Substrate Binding and Reaction Intermediates of Glutamine Synthetase (Escherichia coli W) as Studied by Isotope Exchanges*

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SUMMARY

Substrate concentration effects on isotopic exchange rates at equilibrium have been measured with Escherichia coli glutamate synthetase (adenylated form) with 32P, 14C, 18O, and 15N. A new test for possible compulsory substrate-binding orders is presented, involving measurement of equilibrium exchange rates while increasing concentration of all substrates. This test shows random substrate-binding patterns for the enzyme. Inhibition of some equilibrium exchange rates while various pairs of substrates were increased in concentration appears to result from competitive rather than compulsory binding order effects. The relative rates of equilibrium exchanges were (glutamate ⇔ glutamine) > (NH3 ⇔ glutamine) > (Pi ⇔ ATP) = (ADP ⇔ ATP). The inequalities show that interconversion of bound substrates is not the only rate-limiting step, and allow deductions about relative association-dissociation rates of various substrates.

Glutamine synthetase from E. coli does not catalyze any detectable ADP ⇔ ATP, Pi ⇔ ATP, glutamate ⇔ glutamine, or NH3 ⇔ glutamine exchanges unless all substrates are present. The absence of ADP ⇔ ATP exchange in presence of glutamate or of NH3 ⇔ glutamine exchange in presence of Pi, together with lack of compulsory substrate-binding orders, does not give support to formation of γ-glutamyl phosphate as an enzyme-bound intermediate. No phosphoryl enzyme, glutamyl enzyme, or amido enzyme could be detected by isotopic labeling and isolation procedures. The results suggest further consideration of a concerted reaction mechanism requiring all substrates present in the active site.

Elegant studies of Stadtman et al. (1). Their work has resulted in an understanding of the main features of the enzyme's structure and of its intricate control by a variety of metabolic products derived in part from glutamine. Information about substrate-binding patterns and about possible intermediates in covalent interconversion are obvious requirements for satisfactory understanding of the enzyme's action. This paper reports the results of investigations into these questions by measurement of partial reactions and equilibrium exchange rates with isotopic probes.

Since the initial experiments by Doudoroff et al. (2) with sucrose phosphorylase, the demonstration of pertinent partial reactions has become increasingly recognized as an important means of revealing covalent intermediates in enzyme catalyses. Such searches with E. coli glutamine synthetase have not been reported in any depth. With respect to elucidation of substrate-binding orders, over the past decade measurements of isotopic exchange rates at chemical equilibrium in multisubstrate enzyme systems has developed as an important approach (for recent examples, see References 3 to 8). Although the theoretical treatments for such systems (9, 12) have assumed the applicability of the usual Michaelis-Menten saturation kinetics, the prominent diagnostic features for compulsory binding orders from equilibrium exchange rates at chemical equilibrium in multisubstrate enzyme systems has developed as an important approach (for recent examples, see References 3 to 8). Although the theoretical treatments for such systems (9, 12) have assumed the applicability of the usual Michaelis-Menten saturation kinetics, the prominent diagnostic features for compulsory binding orders from equilibrium exchange rates at chemical equilibrium in multisubstrate enzyme systems has developed as an important approach (for recent examples, see References 3 to 8). Although the theoretical treatments for such systems (9, 12) have assumed the applicability of the usual Michaelis-Menten saturation kinetics, the prominent diagnostic features for compulsory binding orders from equilibrium exchange rates at chemical equilibrium in multisubstrate enzyme systems has developed as an important approach (for recent examples, see References 3 to 8).

EXPERIMENTAL PROCEDURE

Materials—Glutamine synthetase was prepared according to the method of Woolfolk et al. (13). E. coli W were grown on a
rich glutamate-glycerol medium 2 hours into stationary phase. Minor modifications of the purification were that the first ammonium sulfate precipitation of the enzyme was carried out at pH 4.60, not pH 4.40. Also, in Step 7 complete recovery of all activity required repeated extractions with buffer of the pH 5.15 precipitate. The purified enzyme showed a single major (>99%) band upon disc gel electrophoresis. After dialysis to remove ammonium sulfate, the enzyme was stored at pH 7 in 0.01 M imidazole, 0.01 M MnCl₂ buffer at 4°C. Enzyme used in experiments to probe for partial reactions and intermediates was chromatographed on a column of Sephadex G-50 (bead), reprecipitated as in Step 7, redissolved, and dialyzed against the pH 7 imidazole-MnCl₂ buffer. Then 5 ml of the dialyzed solution containing 50 mg of enzyme were then passed through a bed of Dowex 1-chloride resin, 1 × 5 cm, pH 7.0. This is referred to in the text as “Dowex-treated” enzyme. A similar technique was used previously (14) to show the absolute requirement of sub-mitochondrial particles for ADP in the H₂O → P₃ exchange during oxidative phosphorylation.

