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A Positional Isotope Exchange Study of the Argininosuccinate Lyase Reaction†

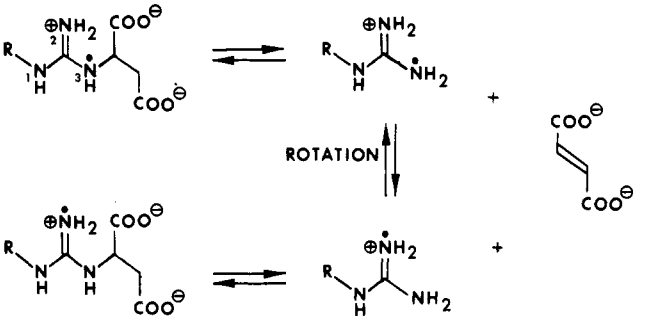
Frank M. Raushel* and Lisa J. Garrard

ABSTRACT: ^{15}N nuclear magnetic resonance spectroscopy was used to follow the positional isotope exchange reaction of bovine liver argininosuccinate lyase. The enzyme was shown to catalyze the N-3–N-2 positional nitrogen exchange in [3-^{15}N]argininosuccinate in the presence of excess arginase. The ratio of the positional isotope exchange rate and the rate for net substrate turnover is less than 0.15 at low levels of fumarate but increases to a limiting value of 1.8 at high fumarate. These data have been interpreted to mean that the dissociation of fumarate and arginine from the ternary enzyme complex is random although fumarate is released at least an order of magnitude faster than is arginine from this complex. The rate constant for the release of fumarate from enzyme–arginine–fumarate is at least 6 times faster than the turnover number of the reverse reaction of argininosuccinate lyase. The lower limit for the release of arginine from this same complex is 0.5.

The positional isotope exchange (PIX) technique was first developed by Midelfort & Rose (1976) to investigate the kinetic competence of γ-glutamyl phosphate as a reactive intermediate in the glutamine synthetase reaction. More recently, this technique has also been applied to the reactions catalyzed by carbamyl-phosphate synthetase (Wimmer et al., 1979; Raushel & Villafranca, 1980), farnesylpyrophosphate synthetase (Mash et al., 1981), pyruvate kinase (Lowe & Sproat, 1978), and others. Thus far, this method has been primarily used to determine whether these enzyme-catalyzed reactions proceeded via stepwise or concerted reaction pathways. For example, in the carbamyl-phosphate synthetase reaction the kinetic competence of both carboxy phosphate (Wimmer et al., 1979; Raushel & Villafranca, 1980) and carbamate (Raushel & Villafranca, 1980) was demonstrated via positional isotope exchange with [*β,γ-bridge*-^{18}O]ATP and carbamyl [*bridge*-^{18}O]phosphate, respectively.

The positional isotope exchange technique can also be used to conveniently measure the partitioning of enzyme-bound intermediates and complexes and to measure individual rate constants or ratios of rate constants in these mechanisms. These results can then be combined with other techniques such as stopped-flow or rapid-quench experiments to obtain individual rate constants and information on the rate-limiting steps. In the carbamyl-phosphate synthetase reaction, the positional isotope exchange data were used to establish that the formation of the intermediate carboxy phosphate was probably rate limiting for the overall chemical reaction.

Scheme I



All of the PIX reactions examined to date have followed the isotopic exchange of ^{18}O and ^{16}O atoms in phosphoryl or carboxylate groups. A previously unrecognized group in which a positional isotope exchange reaction can occur is the guanidino group of arginine and creatine. Shown in Scheme I is an outline for the detection of positional isotope exchange in the argininosuccinate lyase reaction. When argininosuccinate that is labeled with ^{15}N at N-3 is cleaved by argininosuccinate lyase to enzyme-bound arginine and fumarate, the two guanidino amino groups are able to equilibrate via rotation about the carbon–nitrogen bond. Resynthesis of argininosuccinate will form, with a 50% probability, argininosuccinate labeled with ^{15}N at the N-2 position. To ensure that this exchange reaction is occurring only via enzyme-bound ligands, arginase can be added to the reaction mixture to keep the free arginine concentration at zero.

