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Reaction of argininosuccinase with bromomesaconic acid: Role of an essential lysine in the active site

(enzyme modification/active site lysine/active site species similarity/urea cycle)

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ABSTRACT **We have undertaken studies on bovine liver argininosuccinase (L-argininosuccinate arginine-lyase, EC 4.3.2.1) with the active site-directed reagent bromo[U-^{14}C]mesaconic acid, an analogue of fumaric acid. Reactivity, measured by enzyme inactivation, followed pseudo-first-order kinetics, and the rate increased with reagent concentration. Argininosuccinate completely protected the enzyme against inactivation, but neither arginine nor fumarate was protective. A plot of the degree of inactivation as a function of alkyl groups incorporated was extrapolated to 4 mol per mol of enzyme, or 1 mol per active site. After large-scale alkylation of the enzyme (and digestion with trypsin), two ^{14}C-labeled tryptic peptides were isolated. These were chemically sequenced by the Edman method. The amino acid sequences proved to be identical with regions of the deduced amino acid sequences of argininosuccinases from human and yeast sources [O'Brien, W. E., McInnes, R., Kalumuck, K. & Adcock, M. (1986) *Proc. Natl. Acad. Sci. USA* 83, 7211–7215; Beacham, I. R., Schweitzer, B. W., Warrick, H. M. & Carbon, J. (1984) *Gene* 29, 271–279]. The ^{14}C-labeled tryptic peptide in the active site region had the sequence Gly-Leu-Glu-Xaa-Ala-Gly-Leu-Leu-Thr-Lys; Xaa represents an unknown phenylthiohydantoin derivative detected in cycle 4. The corresponding amino acid was identified as lysine-51 on the basis of sequence similarity with human and yeast amino acid sequences in this region. The reaction of the enzyme with the alkylating agent and the specific protection against inactivation by argininosuccinate suggest that this lysine residue has an essential role in the binding of argininosuccinate to the enzyme and, consequently, is essential for catalysis.**

Argininosuccinase (L-argininosuccinate arginine-lyase, EC 4.3.2.1) catalyzes the following reaction:

$$\text{L-argininosuccinate} \rightleftarrows \text{L-arginine} + \text{fumarate}$$

In the forward direction a N—C bond is cleaved by β-elimination, involving the abstraction by a nucleophile of a proton from C-3 in the succinate moiety, to form fumarate, and the donation of a proton to the amidino group of argininosuccinate, to form arginine (1). Since the enzyme lacks a prosthetic group (2, 3) and the guanidino nitrogen is strongly basic, N—C bond cleavage must be promoted by an electrophilic displacement at N—C$^{\alpha}$, proceeding through a symmetrical transition state (3). Interest in the reaction mechanism of the enzyme has more recently focused on the acid–base requirements from which to deduce the number of proton acceptor and donor residues (4). This kinetic analysis has led to reversible inactivation with diethylpyrocarbonate and the suggestion that a histidine residue and a carboxyl group, probably on another amino acid residue, may participate in the catalytic mechanism (4).

We have pursued modification of argininosuccinase through the use of active site-directed agents. The oligomeric structure of the enzyme is tetrameric and the four subunits (each 50 kDa) are identical (5). As shown by Rochovansky (6), the enzyme has four binding sites for argininosuccinate, one per subunit; each binding site is presumably equivalent to one active site. In the present paper we describe studies in which the substrate analogue bromomesaconic acid was used as an active site-directed alkylating agent. These studies are a first step in the characterization of the active site region. The enzyme is subject to genetic mutation in humans more frequently than several other urea cycle enzymes. Clinical symptoms become evident neonatally. Knowledge of the critical amino acid residues involved in catalysis is important in characterizing the genetic lesions. The identification of active site residues is also valuable in the design of *in vitro* site-directed mutagenesis of the human enzyme.

MATERIALS AND METHODS

Materials. [U-^{14}C]Glutamate was purchased from New England Nuclear; acetonitrile, from Burdick and Jackson (Muskegon, MI); trifluoroacetic acid, standard amino acids, triethylamine, and phenylisothiocyanate, from Pierce.

Preparation and Assay of Argininosuccinase. The enzyme was isolated from bovine liver according to the procedures published from this laboratory (5–7). The specific activity was 1430 μmol of substrate cleaved per hr per mg of protein at 38°C (5). Rates of argininosuccinate cleavage were followed at 23°C by a continuous assay, from the increase in absorbance at 240 nm as fumarate is formed (8). For use in the present study the enzyme was dialyzed at room temperature against 0.05 M Tris/Tris·HCl buffer (pH 7.5).