The observed specific activity of the purified enzyme agreed reasonably well with published values (13), as determined by biosynthetic or transferase assays. The degree of adenylylation was determined by ultraviolet absorption spectra, comparing A₄₅₀ and A₆₅₀, and by differential kinetic assays with MnCl₂ and MgCl₂ (15). The average number of AMP moieties per 12 subunits was found to be 10, the enzyme thus being designated as E₄₀.

L-[¹⁴C]Glutamic acid was a Schwarz product, purified by DEAE-cellulose chromatography (see "Methods"), and was shown to be free of glutamine and pyrrolidone carboxylate by paper chromatography with 1-butanol-acetic acid-water (4:1:1).

L-[¹⁴C]Glutamate was produced from L-[¹⁴C]glutamate by a biosynthetic reaction with glutamine synthetase, NH₃, and ATP. Purification was carried out by DEAE-cellulose chromatography.

[¹⁴C]Pyrrolidone carboxylate was produced by the reaction of [¹⁴C]glutamate, 100 μmol (0.010 μCi per μmol), in aqueous medium, pH 4.0, in the presence of 200 μmol of phosphate for 48 hours at 100°C (16). Separation of product from reactants was by paper chromatography and indicated a yield of 35%.

L-Glutamate and L-glutamine were Schwarz products, recrystallized from ethanol-water. Nucleotides were from P-L Biochemicals. 3,3-Dimethylglutaric acid was an Aldrich product, m.p. 100°C. All other compounds were reagent grade. Deionized water, twice glass distilled, was used for all solutions.

Methods—Separation of all substrates in a reaction mixture was obtained by column chromatography with DEAE-cellulose (formate), usually 1 × 20 cm. Typically, a 1.0-m reaction mixture was diluted to 3 ml, applied to the column, and followed by 2 ml of distilled water. Sequential elution was accomplished by a linear gradient of pH 3.65 formate, formed from 20 ml of water and 20 ml of 0.8 M formate. The order of elution was (ammonia), glutamine, glutamate, phosphate, ATP, then ATP. Amino acid peaks were routinely located by ninhydrin spray tests of 5-μl portions of each fraction (usually 1.0 ml each) spotted on filter paper strips. Phosphate was located by ²³⁰P tracer. Nucleotides were located by A₆₅₀. Where exchanges had occurred, radioactivity could be used as an additional check on location and separation of peaks.

Selection of reaction conditions was based on reported characteristics of the enzyme plus some additional experimental evaluations. Imidazole buffer was avoided since it apparently can suppress enzyme activity as can L-histidine. Most studies were made at pH 6.50 because the adenylylated enzyme has maximal activity at this pH in the presence of manganese ion.

The equilibrium constant for the reaction at pH 6.50, 37°C, μ = 0.25 M, was determined by carrying out the reverse reaction with ³²P-labeled phosphate. Since the micromoles of ATP formed as measured by ³²P incorporation also equals the NH₃ and glutamate formed as well as the glutamine, P₃, and ADP depleted, the calculation of the apparent Kₑ₄₃:

\[ Kₐ₄₃ = \frac{(NH₃)(glutamate)(M-ATP)}{(glutamine)(P₃)(M-ADP)} \]

where M-ATP and M-ADP are the metal complexes, was relatively simple. Triplicate determinations yielded an average value of 460 ± 30, falling within the range of published values at pH 6 and 7 (17). P₃ and ATP were routinely separated by extraction of the neutral phosphomolybdate complex from acid solution into isobutyl alcohol-benzene (1:1) or 4-methyl-2-pentanone; ATP remained in aqueous phase.

