

Determination of the Mechanism of the Argininosuccinate Synthetase Reaction by Static and Dynamic Quench Experiments[†]

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ABSTRACT: The reactions catalyzed by argininosuccinate synthetase have been examined by the use of static and dynamic quench techniques. The time course of the forward reaction (22 °C) at pH 8.0 is characterized by a "burst" of AMP formation upon quenching with acid that is equivalent to 0.59 mol of enzyme. The pre-steady-state rate is followed by a slower steady-state rate of 0.60 s⁻¹. The rate constant for the transient phase is 9.7 s⁻¹. The time course for the formation of argininosuccinate is linear and shows neither a "lag" nor a burst phase. These results have been interpreted to mean that the mechanism for the formation of argininosuccinate consists of at least two distinct chemical steps with the formation of citrulline adenylate as a reactive intermediate. In the presence of aspartate the rate constant for the formation of citrulline adenylate (6.2 s⁻¹) from ATP and citrulline is 7 times faster than the rate of formation of argininosuccinate from aspartate and citrulline adenylate (0.9 s⁻¹). This suggests that the second step is predominantly rate limiting. The rate constant for the formation of citrulline adenylate in the absence of enzyme-bound aspartate (0.01 s⁻¹) is 600 times slower than when aspartate is present. This indicates that the binding of aspartate to the enzyme regulates the formation of the intermediate. These results are in complete accord with our previously published steady-state kinetic scheme showing sequential addition of substrates [Raushel, F. M., & Seigle, J. L. (1983) *Arch. Biochem. Biophys.* 225, 979-985].

In the preceding paper (Hilscher et al., 1985) we developed and tested a strategy for probing the argininosuccinate synthetase reaction for the formation of citrulline adenylate as a reactive intermediate. The results indicated that the enzyme was unable to catalyze at a significant rate the positional exchange of oxygen-18 within [¹⁸O]ATP in the presence of citrulline but in the absence of aspartate. Although these results do not support the proposal that citrulline adenylate is a kinetically competent intermediate, they cannot be used to prove that this intermediate is not formed during the reaction sequence.

At least three factors may have contributed to the very slow rate of positional isotope exchange with argininosuccinate synthetase. Since the kinetic mechanism for this enzyme is ordered (Raushel & Seigle, 1983), the concentration of citrulline used in those experiments may have suppressed the PIX¹ reaction by preventing any release of ATP from the active site. The intermediate may be required to form but only after aspartate has bound to the active site. The extremely tight binding of the intermediate and pyrophosphate to the enzyme and divalent cation(s) may have hindered P-O bond rotation to the extent that no exchange would be detected even if the intermediate was rapidly being formed.

In this paper two additional approaches have been developed for probing the mechanism of argininosuccinate synthetase. If citrulline adenylate and pyrophosphate were to form and remain bound to the active site of argininosuccinate synthetase after incubation with ATP and citrulline, then it should be possible to determine the extent of that reaction by quenching the mixture with acid. The labile citrulline adenylate intermediate should be immediately hydrolyzed at low pH to AMP and citrulline. The formation and subsequent isolation of

AMP or PP_i would then serve as a direct measure of citrulline adenylate synthesis. This approach assumes that the equilibrium constant for the formation of the enzyme-bound intermediate is not so low as to prevent the quantitation of the expected products by conventional techniques. The second approach is to measure and compare the pre-steady-state rate of formation of AMP or PP_i and the ultimate product, argininosuccinate. Significant differences in the time courses for the formation of these two products would require at least two distinct chemical steps.

The results from the static and dynamic quench experiments clearly indicate that an intermediate is formed from ATP and citrulline at the active site prior to reaction with aspartate. Moreover, the binding of aspartate to the enzyme increases the rate of formation of this intermediate by at least 600-fold.