Preparation of [U-^{14}C]Mesaconic Acid. Mesaconic acid was prepared from glutamate with the glutamate mutase system of *Clostridium tetanomorphum* as described by Barker (9), under conditions favoring mesaconate accumulation. Minor modifications were made in the preparation of the incubation medium. The cold extract was then added to the medium; addition of 25 ml of 0.40 M glutamate (D and L isomers, 1:1) initiated the reaction at 38°C. For ^{14}C-labeling, 0.5 mCi (1 Ci = 37 GBq) of L-[U-^{14}C]glutamate was included. The rate of mesaconic acid formation was monitored on small aliquots read at 240 nm against a control. At 90 min, the reaction was terminated with H_2SO_4 to pH 6, and then to pH 2, the precipitate was removed, and the supernatant fluid was extracted with ether. Four such batches were prepared. The combined residue obtained after removing the ether was mesaconic acid, about 80% pure, in 24% yield. Upon addition of 1.4 g of unlabeled mesaconic acid, the mixture was

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crystallized in 50% yield (2.1 g, specific radioactivity 25.5 cpm per nmol).

Three-Step Preparation of Bromo[U-^{14}C]Mesaconic Acid. The labeled acid (2.0 g), dissolved in a large excess of anhydrous ethyl alcohol, was converted by acid catalysis (10) to the diethyl ester. Excess alcohol was recovered under reduced pressure in the presence of Na_2CO_3. The ester was distilled in 85% yield (11) through a short-path minicolumn still; at 500 μm Hg (1 μm Hg = 0.133 Pa), the boiling point was 62°C. Alkaline saponification of a small sample gave mesaconic acid.

Bromination was carried out in CCl_4 under decreased illumination by reaction with a limited excess of *N*-bromosuccinimide (2.66 g), each half added with 31 mg of dibenzoyl peroxide and 21 mg of MgO (12, 13) with rapid mixing under reflux. After suitable treatment to remove excess reagents, bromomesaconic diethyl ester was distilled at 95°C in 75% yield at 200 μm Hg. It was subjected to acid hydrolysis as follows: 2.70 g of bromo[U-^{14}C]mesaconic acid in 20 ml of 90% formic acid and 0.68 ml of methanesulfonic acid was slowly refluxed in the dark for 6 hr. Bromomesaconic acid crystallized at 4°C after the volume was reduced *in vacuo* to about 10 ml. The yield was 0.56 g before, and 0.39 g after, recrystallization. The melting point (170–180°C) agreed with an unlabeled sample kindly supplied by R. A. Laursen (14, 15) and the mixed melting point was unchanged; the two IR spectra coincided. The specific radioactivity was 30 cpm/nmol in dioxane and 25 cpm/nmol in aqueous medium.

Enzyme Alkylation. In aqueous buffers at neutral pH, bromomesaconate cyclizes to form the sodium salt of the lactone, aconic acid, as HBr (NaBr) splits out without a net change in pH. The half-life at pH 7.0 is 15 min (14, 15). To maintain the concentration of reagent near the initial level for studies of reaction rate and ^{14}C incorporation at 23°C, several additions of reagent were made to the incubation mixture during the progress of enzyme inactivation. Incorporation of ^{14}C with the protein was determined on 100-μl aliquots of the reaction mixture pipetted into 0.50 ml of cold 10% (wt/vol) trichloroacetic acid; the precipitate was collected on a Millipore filter and then washed with 6% trichloroacetic acid and diethyl ether. Radioacivity was determined on the washed filter by scintillation counting and was also determined directly on aliquots of solutions of the inactivated enzyme after ammonium sulfate precipitation and dialysis. Specific conditions of alkylation are given in the figure legends.

Preparation of Alkylated Tryptic Peptides. For large-scale preparations, 20 mg of the enzyme, dialyzed at 25°C against 0.05 M Tris/Tris·HCl (pH 7.5), was alkylated as described above over a 3-hr period to 75% inactivation. The protein was precipitated with ammonium sulfate and repeatedly dialyzed against 0.05 M Tris/Tris·HCl (pH 7.5) at room temperature, and ^{14}C incorporation was determined on a sample of the protein. The enzyme was denatured in 6 M guanidinium chloride and reduced with dithiothreitol, and the cysteine residues were blocked chemically by conversion to *S*-β-(4-pyridylethyl)cysteine side chains. The protein was dialyzed exhaustively against 0.05 M NH_4HCO_3 (pH 8.5) and digested with trypsin (24 hr, 25°C; trypsin/argininosuccinase weight ratio 1:50). Aliquots were lyophilized and stored dry at −20°C.

