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MAMMALIAN ADENYLOSUCCINATE LYASE

PARTICIPATION IN THE CONVERSION OF 2′-dIMP and β-D-ARABINOSYL-IMP TO ADENINE NUCLEOTIDES

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Summary

Adenylosuccinate synthetase (EC 6.3.4.4) from rabbit muscle efficiently catalyzes the formation of 2′-deoxyadenylosuccinate and β-D-arabinosyladenylosuccinate from 2′-dIMP and β-D-arabinosylIMP (Spector, T. and Miller, R.L. (1976) Biochim. Biophys. Acta 445, 509—517). These novel analogs of adenylosuccinate were synthesized with this enzyme and their kinetic constants were determined with adenylosuccinate lyase purified from Ehrlich ascites cells. 2′-Deoxyadenylosuccinate and β-D-arabinosyladenylosuccinate were readily cleaved to 2′-dAMP and β-D-arabinosylAMP, respectively. Their K_m values were similar to that of adenylosuccinate (3—6 μM) and their substrate efficiencies (V/K_m) were 120 for 2-deoxyadenylosuccinate and 32 for β-D-arabinosyladenylosuccinate, compared to a value of 100 for adenylosuccinate. The products of the reactions, 2′-dAMP and β-D-arabinosylAMP, were competitive inhibitors with K_i values of 5 and 87 μM, respectively. ATP and ADP were considerably weaker competitive inhibitors with K_i values of 200—300 μM. IMP, GMP, xanthosine 5′-monophosphate, 6-thioIMP and 6-thioGMP had K_i values >200 μM.

Introduction

The bioconversion of IMP to AMP occurs via a two step process. In the first reaction, catalyzed by adenylosuccinate synthetase (EC 6.3.4.4), IMP is condensed with aspartic acid to form adenylosuccinate. This intermediate is then cleaved by adenylosuccinate lyase (EC 4.3.2.2) to form AMP and fumarate. A recent investigation of the metabolism of analogs of IMP has revealed the narrow substrate specificity of adenylosuccinate synthetase [1]. Ten analogs of IMP were studied, but only three were found to be substrates. β-D-arabinosyl-

IMP, 2′dIMP and 6-methoxypurine nucleotide were converted to β-D-arabinosyladenylosuccinate, 2′-deoxyadenylosuccinate and adenylosuccinate, respectively. In the study presented here, the two novel intermediates were synthesized and the kinetic constants for their conversion to adenine nucleotides by adenylosuccinate lyase were determined. The ability of these adenine nucleotides and other purine nucleotides to inhibit this enzyme was also studied.

Materials and Methods

Enzymes. Adenylosuccinate lyase was purified from the Ehrlich ascites fluid of white male mice that were inoculated 6 days earlier with 0.1 ml of tumor tissue. The purification procedure and standard assay condition described by Brox [2] was used in this study. The preparation contained no detectable AMP deaminase, AMP kinase or ATPase. The assay mixtures contained 20 mM Tris · HCl, pH 7.8, 20 mM EDTA and enzyme. The substrates and inhibitors varied for each experiment. Reactions with adenylosuccinate or its pentose-modified analogs were monitored spectrophotometrically [3] [$\Delta\epsilon_{280} = -10.7\ mM^{-1} \cdot cm^{-1}$] at 37° C. Kinetic constants were determined by the direct-fit method of Wilkinson [4]. Units (μmol of adenylosuccinate cleaved/min) were determined at 60 μM adenylosuccinate as previously described [2].

Adenylosuccinate synthetase was purified from rabbit muscle through the phosphocellulose column step of the procedure of Mirhead and Bishop [5].

Nucleotides. β-D-ArabinosylIMP was prepared by enzymatically deaminating β-D-arabinosylAMP according to the method of Miller and Adamczyk [6]. The reaction was incubated to completion in 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethansulfonic acid (HEPES), pH 6.5. The deaminated product was used for the synthesis of β-D-arabinosyladenylosuccinate without further purification since adenylosuccinate was found not to be deaminated by AMP deaminase.

