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Isotope Exchange at Equilibrium Studies with Rat Muscle Adenylosuccinate Synthetase†

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ABSTRACT: The kinetic mechanism of rat muscle adenylosuccinate synthetase was studied by determining the rates of isotope exchange at equilibrium. A random sequential binding mechanism was indicated for both the forward and reverse reactions. Aspartate, adenylosuccinate, GDP, and P_i were determined to bind in rapid equilibrium. GTP exchanges with both GDP and P_i at the same rate, which is similar to the exchange rate of IMP with adenylosuccinate. Aspartate exchanges with adenylosuccinate at a higher rate than does IMP over the range of concentrations tested. The slower IMP and GTP exchange rates suggest a forward binding mechanism containing a preferred path in which the quaternary complex is most often formed by aspartate binding to the E–GTP–IMP complex. This preferred path is consistent with an interaction between IMP and GTP in the absence of aspartate as determined by isotope scrambling experiments [Bass, M. B., Fromm, H. J., & Rudolph, F. B. (1984) *J. Biol. Chem. 259*, 12330–12333]. However, the products of such an interaction are tightly bound to the enzyme as no partial exchange reactions between adenylosuccinate and aspartate in the presence or absence of P_i were detected.

Adenylosuccinate synthetase [IMP:L-aspartate ligase (GDP-forming), EC 6.3.4.4] catalyzes the formation of adenylosuccinate from aspartate and IMP while converting GTP to GDP and inorganic phosphate (P_i):[1]

$$\text{GTP} + \text{aspartate} + \text{IMP} \rightleftharpoons \text{adenylosuccinate} + \text{GDP} + P_i \quad (1)$$

Initial rate kinetic studies (which include product inhibition and competitive inhibitor studies of adenylosuccinate synthetase from a variety of sources) are consistent with a random Ter Ter sequential mechanism but do not distinguish among proposed reaction mechanisms (Van der Weyden &

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[1] Abbreviations: P_i, inorganic phosphate; IMP, inosine monophosphate; GTP, guanosine triphosphate; GDP, guanosine diphosphate; EDTA, ethylenediaminetetraacetate; HPLC, high-performance liquid chromatography; PEI–cellulose TLC, poly(ethylenimine)–cellulose thin-layer chromatography; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate. In this paper, "substrates" refers to GTP, IMP, and aspartate; "products" refers to adenylosuccinate, GDP, and P_i; and "reactants" refers to all the reacting components (substrates and products). Since every reactant concentration is varied simultaneously, solutions are conveniently identified as a factor (f) by which the reference concentrations are multiplied.

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Kelly, 1974; Rudolph & Fromm, 1969; Nagy et al., 1973; Clark et al., 1977; Bauger, 1980). During the catalysis the C-6 oxygen of IMP is quantitatively transferred to the released P_i (Lieberman, 1956). The release of P_i from GTP proceeds by a single direct, in-line displacement of GDP by the C-6 oxygen of IMP (Webb et al., 1984). A phosphoryl-IMP intermediate is thus formed at some step in the reaction although its role in the reaction mechanism has not been fully described. The question of whether the interaction between IMP and the γ-phosphoryl of GTP occurs before, during, or after the amino nitrogen of aspartate attacks the C-6 position of IMP has led to three proposed reaction mechanisms for the synthetase. In the first mechanism, IMP is phosphorylated at the C-6 position by GTP, and a subsequent nucleophilic attack by the amino nitrogen of aspartate releases the inorganic phosphate (Lieberman, 1956; Fromm, 1958; Bass et al., 1984). In the second mechanism, there is a concerted reaction with all the substrates participating simultaneously (Miller & Buchannan, 1962). In a third mechanism, aspartate attacks the C-6 position of IMP, thereby activating the oxygen atom for attack on the γ-phosphoryl group. This mechanism is supported by GTP analogue studies (Markham & Reed, 1978) and may be valid for those analogues. The intermediates of any mechanism must be tightly bound in the active site since no partial exchange reactions have been found for this reaction (Fromm, 1958; Miller & Buchanan, 1962).

