Mechanisms of Substrate Binding with Glutamine Synthetase

EQUILIBRIUM ISOTOPE EXCHANGES WITH THE OVINE BRAIN, PEA SEED, AND ESCHERICHIA COLI ENZYMES*

(Received for publication, April 16, 1973)

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SUMMARY

Equilibrium isotope exchange kinetics were used to investigate the sequences of substrate binding with ovine brain, pea seed, and Escherichia coli glutamine synthetases. Without exception, the relative rates of exchange are (glutamate ⇄ glutamine) > (NH₃ ⇄ glutamine) > (Pᵢ ⇄ ATP) ≈ (ADP ⇄ ATP). This suggests that the rate of net turnover at saturating substrate levels is limited more strongly by the rate of nucleotide release than by the rate of covalent interconversion. With the ovine brain enzyme, the kinetic patterns of equilibrium exchange are consistent with a partially ordered sequence of substrate binding, where ATP binds before NH₃, glutamate leaves before ADP, but glutamate and phosphate are bound and released randomly on their respective sides of the reaction. This particular binding order does not permit observation of those partial exchange reactions expected if an enzyme-bound γ-glutamyl phosphate intermediate were formed. Upon variation of the concentration of all substrates in constant ratio at equilibrium, the pea seed, the Mn²⁺-activated adenylylated and the Mg²⁺-activated unadenylylated E. coli enzymes exhibit exchange kinetic patterns consistent with random substrate addition and release. The kinetic data also suggest synergism of substrate binding between glutamate and ATP. A brief section on theory and interpretation of kinetic patterns of equilibrium exchanges as related to substrate-binding order is presented.

Recent studies on glutamine synthetase in Escherichia coli (1–4) have led to an understanding of the central role this enzyme plays in regulation of nitrogen metabolism. The reaction catalyzed is

\[ \text{NH}_3 + \text{L-glutamate} + \text{ATP} = \text{L-glutamine} + \text{ADP} + \text{P}_i \]  

Enzymes from divergent sources are known to differ in their properties, for example, metal ion and substrate specificity, pH activity profiles, quaternary structures, and responses to feedback modifiers (5–9). The research described here is part of an investigation of differences in mechanism of action among the enzymes from bacterial, plant, and mammalian sources. Specifically, this study investigates whether the mechanisms of substrate binding differ markedly, as suggested by earlier data. With the ovine brain enzyme, the observed enhancement of glutamate binding by added ATP has led Meister and co-workers (10, 11) to propose that reactant's binding is ordered: first ATP binds, then glutamate, then ammonia. The tight (E·ATP·Glu) complex in ammonia-free solution appears to exist as (E·ATP·Glu-P). ADP is not released, however, and hence an independent ADP ⇄ ATP exchange is not observed. This suggests that product release also may be ordered.

With the pea seed enzyme, the equilibrium exchange data of Graves and Boyer (12), observed upon variation of substrate pairs, indicated a partially compulsory order of binding: preferential binding of NH₃ after L-glutamate and compulsory release of ADP before glutamine and possibly before Pᵢ. The kinetic patterns that led to these conclusions might be attributable in some cases to competitive substrate interactions, however, as found in similar experiments with the E. coli enzyme (13). Previous kinetic data (13) with adenylylated E. coli glutamine synthetase (E₄) were consistent with a random order substrate-binding scheme, but were obtained with a mixture of Mg²⁺ and Mn²⁺. Random substrate binding is verified in the present study for both the adenylylated and unadenylylated forms, activated purely by Mn²⁺ and Mg²⁺, respectively.

The technique employed in this study is isotopic exchange kinetics at chemical equilibrium, a powerful and incisive approach to enzyme mechanisms (see under "Theory"). The sorts of kinetic patterns that can result from synergistic substrate binding, as opposed to those characteristic of ordered substrate binding, are defined by computer-derived model studies.

