Stereochemical Probes of the Argininosuccinate Synthetase Reaction†

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ABSTRACT: The stereochemical course of the argininosuccinate synthetase reaction has been determined. The S_P isomer of [α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]ATP is cleaved to (S_P)-[^{16}O,^{17}O,^{18}O]AMP by the action of argininosuccinate synthetase in the presence of citrulline and aspartate. The overall stereochemical transformation is therefore net inversion, and thus the enzyme does not catalyze the formation of an adenylylated enzyme intermediate prior to the synthesis of citrulline adenylate. The R_P isomer of adenosine 5′-*O*-(2-thiotriphosphate) (ATPβS) is a substrate in the presence of Mg^{2+}, but the S_P isomer is a substrate when Cd^{2+} is used as the activating divalent cation. Therefore, the Λ screw sense configuration of the β,γ-bidentate metal–ATP complex is preferred by the enzyme as the actual substrate. No significant discrimination could be detected between the R_P and S_P isomers of adenosine 5′-*O*-(1-thiotriphosphate) (ATPαS) when Mg^{2+} or Mn^{2+} are used as the divalent cation. Argininosuccinate synthetase has been shown to require a free divalent cation for full activity in addition to the metal ion needed to complex the ATP used in the reaction.

Argininosuccinate synthetase catalyzes the reversible formation of argininosuccinate, pyrophosphate, and AMP from ATP, citrulline, and aspartate. It has been shown that the kinetic mechanism is ordered (Raushel & Seiglie, 1983). Thus, no products are released into solution until all substrates have bound to the enzyme. However, we have recently obtained rapid quench data (Ghose & Raushel, 1985) to support the original suggestion by Ratner (1973) that citrulline adenylate is a kinetically competent intermediate in this reaction. Although citrulline adenylate and pyrophosphate can be formed from ATP and citrulline in the absence of enzyme-bound aspartate, the addition of aspartate to the active site increases the rate of formation of this intermediate by 600-fold (Ghose & Raushel, 1985).

There is no evidence to indicate that the enzyme becomes adenylylated prior to the formation of citrulline adenylate. Since ATP is the first substrate to bind to the enzyme, it would be possible for a nucleophilic group of the protein to attack the α-P of ATP to form an adenylylated enzyme and bound pyrophosphate. If the pyrophosphate is firmly bound to the enzyme and/or if the partial reaction is significantly accelerated by citrulline or aspartate, then this mechanistic feature will not be apparent from an analysis of the steady-state or rapid-quench experiments. Since we have already shown that the formation of citrulline adenylate is greatly stimulated by aspartate, the question as to whether an adenylylated enzyme intermediate is also formed must at this time remain unresolved. However, this ambiguity in the actual mechanism can be solved through an analysis of the stereochemical course of the reaction at the α-P of ATP. An adenylylated enzyme intermediate would result in net retention of configuration at phosphorus in the product AMP while a direct attack at the α-P of ATP by the ureido oxygen of citrulline would result in net inversion (Knowles, 1980). In this paper we have synthesized chiral ATP and have determined the relative stereochemistry of the AMP produced by the catalytic action of argininosuccinate synthetase.

The binding stereochemistry of divalent metal to ATP is another unresolved aspect of the reaction catalyzed by this enzyme. The substrate activities of thio-ATP analogues with various metal ions were measured in an attempt to determine the configuration of the metal–nucleotide complex that is the actual substrate for argininosuccinate synthetase. During the course of these studies we also obtained evidence to indicate that this enzyme requires a free divalent cation for maximal activity in addition to the divalent cation that complexes the polyphosphate moiety of ATP.

Materials and Methods

Materials. Argininosuccinate synthetase was isolated from bovine liver by using a slight modification of the procedure described by Rochovansky et al. (1977). Adenosine 5′-*O*-(thiomonophosphate), adenosine 5′-*O*-(2-thiodiphosphate), adenosine 5′-*O*-(3-thiotriphosphate), 5′-adenylyl imidodiphosphate, and 5′-adenylyl methylenediphosphate were obtained from Boehringer. [^{17}O]Water (45% ^{17}O) and [^{18}O]-water (95% ^{18}O) were purchased from Cambridge Isotope Laboratories. The S_P and R_P isomers of adenosine 5′-*O*-(2-thiotriphosphate) were synthesized according to the method of Eckstein and Goody (1976). The S_P and R_P isomers of adenosine 5′-*O*-(1-thiotriphosphate) were synthesized according to the method of Sheu and Frey (1977).

Synthesis of [$^{18}O_2$]*AMP*α*S*[1] (*I*). [$^{18}O_2$]AMPαS was synthesized as described by Richard and Frey (1982). From 0.5 g of adenosine and 0.235 mL of $PSCl_3$ we obtained 690 μmol of [$^{18}O_2$]AMPαS after chromatography on DE-52. The compound was pure as indicated by HPLC. The enrichment of ^{18}O was 85% as determined from ^{31}P NMR.

