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INHIBITION OF ADENYLOSUCCINATE LYASE BY L-ALANOSYL-5-AMINOIMIDAZOLE-4-CARBOXYLIC ACID RIBONUCLEOTIDE (ALANOSYL-AICOR)

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Abstract—L-Alanosyl-5-aminoimidazole-4-carboxylic acid ribonucleotide (alanosyl-AICOR) has been synthesized enzymatically using 4-(*N*-succino)-5-aminoimidazole-4-carboxamide ribonucleotide (SAICAR) synthetase in conjunction with 5-aminoimidazole-4-carboxylic acid ribonucleotide and L-2-amino-3-(*N*-hydroxy-*N*-nitrosoamino)propionic acid (alanosine). The product was characterized by chromatography, ultraviolet spectrum and NMR spectrum at 300 MHz. Alanosyl-AICOR was not a substrate of adenylosuccinate lyase from rat skeletal muscle, but it was an apparent competitive inhibitor in both of the reactions catalyzed by the enzyme. The K_I values for alanosyl-AICOR were ~1.5 and 1.3 μM in the SAICAR and adenylosuccinate cleavage reactions respectively. These K_I values were essentially the same as the K_m values for the two substrates of adenylosuccinate lyase. They compare with an accumulation of 70 μM alanosyl-AICOR in leukemic nodules of mice treated with alanosine [A. K. Tyagi and D. Cooney, *Cancer Res.* **40**, 4390 (1980)]. Thus, inhibition of adenylosuccinate lyase may account for much of the inhibitory effect exerted by alanosyl-AICOR *in vivo*. We confirmed the previous observation that alanosyl-AICOR is an inhibitor of adenylosuccinate synthetase.

Alanosine† is an antitumor antibiotic found in *Streptomyces alanosinicus* [1]. Its pharmacology, metabolism, and mechanism of action have been reviewed [2–4]. Alanosine is an analog of aspartate, which it can replace in several enzymatic reactions [5]. For example, alanosine can be used in place of aspartate in the SAICAR synthetase reaction. The resulting product is alanosyl-AICOR, which has been reported to be a potent inhibitor of adenylosuccinate synthetase [5, 6] (Scheme 1). Alanosyl-AICOR is believed to be the compound responsible for inhibiting the *de novo* synthesis of purine nucleotides [7] and the conversion of IMP to AMP [8] observed in cultured cells treated with alanosine. The toxic effects of alanosine can be prevented or reversed by addition to the culture medium of adenine, but not hypoxanthine or aspartate [6, 8]. On the basis of these and other observations [2], it has been proposed that alanosyl-AICOR is the compound most likely to be responsible for the therapeutic and toxicologic properties of alanosine [6].

Adenylosuccinate lyase is a bifunctional enzyme in the pathway of purine biosynthesis; it catalyzes the *trans*-elimination of fumarate from both adenylosuccinate and SAICAR (Scheme 1) [9]. It has been proposed that adenylosuccinate lyase modulates the concentration of alanosyl-AICOR in tissues by degrading this antimetabolite [2, 10], but the proposed cleavage of alanosyl-AICOR by adenylosuccinate lyase has been disputed [11]. We now report a study of the effects of alanosyl-AICOR on pure adenylosuccinate lyase.

EXPERIMENTAL PROCEDURES

Purification of enzymes. Adenosylosuccinate synthetase and adenylosuccinate lyase were purified from high speed supernatant fractions of rat skeletal muscle extract by ion-exchange and affinity chromatography.‡ The specific activities at 25° of the adenylosuccinate synthetase and adenylosuccinate lyase used in the experiments reported here were 2 and 11 μmoles/mg protein/min respectively.

SAICAR synthetase was purified by a modification of the method of Patey and Shaw [12]. The steps used by us included extraction of the enzyme from chicken liver acetone powder, preparation of a 30% to 60% $(NH_4)_2SO_4$ fraction, and chromatography on carboxymethyl cellulose. The specific activity at 25° of the enzyme so obtained was 0.17 μmole/mg protein/min.

