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Isotope-Exchange Enhancement Studies of *Escherichia coli* Glutamine Synthetase†

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ABSTRACT: Isotope-exchange enhancement studies, a variation on positional isotope-exchange enhancement as described by Raushel and Garrard [Raushel, F. M., & Garrard, L. J. (1984) *Biochemistry 23*, 1791–1795], are used to establish the point in the biosynthetic reaction of *Escherichia coli* glutamine synthetase at which γ-glutamyl phosphate is formed. In these experiments, the behavior of the reverse biosynthetic reaction, i.e., the reaction of ADP, L-glutamine, and phosphate to form NH_4^+, L-glutamate, and ATP, is examined as a function of the concentration of ammonium ion. By varying the concentration of NH_4^+, the ratio of the velocity of isotope exchange to the velocity of net reaction, as measured by the rate of ^{18}O depletion from labeled phosphate and the rate of production of L-glutamate, respectively, can be modulated in a mechanism-dependent manner. Evidence is presented demonstrating the presence of a branch point in the mechanism. The enzyme–ATP–glutamate complex may partition in two ways, one involving binding of ammonium ion and the other involving the chemical transformation to form the enzyme–ADP–γ-glutamyl phosphate complex. The alternate pathways then rejoin upon formation of the enzyme–ADP–NH_4^+–γ-glutamyl phosphate complex. Because of the branch point, there is no absolute requirement that ammonium ion be absent or present in order for the formation of γ-glutamyl phosphate to occur. At high concentrations of ammonia, one pathway through the branch can be eliminated, effectively making that portion of the pathway ordered, with ATP, L-glutamate, and NH_4^+ binding consistent with our previously reported steady-state kinetic mechanism [Meek, T. D., & Villafranca, J. J. (1980) *Biochemistry 19*, 5513–5519].

Isotope-exchange methods have long been used in biochemistry as a means of demonstrating the existence or absence of reaction intermediates and to observe flux rates through portions of a reaction mechanism. The introduction of positional isotope exchange (PIX)[1] techniques by Midelfort & Rose (1976) provided a powerful new method for analysis of isotope-exchange experiments. Recently, Raushel & Garrard (1984) have extended this technique, describing the use of positional isotope-exchange enhancement (PIXE) as a probe of branching in enzyme mechanisms.

This paper discusses the application of isotope-exchange enhancement (IXE) in an effort to illuminate an aspect of the mechanism of *Escherichia coli* glutamine synthetase [L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2]. Instead of detecting possible branching, our studies are designed to detect where formation of a reaction intermediate takes place.

It has long been postulated that γ-glutamyl phosphate is an intermediate in the biosynthetic reaction (eq 1) catalyzed

$$\text{ATP} + \text{L-glutamate} + NH_4^+ \rightleftharpoons P_i + \text{L-glutamine} + \text{ADP} \quad (1)$$

by the enzyme. Meister and co-workers proposed the formation of γ-glutamyl phosphate on the enzyme in the ATPase reaction (eq 2) in the absence of ammonia using isotope-trapping methods with the sheep brain enzyme (Krishnaswamy et al., 1962; Meister, 1698; Tsuda et al., 1971). Showing the presence of the intermediate in the full biosynthetic reaction proved more difficult. In fact, on the basis of equilibrium isotope exchange studies, Wedler & Boyer (1972) proposed that the biosynthetic reaction was concerted, involving no

$$\text{ATP} + \text{L-glutamate} \rightarrow \text{ADP} + P_i + \text{pyrrolidonecarboxylate} \quad (2)$$

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NADH, dihydronicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate.

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formation of an acyl phosphate intermediate. Later work by Midelfort & Rose (1976) using the PIX technique concluded that γ-glutamyl phosphate was a viable chemical intermediate in both pathways.

The steady-state kinetic mechanism did not shed light on the timing of the formation of γ-glutamyl phosphate since ATP, glutamate, and NH_4^+ bind sequentially followed by product release (Meek & Villafranca, 1980). More recent work from this laboratory, involving rapid-quench and stopped-flow techniques, demonstrated that γ-glutamyl phosphate is a *kinetically* competent intermediate of both reaction pathways (eq 1 and 2) (Meek et al., 1982). The studies reported herein were undertaken to determine at what step in the pathway the formation of γ-glutamyl phosphate takes place. Specifically, must the enzyme have NH_4^+ bound to its surface in order for the transformation to occur?