Recent support for the first mechanism was obtained from the results of positional isotope exchange studies showing the β–γ bridge oxygen of GTP is exchanged in the absence of aspartate (Bass et al., 1984). The positional exchange occurs only by cleavage of that bond, suggesting the phosphoryl transfer to IMP can precede the binding of aspartate. Such positional exchange experiments required long incubations, and the contribution of such a pathway was not established. However, transfer of the phosphoryl group should represent a committing step in the reaction and should lead to IMP and GTP being simultaneously committed to product formation, and it may represent a preferred path to product formation. Equilibrium isotope exchange studies can detect such preferred pathways of reactant binding even in sequential binding mechanisms may be the most sensitive measure for the influence of the binding of one substrate on the binding of another (Boyer, 1978). The information from isotope exchange studies is obtained by conversion of a substrate into its product and is a measure of the physiological pathway. An equilibrium isotope exchange study was undertaken to investigate the role of the phosphorylated IMP intermediate in the reaction mechanism of adenylosuccinate synthetase. The results are consistent with a random sequential binding mechanism where IMP and GTP bind prior to aspartate on the preferred pathway and with the formation of the E–IMP–GTP complex contributing to the overall reaction rate.

Materials and Methods

GTP, GDP, IMP, aspartate, EDTA, and dithiothreitol were purchased from Sigma. HEPES from the Calbiochem-Behring. [U-^{14}C]AMP (specific activity, 538 mCi/mmol), [U-^{14}C]GTP (specific activity, 450 mCi/mmol), and [U-^{14}C]aspartate (specific activity, 224 mCi/mmol) were from Amersham. [γ-^{32}p]GTP (specific activity, 650 mCi/mmol) was obtained from ICN. HPLC and PEI–cellulose thin-layer chromatography as described below were used to check that the radioactivity of each labeled compound comigrated with cold standard. Radioactive decay was monitored by using a Beckman 2801 scintillation counter. Quantifluor scintillation cocktail from Mallinckrodt served as the scintillant.

Adenylosuccinate synthetase was prepared from the muscle tissue of Sprague-Dawley rats and assayed as described by Baugher (1980). The purified enzyme was placed in a buffer of 20 mM HEPES (pH 7.0), 1 mM dithiothreitol, and 0.5 mM EDTA and concentrated to 3.1 mg/mL by using CF-25 Centriflo ultrafiltration membranes from Amicon. The specific activity of the enzyme solution used in this study was 4.5 IU/mg and was apparently homogeneous as evidenced by a single electrophoretic band on SDS electrophoresis.

Separations. Isotope exchange rates are measurements of the rate of equilibration of a radiolabel between related substrate and product pairs. Transfer of the label can only be measured by separation of the reactants from the reaction mixture. HPLC allowed quantitation as well as excellent resolution of the reactants. Separations were achieved by isocratic elution from an ODS11 (0.46 × 23 cm) column from Custom LC (Houston). A Glenco (Houston) single-piston pump was used with a flow rate of 1 mL/min, and the effluent was monitored at 280 nm by an LDC UVIII monitor (a 254-nm filter was used when recovery of GTP and GDP was being monitored). Absorbance changes were recorded on a Linear chart recorder. GTP, GDP, and P_i were resolved with 65 mM KH_2PO_4 (pH 4.6) and 1 mM tetrabutylammonium phosphate in a 10.5% methanol solution (Jahngen & Rossomando, 1984). The same buffer in a 14% methanol solution was used when only the resolution of IMP, adenylosuccinate, and aspartate was required. The small charged reactants, aspartate and inorganic phosphate, were not retained on the column, and both eluted at the injection artifact peak of either buffer. From a 1-mL reaction mixture applied to the ODS11 column, the separated compounds were recovered in 1.5–2.5-mL fractions. Aliquots (0.5 mL) of the fractions were taken to dryness in scintillation vials at 40 °C prior to the addition of scintillant. The HPLC separation of an isotope exchange reaction allowed up to 20% of the total radioactive label from the reaction mixture to be added to a scintillant vial. Quantitation of the reactants was achieved by measuring the areas under the peak standardized by internal or external standards. The area was measured by gravimetric integration.

