Inhibition of Adenylosuccinase by Adenylophosphonopropionate and Related Compounds†

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ABSTRACT: Adenylosuccinase from muscle, liver, and yeast is strongly inhibited by the substrate analogue adenylophosphonopropionate (N^6-(DL-1-carboxy-2-phosphonoethyl)-adenosine 5′-monophosphate). The inhibition is freely reversible and of the competitive type, with apparent K_I values between 5.4 and 86 nM depending on the source of enzyme. Ratios of K_m/K_I with adenylosuccinate as substrate fall in the range of 44 to 1350. Comparison of four carboxyl analogues of adenylosuccinate with the corresponding phosphonate analogues shows that the phosphonates are much better inhibitors. Adenylosuccinate analogues in which the β-carboxyl is replaced by other functional groups are much poorer inhibitors. The exceptionally high affinity of adenylosuccinase for adenylophosphonopropionate appears to involve the dianion of the phosphonate group.

Adenylosuccinase (adenylosuccinate lyase, EC 4.3.2.2) is an enzyme that catalyzes two separate reactions in the de novo synthesis of AMP (Brox, 1973; Miller et al., 1959; Woodward & Braymer, 1966):

$$\text{adenylosuccinate} \leftrightarrow \text{AMP} + \text{fumarate} \quad (1)$$

$$\text{aminoimidazole succinocarboxamide ribonucleoside 5′-phosphate} \leftrightarrow \text{aminoimidazolecarboxamide ribonucleoside 5′-phosphate} + \text{fumarate} \quad (2)$$

In addition to its role in purine biosynthesis, adenylosuccinase functions in the production of ammonia from aspartate via the purine nucleotide cycle (Bogusky et al., 1976; Goodman & Lowenstein, 1977; Moss & McGivan, 1975; Schultz & Lowenstein, 1976; Tornheim & Lowenstein, 1972). The present paper describes the inhibition of the enzyme by adenylosuccinate analogues. Adenylophosphonopropionate,[1] in which the aspartate moiety of adenylosuccinate is replaced by 3-phosphonoalanine, is shown to be an exceptionally powerful competitive inhibitor of the enzyme.

Materials and Methods

Preparation of Substrates. Adenylosuccinate and DL-mercaptopurinosuccinate were synthesized chemically by the condensation of 6-chloropurine riboside 5′-phosphate with L-aspartate and DL-mercaptosuccinate, respectively, according to Hampton (1962). Substitution of other amino acids for L-aspartate in this method permits facile preparation of the corresponding adenylosuccinate analogues. The compounds prepared in this fashion displayed spectra very similar to that of adenylosuccinate, with λ_{max} at 266–267 nm and yielded equimolar amounts of purine base, ribose, and alkaline phosphatase labile orthophosphate. Treatment of each analogue with 1 N HCl at 100 °C according to Carter (1957) liberated the expected free amino acid. The preparation and characterization of adenylophosphonopropionate are described in detail as an example. Four hundred milligrams (2.37 mmol) of DL-2-amino-3-phosphonopropionic acid (Calbiochem) was added to 8.0 mL of aqueous dimethylformamide (40%, v/v) containing 250 mg (0.62 mmol) of the ammonium salt of 6-chloropurine riboside 5′-phosphate, (P-L Biochemicals), and the pH was brought to approximately 8.5 by addition of 1.25 g of sodium bicarbonate. The mixture was incubated at 23 °C without stirring for 13 days, the supernatant was decanted, the residue was washed once with 2.0 mL of 40% aqueous dimethylformamide, and the solutions were combined and taken to dryness in vacuo. The residue was dissolved in 1.0 mL of 40% aqueous dimethylformamide and the resulting solution was streaked on sheets of Whatman 3 MM paper. Following ascending chromatography in 1-butanol:acetic acid:H_2O (10:4:7), the paper was allowed to dry, and the product was located on the chromatogram by its ultraviolet absorbance (R_f = 0.18) and was eluted with water. Other components of the reaction mixture detected on the chromatogram were 6-chloropurine riboside 5′-phosphate (R_f = 0.45), IMP (R_f = 0.28), and the amino acid (R_f = 0.25). The yield after rechromatography and reelution was 55 mg. The ultraviolet absorption maximum at pH 5.0 occurred at 266.5 nm. An extinction coefficient of 19.9 mM^{-1} cm^{-1} was obtained based on chemically assayed content of ribose (Dische, 1953). The proton NMR spectrum of a 70 mM sample in D_2O was obtained on a Bruker 90 MHz (Model HFX-10) instrument.[2] The spectrum was consistent with that expected for a composite of the purine ribotide and 2-amino-3-phosphonopropionate: δ 2.32 and 2.44 (poorly resolved multiplets, 2 H, $-CH_2PO_3^{2-}$), 6.01 (doublet, J = 4.8 Hz, 1 H, ribosyl 1′-H), 8.15 (singlet, 1 H, purine 2-H), 8.32 (broadened singlet, 1 H, purine 8-H).