Levels of Mn⁴⁺ and Mg²⁺ were selected to insure optimal formation of enzyme-manganese and nucleotide-magnesium complexes but with limited formation of enzyme-magnesium and nucleotide-manganese complexes. The reasons for this were that Mg²⁺ has been noted to inhibit noncompetitively the Mn⁴⁺-dependent catalytic activity of adenylylated enzyme subunits and that equilibrium calculations assume (see equation above) complete formation of metal (manganese or magnesium) complexes. Manganese ion in excess of 1 mM at pH 6.5 formed colloidal precipitates in reaction mixtures. Thus Mg²⁺ was added, equivalent to the ATP + ADP concentration. Because of the large association constants for Mg²⁺ and Mn⁴⁺-nucleotides (18), the level of free Mg²⁺ was quite low. Because glutamine synthetase binds Mn⁴⁺ some 1000-fold more tightly than Mg²⁺ at pH 6.50 (19) essentially no inhibitory enzyme-magnesium complex was formed. Further evidence of the enzyme’s lack of sensitivity to Mg²⁺ at pH 6.50 was the observation that addition of a slight excess of Mg²⁺ above total nucleotide at pH 6.5 did not appreciably alter either exchange activity at chemical equilibrium or biosynthetic (initial velocity) activity.

In a typical procedure for measurement of isotopic exchange reactions, substrates, buffer, and metal ions were added at concentrations at or close to equilibrium values. The pH was adjusted, if necessary, the enzyme added, and the mixture incubated long enough to assure establishment of equilibrium. The exchange reactions were then started by addition of very low amounts of highly labeled compounds, so that equilibrium was not perturbed. The reactions were stopped after an appropriate interval of time, usually in the range of 10 to 30 min, by addition of 1 N HCl to bring the pH to 4.0 to 4.5, followed by freezing until ready for chromatographic separation of the substrates.

The following procedures was used to allow variation in levels of substrate pairs or modifiers without altering pH, ionic strength, or other crucial parameters. Two stock solutions were prepared.

1 In this paper, the designation "substrates" refers to NH₃, glutamate, ATP, glutamine, P₃, and ADP. "Reagents" refer to the first three; "products" to the latter three. The designation "reaction components" includes the six substrates plus metal ions, salts, buffer, etc.

2 E. R. Stadtman, private communication.
Glutaminase activity with the purified, Dowex-treated enzyme (20 units) was negligible: when 5 mM [14C]glutamine (0.02 µCi per µmol) was incubated with MnCl2, KCl, and buffer at pH 6.5, 37° no appearance of 14C label in glutamic acid could be detected at a time when >50% glutamate would have been produced if P; and ADP had been present.

ATPase activity under similar conditions, however, was appreciable but not prohibitive. Release of 32P; from [γ-32P]ATP with only ATP present occurred at about 0.1% of the net initial rate of the complete reaction. Thus inherent ATPase activity of the enzyme could interfere in prolonged incubations at equilibrium. Exchange reactions were usually carried out to only 10 to 20% of approach to isotopic equilibrium, which avoided any appreciable error resulting from ATP hydrolysis.

RESULTS

Partial Exchange Reactions—An isotopic exchange indicative of a partial reaction is a quite sensitive probe for formation of covalent intermediates from a given substrate. The intermediate may involve either an enzyme-functional group or a second substrate to give a noncovalently bound moiety. Such examples may be represented as

\[ \text{E} \rightarrow \text{E-A} \]

In either case the AR \( \Rightarrow R \) isotopic exchange is indicative of intermediate formation, but in the former case involving E-A the A-B \( \Rightarrow B \) exchange occurs independent of added X.

Table I presents the results of tests for possible phosphoryl enzyme or adenosine diphosphoryl enzyme intermediates, as probed by [14C]ADP \( \Rightarrow \) ATP and 32P; \( \Rightarrow \) ATP exchanges. In these experiments, sufficient radioactive label was present to have allowed detection of any exchange occurring even at a rate only 4 that of the rate with all components present. That neither exchange occurs above this lower limit suggests that neither phosphoryl enzyme nor adenosine diphosphoryl enzyme moieties occur as kinetically significant, covalently distinct intermediates on the reaction pathway.

Separate additions of NH3-free glutamate (recrystallized, Dowex-K+ treated) or of NH3 gave no observable stimulation of these exchange activities. Hence, no enzyme-bound phosphorylated or adenosine diphosphoryl derivatives of these compounds are likely as reaction intermediates, most significantly not γ-glutamyl phosphate.

