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Kinetic Mechanism of Argininosuccinate Synthetase[1]

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The kinetic mechanism of bovine liver argininosuccinate synthetase has been determined at pH 7.5. The initial velocity and product and dead-end inhibition patterns are consistent with the ordered addition of MgATP, citrulline, and aspartate, followed by the ordered release of argininosuccinate, MgPP_i, and AMP. The mechanism is also in accord with the formation of citrulline-adenylate as a reactive intermediate [O. Rochovansky, and S. Ratner, (1967) *J. Biol. Chem.* **242**, 3839-3849]. No evidence was obtained for nonlinear double-reciprocal plots with any of the three substrates.

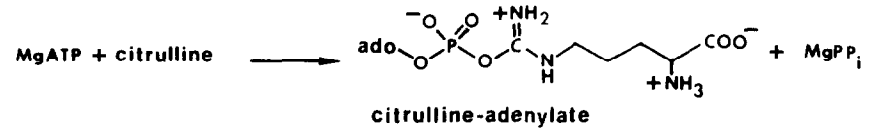
Argininosuccinate synthetase (EC 6.3.4.5) catalyzes the following reaction:

ATP + citrulline + aspartate →

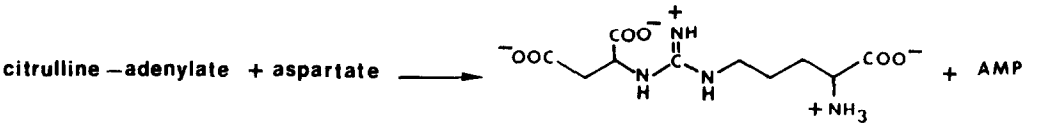
AMP + PP_i + argininosuccinate [1]

This enzyme catalyzes the rate-limiting step in the biosynthesis of urea in the liver of many ureotelic species (1). Ratner and her colleagues have extensively studied the enzyme from beef liver and have succeeded in purifying it to homogeneity (2). The enzyme from human liver has also recently been purified by O'Brien (3). The molecular weight of the beef liver enzyme is 185,000 and is composed of four apparently identical subunits (2).

Based on oxygen-18 labeling experiments and some early pulse-chase experiments, Rochovansky and Ratner (4, 5) have proposed that the function of the ATP used in the reaction is to activate citrulline to form enzyme-bound citrulline-adenylate which is subsequently attacked by aspartate to form argininosuccinate as shown in the equations



citrulline-adenylate [2]



argininosuccinate [3]

Unlike a number of other synthetases, this enzyme does not catalyze a PP_i-ATP exchange in the presence of citrulline but

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TABLE I

INITIAL-VELOCITY PATTERNS[a,b]

Variable substrates	Fixed substrate (mM)	Pattern type	Apparent K_m (μM)			Apparent K_i (μM)		
			MgATP	Citrulline	Aspartate	MgATP	Citrulline	Aspartate
MgATP vs citrulline	Aspartate, 1	Intersecting	45 ± 5	70 ± 7		180 ± 30	290 ± 50	
Citrulline vs aspartate	MgATP, 1	Intersecting		53 ± 7	36 ± 7		1400 ± 300	980 ± 150
MgATP vs aspartate	Citrulline, 0.25	Intersecting	57 ± 6		180 ± 10	80 ± 10		250 ± 50
MgATP vs aspartate	Citrulline, 2.5	Parallel	64 ± 3		57 ± 3			

[a] pH 7.5, 100 mM KCl, 10 mM excess Mg^{2+}.
[b] The double-reciprocal plots can be found in Figs. 1-6.

absence of aspartate (5). This suggests that if citrulline-adenylate is formed, the products are tightly bound and do not exchange with those in solution unless the aspartate site is occupied.

Notwithstanding the importance of this enzyme in the biosynthesis of urea, the kinetic mechanism of argininosuccinate synthetase, is, as yet, unknown. However, some preliminary inhibition and initial-velocity experiments have appeared in the literature (2, 5). As a prelude to establishing the kinetic competence of citrulline-adenylate and the overall chemical mechanism of this enzyme, we have determined the kinetic mechanism of the enzyme using initial velocity and product and dead-end inhibition experiments. The results indicate that the reaction occurs by the ordered addition of ATP, citrulline, and aspartate followed by the ordered release of argininosuccinate, PP_i, and AMP. This mechanism is fully compatible with the two-step mechanism as suggested by Ratner.