R_f values of other compounds synthesized by this method were as follows: L- and D-adenylosuccinate, 0.36; adenylo-

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[1] A list of trivial names used in the text is given in Table I. In addition the following trivial names are used: mercaptopurinosuccinate, S^6-(DL-1,2-dicarboxyethyl)-6-mercaptopurine ribonucleoside 5′-phosphate; adenylophosphonobutyrate, N^6-(DL-1-carboxy-3-phosphonopropyl)-AMP; adenyloethylphosphonate, N^6-(2-phosphonoethyl)-AMP; and phosphonopropionoadenosine, N^6-(DL-1-carboxy-2-phosphonoethyl)adenosine. The nonstandard abbreviations used are: AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside 5′-phosphate; SAICAR, 5-aminoimidazole-4-(*N*-succino)carboxamide ribonucleoside 5′-phosphate.

[2] We wish to thank Joan Goverman for running the NMR spectra.

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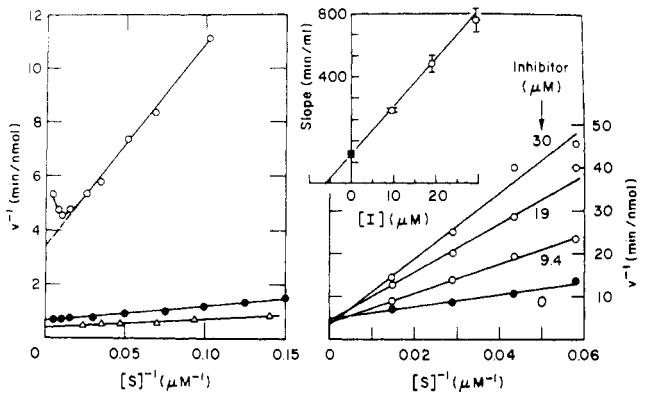

FIGURE 1: (Left) Cleavage of substrates by adenylosuccinase. The enzyme was assayed with adenylosuccinate (●), SAICAR (△), or mercaptopurinosuccinate (○), as described in the Materials and Methods section under Enzyme Assays and Kinetic Studies. (Right) Inhibition of mercaptopurinosuccinate cleavage by adenylosuccinate. The straight lines show the least-squares fit of the data (Cleland, 1967). The inset shows the slopes of these lines vs. [I].

glutarate, 0.39; adenylosulfonopropionate, 0.22; adenylosulfinopropionate, 0.18; adenylopropionate, 0.39; adenylomalate, 0.39; adenylocyanopropionate, 0.40; adenylomethylsulfoxypropionate, 0.32; adenylophosphonobutyrate, 0.23; and adenyloethylphosphonate, 0.23.

The corresponding nucleoside analogues were prepared by the procedure of Hampton (1962), except that 40% aqueous dioxane was used in place of 40% aqueous dimethylformamide. The R_f values of succinoadenosine and phosphonopropionoadenosine were 0.41 and 0.34, respectively, in 1-butanol:acetic acid:water (5:2:3).

SAICAR was prepared enzymatically from AICAR as follows. Reaction mixtures contained 15 μmol of 5-aminoimidazole-4-carboxamide ribonucleoside 3′,5′-monophosphate (Boehringer Mannheim Corp.), and 1 μmol of $MgSO_4$ in 0.5 mL of 120 mM Tris buffer, pH 7.9. The reaction was started by adding 0.3 mg (approximately 0.075 unit) of bovine heart cyclic nucleotide phosphodiesterase (Boehringer Mannheim Corp.); cleavage was complete after 7 h. Under identical conditions cAMP required about 3 h for complete cleavage. The resulting solution containing AICAR was used directly for preparing SAICAR by the method of Lukens & Flaks (1963). SAICAR was purified by ascending paper chromatography on Whatman 3 MM paper using 1-propanol:concentrated NH_4OH:H_2O (10:4:5) as solvent. R_f values in this solvent were: cAMP, 0.60; cyclic AICAR, 0.55; AMP, 0.44; AICAR, 0.38; adenylosuccinate, 0.33; and SAICAR, 0.30. The ultraviolet spectrum of SAICAR was very similar to that of AICAR, with λ_{max} at 267 nm.