Enzyme assays. Adenylosuccinate synthetase was assayed as described elsewhere [13] except that HEPES buffer was used instead of imidazole-HCl and that the assay included 1 unit of pyruvate kinase. The cleavage of adenylosuccinate by adenylosuccinate lyase was assayed by the decrease in

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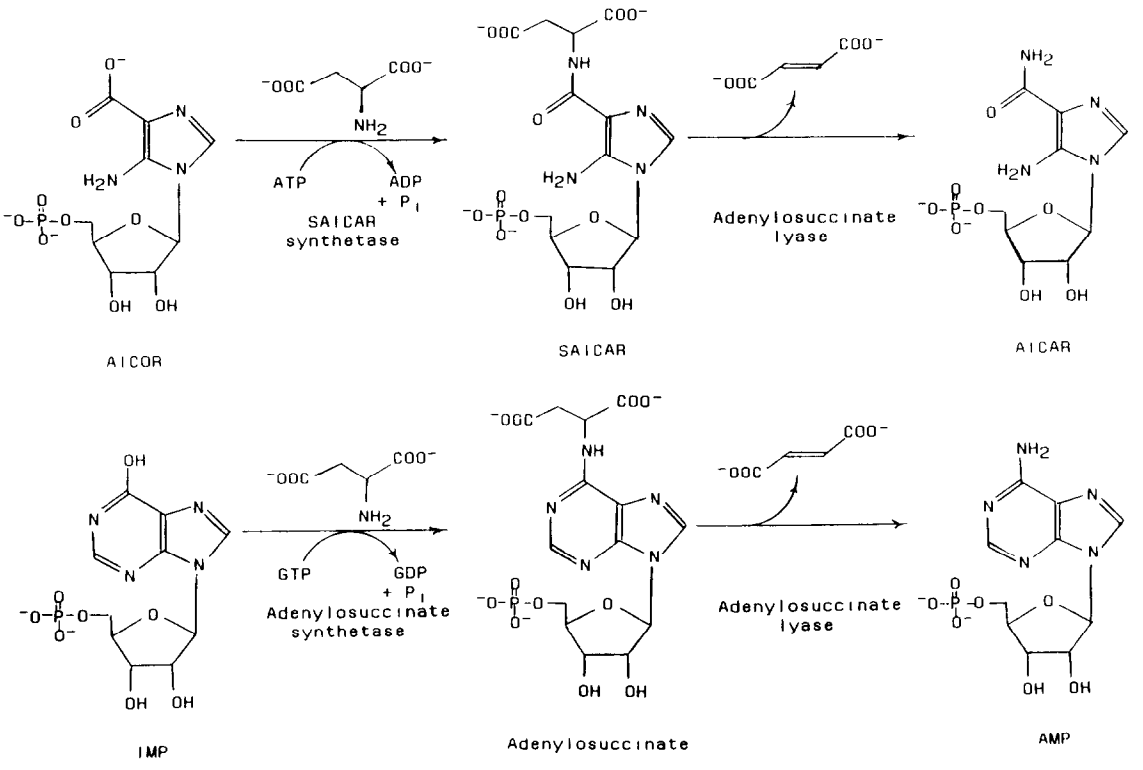
† Abbreviations and trivial names: Alanosine, L-2-amino-3-(*N*-hydroxy-*N*-nitrosoamino)propionic acid; alanosyl-AICOR, L-alanosyl-5-aminoimidazole-4-carboxylic acid ribonucleotide; AICOR, 5-aminoimidazole-4-carboxylic acid ribonucleotide; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; SAICAR, 4-(*N*-succino)-5-aminoimidazole-4-carboxamide ribonucleotide; HPLC, high performance liquid chromatography; and HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

‡ P. J. Casey and J. M. Lowenstein, manuscripts in preparation.