Slight overlap of the GDP and GTP peaks occurred with HPLC at high concentrations ($f > 5$) so PEI–cellulose TLC was used to confirm the results of the separation of GTP and GDP or P_i. PEI–cellulose TLC plates from EM reagents were prewashed by the method of Crabtree and Henderson (1971). The plates were developed for 12 cm in 2 N sodium formate buffer (pH 3.4). Inorganic phosphate migrated near the solvent front, so spreading was prevented by making 1 cm wide lanes on the plates by removing a strip of the TLC coating between the lanes. Separation of adenylosuccinate and IMP was achieved by using 1 N sodium formate (pH 3.4). Cold nucleotide standards were located by visualization under a UV lamp. The 1 cm wide lane was cut into 1-cm segments and placed in 7-mL of scintillant for counting. Each nucleotide spot was included in one segment.

Synthesis of Adenylosuccinate. The most efficient means of following the isotope exchange is to monitor the movement of label from the lower concentration reactant to the higher concentration reactant. In these studies the exchange of IMP or aspartate with adenylosuccinate was measured by the equilibration of label from adenylosuccinate. [^{14}C]Adenylosuccinate was synthesized enzymatically from [U-^{14}C]aspartate or [U-^{14}C]AMP. Incorporation of labeled aspartate occurred in a 1-mL solution containing 20 mM HEPES (pH 7.0), 5.0 mM magnesium acetate, 1 mM IMP, 0.5 mM GTP, 1 mM phosphoenolpyruvate (PEP), 55 μM [U-^{14}C]aspartate, 0.084

Table I: Concentration of Reactants for an Equilibrium Solution for Adenylosuccinate Synthetase

reactant	K_m (μM)	concn at $f = 1$ (μM)
GTP	5[a]	10
aspartate	360[a]	175
IMP	200[a]	100
GDP	20[b]	20
adenylosuccinate	5[b]	10
P_i	8000[b]	8750
K_{eq}[c]	6	10

[a] Values from Baugher (1980). [b] Values from Markham (1977). [c] K_{eq} = [GDP][P_i][adenylosuccinate]/[GTP][IMP][aspartate].

IU of adenylosuccinate synthetase, and 4.0 IU of pyruvate kinase. Pyruvate kinase and phosphoenolpyruvate were included as a recycling system from GTP to ensure the reaction would go to completion. After 10 h of incubation at 32 °C, 97% of the ^{14}C label eluted as adenylosuccinate.

The synthesis of adenylosuccinate with the IMP portion labeled required an additional enzyme to convert AMP to IMP. The 1-mL reaction solution contained 20 mM HEPES (pH 6.7), 5.0 mM magnesium acetate, 100 mM potassium acetate, 100 μM GTP, 500 μM aspartate, 200 μM PEP, 3.7 μM [U-^{14}C]AMP, 17.5 IU of AMP deaminase, 0.084 IU of adenylosuccinate synthetase, and 4.0 IU of pyruvate kinase. Although essentially all the AMP was converted to IMP within 4 h, only about 60% of the label was converted to adenylosuccinate in 24 h, and addition of fresh enzymes after that time produced no further increase in yield. Presumably, the low yield resulted from inhibition of the synthetase by the accumulated adenylosuccinate.

Isolation of the synthesized adenylosuccinate was achieved in two HPLC steps. After the reaction mixture was brought to pH 3.0 with 1 N HCl, the entire 1 mL of reaction mixture was loaded onto the ODS11 column. Adenylosuccinate was eluted with the 14% methanol solution previously described for the HPLC separations. The methanol was removed under a stream of nitrogen, and the remaining adenylosuccinate solution was injected onto a dry-packed 0.5 × 9.0 cm Synchroprep AX-300 column equilibrated with water. The AX-300 column was washed with 10 mL of water and eluted with 0.1 M triethylammonium bicarbonate solution of pH 7.8 (Greenhut & Rudolph, 1985). The peak corresponding to adenylosuccinate was collected and lyophilized. The residue was dissolved in a minimum of water and stored in a neutral solution at −20 °C. The concentration of adenylosuccinate was determined spectrophotometrically ($\epsilon_{280nm} = 11.7 \times 10^3$ M^{-1} cm^{-1}). The second column removed a small contamination of GTP and placed the adenylosuccinate in a volatile buffer for lyophilization.