Results of probes for amido enzyme or glutamyl enzyme intermediates are presented in Table II. Once again, neither predictable exchange glutamate \( \leftrightarrow \) glutamine or NH3 \( \leftrightarrow \) glutamine, is observable within the limits of detection. Added phosphate produces no stimulatory effects, arguing against bound glutamyl phosphate formation from the glutamine side of the reaction. Such formation would likely give detectable NH3 \( \Rightarrow \) GluNH2 exchange in presence of P;.

\[ \text{E} \cdot \text{X} \rightarrow \text{E-A} \]

\[ \text{A-B} \rightarrow \text{B} \]

\[ \text{NH}_3 \text{GlU Gln ADP} \rightarrow \text{ATP Pi} \rightarrow \text{ATP} \]

TABLE I

| Additions | Relative exchange activity* |
|-----------|-----------------------------|
| NH3 | Gla | Gln | ADP \( \Rightarrow \) ATP | P; \( \Rightarrow \) ATP |
| µmole | | | | |
| 0.5 | 1.0 | 5.0 | 1.00 | 1.00 |
| 0 | 0 | 0 | <0.0001 | <0.0001 |
| 0.9 | 0 | 0 | <0.0001 | <0.0001 |
| 0 | 0 | 0 | <0.0001 | <0.0001 |

* With ADP \( \Rightarrow \) ATP taken as 1.00.
The treatment of the enzyme with Dowex 1 (see "Methods") was necessary to abolish a low level of glutamate \(\rightarrow\) GluNH\(_2\) exchange activity. The Dowex exposure did not alter the specific exchange activity of the enzyme with all reaction components present, and presumably removed traces of bound nucleotides or P\(_i\) from the enzyme.

**Search for Isolable Intermediates**—The lack of exchanges resulting from partial reaction systems, although quite indicative, cannot absolutely rule out the formation of covalent intermediates. Thus tests were made for direct isolation or detection of such possible intermediates. Table III presents the results of the search for phosphoryl enzyme and for amido or glutamyl enzyme forms.

The search for phosphoryl enzyme involved incubation of enzyme with an equilibrium reaction mixture containing \(^{32}\)P\(_i\), which was then quenched by extraction of the protein into phenol. This procedure (22) has proven successful in isolating phosphorylated succinyl-CoA synthetase (23). The searches for glutamyl and amido enzyme involved incubation of an identical reaction at equilibrium with \(^{34}\)Cl-glutamate and \(^{15}\)N-glutamine added. In this case, following one of several quench techniques, rapid filtration on Sephadex G-50 was used to separate protein from the reaction mixture. In no case was it possible to observe or detect incorporation of radioactive label or heavy isotope into the protein by these procedures.

Several attempts were made to detect the possible formation or utilization of either \(\gamma\)-glutamyl phosphate or the cyclic derivative, pyridoxal carboxylate by the enzyme. Quenching a reaction mixture containing \(^{14}\)C-glutamate with an equal volume of cold ethanol, followed by paper chromatography or passage through Dowex 50(H\(^+\)) according to Krishnaswamy et al. (24) revealed no \(^{14}\)C moieties identifiable as pyridoxal carboxylate or \(\gamma\)-glutamyl phosphate. Chromatography on DEAE-cellulose (formate) revealed no \(^{14}\)C peaks other than those which gave ninhydrin-positive tests (glutamate and glutamine). The Cl\(_2\), or \(\gamma\)-glutamyl phosphate. Chromatography on DEAE-cellulose revealed no \(^{14}\)C moiety identifiable as pyrrolidone carboxylate through Dowex 50(H\(^+\)) according to Krishnaswamy et al. (24) and the experiment with \(^{32}\)P\(_i\) was carried out separately from that with \(^{14}\)Cl-glutamate and \(^{15}\)N\(_2\)H\(_2\).

### Table II

| Additions | Relative exchange activity \(\mu\)moles |
|-----------|---------------------------------|
| P\(_i\)   | ADP    | ATP    | Glu \(\rightarrow\) Gln | NH\(_3\) \(\rightarrow\) Gln |
| 10        | 4      | 1      | 3.6                      | 2.1                          |
| 0         | 0      | 0      | <0.0001                  | <0.001                       |

\(a\) With ADP \(\rightarrow\) ATP taken as 1.00 (cf. Table I).

### Table III

| Additions | Observed E-P per subunit \(\mu\)moles |
|-----------|---------------------------------|
| NH\(_3\)   | ATP    | ADP    |
| +         | +      | +      | <0.0001                  |
| +         | -      | -      | <0.0001                  |

*Control* by Sephadex separation

| Stopping procedure | E-Glu per subunit | E-NH\(_2\) per subunit |
|--------------------|------------------|------------------------|
| Control            | 0.0000           | 0.000                  |
| pH decrease to 5   | <0.0001          | <0.0001                |
| pH increase to 8.5 | <0.0001          | <0.0001                |
| Rapid freezing     | <0.0001          | <0.0001                |

\(a\) Observed counts per min per theoretical counts per min.

