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Nitro Analogs of Substrates for Argininosuccinate Synthetase and Argininosuccinate Lyase[1]

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The nitro analogs of aspartate and argininosuccinate were synthesized and tested as substrates and inhibitors of argininosuccinate synthetase and argininosuccinate lyase, respectively. The V_{max} for 3-nitro-2-aminopropionic acid was found to be 60% of the maximal rate of aspartate utilization in the reaction catalyzed by argininosuccinate synthetase. Only the nitronate form of this substrate, in which the C-3 hydrogen is ionized, was substrate active, indicating a requirement for a negatively charged group at the β carbon. The V/K of the nitro analog of aspartate was 85% of the value of aspartate after correcting for the percentage of the active nitronate species. The nitro analog of argininosuccinate, N^3-(L-1-carboxy-2-nitroethyl)-L-arginine, was a strong competitive inhibitor of argininosuccinate lyase but was not a substrate. The pH dependence of the observed pK_i was consistent with the ionized carbon acid (pK = 8.2) in the nitronate configuration as the inhibitory material. The pH-independent pK_i of 2.7 μM is 20 times smaller than the K_m of argininosuccinate at pH 7.5. These results suggest that the tighter binding of the nitro analog relative to the substrate is due to the similarity in structure to a carbanionic intermediate in the reaction pathway.

It has recently been demonstrated (1–5) with a number of enzyme systems that the substitution of a nitro group for a carboxylate group produces a very good analog for carbanion intermediates in enzyme-catalyzed reactions. The deprotonated nitro compounds (pK ~ 7.5–10.5) in the nitronate configuration are isosteric with a carbanion that is α to a carboxylate in the aci form (1). To date, nitro analog inhibitors have been synthesized and tested with aspartase (1), fumarase (1), adenylosuccinate lyase (3), aconitase (2), and isocitrate lyase (4). In all cases the nitro analogs were strong competitive inhibitors versus the natural substrate, and the ratio of K_m to K_i was very large (26–72,000). These results have suggested that the reaction mechanisms of these enzymes involve the intermediate formation of a carbanionic species (E1cB mechanism).

Argininosuccinate lyase (EC 4.3.2.1.) catalyzes the cleavage of argininosuccinate (I) to arginine and fumarate. The overall reaction is thus very similar to those catalyzed by aspartase and adenylosuccinate lyase. The details of the catalytic process for argininosuccinate lyase are, as yet, unknown. Therefore, the nitro analog of argininosuccinate (III) was synthesized and tested as an inhibitor of argininosuccinate lyase. The deprotonated form of the nitro analog (IV) should be a close analog of the carbanion of argininosuccinate in the aci form (II). The interaction of the nitro analog of argininosuccinate with argininosuccinate lyase as a substrate is also of some interest. The cleavage of the nitro analog of argininosuccinate would produce 3-nitroacrylic acid, which is potentially a powerful alkylating agent. Therefore, the nitro analog of argininosuccinate will pos-

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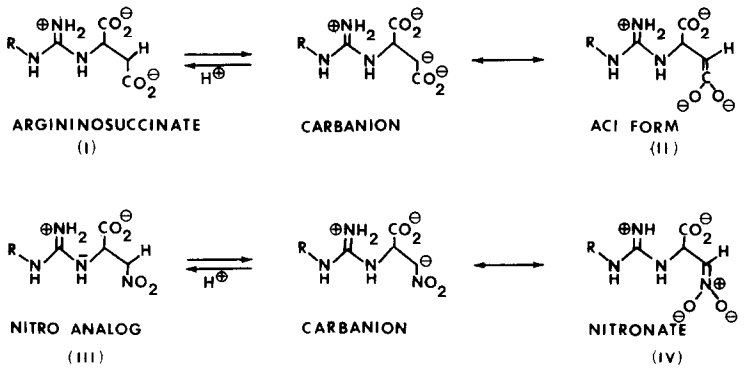

sibly be a suicide inactivator of the lyase as well.

Since a stereospecific synthesis of the nitro analog of argininosuccinate did not appear feasible, the formation of this compound was attempted enzymatically with argininosuccinate synthetase using DL-3-nitro-2-aminopropionic acid as an alternate substrate for L-aspartate. The interaction of DL-3-nitro-2-aminopropionic acid with the synthetase is also described.

EXPERIMENTAL PROCEDURES

Materials. Argininosuccinate synthetase and argininosuccinate lyase were isolated from beef liver using slightly modified procedures of Rochovansky *et al.* (6) and Schulze *et al.* (7), respectively. DL-3-Nitro-2-aminopropionic acid was a generous gift of Dr. David Porter (University of Pennsylvania) who synthesized this compound from acrylic acid (1). All other materials were obtained from either Sigma or Aldrich.

