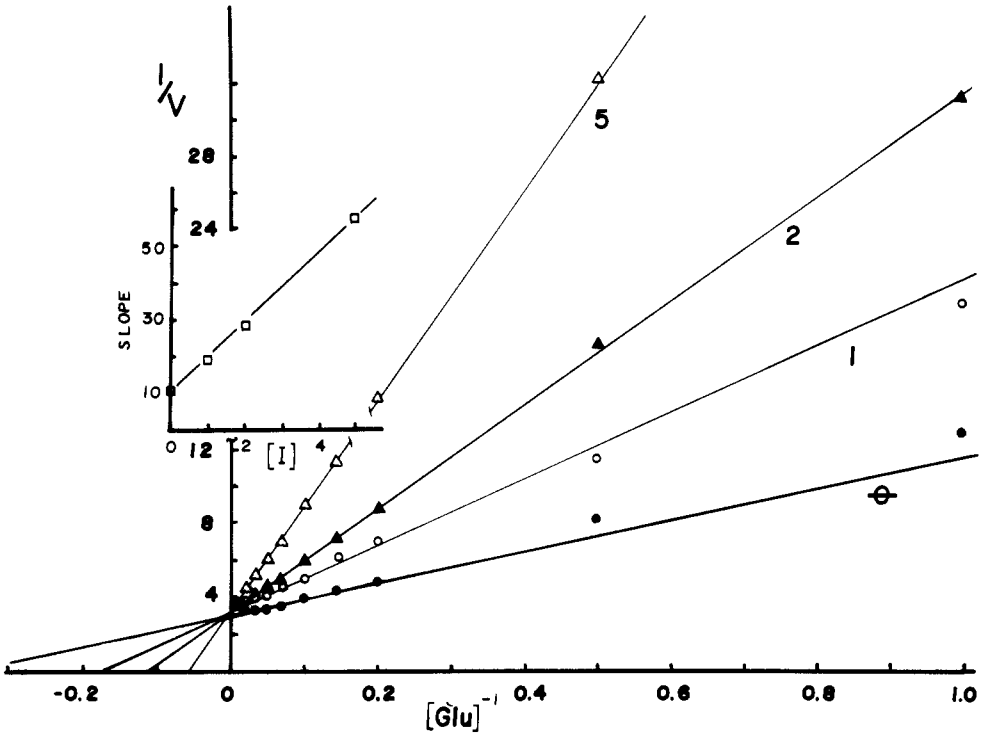


FIGURE 1: Steady-state inhibition kinetics of *E. coli* glutamine synthetase for L-Met(O)(NH)-*S* vs. L-glutamine in the biosynthetic assay, pH 7.5, 37 °C (see Experimental Procedures). The concentrations of L-Met(O)(NH)-*S* used are known (in μM) beside each set of points. (Inset) Replot of slopes vs. [Met(O)(NH)-*S*].

pelleted, the supernatant was sampled (25 μL) and counted. The counts present prior to centrifugation gave the total (bound + free) ligand, whereas those after centrifugation represented the free ligand concentration, from which the calculated difference gave the amount of bound ligand. Determination of bound ligand at each concentration of ligand was performed at least in triplicate.

Results

Binding of Met(O)(NH) to Glutamine Synthetase. (A) Steady-State Kinetics. Purified L-methionine (*S*)-sulfoximine was used in competition vs. L-glutamate in the biosynthetic assay, pH 7.5, 37 °C, with Mg^{2+}-unadenylylated *E. coli* glutamine synthetase (65). The results of these experiments, shown in Figure 1, indicated that L-Met(O)(NH)-*S* is a linear competitive inhibitor vs. L-Glu, with K_{is} = 0.96 μM. At [Met(O)(NH)] above 5 μM in these assays, strongly noncompetitive effects were observed, due to an irreversible inhibition of enzyme activity that was time dependent (Wedler & Horn, 1976; Rhee et al., 1981). Similar experiments (not shown) were carried out with L-Met(O)(NH)-*R*, which was also linearly competitive vs. L-Glu but with K_{is} = 42 μM. These data, along with those of Rhee et al. (1981) on isomeric specificity of L-Met(O)(NH) in the irreversible inhibition reaction with ATP, indicate that the *S* isomer is the one that both binds most tightly to *E. coli* GS and reacts specifically with ATP in the active site. Comparable stereospecificity, observed by different techniques, had been reported previously by Weisbrod & Meister (1973).

Steady-state kinetics were also carried out with L-Met(O)(NH)-*S* vs. the substrates NH_3 and ATP [not shown; see Figure S1 of supplemental material (see paragraph at end of paper regarding supplementary material)]. NH_3 was a weak linear competitive inhibitor vs. Met(O)(NH) in the biosynthetic assay with Met(O)(NH) at 5 mM or less, with an estimated K_{is} = 12 μM, calculated from replots of the slopes. The kinetics of Met(O)(NH)-*S* vs. ATP indicate that the K_m (ATP) shows a very slight decrease with increasing concentrations of Met(O)(NH), indicating some substrate synergism of binding for Met(O)(NH) and ATP. Synergistic binding effects between ATP and either L-glutamate or Met(O)(NH) had been noted previously by other kinetic and spectroscopic techniques (Timmons et al., 1974; Villafranca et al., 1976; Wedler, 1974). Much stronger effects are observed in experiments described below.

In the γ-glutamyltransferase assay, pH 7.5, 37 °C (see Experimental Procedures), L-glutamine was used vs. L-Met(O)(NH)-*S* in steady-state kinetics. Surprisingly, although L-Met(O)(NH)-*S* was a linear competitive inhibitor vs. L-glutamine, a value of K_{is} = 380 μM was obtained.

(B) Kinetics of Irreversible Inhibition. The inactivation kinetics induced by Met(O)(NH)-*S* in the presence of ATP were observed with Mg-unadenylylated *E. coli* GS at pH 7.5, 20 °C, similar to previous studies (Rhee et al., 1981; Wedler & Horn, 1976). The kinetics were followed by removal, quenching, and assay of small samples of enzyme from the Met(O)(NH)–ATP incubation mixture at a series of times (see Experimental Procedures). Although first-order plots of the loss of activity were linear only up to ca. 60% of the original activity (Rhee et al., 1981), the *initial* velocity of inactivation was obtained accurately from these plots. Figure 2 shows the dependence of the initial velocity of irreversible inhibition upon the concentration of Met(O)(NH)-*S* in the incubation mixture. Clearly, with fully active (100%) enzyme the plot is nonhyperbolic. A replot of log $[v/(V_{max} - v)]$ vs. log [Met(O)(NH)] shows a Hill value of n_H = 2.2 and a half-saturation value of $I_{0.5}$ = 25 μM (see inset).