DL-2-Hydroxy-3-phosphonopropionate (DL-3-phosphonolactate), previously synthesized by Pfeiffer et al. (1974), was prepared from DL-3-phosphonoalanine by deamination with nitrous acid using the procedure of Schimerlik et al. (1975). Yield of the trisodium salt was 93%. The product gave a negative ninhydrin test, and its NMR spectrum was very similar to that of the amino acid. The phosphonoamino acids used in the synthetic procedures were obtained from Calbiochem.

Enzymes. Adenylosuccinase was partially purified from skeletal muscle of male rats (Charles River). Animals were decapitated and leg muscle was quickly excised, chilled in 0.9% NaCl, and finely minced with scissors. The muscle was homogenized with a motor-driven Potter Elvehjem apparatus in 4 volumes of a mixture containing 90 mM potassium phosphate buffer, pH 6.8, 180 mM KCl, and 1 mM dithiothreitol. The homogenate was centrifuged at 30 000*g* for 40 min. The clear supernatant, which had an activity of 8–12 nmol per mg of protein per min, was fractionated with saturated ammonium sulfate at 0 °C. The fraction obtained between 37% and 50% saturation was suspended in extraction buffer. The resulting solution had an activity of 40–50 nmol per mg of protein per min; it contained no 5′-nucleotidase activity and was satisfactory for use in inhibition and kinetic studies. The preparation is unstable to freezing and thawing and was prepared freshly on the day of use.

Adenylosuccinate synthetase was purified from rat skeletal muscle starting with the same extraction procedure. Ammonium sulfate fractionation of the extract between 44% and 65% saturation at 0 °C resulted in an activity of 8–15 nmol per mg of protein per min, equivalent to a fivefold purification. Three cycles of freezing and thawing removed nearly all adenylosuccinase activity.

Aspartate aminotransferase, alanine dehydrogenase, and glutamate dehydrogenase were obtained from Boehringer. Aspartase was obtained from Sigma. These enzymes were used in kinetic studies without further purification.

Enzyme Assays. Adenylosuccinase was assayed as described previously (Schultz & Lowenstein, 1976). Reaction mixtures contained 20 mM potassium phosphate, pH 7.4, adenylosuccinate, and enzyme in a volume of 0.52 mL. Adenylosuccinate disappearance was followed by measuring the change in absorbance at 310 nm minus 282 nm using a double-beam spectrophotometer (Perkin-Elmer, Model 356). A difference extinction coefficient of 10.0 mM^{-1} cm^{-1} at 282 nm was used to convert absorbance changes to concentrations (Tornheim & Lowenstein, 1972). Assays of mercaptopurinosuccinate cleavage to 6-mercaptopurine riboside 5′-phosphate were performed in the same way except that the reaction was measured at 318 nm minus 298.5 nm, the latter wavelength being the substrate–product isosbestic point (Hampton, 1962). For assays of mercaptopurinosuccinate cleavage in the presence of adenylosuccinate, product formation was followed at 318 nm minus 400 nm; neither adenylosuccinate, nor AMP, nor IMP absorbs significantly at these wavelengths. An extinction coefficient at 318 nm of 20.2 mM^{-1} cm^{-1} was used to convert absorbance changes to concentrations (Hampton, 1962). Cleavage of SAICAR to AICAR was assayed under the same conditions using the spectrophotometric method of Woodward & Braymer (1966). The reaction was followed at 310 nm minus 267 nm. A difference extinction coefficient of 0.7 mM^{-1} cm^{-1} at 267 nm was used to convert absorbance changes to concentrations.

Adenylosuccinate synthetase was assayed as described by Bogusky et al. (1976), except that the buffer was *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid. This buffer is an inhibitor of adenylosuccinase (Muirhead & Bishop, 1974). The following enzymes were assayed according to published procedures: aspartate aminotransferase (Sizer & Jenkins, 1962), aspartase (Williams & Lartique, 1967), alanine dehydrogenase (Yoshida & Freese, 1964), glutamate dehydrogenase (Schultz & Lowenstein, 1976), malate dehydrogenase (Kitto, 1969), and malic enzyme (Ochoa, 1955).