MATERIALS AND METHODS

Argininosuccinate synthetase was isolated from beef liver according to a slightly modified method of Rochovansky et al. (1977). The specific activity of the preparations of the purified enzyme averaged 1.7 μmol of product (mg of protein)⁻¹ (min)⁻¹ at 25 °C and pH 7.5. The concentration of argininosuccinate synthetase was determined with an extinction coefficient of 6.8 × 10⁴ M⁻¹ cm⁻¹ at 280 nm (Ratner, 1982). Argininosuccinate lyase was isolated from beef liver according to a modified procedure of Havir et al. (1965) and Schulze et al. (1970). All other compounds were obtained from either Sigma or Aldrich.

Enzyme Assays. Argininosuccinate synthetase activity was assayed spectrophotometrically as described previously

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¹ Abbreviations: PIX, positional isotope exchange; MES, 2-(N-morpholino)ethanesulfonate; HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonate; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonate; Tris, tris(hydroxymethyl)aminomethane; HPLC, high-performance liquid chromatography.

(Raushel & Seigle, 1983). Argininosuccinate lyase activity was assayed by monitoring the formation or utilization of fumarate at 240 nm (Raushel & Nygaard, 1983).

Static Quench Experiments. Argininosuccinate synthetase, ATP, citrulline, 10 mM MgCl_2 , 100 mM KCl, 100 mM buffer, and any additional compounds were incubated together at 25 °C in a volume of 150 μL . After being incubated for at least 150 s, the reaction was quenched by the addition of 15 μL of 1 M H_2SO_4 . After centrifugation to remove any precipitated protein the amount of AMP formed after the acid treatment was quantitated by HPLC. Twenty microliters of the quenched reaction mixture was applied to a Whatman Partisil-10 SAX anion-exchange column (25 cm \times 4.6 mm) and eluted with 40 mM KH_2PO_4 (pH 3.5). The observed retention time for AMP at a flow rate of 1.0 mL/min was 5.5 min. The absorbance was monitored at 260 nm, and the amount of AMP was determined by integration of the chromatogram with a Gilson data analysis system. The buffers used in these experiments were MES (pH 6.0), HEPES (pH 7.0), Tris (pH 8.0), and TAPS (pH 9.0). A blank contained everything but enzyme.

Dynamic Quench Experiments. The time course for the pre-steady-state reaction catalyzed by argininosuccinate synthetase was measured with a Dionex D-133 Multi-mixing apparatus. A solution containing argininosuccinate synthetase and 150 mM Tris, pH 8.0, was rapidly mixed with an equal volume of a solution containing 50 mM Tris, pH 8.0, 5.0 mM citrulline, 2.0 mM ATP, 20 mM MgCl_2 , and various other compounds at 22 °C. At varying times 2 volumes of 250 mM H_2SO_4 was rapidly mixed with the reaction solution to quench the reaction. The concentration of AMP formed after the addition of acid was determined by HPLC analysis as described above. When aspartate was used as the third substrate, the concentration of argininosuccinate was determined by enzymatically cleaving the argininosuccinate to arginine and fumarate. The quenched reaction mixture was centrifuged to remove precipitate protein, and 100 μL of this solution was added to 75 μL of 1 M Tris to raise the pH above 7. Argininosuccinate lyase (25 μL) was then added to enzymatically produce fumarate. The fumarate was isolated by chromatography on a Whatman Partisil-10 SAX anion-exchange column and eluted with 75 mM KH_2PO_4 (pH 3.5). The observed retention time for fumarate at a flow rate of 1.0 mL/min is 10.8 min. The absorbance was monitored at 210 nm, and the amount of fumarate was determined by integration with a Gilson data analysis system.

RESULTS

Static Quench Experiments. AMP is formed when argininosuccinate synthetase is incubated for various lengths of time with ATP and citrulline and the reaction solution is quenched with H_2SO_4 . After ~ 1 min of incubation, AMP is produced in an amount that is proportional to the concentration of enzyme used, and this value remains constant for at least 10 min. No AMP is detected when either MgATP or citrulline is omitted from the incubation solution. The amount of AMP produced with 34 and 160 μM enzyme is 2.8 and 12.2 μM , respectively. These results are consistent with the formation of citrulline adenylate at the active site of argininosuccinate synthetase that is hydrolyzed to AMP and citrulline upon addition of acid. This experiment also demonstrates that this intermediate remains tightly bound to the enzyme since there is essentially no additional turnover of products in the absence of the third substrate.