In this paper, we have applied the positional isotope exchange technique to the argininosuccinate lyase reaction. By measuring the positional isotope exchange rate relative to the net chemical turnover of argininosuccinate as a function of

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the concentration of added fumarate, we have shown that the results can be diagnostic for the type of kinetic mechanism of the enzymatic reaction. This new application of the positional isotope exchange technique is also able to determine the individual rate constants (k_{off}) for the dissociation of products from the central complexes (enzyme–fumarate–arginine). This new technique is general and can be used with any enzyme in which a positional isotope exchange reaction can be measured providing the V_{max} of the reverse reaction is significant relative to product dissociation (i.e., potentially all kinases).

Materials and Methods

Argininosuccinate lyase was isolated from beef liver according to the procedure of Havir et al. (1965) and Schulze et al. (1970). The method of Rochovansky et al. (1971) was used to isolate argininosuccinate synthetase from beef liver. [α-^{15}N]Aspartic acid and [2,3-*guanidino*-$^{15}N_2$]arginine hydrochloride were purchased from KOR Isotopes. [^{15}N]Ammonium chloride was purchased from Merck Sharp & Dohme. All other reagents were acquired from either Sigma or Aldrich.

Preparation of [3-^{15}N]Argininosuccinate (I). Argininosuccinate labeled with nitrogen-15 exclusively at N-3 was made enzymatically with argininosuccinate synthetase. The reaction mixture contained 50 mM phosphate buffer, pH 7.5, 11 mM $MgCl_2$, 11 mM ATP, 10 mM citrulline, 10 mM [α-^{15}N]aspartate, 5 units of inorganic pyrophosphatase, and 5 units of argininosuccinate synthetase in a volume of 100 mL. The progress of the reaction was monitored by thin-layer chromatography on poly(ethylenimine) (PEI)–cellulose plates (Baker) and was terminiated after 5 days. The argininosuccinate synthetase and pyrophosphatase were removed by passage of the sample through a YM10 ultrafiltration membrane (Amicon). Metal ions were removed by passage through a Dowex 50 (Na^+) column (1 × 4 cm). The sample was concentrated by rotoevaporation to a volume of 8 mL and was used without further purification. The concentration of argininosuccinate was determined by an end-point assay with argininosuccinate lyase (Havir et al., 1965). ^{15}N nuclear magnetic resonance (NMR) analysis (see Results) showed only one ^{15}N resonance.

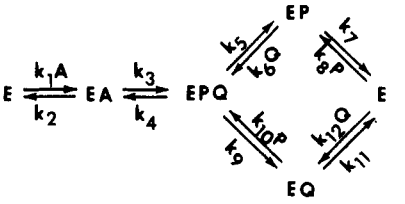
Preparation of [2,3-$^{15}N_2$]Argininosuccinate (II). Argininosuccinate labeled with nitrogen-15 at N-3 and N-2 was synthesized via the reverse reaction of argininosuccinate lyase. The reaction mixture contained 56 mM phosphate buffer, pH 7.5, 11% D_2O, 0.11 mM ethylenediaminetetraacetic acid (EDTA), 11 mM [2,3-*guanidino*-$^{15}N_2$]arginine, 74 mM fumarate, and 0.1 unit of argininosuccinate lyase in a volume of 1.8 mL. The reaction was allowed to proceed for 2 days. The argininosuccinate lyase was removed by centrifugation of the sample through a CF25 Centriflo ultrafiltration membrane cone (Amicon). ^{15}N NMR analysis showed only two ^{15}N resonances (see Results).

^{15}N Nuclear Magnetic Resonance Measurements. ^{15}N NMR spectra were obtained on a Varian XL-200 multinuclear spectrometer operating at a frequency of 20.3 MHz. Typical acquisition parameters were 2400-Hz sweep width, 2.0-s acquisition time, 0.50-s delay between pulses, 10-μs pulse width (26°), broad-band proton decoupling, and 2.0-Hz line broadening. All spectra were referenced to an internal or external standard of $^{15}NH_4Cl$ at pH 7.5. Up to 60 000 transients were collected and Fourier transformed.