EXPERIMENTAL PROCEDURE

Enzymes and Materials—Ovine brain glutamine synthetase was prepared by procedures of Rowe et al. (11, 14). Final specific activity was 100 units per mg. The enzyme from green peas (stewart Blue Bantam) was prepared as described by Elliott (15), with the last step repeated twice, followed by passage through a column of Bio-Gel agarose 0.5m. This gave an ~4000 X purified preparation.

1 In this paper, the term "substrates" refers to all reacting species, e.g. for glutamine synthetase: NH₃, L-glutamate, ATP, L-glutamine, ADP, and Pᵢ. "Reactants" refers to NH₃, L-glutamate and ATP, while "products" refers to L-glutamine, ADP, and Pᵢ. The designation "reaction components" includes all substrates plus metal ions, salts, buffer, etc.
with specific activity of 485 units per mg, as compared to 220 units per mg in the original (15) preparation. Both enzyme preparations were free of adenylate kinase, adenosine triphosphatase, and glutaminase activities. Both enzymes showed single bands upon electrophoresis on polyacrylamide gels at pH 8.5 and also in procedures with enzyme in 0.1% sodium dodecyl sulfate (16). The freezing or lyophilization steps were replaced by storage at 4° in 50% glycerol-buffer (pH 7, 20 mM Tris, 2 mM EDTA, and 2 mM diethanol). One unit of activity was defined as in the preparative procedures (14, 15). R. sili glutamine synthetase, adenyllylated and unadenyllylated, was purified to homogeneity by the procedures of Shapiro and Stadtman (2).

The reaction components l-glutamate, l-glutamine, ATP, and ADP were of highest quality obtainable, supplied by Sigma Chemical Co. Radioactive substrates were obtained from New England Nuclear. [31P]Adenosine (99%) was from Bio-Rad Laboratories.

DEAE-cellulose (formate) used for separation or reaction components was prepared from Whatman DE52 by batchwise washing in 0.5 M formic acid, then washing to neutrality with double distilled water.

Methods—The procedures for preparing a series of reaction solutions at chemical equilibrium, maintaining ionic strength and pH constant, have been described elsewhere (15). The series of solutions usually 1.0 ml each, was then thermostated, a constant amount of enzyme added to each, and allowed to equilibrate for 10 min to establish chemical equilibrium exactly. The isotopic exchange reaction was then initiated upon addition of a low level (<1% of the total pool size) of high specific activity substrate, e.g. l-[^15N]glutamate. The reactions were then incubated for a specific time period, usually 30 min or less, then the reactions were stopped by addition of EDTA (20% excess over metal ion present), dilution to 3.0 ml, then chilling to 0° or freezing prior to separation of the reactants undergoing isotopic exchange.

Reactions involving ovine brain and pea enzymes were carried out at 37° and 30°, respectively, all at pH 6.5 with 50 mM MgCl₂, since the results of Monder (17) and Elliott (15) indicate that each of these systems exhibit optimal activities under these conditions. The equilibrium constant for the reaction at 30° and 37° was verified by procedures described elsewhere (13) to be 460 ± 30. Kinetics with the unadenyllylated E. coli (Eii) enzyme employed reaction conditions satisfying a Kₘ = 1200 (18) at pH 7.0, 37°, with Mg²⁺ present. The Eii enzyme exhibits ~50% activity under initial velocity conditions at pH 7.0, compared to that at pH 7.5, the pH optimum (1–4). The adenyllylated enzyme (Eii) was studied at pH 6.5, 37°, with [Mn⁺] = [ATP] + [ADP] + 1 mM.

Separation of substrate pools following each set of exchange reactions was accomplished by chromatography on DEAE-cellulose (formate). The gradient elution procedure described by Wedler and Boyer (13) was modified to three separate stepwise elution procedures. Each stopped and diluted reaction mixture was applied to a column (0.7 X 13 cm) of DEAE-cellulose. Elution with first 20 ml of water, then 20 ml of 0.5 M HCl, separated glutamine and NH₄⁺ from glutamate. Phosphate and ATP were separated by washes with 20 ml of 0.05 M HCl, then 20 ml of 0.5 M HCl, respectively. ADP and ATP were separated by washes with 25 ml of 0.05 M HCl and 20 ml of 0.5 M HCl, respectively. Samples of the pools thus separated were counted by liquid scintillation techniques. From calculated disintegrations per min values, the number of micromoles exchanged between pools per unit time was calculated by equations presented previously (13).