The effect of varying the concentration of ATP and citrulline on the amount of the intermediate formed at equilibrium is

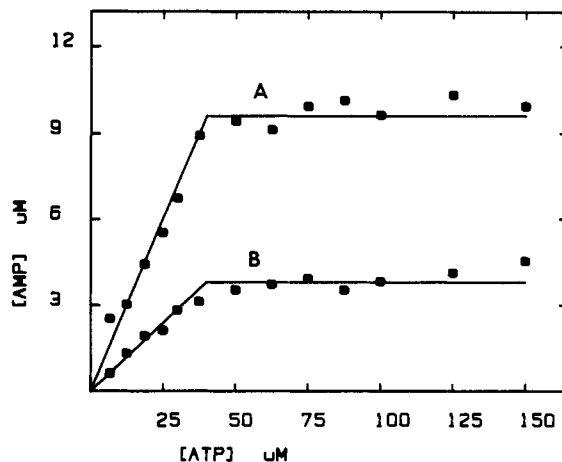


FIGURE 1: Determination of the equilibrium constant for the formation of the intermediate in the presence of malate. Argininosuccinate (39 μM) was mixed with 50 mM citrulline, 10 mM MgCl_2 , 100 mM Tris, pH 8.0, and the indicated levels of ATP and then quenched with acid. (A) 10 mM malate; (B) no other additions. Additional details are given in the text.

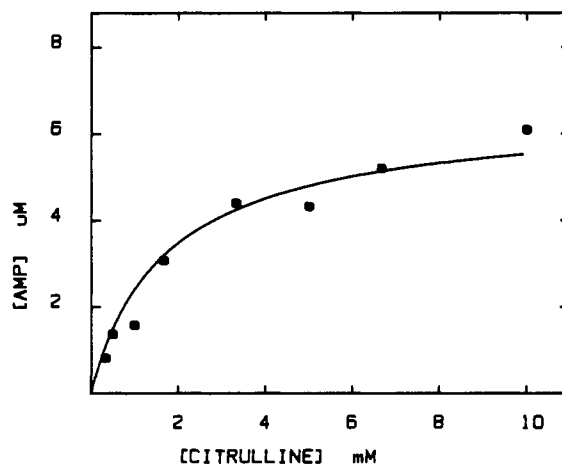


FIGURE 2: Determination of the dissociation constant for citrulline from the E-ATP-citrulline complex. Argininosuccinate synthetase (67 μM) was mixed with 0.15 mM ATP, 10 mM MgCl_2 , and 100 mM Tris, pH 8.0, at the indicated levels of citrulline and then quenched with acid. The solid line is drawn for a dissociation constant of 0.88 mM for citrulline from E-ATP-citrulline. It is assumed that the dissociation constant for ATP from E-ATP is 130 μM and the equilibrium constant for the formation of citrulline adenylate is 0.10. Additional details are given in the text.

illustrated in Figures 1B and 2. At pH 8.0 when the concentration of ATP is varied at saturating citrulline (50 mM), the amount of AMP after the acid quench increases linearly until saturation is reached and no additional AMP is produced (Figure 1B). The slope is equal to 0.089. Similar graphs are obtained at pH 6.0, 7.0, and 9.0, but the slopes are 0.13, 0.11, and 0.08, respectively. Shown in Figure 2 is the effect of varying the concentration of citrulline at a fixed level of ATP (150 μM). The curve through the data points is for a dissociation constant of 880 μM (see below).