Positional Isotope Exchange. Argininosuccinate lyase was incubated with 50 mM 2-amino-2-(hydroxymethyl)-1,2-propanediol, pH 8.0, 100 mM KCl, 10 mM [3-^{15}N]argininosuccinate (I), 10% D_2O, 125 units of bovine liver arginase, and various amounts of fumarate in a volume of 2.5 mL. After the chemical reaction had proceeded to 40–60% completion, the reaction was terminated by centrifuging the reaction mixture through a CF25 Centriflo ultrafiltration membrane cone (Amicon) to remove the protein. The samples were then frozen at −20 °C until they were analyzed by ^{15}N NMR spectroscopy.

Enzyme Assays. Enzyme assays and absorbance measurements were made with a Gilford 260 UV–vis spectrophotometer and a Linear 255 recorder. A unit of argininosuccinate lyase activity is defined as the amount of enzyme needed to catalyze the formation of 1 μmol of fumarate/min at 25 °C and pH 7.5 at saturating argininosuccinate.

Scheme II



Theory

The simplest general mechanism that can be written for the argininosuccinate lyase reaction is found in Scheme II where A = argininosuccinate, P = fumarate, and Q = arginine. In this mechanism, all of the chemistry occurs between EA and EPQ in one step and any possible conformational changes have been omitted. Measurement of the positional isotope exchange reaction relative to the net chemical turnover of argininosuccinate is then a direct determination of the partitioning of the complex EPQ. The steps for arginine addition (k_6 and k_{12}) can be neglected because the excess arginase will keep the free arginine concentration essentially zero. The partitioning of the complex EPQ can therefore be presented in terms of individual rate constants by using the theory of net rate constants of Cleland (1975). In the above mechanism, the partitioning of EPQ forward (k_f) can be presented as

$$k_f = [k_5 + k_9k_{11}/(k_{11} + k_{10}[P])][EPQ] \quad (1)$$

and the partitioning of EPQ backward toward starting materials can be given as

$$k_r = [k_2k_4/(k_2 + k_3)][EPQ] \quad (2)$$

Therefore, the ratio of the PIX rate relative to the chemical rate (k_r/k_f) is

$$\frac{k_r}{k_f} = \frac{k_2k_4/(k_2 + k_3)}{k_5 + k_9k_{11}/(k_{11} + k_{10}[P])} \quad (3)$$

Depending on the relative ratios of the rate constants in eq 3, a plot of k_r/k_f vs. the concentration of added fumarate ([P]) can be very diagnostic for the amount of flux through each reaction pathway.

Ordered Release of Fumarate before Arginine ($k_5 = 0$). At [P] = 0, the ratio of the PIX rate to the chemical rate is

$$k_r/k_f = [k_2k_4/(k_2 + k_3)]/k_9 \quad (4)$$

Since the turnover number in the reverse direction (V_2/E_t) is equal to $k_2k_4/(k_2 + k_3 + k_4)$, the lower limit for the ratio of k_9 to V_2/E_t can be determined as shown in eq 5. When P

$$k_9/(V_2/E_t) = k_f/k_r + k_9/k_2 \quad (5)$$

(fumarate) is added to the system, the value of k_r/k_f gets larger in a linear fashion as shown schematically in Figure 1. The

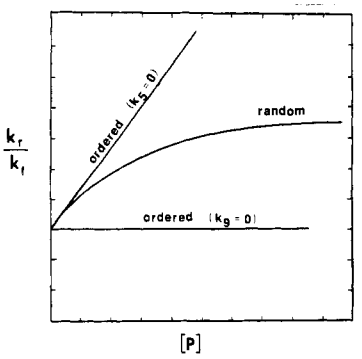


FIGURE 1: Enhancement of the ratio of the positional isotope exchange rate and the net rate of chemical turnover (k_r/k_f) as a function of added product inhibitor. Plotted according to eq 3.

value of k_{10}/k_{11} is given by the slope/intercept.