At this point one must explain the basis for the difference between the K_{is} (MSOX) $\simeq$ 1 μM vs. L-glutamate (Figure 1) and the $I_{0.5}$ value = 25 μM (Figure 2). This was investigated first by the construction of several appropriate kinetic models.

(C) Models. First, *single-site* models of two pathway types were considered.

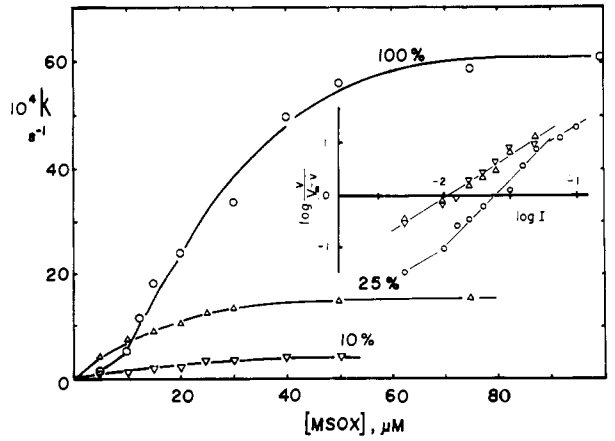
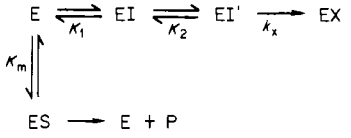


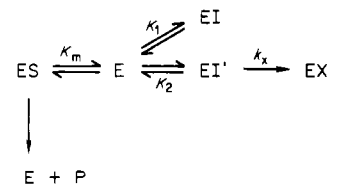
FIGURE 2: Rate of irreversible inhibition of *E. coli* GS ($E_{(2)}$) by L-Met(O)(NH)-*S* and ATP as a function of inhibitor concentration, pH 7.5, 20 °C. The rates of inhibition were assayed by removal of aliquots and quenching at a series of times from an incubation mixture containing 50 mM Hepes (pH 7.5), 100 mM KCl, 50 mM $MgCl_2$, 5 mM ATP, and various amounts of L-Met(O)(NH)-*S* (see Experimental Procedures). The enzyme was 0.05 mg/mL (10^{-6} M) or less in the incubation mixture. Enzyme partially inhibited by MSOX-*S* and ATP was isolated and characterized by biosynthetic assay, so that 25% residual activity corresponded to $EX_{(6.5)}$ and 10% to $EX_{(8)}$ [determined from Figure 2 of Rhee et al. (1981)]. (Inset) Hill plot of the data, from which $n_H = 2.2$, $I_{0.5} = 25$ μM for 100% GS ($EX_{(0)}$) and $n_H = 1.3$, $I_{0.5} = 13$ μM for 25% and 10% GS ($EX_{(6.5)}$ and $EX_{(8)}$). At <10% or >90% saturation, the slope of the Hill plot becomes equal to 1.0 but assumes its maximum value between these values of percent saturation.

(1) One is the *sequential* model, in which inhibitor (I) is competitive with substrate (S), but after initial, tight inhibitor binding to the enzyme (E) at the K_1 step, a conformational change (K_2 step) must occur prior to irreversible inhibition to form EX:



For this case, derivation of the kinetic expression for k_x as a function of K_m, S, K_1, K_2, and I leads to the conclusion that the value of the apparent $I_{0.5}$ depends mainly upon K_1 rather than K_2, since a value of K_2 that favors EI' ≫ EI can only make the $I_{0.5}$ value smaller than or equal to but not larger than K_{is}. A K_2 value such that EI' ≫ EI can only make $I_{0.5}(\text{obsd}) \simeq K_1$.

(2) The other is the *alternative* model, in which inhibitor is competitive with substrate but can bind in two different conformational modes to the enzyme, one of which is tight (K_1) but does not allow for irreversible inhibition and the other one of which is looser (K_2) but does lead to EX:



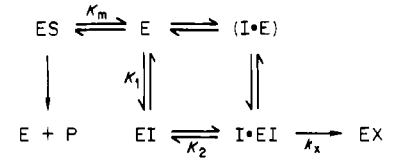
For this case, one can derive that

$$I_{0.5}(\text{obsd}) = \frac{K_1 K_2}{K_1 + K_2}$$

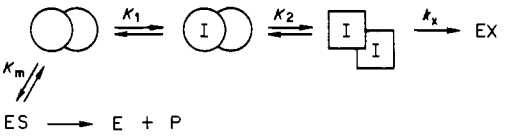
so that if $K_2 > K_1$ by 10-fold or more, then $I_{0.5}(\text{obsd}) \simeq K_1$, which is the opposite of what was observed above.[2]

Therefore, since neither of these single-site models predicts nor simulates the kinetics of Figures 1 and 2, *multiple-site models* of two types must be considered.

(3) One is the *intrasubunit* model, in which each subunit can bind more than one molecule of inhibitor, the first tightly (K_1) and in a manner competitive with substrate and the second loosely (K_2) at a separate noncatalytic site that promotes the formation of EX:



(4) The other one is the *intersubunit* model, in which each subunit can bind only one molecule of inhibitor. In a dimeric model the first I binds tightly (K_1) and is competitive vs. S, and then a second I binds less tightly (K_2) to an adjacent subunit, forcing a conformational change at the first subunit that promotes the formation of EX:



One can derive that for *both* cases 3 and 4 the expression for irreversible inhibition is

$$k_x(\text{obsd}) = k_x \frac{[I^2/(K_1 K_2)]}{\left(1 + \frac{I}{K_1}\right)\left(1 + \frac{I}{K_2}\right)}$$

so that if $K_2 > K_1$ and $I > K_1$ by 10-fold, this equation reduces to

$$k_x(\text{obsd}) \simeq k_x\left(\frac{I}{K_2 + I}\right)$$

The expression agrees with and predicts the observed data in Figures 1 and 2, namely, that $K_{is} = K_1 \simeq 1$ μM, but $I_{0.5} = K_2 \geq 10$ μM, and that k_x depends upon K_2, not K_1. Therefore, one is left with the problem of distinguishing between cases 3 vs. 4, i.e., whether there is 1 vs. 2 mol of Met(O)(NH) bound per subunit. This question has been specifically addressed in the following experiment.