Experimental Procedures

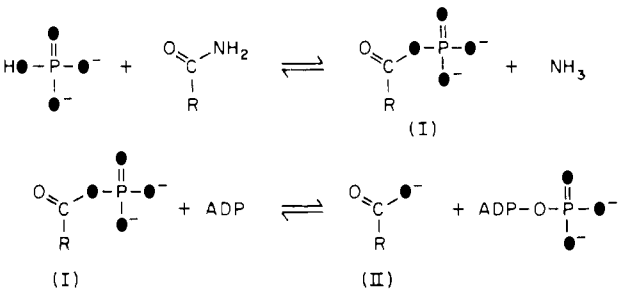
Glutamine synthetase was isolated from *E. coli* in a state of low adenylylation, between 0.8 and 3.0 adenyl group per dodecamer, by the method of Miller et al. (1974) or Woolfolk et al. (1966). For the experimental conditions described, no difference in enzyme behavior was noted over this small range of adenylylation states. Enzyme activity was assayed by coupling the production of ADP to the oxidation of NADH with pyruvate kinase and lactate dehydrogenase and observing the decrease in absorbance at 339 nm. Activity assays of the reverse reaction were accomplished by coupling the production of ATP to the reduction of NADP with hexokinase and glucose-6-phosphate dehydrogenase and observing the absorbance increase at 339 nm. Protein concentrations were determined by the method of Lowry et al. (1981) and by spectral measurements as described by Ginsburg et al. (1970).

$KH_2P^{18}O_4$ was synthesized by the method of Risley & Van Etten (1978). The material used in our experiments was between 95% and 88% $KH_2P^{18}O_4$, the remainder being $KH_2P^{18}O_3{}^{16}O$. The rates of exchange were determined by monitoring the ^{18}O distribution by the ^{31}P NMR technique of Cohn & Hu (1978). Because of the high isotopic purity of the phosphate, rates were calculated directly from the change in the height of the $P^{18}O_4$ peak (DeBrosse & Villafranca, 1983).

The IXE studies described in this paper were designed to examine the ratio of v_{ex} to v_{rxn} of the reverse biosynthetic reaction, i.e., the formation of NH_4^+ L-Glu, and ATP from ADP, L-Gln, and P_i. In a typical reaction, a cuvette contained 2.5 mL of assay solution with 50 mM HEPES, 100 mM KCl, 2 mM ADP, 70 mM L-Gln, 15 mM $KH_2P^{18}O_4$, and 17 mM $MgCl_2$. D-Glucose (100 mM) and 9 units/mL hexokinase (yeast) were present to remove any ATP formed during the course of the reaction. The concentration of NH_4Cl was varied between 0 and 10.0 mM. For those reactions at low ammonium ion concentration (less than 3.0 mM), glutamic dehydrogenase (20 units/mL, bovine liver) and 0.5 mM NADP were present to remove any L-Glu formed and as a means of following the progress of the reaction. At higher ammonium ion concentrations, glucose-6-phosphate dehydrogenase (yeast) at a concentration of 25 units/mL replaced glutamic dehydrogenase. In this case, the reaction was followed by observing the rate of ATP formation.

Reactions were initiated by adding sufficient glutamine synthetase to bring the concentration of unadenylylated active sites into the range of 100–500 nM. All reactions were run at 25 °C, pH 7.5. After initiating the reaction by addition of glutamine synthetase, the reaction was followed spectrophotometrically by observing the reduction of NADP at 339 nm until 4–9% of the limiting substrate (either NH_4^+ or NADP for the coupling reaction) had been reacted. In those experiments where no ammonia was initially present, the reaction was allowed to proceed to the same extent as or less than the sample with the lowest non-zero initial NH_4^+ concentration. The reaction was then stopped by addition of 2.0 mL of 0.1 M Na_2EDTA in D_2O. The pH of the sample was then adjusted to between 9 and 10 with KOH. The velocity of net reaction, v_{rxn}, was calculated from the spectrophotometric observations. The velocity of exchange, v_{ex}, was calculated from the rate of ^{18}O washout from the labeled phosphate as determined by ^{31}P NMR. Samples that could not be analyzed immediately were stored frozen at −4 °C for later analysis. ^{31}P NMR spectra were obtained on a Bruker WM360 8.455-T (145-MHz) ^{31}P NMR spectrometer.