Ordered Release of Arginine before Fumarate ($k_9 = 0$). In this mechanism, the lower limit for the value of k_5 relative to V_2/E_t is determined from the ratio of the PIX rate and the chemical rate as shown in eq 6. The addition of P will have

$$k_5/(V_2/E_t) = k_f/k_r + k_5/k_2 \quad (6)$$

no effect on this ratio as illustrated in Figure 1.

Random Release of Arginine and Fumarate. For this general mechanism, the value of k_r/k_f as a function of P is given by eq 3, and a plot of k_r/k_f vs. [P] is shown in Figure 1. The intercept on the vertical axis at [P] = 0 is

$$k_r/k_f = [k_2k_4/(k_2 + k_3)]/(k_5 + k_9) \quad (7)$$

As the concentration of P is increased, the flux through the lower pathway diminishes until the net chemical reaction is only occurring via the upper pathway. A plateau occurs at a value of k_r/k_f equal to $[k_2k_4/(k_2 + k_3)]/k_5$. The combination of these limiting values of k_r/k_f with the equation for V_2/E_t permits the determination of the lower limit for both k_5 and k_9 relative to V_2/E_t and the exact value of k_5/k_9.

It is clear from the above examples that the determination of the positional isotope exchange rate relative to the net chemical turnover can be very diagnostic as to the kinetic mechanism. Not only can the mechanism be identified as ordered or random but also the lower limit for k_{off} of both products from the central complex can be directly measured.

Results

The strategy for preparing argininosuccinate labeled with ^{15}N at positions N-3 and/or N-2 is shown in Scheme III. The ^{15}N NMR spectra of the isolated material as prepared under Materials and Methods are shown in Figure 2A,B. The argininosuccinate prepared from [2,3-$^{15}N_2$]arginine and excess fumarate by argininosuccinate lyase shows two resonances at

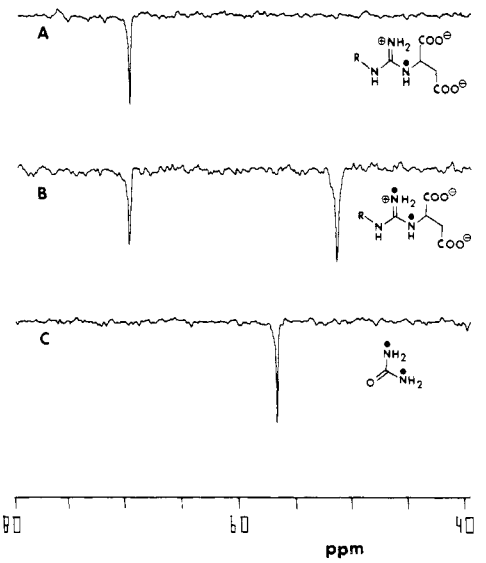


FIGURE 2: ^{15}N NMR spectra of argininosuccinate and urea: (A) [3-^{15}N]argininosuccinate; (B) [2,3-$^{15}N_2$]argininosuccinate; (C) [1,2-$^{15}N_2$]urea.

69 and 51 ppm downfield from NH_4^+. The argininosuccinate prepared from [α-^{15}N]aspartate with argininosuccinate synthetase shows only one resonance at 69 ppm. Therefore, the chemical shift positions for N-3 and N-2 can be assigned to 69 and 51 ppm, respectively. Also, shown in Figure 2C is the spectrum of urea prepared from the hydrolysis of [2,3-$^{15}N_2$]arginine by arginase. The chemical shift position is 56 ppm.

When argininosuccinate lyase is added to a solution of [3-^{15}N]argininosuccinate and excess arginase and the reaction followed by ^{15}N NMR spectroscopy, one can observe the disappearance of the resonance at 69 ppm due to the loss of argininosuccinate and the appearance of a resonance at 56 ppm due to the synthesis of urea. Any positional isotope exchange from N-3 to N-2 will result in the formation of a new resonance at 51 ppm. The inclusion of excess arginase ensures that all of the observed PIX originates from arginine bound to the protein. Shown in Figure 3 is a series of NMR spectra taken at various times after argininosuccinate lyase has been added to a solution of 10 mM [3-^{15}N]argininosuccinate, arginase, and 20 mM fumarate. The formation of [2-^{15}N]argininosuccinate can be clearly observed after 3 and 5 h.