Scheme 1. Steps in purine biosynthesis resulting in the use of aspartate as a nitrogen donor.

absorbance at 282 nm minus 320 nm using a difference extinction coefficient of 10.0 mM^{-1} cm^{-1} [13]. The reaction mixture contained 20 mM potassium HEPES, pH 7.4, 50 mM KCl, 0.2 mg of bovine serum albumin, adenylosuccinate, and enzyme in a total volume of 1.0 ml. The cleavage of SAICAR by adenylosuccinate lyase was assayed using the same reaction mixture except that SAICAR replaced adenylosuccinate, and the decrease in absorbance was measured at 267 nm minus 320 nm using a difference extinction coefficient of 0.7 mM^{-1} cm^{-1} [14]. SAICAR synthetase was assayed spectrophotometrically by measuring the decrease in absorbance at 282 nm minus 320 nm using a difference extinction coefficient of 7.0 mM^{-1} cm^{-1} [15]. The complete reaction mixture contained 50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 250 mM $KHCO_3$, 2.4 mM L-aspartate, 1.5 mM ATP, 0.6 mM AICOR and enzyme in a total volume of 0.6 ml. The light path length was 1 mm.

Preparation of compounds. AICOR was synthesized by the hydrolysis of AICAR in 6 N NaOH as previously described [16]. AICOR exhibits an absorption maximum at 248 nm [17] and migrates as a single spot with an R_f of 0.50 on silica thin-layer plates in solvent A (see below) [16]. SAICAR was prepared enzymatically from AICOR and L-aspartate. The reaction mixture contained 12 μmoles of AICOR, 20 μmoles of L-aspartate, 30 μmoles of ATP, 150 μmoles of $KHCO_3$, 30 μmoles of $MgCl_2$, 150 μmoles of Tris–HCl, pH 8.0, and 0.05 units of SAICAR synthetase in a total volume of 2.7 ml. After incubation at 37° for 18 hr, the protein was removed by centrifuging the mixture through a Centricon 10 microconcentrator. The filtrate was diluted 2-fold with distilled water and injected onto a column of AGMP-1 ion exchange resin (0.5 × 25 cm) [18]. After a 5 ml wash with distilled water, the adsorbed compounds were eluted with a 30-ml gradient from 0 to 90 mM trifluoroacetic acid followed by 10 ml of 90 mM trifluoroacetic acid. The flow rate was 1 ml/min. SAICAR emerged with ADP at 26 min. The solvent was removed by evaporation under reduced pressure, and the residue was dissolved in distilled water and streaked on a sheet of Whatman 3-mm paper. The chromatogram was developed in the ascending mode using solvent B (see below). In this system, the R_f of ADP is 0.45, while that of SAICAR is 0.15. The band containing SAICAR was cut out and the compound was eluted with water. SAICAR purified by this method had an absorbance maximum at 268 nm and migrated as a single spot with an R_f of 0.38 on paper chromatography in solvent C (see below) [19, 20].

Alanosyl-AICOR was prepared by the same method as SAICAR except that the reaction mixture contained 100 μmoles of L-alanosine in place of aspartate. The product emerged from the AGMP-1 column (see above) at 25 min. The solvent was removed by evaporation under reduced pressure, the residue was dissolved in water, and the resulting solution was streaked in a line on a sheet of Whatman 3-mm paper. The chromatogram was developed in the ascending mode using solvent C. In this system,

the R_f of ADP is 0.23 while that of alanosyl-AICOR is 0.33. The band containing alanosyl-AICOR was cut out and eluted with water. The compound was then rechromatographed on the AGMP-1 column, freeze-dried, and dissolved in distilled water. In order to take a proton NMR spectrum, the pH of the solution of alanosyl-AICOR was adjusted to 5 with NaOH and the solution was passed through a column of Chelex 100 resin (0.7 cm × 5.0 cm) to remove metal ions, if any were present. The solution was freeze-dried, and the compound was dissolved in D_2O.