Kinetic Studies. Values for K_m, V_{max}, and K_I were determined from double-reciprocal plots using the computer programs of Cleland (1967) to perform a weighted fit of the data. p*K* values were calculated using a computer program kindly provided by Dr. W. W. Cleland.

Results

Substrate Specificity of Adenylosuccinase. The natural substrates of adenylosuccinase are adenylosuccinate and SAICAR. The enzyme from rat skeletal muscle shows Mi-

TABLE I: Some Inhibitors of Adenylosuccinase.

Systematic name	Trivial name	Parent amino acid	K_I (μM)[a]	K_I/K_m[b]
N^6-(DL-1-Carboxy-2-phosphonoethyl)-AMP	Adenylophosphonopropionate	DL-3-Phosphonoalanine	0.022 ± 0.001	0.003
N^6-(DL-1,2-Dicarboxy-*threo*-2-hydroxyethyl)-AMP	Adenylomalate	DL-*threo*-3-Hydroxyaspartate	3.3 ± 0.07	0.26
N^6-(L-1-Carboxy-2-sulfinoethyl)-AMP	Adenylosulfinopropionate	L-Cysteine sulfinic acid	16 ± 3	1.3
N^6-(L-1-Carboxy-2-sulfonoethyl)-AMP	Adenylosulfonopropionate	L-Cysteic acid	58 ± 3	4.6
N^6-(L-1,3-Dicarboxypropyl)-AMP	Adenyloglutarate	L-Glutamate	44 ± 1	3.5
N^6-(D-1,2-Dicarboxyethyl)-AMP	D-Adenylosuccinate	D-Aspartate	100 ± 5	7.9
N^6-(2-Carboxyethyl)-AMP	Adenylopropionate	β-Alanine	700 ± 34	55
N^6-(L-1-Carboxy-2-methylsulfoxyethyl)-AMP	Adenylomethylsulfoxypropionate	L-*S*-Methylcysteine sulfoxide	180 ± 12	14
N^6-(L-1-Carboxy-2-cyanoethyl)-AMP	Adenylocyanopropionate	L-3-Cyanoalanine	290 ± 60	23
N^6-(L-1,2-Dicarboxyethyl)adenosine	Succinoadenosine	L-Aspartate	780 ± 81	61
L-aspartate			16 000 ± 5000	1260

[a] Mean ± SEM of regression line. [b] K_m value for L-adenylosuccinate of 12.7 ± 0.5 μM determined as described in text. Measurements were made and evaluated as described in the Material and Methods section under Enzyme Assays and Kinetic Studies.

chaelis–Menten kinetics with both substrates (Figure 1). K_m values for adenylosuccinate and SAICAR are 13 ± 1 μM and 8.1 ± 0.4 μM, respectively; V_{max}(SAICAR)/V_{max}(adenylosuccinate) is 1.8. The sulfur-containing analogue of adenylosuccinate, mercaptopurinosuccinate, which had previously been shown to be a substrate of the yeast enzyme (Hampton, 1962), is cleaved by the rat muscle enzyme. The K_m for the racemic compound is 20 ± 1 μM and V_{max}(mercaptopurinosuccinate)/V_{max}(adenylosuccinate) is 0.14. Unlike the natural substrates, this compound displays excess substrate inhibition (Figure 1), with a K_I of 350 μM. The apparent K_I value for the competitive inhibition of mercaptopurinosuccinate cleavage by adenylosuccinate is 7.0 ± 0.3 μM, which is similar to the K_m value (Figure 1).

Inhibition by Substrate Analogues. None of the adenylosuccinate analogues prepared by condensation of various amino acids with 6-chloropurine riboside 5′-phosphate displays measurable activity as substrate when substituted for adenylosuccinate in the standard assay system. However, all of the compounds tested are competitive inhibitors of the rat muscle enzyme with respect to adenylosuccinate (Table I). The sulfur isostere which contains L-cysteine sulfinate instead of the aspartate moiety has an affinity for the enzyme similar to that of adenylosuccinate, but the L-cysteine sulfonate analogue has a much lower affinity for the enzyme. The D isomer of adenylosuccinate is a relatively weak inhibitor. Analogues containing monocarboxylic acids on the 6-amino group of AMP are very weak inhibitors, as is the nucleoside of adenylosuccinate (Table I). Replacement of the aspartate moiety with DL-β-phosphonoalanine yields adenylophosphonopropionate, an inhibitor with a K_I value in the range 22–65 nM (Figure 2). Because the L-aspartate-containing isomer of adenylosuccinate binds to the enzyme about eight times as tightly as the D-aspartate containing isomer, it can be inferred that the corresponding L isomer of the phosphonate analogue has a K_I value in the range 11–33 nM. The analogue also inhibits adenylosuccinase from other sources (Table II), with K_m/K_I ratios ranging from 44 for the yeast enzyme to 1350 for the chicken liver enzyme. Preincubation of the rat muscle enzyme with 3.6 μM adenylophosphonopropionate for 15 min, followed by addition of 400 μM adenylosuccinate, did not result in a greater degree of inhibition than when substrate and inhibitor were added simultaneously to enzyme preincubated with buffer alone. Adenylomalate, which is the β-hydroxy analogue of