Analogues of aspartate that cannot react with the proposed intermediate were added to the reaction mixtures in an attempt to demonstrate whether the binding of ligands to the aspartate site can shift the equilibrium more toward intermediate formation. Shown in Figure 1A are the results when 10 mM L-malate is added to a reaction mixture identical with those presented in Figure 1B. The slope of the plot in Figure 1A in the presence of malate at low levels of ATP is 0.23. In the presence of 10 mM succinate (data not shown) the slope is 0.29. The maximum amount of AMP after the acid quench

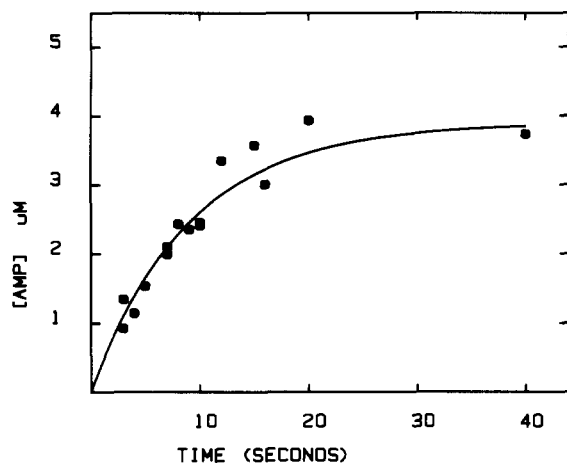


FIGURE 3: Time course for the formation of citrulline adenylate. The reaction mixture contained 45 μM argininosuccinate synthetase, 0.15 mM ATP, 10 mM MgCl_2 , 50 mM citrulline, and 100 mM Tris, pH 8.0. Additional details are given in the text.

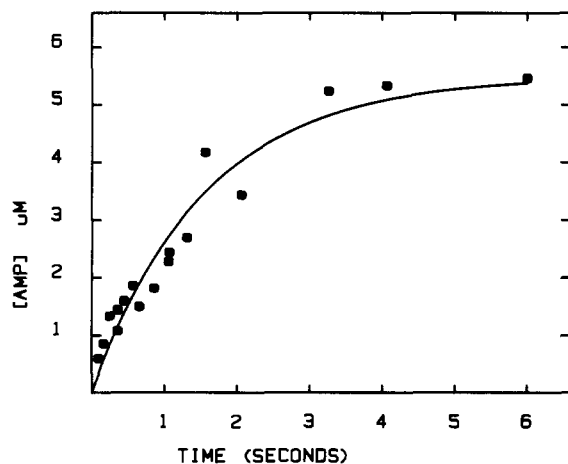


FIGURE 4: Time course for the formation of citrulline adenylate in the presence of α -methyl aspartate. The reaction mixture contained 20 μM argininosuccinate synthetase, 1.0 mM ATP, 10 mM MgCl_2 , 2.5 mM citrulline, and 10 mM α -methyl DL-aspartate. Additional details are given in the text.

is not further increased by higher levels of either succinate or malate.

Dynamic Quench Experiments. The time course for the formation of citrulline adenylate at saturating levels of citrulline and MgATP is shown in Figure 3. The data can be fit to a single exponential and a first-order rate constant of 0.11 s^{-1} . In this experiment the reaction was initiated by manually mixing the reaction components since the rate was too slow for the rapid-mixing device. In the presence of 10 mM α -methyl aspartate the first-order rate constant for intermediate formation is 0.64 s^{-1} (Figure 4). The ratio for the maximum amount of intermediate formed and the amount of enzyme used is 0.27.