Exchange reactions involving l[^31P]labeled ammonium were carried out with l[^15N]glutamate also present for comparison of exchange rates. After separation of NH₄⁺ and glutamate from glutamate, as described above, and determination of [15N]glutamate ± glutamine, glutaminase was rendered free of NH₄⁺ by passage through Dowex 50 (K⁺). Chemical procedures for conversion of NH₄⁺ to N₂ are described elsewhere (13, 19). Masses 0 to 50 were scanned for each sample with an Electro dynamically Corp. model 21130 mass spectrometer. Atom percent excess [31P] in N₂ (14N) was calculated as micromoles of 14N exchanged from NH₄⁺ to glutamine.

Theory

Consider first a two-reactant, two-product system in which random order substrate binding occurs, represented in the nomenclature of Clossen (20) by Equation 1.

\[ \text{[D, Q]} \]

\[ \text{[A, P]} \] const.

\[ \text{R \ (B + Q)} \]

\[ \text{R \ (A + P)} \]

\[ \text{SCHME 1} \]

This pattern is one expected for the binding mechanism of Equation 3. Note that R² rises smoothly to a maximum, but R peaks then falls toward zero. The basis for this behavior is that in a random order system A and P can escape from the complexes EA, EP, EAB, and EPQ, but, in the ordered system of Equation 3, A and P are released only from EA and EP. Thus in an ordered system (not but in a random one) changes in the levels of EA and EP can control the rate of A ⇔ P exchange. At high [B] and [Q], the complexes EA and EP are drawn over into EAB and EPQ and A ⇔ P is diminished for the ordered system of Equation 3.

Variation in [A, P] with constant [B] and [Q] gives an exchange pattern where both R and R² rise to a maximum without inhibition. This lack of inhibition can be used to exclude the binding of A after B or the release of Q before P.

In addition, a comparison of relative equilibrium exchange rates at saturating substrate levels also can be used to exclude certain orders of substrate binding. In an ordered binding situation, the exchange rate between the first substrates to dissociate from the central complexes (B ⇔ Q in Equation 3) must be as fast as or faster than all other exchanges. If all exchange rates are equal, then covalent interconversion is likely to be definitively rate-limiting. If the exchange rates are unequal, as is

\[ ^{1} \text{This nomenclature differs from that of Clossen (20) in that Q is released before P, but is used elsewhere (26) deliberately in equilibrium exchange derivations to indicate that P is the first product to bind in the reverse reaction or at equilibrium.} \]
most often observed with actual enzyme-catalyzed exchanges (13, 23), covalent interconversion cannot be the slowest step, and individual substrate dissociation rates determine exchange rates.

Finally, it has been shown (24) that feedback modifiers may produce distinctive changes in $R$ and $R'$ with random versus compulsory order-binding systems. In some cases, therefore, modifier-induced inhibition patterns can be used as evidence to support or exclude ordered binding (25).

RESULTS

Ovine Brain Enzyme—Variation of all substrates in constant ratio at equilibrium produces the kinetic patterns seen in Fig. 1. Substrates were raised to values above their published $K_m$ values (26). The rate of glutamate $\leftrightarrow$ glutamine exchange is faster than the rates of $P_i \leftrightarrow$ ATP or ADP $\leftrightarrow$ ATP at all levels of substrates, and the rate of glutamate $\leftrightarrow$ glutamine exchange rises smoothly toward a maximum. This argues that none of the other four substrates bind after glutamate or glutamine in a compulsory manner. However, the $P_i \leftrightarrow$ ATP and ADP $\leftrightarrow$ ATP exchanges first rise to a maximum then fall toward zero as substrate levels increase. These patterns may result from ordered substrate binding, or alternatively from substrates acting as negative modifiers at noncatalytic sites. Variation of all substrates in constant ratio blocks out competitive effects at the catalytic site. If ordered binding is the principal effect, then either NH$_3$ or l-glutamate bind after ATP, or glutamine is released before ADP or $P_i$, or both may be true. Alternative explanations for such peaking and inhibition patterns, other than ordered substrate binding, are presented under “Discussion.”