MATERIALS AND METHODS

Argininosuccinate synthetase was isolated from beef liver according to the method of Rochovansky *et al.* (2) to a specific activity of 1 μmol/min/mg at 25°C. The buffers used in the purification contained 0.1 mM EDTA and 1.0 mM dithiothreitol. All other compounds and enzymes were obtained from Sigma, Boehringer, or Aldrich.

Enzyme assays. Argininosuccinate synthetase activity was measured spectrophotometrically using an adenylate kinase-pyruvate kinase-lactate dehydrogenase coupling system. A Gilford 260 spectrophotometer and a Linear 10-mV recorder were used to follow the reaction at 340 nm. For activity measurements of the forward reaction in the absence of added AMP, each 3.0-ml cuvette contained 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes),[3] pH 7.5, 33 μg each of salt-free lactate dehydrogenase and pyruvate kinase, 5 units of adenylate kinase, 5 units of inorganic pyrophosphatase, 1 mM phosphoenolpyruvate, 0.2 mM NADH, 100 mM KCl, 10 mM $MgCl_2$, and various amounts of the different substrates and inhibitors. The 10 mM Mg^{2+} was required for maximal activity of argininosuccinate synthetase. No inhibition of argininosuccinate synthetase by Mg^{2+} was observed at concentrations up to at least 20 mM.

Activity measurements in the presence of added AMP were made by monitoring the production of PP_i with inorganic pyrophosphatase (6). Each 3-ml cuvette contained 50 mM Hepes, pH 7.5, 100 mM KCl, 5 units of pyrophosphatase, 5 mg glycogen, 0.5 mM NADP, 5 units of phosphorylase *a*, 5 units of glucose-6-phosphate dehydrogenase, 5 units of phosphoglucomutase, 10 mM $MgCl_2$, 15 μM Ap_5A[P^1,P^5-di(adenosine-5') pentaphosphate], and various amounts of substrates and inhibitors. The Ap_5A was added to inhibit any adenylate kinase that may have been present in the coupling-enzyme preparations (7). Ap_5A does not inhibit argininosuccinate synthetase. All assays were conducted at either 25 or 30°C and were initiated by the addition of argininosuccinate synthetase (0.02–0.1 unit) with the aid of an adder-mixer.

Data analysis. The kinetic data were analyzed using the Fortran programs of Cleland (8). Intersecting and parallel initial-velocity data were fitted to Eqs. [4] and [5], respectively. Competitive, uncompetitive, and noncompetitive inhibition experiments were fitted to

[3] Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ap_5A, P^1,P^5-di(adenosine-5')pentaphosphate.

