Finally, although the reactions of cytosolic glutathione *S*-transferases proceed with inversion of configuration (Mangold & Abdel-Monem, 1982) and with regioselectivity (Monks et al., 1982; Watabe et al., 1983), the present study is the first to demonstrate the induction of a chiral center by the microsomal glutathione *S*-transferases. Studies on the regio- and stereospecificities of the microsomal glutathione *S*-transferases are warranted and may reveal information on the mechanism of the reaction.

Registry No. Chlorotrifluoroethene, 79-38-9; glutathione *S*-transferase, 50812-37-8; glutathione, 70-18-8; (*R*)-*S*-(2-chloro-1,1,2-trifluoroethyl)glutathione, 97058-30-5; (*S*)-*S*-(2-chloro-1,1,2-trifluoroethyl)glutathione, 97058-31-6.

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Carbon Isotope Effect on Dehydration of Bicarbonate Ion Catalyzed by Carbonic Anhydrase†

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ABSTRACT: The carbon-13 kinetic isotope effect on the dehydration of HCO_3^- by bovine carbonic anhydrase has been measured. To accomplish this, bicarbonate was added to a buffer solution at pH 8 containing carbonic anhydrase under conditions where purging of the product CO_2 from the solution is rapid. Measurement of the isotopic composition of the purged CO_2 as a function of the concentration of carbonic anhydrase permits calculation of the isotope effect on the enzymic reaction. The isotope effect on the dehydration is $k^{12}/k^{13} = 1.0101 \pm 0.0004$. This effect is most consistent with a ping-pong mechanism for carbonic anhydrase action, in which proton transfer to or from the enzyme occurs in a step separate from the dehydration step. Substrate and product dissociation steps are at least 2–3-fold faster than the hydration/dehydration step.

Carbonic anhydrase catalyzes the hydration of carbon dioxide and its reverse, the dehydration of bicarbonate ion (Pocker & Sarkanen, 1978; Silverman & Vincent, 1984). The enzyme is nearly ubiquitous in living systems, playing a key role both in animal (Pocker & Sarkanen, 1978) and in plant (Reed & Graham, 1981) metabolism. Structurally, carbonic anhydrase is a simple enzyme, having a single subunit with a molecular weight near 30 000. All forms of the enzyme studied to date contain zinc, but there are no other cofactors (Pocker & Sarkanen, 1978). X-ray crystal structures have been reported for several forms of the enzyme (Liljas et al., 1972; Kannan et al., 1975).

The turnover number for carbonic anhydrase from mammalian erythrocytes is among the highest known for any enzyme. The overall reaction rate may be limited at least in part

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by rates of diffusion of substrates and products on and off the enzyme. The overall stoichiometry of the reaction requires a proton transfer:

$$CO_2 + H_2O \rightarrow HCO_3^- + H^+$$

The timing of this proton transfer has been the subject of vigorous debate. The enzyme shows a very large solvent isotope effect on V_{max} but no solvent isotope effect on V/K (Silverman & Vincent, 1984). This observation has often been interpreted in terms of a two-step, "ping-pong" mechanism. The first step in this mechanism is hydration of CO_2 and release of HCO_3^- to the solvent, leaving the enzyme with an extra proton:

$$CO_2 + E\text{–}H_2O \rightarrow HCO_3^- + E\text{–}H^+$$

In the second step, the extra proton is released to the medium in a reaction that shows a large solvent isotope effect:

$$E\text{–}H^+ \rightarrow E\text{–}H_2O + H^+$$

Buffer catalysis of this proton transfer can be observed under certain circumstances (Silverman & Vincent, 1984). Although considerable evidence favors this ping-pong sequence, alternative reaction schemes in which CO_2 hydration and proton release occur within the same enzyme–substrate complex are still under consideration (Pocker & Deits, 1983).

Heavy atom isotope effects are proving increasingly useful in studies of enzymic reaction mechanisms (O'Leary, 1978; Cleland, 1982). Particularly for reactions in which CO_2 is a substrate or product, these isotope effects can be used to determine details of transition-state structure and relative rates of various steps in the overall reaction sequence. Intrinsic isotope effects[1] for carboxylations and decarboxylations are generally in the range $k^{12}/k^{13} = 1.03$–1.07. Observed isotope effects are often smaller than these values because steps other than the carboxylation or decarboxylation step may be partially rate determining.