The saturation curves in Fig. 1 show sigmoidal character at low substrate, especially in the glutamate $\leftrightarrow$ glutamine exchange. It was found that by repeating the experiment with different absolute levels and ratios of glutamate, glutamine, and $P_i$ it was possible to enhance or abolish the sigmoidicity in glutamate $\leftrightarrow$ glutamine and increase or decrease the maximal rate as well. These phenomena were not investigated further, although a likely explanation seems to be competitive interactions between glutamate and glutamine, leading to dead-end complexes such as ($E\cdot$Glu$\cdot$ADP$\cdot$P$_i$) or ($E\cdot$NH$_3\cdot$Gln$\cdot$ATP). Other explanations for the sigmoidal effects are considered below.

Fig. 2 presents the results of varying first (A) the pair l-glutamate and l-glutamine, and then (B) the pair $P_i$ and ATP. In all cases the kinetic responses of the glutamate $\leftrightarrow$ glutamine and $P_i \leftrightarrow$ ATP exchanges are all distinctly hyperbolic and rise smoothly to a maximum. The absence of inhibition patterns in any exchange rate curve even at high substrate implies that the sequences of binding of glutamate relative to ATP and of glutamine relative to $P_i$ are completely random.

The data in Fig. 2 also indicate that there may be some mutual synergism of binding between amino acid and nucleotide substrates under dynamic conditions. Initial velocity kinetic data indicate a $K_m$ value for ATP of 1.5 mM at pH 6.5, 37°, with 50 mM MgCl$_2$ present. In the experiment of Fig. 2A, ATP was used at fixed levels above and below this value. This altered the half-saturation level of glutamate and glutamine in the glutamate $\leftrightarrow$ glutamine exchange. In this same figure, $P_i \leftrightarrow$ ATP shows a half-saturation value below that for glutamate $\leftrightarrow$ glutamine. In Fig. 2B, upon varying $P_i$ and ATP with fixed glutamate and glutamine levels, the glutamate $\leftrightarrow$ glutamine exchange half-saturates at a lower substrate level than does $P_i \leftrightarrow$ ATP.

Fig. 3 presents the effects on exchange kinetics of varying (A) glutamate and glutamine levels, and (B) $P_i$ and ATP levels, in constant ratio at equilibrium upon glutamate $\leftrightarrow$ glutamine and glutamine exchange catalyzed by ovine brain glutamine synthetase, pH 6.50, 37°, 30 min. At maximal levels (O) each 1.0 ml contained the same reaction component and enzyme levels as in Fig. 1. The glutamate $\leftrightarrow$ glutamine exchange also was observed (●) with ATP and ADP at 1.0 and 2.5 mM, respectively.
the ATP and ADP pair, and (B) the NH₃ and L-glutamine pair. In Fig. 3A, P₁ ⇌ ATP rises smoothly to a maximum, indicating that ADP is not released before P₁. The glutamate ⇌ glutamine exchange, however, rises to a peak, then is partially inhibited. Such partial inhibition is usually explained in terms of a random sequence of binding (see Equation 2) with one branch kinetically preferred. However, since (glutamate ⇌ glutamine) > (P₁ ⇌ ATP) under all conditions, it is not logically consistent that glutamate binds preferentially before ATP, or that glutamine is released after ADP (see under “Theory”). Tight complexation of amino acid and nucleotide substrates under saturating conditions, due to the mutual synergism of binding noted above, might be another possible explanation for this partial inhibitory effect. Kinetic models designed to test this possibility are derived and discussed below.