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[1] Abbreviations: ADPαS, adenosine 5′-*O*-(2-thiodiphosphate); AMPαS, adenosine 5′-*O*-(thiomonophosphate); AMP-PCP, 5′-adenylyl methylenediphosphate; AMP-PNP, 5′-adenylyl imidodiphosphate; ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); ATPβS, adenosine 5′-*O*-(2-thiotriphosphate); ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); cAMP, adenosine cyclic 3′,5′-phosphate; DE-52, diethylaminoethyl-cellulose (Whatman); FAB-MS, fast atom bombardment mass spectrometry; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; MES, 2-(*N*-morpholino)ethanesulfonic acid; NBS, *N*-bromosuccinimide; PEP, phosphoenolpyruvate.

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Synthesis of (S_P)-[$\alpha,\alpha\beta$-$^{18}O_2$]*ATP*α*S* (*II*). The labeled ATPαS was synthesized from [$^{18}O_2$]AMPαS by the combined activities of pyruvate kinase and adenylate kinase (Brody & Frey, 1981). The reaction mixture contained 10 mM [$^{18}O_2$]AMPαS, 10 mM Mg^{2+}, 100 mM HEPES, pH 8.0, 25 mM PEP, 0.5 mM ATP, 5.0 units/mL pyruvate kinase, and 5.0 units/mL adenylate kinase in a volume of 54 mL. The reaction was terminated by passage through an Amicon CF25 ultrafiltration cone. A total of 432 μmol of (S_P)-[$\alpha,\alpha\beta$-$^{18}O_2$]ATPαS was recovered after chromatography on DE-52.

Synthesis of (S_P)-[$\alpha,\alpha\beta$-$^{18}O_2$]*ADP*α*S* (*III*). Hexokinase was used to synthesize (S_P)-[$\alpha,\alpha\beta$-$^{18}O_2$]ADPαS from the purified (S_P)-[α,α-$^{18}O_2$]ATPαS. The reaction mixture contained 2.0 mM (S_P)-[$\alpha,\alpha\beta$-$^{18}O_2$]ATPαS, 10 mM glucose, 50 mM HEPES, pH 8.0, 5.0 mM Mg^{2+}, and 1.0 unit/mL yeast hexokinase in a volume of 164 mL. The progress of the reaction was followed with HPLC. The yield after purification with a column of DE-52 was 265 μmol.

Synthesis of (S_P)-[α-$^{17}O,\alpha$-$^{18}O,\alpha\beta$-^{18}O]*ADP* (*IV*). The sulfur in the labeled ATPαS was displaced with NBS in [^{17}O]water–dioxane according to the procedure developed by Connolly et al. (1982). (S_P)-[$\alpha,\alpha\beta$-$^{18}O_2$]ADPαS (265 μmol) was evaporated to dryness. The residue was dissolved in 100 μL of [^{17}O]water and evaporated again. This was repeated. The residue was dissolved in 500 μL of [^{17}O]H_2O and 2.5 mL of dry dioxane. *N*-Bromosuccinimide (200 mg) was added and allowed to react for 5 min. The reaction was quenched with 500 μL of β-mercaptoethanol and 6 mL of H_2O. The yield of (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]ADP was 90 μmol after chromatography on DE-52.

Synthesis of (S_P)-[α-$^{17}O,\alpha$-$^{18}O,\alpha\beta$-^{18}O]*ATP* (*V*). Pyruvate kinase and PEP were used to phosphorylate (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]ADP to (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]ATP. The reaction mixture contained 2.0 mM (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]ADP, 3.0 mM PEP, 10 mM Mg^{2+}, 50 mM HEPES, pH 8.0, and 1.0 unit/mL pyruvate kinase in a volume of 36 mL. The progress of the reaction was monitored by HPLC. The protein was removed by centrifugation through an Amicon CF25 ultrafiltration cone. The yield after purification on a column of DE-52 was 68 μmol.

Formation of Chiral [$^{16}O,^{17}O,^{18}O$]*AMP*. (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]ATP was cleaved to AMP by the action of argininosuccinate synthetase in the presence of citrulline and aspartate. The reaction mixture contained 1.5 mM (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]ATP, 4.0 mM aspartate, 4.0 mM citrulline, 10 mM Mg^{2+} 100 mM HEPES, pH 8.5, 2 units/mL inorganic pyrophosphatase, and 4 units of purified argininosuccinate synthetase in a volume of 45 mL. After 2 h the reaction was complete as determined by HPLC. The argininosuccinate was removed by centrifugation through an Amicon CF25 ultrafiltration membrane cone. The yield after purification on a column of DE-52 was 60.0 μmol. Fast atom bombardment mass spectroscopic analysis indicated that the ^{16}O:^{17}O:^{18}O ratio was 1.6:0.4:1.0 (Hilscher et al., 1985).

