# Kinetic Mechanism of Bovine Liver Argininosuccinate Lyase<sup>1</sup>

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The kinetic mechanism of bovine liver argininosuccinate lyase has been determined at pH 7.5, 25°C. Fumarate and arginine are both noncompetitive inhibitors versus argininosuccinate. The dead-end inhibitor, succinate, is competitive versus fumarate and argininosuccinate, but noncompetitive versus arginine. Citrulline is competitive versus arginine and noncompetitive versus argininosuccinate and fumarate. The results are consistent with a random mechanism with the formation of two dead-end complexes:  $E \cdot argininosuccinate \cdot fumarate and E \cdot argininosuccinate \cdot arginine. No evidence$ was obtained for nonlinear reciprocal plots. The equilibrium constant was found to be<math>3.7 mM.

Argininosuccinate lyase (EC 4.3.2.1) catalyzes the following reaction:



The enzyme from beef liver has been extensively studied by Ratner and her colleagues (1-4). They have purified the enzyme to apparent homogeneity and have shown the protein to be a tetramer of molecular weight 202,000 (2). The enzyme is cold-labile and is protected from cold inactivation by the substrates or 50 mM phosphate buffer (2). Although this protein is a key enzyme in the metabolism of nitrogen via the urea cycle, a complete analysis of the kinetics of the reaction catalyzed by argininosuccinate lyase has not appeared in the literature. Some preliminary inhibition experiments have been reported but no details have appeared (5, 6).

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We have therefore initiated a complete steady-state initial velocity and product inhibition study of argininosuccinate lyase and have established that the kinetic mechanism is random.

### MATERIALS AND METHODS

Argininosuccinate lyase was isolated from beef liver according to the procedure of Havir *et al.* (2) and Schulze *et al.* (7) through the DEAE-cellulose step to a specific activity of 4.0  $\mu$ mol/min/mg at 25°C. All buffers used in the purification contained 0.1 mM EDTA and 1.0 mM dithiothreitol. Argininosuccinate was obtained from Sigma as its barium salt and was converted to the potassium salt and assayed as described by Havir *et al.* (2). All other reagents were obtained from either Sigma or Aldrich.

Enzyme assays. Argininosuccinate lyase activity was assayed in the forward or reverse direction by monitoring the appearance or disappearance of fumarate at 240 nm with a Gilford 260 spectrophotometer and a 10 mV Linear recorder. All assays were

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FIG. 1. Variation of activity with argininosuccinate concentration. Conditions: 50 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid, pH 7.5, 100 mM KCl, 25°C. The velocities are in arbitrary units.

done at 25°C. Each 3-ml cuvette contained 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.5, 100 mM KCl, and substrates and inhibitors as indicated in the individual experiments. All reactions were initiated by the addition of argininosuccinate lyase (0.05-0.15 units) with the aid of an adder-mixer.

Data analysis. Reciprocal velocities were plotted graphically against reciprocals of substrate concentration and the data were fitted to Eq. [2], assuming equal variances for the velocities and using the Fortran programs of Cleland (8). Data for a sequential initial velocity pattern were fitted to Eq. [3], for competitive inhibition to Eq. [4], for noncompetitive inhibition to Eq. [5] and for uncompetitive inhibition to Eq. [6]. The nomenclature used in this paper is that of Cleland (9)

$$v = \frac{VA}{K+A}$$
[2]

$$v = \frac{VAB}{K_{ia}K_b + K_bA + K_aB + AB}$$
[3]

$$v = \frac{VA}{K(1 + (I/K_{is})) + A}$$
[4]

$$v = \frac{VA}{K(1 + (I/K_{is})) + A(1 + I/K_{ii}))}$$
[5]

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

## RESULTS

Variation of argininosuccinate. The variation of activity with argininosuccinate concentration is shown in Fig. 1. As can be seen from the data, the double reciprocal plot is linear from 30  $\mu$ M to 1.0 mM argininosuccinate. The Michaelis constant from a fit to Eq. [2] is  $51 \pm 5 \mu M$ .