We have recently reported measurement of carbon kinetic isotope effects on the nonenzymic interconversion of CO_2 and HCO_3^- (Marlier & O'Leary, 1984). For study of CO_2 hydration, the product HCO_3^- was converted to malate by means of phosphoenolpyruvate carboxylase and malate dehydrogenase. This malate was degraded by means of malic enzyme, and the isotope effect was calculated by comparison of the isotopic composition of the resulting CO_2 with that of the starting CO_2. For study of HCO_3^- dehydration, HCO_3^- was added to a pH 8 buffer in the presence of an efficient nitrogen purge, such that CO_2 was swept from the solution as rapidly as it was formed. The isotope effect in the hydration direction was $k^{12}/k^{13} = 1.0069$ at 24 °C, pH 7.5. In the dehydration direction, the effect was $k^{12}/k^{13} = 1.0147$ at pH 8.2. The ratio of the two isotope effects gives the equilibrium isotope effect for CO_2 hydration, as expected (Mook et al., 1974). These results were interpreted in terms of a single-step mechanism for HCO_3^- dehydration in which proton transfer to the departing oxygen is synchronous with carbon–oxygen bond breaking. Because the isotope effect is smaller than intrinsic isotope effects ordinarily observed in reactions involving CO_2, we believe that the transition state is very reactant-like.

The carbon isotope effect on the enzymic dehydration of HCO_3^- can provide useful information about the mechanism of action of carbonic anhydrase, particularly in connection with studies of the solvent deuterium isotope effect. However, such an experiment is made more difficult because of the possibility that isotopic equilibration between CO_2 and HCO_3^- will occur rapidly compared to release of CO_2 from the solution, particularly at high concentrations of carbonic anhydrase. In this paper we report a method for obtaining this isotope effect and a method for estimating the importance of this equilibrium reaction.

[1] An intrinsic isotope effect is the isotope effect for a single isotope-sensitive step, in the absence of contributions from other steps. Intrinsic isotope effects represent the upper limit for observable isotope effects.

Experimental Procedures

Materials. Carbonic anhydrase from bovine erythrocytes and *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid were obtained from Sigma Chemical Co. $NaHCO_3$ was obtained from Columbus Chemical Industries, Inc., and the same bottle was used throughout, for isotopic consistency. Water was purified with a Millipore Super-Q water purification system.

Methods. Carbonic anhydrase was assayed by the colorimetric method of Roughton and Booth (Waygood, 1955). Isotope effect experiments were carried out by the nitrogen purge method of Marlier & O'Leary (1984), with the addition of a measured amount of carbonic anhydrase. All experiments were conducted at 25 °C in 0.5 M *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid buffer, pH 8.2.

Isotope ratios were measured on a Finnigan Delta-E isotope ratio mass spectrometer. All ratios were corrected for oxygen-17 (Craig, 1957). Individual isotope ratios are estimated to be reproducible to ±0.05‰.

Oxygen Exchange. Dehydration of HCO_3^- was carried out as described above except with water containing ^{18}O [$\delta(^{18}O) = 1000‰$], and the CO_2 produced was analyzed for ^{18}O by isotope-ratio mass spectrometry. The presence of ^{18}O above natural abundance in the CO_2 was evidence of reversion of dissolved CO_2 to HCO_3^- prior to purging from the solution.

In a similar experiments, the reaction was conducted with $HC^{18}O_3^-$ in normal water, and the ^{18}O content of the product CO_2 was examined. Oxygen exchange was indicated by a difference between the isotopic content (after correction for statistical factors) between the starting HCO_3^- and the product CO_2.

Theory

If a solution of $NaHCO_3$ at high pH is added to a buffer solution with a pH of near 8, then over the course of several seconds a small fraction of the HCO_3^- will be converted to CO_2. If this CO_2 can be removed from solution before it is reconverted to HCO_3^-, then its isotopic composition can be used to calculate the carbon isotope effect associated with dehydration of HCO_3^- according to

$$k^{12}/k^{13} = \ln (1 - f)/\ln (1 - f[R(HCO_3^-)/R(CO_2)])$$

where $R(HCO_3^-) = [H^{13}CO_3^-]/[H^{12}CO_3^-]$, $R(CO_2) = [^{13}CO_2]/[^{12}CO_2]$, and f = fraction of HCO_3^- consumed. In practice, $R(HCO_3^-)$ is measured by acidifying a sample of the HCO_3^- used and quantitatively collecting the CO_2 so formed for isotopic analysis.

The success of this method requires that CO_2 be removed from the solution rapidly compared to the rate at which it is reconverted to HCO_3^-. We have previously demonstrated that purging with nitrogen efficiently removes CO_2 (Marlier & O'Leary, 1984), and the same phenomenon has been noted by others (Pocker & Deits, 1983). Additional evidence relevant to this point is given below.