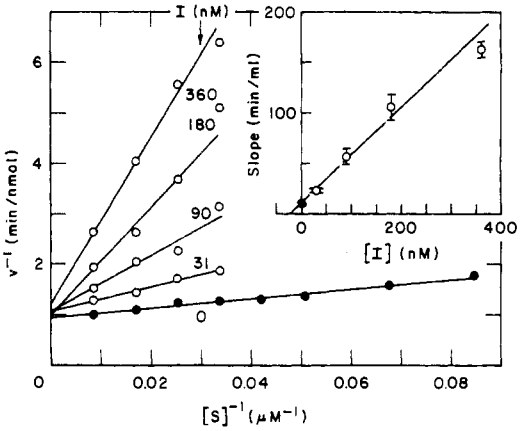

FIGURE 2: Inhibition of adenylosuccinase from rat skeletal muscle by adenylophosphonopropionate (I). The inset shows a plot of the slopes. The straight lines show the least-squares fit of the data (Cleland, 1967). The calculated K_I = 22 ± 1 nM for the inhibitor made from DL-phosphonoalanine. Reaction mixtures contained about 1 mUnit adenylosuccinase. For other details, see Enzyme Assays and Kinetic Studies in the Materials and Methods section.

adenylosuccinate, is a strong inhibitor of the enzyme from every species examined, with K_I/K_m ratios ranging from 3 to 6.

With SAICAR as substrate for adenylosuccinase from rat muscle, adenylophosphonopropionate yielded a K_I value of 85 nM (not shown in Table I).

Mechanism of Inhibition by Phosphonates. The K_m of adenylosuccinase for adenylosuccinate increases with pH (Figure 3), as does the K_I for the phosphonate inhibitor. Although K_m and K_I in general vary with pH in a complex manner reflecting conformational as well as ionization changes in the enzyme and substrates, in the present case pH dependence of the ratio K_m/K_I seems to reflect primarily binding differences between adenylosuccinate and its phosphonate analogue due to protonation of the β-phosphonate moiety of adenylophosphonopropionate. The K_m/K_I ratio increases with increasing pH and shows a pK of 6.6 ± 0.2 (Figure 3), consistent with pK_2 of 6.6–6.8 for 3-phosphonoalanine (Chambers & Isbell, 1964). The pH dependence of the maximum reaction velocity V displays similar behavior, with a calculated pK of 6.5 ± 0.1. The pH dependence of V/K_m indicates that deprotonation of an

TABLE II: Competitive Inhibition of Adenylosuccinase from Various Species by Adenylophosphonopropionate and Adenylomalate.[a]

Enzyme source[b]	A Adenylo-succinate K_m (μM)	B Adenylophos-phonopropionate K_I (μM)	C Adenylo-malate K_I (μM)	D K_m/K_I (A/B)
Rat muscle	13 ± 0.5	0.065 ± 0.001	3.3 ± 0.1	200
Rat liver	10 ± 0.4	0.045 ± 0.001	1.7 ± 0.1	222
Mouse liver	10 ± 0.2	0.025 ± 0.001		400
Chicken muscle	5.3 ± 0.5	0.015 ± 0.002	1.4 ± 0.1	353
Chicken liver	7.3 ± 0.1	0.0054 ± 0.0002	1.2 ± 0.1	1352
Eel muscle	6.6 ± 0.2	0.044 ± 0.002	1.9 ± 0.05	150
Eel liver	8.1 ± 0.1	0.082 ± 0.002	2.9 ± 0.03	99
Brewers' yeast	3.8 ± 0.3	0.086 ± 0.003		44

[a] Trivial names are explained in Table I. [b] Tissue extracts were prepared as described for muscle. Yeast enzyme was purified by the method of Carter & Cohen (1956). Measurements were made and evaluated as described in the Materials and Methods section under Enzyme Assays and Kinetic Studies.