Yet another explanation is that nucleotide binds to a non-catalytic, separate modifier site. This is supported by observations of Wellner and Meister (27) that the stoichiometry of ADP binding to ovine brain enzyme subunits is at least 2:1. The mode of modifier action likely to produce the pattern in Fig. 3A is a differential, partial inhibition of glutamate or glutamine dissociation. Present data do not support or disprove this idea.

In Fig. 3B, upon raising the levels of NH₃ and glutamine, the glutamate ⇌ glutamine exchange rises smoothly to a maximum, which indicates that NH₃ does not bind after glutamate. The P₁ ⇌ ATP exchange, however, shows a rise, then strong inhibition. This could, a priori, result from NH₃ binding after ATP, or glutamine being released before P₁, or both. The data of Fig. 2, however, showed that P₁ and glutamine bind randomly relative to each other. The inhibition of P₁ ⇌ ATP in Fig. 2B appears to result from NH₃ binding after ATP in a compulsory manner. Next, experiments were conducted to determine whether either glutamine or P₁ is released in an ordered manner before ADP on the product side of the reaction. Thus, the ADP ⇌ ATP exchange kinetics were observed upon varying the levels of the substrate pairs (A) ATP and Pi, (B) glutamate and L-glutamine, and (C) NH₃ and L-glutamine. Fig. 4 shows the results of these experiments.

In Fig. 4A, varying the levels of Pi and ATP simply allows the ADP ⇌ ATP exchange to rise smoothly to a maximum. This argues that P₁ is not released before ATP in a compulsory manner. Also, the data in Fig. 3A showed that ADP is not released before P₁. Thus, P₁ and ADP are released randomly relative to each other. As noted above, P₁ is also released randomly relative to glutamine.

Interestingly, Fig. 4B shows that glutamate and glutamine produce a definitive inhibition pattern in the ADP ⇌ ATP exchange. This could result from glutamate binding after ATP...
or from glutamine being released before ADP. The first of these
two possibilities is excluded, since it was shown in Fig. 2A that
glutamate does not bind after ATP in a compulsory manner.
This strongly suggests compulsory release of glutamine before
ADP. The reciprocal plot for fractional inhibition (1/i), teresus
(\text{Glu})^{-2} indicates complete inhibition of nucleotide release at
infinite glutamate and glutamine levels. In Fig. 4C, variation
of NH$_3$ and glutamine also produces strong, complete inhibition
of ADP $\rightleftharpoons$ ATP at saturation, as would be expected for NH$_3$
binding after ATP and glutamine release before ADP. This
partially ordered binding is the one most consistent with all
present data.

A logical consequence of this particular binding pattern is that
the independent exchanges indicative of $\gamma$-glutamine-P will not
be observable. The observation of a nucleotide-independent,
P$_i$-dependent NH$_3$ $\rightleftharpoons$ glutamine exchange is not possible since
ATP must bind before NH$_3$ in the forward reaction, or ADP must
bind before glutamine in the reverse reaction. The observation of
an NH$_3$ and glutamine-independent, glutamate-dependent
ADP $\rightleftharpoons$ ATP exchange is not possible, since glutamine must be
released before ADP$'$, and glutamine is not formed in the NH$_3$
-free partial system. These predictions were tested by our at-
ttempting to observe these exchanges with the complete system
and a variety of partial reaction systems. No exchange reactions
were catalyzed by the ovine brain enzyme at appreciable rates
unless all substrates were present. These results agree with an
earlier report (10) of failure to observe independent ADP $\rightleftharpoons$ ATP
or P$_i$ $\rightleftharpoons$ ATP exchanges under somewhat different conditions.