Isolation of ^{14}C-Labeled Peptides by Reversed-Phase HPLC. The mixture of tryptic peptides was resolved by high-performance liquid chromatography with Waters Associates equipment. Initial separations were made on a μBondapak C_{18} analytical column with 0.07 trifluoroacetic acid and 80% acetonitrile as eluants A and B, respectively. Elution was with a 3-hr gradient (0–60% eluant B at 1 ml/min) and was monitored at 220 nm. The peptides were collected manually and radioactivity was determined on small aliquots. Two or three peptides with significant ^{14}C were located. The ^{14}C-labeled peptides were isolated from the remaining mixture of tryptic peptides by repeated runs on a high-capacity column (4-fold larger) of the same kind. The two ^{14}C-labeled peptides nos. 3129 and 3230 were each pooled, divided, lyophilized, and stored at −20°C. The final purification step made use of a Whatman analytical 5P-ODS-3 column with a small guard column and a gradient mixture resembling eluants A and B described above; homogeneity was assessed during numerous trials by recovery of cpm and peak symmetry. The injection volume (and load) was kept small (50–60 μl) to retain good resolution, as judged from the symmetry of the eluted peak; thus, six or seven repeated runs were necessary. One-tenth of the pool was used for measurement of cpm. Both fractions 3129 and 3230 were purified in this way. The two ^{14}C-labeled bovine tryptic peptides were sequenced using an Applied Biosystems (Foster City, CA) gas-phase sequencer.

RESULTS AND DISCUSSION

Preliminary active site studies with bromo analogues suggested that neither arginine nor succinate analogues were sufficiently reactive. The fact that fumarase has a substrate in common with argininosuccinase suggested that certain site-reactive alkylating agents found to be effective for fumarase—e.g., iodoacetate and bromocrotonate, described by Hill and his group (12, 13, 16), and bromomesaconic acid, described by Laursen *et al.* (14, 15)—might be potential site-reactive alkylating reagents. These compounds were found to inactivate 50 μM solutions of argininosuccinase. For the series N^{α}-bromoacetyl-L-arginine, DL-bromosuccinate, bromocrotonate, and bromomesaconate, each used at 10 mM, the rates of inactivation, expressed as the apparent first-order constant (k), were 0.00036, 0.0114, 0.078, and 0.162 hr^{-1}, respectively. Full protection against inactivation was afforded by 2 mM argininosuccinate. The results clearly indicated that the enzyme was far more accessible to reactive analogues of fumaric acid than to analogues of moieties of argininosuccinate. Bromomesaconate proved to be the most reactive, for the rate of inactivation, 0.162 hr^{-1}, could be appreciably increased by multiple additions of the reagent owing to its brief half-life (see *Materials and Methods*).

On the addition of bromomesaconic acid to argininosuccinase, the enzyme was inactivated at a rapid rate according to pseudo-first-order kinetics (Fig. 1). Argininosuccinate (3 mM) fully protected against inactivation. As seen in Fig. 1, the rate of inactivation was influenced by enzyme concentration. However, when enzyme concentration was held constant, inactivation rates increased with increases in reagent concentration, in accord with expectation (data not shown). After very long incubation with the reagent (7 hr), some nonspecific interaction occurred with -SH groups. The amount of ^{14}C liberated by titration with 5,5′-dithiobis(nitrobenzoic acid) served to exclude significant incorporation with cysteine side chains.

Bromo[U-^{14}C]mesaconic acid, prepared uniformly labeled in all six carbons (see *Materials and Methods*) to increase experimental sensitivity, was used to determine the stoichiometry of incorporation and to identify the alkylated amino acid residue. The extent of incorporation of [U-^{14}C]mesaconate, plotted as a function of the rate of inactivation, displays a direct correlation; the curve extrapolates to 4 mol of reagent per mol of enzyme, or 1 mol per active site (Fig. 2). The protection against inactivation afforded by argininosuccinate (Fig. 1) is highly specific, for neither arginine nor fumarate was found to exert protection. The stoichiometry of incorporation, and the fact that protection afforded by argininosuccinate is highly specific, suggests that the reagent is also specific for the active site.