The pre-steady-state time course for the overall reaction in the presence of saturating aspartate is shown in Figure 5. The formation of AMP after the acid quench is characterized by a burst followed by a slower steady-state rate. These data were fit to

$$P/E_0 = k_{\text{cat}}t + \beta(1 - e^{-\lambda t}) \quad (1)$$

where P is the concentration of product, E_0 is the total enzyme, k_{cat} is the steady-state rate constant, t is time, β is the burst height, and λ is the transient burst rate constant. In Figure 5 the steady-state rate constant is 0.60 s^{-1} , λ is 9.7 s^{-1} , and

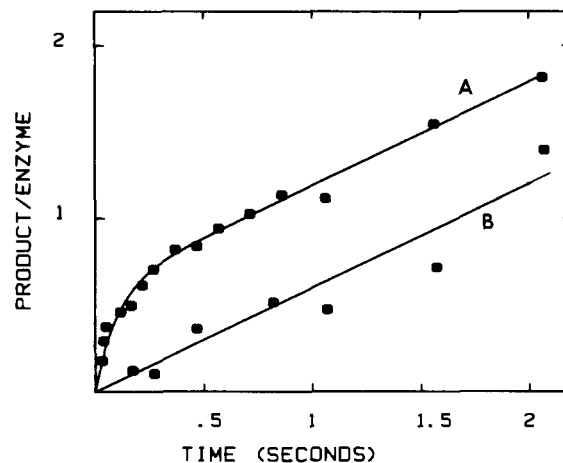
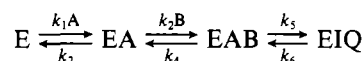


FIGURE 5: Time course for the formation of argininosuccinate and AMP in the presence of aspartate. The reaction mixture contained 17 μM argininosuccinate synthetase, 2.5 mM citrulline, 1.0 mM ATP, 10 mM MgCl_2 , 100 mM Tris, pH 8.0, and 2.5 mM aspartate. (A) Formation of AMP; (B) formation of argininosuccinate. Additional details are given in the text.

$\beta = 0.59$. No detectable burst or lag is apparent in the formation of argininosuccinate.

DISCUSSION

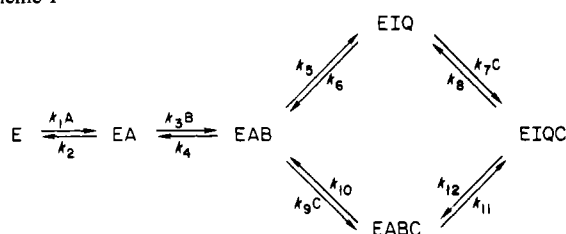
Static Quench Experiments. When a solution of argininosuccinate synthetase, MgATP, and citrulline is briefly incubated and then quenched with acid, less than 1 enzyme equiv of AMP is formed. This result demonstrates that in the presence of citrulline and enzyme a bond to the α -phosphorus of ATP is labilized, and it is thus fully consistent with the formation of citrulline adenylate at the active site. Similar results have been previously obtained by Rochovansky & Ratner (1967), who were able to detect the formation of less than 1 enzyme equiv of pyrophosphate. The minimal kinetic scheme for the formation of citrulline adenylate by argininosuccinate synthetase is



where E = enzyme, A = MgATP, B = citrulline, I = citrulline adenylate, and Q = pyrophosphate. This model is based on the observation that the binding of MgATP and citrulline is ordered, as determined by a steady-state kinetic analysis of the overall synthetic reaction in the presence of aspartate (Rauschel & Seigle, 1983). The complex EIQ is depicted without the possible release of pyrophosphate into solution because we have previously shown that PP_i is not released until the aspartate site is occupied (Rauschel & Seigle, 1983), and Rochovansky & Ratner (1961) have demonstrated that there is no citrulline-dependent ATP- PP_i exchange. Thus, both citrulline adenylate and pyrophosphate are firmly bound to the enzyme. This conclusion is confirmed by the observation that no significant turnover is detected for at least 10 min after initial intermediate formation.