Enzyme added after pH decreased to 5.

**Attempts to label and isolate possible covalent enzyme intermediates formed at chemical equilibrium**

Each 1.0-ml reaction at pH 6.50, 37\(^\circ\), contained (in micromoles): 0.34 NH\(_4\)Cl (\(^{16}\)N-99.1\% enrichment), 1 glutamate (\(^{14}\)C-0.04 \mu\)Ci), 5 glutamine, plus the enzyme, salt, and buffer levels indicated in Table I. MgCl\(_2\) was never in excess of nucleotide. Other substrates added are listed below. A control reaction was carried out, omitting enzyme.

### A. Possible phosphoryl enzyme (E-P) by phenol extraction

| Additions | Observed E-P per subunit \(\mu\)moles |
|-----------|---------------------------------|
| NH\(_3\)   | ATP    | ADP    |

### B. Possible glutamyl enzyme (E-Glu) or amido enzyme (E-NH\(_2\)) by Sephadex separation

- **Effects of Increase in Concentration of Substrate Pairs on Exchange Rates**—Comparison of the relative rates of exchange for ADP \(\leftrightarrow\) ATP and P\(_i\) \(\leftrightarrow\) ATP (Table I) reveals that they are essentially equal. These rates, however, are not equal to the rates of glutamate \(\leftrightarrow\) glutamine and NH\(_3\) \(\leftrightarrow\) glutamine exchange (Table II), which are themselves unequal. Arranged in decreasing order of velocity the exchange rates are:

- glutamate \(\leftrightarrow\) glutamine
- NH\(_3\) \(\leftrightarrow\) glutamine
- (P\(_i\) \(\leftrightarrow\) ATP) - (ADP \(\rightarrow\) ATP)
glutamine synthetase at pH 6.5, 37°. The lack of detectable partial reactions or isolable intermediates points to a concerted mechanism for covalent interconversion and argues against any "ping-pong" mechanism. If this is so, the relative rates of exchange between substrate pools allow one to exclude certain order of binding. In a compulsory binding sequence, the most rapid exchange must occur between the last reactant to bind and the first product to dissociate; all other exchanges must be slower or equal in rate.

With three reactants and three products, the glutamine synthetase reaction offers various reaction component pairs for which the levels may be varied at equilibrium and between which isotopic exchanges may be observed, to give information about possible orders. For example, one may vary the structurally similar pairs of ATP and ADP, glutamate and glutamine, P1 and ATP, and NH3 and ADP. Less similar pairs which may be true) or from a direct competition of glutamate or glutamine may bind after Pi in the reverse direction, or both may bind at the same site.

As noted in Fig. 1A, with increase in the glutamate and glutamine concentrations, the P1 \( \rightleftharpoons \) ATP exchange is first stimulated, then suppressed strongly. This effect could result from a compulsory binding order (glutamate may bind after ATP, or glutamine may bind after P1 in the reverse direction, or both may bind at the same site). Less similar pairs which may induce nonproductive binding modes. Alternatively, substrates may bind to noncatalytic sites and induce thereby some weak activation or inhibition effects.

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Fig. 2. The effects of ATP and ADP (A) and NH3 and glutamine (B) upon [14C]glutamate \( \rightleftharpoons \) glutamine (●) and ADP \( \rightleftharpoons \) ATP (△) exchanges at chemical equilibrium. Each 1.0-ml reaction at pH 6.50, 37°, contained the levels of enzyme, buffer, and salts noted in Fig. 1. (A) contained (in micromoles) 1 NH3, 2 glutamate, 20 glutamine, and 10 P1, plus varied amounts of ADP and ATP. (B) contained (in micromoles) 2 glutamate, 0.5 ATP, 20 P1, and 4 ADP, plus varied amounts of NH3 and glutamine.

P1 \( \rightleftharpoons \) ATP exchange are presented in Fig. 1A. The glutamate \( \rightleftharpoons \) glutamine exchange rises to a maximum, but with some slight inhibitory or discontinuous behavior. The data points of Fig. 1A were found to be reproducible in multiple experiments with less than 5% error. An error of 20%, especially in the third data point at 1.25 mM glutamate would be necessary for the curve to appear hyperbolic. The nonhyperbolic behavior may arise from one of several possible effects. Anticooperative substrate binding may occur, or high levels of glutamate and glutamine may induce nonproductive binding modes. Alternatively, substrates may bind to noncatalytic sites and induce thereby some weak activation or inhibition effects.