Configurational Analysis of [$^{16}O,^{17}O,^{18}O$]*AMP*. [^{16}O,^{17}O,^{18}O]AMP was cyclized to cAMP by diphenyl phosphorochloridate and potassium *tert*-butoxide according to the method developed by Jarvest et al, (1981). From 41 μmol of [^{16}O,^{17}O,^{18}O]AMP we recovered 6.0 μmol of labeled cAMP after chromatography on DE-52. The labeled cAMP was then methylated with CH_3I/18-crown-6 to give *N*′-methyladenosine cyclic 3′,5′-phosphate methyl esters as described previously by Jarvest et al. (1981).

Assay of Argininosuccinate Synthetase Activity. Argininosuccinate synthetase activity in the forward direction was determined spectrophotometrically according to the procedure of Raushel and Seiglie (1983). Each 3-mL cuvette contained 50 mM HEPES, pH 8.0, 2.0 mM aspartate, 2.0 mM citrulline, 0.2 mM NADH, 0.5 mM PEP, and 10 units each of adenylate kinase, pyruvate kinase, pyrophosphatase, and lactate dehydrogenase. The reaction mixture also contained variable amounts of divalent metal and ATP. Substrate activity for the sulfur-substituted ATP molecules was determined with HPLC. Each 1.0- or 0.5-mL incubation mixture contained 1.0 mM nucleotide triphosphate, 2.0 mM aspartate, 2.0 mM citrulline, 1.0 unit of inorganic pyrophosphatase, 50 mM HEPES, pH 8.0, 100 mM KCl, and various amounts of divalent cations. The reaction was initiated by the addition of an appropriate amount of argininosuccinate synthetase. At various times aliquots of the reaction mixture were applied to the HPLC column and the amount of AMP or AMPαS was quantitated by a Gilson data analysis system using UV detection at 260 or 254 nm. Substrate activity in the reverse direction was assayed by using the HPLC system. Each 1.0-mL reaction mixture contained 10 mM Mg^{2+}, 2.0 mM pyrophosphate, 2.0 mM argininosuccinate, 50 mM MES, pH 6.0, 100 mM KCl, and either 1.0 mM AMP or 1.0 mM AMPαS. The reaction rate was determined by integration of either the ATP or ATPαS that was synthesized.

Data Analysis. The kinetic data were analyzed by using the FORTRAN programs of Cleland (1967) that have been translated into BASIC. Initial velocity data conforming to an intersecting pattern were fit to eq 1. Competitive inhibition patterns were fit to eq 2. The nomenclature used in this paper is that of Cleland (1963).

$$v = \frac{VAB}{K_{ia}K_b + K_bA + K_aB + AB} \quad (1)$$

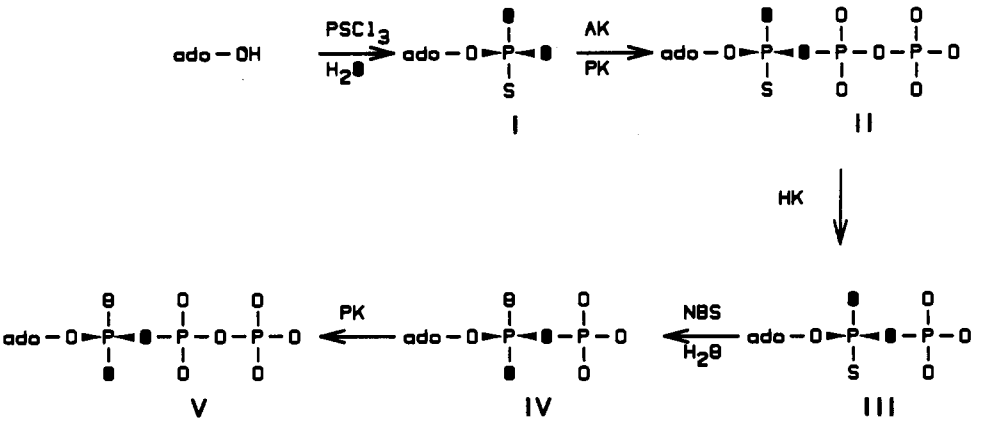
$$v = \frac{VA}{K[1 + (I/K_{is})] + A} \quad (2)$$