Preparation of N^3-(L-1-carboxy-2-nitroethyl)-L-arginine (III). The nitro analog of argininosuccinate was prepared by incubating 5.0 mM L-citrulline, 5.0 mM ATP, 10 mM $MgCl_2$, 10 mM DL-3-nitro-2-aminopropionic acid, 50 m*M* Pipes,[2] pH 7.0, 5 units of inorganic pyrophosphatase, and 2.5 units of argininosuccinate synthetase in a volume of 1.0 ml for 4 h at 25°C. The progress of the reaction was followed using HPLC. Aliquots were applied to a Whatman SCX cation-exchange column, eluted with 20 mM ammonium phosphate buffer, pH 2.5, and monitored at 205 nm. The observed retention times for 3-nitro-2-aminopropionic acid and the nitro analog of argininosuccinate were 3.9 and 8.2 min, respectively.

Enzyme assays. For kinetic studies argininosuccinate synthetase was assayed using a pyruvate kinase–lactate dehydrogenase–adenylate kinase coupling system (8). Each 3.0-ml cuvette contained 1.0 mM citrulline, 1.0 mM ATP, 10 mM $MgCl_2$, 10 units each of pyruvate kinase, lactate dehydrogenase, pyrophosphatase, and adenylate kinase, 1.0 mM PEP, 0.16 mM NADH, 50 mM buffer, and variable amounts of either L-aspartate or DL-3-nitro-2-nitropropionate. The buffers at pH 7.1 and 8.3 were Pipes and Taps, respectively. The change in absorbance at 340 nm was monitored with a Gilford 260 UV-VIS spectrophotometer and a Linear 255 recorder.

Argininosuccinate lyase was assayed by following the formation of fumarate at 230 nm (9). Each 3.0-ml cuvette contained 100 mM buffer, argininosuccinate (0.036–0.73 mM), and various amounts of the nitro analog of argininosuccinate. The reaction was initiated with the addition of argininosuccinate lyase with the aid of an adder-mixer. Buffers used in these studies included Mes (pH 6.25), Pipes (pH 6.75), Hepes (pH 7.25 and 7.75), Taps (pH 8.25 and 8.75), and Ches (pH 9.25).

Data processing. Values of kinetic constants were determined by fitting velocity and concentration data to the appropriate rate equation by the least-squares method using the Fortran programs of Cleland (10) that have been translated into Basic. Substrate saturation curves were fitted to

$$v = \frac{VA}{K + A}, \qquad [1]$$

and competitive inhibition data were fitted to

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

[2] Abbreviations: Pipes, 1,4-piperazinediethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Taps, 3-{[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino}-1-propanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; PEP, phosphoenolpyruvate.

where v is the experimentally determined velocity, V is the maximum velocity, A is the substrate concentration, K is the Michaelis constant, I is the inhibitor concentration, and K_{ii} is the slope inhibition constant. The K_i values of the nitro analog of argininosuccinate as a function of pH were fitted to

$$pK_i = \log \frac{C}{1 + H/K_a + K_b/H}, \quad [3]$$

where $1/C$ is the pH-independent K_i value, K_a and K_b are dissociation constants of groups that ionize, and H is the hydrogen ion concentration.

RESULTS

Argininosuccinate synthetase. The nitro analog of aspartate was an excellent alternate substrate for bovine liver argininosuccinate synthetase. The rate of this reaction was determined by spectrophotometrically following the formation of AMP in a coupled enzyme system. The progress of the reaction was also observed by monitoring the formation of the nitro analog of argininosuccinate via HPLC. The kinetic constants from fits of the data to Eq. [1] for the reaction of DL-3-nitro-2-aminopropionic acid catalyzed by argininosuccinate synthetase at pH 7.1 and 8.3 are shown in Table I. The observed constants for aspartate are also shown for comparison.

It has been previously shown that argininosuccinate synthetase is specific for the L configuration of aspartate (11). However, since only a racemic mixture of the nitro analog of aspartate was available to us, we tested the DL-mixture in an attempt to show that only 50% of this material was converted into product. When argininosuccinate synthetase was added to a solution containing 1.0 mM ATP, 1.0 mM citrulline, 83 μM DL-3-nitro-2-aminopropionate, excess pyrophosphatase, and the reaction allowed to go to completion, the final concentration of AMP was 42 μM. This indicates that the synthetase is specific for only one of the stereoisomers and, by analogy with the results for aspartate, it is assumed that it is the L configuration that is active.

