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MAMMALIAN ADENYLOSUCCINATE SYNTHETASE

NUCLEOTIDE MONOPHOSPHATE SUBSTRATES AND INHIBITORS

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Summary

A spectrophotometric assay was developed to investigate the substrate and inhibitor specificity of adenylosuccinate synthetase (IMP:L-aspartate ligase (GDP-forming), EC 6.3.4.4) from rabbit muscle. Ten analogs of inosine 5'-monophosphate (IMP) were studied. Only three of these analogs were substrates of this enzyme. The substrates, listed in order of their substrate efficiency (V/K_m) relative to IMP (100), are: 2'-dIMP (61); β-D-arabinosylIMP (8.8); 6-methoxypurine nucleotide (approx. 0.9).

While the non-substrate analogs of IMP were weak inhibitors of this enzyme, GMP and four of its analogs had K_i values ranging from 30 to 80 μM. The GMP analogs (8-azaGMP, 7-deaza-8-azaGMP, 2'-dGMP and β-D-arabinosylGMP) and GMP were competitive inhibitors with respect to GTP.

Introduction

Inosine 5'-monophosphate (IMP) is the pivotal compound for the metabolic balance between adenine and guanine nucleotides. Many clinically useful base and nucleoside analogs of purines that are metabolized in vivo to compounds that structurally resemble IMP have been investigated with respect to their interactions with the enzymes involved in the bioconversion of IMP to GMP [1,2]. However, very little information is available concerning the ability of these compounds to interact with the enzymes involved in the conversion of IMP to AMP.

Abbreviations used: IMP, inosine 5-monophosphate; allopurinol, 4-hydroxypyrazolo[3,4-d]-pyrimidine; oxipurinol, 4,6-dihydroxypyrazolo[3,4-d]pyrimidine; allopurinol nucleotide, 1-ribosylallopurinol 5'-phosphate; 7-deaza-8-azaGMP, 1-ribosyl-4-hydroxy-6-aminopyrazolo[3,4-d]pyrimidine-5'-phosphate; XMP; xanthosine 5'-phosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid.

The study presented here deals with the first unique step of the latter pathway. The reaction involves the condensation of IMP and L-aspartate and is catalyzed by adenylosuccinate synthetase *. A sensitive spectrophotometric assay was developed to assess the substrate activity of ten analogs of IMP with this enzyme. The inhibitor activity of the non-substrate analogs of IMP and six analogs of GMP were also studied.

Materials and Methods

Adenylosuccinate synthetase. The enzyme was purified from rabbit muscle according to the method of Muirhead and Bishop [3]. Their procedure was followed through the gel filtration step, but Sephadex G-100 gel was substituted for Sephadex G-150. The final enzyme product had a specific activity (as defined in ref. 3) of 4 units/mg. In contrast to their report, the enzyme was found to be completely stable to freezing, thawing and refreezing and was therefore stored at —70° C with no loss of activity during a period of 1 year.

Standard assay conditions. The concentration of reagents described by Muirhead and Bishop [3] were also used in the present studies. These "standard concentrations" were: 50 mM HEPES at pH 7.0; 8 mM $MgCl_2$; 0.125 mM GTP; 4 mM aspartic acid; 0.25 mM IMP. Since GDP is a powerful inhibitor of adenylosuccinate synthetase [3], it was advantageous to include a system that phosphorylates the GDP that was prevalent in the commercial preparation of GTP and/or produced during the enzymatic reaction. This system was either provided by the components of the "coupled assay" or by the following "GTP regeneration reagents"; 2 I.U./ml pyruvate kinase; 0.4 mM phospho*enol*pyruvate; 8 mM KCl. All coupling enzymes were exhaustively dialyzed against 10 mM Tris · HCl, pH 7.6, to remove the $(NH_4)_2SO_4$ that was present in the stock solutions.

Reactions were incubated at 25° C and were initiated with either adenylosuccinate or neutralized aspartic acid. Enzyme units were determined under these conditions and correspond to the amount of enzyme that will catalyze the formation of 1 μmol of product/min. Velocities are expressed as μmol of product formed/min/per unit of adenylosuccinate synthetase and always represent the initial linear portions of the reactions. Kinetic data were analyzed by direct fit to the Michaelis-Menten equation according to the method of Wilkinson [4].