The ^{15}N chemical shift position for [2,3-$^{15}N_2$]arginine is 51 ppm and thus resonates in exactly the same position as N-2 of argininosuccinate. Therefore, it is essential that the arginase concentration be sufficiently high so as to not give a false indication of positional isotope exchange. To ensure that sufficient arginase has been added to our reaction mixtures, we have added greater than a 100-fold excess of arginase over

Scheme III

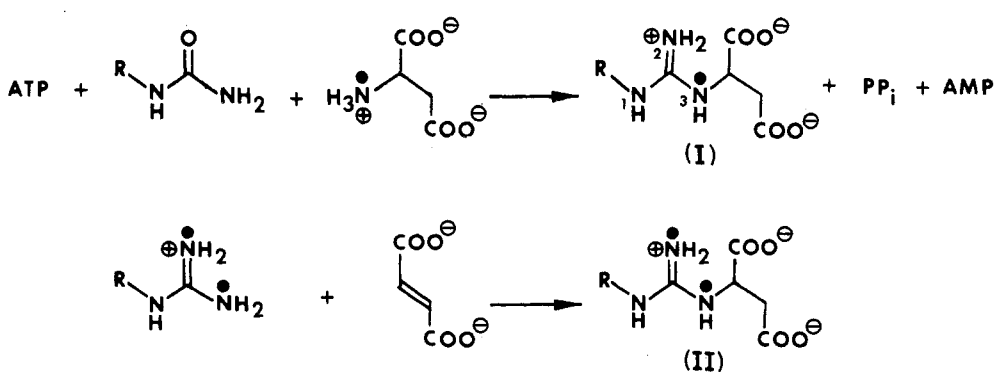


Table I: Argininosuccinate Guanidino Nitrogen Positional Isotope Exchange Reaction of Argininosuccinate Lyase

fumarate (mM)	enzyme (units)	time (h)	X^a	F^b	V_{ex} (μmol/h)^c	V_{chem} (μmol/h)^d	$k_r/k_f{}^e$
0	0.12	3	0.48	<0.10	<0.6	4.0	<0.15
10	0.12	8	0.42	0.32	0.93	1.3	0.70
20	0.39	3	0.42	0.51	4.6	3.5	1.30
30	0.39	4	0.43	0.63	4.8	2.7	1.8
40	0.39	6	0.54	0.70	3.4	2.2	1.6
50	0.36	7	0.41	0.64	2.8	1.5	1.8
60	0.26	11	0.38	0.64	1.7	1.0	1.7

[a] Fraction of change of the original argininosuccinate pool. [b] Fraction of equilibrium value for the exchange reaction. [c] Calculated by using eq 8. [d] Rate of urea production. [e] V_{ex}/V_{chem}.

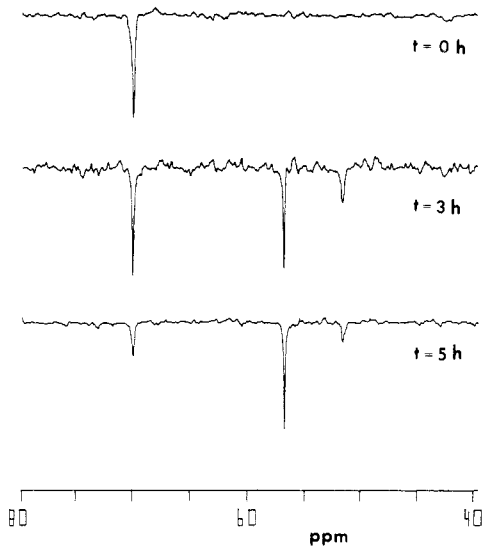


FIGURE 3: ^{15}N NMR spectra taken at the indicated times after the addition of argininosuccinate lyase to a solution containing 10 mM [3-^{15}N]argininosuccinate, 20 mM fumarate, and 125 units of arginase. Additional details are given in the text.