To determine whether the synthetase was specific for the carbanion form of the nitro analog of aspartate, the enzyme was added to a reaction mixture in which the nitro analog was present predominantly as the nitronate form, and also when the nitro analog was fully protonated. The time courses of these reactions were then compared with an equilibrated system at pH 7.1. This experiment is feasible because Porter and Bright (1) have previously shown that the $t_{1/2}$ for protonation and deprotonation is approximately 60 s under these conditions. The level of the nitro analog present was 83 μM, which is considerably below the K_m of 730 μM at pH 7.1. Therefore, any changes in the percentage of active substrate will result in dramatic changes in the time courses of these reactions. The unprotonated nitronate was prepared by preincubation of the nitro analog of aspartate in pH 10.5 buffer, while the fully protonated analog was prepared by preincubation at pH 4.0 for approximately 1 h. The reactions were then initiated by adding the nitro analog to a solution of synthetase, ATP, citrulline, and coupling system at pH 7.1. The time courses of these reactions are shown in Fig. 1.

The pK value of the nitro analog of argininosuccinate was determined by spectrophotometric titration at 240 nm (1). The nitro analog was isolated by HPLC and was titrated with 0.1 N NaOH. The change in absorbance at 240 nm and the pH of the solution were recorded after each addition. The pK was determined to be 8.15.

TABLE I

KINETIC CONSTANTS OF DL-3-NITRO-2-AMINOPROPIONATE AND L-ASPARTATE WITH ARGININOSUCCINATE SYNTHETASE[a]

Kinetic constant	Aspartate		3-Nitro-aminopropionate	
	pH 7.1	pH 8.3	pH 7.1	pH 8.3
K (μM)	73	174	730	444
Rel V	100	153	64	91
Rel V/K	100	64	10.4	16

[a] From fits of the data to Eq. [1] at 25°C; 1.0 mM ATP, 1.0 mM citrulline, 10 mM Mg^{2+}.

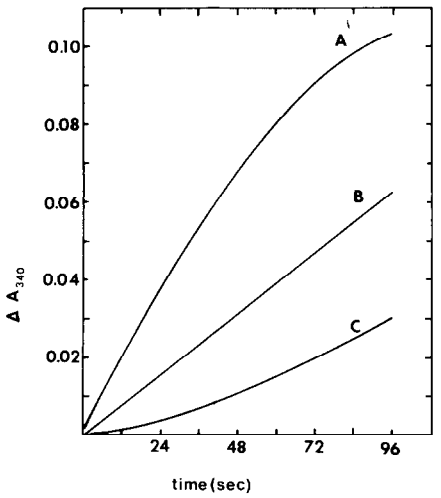

FIG. 1. Assay time courses for the reaction of DL-3-nitro-2-aminopropionic acid catalyzed by argininosuccinate synthetase. The reactions were initiated by the addition of the 3-nitro-2-aminopropionic to the rest of the reaction mixture at pH 7.1. The 3-nitro-2-aminopropionic acid was preincubated at pH (A) 10.5; (B) 7.1; and (C), 4.0. Additional details are given in the text.

Argininosuccinate lyase. The isolated nitro analog of argininosuccinate was tried as a substrate and inhibitor of argininosuccinate lyase. By monitoring the reaction at 230 nm no indication was given that the nitro analog of argininosuccinate could be enzymatically cleaved to arginine and nitroacrylic acid. The analog was, however, a good competitive inhibitor versus argininosuccinate at pH values from 6.25 to 9.25. The time courses of these inhibition experiments were linear, and there was no indication of any time-dependent changes in the affinity of the inhibitor with the lyase. The rate of the reaction was also the same whether the reaction was initiated by the addition of enzyme alone and when the enzyme was preincubated with inhibitor for 30 min.

The competitive inhibition data were fitted to Eq. [2]. A plot of pK_i versus pH is shown in Fig. 2. The data were fitted to Eq. [3]. The two pK values from this analysis are 8.2 ± 0.1 and 8.8 ± 0.1. The pH-independent value of K_i is 2.7 μM.

DISCUSSION

The deprotonated form of the nitro analog of aspartate appears to be the active substrate for argininosuccinate synthetase. This is most readily apparent from Fig. 1, which compares the reaction time courses when predominantly deprotonated and protonated DL-3-nitro-2-aminopropionic acid are added to argininosuccinate synthetase and the rest of the reaction mixture at pH 7.1. The deprotonated material clearly shows a burst of activity, and the fully protonated material proceeds with a definite lag phase when compared with the equilibrated material. The burst and lag phase can be attributed to the relatively slow protonation and deprotonation of the nitro analog ($t_{1/2} \sim 60$ s). The synthetase, therefore, requires a net negative charge at the β carbon of the substrate.