Coupled enzyme assay. The conversion of IMP to adenylosuccinate and concomitant production of GDP was coupled to the oxidation of NADH as mediated via pyruvate kinase and lactate dehydrogenase. These assays were monitored at 340 nm ($\Delta\epsilon = 6.22\ mM^{-1} \cdot cm^{-1}$) or at 360 nm in the presence of thiopurines [6] ($\Delta\epsilon = 4.05\ mM^{-1} \cdot cm^{-1}$) on the 0—0.1 scale of a Gilford recording spectrophotometer equipped with a thermostated cell compartment. The coupling system had the capacity to detect the substrate activity of the IMP analogs without predetermining individual extinction coefficient changes.

* Adenylosuccinate synthetase (IMP:L-aspartate ligase (GDP-forming), EC 6.3.4.4) catalyzes the following reaction:

$$IMP + GTP + L\text{-aspartate} \overset{Mg^{2+}}{\rightleftharpoons} adenylosuccinate + GDP + P_i.$$

Furthermore, the high ultraviolet absorbance of some of the analogs and the stray light problems associated with the direct assay at 280 nm [5] were circumvented. The concentration of the reagents of the 'coupling system' were 5 I.U./ml pyruvate kinase, 10 I.U./ml lactate dehydrogenase, 0.5 mM phospho*enol*pyruvate, 0.2 mM NADH, and 8 mM KCl. All coupling enzymes were exhaustively dialyzed as described above. The properties of this coupling system are presented in Results.

Radiochemical assay. This assay was used for the product confirmation and some of the inhibitor studies. The reaction conditions were standard with the exceptions that the concentration of IMP is indicated for each experiments and uniformly labeled [^{14}C]aspartic acid (2 Ci/mol) was used. Reactions were performed in a total volume of 60 μl and were terminated by addition of 5 μl of 0.2 M EDTA, pH 7, containing 5 mM adenylosuccinate carrier. Samples of the mixtures (20—40 μl) were then subjected to high voltage electrophoresis at 3500 V for 40 min in 50 mM citrate at the pI of aspartic acid, pH 2.8. A typical chromatogram resulted in [^{14}C]aspartic acid remaining at the origin and [^{14}C]adenylosuccinate traveling 9.6 cm towards the anode. IMP, GDP and GTP also migrated in the anodal direction traveling 12.4, 15.3 and 18.1 cm, respectively. The [^{14}C]aspartic acid was visualized with ninhydrin spray and the [^{14}C]adenylosuccinate by ultraviolet light. The spots were either cut out and counted in a liquid scintillation counter as previously described [6], or the chromatograms were scanned with a Nuclear-Chicago Actigraph III radioscanner.

Nucleotides. Allopurinol nucleotide was a product of Kyowa Hakko Kogyo Co., Tokyo, Japan. IMP, GMP, 2′-dGMP, 2′-dIMP, 8-azaGMP, 6-thioIMP (converted to the sodium salt on a Dowex 50-X8 (sodium) column) and 6-chloropurine nucleotide were purchased from P-L Biochemicals; and 3′-5′-cyclic IMP and 3′-5′-cyclic GMP from Sigma Chemical Co.

β-D-ArabinosylIMP, β-D-arabinosylGMP, and 8-azaIMP were synthesized according to the procedure described by Miller and Adamczyk [1]. 6-Methylthiopurine nucleotide was synthesized by L.M. Beacham, III of these laboratories by a published procedure [7]. 6-Methoxypurine nucleotide was prepared by Wayne Miller of these laboratories using a known procedure [8].

6-ThioGMP was chemically synthesized and 7-deaza-8-azaGMP was enzymaticlly synthesized according to the procedures previously described [6], but the latter compound was isolated as the lithium salt by the following method. Protein was removed from the reaction mixture by precipitation with trichloroacetic acid (3%) and centrifugation; and trichloroacetic acid was removed by extraction with five volumes of ether. The nucleotide was then adsorbed onto a Dowex 1-X8 column (2 × 20 cm) and eluted with a linear gradient of 0—1 M LiCl containing 1 mM HCl. The fractions containing 7-deaza-8-azaGMP (R_f 0.8; cellulose thin-layer chromatography in 5% $(NH_4)SO_4$/isopropanol (19 : 1, v/v)) were neutralized with LiOH and lyophilized. LiCl was removed by extraction with acetone/methanol (9 : 1, v/v). The remaining precipitate was then redissolved, applied to a Bio-Rad P-2 column (2.5 × 90 cm) equilibrated with 2 mM LiCl, pH 6.8, and eluted with the same solvent. Fractions containing the nucleotide were lyophilized and the LiCl was again extracted as described above. The final product had a ribose : phosphate ratio of 1.0 : 1.0, was de-

phosphorylated by 5′-nucleotidase and appeared >98% homogeneous when analyzed as described below.