Isotope Exchange. Isotope exchange measurements were made over a 20-fold concentration range of each reactant. The reactant levels were held in a constant ratio described by their respective K_m values (Wedler & Boyer, 1972). Because this ratio was maintained throughout the experiment, it was convenient to normalize the reactant concentrations to a preset concentration and express the levels as a factor (f) by which the normal concentration is multiplied. Table I lists published K_m values and the concentration at $f = 1$ for each reactant. Up to a 2-fold variance from the K_m values for some reactants was necessary to achieve a K_{eq} of 10. GTP and adenylosuccinate concentrations were raised to allow sufficient radioactive label to be added to the reaction mixture of $f = 0.5$ without disturbing the equilibrium.

A solution of the highest concentration ($f = 10$) was made, and dilutions with 20 mM HEPES and 2 mM magnesium acetate achieved the f values of 0.5, 1, 2, 3, 4, 5, and 10. Reaction mixtures of 0.5 mL were incubated for 1 h at 32 °C with 0.005 IU of adenylosuccinate synthetase to ensure that equilibrium was attained. No loss of enzyme activity occurred during this incubation. The radioactive compound was added in a 6-μL volume to initiate the exchange reaction. The concentration of the labeled reactant was less than 1% of that of the corresponding unlabeled compound in the $f = 0.5$ solution. The reaction was quenched after 30 min with 10–25 μL of 2 N HCl, bringing the solution to pH 3.0. Samples were stored at −40 °C until just prior to the separation of the components by HPLC or PEI–cellulose TLC. Radioactivity associated with each component of the exchange pair was determined as previously described. The distribution of radiolabel in a given compound expressed as a percentage of the total radioactivity was used to calculate A in eq 2 as described below. Controls of HCl added before the enzyme, of HCl added immediately before the addition of the labeled compound, and of no enzyme added were included. Solutions of $f = 0.5$ and $f = 5$, to which the isotopic label had been added, were incubated with 0.05 IU of adenylosuccinate synthetase for 1.5 h to confirm the theoretical isotopic equilibrium used to determine the fractional attainment of equilibrium (A) in eq 2.

The rate of isotope exchange was calculated from

$$R = -xy[\ln (1 - A)]/[t(x + y)] \quad (2)$$

where x is the nanomoles of the exchanging substrate, y is the nanomoles of exchanging product, A is the fractional attainment of isotopic equilibrium calculated by dividing the measured percent of isotope found in the respective product by the percent of the theoretical labeling of that product at isotopic equilibrium, and t is the time in hours (Purich & Allison, 1980).

Partial Exchange Reactions. Occurrence of partial reactions was evaluated by incubating 0.01 IU of adenylosuccinate synthetase with 2 mM aspartate, 18 μM adenylo[^{14}C]-succinate, and 2 mM magnesium acetate in a 250-μL reaction solution buffered with 20 mM HEPES (pH 7.0). A similar solution was prepared that also contained 10 mM P_i. Identical solutions without enzyme were incubated as controls. At 0, 4 and 8 h after addition of the enzyme, 50-μL aliquots were chromatographed by reverse-phase HPLC, and radioactivity in the respective fractions was determined as previously described.