The equilibrium constant for the formation of the bound intermediate (k_5/k_6) can be determined by saturation with MgATP and citrulline. This is most easily achieved by saturating the enzyme with citrulline while the concentration of MgATP is varied (Figure 1) to obtain the equilibrium constant and an independent measure of the total enzyme concentration. Plots of this type are characterized by a linear increase in the concentration of the intermediate until the total ATP concentration equals the number of enzyme active sites. At this point there is no further increase in the amount of intermediate

Scheme I



formed. Since the mechanism for the binding of ATP and citrulline is ordered, it is assumed that all of the ATP is bound to the enzyme at saturating citrulline. The equilibrium constant can be calculated from

$$\text{slope} = K_{eq}/(1 + K_{eq}) \quad (2)$$

where the $K_{eq} = k_5/k_6$. The results indicate that the equilibrium constant at pH 6, 7, 8, and 9 is 0.14, 0.12, 0.10, and 0.09, respectively.

Since the intermediate can form before all substrates have bound to the enzyme, it is of interest to determine whether the third substrate (aspartate) is required to bind directly to the E-ATP-citrulline complex (EAB) or whether the binding site for this substrate is only made available after intermediate formation. With static quench experiments this cannot be directly tested with aspartate because of the subsequent product turnover. However, analogues of aspartate can be tested for this purpose as illustrated in Scheme I, where C represents aspartate or an analogue of aspartate. In this scheme if the analogue can only bind to the enzyme complex with the preformed intermediate (EIQ), then saturation with C will drive all of the available enzyme into EIQC via the upper pathway. However, if C can bind to the EAB complex, then saturation with C will result in the formation of the intermediate with an equilibrium constant that is determined by the ratio of k_{11} and k_{12} . In the presence of L-malate the equilibrium constant for intermediate formation can be calculated from the data in Figure 1B and eq 2 to be 0.30. In the presence of succinate the equilibrium constant is 0.41. These results demonstrate that the aspartate analogues are not required to bind to the EIQ complex since the apparent equilibrium constant is not infinity. However, the binding of these inhibitors (and presumably aspartate) to the enzyme dramatically shifts the equilibrium more toward intermediate formation probably through an induced protein conformational change.

One additional piece of information can be obtained from the static quench experiments. The dissociation constant for the release of citrulline from the E-ATP-citrulline complex can be obtained from a titration of citrulline at fixed ATP if the dissociation constant of ATP from E-ATP and the equilibrium constant for intermediate formation is known by using the above models. The data in Figure 2 correspond to a dissociation constant of 880 μM when an equilibrium constant of 0.10 for intermediate formation and a dissociation constant for ATP of 130 μM (Raushel & Seigle, 1983) are used. This value for k_4/k_3 is consistent with an independent determination of $1400 \pm 300 \mu\text{M}$ obtained from initial velocity studies (Raushel & Seigle, 1983).

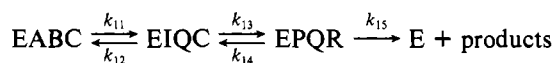
Dynamic Quench Experiments. The rate of formation of the labile intermediate under a variety of conditions was measured with the Dionex rapid-mixing apparatus in order to determine the size of the individual kinetic constants. If it is assumed that the formation of the enzyme-substrate complexes are fast relative to the bond-making and -breaking steps, then the observed rate constant for the formation of

citrulline adenylate (Figure 3) will be the sum of the rate constants k_5 and k_6 (Fersht, 1977). Since the ratio of k_5 and k_6 is already known from the static quench experiments, this enables us to calculate k_5 and k_6 as 0.010 and 0.10 s^{-1} , respectively. The microscopic rate constant for intermediate formation, k_5 , is almost 2 orders of magnitude slower than k_{cat} ($\sim 1 \text{ s}^{-1}$) for the overall synthesis of argininosuccinate. Since no individual forward rate constant can be slower than k_{cat} , this result dramatically demonstrates that virtually all of the flux under saturating substrate conditions flows through the lower pathway as depicted in Scheme I. The upper pathway is simply too slow to account for any significant turnover. This suggests that the binding of aspartate to the active site must significantly increase the magnitude of the rate constant for citrulline adenylate formation (k_{11}).