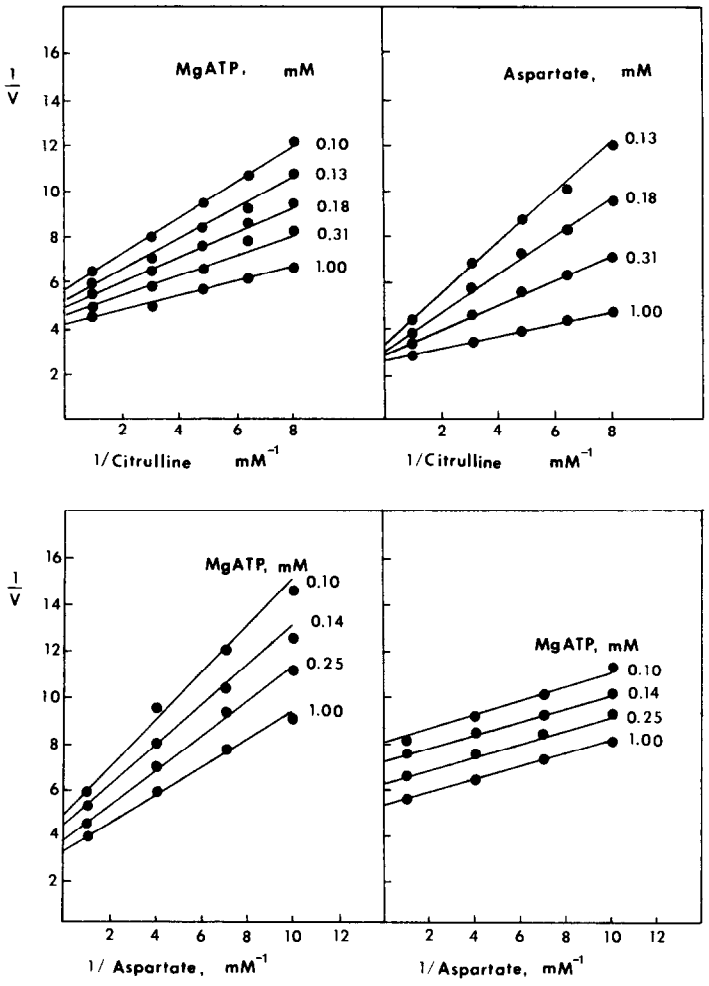

FIG. 1. Initial-velocity double-reciprocal plots.

Eqs. [6], [7], and [8], respectively. When the type of pattern was in doubt (parallel or intersecting), both possible fits were tried and a comparison of the σ values (square root of residual least-squares) was made to determine the best fit. In no case did a set of data fit both patterns equally well. The nomenclature used in this paper is that of Cleland (9).

$$v = \frac{VAB}{K_{ia}K_b + K_bA + K_aB + AB} \quad [4]$$

$$v = \frac{VAB}{K_bA + K_aB + AB} \quad [5]$$

$$v = \frac{VA}{K(1 + (I/K_{is})) + A} \quad [6]$$

$$v = \frac{VA}{K + A(1 + (I/K_{ii}))} \quad [7]$$

$$v = \frac{VA}{K(1 + (I/K_{is})) + A(1 + (I/K_{ii}))} \quad [8]$$

RESULTS

Initial-velocity patterns—forward reaction. The initial-velocity results from fits of the data to Eqs. [4] and [5] for the forward reaction of argininosuccinate synthetase appear in Table I. Citrulline gives intersecting patterns vs either MgATP or aspartate at a concentration of the nonvaried substrate of 1.0 mM. The MgATP vs aspartate initial-velocity pattern is intersecting at low concentrations of citrulline but becomes parallel at 2.5 mM citrulline ($40 \times K_m$). Over the concentration range

of 0.1–1.0 mM, there was no indication of nonlinearity in any of the double-reciprocal plots of aspartate, citrulline, or MgATP (Fig. 1).

Product inhibition. Product inhibition data from the forward reaction are shown in Table II from fits to Eqs. [6]–[8]. PP_i is uncompetitive vs aspartate or citrulline but noncompetitive vs MgATP. Argininosuccinate is noncompetitive vs MgATP, aspartate, and citrulline. The AMP-vs-MgATP pattern is competitive. The inhibition of AMP vs citrulline or aspartate is noncompetitive (Figs. 2–4).

Dead-end inhibition. Arginine and α-methyl-DL-aspartate were used as dead-end inhibitors of the forward reaction. As expected, arginine is competitive vs citrulline, and α-methyl-DL-aspartate is competitive vs aspartate. Rochovansky and Ratner (5) have previously shown that α-methyl-DL-aspartate is not a substrate for argininosuccinate synthetase. Arginine is noncompetitive vs aspartate and uncompetitive vs MgATP. Uncompetitive inhibition patterns are obtained vs either MgATP or citrulline by α-methyl-DL-aspartate. The kinetic constants from fits of

TABLE II

PRODUCT AND DEAD-END INHIBITION PATTERNS[a,b]