**Pea Seed Enzyme**—Equilibrium exchange data reported earlier
by Graves and Boyer (12) were interpreted as consistent with a
partially ordered substrate binding mechanism for this enzyme
(see introductory remarks). Competitive effects among sub-
strates might account for some of these inhibition patterns, as
shown with the *E. coli* enzyme (13). Therefore, to block out
such effects at the active site, the concentration of all substrates
was varied in constant ratio at equilibrium. The results of this
experiment on the kinetics of glutamate $\rightleftharpoons$ glutamine, P$_i$ $\rightleftharpoons$
ATP, and ADP $\rightleftharpoons$ ATP are shown in Fig. 5A. Substrate levels
were at or above those used previously (12), and above those
reported as $K_m$ values (15, 28). All exchange rates rise smoothly
to a maximum without inhibition. This excludes ordered bind-
ing among the components involved in these exchanges: glu-
tamate, ATP, ADP, glutamine, and P$_i$. The NH$_3$ $\rightleftharpoons$ gluta-
mine exchange was not observed in this experiment, so it is possi-
able that ATP or glutamate bind after NH$_3$. However, since
NH$_3$ $\rightleftharpoons$ glutamine is faster than ADP $\rightleftharpoons$ ATP (see Table I),
ATP cannot bind after NH$_3$. Again, at subsaturating substrate
levels, some sigmoidal character is apparent in the exchange rate
curves of Fig. 5A. This was not observed upon variation of
substrate pairs (12).

**E. coli Enzymes**—The order of substrate binding to unadenyl-
ylated (E$_{\text{E}}$) glutamine synthetase from *E. coli* was probed by
variation of all substrates at equilibrium, 37$^\circ$, pH 7.0, with
[Mg$^{2+}$] = [ATP] + [ADP] + 10 mM. The results are shown in
Fig. 5B. The saturation curves show no inhibitory pat-
tterns indicative of ordered binding among these substrates.

**TABLE I**

| Enzyme source          | Condition* | 1   | 2   | 3  | 4  | Ratio (2:3) |
|------------------------|------------|-----|-----|----|----|------------|
| *Escherichia coli* [E$_{\text{E}}$] | a, c, e        | 1.0 | 0.77| 0.31| 0.31| 1.3         |
| *E. coli* [E$_{\text{E}}$] | b, c, f        | 0.59| 1.0 | 0.31| 0.31| 3.2         |
| Pea seed               | b, d, g        | 0.26| 1.0 | 0.50| 0.50| 2.0         |
| Sheep brain            | b, c, g        | 0.70| 1.0 | 0.27c| 0.27| 3.7         |

* Conditions: (a) pH 7.0, (b) pH 6.5, (c) 37$^\circ$, (d) 30$^\circ$, (e) [Mg$^{2+}$] = [ATP] + [ADP] + 10 mM, (f) [Mg$^{2+}$] = [ATP] + [ADP] + 1
mm, and (g) [Mg$^{2+}$] $= 50$ mm.

† Fig. 4 and Tables I and II of Ref. 13.

* Taken at $f = 0.5$ in Fig. 4.
Glutamate ⇔ glutamine is faster than P₁ ⇔ ATP, and some sigmoidicity in P₁ ⇔ ATP rate is seen.

In an earlier study with adenylylated enzyme (E₈) the substrate binding order was determined to be random at pH 6.50 and 37° (19). A mixture of metal ions was used in these experiments, so as to produce mainly MgATP and Mn-enzyme. The use of mixed metal ions, however, might introduce some doubt regarding detailed kinetic behavior of this complex system, since, e.g., Mg²⁺ can noncompetitively block adenylylated enzyme activity. Thus, the experiment involving variation of all substrates at equilibrium was repeated using Mn²⁺ only, with [Mn²⁺] = [ADP] + [ATP] + 1 mM and E₈. The kinetic pattern obtained was essentially the same as that reported earlier (13). Neither exchange is inhibited, and some sigmoidicity is seen in P₁ ⇔ ATP at f ≤ 0.2. The relative maximal values of (glutamate ⇔ glutamine)/(P₁ ⇔ ATP) rates are unchanged from the previous experiment.