The observed rate constant for citrulline adenylate formation in the presence of the aspartate analogue α -methyl aspartate² is 0.64 s^{-1} . This observed rate constant is the sum of k_{11} and k_{12} in Scheme I. With an equilibrium constant of 0.37 the values of k_{11} and k_{12} in the presence of α -methylaspartate are 0.17 and 0.46 s^{-1} , respectively. These results indicate that the presence of an aspartate analogue can significantly increase the rate of intermediate formation. α -Methyl aspartate enhances the rate of formation almost 20-fold.

The pre-steady-state time courses for the overall synthetic reaction indicate a burst for the formation of acid-labile intermediate and AMP but no detectable burst or lag for the formation of argininosuccinate. The steady-state rates are identical. These results demonstrate that the mechanism for the formation of argininosuccinate is at least a two-step process in complete accord with the static quench experiments. In a concerted mechanism the time course for each product must be identical. These results also provide an indication as to the location of the rate limitation. The size of the burst for the formation of the acid-labile intermediate indicates that the slow step(s) is (are) after the formation of citrulline adenylate. This could be either the attack of the α -amino group of aspartate at the carbon of the intermediate or release of one of the three products into the bulk solution. If product-release steps are contributing significantly to the overall rate, then a burst would also be apparent for the appearance of argininosuccinate, but none is observed. Therefore, the overall rate-limiting step is either the formation of argininosuccinate or a protein conformational step that immediately precedes this step. The release of products must be fast.

The minimal scheme for the formation of argininosuccinate by the enzyme is



where P = argininosuccinate, R = AMP, and the rest of the terms have been previously defined. Assuming that the release of products is very fast, then the following relationships hold

² α -Methyl aspartate has been reported by Rochovansky & Ratner (1967) not to be a substrate for argininosuccinate synthetase. However, in our hands this compound appears to be a very slow substrate. The rate of formation of AMP upon incubation with enzyme, citrulline, and α -methyl aspartate is about 2% of the rate of the reaction with aspartate at pH 8.5. The K_m value for α -methyl DL-aspartate is 0.7 mM. When the reaction is followed by proton NMR, the methyl group of α -methyl DL-aspartate (1.39 ppm from Me_4Si) diminishes with time, and a new resonance (presumably the methyl signal for α -methyl argininosuccinate) appears at 1.45 ppm. At equilibrium the integrated areas are approximately equal. This would indicate that α -methyl DL-aspartate is not simply promoting the pyrophosphorolysis of ATP and suggests that only one of the stereoisomers of the racemate is a substrate for argininosuccinate synthetase.

Scheme II

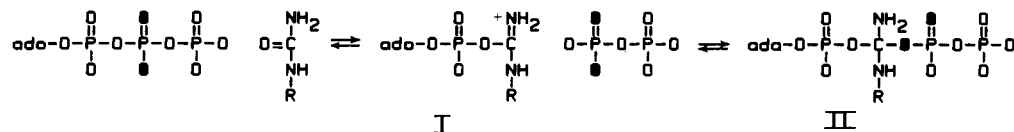


Table I: Summary of Equilibrium and Rate Constants

equilibrium constants	rate constants
$k_2/k_1 = 0.13 \text{ mM}$	$k_5 = 0.01 \text{ s}^{-1}$
$k_4/k_3 = 0.88 \text{ mM}$	$k_6 = 0.10 \text{ s}^{-1}$
$k_5/k_6 = 0.10$	$k_{11} = 6.2 \text{ s}^{-1}$
$k_{11}/k_{12} = 2.5$	$k_{12} = 2.5 \text{ s}^{-1}$
	$k_{13} = 0.90 \text{ s}^{-1}$

for the time course of all enzyme forms containing AMP after the addition of acid (EIQC, EPQR, and products).

$$k_{\text{cat}} = (k_{11}k_{13})/(k_{11} + k_{12} + k_{13}) \quad (3)$$

$$\beta = k_{11}(k_{11} + k_{12})/(k_{11} + k_{12} + k_{13}) \quad (4)$$

$$\lambda = k_{11} + k_{12} + k_{13} \quad (5)$$

Substitution of the experimentally determined values for k_{cat} , β , and λ permits the calculation of all three rate constants. The rate constants k_{11} , k_{12} , and k_{13} are thus determined to be 6.2, 2.5, and 0.9 s^{-1} , respectively.