Scheme I: Mechanism of ^{18}O Depletion of Labeled Phosphate in the Reverse Biosynthetic Reaction of Glutamine Synthetase[a]



[a] The filled O's represent ^{18}O. Labeled phosphate and glutamine react to form the acyl phosphate, γ-glutamyl phosphate (I). I can then react with ADP to form glutamate (II) and ATP. Because the two oxygens in II are torsionally equivalent due to free rotation about the C–R bond as shown by Bild & Boyer (1980) and Balakrishnan et al. (1978), there is a 50% chance that ^{16}O will be incorporated into phosphate if the reaction reverses.

Theory

One essential feature of PIXE and IXE experiments is the introduction of varying amounts of a reaction product that binds to the enzyme at a point in the mechanism before net chemical reaction is detected. Another requirement is that the added product binds either before or after the step at which isotope exchange occurs, depending upon the particular models that are being tested. Thus, the ratio of the velocity of exchange (v_{ex}) to the velocity of net reaction (v_{rxn}) can be modulated in a mechanism-dependent manner. Raushel & Garrard (1984) first devised this method to test for branching in the pathway of argininosuccinate lyase.

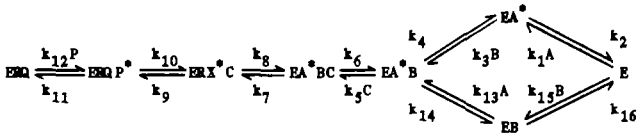
For our studies, we perturbed the reverse biosynthetic reaction of glutamine synthetase, i.e.

$$\text{ADP} + \text{L-Gln} + P_i \rightarrow NH_4^+ + \text{L-Glu} + \text{ATP} \quad (3)$$

The ratio v_{ex}/v_{rxn} was modulated by adding varying amounts of NH_4^+. The isotope traced was ^{18}O, introduced into the reaction in the form of $KH_2P^{18}O_4$. As the reaction proceeded, ^{18}O was be depleted due to the reactions depicted in Scheme I.

The formation of γ-glutamyl phosphate from ATP and L-glutamate may occur in one of three ways. It may form only when NH_4^+ is bound to the enzyme surface or only when NH_4^+ is absent from the enzyme, or there may be no requirement for the presence or absence of NH_4^+ at all. In addition, these three cases may be further subdivided to account for random or ordered binding of ATP and L-glutamate. Kinetic studies have indicated that the glutamine synthetase reaction is ordered (Meek et al., 1980) while isotope-exchange experiments have detected apparent random behavior in the

Scheme II: Mechanism in Which γ-Glutamyl Phosphate Can Be Formed Only While NH_4^+ Is Bound to the Enzyme[a]



[a] The substrates and products are abbreviated for clarity. E is glutamine synthetase, A is ATP, B is L-Glu, C is NH_4^+, X is γ-glutamyl phosphate, P is P_i, Q is L-Gln, and R is ADP. The flow of the labeled species is represented by an asterisk. In the strictly ordered mechanism, the bottom branch is not present; i.e., $k_{14} = k_{15} = 0$ and $k_{13} = k_{16} = \infty$. The mechanism is essentially similar for the case in which γ-glutamyl phosphate can be formed in the absence of enzyme-bound NH_4^+. However, the E·ATP·Glu·NH_4^+ complex would be replaced by E–ADP–glutamyl phosphate and the binding of ammonium ion would occur at the step associated with k_7 instead of k_5.

binding of ATP and L-Glu (Welder & Boyer, 1972).

First, let us consider the case in which γ-glutamyl phosphate can be formed only by the enzyme form containing bound NH_4^+. The mechanism is depicted in Scheme II in which E·R·Q·P* represents E·ADP·Gln·P_i, the enzyme complex that undergoes chemical reaction. If the mechanism is strictly ordered (i.e., the lower branch in Scheme II is not present, $k_{14} = k_{15} = 0$ and $k_{13} = k_{16} = \infty$), then in the simplest experiment, ADP and L-Gln can be held at saturating levels while P_i is subsaturating. The NH_4^+ concentration will be varied while the concentration of ATP and L-Glu is maintained at 0 by the presence of hexokinase and glutamic dehydrogenase. Under these conditions, we can use Cleland's concept of net rate constants (Cleland, 1975) to derive the ratio v_{ex}/v_{rxn}:

$$\frac{v_{ex}}{v_{rxn}} = \frac{k_{7'}}{k_{6'}} = \frac{k_{7'}}{k_6} + \frac{k_{7'}k_5}{k_4k_6}[NH_4^+] \qquad (4)$$