Glutamate has been found by others to induce negative interactions and nonhyperbolic responses (27). Stadtman has also observed some antagonism in the binding of glutamate and NH3 under certain conditions. As will be discussed, glutamate, or glutamine, or both, apparently competes with P1, or ATP, or both, and since all components are apparently required in the active site for activity, the suppression of P1-ATP exchange by competitive effects may also suppress glutamate-glutamine to some extent at high glutamate and glutamine.

The effects of varying the levels of glutamate and glutamine in constant ratio upon the rates of glutamate \( \rightleftharpoons \) glutamine and
avoided in Fig. 2A because both ATP and ADP were varied together.

Increased levels of NH₃ and glutamine produce the striking effects shown in Fig. 2B: both exchanges are initially stimulated, then suppressed strongly. One may interpret the effects on the glutamate ⇌ glutamine exchange as either compulsory binding of NH₃ after glutamate, or as direct competition of NH₃ for a glutamate- or glutamine-binding locus. The interpretation of the suppression of the P₁ ⇌ ATP exchange rate is more complex. It may be attributed to either compulsory binding of NH₃ after ATP, glutamine after P₁, or both. Alternatively, or in addition, NH₃ or glutamine, or both, may compete directly for an ATP- or P₁-binding locus.

The results thus far presented show that nucleotides and phosphate do not appear to bind after amino acid or NH₃ substrates in a compulsory manner, nor do they appear to compete with the latter for binding sites, but that the reverse may be true. The amino acids and NH₃ do exert inhibitory effects upon the exchanges between ATP and P₁, and NH₃ can exert similar effects on the glutamate ⇌ glutamine exchange. To help discern among possibilities, an additional isotopic probe, the ATP ⇌ ADP exchange, was measured. Fig. 3A shows the effect of increased ATP and P₁ upon the ATP ⇌ ADP exchange: the rate rises smoothly to a maximum. Thus P₁ either dissociates after or randomly relative to ADP. Since the conclusion from Fig. 2B was that ADP could not bind after P₁, ADP and P₁ dissociate randomly relative to each other.

In Fig. 3B the increased levels of glutamate and P₁ are shown to exert only partial inhibitory effects upon the ATP ⇌ ADP exchange. Since ADP and P₁ dissociate randomly relative to each other, the possible explanations for this phenomenon include either preferential but not compulsory binding of glutamate after ATP, or a competition of glutamate for an ATP-binding site. Also possible is an action of glutamate as a weak negative modifier.

The effect of variation in glutamate and ADP as a pair, Fig. 3C, produces more complete suppression of ATP ⇌ ADP exchange, but under these conditions (low fixed level of ATP) likely ADP competition for the ATP-binding site occurs, possibly added to the effects observed with glutamate in Fig. 3B.

Effect of Increasing Concentrations of all Substrates on Exchange Rates—The results given above, although eliminating some possibly compulsory binding orders, show inhibitions characteristic of certain compulsory and perhaps preferential orders, or of direct or indirect inhibitory effects, especially with the amino acid and ammonia substrates. Further clarification was thus desirable.

One of the most definitive and direct experiments to substantiate or negate some of the above alternatives involves varying the levels of all substrates simultaneously in constant ratio. If either noncompetitive effects or compulsory binding orders occur, inhibition of the appropriate exchanges should still be observed when the concentration of all substrates is increased. However, if only competitive effects between substrates are the causes for inhibition when substrate pairs are increased, such competitive inhibitory effects should be absent when concentrations of all substrates are increased with constant substrate ratios.

Fig. 4 presents the results of varying all substrate levels simultaneously in constant ratio at chemical equilibrium, as probed by the glutamate ⇌ glutamine and P₁ ⇌ ATP exchanges. Both exchanges rise smoothly to a maximum. The concentration range for each substrate extends beyond that which gave inhibitory effects reported in Figs. 1A, 2B, 3B, and 3C. In addition, the maximum concentrations were considerably above the reported Kₘ values. The results rule out compulsory binding orders or noncompetitive effects as responsible for the previously observed inhibitions.
DISCUSSION

One prominent feature of the present data is the demonstration of random binding and release of substrates. The simple experiment reported in Fig. 4 appears to represent a new and powerful approach to test for compulsory binding orders. In this experiment, concentrations of all substrates were increased by the same ratio. Any competition between substrate for binding sites thus remain unaltered. The absence of decline in exchange rates as concentration of all substrates is increased eliminates compulsory substrate-binding orders or a noncompetitive inhibition by a particular substrate at a control site.