TABLE III: Competitive Inhibition of Rat Muscle Adenylosuccinase by Phosphonate-Containing Substrate Analogues.

Compound	K_I (μM)	Ratio K_I(phosphonate)/ K_I(carboxylate)
Adenylosuccinate[a]	7.0	0.0030
Adenylophosphonopropionate[b]	0.02	
Adenyloglutarate	44	0.0086
Adenylophosphonobutyrate[b]	0.38	
Adenylopropionate	700	0.024
Adenyloethylphosphonate	17	
Succinoadenosine	780	0.090
Phosphopropionoadenosine[b]	70	
L-Aspartate	16 000	0.13
DL-3-Phosphonoalanine	2 000	

[a] K_I determined with mercaptopurinosuccinate as substrate. [b] DL with respect to phosphonoamino acid moiety. Measurements were made and evaluated as described in the Materials and Methods section under Enzyme Assays and Kinetic Studies.

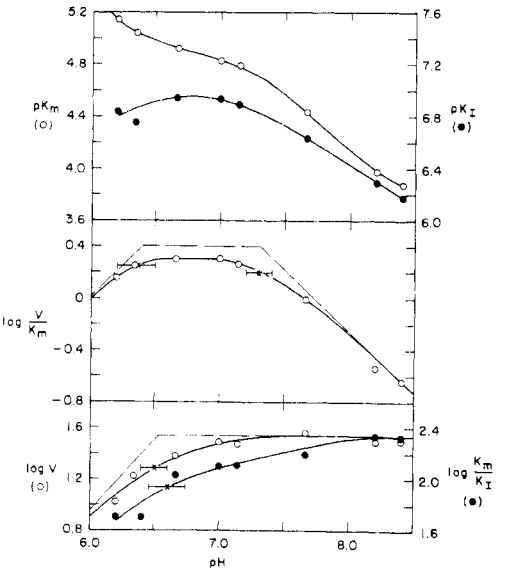

FIGURE 3: Effect of pH on kinetics of adenylosuccinase. Assays were performed using adenylosuccinate as substrate and 20 mM Tricine–pyrophosphate buffer. The curves were calculated according to the equation $V_{max} = V'_{max}/(1 + [H^+]/K_a + K_b/[H^+])$ (Dixon & Webb, 1967) to give the weighted best fit of the experimental points (Cleland, 1967). The pK values calculated by the same method are shown by the symbol X with error bars showing the standard error.

enzyme or substrate moiety with a pK value of 6.3 ± 0.2 results in an increased affinity for the substrate, and that deprotonation of a moiety with a pK value of 7.3 ± 0.1 results in a decreased affinity for the substrate. Adenylosuccinate has no ionizable group with pK = 7.3; however, its 5′-phosphate has a pK_2 in the region of 6.3. Thus the fully ionized form of adenylophosphonopropionate is the species that appears to bind most strongly to adenylosuccinase.

The involvement of the phosphonate moiety in the high affinity binding is indicated by comparing the interaction of adenylosuccinase with several carboxylate inhibitors and their corresponding phosphonate analogues (Table III). In every case the K_I of the phosphonate inhibitor is considerably lower than that of the carboxylate inhibitor. Even the poor inhibitor DL-3-phosphonoalanine displays a lower K_I than L-aspartate, of which it is the phosphonate analogue. Thus the dianion of the phosphonate moiety appears to be largely responsible for the very low K_I observed for adenylophosphonopropionate.

Discussion

Previous studies showed that when the carboxyl group of a substrate or inhibitor is replaced by a phosphonate group the resulting analogues are weak inhibitors with K_I or K_m values at least an order of magnitude greater than the K_m for the corresponding carboxylates (Table IV). The powerful inhibition of adenylosuccinase by adenylophosphonopropionate is thus unprecedented. This observation prompted us to test whether aspartase (L-aspartate ammonia lyase), which catalyzes a reaction very similar to adenylosuccinase, is inhibited by the β-phosphonate analogue of aspartate. Table IV shows that DL-3-phosphonoalanine is a competitive inhibitor with a ratio of $K_I/K_m = 0.16$. Most phosphonates found to be powerful enzyme inhibitors have been methylene phosphonate isosteres of the analogous phosphate esters and anhydrides; examples are 3,4-dihydroxybutyl-1-phosphonate, an inhibitor of α-glycerophosphate dehydrogenase (Cheng et al., 1975); the α,β-methylene phosphonate analogue of ATP, an inhibitor of adenylcyclase (Krug et al., 1973); and the α,β-methylene phosphonate analogue of ADP, an inhibitor of 5′-nucleotidase (Burger & Lowenstein, 1975). Other examples are N-(phosphonoacetyl)-L-aspartate and 4,5-dicarboxy-2-ketopentylphosphonate which are inhibitors of aspartate transcarbamylase (Hoogenraad, 1974; Swyryd et al., 1974).