If a small amount of carbonic anhydrase is added in this experiment, then part of the substrate is dehydrated spontaneously and part is dehydrated enzymically. Under these

conditions, the isotope effect is a weighted average of the isotope effects for the spontaneous and enzymic reactions. If the amount of carbonic anhydrase is increased, the contribution from the enzymic pathway increases, and the isotope effect approaches that for the enzymic reaction. However, as the amount of carbonic anhydrase increases, rehydration of dissolved CO_2 (which occurs at a negligible rate in the absence of enzyme) will begin to compete with purging. When this occurs, the observed isotope effect obtained by comparing the purged CO_2 with the starting HCO_3^- will approach the equilibrium isotope effect on bicarbonate dehydration. Thus, a plot of the observed isotope effect as a function of carbonic anhydrase concentration starts at the nonenzymic effect, progresses rapidly at low carbonic anhydrase concentrations to a value near that of the enzymic reaction, and then at higher concentrations approaches the equilibrium isotope effect. If there is a reasonably large range in which the purging of CO_2 is fast compared to the reverse reaction, the isotope effect can be approximated by extrapolation of the straight line portion of the curve to zero carbonic anhydrase. However, the isotope effect can more accurately be obtained by curve fitting of the observed isotope effect vs. enzyme concentration, with the known nonenzymic isotope effect and the equilibrium isotope effect as input parameters.

The data were fitted as follows: The reaction is given by

$$HCO_3^-(aq) \underset{k_r}{\overset{k_f}{\rightleftharpoons}} CO_2(aq) \xrightarrow{k_g} CO_2(g)$$

where $k_f = k_{u,f} + k_{c,f}[E]$ and $k_r = k_{u,r} + k_{c,r}[E]$. The isotope effects on the individual rate constants, α, are given by

$$\alpha_{u,f} = k^{12}{}_{u,f}/k^{13}{}_{u,f}, \text{ etc.}$$

the subscripts f and r refer to forward and reverse reactions, respectively. The subscripts u and c refer to uncatalyzed and catalyzed reactions, respectively, and [E] is enzyme concentration. The rate of purging of CO_2 from the aqueous solution, k_g, is assumed to be independent of enzyme concentration and is irreversible.

The observed isotope effect at a particular concentration of enzyme, α_{obsd}, is given by

$$\alpha_{obsd} = \frac{C_f(\alpha_{u,f} - \alpha_{c,f}) + \alpha_{c,f}}{p[C_r(\alpha_{u,r} - \alpha_{c,r}) + \alpha_{c,r} - 1] + 1}$$

where $C_f = k_{u,f}/k_f$, $C_r = k_{u,r}/k_r$, and $p = 1/(1 + C_r k_g/k_{u,r})$. The observed isotope effect thus becomes a nonlinear function of enzyme concentration. The isotope effects in both directions for the uncatalyzed reaction are already known. The isotope effects in the two directions for the catalyzed reaction are related by the known equilibrium isotope effect. The observed isotope effects were then fit to the optimum values of the individual isotope effects and the partitioning parameter p by a least-squares procedure.

An independent confirmation of the validity of this procedure is obtained by conducting the reaction with $H_2{}^{18}O$ and measuring the amount of ^{18}O in the product. The partitioning of aqueous CO_2, p, which is needed for use in the curve-fitting procedure described above, is obtained from this labeling experiment.

Results

Purging of CO_2 from a solution of bicarbonate at pH 8.2, 25 °C, gives CO_2 that has an isotopic composition different from that of the starting bicarbonate corresponding to an isotope effect of $k^{12}/k^{13} = 1.0151$, consistent with our earlier studies (Marlier & O'Leary, 1984). The adequacy of the purging method can be tested by ^{18}O labeling. If this experiment is conducted with water containing a small amount of ^{18}O, then the presence of any excess ^{18}O in the product CO_2 is indicative of rehydration of dissolved CO_2 prior to purging. We find that about 3% of the CO_2 formed has exchanged one ^{18}O with water under these circumstances. This is a sufficiently small value that the correction for rehydration of aqueous CO_2 is negligible in the spontaneous reaction.