(*D*) *Equilibrium Binding.* Figure 3 clearly indicates that the maximum number of [Me-^{3}H]Met(O)(NH) molecules that can bind per subunit of Mg-unadenylylated GS at pH 7.5, 30 °C, in the presence of the unreactive ATP analogue, AMP-PNP, is very close to one. The data also illustrate that the Scatchard plot is nonlinear, being composed of two binding isotherms with $K_1 \simeq 16$ μM and $K_2 \simeq 220$ μM. A more rigorous quantitative fitting of those points was deliberately avoided here, due to errors inherent in the data.

Experiments also were carried out in which [Me-^{3}H]Met-(O)(NH) plus cold ATP or [8-^{14}C]ATP plus cold Met(O)-(NH)-*S* was allowed to react, followed by removal of unbound small molecules. The stoichiometry of Met(O)(NH) or ATP irreversibly bound per subunit was 0.9–1.0.

[2] Kinetic expressions analogous and formally identical with those derived for such single site models have been considered for "wrong-way" binding of substrates with α-chymotrypsin (Hess, 1971; Segel, 1975, and references therein).

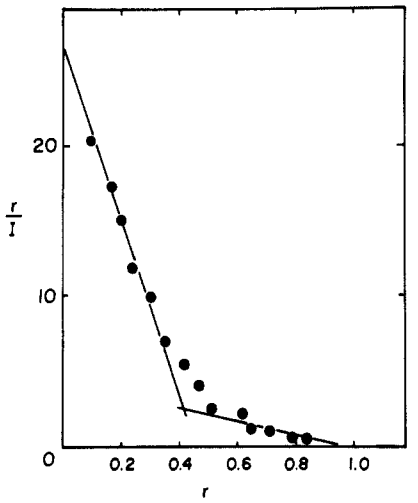
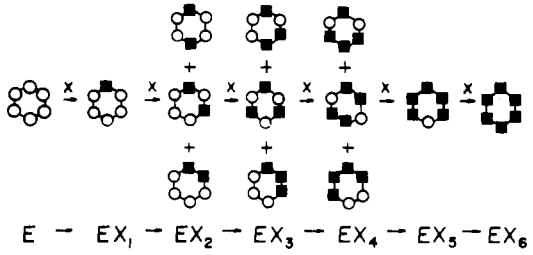


FIGURE 3: Equilibrium binding of Met(O)(NH)-*SR* by *E. coli* glutamine synthetase in the presence of 5 mM AMP-PNP at pH 7.5, 30 °C, with 50 mM Hepes, 100 mM KCl, and 50 mM $MgCl_2$. Calculated values for K_1 and K_2 at low and high Met(O)(NH) were 16 μM and 220 μM, respectively.

Scheme I



Overall, these data are consistent with (intersite) model 4 presented in the preceding section and are not consistent with any of the other three models. Therefore, it seems likely that subunit interactions are important in the binding and irreversible inhibition of GS with Met(O)(NH). The relation of these effects to enzyme catalysis will be examined in the following sections. It is noteworthy at this point that the binding of the fluorescent dye TNS [2-(*p*-toluidinyl)-naphthalene-6-sulfonate] to *E. coli* GS also exhibited biphasic saturation (Wedler et al., 1977) and that subsequent experiments[3] have revealed that only one TNS binds per subunit.

Glutamine Synthetase Partially Inactivated by Met(O)-(NH) and ATP. (A) Model Scheme. The progressive inactivation of enzyme subunits by L-Met(O)(NH)-*S* and ATP is represented in Scheme I, in this case for a hexameric enzyme. For the species EX_2, EX_3, and EX_4, several geometric isomeric forms are possible, each of which may have different intrinsic percent activities, depending upon the extent to which inactivation of a given subunit affects the activity of its nearest-neighbor subunits.

The very nonlinear first-order kinetic plots observed by Rhee et al. (1981) for the MSOX inactivation of unadenylylated GS suggest that the effects transmitted from one subunit to another are fairly strong. The concentration of Met(O)(NH) in these previous experiments was fairly high (43 μM) relative to K_{is}, $I_{0.5}$, or [E]. It was not determined in these earlier experiments whether the deviations from first-order kinetics were due to a decreased value for the k_{max} for inactivation or to increases in the binding constants for Met(O)(NH) or ATP. The experiments that follow were designed to answer several specific questions. How many subunits must be inactivated for these effects to occur? What are the changes that occur with regard to V_{max} and the K_m values of each substrate, as a function of the fraction of subunits inhibited? Are the changes in K_m, if any, more strongly exhibited toward certain substrates in the active site? What changes occur in the parameters for binding and reactivity of Met(O)(NH), e.g., K_{is}, k_{max}, $I_{0.5}$, and the Hill n_H, for the inactivation reaction? In what way are these effects related to the normal catalytic (biosynthetic) reaction?

The percent activity has already been found to be diminished to a greater extent than the fraction of subunits that were occupied by the Met(O)(NH)-P·ADP inactive complex (Rhee et al., 1981). Thus, the enzyme that was 50% active was designated EX_5, that 25% active as $EX_{6.5}$, and that 10% active as EX_8, where the subscript denotes the number of subunits out of 12 irreversibly inhibited in the dodecamer (to the nearest 0.50).

(B) Biosynthetic Parameters in EX_n Species. The V_{max} and K_m values of enzyme inhibited 50%, 75%, and 90% by Met-(O)(NH)-*S* in the presence of ATP were determined by steady-state kinetics. In these experiments one is essentially looking at the properties of the subunit active sites that are *not* occupied by Met(O)(NH)-P·ADP. Thus 90% inactive = 10% active = EX_8, etc. The double-reciprocal plots from which these data in Figure 4 were calculated are presented in the supplemental material and Figure 5.

As seen in Figure 4 (top) and Figure S2 the K_m for L-glutamate was only increased approximately 2-fold in the enzyme that was 90% inactive, EX_8.