Variable substrate	Inhibitor	Fixed substrates (mM)	Inhibition pattern[c]	K_{ii} (mM)	K_{is} (mM)
MgATP	AMP	Citrulline, 1.0 Aspartate, 1.0	C	0.47 ± 0.04	
Citrulline	AMP	MgATP, 0.25 Aspartate, 1.0	NC	3.2 ± 0.5	1.0 ± .3
Aspartate	AMP	MgATP, 0.25 Citrulline, 0.25	NC	0.82 ± 0.13	0.47 ± .10
MgATP	PP_i	Citrulline, 0.25 Aspartate, 0.25	NC	0.67 ± .10	0.25 ± 0.7
Citrulline	PP_i	MgATP, 2.5 Aspartate, 0.25	UC	0.53 ± 0.04	
Aspartate	PP_i	MgATP, 2.5 Citrulline, 0.25	UC	0.53 ± 0.03	
MgATP	Argininosuccinate	Citrulline, 1.0 Aspartate, 1.0	NC	4.0 ± 1.0	0.11 ± 0.01
Citrulline	Argininosuccinate	MgATP, 1.0 Aspartate, 1.0	NC	1.1 ± 0.2	0.13 ± .01
Aspartate	Argininosuccinate	MgATP, 1.0 Citrulline, 1.0	NC	3.1 ± 0.6	0.21 ± 0.01
MgATP	Arginine	Citrulline, 0.25 Aspartate, 1.0	UC	1.9 ± 0.1	
Citrulline	Arginine	MgATP, 1.0 Aspartate, 1.0	C		.33 ± 0.03
Aspartate	Arginine	MgATP, 1.0 Citrulline, 0.25	NC	2.6 ± 0.5	0.46 ± 0.07
MgATP	α-Methylaspartate	Citrulline, 1.0 Aspartate, 0.25	UC	6.4 ± .4	
Citrulline	α-Methylaspartate	MgATP, 1.0 Aspartate, 0.25	UC	8.0 ± 0.3	
Aspartate	α-Methylaspartate	MgATP Citrulline	C		1.7 ± 0.1

[a] At pH 7.5, 100 mM KCl, 10 mM excess Mg^{2+}.

[b] The double-reciprocal plots can be found in Figs. 1–6.

[c] C, competitive, UC, uncompetitive; NC, noncompetitive.

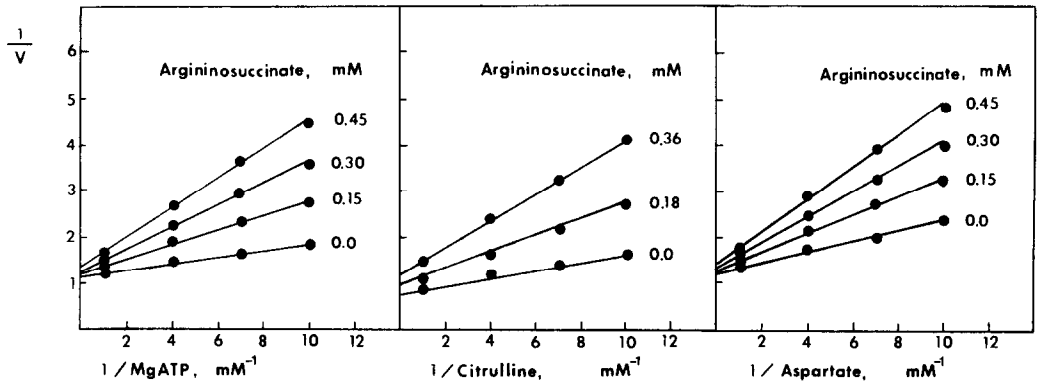

FIG. 2. Product inhibition by arginosuccinate.

the data to Eqs. [6]–[8] appear in Table II and (Figs. 5 and 6).

DISCUSSION

The double-reciprocal plots for all three substrates in the forward reaction of argininosuccinate synthetase were found to be linear over the concentration range used in this study. This result is at variance with the report by Rochovansky *et al.* (2). They found significant deviations from linearity in the double-reciprocal plots for all three substrates, although O'Brien has recently found *linear* double-reciprocal plots with the enzyme from human liver (3). The reason for this difference is not clear but it does not affect the conclusions concerning the kinetic mechanism of this enzyme.

Kinetic mechanism. The following kinetic mechanism is consistent with all of the initial-velocity and inhibition data of argininosuccinate synthetase.

MgATP	citrulline	aspartate	argininosuccinate	$MgPP_i$	AMP
↓	↓	↓	↑	↑	↑

A full rate equation for this mechanism can be found in Segal (10).