**Rate-limiting Steps**—A comparison of the relative rates of equilibrium exchange between substrate pools catalyzed by various glutamine synthetase enzymes (Table 1) reveals several effects. Without exception, glutamate ⇔ glutamine and NH₃ ⇔ glutamine are faster than P₁ ⇔ ATP or ADP ⇔ ATP. This suggests that the rate of nucleotide release contributes more significantly to the rate of net runover than do the covalent interconversion steps under saturating substrate conditions. Second, the ratio of rates (glutamate ⇔ glutamine)/(P₁ ⇔ ATP), differs among these enzymes. This may suggest subtle but important differences in active site steric adaptation to bound substrate, resulting in different rates of substrate release.

**DISCUSSION**

**Ovine Brain Enzyme**—The observed equilibrium exchange kinetic patterns can be interpreted to exclude unambiguously certain substrate binding mechanisms. Glutamate and phosphate do not bind in an ordered manner relative to other substrates, and ADP is not released before glutamine, as argued under “Results,” due to lack of inhibition patterns diagnostic of such ordering. The inhibitions observed in Figs. 1 to 4, however, strongly suggest but do not absolutely prove that NH₃ binds after ATP, and glutamine must be released before ADP. This partially ordered binding scheme may be represented by Equation 4.

\[
\text{Glu} \quad \text{ATP} \quad \text{NH}_3 \quad \text{ADP} \quad \text{E} \quad (\text{EABC} = \text{EPQR}) \quad \text{Gln} \quad \text{P₁} \quad \text{E}
\]

Alternative explanations for the observed peaking and inhibition patterns of Figs. 1 to 4 exist and should be considered. (a) Formation of dead-end complexes should effectively remove active enzyme from solution and thus inhibit all exchanges. Such inhibition is clearly not observed in any of the experiments reported here. Earlier observations upon variation of NH₃ and glutamine with E. coli, (E₉) enzyme (Ref. 13, Fig. 2B) may, however, provide one example of this effect. (b) Substrates bound to noncatalytic modifier sites could potentially block either catalytic steps or the association-dissociation steps for certain substrates. As already shown in detail by model kinetics (24), inhibition of catalysis effectively removes active enzyme and thus inhibits all exchanges in constant ratio. This is not observed in Figs. 1 to 4. However, if a modifier differentially blocks dissociation or both association and dissociation for one substrate but not others, in a random binding system, one exchange would be suppressed preferentially. Present equilibrium exchange kinetic techniques are not capable of distinguishing this effect from ordered binding effects. Under initial velocity conditions the pea seed enzyme exhibited substrate inhibition by ammonia that might be interpreted in this manner (28).

Thus, for the ovine brain system, Equation 4 represents the substrate binding mechanism most consistent with all present data. The above discussion is not intended to indicate proof of a single substrate binding order for the ovine brain enzyme. As with analogous systems, e.g., succinyl-CoA synthetase (29), a complex subarray of minor pathways is almost certain to exist.

**Pea Seed and E. coli Enzymes**—Upon comparison with model kinetic patterns (cf. “Theory”), the data of Fig. 5 clearly show that these enzymes exhibit random binding of glutamate, glutamine, ATP, P₁, and probably ADP. These results argue against a previous interpretation for the pea seed enzyme, for which a partially ordered system had been postulated (12). With the E. coli enzymes, it is now clear that the random nature of substrate binding is not a function of pH, metal ion, or state of adenylylation.

Explanations for the sigmoidal responses of exchange rates observed in Figs. 1 and 5, A and B, should be considered. The apparent cooperativity of binding of substrates is not attributable to either homotropic (30) or heterotropic interactions, nor to modifiers acting as positive modifiers at noncatalytic sites, since such effects do not occur in experiments in which pairs of substrates were varied (as in Figs. 2 to 4, and the figures in Refs. 12 and 13). Also, initial velocity kinetics carried out in this laboratory failed to detect any homotropic cooperativity in the binding of either L-glutamate or ATP with any of the four enzymes of this study.