RESULTS

Synthesis of (S_P)-[α-$^{17}O,\alpha$-$^{18}O,\alpha\beta$-^{18}O]*ATP*. The strategy for synthesizing chiral ATP for use as a stereochemical probe of the argininosuccinate synthetase reaction is illustrated in Scheme I. The synthetic sequence is dependent on the previously determined stereochemical transformations of both chemical and enzymatic reactions. The synthesis starts with the enzymatic pyrophosphorylation of [$^{18}O_2$]AMPαS by the enzymes adenylate kinase and pyruvate kinase. These reaction conditions are known to pyrophosphorylate the *pro-R* oxygen of AMPαS to produce (S_P)-[α-^{18}O,$\alpha\beta$-^{18}O]ATPαS (Bryant & Benkovic, 1979). The γ-phosphoryl group is next removed with hexokinase to produce (S_P)-[α-^{18}O,$\alpha\beta$-^{18}O]ADPαS. The sulfur at the α-P of ADPαS is subsequently displaced by [^{17}O]water after activation by NBS (Connolly et al., 1982). This reaction has been shown to proceed with inversion of configuration, and thus the S_P isomer of [α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]ADP is formed. This displacement reaction cannot be effectively utilized directly with ATPαS because of scrambling of the isotope between the α- and γ-phosphoryl sites (Connolly et al., 1982). The labeled ADP is then rephosphorylated in high yield by pyruvate kinase to produce the labeled ATP.

Configurational Analysis of Chiral [$^{16}O,^{17}O,^{18}O$]*AMP Produced by Argininosuccinate Synthetase*. The strategy for analyzing the chirality of the [^{16}O,^{17}O,^{18}O]AMP produced by the action of argininosuccinate synthetase on (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]ATP is illustrated in Scheme II (Jarvest et al., 1981). The [^{16}O,^{17}O,^{18}O]AMP is cyclized to cAMP by the combined action of diphenyl phosphorochloridate and *tert*-

Scheme I



Scheme II

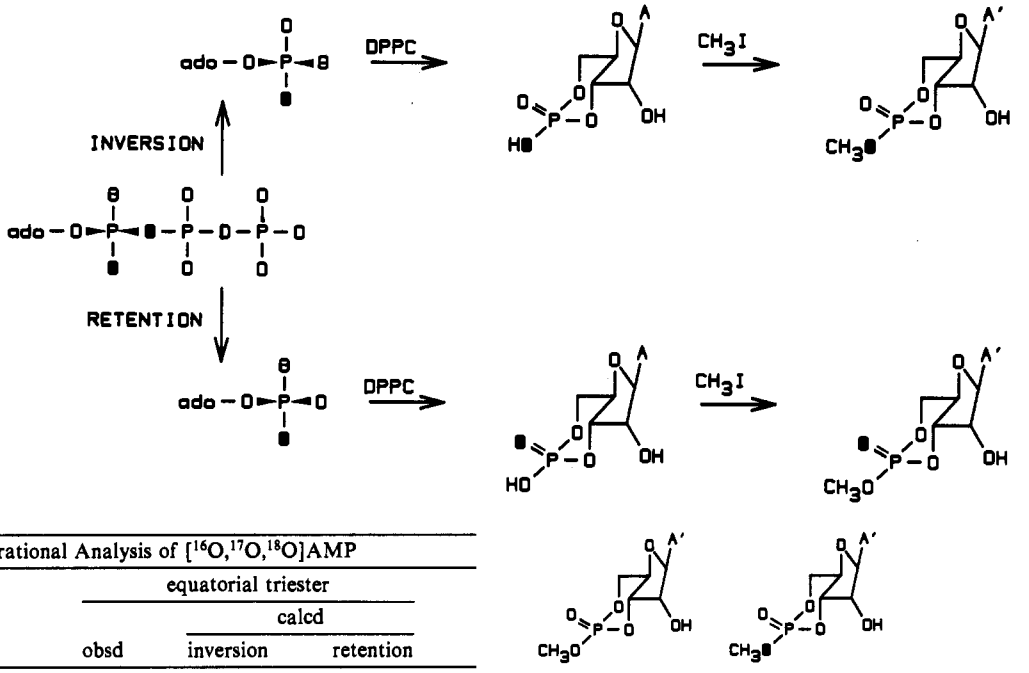


Table I: Configurational Analysis of [^{16}O,^{17}O,^{18}O]AMP

	equatorial triester		
		calcd	
	obsd	inversion	retention
MeO—P═O	0.96	0.80	0.80
Me●—P═O	1.00	1.00	0.64
MeO—P═●	0.52	0.64	1.00
Me●—P═●	0.28	0.19	0.19

butoxide. This reaction has been shown to proceed with inversion of configuration. Any species containing ^{17}O will not appear in the ^{31}P NMR spectrum because of the large electric quadrupolar relaxation (Tsai, 1979). Thus only those species containing ^{16}O or ^{18}O are depicted. The cAMP is methylated by CH_3I to produce both the equatorial and axial triesters (only the equatorial triesters are shown). The proton-decoupled high-resolution ^{31}P NMR spectrum of the equatorial triester is illustrated in Figure 1. From the known distribution of all the major species in the isolated [^{16}O,^{17}O,^{18}O]AMP the expected relative peak intensities can be calculated (Jarvest et al., 1981). The calculated and experimental peak intensities expected for both the retention and inversion of configuration are shown in Table I. The experimental results are consistent with an overall inversion of configuration.