The reasoning used to arrive at the above mechanism is as follows (9):

1. The initial-velocity patterns for MgATP vs citrulline and citrulline vs aspartate are intersecting. The MgATP-vs-

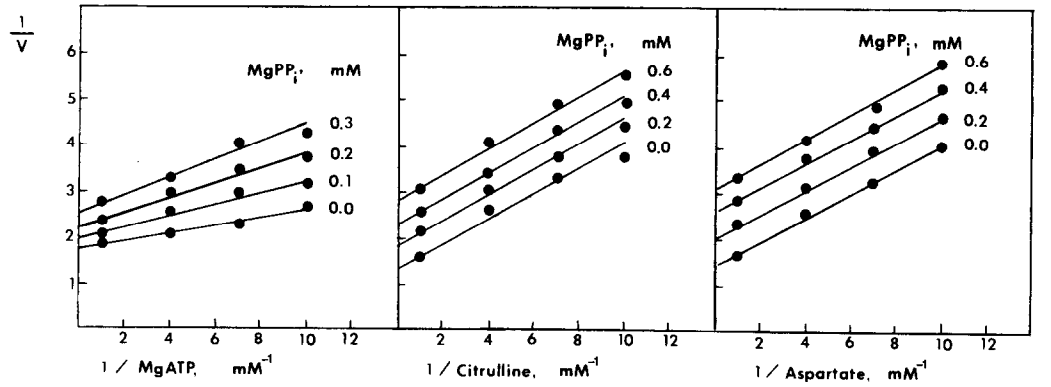

FIG. 3. Product inhibition by pyrophosphate.

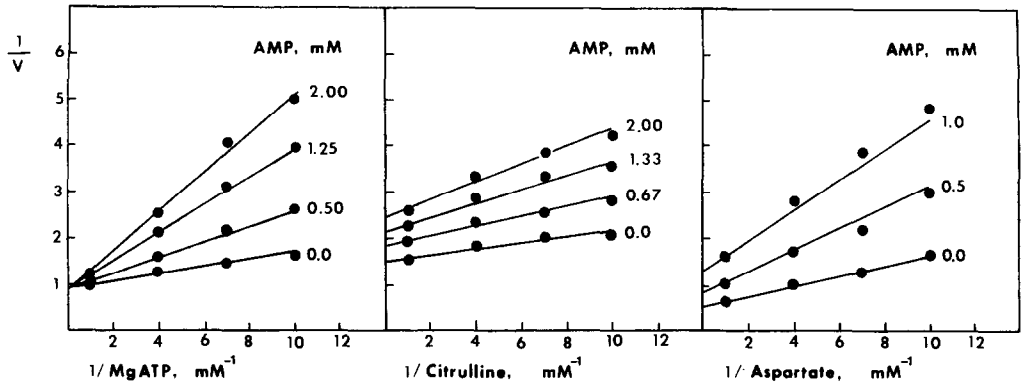

FIG. 4. Product inhibition by AMP.

aspartate pattern is intersecting at low citrulline but becomes parallel at saturating citrulline. These results demonstrate that no products are released before all of the substrates have added to the enzyme. These data also show that citrulline binds between MgATP and aspartate.

2. The dead-end inhibitor used to mimic aspartate, α-methyl-DL-aspartate, is competitive vs aspartate but uncompetitive vs the other two substrates. This indicates that aspartate binds only after the addition of MgATP and citrulline.

3. Arginine, the dead-end inhibitor used to mimic citrulline, is competitive vs citrulline, uncompetitive vs MgATP, and noncompetitive vs aspartate. The results indicate that citrulline binds before aspartate but after MgATP. The initial-velocity and dead-end inhibition data are therefore consistent with the ordered addition of MgATP, citrulline, and aspartate.

4. MgPP_i is uncompetitive vs aspartate and citrulline while noncompetitive vs MgATP. The two uncompetitive patterns suggest that there is a product released immediately before and after the release of MgPP_i. These results would suggest that the inhibition by PP_i vs MgATP should also be uncompetitive. The noncompetitive pattern can be rationalized by assuming that MgPP_i also has some affinity for free enzyme. Therefore a slope effect will be observed from the competition for free enzyme with MgATP and an intercept effect will arise due to binding with E-AMP.