A much more dramatic decrease in the binding of NH_4^+ is observed for the enzyme that was 100% active, compared to species with 25% or 10% residual activity, i.e., $EX_{6.5}$ or EX_8. In Hill plots of these data (not shown), the $S_{0.5}$ values increased at least 6-fold for EX_8 compared to EX_0. In the less tight phase of the biphasic saturation plots (Figure 5), the K_m for NH_4^+ increased at least 5–7-fold. These $S_{0.5}$ and K_m values agreed reasonably well and so are plotted as K_m in Figure 4. Much less dramatic changes in K_m were evident in the tighter phases of Figure 5. The biphasic nature of the NH_4^+ binding, observed previously by Meek & Villafranca (1980), is strongly enhanced either at low L-Glu or upon inactivation by Met-(O)(NH) plus ATP, and in the latter case became observable even at high (50 mM) L-Glu.

A study of the pH dependence of the K_m of NH_4^+ to fully active Mg-unadenylylated GS was carried out (Figure 6). The decrease in K_m with increasing pH is indicative of preferential binding of NH_3 over NH_4^+, since the data fit a line with slope of unity in this plot of log K_m vs. pH. An alternative explanation is that the binding of NH_4^+ depends upon a single protein group in its deprotonated form. Similar pH studies with complex enzymes have been carried out by Rife & Cleland (1980), who have presented the relevant equations for interpretation of experiments. The half-maximal value for the data in Figure 6 is near pH 7.5–8.0, fully 1 pH unit below the pK_a for the NH_4^+ ion. This may result from perturbation of the equilibrium constant for ionization by preferential binding of NH_3.

Interestingly, the K_m for ATP (Figure 4, top) decreases with increasing inactivation by Met(O)(NH) and ATP, at least 3-fold as one goes from EX_0 to EX_8. Synergism in the binding of Met(O)(NH) and ATP had been observed from spectroscopic titrations (Rhee et al., 1981; Timmons et al., 1974; Villafranca et al., 1976), but this was previously assumed to be strictly an intrasite effect. The present data indicate that Met(O)(NH) irreversibly bound as Met(O)(NH)-P·ADP on

[3] P. B. Chock, personal communication.

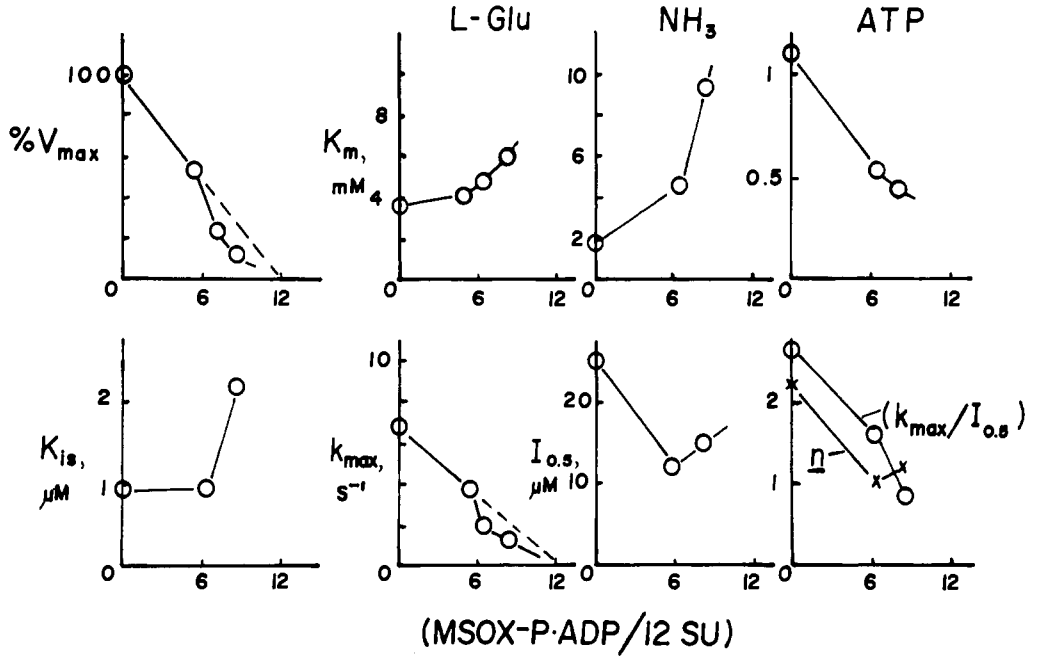


FIGURE 4: Kinetic properties of *E. coli* glutamine synthetase, partially inactivated by L-Met(O)(NH)-*S* and ATP, pH 7.5, 37 °C (see Experimental Procedures and text): % V_{max} and K_m's for substrates in the biosynthetic reaction from Figures 5, S2, and S-3; K_{is} for Met(O)(NH) vs. L-glutamate by steady-state kinetics (see Figure 2 for $EX_{(0)}$); k_{max} and $I_{0.5}$ for L-Met(O)(NH) irreversible inhibition with ATP, plus calculated Hill n_H values and ratio of $(k_{max}/I_{0.5})$ (see Figure 2). K_m values for NH_3 were in fact $S_{0.5}$ values from Hill plots of the data in Figure 5 at 3 mM L-Glu.