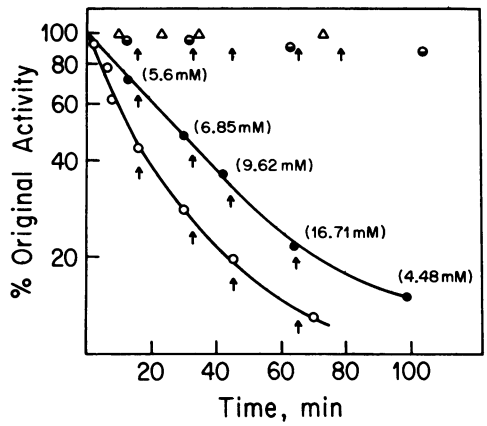


FIG. 1. Effects of protein concentration and multiple additions of bromomesaconate on inactivation of argininosuccinase with bromomesaconate. Argininosuccinase in 0.05 M Tris (pH 7.5) was inactivated in the dark at 23°C with 10 mM bromomesaconate. The loss of enzyme activity was determined from assays on two 10-μl aliquots of the reaction mixture. At the times indicated by arrows, calculated amounts of freshly neutralized (pH 6) bromomesaconate were added to the reaction mixture to restore the bromomesaconate concentration to 10 mM. The values in parentheses refer to the calculated concentration of bromomesaconate at the time of assay. Percent original activity was based on the activity of a control sample to which an equal amount of buffer had been added. The initial concentration of argininosuccinase active sites in the reaction mixture was 12.2 μM (●-●), 12.2 μM plus 4 mM argininosuccinate (◒), 120 μM (○-○), or 120 μM plus 5.7 mM argininosuccinate (△).

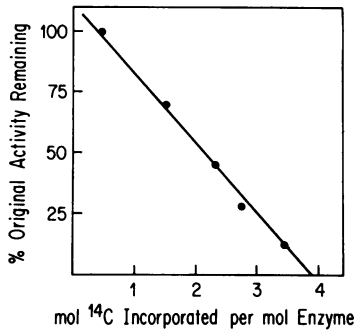


FIG. 2. Inactivation of argininosuccinase as a function of bromo[^{14}C]mesaconate incorporation. Argininosuccinase (120 μM in active sites) in 0.05 M Tris/Tris·HCl (pH 7.5) was alkylated at 23°C in the dark with 10 mM bromo[U-^{14}C]mesaconate (25 cpm per nmol). A second sample (116 μM in active sites), containing 5.7 mM argininosuccinate, was treated in the same manner. Enzyme activity was followed, as in Fig. 1, on 2-μl aliquots. Protection by substrate and the conditions and time course of the inactivation were similar to the lower curve in Fig. 1. Incorporation of bromo[U-^{14}C]mesaconate was determined from 100-μl aliquots of the reaction mixtures as described in *Materials and Methods*.

The incorporated alkyl groups remain bound after tryptic hydrolysis. The resulting mixture of peptides were eluted through a Dowex-50 column with dilute NH_4OH without loss of counts. Peptide map data indicated that a major band containing ^{14}C was associated only with the peptides. No strongly acidic materials such as [^{14}C]mesaconic or [^{14}C]aconic acid were detected.

Isolation of ^{14}C-Alkylated Tryptic Peptides. With these assurances, enzyme alkylations were carried out on a large scale under the conditions described in *Materials and Methods*. Several batches were subjected to the steps described, leading to resolution of the tryptic peptides by reversed-phase HPLC. Close to 49 tryptic peptides were expected from the total number of arginine and lysine residues per subunit of argininosuccinase (17). About 50–55 peaks emerged on elution and were numbered sequentially. Of these, only two or three contained significant radioactivity. After final purification, each of these contained ≈50 cpm (yield per 10 mg of enzyme). However, the number of peptide bonds judged by the absorbance at 220 nm differed by a factor of about 20. Only a few amino acids (tryptophan and histidine) have high absorbance in this region. The low specific radioactivity of the bromo[U-^{14}C]mesaconic acid (25 cpm/nmol) and the small amount of purified material available limited further experiments to highly sensitive methods. Nevertheless, even when 200-pmol of peptide was acid-hydrolyzed (6 M HCl, 24 hr, 110°C) and then analyzed by chromatography on a Waters Pico-Tag column, no unknown phenylthiocarbamoyl derivative (corresponding to the alkylated residue) or statistically significant radioactive peak was detected. To avoid acid hydrolysis, we turned to chemical sequencing.