Chromatographic methods. HPLC system 1 was the AGMP-1 system described for the purification of SAICAR. HPLC system 2 consisted of a Varian Micro-Pak AX-5 anion exchange column, 0.4 cm × 30 cm, equilibrated with 150 mM potassium phosphate, pH 2.1. Samples were injected onto the column at pH 2.1 and were eluted with a 20-ml linear gradient from 150 to 750 mM potassium phosphate, pH 2.1. Paper and thin-layer chromatography solvents were as follows: (A) *n*-butanol–acetic acid–H_2O (5:3:2); (B) isobutyric acid–NH_4OH–H_2O (66:1:33); (C) *n*-butanol–acetic acid–H_2O (10:4:7); and (D) *tert*-amyl alcohol–formic acid–H_2O (3:2:1).

Other methods. NMR spectra were taken on a Varian XL-300 spectrometer. HPLC analyses were carried out on a Beckman model 334 gradient system using an Hitachi model 100-40 spectrophotometer at 268 nm. A Perkin–Elmer model 557 dual wavelength spectrophotometer was used for all other spectrophotometric studies. Purine compounds on paper and thin-layer chromatograms were visualized under ultraviolet light; amino acids were visualized by spraying with 0.2% ninhydrin in ethanol.

Materials. L-Alanosine was obtained from the National Cancer Institute. AGMP-1 (macroporous Dowex-1) was a gift of Bio-Rad Laboratories. Chelex 100 was obtained from Bio-Rad. Cellulose thin-layer plates (Eastman 13254) were obtained from Eastman Kodak. Centricon 10 microconcentrators were obtained from Amicon. Deuterium oxide (99.96 atom %) was obtained from Aldrich. All nucleotides were obtained from Sigma.

RESULTS

Characterization of alanosyl-AICOR. The combination of HPLC system 1 and paper chromatography for the purification of alanosyl-AICOR resulted in an apparently pure compound without any contamination by ADP. Alanosyl-AICOR so obtained had a broad absorption maximum from 261 to 265 nm and migrated as a single spot on cellulose thin-layer plates in solvent D and as a single peak on HPLC systems 1 and 2. The extinction coefficient was 11.0 mM^{-1} cm^{-1} at 263 nm, based on the chemically assayed content of ribose [21] after hydrolysis of the compound in 0.05 M H_2SO_4 [22]. This is much lower than that of 57.5 mM^{-1} cm^{-1} reported previously [6], but is in reasonable agreement with that recently reported for the chemically synthesized dephospho alanosyl-AICOR of 15.6 mM^{-1} cm^{-1} [23] and with the extinction coefficients of a number of compounds of the 5-aminoimidazole-4-carboxamide type [24, 25]. The proton NMR spectrum of the alanosyl-AICOR prepared enzymatically by us was consistent with that obtained for the chemically prepared dephospho compound [23]. The assignments were: δ 3.81, 3.95, 4.40, and 4.65 (5H, multiplets, ribose hydrogens), δ 4.33 and 4.48 (2H, multiplets, C_β hydrogens), δ 4.82 (1H, multiplet, $C_\alpha H$), δ 5.53 (1H, d, J = 6 Hz, ribose 1'H), and δ 7.43 (1H, s, imidazole-H).

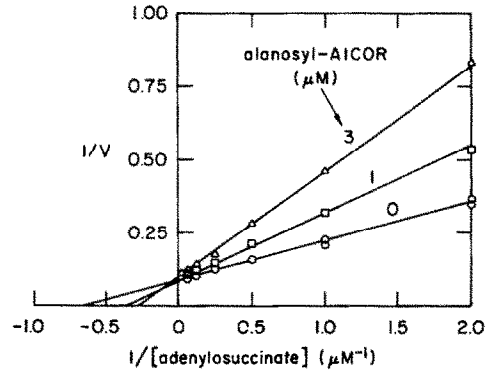


Fig. 1. Inhibition of adenylosuccinate cleavage by alanosyl-AICOR. Assays were conducted in 20 mM HEPES, pH 7.4, 50 mM KCl, 0.02% BSA, 0.5 to 32 μM adenylosuccinate, and alanosyl-AICOR as indicated. The reciprocal of the initial velocity (μmoles/mg protein/min) is plotted versus the reciprocal of adenylosuccinate concentration.