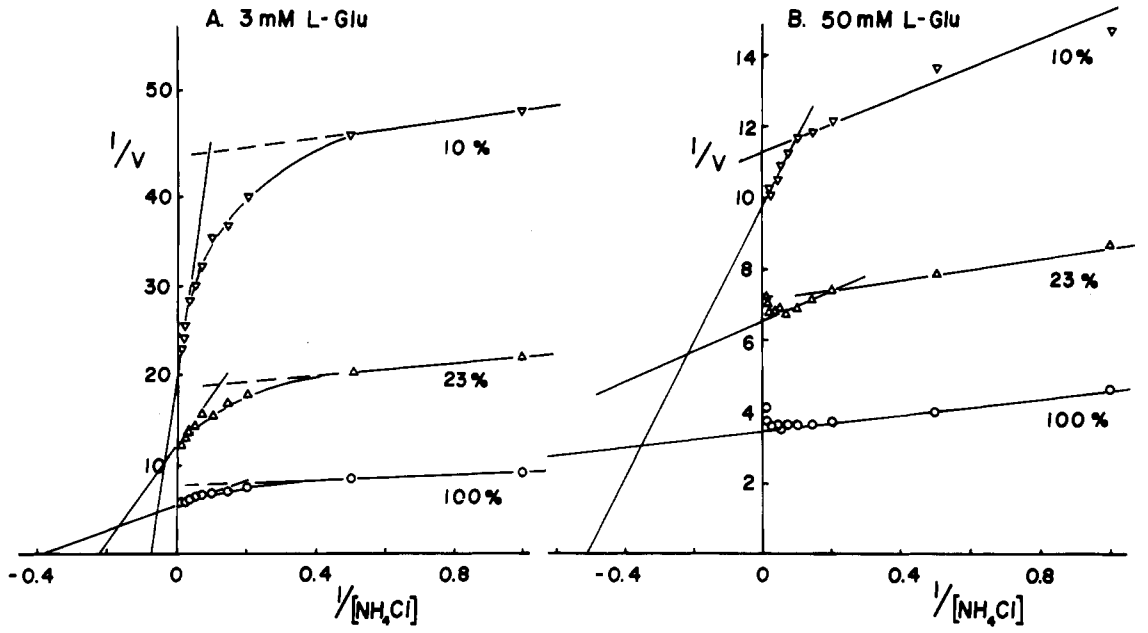


FIGURE 5: Double-reciprocal plots of velocity vs. [NH_4Cl] at (A) 3 mM L-glutamate and (B) 50 mM L-glutamate for *E. coli* glutamine synthetase partially inactivated by L-Met(O)(NH)-*S* and ATP: $EX_{(0)}$, 100%; $EX_{(6.5)}$, 25%; $EX_{(8)}$, 10% residual activity. Biosynthetic assays were carried out at pH 7.5, 37 °C (see Experimental Procedures). Calculated K_m or $S_{0.5}$ values are plotted in Figure 4 (see text).

one subunit diminishes the K_m for ATP on adjacent subunits, i.e., the synergistic effect is transmitted between subunits.

Changes in the V_{max} (biosynthetic reaction) and the k_{max} (irreversible inhibition reaction) plotted in Figure 4 indicate that neither of these parameters deviate significantly from a linear decrease in activity until approximately EX_5 is reached. Beyond this point, fairly strong deviations from linearity in both parameters occur. Therefore, one must conclude that the marked deviations from first-order kinetics of the irreversible inhibition reaction observed by Rhee et al. (1981) were due to changes in *both* the k_{max} and the ligand K_m values, but mainly with k_{max} since the enzyme was saturated with Met-(O)(NH).

(C) Irreversible Inhibition Kinetics in EX_n. Figure 2 indicates that the maximal velocity of irreversible inhibition of Mg-unadenylylated *E. coli* GS at 20 °C, k_{max}, is inhibited markedly in $EX_{6.5}$ and EX_8 (the 25% and 10% active species, respectively). The Hill plots of these velocities as a function of [Met(O)(NH)], Figure 2 (inset), indicate that $I_{0.5}$ decreases from 25 to ca. 13 μM and n_H from 2.2 to 1.3 from EX_0 to $EX_{6.5}$ or EX_8. These values are also plotted in Figure 4 along with the ratio of $(k_{max}/I_{0.5})$, which decreases markedly.

These data for $I_{0.5}$ and n_H are consistent with a model (see Scheme I) in which $I_{0.5}$, the half-saturation value for Met-(O)(NH) in the irreversible inhibition reaction, becomes lower as enzyme subunits become progressively more inactivated.

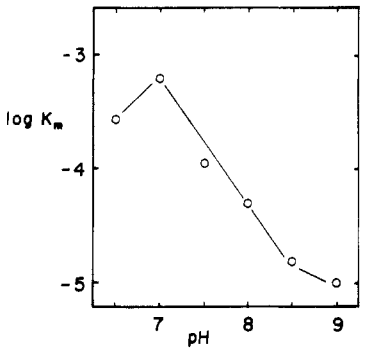
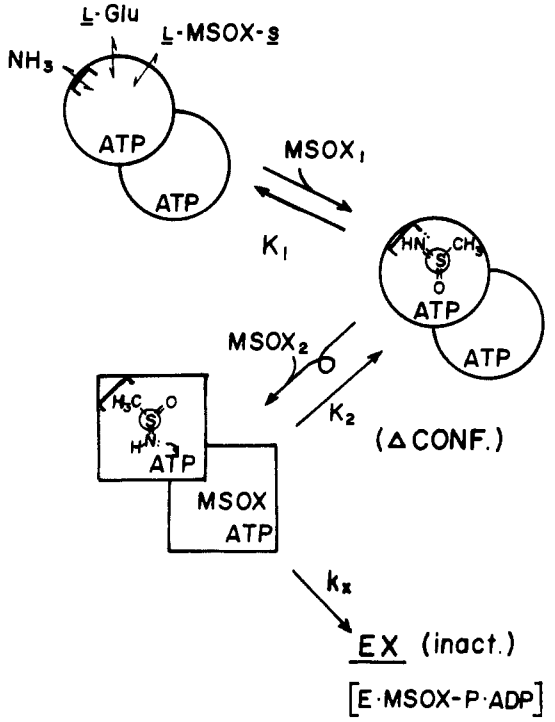


FIGURE 6: Dependence of the K_m of NH_4Cl upon pH with *E. coli* glutamine synthetase. The slope of the straight line between pH 7.0 and 8.5 was calculated from the equation $\log y = \log [C(1 + K/H)]$; see text. The L-glutamate concentration was 50 mM or higher.

Scheme II



In this model, as the percent inactivation increases, the extent of the conformational change in the still-active subunits increases so that the positive cooperativity is significantly diminished. That is, the enzyme is shifted further toward a conformation that binds inhibitor more tightly. In EX_8, $I_{0.5}$ = 13 μM and $K_{is} \simeq 2$ μM. Thus, $K_{is}/I_{0.5}$ is only ca. 6 for EX_8 whereas it is ca. 26 in EX_0.

Hypothesis. A hypothetical scheme that provides an integrated explanation for the observed differences between K_{is} and $I_{0.5}$ for Met(O)(NH), the positive cooperativity in $I_{0.5}$, and the negative cooperativity in equilibrium binding and titrations with Met(O)(NH) is shown in Scheme II. The initial step (i.e., K_{is}) involves competition of NH_3 and L-Glu vs. Met(O)(NH) in the biosynthetic reaction, with tight binding of Met(O)(NH) in a mode sterically unfavorable for reaction with ATP. Next, binding of a second molecule of Met(O)(NH) to an adjacent subunit drives an energetically unfavorable conformation change (i.e., $I_{0.5}$): rotation of the imino group of the sulfoximine toward the γ-phosphoryl of ATP, which allows reaction with ATP to form the inactive Met(O)(NH)-P·ADP complex. Experiments with analogues of Met(O)(NH) specifically designed to test this hypothesis are described below.