Amino Acid Sequence of the ^{14}C-Labeled Alkylated Peptides. The two labeled peptides were each sequenced through 10 or 11 cycles by the Edman method, using a gas-phase sequencer (23). The amino acid sequence of peptides 3129D and 3230D-a and -b are shown in Table 1, where they are compared with argininosuccinase sequences from two other sources. The entire amino acid sequence of argininosuccinase has been deduced from the cDNA sequence of human argininosuccinase mRNA by O'Brien *et al.* (18). The amino acid sequence deduced from the DNA sequence of the yeast *ARG4* gene, described by Carbon and his group (19), is now also available. Complete similarity is evident between the bovine peptide 3129D and residues 48–57 of the human sequence. Similarity between the yeast and bovine sequence is almost as complete; the exceptions are glutamine vs. glutamic acid at position 50 and leucine vs. alanine at position 52. The entire yeast gene sequence shows only 57% amino acid homology to the human gene, but in the region shown in Table 1 the homology is higher: 83% for the 12 amino acid (residues 48–59).

The several amino acids in the human enzyme that precede and follow the alkylated peptide sequence 3129D indicate that this bovine peptide was indeed an intact tryptic peptide, since the chemical sequence began after arginine and terminated after lysine. The unknown phenylthiohydantoin derivative that appears in cycle 4 may therefore be construed to be an alkylated lysine residue and to account for the presence of radioactivity in peptide 3129D. The sample used for sequence analysis contained, at most, 8 cpm, in agreement with the phenylthiohydantoin peak area. That this lysine residue was blocked by alkylation is further supported by the fact that it was not susceptible to tryptic cleavage.

The second peptide fragment (3230D) containing radioactivity was also subjected to sequence analysis. The sample proved to contain two tryptic peptides, designated 3230D-a and 3230D-b in Table 1. The successive yields of the phenylthiohydantoin derivatives of each pair of stable amino acids sequenced were plotted. The plots were linear with two distinct slopes, consistent with expectation for two separate tryptic peptides.

Peptide 3230D-a, run for 11 cycles, terminated in lysine and began after arginine, whereas the shorter peptide 3230D-b began after lysine and terminated with arginine. Through homology, then, the three isolated bovine sequences are established as being tryptic peptides. The sequence of peptide 3230D-a is identical to the human sequence at residues 12–24, except for one minor exception (threonine vs. alanine at position 16). The two are considered to be closely related. The third tryptic peptide sequence (3230D-b) is identical to the human sequence at residues 289–297. The similarities between the human and yeast sequence for 14 amino acids are 57% and

Table 1. Amino acid sequences of ^{14}C-alkylated tryptic peptides of bovine argininosuccinase compared to similar regions of human and yeast argininosuccinases

Species	Sequence
	1 2 3 4 5 6 7 8 9 10
Bovine (peptide 3129D)*	Gly-Leu-Glu-Xaa-Ala-Gly-Leu-Leu-Thr-Lys
Human (residues 46–59)†	Ser-Arg-Gly-Leu-Glu-Lys-Ala-Gly-Leu-Leu-Thr-Lys-Ala-Glu
Yeast (residues 46–59)‡	Thr-Ala-Gly-Leu-Gln-Lys-Leu-Gly-Leu-Leu-Thr-Glu-Thr-Glu
	1 2 3 4 5 6 7 8 9 10 11
Bovine (peptide 3230D-a)*	Phe-Val-Gly-Thr-Val-Asp-Pro-Ile-Met-Glu-Lys
Human (residues 11–24)†	Gly-Arg-Phe-Val-Gly-Ala-Val-Asp-Pro-Ile-Met-Glu-Lys-Phe
Yeast (residues 11–24)‡	Gly-Arg-Phe-Thr-Gly-Gly-Thr-Asp-Pro-Leu-Met-His-Leu-Tyr
	1 2 3 4 5 6 7 8 9
Bovine (peptide 3230D-b)*	Asn-Pro-Asp-Ser-Leu-Glu-Leu-Ile-Arg
Human (residues 287–300)†	Lys-Lys-Asn-Pro-Asp-Ser-Leu-Glu-Leu-Ile-Arg-Ser-Lys-Ala
Yeast (residues 287–300)‡	Lys-Lys-Asn-Ala-Asp-Ser-Leu-Glu-Leu-Leu-Arg-Gly-Lys-Ser

*Sequence obtained by Edman degradation; numbers above bovine residues indicate degradation cycles. Xaa represents an unknown phenylthiohydantoin amino acid derivative observed in cycle 4 for peptide 3129D.
†Sequence from the amino acid sequence deduced from cDNA for human argininosuccinase (18).
‡Sequence from the amino acid sequence deduced from the yeast (*Saccharomyces cerevisiae*) argininosuccinase gene *ARG4* (19).