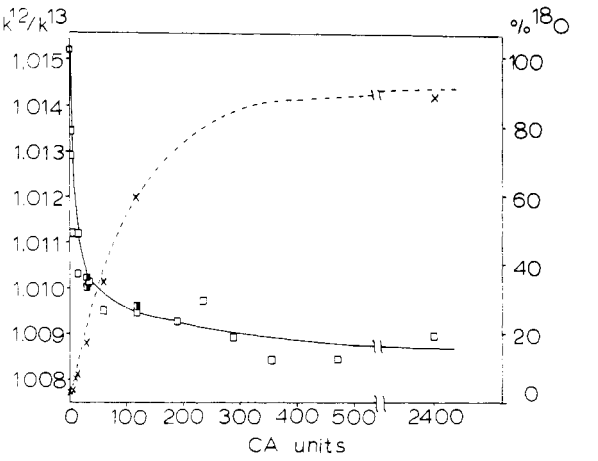

FIGURE 1: Observed isotope effect for the dehydration of HCO_3^- vs. carbonic anhydrase concentration at pH 8.2, 25 °C. Open squares correspond to measured values. Half-filled squares represent two measurements. The solid line shows the best multiparameter fit to the experimental data. Crosses represent the percent of ^{18}O incorporation into CO_2 in experiments carried out in $H_2{}^{18}O$.

In experiments with added carbonic anhydrase, the apparent isotope effect was measured as a function of enzyme concentration, keeping all else constant (Figure 1). The plot divides into three parts: (1) At zero carbonic anhydrase, the isotope effect equals the previously measured nonenzymic value. (2) At low enzyme concentrations (0–30 units of carbonic anhydrase), the observed isotope effect is a combination of enzymic and nonenzymic effects. As carbonic anhydrase concentration increases, the observed isotope effect approaches the enzymic isotope effect. (3) At still higher carbonic anhydrase levels (30–300 units of enzyme), the observed isotope effect decreases as a result of partial equilibration of CO_2 and HCO_3^- prior to purging of CO_2 from the solution. At high enzyme concentrations (above 300 units), the observed isotope effect approaches the equilibrium isotope effect (1.009; Mook et al., 1974). Extrapolation of the third portion of the curve to zero carbonic anhydrase suggests that the isotope effect on the enzymic reaction is about 1.010.

The rate of the rehydration of dissolved CO_2 was independently estimated by conducting the reaction in $H_2{}^{18}O$ and calculating the fraction of oxygen exchange from the ^{18}O content of the CO_2 produced (Figure 1). At low carbonic anhydrase concentration, the contribution of the rehydration of aqueous CO_2 to the overall reaction is small. At higher carbonic anhydrase concentrations, the rehydration rate increases. In the range of carbonic anhydrase concentrations most important for calculation of the isotope effect, the rehydration has only a small effect on the observed isotope effect.

Least-squares fitting of the plot of observed isotope effect and isotope exchange vs. the carbonic anhydrase concentration using the known nonenzymic isotope effect and the equilibrium isotope effect as input parameters gives an isotope effect of $k^{12}/k^{13} = 1.0101 \pm 0.0004$.

Discussion

The transition state for the dehydration of HCO_3^- resembles the transition state for decarboxylation reactions, in that a

departing CO_2 is weakly bonded to some other group

$$R\cdots CO_2^{\delta-}$$

although the degree of bonding between R and CO_2 may vary. If the degree of bonding is the same, we would expect that decarboxylations and HCO_3^- dehydration would show similar intrinsic isotope effects. However, the carbon isotope effects on both the enzymic and the nonenzymic dehydration of HCO_3^- are small compared to intrinsic carbon isotope effects obtained in decarboxylation reactions (O'Leary, 1977, 1978; Cleland, 1982). Available evidence suggests that the spontaneous reaction proceeds in a single step, presumably general acid catalyzed loss of water from HCO_3^- (Marlier & O'Leary, 1984). Thus, the observed carbon isotope effect for the nonenzymic reaction is equal to the intrinsic isotope effect for the reaction. The small size of the isotope effect indicates that the transition state resembles the starting state. The large inverse solvent isotope effect in the nonenzymic reaction (Pocker & Bjorkquist, 1977) is also consistent with this mechanism.

How should the magnitude of the intrinsic isotope effect for the carbonic anhydrase reaction compare with that for the spontaneous reaction? We expect that the transition states for the dehydration steps in the two cases are similar:

$$A\cdots OH\cdots CO_2^{\delta-}$$

where A = H^+ for the spontaneous reaction and, probably, Zn^{2+} for the enzymic reaction. The difference is sufficiently remote from the site of isotopic substitution that, provided the degree of carbon–oxygen bond breaking is similar in the spontaneous and enzymic reactions, the two intrinsic isotope effects should be similar, though not necessarily identical. Differences in solvation may cause small changes in the intrinsic isotope effect, but this should not be sufficient to undermine the basic similarity of the two isotope effects. If the two intrinsic effects are similar, then it follows that in the enzymic reaction the dehydration step is largely rate limiting. This conclusion does not depend on the precise equality of the intrinsic isotope effects for the enzymic and nonenzymic reactions. In order for this conclusion to be false, the intrinsic isotope effect for the enzymic reaction would have to be significantly (i.e., 2-fold or more) larger than the intrinsic isotope effect for the spontaneous reaction, and there is no precedent that leads us to believe that such a thing is likely.