β-D-Arabinosyladenylosuccinate and 2′-deoxyadenylosuccinate were prepared by incubating 100 mg of 2′-dIMP or β-D-arabinosylIMP with 1.7 I.U. of adenylosuccinate synthetase, 0.8 mM GTP, 6.5 mM aspartate, 1.3 mM phosphoenolpyruvate, 100 I.U. pyruvate kinase (Boehringer Mannheim, 200 I.U./mg), 1.7 mM KCl, 8 mM $MgCl_2$, 50 mM HEPES, pH 7.0 in a 300 ml volume at 33° C. This mixture was frozen and lyophilized after the A_{280nm} of the solution stopped increasing. The powder was redissolved in 5 ml H_2O and the solution was filtered. The filtrate was then layered onto a <400 mesh polyacrylamide (Bio-Rad P-2) column (2.5 × 100 cm), equilibrated with H_2O. The products, as detected by thin-layer chromatography (see below) in Solvent 1, eluted with H_2O in the following order: adenylosuccinate analog; IMP analog; GTP. Aspartate overlapped from the end of the adenylosuccinate peak to the beginning of the GTP peak. The fractions containing the desired product were pooled and concentrated by lyophilization to a volume of 1 ml. This solution was applied onto a H_2O-equilibrated Sephadex G-10 column (2.5 × 100 cm) and the column was eluted with H_2O. Fractions containing the adenylosuccinate analog, detected as described above, were pooled, lyophilized and stored as a white hygroscopic powder at −25° C. In both cases, 20—25 mg of product was obtained. These compounds were >95% pure, as judged by high pressure liquid chromatography analysis [7]. These products had ultraviolet spectra that

TABLE I

THIN-LAYER CHROMATOGRAPHY R_F VALUES

Solvent 1 [8]: acetic acid/H_2O/acetone (10 : 40 : 100, v/v); cellulose sheets (Eastman No. 13255). Solvent 2: 0.2 M ammonium formate, pH 5.0; DEAE-cellulose sheets (Eastman cel 300 DEAE). Solvent 3: *n*-butanol/NH_4OH/H_2O (86 : 5 : 14, v/v); cellulose sheets. Solvent 4: Tris/boric acid/H_2O (10.1 g : 1.9 g : 250 ml); cellulose sheets.

Compound	Solvent			
	1	2	3	4
2'-dIMP	0.24			
β-D-ArabinosylIMP	0.20			
GTP	0.0			
Aspartate	0.24			
Adenylosuccinate	0.36	0.13		
2'-Deoxyadenylosuccinate	0.42	0.13		
β-D-Arabinosyladenylosuccinate	0.38	0.13		
AMP		0.48		
2'-dAMP		0.48		
β-D-ArabinosylAMP		0.48		
Adenosine			0.28	0.65
2'-Deoxyadenosine			0.41	0.49
β-D-Arabinosyladenine			0.28	0.48
Succinyladenosine			0.0	0.95
Succinyl-2'-deoxyadenosine			0.0	0.95
Succinyl-β-D-arabinosyladenine			0.0	0.95

were identical to that of adenylosuccinate. The base : phosphate ratio was 1 : 1.04 for 2'-deoxyadenylosuccinate and 1 : 1.1 for β-D-arabinosyladenylosuccinate. Both products co-chromatographed with adenylosuccinate in Solvent 2 and after high voltage electrophoresis [1], but were distinguishable in Solvent 1 (see Table I).

ATP (containing <4% ADP and no AMP), AMP, 2'-dAMP, GTP, 2'-dIMP, IMP, xanthosine 5'-monophosphate, and *Escherichia coli* alkaline phosphatase, 20 I.U./mg were purchased from P.L. Biochemicals; adenylosuccinate from Sigma; β-D-arabinosylAMP from Terra Marine Biochemicals. The sources and purity of the other nucleotides are reported elsewhere [1].

Thin-layer chromatography. Chromatography conditions and R_F values are presented in Table I.

Results

Kinetic constants for the cleavage of β-D-arabinosyladenylosuccinate and 2'-deoxyadenylosuccinate. Both β-D-arabinosyladenylosuccinate and 2'-deoxyadenylosuccinate were rapidly cleaved by adenylosuccinate lyase. The kinetic constants for these compounds and adenylosuccinate are presented in Table II. The K_m value for adenylosuccinate (4.7 ± 0.3 μM) is just slightly lower than that (8.4 ± 1.7 μM) determined by Brox [2] under identical conditions.