5. The inhibition by argininosuccinate is noncompetitive vs MgATP, citrulline, and aspartate. Since MgATP is the first substrate to add in the reaction sequence,

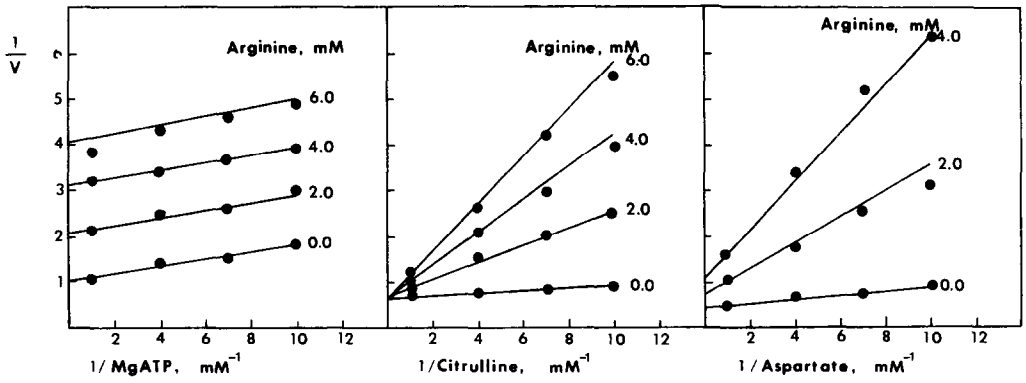

FIG. 5. Dead-end inhibition by arginine.

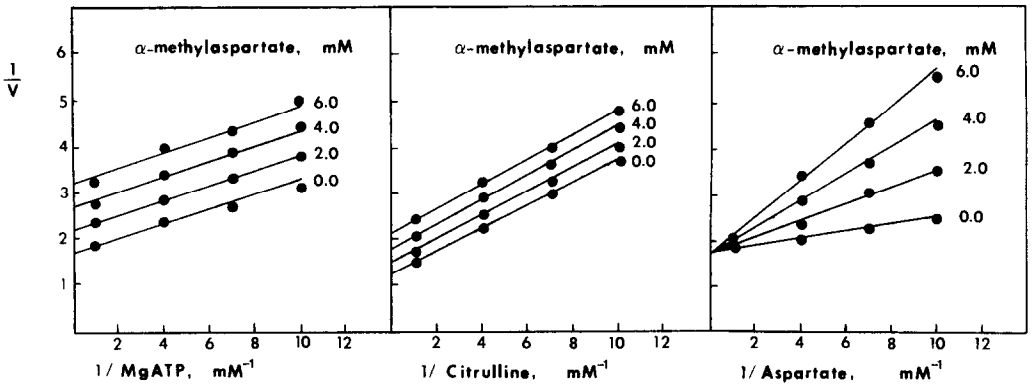

FIG. 6. Dead-end inhibition by α-methylaspartate.

this result is consistent with argininosuccinate as the first product to leave the enzyme.

6. AMP is competitive vs ATP, showing that these compounds compete for the same enzyme form and confirming the order of release from the enzyme as argininosuccinate, PP_i, and finally AMP.

The proposed kinetic mechanism is consistent with the intermediate formation of citrulline-adenylate as proposed by Rochovansky and Ratner (4, 5). However, it cannot be determined whether the binding of aspartate to the enzyme facilitates or promotes the formation of citrulline-adenylate through a substrate-induced conformational change. The lack of a PP_i-ATP exchange in the absence of aspartate is also consistent with the above mechanism since PP_i is not released until after argininosuccinate has left the enzyme. This would indicate that PP_i and citrulline-adenylate are tightly bound to the enzyme and do not exchange with those compounds in solution. A similar situation also occurs with glutamine synthetase from *Escherichia coli* (11). ATP and glutamine bind to the enzyme and react to form γ-glutamyl-phosphate and ADP (12). These compounds are also tightly bound and not readily exchangeable. Rapid-quench and/or positional isotope-exchange (PIX) experiments (13) will be needed to assess the kinetic competence of citrulline adenylate in the argininosuccinate synthetase reaction.

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