Variation of Divalent Cation. Shown in Figure 2 is a plot of relative activity vs. the concentration of various divalent cations as activators of the argininosuccinate synthetase reaction. At optimal levels of the divalent cation the order of activity is Mg > Mn > Cd > Co > Zn. Nickel did not produce significant substrate turnover under these conditions.

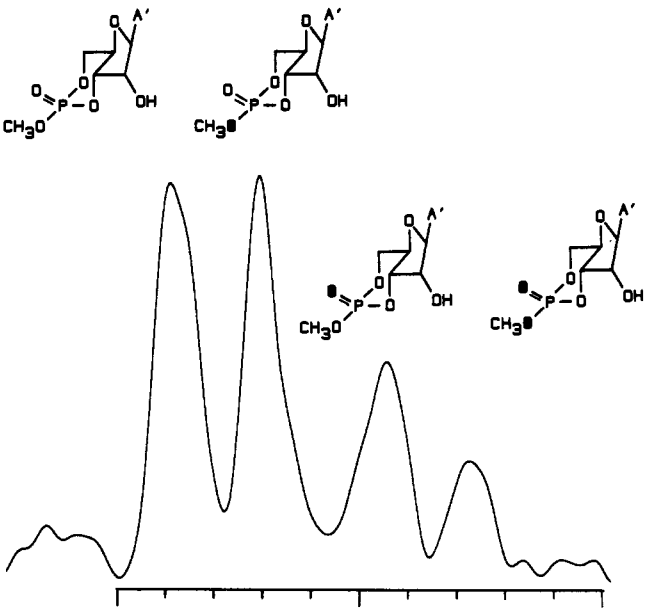


FIGURE 1: 162-MHz NMR spectrum of the equatorial triester derived by cyclization and methylation of [^{16}O,^{17}O,^{18}O]AMP obtained from the action of argininosuccinate synthetase on (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]ATP. The spectrum was obtained in Me_2SO-d_6. The following parameters were used to obtain the spectrum: sweep width, 1000 Hz; acquisition time, 3.0 s; pulse angle, 90°; 10371 transients; resolution enhancement, 0.15; Gaussian multiplication, 0.40 s; scale, 0.01 ppm/division.

The optimal concentration of the divalent cation appears to occur at a concentration equivalent to the ATP concentration

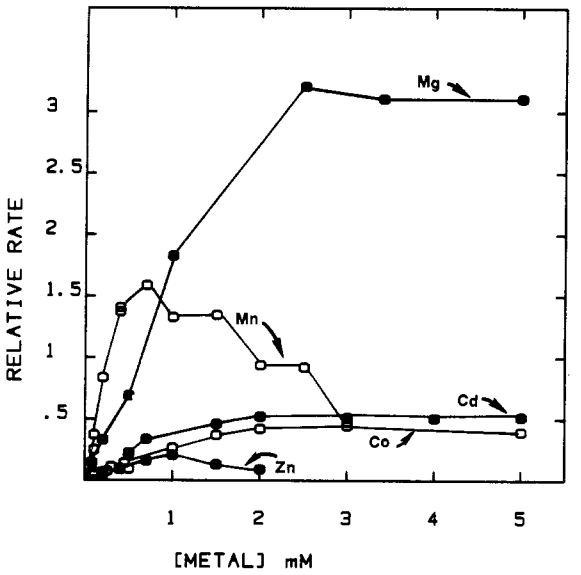


FIGURE 2: Variation of enzymatic activity with various concentrations of added divalent cations. Experimental conditions: 1.0 mM ATP, 2.0 mM aspartate, 2.0 mM citrulline, 50 mM HEPES (pH 8.0), an 10 units/mL pyrophosphatase.

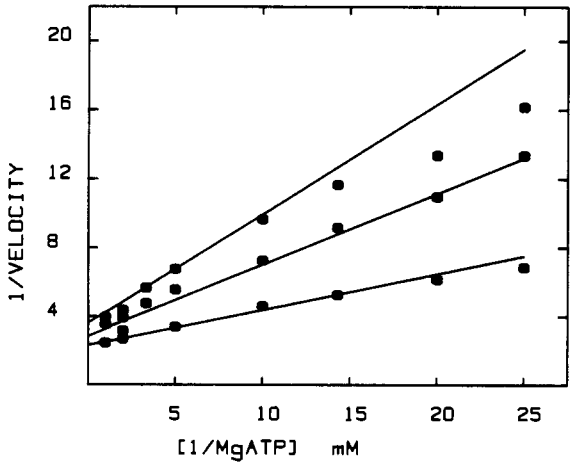


FIGURE 3: Variation of enzymatic activity with concentration of Mg^{2+}_{free} and MgATP. The data were fit to eq 1. The velocities are in arbitrary units.

used in these experiments (1.0 mM). Mg^{2+} is an exception. Significantly higher levels of Mg^{2+} are required to produce maximal activity. This suggests that the enzyme requires an additional divalent cation for full activity.