Table I: Summary of K_{is}, $I_{0.5}$, k_{max}, and n_H Values for Met(O)(NH) and Its Analogues (See Text) from Steady-State Kinetics

inhibitor	vs.	K_{is} (μM)	$I_{0.5}$ (μM)	k_{max} (s^{-1})	n_H
L-Met(O)(NH)-*S*	L-Glu	0.96	25	0.0062	2.2
	NH_4Cl	12			
	L-Gln	380			
L-Met(O)(NH)-*R*	L-Glu	42		NR[c]	
L-Eth(O)(NH)-*SR*[a]	L-Gln	1700	8400	0.041	1.4
cyclo-Met(O)(NH)[b]					
crystal form I	L-Glu	29000		NR[c]	
crystal form II	L-Glu	23000		NR[c]	
L-Met(O)$_2$[d]	L-Glu	240		NR[c]	

[a] L-Ethionine (*SR*)-sulfoximine. [b] Cyclo-Met(O)(NH), 3-amino-3-carboxytetrahydrothiopyran sulfoximine. [c] NR, no reaction with ATP to give irreverisble inhibition. [d] L-Methionine sulfone; data from Wedler & Horn (1976).

(*D*) *Analogues of Met(O)(NH).* If the hypothesis in Scheme II is correct, then structural analogues of Met(O)(NH) that are rotationally restricted about the sulfoximine–carbon side-chain bond should fail to participate in the irreversible inhibition reaction or be drastically altered in their kinetics and inhibitory properties. Two such analogues have been synthesized and tested.

(*1*) *Ethionine Sulfoximine.* This analogue has the sterically larger ethyl group in place of methyl on the sulfur atom. Steady-state competition experiments vs. L-glutamate were carried out, as in Figure 1. L-Ethionine (*SR*)-sulfoximine was found to be a linear competitive inhibitor vs. L-glutamate, with K_{is} = 1.7 mM. It was also found to cause irreversible inactivation of the enzyme in the presence of MgATP, pH 7.5. The kinetics of this reaction, as in Figure 2, were found to be slightly sigmoidal, with n_H = 1.4 and $I_{0.5}$ = 8.4 mM with k_{max} = 4.1 × 10^{-2} s^{-1}. Since both K_{is} and $I_{0.5}$ are much larger for ethionine sulfoximine than for Met(O)(NH), obviously the steric size of the group on position 5 is critical in the active site. Interestingly, ethionine sulfoximine reacts 7-fold more rapidly with ATP than methionine sulfoximine and has a $I_{0.5}/K_{is}$ ratio of 5.0, as compared to 26 for Met(O)(NH). This may suggest that the ethyl group sterically favors rotation of the imino group further toward a conformation in which reaction with ATP can occur.

(*2*) *Cyclo-Met(O)(NH).* The two isomers (cis, trans) of 3-amino-3-carboxytetrahydrothiopyran sulfoximine were synthesized and separated as two crystal forms (see Experimental Procedures), but the absolute stereochemistry of each crystal form was not determined. In steady-state kinetics each of the two isomers was linearly competitive vs. L-glutamate, with crystal form I having K_{is} = 29 mM and II having K_{is} = 23 mM, i.e., each binding only about 16-fold less tightly than ethionine sulfoximine, 10-fold less tightly than L-Glu, but much less tightly than methionine sulfoximine. In incubation experiments with enzyme and MgATP, pH 7.5, at 37 °C, no irreversible inhibition of enzyme activity was observed with either crystal form, up to an inhibitor concentration of 200 mM, however. The significance of this latter result is discussed below.

The binding and kinetic constants for Met(O)(NH) and its various analogues with *E. coli* GS, pH 7.5, are summarized in Table I.

Discussion

Even though *E. coli* glutamine synthetase has a dodecameric structure, the discovery of subunit interactions has been a long and arduous process. Complex heterotropic interactions had

been suggested earlier by the cooperativity observed in the equilibrium binding of L-tryptophan (Ginsburg, 1969), along with some interactions between the AMP and L-tryptophan modifier sites, and for L-glutamate binding between adenylylated and nonadenylylated subunits (Ginsburg et al., 1970). The current data, however, bear upon the question of homotropic interactions in relation to the catalytic mechanism.

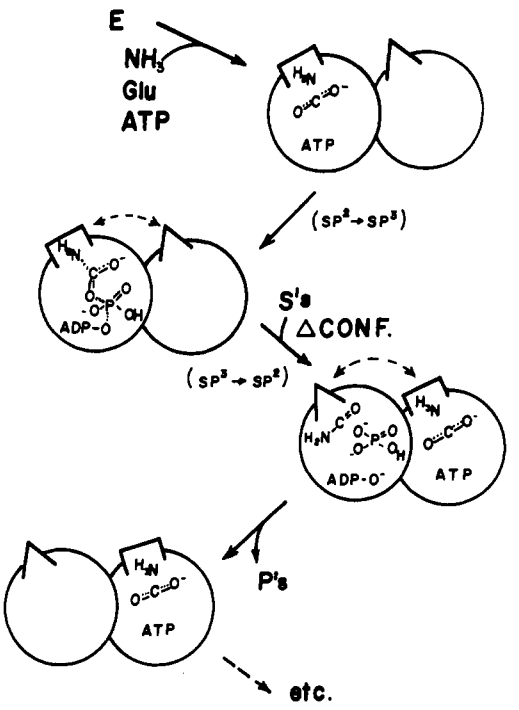
Several lines of evidence clearly demonstrate behavior other than simple monophasic saturation by ligands at the active site of *E. coli* unadenylylated GS: (a) Fluorescence titrations with L-Met(O)(NH)-*S* alone or in the presence of AMP-PNP indicated negative cooperativity, i.e., $n_H < 1$ (Rhee et al., 1981). (b) Ultraviolet difference spectral titrations with L-Met(O)(NH) also showed $n_H < 1$ (Shrake et al., 1980). (c) The kinetics of irreversible inhibition with L-Met(O)(NH) with MgATP deviate strongly from first-order dependence after one half-life or less (Rhee et al., 1981). (d) The concentration dependence of the rate of this latter reaction (Figure 2) shows positive cooperativity, $n_H = 2.2$. Other current evidence includes the following observations: (e) Equilibrium binding of L-Met(O)(NH) is biphasic (Figure 3) but extrapolates to a maximum of one L-Met(O)(NH) per subunit. (f) The percent of subunits inactivated is greater than the percent of subunits occupied by the MSOX-P·ADP complex (Rhee et al., 1981). (g) Enzyme that was only partially inhibited irreversibly by L-Met(O)(NH)-*S* and ATP shows changes in both the catalytic properties and the binding constants for ligands to the subunits that were blocked (Figure 4). In particular, one sees an increased K_m for NH_3 and decreased K_m for ATP (Scheme I and Figure 4). (h) Stopped-flow fluorescence experiments[4] with Met(O)(NH) + enzyme–ATP show saturation of rates as a function of [MSOX] that gives $n_H < 1.0$. (i) Biphasic binding of the fluorescent dye TNS to the enzyme can be interpreted as negative cooperativity (Wedler et al., 1977) since only one TNS is bound maximally per subunit.[3] (j) Both the present results (Figure 2) and those of Meek & Villafranca (1980) indicate apparent negative cooperativity in the kinetics of binding of ammonia to the enzyme.