So v_{ex}/v_{rxn} represents the partitioning of complex EA*BC between the fraction that reverses and the fraction that proceeds to react. This ratio forms a linear relationship with the concentration of NH_4^+. A plot of v_{ex}/v_{rxn} vs. ammonium ion concentration would be a straight line with positive intercept and slope as in curve I of Figure 1A. The intercept on this plot is the partition ratio between the fraction of E·ATP·Glu·NH_4^+ that reverses to form γ-glutamyl phosphate and the fraction from which NH_4^+ dissociates. In addition, the slope of the plot divided by the intercept is k_5/k_4, the partition between the fraction of E·ATP·Glu that combines with ammonium ion and the fraction from which L-Glu dissociates.

If the mechanism is considered to be random in the binding and release of ATP and L-Glu (i.e., the bottom branch in Scheme II is present), the experiment is identical, but the derivation and analysis are slightly different:

$$\frac{v_{ex}}{v_{rxn}} = \frac{k_{7'}}{k_{6'}} = \frac{k_{7'}}{k_6} + \frac{k_{7'}k_5}{k_6(k_4 + k_{14})}[NH_4^+] \qquad (5)$$

$$\text{slope/intercept} = k_5/(k_4 + k_{14}) \qquad (6)$$

The salient point is that the ratio v_{ex}/v_{rxn} is a linear function of the concentration of NH_4^+. The interpretation of the experiment is analogous to the previous case. The intercept represents the partition ratio of the E·ATP·Glu·NH_4^+ complex. The slope of the plot divided by the intercept represents the partitioning of the E·ATP·Glu complex between the fraction that combines with NH_4^+ and the fraction from which either ATP or L-Glu dissociates.

If γ-glutamyl phosphate can be formed only in the absence of enzyme-bound NH_4^+, the reaction scheme would be similar to that depicted in Scheme II, but the E·ATP·Glu·NH_4^+ complex would be replaced by E–ADP–glutamyl phosphate. The binding of ammonium ion would occur in association with k_7 instead of k_5. Again the analysis can be conducted on the basis of whether or not the reaction contains a branch in the mechanism. For an ordered mechanism, the lower branch in Scheme II is not present. If the same experimental conditions as described above apply, then