Results and Discussion

Equilibrium isotope exchange is perhaps the only technique available to distinguish preferred pathways in a mechanism that appears to be random by initial rate analysis. In particular, this study was initiated to evaluate the contribution of the different binding pathways as an investigation into the role of a proposed phosphoryl-IMP intermediate in the reaction of the basic isozyme of adenylosuccinate synthetase from rat muscle tissue. The results of positional isotope exchange experiments have shown the transfer of P_i between GTP and IMP is indeed possible without aspartate (Bass et al., 1984). Yet, such a study cannot unequivocally distinguish whether the exchange occurs during a catalytically competent step or during the formation of a dead-end complex. In an equilibrium isotope exchange experiment the rate of conversion of catalytically competent enzyme complexes is evaluated, providing excellent support for positional isotope exchange studies.

Valid interpretation of equilibrium isotope exchange studies absolutely requires that the substrate and product concentrations remain constant throughout the incubation period.

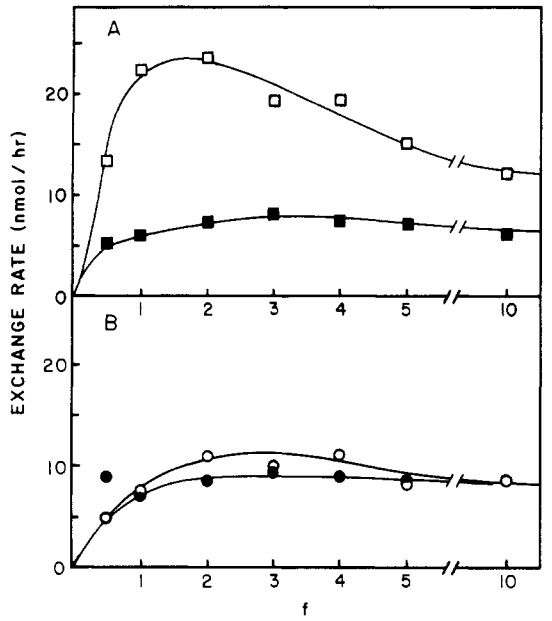


FIGURE 1: Rates of isotope exchange at equilibrium at different reactant concentrations. Composition of the reaction solutions and calculation of exchange rates are described under Materials and Methods under Isotope Exchange. Reactants were held in a constant ratio although the absolute concentrations were varied by a factor (f) from the reference concentration listed as $f = 1$ in Table I. (A) Isotope exchange rates of (■) adenylosuccinate with IMP and (□) adenylosuccinate with aspartate plotted vs. f. The exchange rates were determined by the equilibration of radioactivity from added [U-^{14}C]IMP or adenylo[U-^{14}C]succinate, respectively. (B) Isotope exchange rates of (●) GTP with GDP and (○) GTP with P_i plotted vs. f. The exchange rates were determined by the equilibration of radioactivity from added [U-^{14}C]GTP or [γ-^{32}P]GTP, respectively.

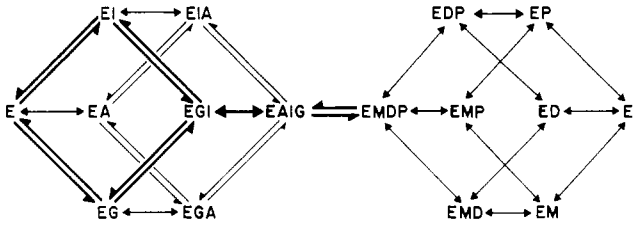
This condition exists only when the concentrations of substrates and products satisfy the equilibrium constant (K_{eq}) of the reaction (eq 3). The equilibrium constant of the reaction has

$$K_{eq} = \frac{[\text{adenylosuccinate}][\text{GDP}][\text{P}_i]}{[\text{IMP}][\text{GTP}][\text{aspartate}]} = 10 \qquad (3)$$

previously been reported to be from 4 to 10 (Fromm, 1958). A K_{eq} of 10 was confirmed for these experiments by the approach to equilibrium method (Purich & Allison, 1980). When enzyme was incubated in reaction solutions that satisfied eq 3, the reactant concentrations varied from the control solutions by less than 2%, which was at the limit of detection of the method. Because HPLC was used for fractionating the exchange solutions, each reaction solution was monitored for any variance of reactant concentration from the control. This control, combined with the 1-h incubation of the reaction solution with the enzyme prior to the addition of labeled reactant, increases our confidence that the exchange rates measured in this study are valid measurements of the equilibrium reaction. The rate of equilibration of label was determined to be linear over at least a 1-h reaction period in all cases by separating identical samples at 20, 40, and 60 min after addition of the labeled reactant.