The application of this approach with glutamine synthetase was particularly useful because the inhibitions noted with some exchanges, as concentrations of reactant pairs were increased while maintaining equilibrium (Figs. 1A and 2B), are consistent with patterns expected when compulsory or partially compulsory binding occur. These inhibitions must have other explanations, such as competitive displacement of a substrate whose concentration is not being increased. In the cell, control of and catalysis by glutamine synthetase probably occur near chemical equilibrium, responding to relatively small perturbations in substrate or modifier levels. The competitive inhibitory effects observed for glutamate, glutamine, and NH₃ may reflect important additional control mechanisms for this already complex enzyme system.

Some conclusions derived from data at chemical equilibrium may not be readily observable under initial velocity conditions. The criticisms by Dalziel (29) of some kinetic derivations by Fromm (30) appear to provide an example: partially compulsory binding order mechanisms may be observable and predictable over limited ranges of substrate concentration under initial velocity conditions, whereas for derivations involving exchanges at chemical equilibrium (9, 10) such binding orders are more clearly predicted and demonstrable in both theory and practice.

A second important feature of the results given in this paper concerns the relative rates of the various steps involved in catalysis and exchange. A random binding order does not imply that substrate dissociation steps to limitation of observed over-all catalytic rates.

An additional important feature of the present data concerns the absence of detectable partial reactions; all reactants or products apparently must be present before any exchange between substrate moieties can occur. Similar behavior has been noted previously for the ADP → ATP and P₁ → ATP exchanges with glutamine synthetase from peas and from brain (25, 27, 31) but the tests reported here were more sensitive. For example, in our experiments, the ADP → ATP exchange in presence of ATP and glutamate but in the absence of ammonia was less than 10⁻⁴ of that observed at equilibrium with the same concentrations of ADP and ATP in the presence of ammonia. The absence of ADP → ATP exchange, even in the presence of added glutamate and also the lack of observable NH₃ → glutamine exchange in the presence of P₁ are of particular importance. Both these exchanges would be expected if γ-glutamyl phosphate formed as a catalytic intermediate in an independent step.

To distinguish definitely between a reaction sequence involving γ-glutamyl phosphate as a catalytic intermediate as contrasted to a concerted mechanism does not appear possible from presently available data. Meister has reviewed findings, principally from his laboratory, that favor γ-glutamyl phosphate participation (32) with the ovine brain enzyme. But none of the results conclusively establishes this mechanistically attractive possibility. For example, Krishnaswamy et al. (24) suggested that the lack of an ADP → ATP exchange in presence of glutamate resulted because both ADP and γ-glutamyl phosphate remained firmly bound to the enzyme. Such a possibility is difficult to accept for E. coli glutamine synthetase in view of the random binding of substrates demonstrated by the present studies. Absence of dissociation of ADP formed from ATP in presence of glutamate would mean that ADP cannot leave the enzyme unless RCONH₂ is bound. This would suggest a compulsory binding order in the reverse relation, in which ADP binds after RCONH₂.

Considerations analogous to the ADP → ATP exchange apply to the absence of an NH₃ → glutamine exchange in presence of P₁. Lack of dissociation of NH₃ must be postulated if γ-glutamyl phosphate is formed from glutamine and P₁. This would suggest a compulsory binding order in the forward reaction with ATP adding before NH₃. Such compulsory binding order is contrary to our present findings.

Although lack of dissociation of NH₃ and of ADP seems un-
likely for E. coli glutamine synthetase, this possibility cannot be eliminated conclusively. In this regard, it is pertinent that Bockovskyans and Ratnher (33) presented convincing evidence for firmly bound pyrophosphate to account for lack of a PP_{i} ATP exchange with arginosuccinate synthetase in presence of ATP and citrulline; firmly bound citrulline adenylate appears to be an intermediate in the reaction.