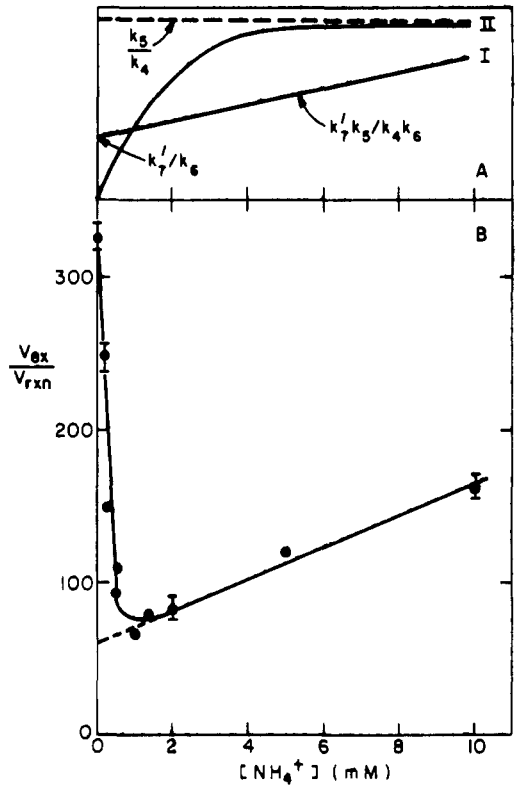


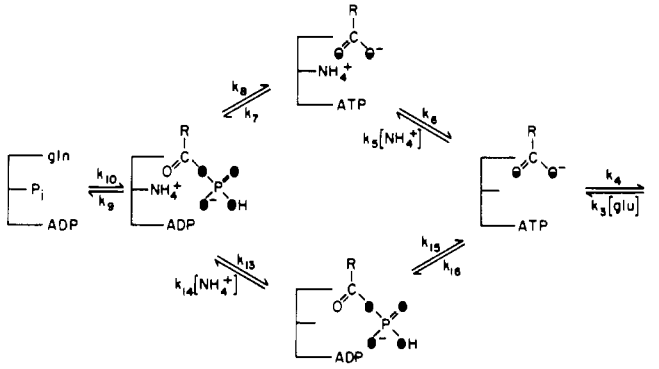
FIGURE 1: (A) Plot of v_{ex}/v_{rxn} as a function of ammonium ion concentration. The curve labeled I assumes that NH_4^+ must be bound to the enzyme before γ-glutamyl phosphate can be formed. The values for the intercept and slope of the line assume ordered binding and release of ATP and glutamate. For the random mechanism, the form of the plot is the same, but the values represented by the slope and intercept consist of different combinations of rate constants. The curve labeled II assumes that NH_4^+ need not be bound to the enzyme surface before γ-glutamyl phosphate can be formed. The values for the asymptote assume ordered binding and release of ATP and glutamate. For a random mechanism, the form of the plot is identical, but the equation for the asymptote is different. (B) Plot of v_{ex}/v_{rxn} as a function of NH_4Cl concentration for a typical experimental as described under Experimental Procedures. In the range of 0–0.5 mM NH_4^+, the concentration range near the K_m for ammonium ion, there is a very steep decline in the ratio. At high concentrations of ammonium ion, the system behaves as if only the mechanism of Scheme II is operating. The dotted line in the figure represents an extrapolation of the rising linear portion of the data back to an NH_4^+ concentration of 0. The value of the slope divided by the intercept for the extrapolated line is 0.17.

$$\frac{v_{ex}}{v_{rxn}} = \frac{k_{5'}}{k_{4'}} = \left(k_5 \frac{k_{7'}[NH_4^+]}{k_6 + k_{7'}[NH_4^+]} \right) \Big/ k_4 \qquad (7)$$

For this mechanism, the relation between v_{ex}/v_{rxn} and ammonium ion concentration is nonlinear. From inspection of eq 7, when the concentration of ammonium ion is 0, no exchange will occur, and v_{ex}/v_{rxn} will also be 0. As the concentration of ammonium ion is increased, the ratio of v_{ex}/v_{rxn} increases to a limiting value:

$$\text{as } [NH_4^+] \to \infty,\ k_{5'} \to k_5,\ v_{ex}/v_{rxn} \to k_5/k_4,\ \frac{d}{d[NH_4^+]}\frac{v_{ex}}{v_{rxn}} \to 0 \qquad (8)$$

Scheme III: Mechanism in Which γ-Glutamyl Phosphate Can Be Formed Whether or Not NH_4^+ Is Bound to the Enzyme Surface[a]



[a] The E-ADP-NH_4^+-γ-glutamyl phosphate complex may either undergo a chemical transformation to E·ATP·Glu·NH_4^+, as in the upper branch, or NH_4^+ may dissociate from the complex, as in the lower branch. The filled O's represent ^{18}O while the partially filled O's represent the equivalent oxygens on the glutamate carboxyl group, which are subject to exchange. The rate constants k_{13}, k_{14}, k_{15}, and k_{16} are different from those used in Scheme II.

Therefore, as the concentration of NH_4^+ increases to saturating levels (effective infinity), the ratio v_{ex}/v_{rxn} increases to a limiting value of k_5/k_4. A plot expected for this type of mechanism is shown in curve II of Figure 1A. The asymptote at k_5/k_4 represents the partitioning of E·ATP·Glu between the fraction that reacts to form γ-glutamyl phosphate and the fraction from which L-Glu dissociates.

If the mechanism is random, the expressions are more complicated but the form of the plot is the same:

$$\frac{v_{ex}}{v_{rxn}} = \frac{k_{5'}}{k_{4,14'}} = \left(k_5 \frac{k_{7'}[NH_4^+]}{k_6 + k_{7'}[NH_4^+]} \right) \Big/ (k_4 + k_{14}) \quad (9)$$

When the concentration of ammonium ion is 0, v_{ex}/v_{rxn} is also 0, producing an intercept at the origin. As the amount of NH_4^+ approaches saturating levels, a limiting value for v_{ex}/v_{rxn} can be found:

$$\text{as } [NH_4^+] \to \infty,\ k_{5'} \to k_5,\ v_{ex}/v_{rxn} \to k_5/(k_4 + k_{14}) \quad (10)$$

The limiting value, $k_5/(k_4 + k_{14})$, represents the partition of the E·ATP·Glu complex between the portion that reacts to form γ-glutamyl phosphate and the portion from which ATP or L-Glu dissociates.