The exchange rates at equilibrium (R) were determined for four pairs ($R_{\text{aspartate-adenylosuccinate}}$, $R_{\text{IMP-adenylosuccinate}}$, $R_{\text{GTP-GDP}}$, and $R_{\text{GTP-P}_i}$) of reactants of adenylosuccinate synthetase, using the method developed by Wedler & Boyer (1972). This protocol eliminates competitive effects by maintaining the reactants in a constant ratio while the absolute concentrations are varied. Our study measured exchange rates over a range of reactant concentrations of approximately $^1/_2$–10 times their respective K_m values. Or, as expressed in normalized terms, concentrations from $f = 0.5$ to 10 were used. Table I lists the concentrations of each reactant at $f = 1$ so that when $f = 5$, the concentrations are 5 times the level at $f = 1$.

Scheme I: Mechanism of Adenylosuccinate Synthetase Defined by Isotope Exchange at Equilibrium[a]



[a] Double arrows represent steady-state steps, and single arrows represent rapid equilibrium steps. Bold arrows indicate the preferred path in the forward reaction. Abbreviations: E, enzyme; A, aspartate; G, GTP; I, IMP; M, adenylosuccinate; D, GDP; P, P_i.

Plots of $R_{\text{aspartate-adenylosuccinate}}$ and $R_{\text{IMP-adenylosuccinate}}$ vs. f are shown in Figure 1A. The plots of $R_{\text{GTP-GDP}}$ and $R_{\text{GTP-P}_i}$ as a function of f are shown in Figure 1B. Both exchanges were measured from the net movement of isotope from GTP labeled with [U-^{14}C]guanosine or ^{32}P in the γ-phosphoryl position. The differences in the two exchange profiles are within the experimental error. $R_{\text{IMP-adenylosuccinate}}$ is essentially the same as the two GTP exchanges over all concentrations tested.

The shape of the curves generated in these experiments can be used to distinguish between random or ordered binding mechanisms. As the reactants reach saturating levels, the curves should either plateau for a random binding mechanism or become completely depressed for an ordered binding mechanism (Purich & Allison, 1980). In our studies, the exchange rates for three of the exchanges reach a plateau as f increases and remains near the maximum level even at the highest concentrations of reactants. Random binding of GTP, GDP, P_i, IMP, and adenylosuccinate is obviously indicated. Despite the small depression of $R_{\text{aspartate-adenylosuccinate}}$, aspartate binding is also random. Because the IMP exchange is not depressed, adenylosuccinate must bind randomly. Therefore, significant depression of $R_{\text{aspartate-adenylosuccinate}}$ would require aspartate binding preferentially to free enzyme. Such a preferred order is inconsistent with aspartate dissociating most rapidly from the quaternary complex as will be discussed later. Although partial depression of the aspartate–adenylosuccinate is apparent, the decrease in the exchange rate likely results from noncompetitive inhibition not defined by these experiments. The protocol of increasing all the reactants in a constant ratio should eliminate competitive effects between reactants (Wedler & Boyer, 1972).

Comparison of the maximum exchange rates attained by each curve shows that $R_{\text{aspartate-adenylosuccinate}}$ exceeds the rates of the other three exchanges by at least a factor of 2 for all concentrations tested. For a random binding mechanism, as shown in Scheme I, the maximum exchange rate (R_m) is determined by (Fromm et al., 1964)

$$R_m = V_{m,\text{forward}} V_{m,\text{reverse}} / (V_{m,\text{forward}} + V_{m,\text{reverse}}) \qquad (4)$$