Initial Velocity Pattern. Shown in Figure 3 is the double-reciprocal plot of velocity vs. [MgATP] at various $[Mg^{2+}]_{free}$. The data were fit to eq 1. The K_m for Mg^{2+}_{free} is 0.60 mM, and the K_i is 2.4 mM.

Synergistic Activity by Mn^{2+} and Mg^{2+}. The divalent cation titrations shown in Figure 2 suggest that an additional uncomplexed divalent cation is required for optimal enzymatic activity. It also appears that this additional divalent cation site binds Mn^{2+} tighter than Mg^{2+}. It should therefore be possible to obtain a synergistic response between Mg^{2+} and Mn^{2+} if the Mn^{2+} can be made to bind primarily to the enzyme site and the Mg^{2+} to the nucleotide. Shown is Figure 4 is a titration of $[Mn^{2+}]_{total}$ at a fixed level of ATP (100 μM). This titration was performed in the presence and absence of 100 μM Mg^{2+}. The slope in the plot of activity vs. $[Mn^{2+}]_{total}$ in the presence of 100 μM Mg^{2+} is 1.3 times larger than the slope in the absence of Mg^{2+}. This indicates that the resultant activity in the presence of Mg^{2+} and Mn^{2+} together is

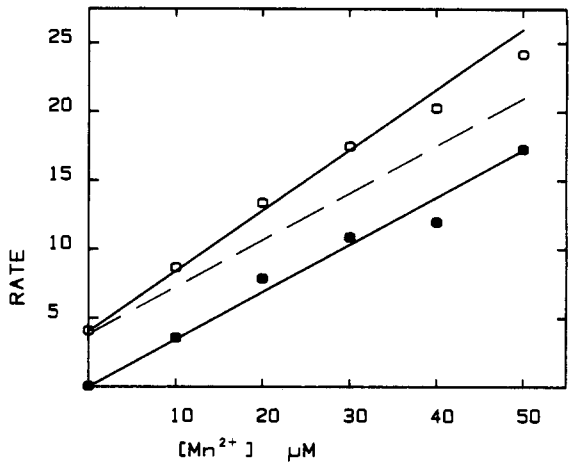


FIGURE 4: Synergistic activity upon addition of Mn^{2+} and Mg^{2+} together. Experimental conditions: 0.10 mM ATP, 2.0 mM aspartate, 2.0 mM citrulline, and 10 units/mL pyrophosphatase. Closed circles: no Mg^{2+} added. Open circles: 0.10 mM Mg^{2+}.

Table II: Substrate Activities of ATP Analogues[a]

compd	inhibition constant[b] (μM)	Mg^{2+}	Mn^{2+}	Cd^{2+}	Zn^{2+}
ATP		100	49	12	14
ATPγS	360	4.5	6.5		
(R_P)-ATPβS	71	0.35	0.08	nd[c]	0.01
(S_P)-ATPβS	160	nd[c]	0.14	0.03	0.01
(R_P)-ATPαS	140	0.02	0.02		
(S_P)-ATPαS	56	0.06	0.06		
AMP-PNP	57				
AMP-PCP	340				

[a] Relative activity at 1.00 mM nucleotide, pH 8.00, 2.0 aspartate, 2.0 mM citrulline, and 10 mM Mg^{2+} or 1.0 mM Mn^{2+}, Cd^{2+}, or Zn^{2+}. [b] From a fit of the data to eq 2. The divalent cation was 10 mM Mg^{2+}. [c] nd, not detectable (<0.01).

measurably greater than the sum of the activities in the presence of Mn^{2+} and Mg^{2+} added separately (dashed line).

Substrate Specificity. The substrate activities of ATP analogues that have sulfur substituted for oxygen at various positions in the polyphosphate region are listed in Table II. These substrate analogues were tested for substrate activity with Mg^{2+}, Mn^{2+}, Zn^{2+}, and Cd^{2+} as the activating cation. These five compounds, as well as AMP-PNP and AMP-PCP, were also tested as inhibitors vs. ATP. All seven compounds were found to be competitive inhibitors vs. ATP. The K_{is} values ranged from 56 μM to 360 μM and are tabulated in Table II. The diastereomeric pair of compounds substituted at the β-P with sulfur show a reversal of activity with Mg^{2+} and Cd^{2+}. The R_P isomer is a substrate with Mg^{2+}, but no activity (<0.01%) could be detected with the S_P isomer. The opposite result is obtained with activation by Cd^{2+}. The analogues containing sulfur at the α-P are very poor substrates and show only limited selectivity with regard to either the S_P or R_P isomer.