Scheme III shows a section of the reaction pathway when there is no absolute requirement for the presence or absence of NH_4^+ during the formation of γ-glutamyl phosphate. In this scheme, either the E–ADP–NH_4^+–γ-glutamyl phosphate complex may undergo a chemical transformation to the E·ATP·Glu·NH_4^+ complex, as depicted in the top branch, or ammonium ion may dissociate, as in the bottom branch. Isotopic exchange occurs in two places in this mechanism: when the E·ATP·Glu·NH_4^+ complex reverses to form E–ADP–NH_4^+–γ-glutamyl phosphate and when the E·ATP·Glu complex reacts to form E–ADP–γ-glutamyl phosphate. Because the chemical transformations occur in a loop in which all steps are reversible, it is not possible to use net rate constants to derive a simple expression for v_{ex}/v_{rxn}. The behavior for this mechanism may be a mixture of the characteristics of the other two, depending on the actual values of the rate constants and the concentration of the modifier.

Results

Figure 1B is a plot of v_{ex}/v_{rxn} vs. NH_4^+, as described under Theory, showing the results of a typical experiment. As the concentration of ammonium ion is increased, both the velocity of exchange and the velocity of net reaction decrease. However, the rate of exchange decreases faster initially than the velocity of net reaction, reaching a minimum in the range of 1–2 mM ammonium ion. This behavior was also observed in other experiments covering lower ammonium ion concentrations. At higher concentrations of NH_4^+, the velocity of exchange and the velocity of net reaction continue to decrease. Now, however, the velocity of reaction decreases faster than the velocity of exchange, so the ratio v_{ex}/v_{rxn} begins to rise. The increase in v_{ex}/v_{rxn} is a linear function of the concentration of NH_4^+.

The value of v_{ex}/v_{rxn} at an ammonium ion concentration of zero does not in fact, represent the ratio in the strict absence of ammonium ion. This is because the reaction itself produces ammonia. In addition, when the glutamic dehydrogenase reaction is used as a coupling reaction, it also produces ammonia. In order for observations to be made, the reaction must run long enough for some ammonia to accumulate in the experimental sample. Therefore, there is always some ammonia present in the reaction mixture during the course of the experiment. Throughout our series of experiments, the apparent value of v_{ex}/v_{rxn} for these reactions at "zero" ammonia was correlated with the amount of ammonia allowed to accumulate during the course of a reaction (data not shown). This extreme sensitivity to NH_4^+ was observed with concentrations of 0.5 mM or less.

Discussion

The data depicted in Figure 1B clearly do not correspond with the predictions for either of the simple mechanisms considered under Theory. Some characteristics of the "mixed" mechanism depicted in Scheme III can be predicted at extremes of NH_4^+ concentration. In the strict absence of ammonium ion, all of the exchange observed must result from the reversal of E·ATP·Glu·NH_4^+ to E–ADP–NH_4^+–glutamyl phosphate. As the concentration of ammonium ion is increased, the bottom branch in the pathway will contribute to the exchange and net reactions. As ammonium ion concentration continues to increase, the bottom pathway eventually will be "shut off" as the ammonia-release steps (the steps associated with rate constants k_6 and k_{13}) become insignificant relative to the ammonia-binding steps. When such conditions apply, the system will behave similarly to the nonbranched mechanism depicted in Scheme II, and the ratio v_{ex}/v_{rxn} will increase as a linear function of the concentration of NH_4^+.

The data in Figure 1B can be interpreted in terms of these predictions. At low ammonia concentrations, v_{ex}/v_{rxn} is very sensitive to small changes in the NH_4^+ concentration. When the concentration increases slightly, increased net reaction occurs through flux through the bottom part of the reaction scheme. It should be mentioned that this region of extreme sensitivity to concentration brackets the K_m value, providing the enzyme with very precise control of reaction rates. As the concentration of NH_4^+ continues to increase, a point is reached where flux through the bottom of the pathway decreases since formation of the E–ADP–glutamyl phosphate complex is inhibited. After the bottom branch is essentially shut off, the mechanism mimics the strictly ordered mechanism in which ammonia binding and dissociation occur only with the enzyme complex containing ATP and L-glutamate. The ratio v_{ex}/v_{rxn} rises as a linear function of ammonia concentration under these conditions, as is depicted in our data.