$V_{m,\text{forward}}$ was determined to be 4.65 nmol/min, and $V_{m,\text{reverse}}$ was 0.85 nmol/min. The calculated R_m is 0.72 nmol/min, compared to the experimental value of 0.4 nmol/min for $R_{\text{aspartate-adenylosuccinate}}$. These calculations show that approximately 56% of the total enzyme exists as competent quaternary complexes at any instant. Since there are 28 different enzyme species possible in the equilibrium mixture, of which 12 are dead-end complexes, the fact that a significant proportion of the enzyme exists as quaternary complexes suggests that the interconversion of the quaternary complexes is a slow step. The binding of the reactants, especially aspartate and adenylo-

succinate, is most likely in rapid equilibrium. Near rapid equilibrium binding is also suggested by validity of Haldane relationships in correctly predicting the K_{eq}, as shown in Table I. Previous kinetic studies have suggested that aspartate binding is rapid equilibrium (Markham & Reed, 1978).

If all the reactants bound in rapid equilibrium, the maximum exchange rates would be equal. Inequality among the exchange rates shown in Figure 1 indicates that there are factors contributing to the overall reaction rate other than the chemical conversion of the quaternary complex (Purich & Allison, 1980). It has previously been stated that a 2-fold difference in exchange rates indicates that the interconversion of the central complex is a rate-determining step but not necessarily the only step that influences the rate of substrate interconversion (Fromm et al., 1964). Because IMP and aspartate exchange into the same product (adenylosuccinate) and aspartate binds in rapid equilibrium, some step involving an E–IMP species must contribute to the reaction rate prior to the binding of aspartate. This step could be IMP binding in steady state or some interaction retaining IMP in the active site independent of aspartate binding.

GTP exchanges with either GDP or P_i at the same rate. It is likely that the exchange rate is influenced by the rate of GTP binding rather than fortuitously similar association and dissociation rates of GDP and P_i. GDP and P_i binding is probably rapid equilibrium, and some E–GTP species makes a small contribution to the reaction rate. Previous studies suggesting that GTP binding is steady state (Markham & Reed, 1978) support this hypothesis.

These results indicate that the quaternary complex is formed in the forward reaction most often by aspartate binding to the E–IMP–GTP complex, indicating a preferred path in the forward reaction scheme. Such a preferred path is quite consistent with the positional isotope exchange results. The similar exchange rates of IMP and GTP further suggest that such an interaction simultaneously commits GTP and IMP to product formation. This interaction, although not the rate-determining step of the reaction, may contribute to the overall reaction rate.

Isotope exchange reactions in an incomplete reaction mixture have demonstrated the presence of covalent intermediates in other amine transfer reactions (Krishnaswamy et al., 1962). The formation of a discrete phosphoryl-IMP intermediate could allow partial exchange reactions between adenylosuccinate and aspartate in the presence of P_i. Partial exchanges between aspartate and adenylosuccinate in the presence or absence of P_i were undetected at a sensitivity of 3.2×10^{-5} times that of the forward initial reaction rate. A similar result has been reported previously (Fromm, 1958) but with a much less sensitive assay system. If the interaction of GTP and IMP is a complete phosphoryl transfer, then the products are tightly bound on the enzyme (Rose, 1980). It is most likely that the interaction between GTP and IMP prior to aspartate binding involves bond stresses with only very transient phosphoryl transfers detected by positional isotope exchange (Bass et al., 1984). This interpretation is also supported by the lack of physical evidence (including NMR specroscopy) of a true phosphoryl-IMP intermediate (Markham, 1977).

In conclusion, the results of this study are 3-fold. First, the binding of the reactants of adenylosuccinate synthetase is random sequential with a preferred path in the forward reaction. That is, the quaternary complex is formed most often by aspartate binding to the E–IMP–GTP complex. Second, simultaneous commitment of GTP and IMP to product formation is consistent with an interaction prior to commitment of aspartate. This supports the first mechanism proposed for adenylosuccinate in which the C-6 oxygen of IMP is transferred to the phosphoryl group prior to the attack by aspartate. Finally, the bindings of the aspartate, adenylosuccinate, GDP, and P_i are rapid equilibrium while the binding of IMP and GTP is best described as steady state.

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