71%, respectively, for residues 11–24 and residues 287–300. The sequence similarities among the three species serve to reinforce the sequence authenticity in the regions under study.

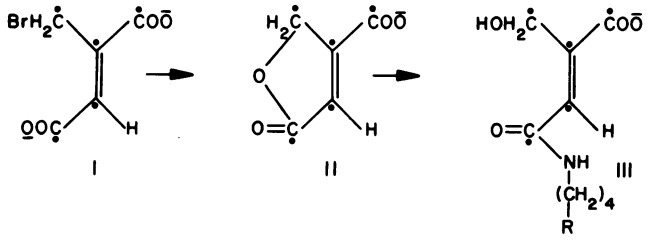
Although alkylated peptide 3230D was isolated from the initial mixture of tryptic peptides on the basis of radioactivity, it markedly contrasted, as noted above, with peptide 3129D in that the ratio of peptide bonds (A_{220}) to radioactivity was 20-fold greater for 3230D than for 3129D; consequently, the aliquot injected contained insufficient radioactivity to allow detection of an unknown phenylthiohydantoin derivative. However, the reactive methionine in peptide 3230D-a should be considered as a possibility. Other investigators have found methionine reactivity with other site-directed alkyl halides for fumarase (13); but in this case, the alkylation, and inactivation, may have been fractional.

Judging from the composition of peptide 3230D-b, its presence might be explained simply on the basis of copurification with peptide 3230D-a.

Evidence for Lysine in the Active Site. The complete similarity of the sequence of tryptic peptide 3129D to the sequences of the human and yeast enzymes is the strongest evidence that lysine is the modified amino acid residue in the active site region. In addition, the relationship of injected radioactivity to peak area (60–70%) speaks for lysine-51 being the site of a modification approaching stoichiometric proportions (cf. Figs. 1 and 2).

Earlier in our study on possible substrate binding sites of argininosuccinase, experiments were conducted by using pyridoxal phosphate as a probe. With a 4-fold excess of pyridoxal phosphate over enzyme active sites, the Schiff base spectrum was obtained and absorbance at 425 nm increased to a maximum at 1 mM pyridoxal phosphate. Separate experiments in which pyridoxal phosphate was followed by $NaBH_4$ reduction showed, under suitably controlled conditions, that inactivation of the enzyme was proportional to the number of pyridoxal phosphate groups bound; a value between one and two groups per active site was obtained. The use of pyridoxal phosphate as a probe (followed by stabilization by reduction) has frequently allowed identification an active site lysine (20–22). Our preliminary findings with pyridoxal phosphate serve to reinforce the presence of lysine in the active site region.

In the absence of direct evidence it is difficult to affirm the mode of modification of the ε-amino group. Alkylation of a lysine side chain of fumarase by reactive bromo derivatives was found in trace amounts in one of the reactions studied by Hill and his group (13), and lysine was not found to be involved in the study of fumarase with bromomesaconic acid by Laursen *et al.* (14, 15). We have considered two possible types of modification in connection with lysine-51 of argininosuccinase: (*i*) by direct alkylation through interaction with bromomesaconic acid and (*ii*) by interaction with [U-^{14}C]aconic acid to form an aconyl amide of the ε-amino nitrogen. As shown in the structures given below, bromo[U-^{14}C]mesaconate (I) at pH 7 has a half-life of 15 min owing to cleavage of HBr (NaBr) to form the lactone (11, 14, 15), [U-^{14}C]aconate (II), by cyclization. This compound, which contains the same original five carbons labeled with ^{14}C (Ċ), but only one carboxyl, accumulates during successive additions of the bromo reagent. As an unsaturated lactone, it may react with the ε-amino side chain of lysine (R) to form a hydroxymesaconyl amide (III) at the ε-nitrogen of the lysine residue.



The amide derivative might be sufficiently stable to survive chemical sequencing conditions but would probably not resist 24-hr hydrolysis with 6 M HCl. The kinetics of inactivation by this route would be complex and a doubtful basis on which to choose either aconate or bromomesaconate as the reactive species during large-scale inactivation.

The reaction of the enzyme with the alkylating agent and the specific protection against inactivation by argininosuccinate suggest that lysine-51 has an essential role in the binding of argininosuccinate to the enzyme and consequently is essential for catalysis.

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