If it is assumed that this linear portion of the graph represents the behavior of the top pathway in the mechanism, it is possible to fit a straight line through that portion of the data.

If that line is extrapolated back to zero ammonium ion, as shown by the dashed line in Figure 1B, we can compute the same quantities as for the simpler, nonbranched mechanism. For the experiment depicted in Figure 1B, the intercept, representing the partition of the E·ATP·Glu·NH_4^+ complex between the fraction that undergoes reaction and the fraction from which ammonia dissociates, is approximately 62 with a standard error of about 6.6. The slope of the line divided by the intercept, representing the partition of the E·ATP·Glu complex, is 0.17.

Although the presence of γ-glutamyl phosphate as a reaction intermediate in the mechanism of the sheep brain enzyme was proposed over 20 years ago (Krishnaswamy et al., 1962), early isotope-exchange studies of the *E. coli* enzyme discounted the possibility that it existed as a chemical intermeidate (Wedler & Boyer, 1972). The rationale was that, if an acyl intermediate is a compulsory step in the reaction mechanism, an ADP ⇌ ATP exchange should be observed in the presence of the substrate glutamate, and the equilibrium isotope-exchange experiments showed no such exchange (Wedler & Boyer, 1972). However, as was pointed out by Buchanan & Hartman (1959), such an exchange may not be observed if ADP does not readily dissociate from the enzyme surface. Midelfort & Rose (1976) developed the positional isotope-exchange technique specifically to overcome this obstacle. This method can detect the ADP ⇌ ATP exchange without requiring that ADP dissociate from the enzyme. Using this new tool, Midelfort and Rose showed that γ-glutamyl phosphate was a reasonable reaction intermediate on chemical grounds.

Subsequent work from this laboratory showed that γ-glutamyl phosphate is also a competent intermediate from a kinetic standpoint (Meek et al., 1982). In those experiments, the ammonium ion concentrations were high enough to effectively turn off the bottom branch of the pathway in Scheme III. It was concluded from those experiments that the formation of E·ATP·Glu·NH_4^+ complex was necessary before the formation of γ-glutamyl phosphate occurred. The results of isotope-exchange enhancement studies reported here demonstrate that not only is γ-glutamyl phosphate a viable chemical and kinetic intermediate but it can be formed in the biosynthetic reaction before or after ammonium ion enters the enzyme active site. The amount of NH_4^+ present largely determines through which pathway most of the chemical flux proceeds. From the point of view of the thermodynamically preferred forward biosynthetic reaction, only ATP and L-glutamate need bind in order for the formation of γ-glutamyl phosphate to occur at low concentrations of ammonium ion. Thus, at low ammonium ion concentrations, γ-glutamyl phosphate is usually formed after binding of ATP and glutamate, and this "primed" enzyme is ready to proceed with the reaction as soon as an ammonium ion is bound. At high ammonium ion concentrations, on the other hand, ammonium ion probably binds to the E·ATP·Glu complex before the formation of γ-glutamyl phosphate has a chance to occur.

Meek & Villafranca (1980) have reported nonlinear kinetics for the biosynthetic reaction when NH_4^+ is the varied substrate at low concentrations of L-glutamate. Our data are consistent with those observations in that the kinetic mechanism of the reaction changes as a function of the ammonium ion concentration. Also, our data indicate a change in mechanism at about the same concentration of ammonium ion as the previous steady-state kinetic work. L-Amino acid oxidase exhibits a roughly analogous mechanism in which the concentration of O_2 influences the pathay the reaction takes (Bright & Porter, 1975).

It also has been reported that the ratio of the turnover number for the forward biosynthetic reaction (V_f/E_t) to the turnover number for the reverse biosynthetic reaction (V_r/E_t) of glutamine synthetase is approximately 8 and that the rate-limiting step for the forward biosynthetic reaction is the release of the product MgADP from the E·MgADP complex (Meek et al., 1982). Our results are consistent with those observations in that the partition of the E·ATP·Glu·NH_4^+ complex proceeds at least 50–60 times faster in the forward direction than in the reverse. Thus, the amount of E·ATP·Glu·NH_4^+ complex that undergoes conversion to E–ADP–NH_4^+–γ-glutamyl phosphate is more than 50 times that from which substrate dissociates. Because the ratio of the forward to reverse reactions for this subsection of the mechanism is so much larger than the ratio of net forward reaction to net reverse reaction, the conversion of ATP and L-glutamate to ADP and γ-glutamyl phosphate cannot be the rate-limiting step in the reaction.