Several lines of evidence are consistent with special active site conformations induced by bound substrates with several of the glutamine synthetase enzymes studied here. The binding of glutamate to the ovine brain enzyme was previously shown to be greatly improved in the presence of ATP (10), although (E-Glu-ATP) yields the nondissociating complex (E-Glu-P-ADP) in ammonium-free solution. Under dynamic conditions the variation in half-saturation levels for exchanges involving glutamate, glutamine, ADP, and ATP seen in Figs. 1 to 3 are interpretable as mutual synergism of binding: glutamate with ATP, and glutamine with ADP. Such synergistic binding may also exist in the bacterial and plant enzymes, based on similar comparisons of kinetic binding curves (12, 13). With the mechanistically analogous succinyl-CoA synthetase, cofactor nucleotide has been observed to induce an enzyme conformation that enhances catalysis rather than substrate binding processes (29, 31, 32).

The probable existence of synergistic substrate binding under dynamic conditions raises several basic questions. (a) If the synergism occurs so as to strongly decrease substrate dissociation rates, could it produce inhibitions of exchange rates that would resemble the peaking and inhibition patterns diagnostic of ordered binding? (b) Are the sigmoidalities observed in the binding curves of Figs. 4 and 5 mainly indicative of multifactor kinetics at subsaturating levels of substrates or also of intrasite synergism of substrate binding?

In an attempt to answer the above questions, the following model for synergism of substrate binding was assumed. The random order, two-reactant, two-product system of Equation 3 can also be written as shown in Equation 5.
Equations for the exchange rates $R(A \rightleftharpoons P)$ and $R'(B \rightleftharpoons Q)$ are derived elsewhere (22, 23). Consider that bound $B$ and $Q$ make the binding of $A$ and $P$ tighter by slowing the dissociation rates from the central complexes $EAB$ and $EPQ$ at the $k_{-4}$ and $k_{-7}$ steps. Fig. 6 shows computer calculations for the $A \rightleftharpoons P$ exchange rate as a function of $[A, B, P, Q]$ and of $[B, Q]$ as $k_{-4}$ and $k_{-7}$ are decreased 2-, 5-, 10-, and 100-fold from their original values. At the point where $k_{-4} = k_{-7} = 0$, the compulsory ordered system of Equation 4 occurs.

First, with a strongly preferred order of substrate release ($k_{-4}$ and $k_{-7}$ reduced 10- to 100-fold), levels of $B$ and $Q$ must be about 5 times their $K_s$ values to produce any inhibition in $R$, the $A \rightleftharpoons P$ exchange rate. Thus, preferential orders of binding would be difficult to detect. Further, substrate synergism in complexation, unless extremely tight, cannot produce equilibrium patterns diagnostic of ordered binding. To produce the definitive peaking and inhibition patterns seen in Figs. 1 to 4, the model shows that $k_{-4}$ and $k_{-7}$ must be essentially zero relative to $k_{-1}$ and $k_{-4}$ so that an essentially compulsory order binding mechanism is operative.

Second, upon variation of all substrates in the model (Fig. 6A), the sigmoidicity of the $R$ and $R'$ responses is observed only at values of $[S]/K_m$ below 0.20. The sigmoidal responses in Figs. 1 and 5 are generally more pronounced at higher $S/K_m$ values. This comparison indicates that intrasite synergism of substrate binding does contribute significantly to the sigmoidal nature of the kinetic curves in Figs. 1 and 5. In Fig. 5B, the observation of sigmoidicity only in $P_1 \rightleftharpoons ATP$ for the E. coli enzyme may indicate synergistic assistance of nucleotide binding by other substrates, but not vice versa.

In conclusion, the present data indicate that important differences exist in the mechanisms of substrate binding and interaction in the active sites of glutamine synthetases from mammalian, plant, and bacterial sources. Other differences in mechanism are the subject of future investigations.

Acknowledgments—Thanks are due to Mr. Steven Kowalczykowski, Miss Terry Lerner, Mr. Tim Flavin, and Mrs. JoAnn Cenusa for their valuable technical assistance during various phases of this work. Dr. Robert Reeves of this department is thanked for providing access to and help with the mass spectrometer. I also thank Dr. P. D. Boyer for a critical reading of the manuscript.

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*J. Biol. Chem.* 1974, 249:5080-5087.

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