Brief examples of possible alternate interpretations of some additional observations discussed by Meister (32) is consistent with γ-glutamyl phosphate participation with the brain enzyme may be helpful. One is a selective formation of [^{14}C]γ-glutamyl hydroxamate upon addition of excess [^{14}C]glutamate and hydroxylamine to a solution containing enzyme, ATP, and [^{14}C]glutamate. This result could, however, mean that the rate of association and reaction of hydroxylamine with enzyme having bound ATP and glutamate is more rapid than the rate of glutamate dissociation. Another observation is the binding and migration of glutamate with the enzyme in presence of ATP. This could reflect allosteric effects of ATP or a compulsory binding order with the brain enzyme. Similarly the apparent formation of and specific binding to the enzyme of ADP and Pi from ATP in the presence of glutamate could result from the weak inherent ATPase activity of the enzyme, rather than from formation of γ-glutamyl phosphate.

Other observations include pyrrolidone carboxylate formation upon heat denaturation or ethanol quenching of reaction mixtures containing enzyme, Mg^{++}, ATP, and glutamine, as well as γ-glutamyl hydroxamate formation from glutamine and NH_{2}OH in presence of ADP and As_{2}. Bound reactants might yield pyrrolidone carboxylate as the enzyme active site is disrupted by protein denaturation. A catalytic site that binds ATP and glutamate for a concerted reaction with NH_{2}OH might also produce γ-glutamyl phosphate very slowly as a side reaction upon substitution of NH_{2}OH for NH_{3}. Also, if NH_{2}OH and As_{2} can substitute for NH_{2} and P_{i}, or the γ-phosphoryl of ATP, or both, transferase reactions might simply involve a rapid reversal and substitution of NH_{2}OH for NH_{3} in the activated complex shown in Fig. 5 rather than “trapping” of an activated covalent glutamate intermediate.

Finally, differences in the E. coli and ovine brain enzymes may be such that the latter can stabilize a γ-glutamyl phosphate and allow for its formation as a discrete substance. It is perhaps significant to note here that the E. coli enzyme does not produce either directly or via γ-glutamyl phosphate any detectable pyrrolidone carboxylate with the conditions of Meister (32) under which the ovine brain enzyme does so. In this sense the enzymes are obviously quite different.

Convincing evidence for γ-glutamyl phosphate as a catalytic intermediate would be demonstration of a steady state level during catalysis, with rates of formation and disappearance that establish its kinetic competence as an intermediate. Such an approach has formidable experimental difficulties.

There remains another possibility not considered by Meister (32) for γ-glutamyl phosphate participation. This is that very pronounced substrate synergism (34) exists, so that γ-glutamyl phosphate formation requires the presence, but not covalent participation of, all reactants, or, conversely, of all products. An absolute requirement of bound NH_{2} for the γ-glutamyl phosphate formation from ATP and glutamate, or a similar requirement of ADP with glutamine and P_{i} as substrates does not seem likely; where quantitation of the effects of other substrates has been possible, as in the observed substrate synergism with succinyl-CoA synthetase (33) and with phosphoribosyl pyrophosphate synthetase (7) only stimulatory but not absolute requirements have been observed. In addition, no such absolute requirement of one substrate for a partial reaction of another substrate with an enzyme has to our knowledge been reported. For example, with phosphoryl-transferring enzymes, some have been shown to involve formation of phosphoryl enzyme intermediates, while others appear to catalyze direct transfer of the phosphoryl group between substrates without phosphoryl enzyme formation. As discussed elsewhere (35), in all instances where phosphoryl enzyme formation has been demonstrated or definitively indicated, the expected partial reactions have been detected by isotopic exchanges.

A concerted mechanism for enzymic reactions analogous to those of glutamine synthetase was suggested some time ago by Buchanan and Hartman (36). A concerted reaction and the associated substrate binding and release steps are indicated for E. coli glutamine synthetase in Fig. 5. Obviously, one way in which an enzyme might favor a concerted reaction is by binding the substrates in appropriate juxtaposition. For example, approach of the lone pair of electrons from the ammonia nitrogen to the γ-carboxyl carbon of glutamate should enhance the ability of one carbonyl oxygen to form a partial bond with the γ-phosphoryl phosphate of ATP, thus enhancing the leaving group capacity of ADP. The partially formed bond between the carboxyl and phosphoryl moieties serves to activate both, allowing ADP or NH_{3} to leave or attack, depending upon the over-all direction of the reaction under consideration. Although the reaction profile may actually involve and reflect participation of more than one transition state form, not just the single form indicated in Fig. 5, such intimate details of the chemical interconversion are difficult to establish by present approaches. The designation “concerted” appears useful, however, when discrete covalent intermediates are not detectable or isolable participants.

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