The partition coefficient we observe for the E–ATP–Glu–NH_4^+ complex (when the alternate pathway is shut off) can be combined with the value of k_7 reported by Meek et al. (1982) to yield an estimate of k_6. The value of k_7 was estimated to be 240 s^{-1} at 25 °C. Since our partition coefficient is between 55 and 65, we estimate k_6 to be between 3.5 and 4.5 s^{-1}. The rate constant for ATP dissociation from the E·ATP complex has been reported as 2.9 s^{-1} (Meek et al., 1982). Both of these rate constants are near the turnover number for the reverse reaction (1.65 s^{-1}; Meek et al., 1982). Thus, at high NH_4^+ concentrations, dissociation of ammonium ion from the E·ATP·Glu·NH_4^+ complex is partially rate limiting for the reverse biosynthetic reaction.

We believe that our extension to the isotope-exchange enhancement technique may be generally applicable to determining the step at which particular chemical transformations occur in multisubstrate enzyme mechanisms. The experimental techniques are simple, the results clear, and the essential mechanistic requirements few. Basically, the putative chemical reaction steps must be separated by a step involving binding or dissociation of a product or substrate or other effector. The concentration of the effector is then varied, and the rates of both net reaction and isotope exchange are monitored. If exchange and net reaction occur on the same side of the effector binding step, as they do in the case of our experiments with glutamine synthetase, the exchange reaction will not change at the same rate that the net chemical reaction does when the concentration of effector is varied. If the exchange reaction occurs on the opposite side of the effector binding step from the net reaction, as in our predictions for the glutamate synthetase reaction if γ-glutamyl phosphate formed only after binding of ammonium ion, the rates of exchange and net reaction should vary in tandem as the concentration of effector is changed. If the binding and exchange processes can occur in a random order, the observed behavior would result from a mixture of the two types of mechanisms. Under those conditions, it may still be possible to uniquely determine such a mechanism.

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Circular Dichroism Studies on Single Chinese Hamster Cells†

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ABSTRACT: The circular dichroism (CD) and circular intensity differential scattering (CIDS) contributions to the CD of single Chinese hamster (CHO) cells have been measured as a function of the position in the cell cycle. The data are analyzed in three main spectral regions: (1) the region above 290 nm (scattering region), (2) the 250-290-nm regions (nucleic acid absorption region), and (3) the region below 240 nm (protein absorption region). The results show that CD/CIDS microspectrophotometry is a good indicator of the cell cycle phase. The results are consistent with the view that chromatin is organized in chiral superstructures which differentially scatter circularly polarized light. These structures appear highly specific and repeatable as the cell passes through its cycle.

Circular dichroism (CD) is a spectroscopic technique sensitive to primary, secondary, and tertiary structures of biological macromolecules. It measures the difference in absorption cross section exhibited by a sample when it is illuminated successively with right- and left-circularly polarized light. The technique has been applied to the study of structures ranging from small chiral molecules, of the order of nucleic acid monomers, to structures as large as intact bacterial cells, cell nuclei, and eukaryotic cells in solutions (Tinoco et al., 1980). Interpretation of results had been reasonably straightforward in cases of simple chiral organizations, such as small optically active molecules. However, results have been complicated by the presence of scattering contributions and distribution effects (Duysens, 1957) in cases of large organizations such as red blood cell membranes, bacteriophages, and intact eukaryotic cells and cell nuclei. These scattering contributions are manifestations of preferential light scattering of right- or left-circularly polarized light, which reflects the higher order chiral structure of the material measured.

The signal measured in a circular dichrograph along the transmitted beam is (Bustamante et al., 1983)

$$\frac{I_R - I_L}{I_R + I_L} = \frac{2.303(\epsilon_L - \epsilon_R)cl}{2} + \frac{\sigma_R(0) - \sigma_L(0)}{2r^2 + \sigma_L(0) + \sigma_R(0)} \quad (1)$$

where I_R and I_L are the intensities transmitted for right- (R) and left-circularly (L) polarized light and $\sigma_R(0)$ and $\sigma_L(0)$ are the scattering cross sections of the sample in the forward direction. r is the distance of the detector from the sample. The first term involves the difference in extinction coefficients, i.e., the circular dichrosim. ϵ_R and ϵ_L are the extinction coefficients for right- and left-circularly polarized light, c is

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