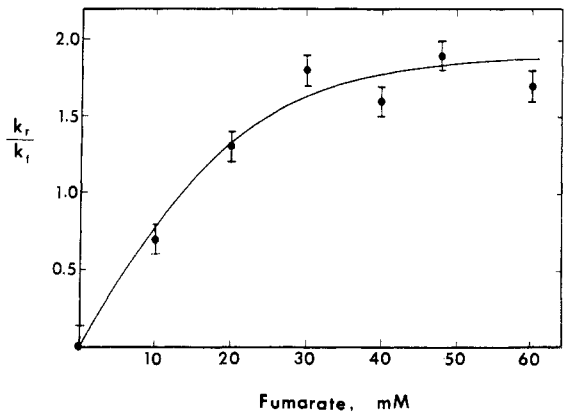


FIGURE 4: Plot of the ratio of the positional isotope exchange rate and the net chemical turnover of argininosuccinate (k_r/k_f) as a function of the concentration of added fumarate. Additional details are presented in the text and in Table I.

the amount of argininosuccinate lyase used in these experiments. In preliminary experiments, we have also shown that decreasing the concentration of arginase by 60% did not change the observed results. Finally, no resonance at 51 ppm was observed when fumarate was omitted from the reaction mixture. This indicates that sufficient arginase has been added to this system to reduce the arginine concentration to below detectable levels even when the net chemical rate for arginine production is the fastest (see Table I).

The rate constants for the positional isotope exchange reaction can be calculated from (Litwin & Wimmer, 1979)

$$\nu_{ex} = \frac{X}{\ln (1 - X)} \frac{A_0}{t} \ln (1 - F) \qquad (8)$$

where X = fraction of change of the original argininosuccinate pool, F = fraction of equilibrium value for exchange attained in the argininosuccinate pool at time t, and A_0 = the concentration of the original argininosuccinate pool. A summary of the data is shown in Table I and plotted as a function of added fumarate in Figure 4. The partitioning of the enzyme complex EPQ as a function of the fumarate concentration can be abstracted from Figure 4. At saturating fumarate, the partitioning ratio (k_r/k_f) is 1.8 ± 0.15, and at low fumarate, this ratio is very close to zero. The estimated upper limit at zero fumarate is 0.15.

Discussion

Positional Isotope Exchange Enhancement (PIXE). When nonequivalent functional groups of an enzyme substrate become rotationally equivalent during the transformation to products, the partitioning of the enzyme–product complex can be determined from the rate of exchange of these groups within the substrate. The detection of this positional isotope exchange (PIX) is dependent on how fast the products dissociate from the enzyme complex relative to how fast the starting material is resynthesized and the substrate dissociates. Clearly, if product release is very fast compared with net turnover in the reverse direction, no positional isotope exchange will be detected. If the dissociation from the enzyme by the product undergoing the positional isotope exchange can be inhibited by inclusion of the nonexchanging product into the reaction mixture, then the PIX rate relative to the chemical rate of the forward reaction will be enhanced.

The enhancement of the PIX rate relative to the chemical rate is dependent on the kinetic mechanism for product dissociation. If the product that undergoes the positional isotope exchange can only leave the enzyme active site after the other product has dissociated, then the addition of the nonexchanging product at high concentration will totally inhibit the release of the product undergoing positional isotope exchange. This causes the ratio of the PIX rate to the chemical rate to increase linearly with the concentration of the other product. If product release is random, then the addition of the other product can only inhibit the flux through the pathway in which the product undergoing positional exchange is released last. The net result is that the PIX rate relative to the chemical rate increases but plateaus at some finite level. There would be no enhancement of the PIX rate if the product undergoing positional exchange is required to be the first product that is released. Therefore, this new technique of enhancing the positional isotope exchange rate relative to the net chemical turnover of substrate is easily able to distinguish ordered from random mechanisms and is also able to tell the order of product release in obligatory kinetic mechanisms.

In enzymatic reactions involving two products, the positional isotope exchange enhancement technique can also quantitatively determine the rate constants for product release from the central enzyme complexes relative to net turnover of the reverse reaction. Therefore, in random kinetic mechanisms the flux through either pathway for product release can be determined. The only other method, besides stopped-flow or rapid-quench experiments, that can provide this same information is the isotope partitioning technique of Rose et al. (1974). However, the PIX enhancement method requires less enzyme and does not use radioisotopes. Unfortunately, not all reactions are amenable to positional isotope exchange.