In terms of the hypothesis of Scheme II for the involvement of subunit interactions in the irreversible inhibition reaction of Met(O)(NH) and ATP, the experiments with rotationally restricted structural analogues of Met(O)(NH) are informative but not absolutely definitive. Ethionine sulfoximine binds much less tightly than Met(O)(NH) but has a K_{is} value nearer to $I_{0.5}$ than does Met(O)(NH). The cyclo-Met(O)(NH) isomers bound rather poorly but were competitive vs. L-Glu. Most significantly, these latter compounds failed to undergo any irreversible inhibition reaction with ATP. This supports the conformational change model of Scheme II, in which failure to rotate the sulfoximine group would prevent reaction with ATP, as observed.

The anomalously high K_i for L-Met(O)(NH)-*S* vs. L-Gln measured in the transferase assay (380 μM) is difficult to rationalize, except in terms of an unfavorable geometry induced in the site by the product analogue arsenate and the substrate analogue hydroxylamine that in turn makes the binding of inhibitor less favorable. Alternatively, the larger steric size of arsenate and NH_2OH compared to the phosphate and NH_3 may simply interfere directly with Met(O)(NH) binding. The sulfoximine group is also larger than the carboxylate or amide of L-Glu or L-Gln.

The fact that NH_3 rather than NH_4^+ ion appears to be the preferred species bound to the enzyme (Figure 6) implies that

Scheme III



GS discriminates between 10^{-5} M NH_3 vs. 55 M H_2O, at least 7 orders of magnitude difference. This cannot be due only to differences in the pK_a's of H_2O vs. NH_4^+ since if the enzyme did not also discriminate between these two in terms of *binding* as well as *catalysis* (basicity, nucleophilicity), NH_3 would compete for binding vs. a 55 M inhibitor. Very specific recognition of NH_3 over H_2O, to prevent Glu-promoted ATPase activity, is therefore implied. The NH_3 site probably consists of protein groups capable of H bonding, at least one of which must be a general base to aid catalytic conversion of NH_3 to the amide NH_2 group.

With regard to the catalytic mechanism, recent evidence suggests that energy from substrate binding may be used to modulate reaction reversal and to promote turnover, the rate-limiting step for which appears to be product dissociation (Stokes & Boyer, 1976; Wedler, 1974; Wedler & Boyer, 1972). Bild et al. (1980) have observed that $[NH_4^+]$ modulated the ^{18}O exchange between L-glutamate and P_i during net turnover but that [ATP] had little effect on the number of reaction reversals prior to product release. Midelfort & Rose (1976) had also observed that ammonia suppressed the positional isotope exchange in β–γ bridge (^{18}O-labeled) ATP. Meek & Villafranca (1980) and we (Figure 5) have observed that double-reciprocal plots of steady-state kinetic data for ammonia binding are nonlinear (concave downward) at limiting L-glutamate, indicating negative cooperativity. Partial inactivation by Met(O)(NH) and ATP enhances this effect (Figure 5). Binding at the ATP subsite produces little or no effect, as indicated by the affinity labeling experiments by Foster et al. (1981) with 5′-[*p*-(fluorosulfonyl)benzoyl]-adenosine in which the inhibition kinetics were first order and showed strictly hyperbolic ($n_H = 1$) saturation. Thus, it appears that especially the NH_3 binding site is critically related to the modulation of the product release rate. Synergism of binding between ligands in the L-glutamate and ATP sites is also clearly indicated, with the data of Figure 4 being the first to indicate that this synergism may be transmitted between subunits. Recently, Hunt & Ginsburg (1980) have found that L-Glu or Met(O)(NH) binding caused increased association constants (K_a) for Mn^{2+} at the tight (n_1) site on *E. coli* GS

[4] S. G. Rhee, P. B. Chock, and F. C. Wedler, unpublished results.

but that L-Gln did not alter K_a.

With the current data, plus these recent findings, it seems appropriate to suggest a mechanism in which NH_3 binding enhances catalytic turnover by promotion of the dissociation of products. This hypothesis is presented in Scheme III, in which the binding of NH_3 promotes both the collapse of the tetrahedral transition state and the dissociation of products, specifically by driving the amino group of L-Gln out of the ammonia subsite. There is no significance to the use of a dimeric model, except for the sake of simplicity and its ability to predict most of the current observations.

This dimeric model in Scheme III is not intended to imply a flip-flop or alternating site model as the only possibility for *E. coli* glutamine synthetase, for which n_H values over the range 0.5–0.9 have been observed. An alternative site model would require $n_H = 0.5$. Other current evidence for *E. coli* glutamine synthetase is not consistent with a half-of-the-sites mechanism for ligand binding, however. At least 50% of enzyme activity must be lost before one observes deviations from linearity in V_{max} (Figure 3), 1:1 stoichiometry of ATP binding (Rhee et al., 1981), or changes in K_m's (Figure 3). In contrast, classical examples of alternating site enzymes include glyceraldehyde-3-P dehydrogenase for which only two of the four subunits can be acylated by substrates or need be cyanylated for 100% activity loss and in which cofactor induces anticooperative binding (Byers & Koshland, 1975; Scheek et al., 1979; Stallcup & Koshland, 1973). With pigeon liver malic enzyme, Hsu & Pry (1980) found that only half the subunits participate in catalysis at any time. Inactivation of the pig heart L-alanine transaminase by Burnett et al. (1980) with the mechanism-based inhibitor L-propargylglycine was accomplished by incorporation of only 1 mol of inhibitor per dimer at 97% inhibition of activity. Interestingly, with *E. coli* glutamine synthetase, such inhibition by Met(O)(NH)-*S* and ATP causes both diminished catalytic efficiency and ligand binding.