The basis for the high affinity of adenylosuccinase for ad-

TABLE IV: Interaction of Phosphonate-Containing Substrate Analogues with Carboxyl Binding Sites of Various Enzymes.[a]

Enzyme	Substrate	Analogue inhibitor	Interaction	K_I (mM)	K_I/K_m	References
Adenylosuccinase	L-Adenylo-succinate	Adenylophosphonopropionate	Competitive	0.000022	0.002	This paper
Aspartase	L-Aspartate	DL-3-Phosphonoalanine	Competitive	8.0	0.16	This paper
Arginase	L-Arginine	DL-1-Amino-4-guanidino-butylphosphonate	Competitive	4.5	0.45	La Coste et al., 1972b
Transamidinase	L-Arginine	DL-1-Amino-4-guanidino-butylphosphonate	Noncompetitive	8.4	2.0	La Coste et al., 1972b
Alanine racemase	L-Alanine	DL-1-Aminoethylphosphonate	Competitive	0.55	20	Adams et al., 1974
Malate dehydrogenase	L-Malate	DL-3-Phosphonolactate	Competitive	12	25	This paper
Aspartate aminotransferase	L-Aspartate	DL-2-Amino-3-phosphono-propionate	Competitive	180	42	This paper
Adenylosuccinate synthetase	L-Aspartate	DL-2-Amino-3-phosphono-propionate	Competitive	13	46	This paper
Alanine dehydrogenase	L-Alanine	DL-1-Aminoethylphosphonate	Competitive	150	63	This paper
Tyrosinase	L-Tyrosine	DL-1-Amino-2-(*p*-hydroxy-phenyl)ethylphosphonate	Substrate[b]	26	520	Cassaigne et al., 1967
Argininosuccinate synthetase	L-Citrulline	DL-1-Amino-4-ureidobutyl-phosphonate	None	Large	Large	La Coste et al., 1972a
Glutamate dehydro-genase	L-Glutamate	DL-2-Aminophosphono-butyrate	None	Large	Large	This paper
Malic enzyme	L-Malate	DL-3-Phosphonolactate	None	Large	Large	This paper

[a] In order of increasing K_I/K_m. [b] K_m value. Measurements were made and evaluated as described in the Materials and Methods section under Enzyme Assays and Kinetic Studies.

enylophosphonopropionate appears to lie in the two negative charges of the phosphonate group. Comparison of the inhibition constants of pairs of substrate analogues differing only by the substitution of a phosphonate for a carboxyl group (Table III) shows that adenylosuccinase has a much higher affinity for phosphonates than for carboxyls, and that this difference carries over even to such weak competitive inhibitors as DL-3-phosphonoalanine which is still a better inhibitor than L-aspartate.

The tetrahedral geometry of the phosphonate group is not a factor in this phenomenon since the L-diastereoisomer of DL-adenylophosphonopropionate binds approximately 2600 times more tightly to the rat muscle enzyme than L-adenylosulfonopropionate (Table I); the latter analogue binds less tightly to the enzyme than the corresponding sulfinic acid analogue, L-adenylosulfinopropionate, the trigonal, planar sulfinyl substituent of which closely approximates the β-carboxyl of adenylosuccinate. The dianionic alkyl phosphonate group and the monoanionic alkyl sulfonate group do not differ substantially in interatomic distances, bond angles, or bulk (Darriet et al., 1975; Hendrickson & Karle, 1971). At the pH of the assay, however, the phosphonate group is doubly charged, while the β substituents of all other analogues tested were either singly charged or uncharged. Analysis of the pH dependence of the inhibition by the phosphonate analogue suggests that protonation of the phosphonate group decreases its binding to adenylosuccinase (Figure 3), a further indication of the participation of the phosphonate dianion in the binding phenomenon.