Support for this proposal also comes from a comparison of the V/K values for the nitro analog of aspartate and aspartate obtained with argininosuccinate synthetase. If the deprotonated material is the actual substrate, the ratio of V/K for aspartate and the nitro analog should become smaller at higher pH because a larger percentage of the nitro analog will be in the correct form at the elevated pH. The pK

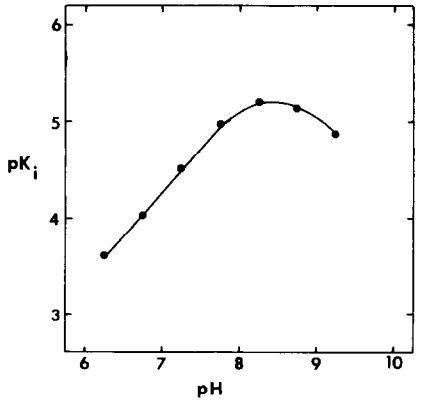

FIG. 2. Variation of pK_i with pH. The pK_i profiles are shown for the nitro analog of argininosuccinate as a competitive inhibitor with argininosuccinate lyase. The solid line represents the fit of the data to Eq. [3]. Additional details are given in the text.

for the proton at C-3 of the nitro analog is 7.75 and 9.27 when the α-amino group is protonated and deprotonated, respectively (1). The ratio of V/K for aspartate and the nitro analog of aspartate is 9.6 at pH 7.1 and 4.0 at pH 8.3 (See Table I). At pH 7.1 the carbanion of the nitro analog comprises 12% of the total material, while at pH 8.3 30% of the nitro analog exists in the carbanion form. The 2.5-fold increase in the amount of carbanion can be directly correlated with the 2.4-fold (9.6/4.0) decrease in the ratio of V/K values at pH 7.1 and 8.3.

The nitro analog of aspartate is one of the few compounds reported to date that can substitute for aspartate in the argininosuccinate synthetase reaction (11). At pH 7.1 and 8.3 the apparent V_{max} of the nitro analog is about 60% of the maximal rate with aspartate. The adjusted V/K values, after correcting for the amount of active substrate, are both about 85% of the measured value of aspartate. It therefore appears that the replacement of the C-4 carboxylate group with a nitro substituent does not significantly alter the enzyme activity providing that deprotonation occurs at C-3. Since the carbanion of 3-nitro-2-aminopropionic acid will exist predominantly in the nitronate configuration, the net charge of the compound is more important than the hybridization of the C–N bond.

The nitro analog of argininosuccinate (**III**) is a very good competitive inhibitor of argininosuccinate lyase. The active form of this inhibitor appears to be in the ionized nitronate configuration (**IV**). This conclusion is primarily based on the pH dependence of the measured competitive inhibition constant. The pH profile of the inhibition data is bell-shaped, and indicates a group that must be unprotonated with a pK of 8.2 and a group that must be protonated with a pK of 8.8. The pK of 8.2 can be directly compared with the measured pK of 8.15 for the ionization of the proton at C-3 of the nitro analog of argininosuccinate.

The origin of the pK appearing at 8.8 is more uncertain. The pH profile for V/K of argininosuccinate shows one group that must be unprotonated and two groups that must be protonated for activity.[3] The unprotonated group and a protonated group with a pK of 8.2 are thought to be involved in acid–base catalysis by the enzyme. The other unprotonated group with a pK of 8.8 is therefore very likely to result from the same group that appears in the pH profiles of the pK_i of the nitro analog. Since this pK does not appear in the pH profiles of V_{max} the effect must certainly result from decreased binding due to ionization of a substrate/inhibitor substituent or an enzyme side-chain group. The only substrate/inhibitor substituent that ionizes in this region is the α-amino group of the arginine moiety of argininosuccinate. The α-amino group of arginine has a measured pK of 9.0 (12). Alternatively, the group could also be a cationic enzyme side chain (lysine) that is responsible for binding one of the carboxylate groups of argininosuccinate.

The calculated pH-independent value of K_i for the nitro analog is 2.7 μM. This is about 20 times below the K_m for argininosuccinate at pH 7.5 (9). The tighter binding of the inhibitor relative to the substrate suggests that the inhibitor is a reaction-intermediate analog (13, 14), as indicated in Scheme I.

Compared with the nitro analogs that have been prepared and tested with other enzymes, the K_m/K_i ratio exhibited by the nitro analog of argininosuccinate is relatively small. For example, the Michaelis constant for isocitrate is 72,000 times larger than the inhibition constant of the nitro analog of isocitrate with aconitase (2). However, with the more closely related enzyme, adenylosuccinate lyase, the ratio of K_m/K_i is only 26 (3). Nevertheless, the tighter binding of the nitro analog of argininosuccinate can be interpreted to mean that the reaction catalyzed by argininosuccinate lyase proceeds via a carbanion intermediate. The weaker binding of the nitro analog of argininosuccinate compared with some of the other nitro analogs may

[3] Unpublished observation by F. M. Raushel and Q. T. N. Bui.

simply reflect the importance of the correct net charge after ionization as well as the correct geometry at C-3.

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