As noted above, the most popular mechanism for carbonic anhydrase is the ping-pong mechanism in which a zinc-bound water or hydroxide acts as the reactive species. Proton transfer between the enzyme and the solvent occurs in a separate step, at a time when neither CO_2 nor HCO_3^- is bound to the enzyme. Under certain circumstances this proton-transfer reaction becomes rate limiting, and large solvent isotope effects are observed. Because the proton-transfer step occurs when neither CO_2 nor HCO_3^- is bound to the enzyme, the rate of the proton-transfer step does not affect the observed carbon isotope effect. Thus, the carbon isotope effect is consistent with the ping-pong mechanism for carbonic anhydrase.

The significant steps in the dehydration half-reaction can be summarized as

$$E + HCO_3^- \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{–}HCO_3^- \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E\text{–}CO_2 \xrightarrow{k_5} E + CO_2$$

and the isotope effect in such a system will be sensitive to these following three steps: (1) Binding of HCO_3^- to the enzyme. We expect that there will be no isotope effect on this step, on the basis of our knowledge of isotope effects on diffusion in aqueous solution (O'Leary, 1984). (2) Dehydration of enzyme-bound HCO_3^-. Although it is possible that this might involve more than a single step, we will assume for the present that there is only one step. Further, let us assume that the isotope effect on this step is the same as that observed in the spontaneous reaction. (3) Dissociation of CO_2 from the enzyme. Again, we expect to see no isotope effect, on the basis of what we know about isotopic effects on diffusion in aqueous solution.

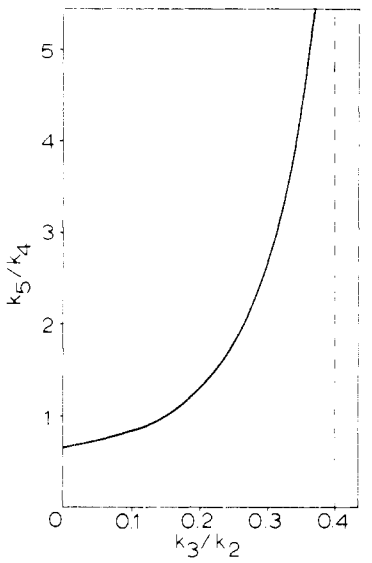

FIGURE 2: Possible values of k_3/k_2 and k_5/k_4.

The overall isotope effect is then given by

$$k^{12}/k^{13} = \frac{\alpha_3/\alpha_4 + \alpha_3 k_5/k_4 + k_3 k_5/(k_2 k_4)}{1 + k_5/k_4 + k_3 k_5/(k_2 k_4)}$$

where $\alpha_3 = k_3^{12}/k_3^{13}$ and $\alpha_4 = k_4^{12}/k_4^{13}$. Thus, the observed isotope effect depends on these two isotope effects and on the rate constant ratios k_3/k_2 and k_5/k_4. The former ratio reflects the relative rates of dehydration and dissociation of enzyme-bound HCO_3^-. The latter ratio reflects the relative rates of dissociation and rehydration of enzyme-bound CO_2. We know approximate values for α_3 and α_4, and we can use the observed isotope effect to estimate possible values of the two partition factors k_3/k_2 and k_5/k_4. There is not a unique solution because there are two adjustable parameters that are interdependent. Given these limitations, we find that k_3/k_2 can be no larger than 0.4 and k_5/k_4 can be no smaller than 0.7. Combinations of possible values of these two ratios are shown in Figure 2.

The carbon isotope effect reported here is consistent with the suggested ping-pong mechanism for carbonic anhydrase. If this mechanism is correct, and if our assumption about the isotope effects on k_3 and k_4 is correct, then the rate-limiting step in the dehydration part of the mechanism is the chemical dehydration step itself, rather than the substrate binding and dissociation steps. However, it appears that the dissociation steps are only a few fold faster than the chemical steps.

If the mechanism of carbonic anhydrase action involves separate dehydration and proton-transfer steps, as proposed, these experiments give no information about the proton-transfer half-reaction. For the hydration/dehydration half-reaction, these results eliminate mechanisms in which the slow step is a conformation change, a proton transfer, or a substrate or product dissociation from the enzyme.

Registry No. HCO_3^-, 71-52-3; ^{13}C, 14762-74-4; carbonic anhydrase, 9001-03-0.

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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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