Interaction of alanosyl-AICOR with adenylosuccinate lyase. The ability of purified rat skeletal muscle adenylosuccinate lyase to use alanosyl-AICOR as a substrate was tested as follows. Alanosyl-AICOR (200 nmoles) was incubated with 1.3 μg of purified adenylosuccinate lyase in 200 μl of 20 mM potassium phosphate, pH 7.4, at 25° for 1 hr. In a parallel experiment, alanosyl-AICOR was replaced by 200 nmoles of SAICAR. Controls were run which lacked enzyme. The reaction mixtures were analyzed for the release of AICAR using HPLC system 1 (see Experimental Procedures). All of the SAICAR was cleaved to AICAR during the incubation period. However, none of the alanosyl-AICOR was cleaved and no AICAR production was observed under these conditions. All of the alanosyl-AICOR starting material eluted from the column in the same position as in the control experiment which

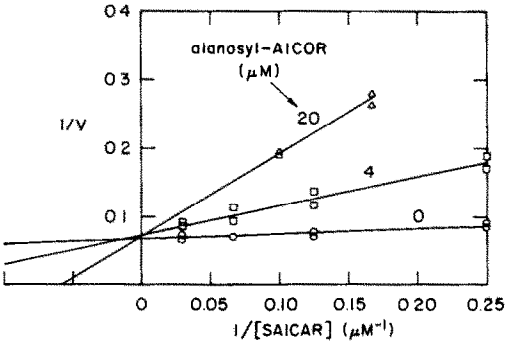


Fig. 2. Inhibition of SAICAR cleavage by alanosyl-AICOR. Assay conditions were as in Fig. 1, except that the SAICAR levels were varied from 4 to 32 μM and alanosyl-AICOR was present as indicated.

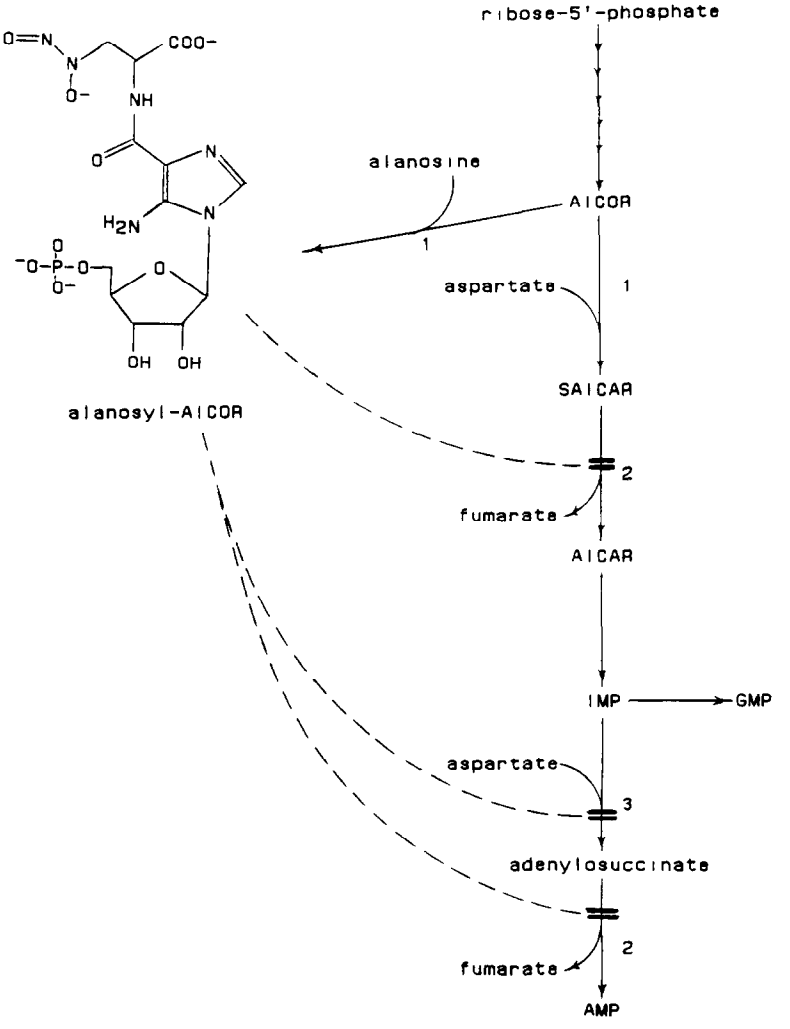
Table 1. Summary of kinetic constants obtained for the reactions catalyzed by adenylosuccinate lyase