Discussion

Activation by Divalent Cations. Argininosuccinate synthetase from bovine liver is activated by divalent cations. Undoubtably, one of the roles for divalent cations in this reaction is in the complexation of the ATP. Other studies have previously shown that binding of metals to ATP facilitates nucleophilic attack at the phosphorus centers (Walsh, 1978). In addition to the metal ion required for nucleotide complexation, argininosuccinate synthetase apparently also requires an additional metal ion for maximal activity. This conclusion

is based on the results presented in Figures 2–4. In the titration of metal ion at a fixed concentration of ATP (Figure 2) saturation with Mg^{2+} is not reached until the metal ion concentration is 2.5 times the total ATP concentration. Assuming a dissociation constant of 26 μM for MgATP (Sillen & Martell, 1971), the ATP should have been >90% complexed at 1.1 mM $[Mg^{2+}]_{total}$ when the $[ATP]_{total}$ = 1.0 mM. However, saturation is not obtained until the $[Mg^{2+}]_{total}$ = 2.5 mM. This suggests that an additional site on the enzyme must bind the uncomplexed Mg^{2+} for maximal activity. Since maximal activity is reached with Mn^{2+} at a concentration equal with the ATP concentration, it appears that Mn^{2+} binds to this enzyme site somewhat tighter than does Mg^{2+}.

The need for an additional metal ion that can bind to the enzyme is confirmed by the double-reciprocal plot of $[Mg^{2+}]_{free}$ vs. [MgATP]. Both the K_m for MgATP and the V_{max} of the forward reaction are dependent on the concentration of uncomplexed Mg^{2+}. The initial velocity pattern for this dependence on $[Mg^{2+}]_{free}$ is intersecting. This rules out an equilibrium-ordered mechanism in which Mg^{2+} must bind before MgATP. The addition of Mg^{2+} and MgATP to the enzyme is probably random, but this has not as yet been proven.

The requirement for an uncomplexed divalent cation is further demonstrated by the titration in Figure 4. Titration of Mn^{2+} at low levels of ATP (100 μM) produces a linear increase in activity up to at least 50 μM Mn^{2+}. When this titration is repeated in the presence of 100 μM Mg^{2+}, a linear increase in activity is also observed with increasing Mn^{2+}. However, the resultant activity in the presence of Mg^{2+} and Mn^{2+} together is measurably greater than if the activity with Mg^{2+} alone is added to the rate when only Mn^{2+} is present. This synergistic effect by Mg^{2+} and Mn^{2+} is the result of tighter binding of Mn^{2+} at the site for uncomplexed metal.

The function of this site for uncomplexed divalent metal cation is as yet unknown. It may play just a structural role in the maintenance of the protein architecture. Alternatively, this divalent cation may be intimately involved in the reaction mechanism. The need for an additional divalent cation in reactions utilizing ATP is not new. It has previously been demonstrated that glutamate synthetase (Hunt et al., 1975), carbamyl phosphate synthetase (Raushel et al., 1979), PEP carboxykinase (Foster et al., 1967), pyruvate kinase (Gupta et al., 1976), and a few other enzymes also have an absolute requirement for a second metal ion site. The function of the uncomplexed metal ion in these enzyme-catalyzed reactions is also unknown.

Stereochemistry of Metal–ATP Complex. The five possible thio analogues of ATP were tested as inhibitors and substrates in the argininosuccinate synthetase reaction in order to define the stereochemical configuration of the metal–nucleotide complex that is active with this enzyme. The sulfur-substituted ATP analogues were found to be generally poor substrates for argininosuccinate synthetase. The best activity was obtained with ATPγS and the worst activity with the isomers of ATPαS. Thus, as the site of the sulfur substitution approaches the focal point of the reaction, the activity drops dramatically. The diminution of reactivity is not due to a lack of binding since all five compounds were found to be good competitive inhibitors vs. MgATP. The observed range of inhibition constants (56–360 μM) is only slightly different than the K_i of 180 μM for the dissociation of MgATP from the enzyme–nucleotide complex (Raushel & Seiglie, 1983).

The diastereomeric pair of compounds that have sulfur substituted at the β-P show a reversal of reactivity when Mg^{2+} and Cd^{2+} are used as the divalent cation. The R_P isomer is active with Mg^{2+} while only the S_P isomer is active with Cd^{2+}. Jaffe and Cohn (1978) have shown that this reversal in specificity is due to the greater affinity of Mg^{2+} for oxygen ligands and the preference of Cd^{2+} for sulfur ligands. The stereochemical configuration of the active metal–nucleotide complex, however, is identical. Thus, argininosuccinate synthetase prefers the β,γ-Λ configuration of the bidentate complex of divalent cation and ATP (Eckstein, 1985). Mn^{2+} shows no such selectivity since Mn^{2+} can bind to either oxygen or sulfur ligands.