Product identification. Reactions individually containing one of the above substrate analogs were incubated with adenylosuccinate lyase for 8 h at 37°C and then for 16 h at 25°C. These mixtures were concentrated 10-fold by lyo-

TABLE II

SUBSTRATES AND PRODUCT INHIBITORS OF ADENYLOSUCCINATE LYASE

Substrate	$V \pm$ S.E. (μmol/min per unit)	$K_m \pm$ S.E. (μM)	V/K_m (relative)	Product	Product inhibition * $K_i \pm$ S.E. (μM)
Adenylosuccinate	1.07 ± 0.01	4.7 ± 0.3	100	AMP	25 ± 4
2'-Deoxyadenylosuccinate	0.82 ± 0.02	3.0 ± 0.3	120	2'-dAMP	5 ± 0.3
Arabinosyladenylosuccinate	0.41 ± 0.01	5.6 ± 0.5	32	ArabinosylAMP	87 ± 12

* The inhibition by AMP, 2'-dAMP and arabinosylAMP was purely competitive with respect to adenylosuccinate.

philization and analyzed for product formation in Solvent 1 (See Table I). This procedure established that these substrate analogs were completely converted to analogs of AMP, but did not distinguish the identities of the products. Product identification was accomplished after converting the reaction products to nucleoside derivatives with alkaline phosphatase. The product of the reaction with 2'-deoxyadenylosuccinate was confirmed as 2'-dAMP since its dephosphorylated derivative co-chromatographed with authentic 2'-deoxyadenosine in Solvent 3. Co-chromatography of the dephosphorylated compound produced from β-D-arabinosyladenylosuccinate with authentic β-D-arabinosyladenine in Solvents 3 and 4 established its identity as β-D-arabinosylAMP.

Inhibition by products and other nucleotides. AMP, the natural product from the adenylosuccinate lyase reaction, was found to compete with either adenylosuccinate, β-D-arabinosyladenylosuccinate or 2'-deoxyadenylosuccinate for binding. Similar K_i values (19—35 μM) were calculated from the data of inhibition experiments with each substrate. These values were also similar to the K_i of 26 μM determined by Brox [2] with adenylosuccinate as the substrate.

β-D-ArabinosylAMP and 2'-dAMP were found to be alternate-product competitive inhibitors of adenylosuccinate lyase. Their inhibition constants are presented in Table II.

Competitive inhibition was also observed with ATP and ADP, but these inhibitors (K_i = 200—300 μM) were weaker than AMP. All the other nucleotides studied, IMP, GMP, xanthosine- 5'-monophosphate, 6-thioGMP and 6-thiIMP, had K_i values >200 μM. Inhibition studies were performed in the absence of Mg^{2+} because no cleavage of adenylosuccinate could be detected in reaction mixtures containing Mg^{2+} at concentrations approaching that of EDTA.

Discussion

An earlier study from this laboratory had demonstrated that although the substrate specificity of adenylosuccinate synthetase was particularly narrow, it was capable of catalyzing the formation of novel adenylosuccinate analogs from reactions with 2'-dIMP or β-D-arabinosylIMP [1]. The present study establishes that these novel analogs, 2'-deoxyadenylosuccinate and β-D-arabinosyl-

adenylosuccinate, can be efficiently converted to 2′-dAMP and β-D-arabinosyl-AMP, respectively, by adenylosuccinate lyase. It was previously demonstrated that 2′-dIMP and β-D-arabinosylIMP can also be converted to 2′-dGMP and β-D-arabinosylGMP, respectively, via sequential oxidation and amination by IMP dehydrogenase [6] and GMP synthetase [9]. However, the substrate efficiencies (V/K_m) of these analogs are significantly lower with the enzymes that convert IMP to GMP than with the enzymes that convert IMP to AMP. The highest relative efficiency is observed with adenylosuccinate lyase. The 2′-deoxyribosyl, β-D-arabinosyl and ribosyl monophosphates of succinyladenine all interact with this enzyme with similar K_m and V values.

It is of interest that both β-D-arabinosylAMP and β-D-arabinosylIMP [10] have been reported to display anti-viral activities. In light of the relatively favorable kinetic constants for the enzymatic conversion of β-D-arabinosylIMP to β-D-arabinosylAMP, it may therefore be possible that β-D-arabinosylIMP can be converted to β-D-arabinosylAMP in vivo.

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