The simplest explanation for the strong interaction of adenylosuccinase with the β-phosphonate analogue is the presence in the β-carboxyl binding domain of two positive charges. These could take the form of a bivalent metal ion, or of two basic amino acids. Two positive charges at the β-carboxyl binding site would facilitate proton abstraction from the β carbon of adenylosuccinate. Interaction of these charges with a phosphonate dianion would be much stronger than with the carboxylate monoanion but would not facilitate proton abstraction from the β carbon. DL-3-Phosphonoalanine is an effective competitive inhibitor of L-aspartase which catalyzes a reaction that is formally similar to the adenylosuccinase reaction (Table III) (Hanson & Havir, 1970; Ratner, 1970). These two enzymes are the only ones presently known that bind phosphonate analogues substantially more tightly than their carboxylate substrates, and they may possess similar β-carboxyl binding sites.

In the case of β-methylaspartase, NMR studies indicate that a carboxyl group of the substrate is coordinated with a bivalent metal ion (Fields & Bright, 1970). The dication is interpreted to assist in delocalizing the electron on the β carbon of β-methyl aspartate and to facilitate production of a carbanion which then eliminates ammonium (Bright, 1967).

Comparison of the interaction of adenylosuccinase with pairs of analogous carboxyl and phosphonate inhibitors (Table III) shows that sites other than the β-carboxyl binding site also play a role in substrate and inhibitor binding. Although the β-phosphonate compounds bind more tightly than the analogous β-carboxyl compounds, the ratio K_I(phosphonate)/K_I(carboxyl) is not the same for different pairs of inhibitors which lack certain functional groups. Elongation of the carboxylate side chain by one carbon atom, as in the case of adenyloglutarate and its phosphonate analogue, does not alter this ratio substantially. However, omission of the α-carboxyl group, as in adenylopropionate and its phosphonate analogue, or omission of the 5′-phosphate, as in succinoadenosine and its phosphonate analogue, or omission of the purine nucleotide moiety, as in L-aspartate and its phosphonate analogue, results in an increased ratio (Table III). The cooperation of various functional groups in promoting binding of the phosphonate group is thus apparent.

The substrate specificity of adenylosuccinase has not been evaluated extensively. In addition to its two natural substrates, adenylosuccinate and SAICAR, the sulfur isostere 6-mercaptopurinosuccinate has been shown to be cleaved by the yeast enzyme (Hampton, 1962). This compound is also a substrate for the mammalian enzyme (Figure 1). None of the other adenylosuccinate analogues tested were substrates. An anionic group at both the α and β positions of the N^6 substituent seems

to facilitate binding since monoanionic N^6 substituents are poor inhibitors (Table I). In contrast, adenylosuccinase from Ehrlich ascites cells displays low specificity for the ribose moiety of the substrate, both the deoxyribosyl and arabinosyl analogues of adenylosuccinate being substrates, with K_m values comparable to adenylosuccinate (Spector, 1977). Similarly, the enzyme from avian liver cleaves the arabinosyl and xylosyl analogues of SAICAR (MacKenzie et al., 1976). Adenylosuccinases from rat muscle (Figure 1) and from chicken liver and Ehrlich ascites cells (Miller et al., 1959; Brox, 1973) have similar affinities for adenylosuccinate and SAICAR. However, adenylosuccinase from yeast has a 100-fold higher K_m for SAICAR than for adenylosuccinate (Miller et al., 1959). The 5′-phosphate is required for binding (Table I), as was reported previously for the yeast enzyme (Hampton, 1962).

Adenylosuccinases from liver and muscle of rat, chicken, and eel (*Anguilla rostrata*) display similar patterns of K_m and K_I values for adenylosuccinate and the inhibitors tested (Table II), indicating that these enzymes are very similar, regardless of their functional specialization for purine biosynthesis de novo, nitrogen elimination via uricogenesis, or the purine nucleotide cycle. The enzyme from chicken liver is significantly more sensitive to inhibition by adenylophosphonopropionate, suggesting that the enzyme from this species differs somewhat in its structural features. Rudolph et al. (1977) reported that adenylosuccinate synthetase from chicken liver displays kinetic properties different from those of the enzyme from chicken muscle and rat liver.

Because adenylophosphonopropionate is a potent and highly specific inhibitor of an enzyme catalyzing two steps in purine biosynthesis, the potential exists for use of this and related compounds as purine antimetabolites.

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