The R_P and S_P isomers of ATPαS are very poor substrates and do not show any significantly different reactivity when either Mg^{2+} or Mn^{2+} are utilized as the activating divalent cation. Moreover, less than 0.01% activity could be detected with either Zn^{2+} or Cd^{2+}. This lack of discrimination between either isomer of the α analogues could suggest that the metal ion is not coordinated to the ATP at the active site (at least during the rate-limiting step). Alternatively, the protein could stereospecifically be coordinating one of the prochiral oxygens of the α-P of ATP. If the preference for this interaction is also greater for oxygen than for sulfur, then the Mg^{2+} could be forced to coordinate to the sulfur, and thus no preference for either diastereomer would be detected. We attempted to determine the stereochemistry of the ATPαS that would be synthesized from AMPαS in the back reaction. However, we were unable to detect any significant formation of either ATP or ATPαS when AMPαS was substituted for AMP even at pH 6.0.

Stereochemical Course of the Argininosuccinate Synthetase Reaction. The stereochemical course of the argininosuccinate synthetase reaction has been determined by the use of ATP chirally labeled with the stable isotopes of oxygen. The results indicate that the AMP produced from the chiral ATP is formed with an inversion of configuration at phosphorus. This is consistent with a single or odd number of displacement reactions at the α-P (Knowles, 1980). Therefore, these results rule out a mechanism in which the enzyme becomes adenylylated prior to the formation of citrulline adenylate. Thus, the steady-state, rapid-quench, and stereochemical experiments are in complete agreement.

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Isotopic Probes of the Argininosuccinate Lyase Reaction†

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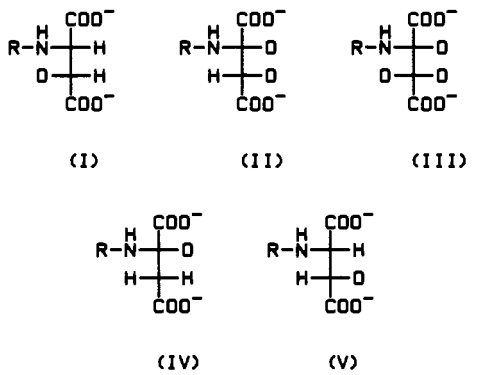
ABSTRACT: The mechanism of the argininosuccinate lyase reaction has been probed by the measurement of the effects of isotopic substitution at the reaction centers. A primary deuterium isotope effect of 1.0 on both V and V/K is obtained with (2*S*,3*R*)-argininosuccinate-*3-d*, while a primary ^{15}N isotope effect on V/K of 0.9964 ± 0.0003 is observed. The ^{15}N isotope effect on the equilibrium constant is 1.018 ± 0.001. The proton that is abstracted from C-3 of argininosuccinate is unable to exchange with the solvent from the enzyme–intermediate complex but is rapidly exchanged with solvent from the enzyme–fumarate–arginine complex. A deuterium solvent isotope effect of 2.0 is observed on the V_{max} of the forward reaction. These and other data have been interpreted to suggest that argininosuccinate lyase catalyzes the cleavage of argininosuccinate via a carbanion intermediate. The proton abstraction step is not rate limiting, but the inverse ^{15}N primary isotope effect and the solvent deuterium isotope effect suggest that protonation of the guanidino group and carbon–nitrogen bond cleavage of argininosuccinate are kinetically significant.

Argininsuccinate lyase catalyzes the cleavage of argininosuccinate to arginine and fumarate. The enzyme is found in the liver where it functions in the biosynthesis of urea. The enzyme from bovine liver has been shown by Lusty and Ratner (1972) to be a tetramer of four identical subunits. No external cofactor is involved, and the enzyme apparently does not require metal ions for catalytic activity.

The details of the catalytic events leading to the chemical transformation of argininosuccinate to fumarate and arginine are largely unknown. Ratner and co-workers have shown that the reaction involves the trans elimination of arginine and the *pro-R* hydrogen at C-3 of argininosuccinate (Hoberman et al., 1965). The kinetic mechanism of the reaction is random (Raushel & Nygaard, 1983), but the release of fumarate from the enzyme–arginine–fumarate complex is at least 10 times faster than the release of arginine (Raushel & Garrard, 1984; Kim & Raushel, 1986). There appears to be a carboxylate group and a histidine residue at the active site of the enzyme that can function as a general base and a general acid in the abstraction of a proton from C-3 and the donation of a proton to the departing arginine (Garrard et al., 1985).

In this paper we report on our efforts to determine the magnitude and the timing of the bond-breaking steps in the conversion of argininosuccinate to arginine and fumarate. The chemical mechanism appears to involve the initial formation of a carbanion intermediate. This proposal is based primarily on the tight binding of the nitro analogue of argininosuccinate (Raushel, 1984) and the enzymatic formation of 2-fluoroargininosuccinate from fluorofumarate (Garrard et al., 1983). The details of the chemical reaction have now been further probed by analyzing the effects of isotopic substitution at the reaction centers.

Chart I



Materials and Methods

Arginase, urease, aspartate aminotransferase, inorganic pyrophosphatase, and glutamate dehydrogenase were purchased from Sigma Chemical Co. Argininosuccinate lyase

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