Argininosuccinate Lyase. The positional isotope exchange data presented in Figure 4 clearly indicate that bovine liver argininosuccinate lyase has a random kinetic mechanism. The partitioning ratio of enzyme–arginine–fumarate is very close to zero at low fumarate and increases to a limiting value of about 1.8 at very high fumarate. Previous studies utilizing initial velocity, product, and dead-end inhibition experiments were also consistent with a random kinetic mechanism for this enzyme (Raushel & Nygaard, 1983). A comparison of the limiting values for the partitioning of enzyme–fumarate–arginine at low and high concentrations of added fumarate permits a more quantitiative evaluation of the flux through each pathway for product release. The rate constant for the release of arginine from enzyme–fumarate–arginine (k_5) can be calculated from the partition ratio of enzyme–fumarate–arginine at saturating fumarate and eq 6. This calculation indicates that $k_5/(V_2/E_t)$ is greater than or equal to 0.5. The rate constant for the release of fumarate from enzyme–fumarate–arginine (k_9) can be calculated from the partition ratio of enzyme–fumarate–arginine at zero fumarate by using eq 6 and 7. These calculations indicate that k_9 is at least 6 times faster than V_2/E_t and that k_9/k_5 is greater than 10. These results show that fumarate is released much faster than is arginine from the ternary enzyme complex. Since the value of V_1 is only 0.6 times as fast as V_2 (Raushel & Nygaard, 1983), we can also conclude that the dissociation of products from the ternary enzyme complex in the forward reaction is not rate limiting (nonsticky products) and, therefore, the slowest step of the forward reaction must precede the actual dissociation of products into the bulk solution.

In the measurement of the positional isotope exchange rates in the argininosuccinate lyase reaction by ^{15}N NMR, it was hoped that the exchange and chemical reactions could be followed continuously from the start to the end of the reaction. We found, however, that the signal to noise ratios that could be obtained by accumulating NMR data over a period of about 1 h were not sufficient to measure the exchange or chemical reactions with the required precision. Therefore, we were forced to stop the reaction at the indicated times and then accumulate NMR data for long periods of time. One drawback to this approach is that the extent of the chemical and exchange reactions may not be optimized in every instance and in some cases the exchange reaction rates required large corrections for recycling via eq 8. We were, however, careful to obtain a sufficient number of independent measurements at the higher concentrations of fumarate to ensure that a plateau had been reached.

The successful application of positional isotope exchange to enzyme systems relies on the assumption that the barrier for bond rotation is small. If the rate of bond rotation is slow relative to the other steps in the reaction mechanism, then the observation of negligible positional isotope exchange may be solely due to slow bond rotation rather than product release. The rotation about the carbon–N-1 bond in arginine can be expected to be somewhat hindered because the guanidino group will impart some double bond character to this system. Kanamori & Roberts (1983) have recently measured the rate of this bond rotation in a H_2O–dimethyl sulfoxide mixture by using ^{15}N NMR line-width measurements. They found a value of approximately 10^3 s^{-1} at 25 °C. The turnover number of the forward reaction of argininosuccinate lyase at 25 °C is approximately 6 s^{-1} (Schulze et al., 1970). Although the guanidino carbon–nitrogen bond rotation rate appears to be sufficiently fast enough to not limit the positional isotope exchange measurements, it is impossible to determine if the enzyme imparts an additional barrier to rotation. Since positional isotope exchange is observed at high concentrations of fumarate, it does not appear that bond rotation is limiting under these conditions.

Summary. We have applied the positional isotope exchange technique to the argininosuccinate lyase reaction. The results indicate that the products, fumarate and arginine, are released randomly from the central complexes and that fumarate is released at least 10 times faster than is arginine.

Registry No. I, 88981-08-2; II, 88981-09-3; argininosuccinate lyase, 9027-34-3; ^{15}N, 14390-96-6.

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