Several recent examples of substrate modulation of catalysis in mechanistically related systems are relevant to this discussion. Bild et al. (1980a,b) observed that $[^{18}O\text{–}P_i] \rightleftarrows$ succinate exchange was modulated on succinyl-CoA synthetase by ATP but not CoA and proposed an alternating site model for ATP promotion of catalysis. The recent elegant results of Wolodko et al. (1981) indicate that succinyl-CoA synthetase operates with alternating sites cooperativity of catalysis. With the rat liver glutamine synthetase, Tate et al. (1972) found only four Met(O)(NH) required for complete inhibition of activity and that the enzyme bound only ca. five ATP per octamer. Recently, Graves et al. (1979) have observed anticooperative binding of L-tryptophan to tryptophan-tRNA synthetase. The recently discovered multiple forms of glutamine synthetase from *Bacillus caldolyticus* also exhibit strong apparent negative cooperativity in the binding of ammonia, suggesting that similar substrate modulation effects may occur with these systems (Wedler et al., 1980).

Acknowledgments

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Supplementary Material Available

Graphs showing the steady-state inhibition kinetics of *E. coli* GS for L-Met(O)(NH)-*S* vs. (A) NH_4Cl and (B) ATP in the biosynthetic assay, pH 7.5, 37 °C (Figure S1), double-reciprocal plots of velocity vs. [L-glutamate] for *E. coli* GS partially inactivated by L-Met(O)(NH)-*S* and ATP (Figure S2), double-reciprocal plots of velocity vs. [ATP] for *E. coli* GS partially inactivated by L-Met(O)(NH)-*S* and ATP (Figure S3), and changes in substrate binding parameters for *E. coli* glutamine synthetase as a function of percent inactivation by L-Met(O)(NH)-*S* and ATP (Figure S4) (4 pages). Ordering information is given on any current masthead page.

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Affinity Labeling of a Guanosine 5′-Triphosphate Site of Glutamate Dehydrogenase by a Fluorescent Nucleotide Analogue, 5′-[*p*-(Fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine†

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ABSTRACT: Bovine liver glutamate dehydrogenase is reversibly inhibited by the fluorescent nucleotide 1,N^6-ethenoadenosine 5′-triphosphate (ϵATP) and reacts irreversibly with the corresponding affinity label 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine. The enzyme is not inactivated by this reaction as measured in the absence of modifiers. However, a time-dependent increase is observed in the catalytic activity of the enzyme, when assayed in the presence of the allosteric inhibitor guanosine 5′-triphosphate (GTP). This change in inhibition by GTP allows determination of a rate constant for reaction with 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine. A nonlinear dependence of the reaction rate on reagent concentration suggests a reversible binding prior to irreversible modification. The rate constant is unaffected by the substrate α-ketoglutarate or high concentrations of reduced diphosphopyridine nucleotide (DPNH) alone and is only slightly lowered by the activator adenosine 5′-diphosphate (ADP). A decrease in the rate constant is caused by added GTP or ϵATP, and a combination of GTP in the presence of reduced coenzyme provides complete protection. The compound 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-etheno[2-^{3}H]-adenosine was synthesized and used to show that 1.28 mol of 5′-(*p*-sulfonylbenzoyl)-1,N^6-ethenoadenosine/mol of subunit is incorporated at 100% change in sensitivity to GTP inhibition. As compared to native glutamate dehydrogenase, the modified enzyme exhibits a decreased affinity for and diminished maximum inhibition by saturating concentrations of GTP, a decreased maximum extent of activation with no change in affinity for ADP, and a normal ability to be inhibited by high DPNH concentrations. In contrast to the 2 mol of GTP bound by native enzyme, only 1 mol of GTP is bound per peptide chain of the modified enzyme in the absence or presence of DPNH. This implies that one of the natural GTP sites is eliminated as a result of reaction with 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine. This study indicates that reaction occurs at a GTP site and suggests that alteration of the N-1 and 6-NH_2 positions of the adenine ring leads to recognition by glutamate dehydrogenase as an inhibitory guanine nucleotide.

The fluorescent nucleotides 1,N^6-ethenoadenosine triphosphate and 1,N^6-ethenoadenosine diphosphate have been widely utilized in probing the environment of binding sites in proteins (Secrist et al., 1972; Yanagida, 1981). These compounds possess two important properties that can be useful in studies of protein structure: first, the fluorescent properties can be employed in the measurement of the distance between sites on a protein by energy transfer (Brand & Witholt, 1967; Horton & Koshland, 1967; Stryer, 1978); second, since the N-1 and 6-NH_2 purine positions are altered by the etheno bridge in the 1,N^6-ethenoadenine moiety of these nucleotides, the importance of these positions in determining the specificity of binding of these nucleotides to particular enzymes can be evaluated.

The activity of the allosteric enzyme bovine liver glutamate dehydrogenase [L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3] is modulated by GTP,[1] which inhibits, ADP, which activates, and DPNH, which inhibits at high concentrations by binding at a site distinct from the catalytic site (Goldin & Frieden, 1972). Glutamate dehydrogenase is composed of six identical subunits with several nucleotide sites per subunit, including a site for ADP, two for GTP, and two for DPNH (one catalytic and one regulatory) (Pantaloni & Dessen, 1969; Goldin & Frieden, 1972; Pal & Colman, 1979). The number of GTP binding sites observed depends on the presence of the reduced coenzyme: in the absence of DPNH, native enzyme has one GTP binding site, whereas in the presence of DPNH, two GTP binding sites are observed that differ in affinity for the guanine nucleotide (Pal & Colman, 1979).

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[1] Abbreviations: 5′-FSBϵA, 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine; ϵATP, 1,N^6-ethenoadenosine 5′-triphosphate; ϵADP, 1,N^6-ethenoadenosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; DPNH, reduced diphosphopyridine nucleotide; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; TPNH, reduced triphosphopyridine nucleotide.

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