Initial velocity patterns. When arginine is varied at changing fixed concentrations of fumarate an intersecting double reciprocal plot is obtained. The kinetic constants from a fit of the data to Eq. [3] are shown in Table I. There is no indication in any of these experiments for nonlinear reciprocal plots. The ratio of maximal velocities for the forward and reverse reactions at pH 7.5 is 0.6.

Product and dead-end inhibition. In the forward reaction both fumarate and arginine were found to be linear noncompetitive inhibitors versus argininosuccinate. Succinate was found to be a competitive inhibitor versus fumarate and argininosuccinate but a noncompetitive inhibitor versus arginine. Citrulline was competitive versus arginine and noncompetitive versus fumarate and argininosuccinate. The kinetic constants from fits of the inhibition data to Eqs. [4] and [5] appear in Table II.

Equilibrium constant. The equilibrium constant for the reaction catalyzed by argininosuccinate lyase was determined at pH 7.5 by making up reaction mixtures that were initially 0.470 mM in fumarate and variable in arginine (2.5-5.0 mM). Enzyme was added and the  $A_{240}$  was monitored until the reaction was complete. The final concentrations of the substrates and products were calculated from the  $\Delta A_{240}$  using a millimolar extinction coefficient of 2.44 for fumarate (9). The equilibrium constant, defined as:

#### TABLE I

KINETIC CONSTANTS FROM INITIAL VELOCITY EXPERIMENTS<sup>a</sup>

| Substrate         | К (тм)            | <i>K</i> i (тм) | Rel V |  |
|-------------------|-------------------|-----------------|-------|--|
| Argininosuccinate | $0.051 \pm 0.005$ |                 | 100   |  |
| Fumarate          | $0.06 \pm 0.01$   | $0.65 \pm 0.1$  | 150   |  |
| L-Arginine        | $0.28 \pm 0.04$   | $2.9 \pm 0.6$   | 170   |  |

 $^{\rm a}$  From fits to Eqs. [2] and [3] of the data at pH 7.5, 25°C, 100 mM KCl.

| Inhibitor  | Variable<br>substrate | Fixed<br>substrate (mm) | <i>К</i> <sub>ів</sub> (тм) | <i>K</i> <sub>ii</sub> (mм) | Inhibition |  |  |
|------------|-----------------------|-------------------------|-----------------------------|-----------------------------|------------|--|--|
| L-Arginine | Argininosuccinate     | -                       | $8.0 \pm 1.5$               | $20 \pm 2$                  | NC         |  |  |
| Fumarate   | Argininosuccinate     | _                       | $0.47 \pm 0.09$             | $0.90 \pm 0.06$             | NC         |  |  |
| Citrulline | Argininosuccinate     | -                       | $39 \pm 3$                  | $640 \pm 60$                | NC         |  |  |
| Succinate  | Argininosuccinate     | _                       | 83 ± 5                      | -                           | С          |  |  |
| Succinate  | Fumarate              | Arginine, 0.75          | $34 \pm 2$                  | -                           | С          |  |  |
| Succinate  | Arginine              | Fumarate, 0.50          | $54 \pm 6$                  | $195 \pm 33$                | NC         |  |  |
| Citrulline | Arginine              | Fumarate, 0.50          | $3.9 \pm 0.2$               |                             | С          |  |  |
| Citrulline | Fumarate              | Arginine, 0.75          | $14 \pm 4$                  | 9 ± 1                       | NC         |  |  |

TABLE II

PRODUCT AND DEAD-END INHIBITION CONSTANTS<sup>a,b</sup>

<sup>a</sup> From fits to Eqs. [4], [5], and [6] of the data at pH 7.5, 25°C. 100 mM KCl.

<sup>b</sup> The double reciprocal plots can be found in the Miniprint Supplement.

is  $3.7 \pm 0.1$  mM from an average of six determinations.