These results clearly show that the binding of aspartate to the enzyme can strongly influence the kinetic and thermodynamic properties of the reaction between ATP and citrulline. In the presence of aspartate the apparent equilibrium constant for intermediate formation is increased from 0.1 to 2.5 while the forward rate constant for the formation of this same intermediate is increased 600-fold (0.01 vs. 6.2 s^{-1}). These changes are probably mediated through an aspartate-induced protein conformational change. The slow step in the formation of argininosuccinate is either the attack of aspartate on the intermediate or a prior conformational change. The rate and equilibrium constants are summarized in Table I.

It is still not clear why argininosuccinate synthetase does not catalyze a citrulline-dependent oxygen-18 positional isotope exchange reaction. We have provided evidence to indicate that the enzyme-bound citrulline adenylate and pyrophosphate are formed in the absence of aspartate, and we have now determined the individual rate constants for this system. Although the rate of formation of citrulline adenylate in the absence of aspartate is almost 3 orders of magnitude slower than in the presence of this substrate, we still should have been able to detect a significant amount of PIX with the amount of the enzyme used. The calculated rate for the positional isotope exchange reaction is approximately 0.01 s^{-1} , but the measured rate of PIX is $\leq 0.0001 \text{ s}^{-1}$ (Hilscher et al., 1985). The level of citrulline was also not a factor since the amount used was not greater than the dissociation constant for this substrate from the E-ATP-citrulline complex. The remaining possibility is a very tight complexation of the oxygen anions of pyrophosphate by the Mg^{2+} and/or the protein. If the β -phosphoryl group in pyrophosphate is unable to rotate, then no positional exchange will be noted because upon re-formation of ATP the same oxygen will always form the bond to the α -phosphoryl group. However, it is difficult to imagine how simple ionic interactions could totally suppress bond rotation. In the absence of aspartate it might be possible for one of the oxygen anions of pyrophosphate to add to the carbon of the intermediate as illustrated in Scheme II. Formation of an adduct

such as II could totally suppress the scrambling of the oxygens of the β -phosphoryl group. However, this proposal would require that the original unlabeled $\alpha\beta$ -bridge oxygen of ATP always find itself back in the same position upon resynthesis of enzyme-bound ATP. Since this oxygen is only five atoms away from the original α -phosphorus atom in structure II, it just may be possible for a partial P-O bond to form before the C-O bond has completely broken. Support for these proposals is currently being sought by probing the environment of the enzyme-bound substrates and intermediates via NMR.

The enhancement of the rate of citrulline adenylate formation by aspartate is similar to the observations of Meek et al. (1982) with glutamine synthetase. Glutamine synthetase has been shown to catalyze the formation of γ -glutamyl phosphate from ATP and glutamine in the absence of NH_3 (Midelfort & Rose, 1976). From isotope partitioning and rapid-quench experiments Meek et al. (1982) were able to show that in the absence of NH_3 the rate constant for the formation of γ -glutamyl phosphate is 6.9 s^{-1} while in the presence of NH_3 the rate of formation is increased to 240 s^{-1} , an enhancement of 35-fold. These results are in contrast to the mechanism of some other synthetase reactions in which the pyrophosphate is released into the bulk solution from the active site before the third substrate reacts with the activated intermediate. In these cases there is no opportunity for an enhancement of the rate of formation by the third substrate via an induced conformational change.

Registry No. Argininosuccinate synthetase, 9023-58-9; argininosuccinate, 2387-71-5; citrulline adenylate, 14415-52-2; aspartic acid, 56-84-8; α -methyl DL-aspartate, 2792-66-7.

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