Activity	K_m (μM)	K_I Alanosyl-AICOR (μM)
SAICAR cleavage	~1	~1.5
Adenylosuccinate cleavage	1.4	1.3

lacked enzyme. Moreover, replacement of SAICAR by 50 μM alanosyl-AICOR in the spectrophotometric assay for adenylosuccinate lyase in the presence of 5 μg of the purified enzyme resulted in no change in the absorbance spectrum of the mixture over a period of 3 hr. We conclude that alanosyl-AICOR is not a substrate for adenylosuccinate lyase from rat skeletal muscle.

Inhibition of adenylosuccinate lyase by alanosyl-AICOR. Initial velocity measurements conducted in the presence of alanosyl-AICOR revealed that this compound inhibited the cleavage of adenylosuccinate (Fig. 1) and SAICAR (Fig. 2) by adenylosuccinate lyase. Alanosyl-AICOR appeared to be a competitive inhibitor, and V_{max} values determined in the absence and presence of the inhibitor were approximately the same. Table 1 shows K_I values for the inhibition of both SAICAR and adenylosuccinate cleavage by alanosyl-AICOR, as well as K_m values determined for both substrates. The values for the SAICAR cleavage reaction are approximate, because the small difference extinction coefficient between SAICAR and its cleavage products does not permit running points at SAICAR concentrations of less than 4 μM.

Inhibition of adenylosuccinate synthetase by alanosyl-AICOR. It has been reported that alanosyl-AICOR is a potent inhibitor of adenylosuccinate synthetase [6, 26], and that this inhibition is competitive with respect to IMP [6]. Initial velocity measurements of the rat skeletal muscle adenylosuccinate synthetase reaction with IMP as the variable substrate gave an apparent K_i of 0.5 μM for alanosyl-AICOR and an apparent K_M for IMP of 0.4 mM (data not shown).



Scheme 2. Effect of alanosyl-AICOR on the pathway of purine biosynthesis. Key: (1) SAICAR synthetase; (2) adenylosuccinate lyase; and (3) adenylosuccinate synthetase.

DISCUSSION

The finding that alanosyl-AICOR is not cleaved by purified adenylosuccinate lyase from rat skeletal muscle led us to examine whether this compound inhibits adenylosuccinate lyase. Alanosyl-AICOR was found to be an inhibitor of both of the reactions catalyzed by adenylosuccinate lyase. In agreement with previous reports [6, 26], alanosyl-AICOR was also found to inhibit adenylosuccinate synthetase. The effects of the incorporation of alanosine into alanosyl-AICOR on purine biosynthesis are summarized in Scheme 2.

When mice bearing L5178Y/AR leukemic nodules are treated with DL-[1-^{14}C]alanosine, the level of alanosyl-AICOR in the nodules rises to 70 μM [6]. Given the K_I values for the inhibition of both of the reactions catalyzed by adenylosuccinate lyase (Table 1), and the fact that the intracellular concentrations of adenylosuccinate and SAICAR are in the submicromolar to micromolar range [27, 28], the concentration of alanosyl-AICOR that accumulates can be expected to cause a strong inhibition of adenylosuccinate lyase and of purine biosynthesis *in vivo*.

Inhibition of adenylosuccinate lyase by high levels of AICAR in mice produces skeletal muscle dysfunction [29]. Deficiency of adenylosuccinate lyase in a number of organs of several autistic children is associated with severe psychomotor delay [30, 31].

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