#### DISCUSSION

Argininosuccinate lyase from beef liver has previously been reported to have nonlinear double reciprocal plots for the cleavage of argininosuccinate to arginine and fumarate (4). With our reaction conditions we have obtained linear plots in all cases for all substrates. The reason for this difference is not clear but the purified enzyme from human liver has recently been shown in two independent reports to exhibit normal Michaelis-Menten kinetics (11, 12). The equilibrium constant for argininosuccinate lyase of 3.7 mM is in good agreement with the value of 3.22 mm as determined by Kuchel et al. (6) but is not consistent with the value of 11.4 mm at 38°C as reported by Ratner (5).

The inhibition experiments with the product and dead-end inhibitors are consistent with a random Uni-Bi kinetic mechanism for bovine liver argininosuccinate lyase. It has previously been shown that compounds that resemble the substrates in structure but do not chemically react can be used to establish the order of addition and release of substrates from the active sites of enzymes (13). In this study succinate has been used as a dead-end inhibitor for fumarate and citrulline as a dead-end inhibitor for arginine. Both of these compounds gave the expected competitive inhibition patterns versus the compounds they were expected to mimic. Succinate and citrulline were also found to be noncompetitive inhibitors versus arginine and fumarate, respectively. This establishes that the order of addition of fumarate and arginine to the enzyme must be random since if there was an obligatory order of addition to the enzyme, one of these inhibition patterns would have been uncompetitive. For example, if arginine added before fumarate then succinate would be expected to be uncompetitive versus arginine.

If the addition or release of arginine and fumarate from the enzyme was completely random then both of these compounds would be expected to be competitive inhibitors versus argininosuccinate since all substrates and products would be able to combine with free enzyme. However, both of these compounds are noncompetitive inhibitors versus argininosuccinate. This indicates that two dead-end complexes are able to form: E. argininosuccinate. arginine and E. argininosuccinate fuarate. The dead-end complex, E · argininouccinate · citrulline, also forms as indicated by the noncompetitive inhibition of citrulline with argininosuccinate as the variable substrate, although the  $K_{ii}$  is greater than 10 times the  $K_{is}$ . Since succinate is a competitive inhibitor versus argininosuccinate, a dead-end complex with

argininosuccinate and succinate is apparently unable to form. The active site of argininosuccinate lyase is thus able to accommodate the binding of argininosuccinate and one of the two products at the same time. This situation is commonly seen with kinases in which both ATP and the phosphorylated product are able to bind at the active site at the same time and thus give noncompetitive inhibition patterns (13, 14).

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Figure 1A: Double reciprocal plots for the initial velocity and product inhibition data that are presented in Tables I and II. Additional details are given in the text. Velocities are in arbitrary units. A: fumarate vs. arginine (a,b,c,d) and e are 1,0,0.31,0.18,0.13 and 0.10 mM fumarate, respectively) B: arginine vs. argininosuccinate (a,b,c) and d are 0,0.3,0.6, and 9.0 mM arginine, respectively) C: fumarate vs. argininosuccinate (a,b,c) and d are 0,0.3,0.6, and 0.9 mM arginine, respectively) C: fumarate vs. argininosuccinate (a,b,c) and d are 0,0.3,0.6, and 0.9 mM fumarate, respectively) D: citrulline vs. argininosuccinate (a,b,c) and d are 0,0.3,0.6, and 0.9 mM fumarate, respectively) D: citrulline vs. argininosuccinate (a,b,c) and d are 0,30,60 and 90 mM succinate, respectively) F: succinate vs. fumarate (a,b,c) and d are 0,30,60 and 90 mM succinate, respectively) F: citrulline vs. arginine (a,b,c) and d are 0,30,60 and 90 mM succinate, respectively) F: citrulline vs. arginine (a,b,c) and d are 0,30,60 and 90 mM succinate, respectively) F: citrulline vs. arginine (a,b,c) and f are 0,2.0,4.0,5.0,8.0 and 10.0 mM citrulline, respectively) F: citrulline vs. fumarate (a,b,c,d) and f are 0,2.5,5.0,7.5 and 10.0 mM citrulline, respectively) F: citrulline vs. fumarate (a,b,c,d) and e are 0,2.5,5.0,7.5 and 10.0 mM citrulline, respectively)