All IMP and GMP analogs that demonstrated either substrate or inhibitor activity with adenylosuccinate synthetase were analyzed for purity by high-pressure liquid chromatography [9]. The approximate percent homogeneity was: IMP, 2′-dIMP, β-D-arabinosylIMP, 8-azaIMP, 6-methoxypurine nucleotide, allopurinol nucleotide, 6-methylthiopurine nucleotide, XMP, 6-thioGMP, GMP, 2′-dGMP, 8-azaGMP β-D-arabinosylGMP and 3′,5′-cyclic GMP > 99; 7-deaza-8-azaGMP > 98; 6-thioIMP > 91; 6-chloropurine nucleotide > 86. These analyses revealed that the nucleotides of 6-chloropurine and 6-thiopurine may be contaminated with 0.01—1% IMP.

Other chemicals. Pyruvate kinase (No. 15744), lactate dehydrogenase (No. 15372) and phospho*enol*pyruvate were purchased from Boehringer Mannheim Corp.; GTP and NADH from P-L Biochemicals; uniformly labeled [^{14}C]aspartic acid (232 Ci/mol) from Amersham/Searle.

Results

Validity of the coupled enzyme assay. When the adenylosuccinate synthetase reaction was coupled to the oxidation of NADH (see Materials and Methods) the observed velocities were linear with respect to both time and the concentration of adenylosuccinate synthetase. Furthermore, these velocities were within 10% of those determined by the direct spectral assay [3] provided that the "GTP-regenerating reagents" were included in the direct assay. As demonstrated in Fig. 1, one mol of NADH was oxidized for every mol of IMP added

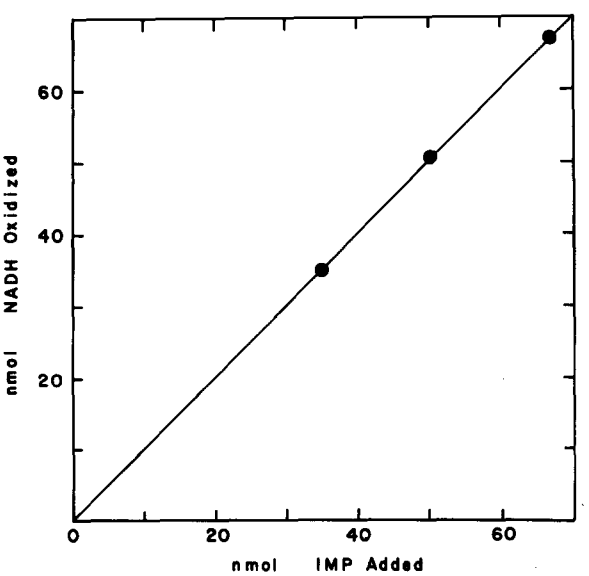

Fig. 1. Stoichiometry of the coupling of the oxidation of NADH to the formaton of adenylosuccinate. The "coupled enzyme" assay was used with the reagents at their "standard concentration" plus the above indicated amounts of IMP and 0.005 unit of adenylosuccinate synthetase. Reactions were initiated with aspartic acid and monitored until completion. The A_{340nm} was recorded before and after the reaction. The slope of the plot is 0.997.

TABLE I

SUBSTRATE SPECIFICITY OF ADENYLOSUCCINATE SYNTHETASE FROM RABBIT MUSCLE

K_m and V values were determined with the other substrates fixed at their "standard concentrations". See text for details.

Nucleotide	K_m (± S.E.) (mM)	V (± S.E.) (μmol/min per unit)	Relative V/K_m
IMP	0.32 ± 0.05	2.23 ± 0.21	100
2′-dIMP	0.26 ± 0.10	1.15 ± 0.30	61
β-D-ArabinosylIMP	0.85 ± 0.19	0.54 ± 0.38	8.8
6-Methyloxypurine nucleotide	>30	>2	Approx. 0.9
XMP	—	<0.001 *	—
8-AzaIMP	—	<0.001 *	—
6-ThioIMP	—	<0.001 *	—
6-Methylthiopurine nucleotide	—	<0.001 *	—
6-Chloropurine nucleotide	—	<0.001 *	—
Allopurinol nucleotide	—	<0.001 *	—
3′,5′-cyclic IMP	—	<0.001 *	—

* No velocity was detected with these compounds at 0.5 or 1.0 mM and adenylosuccinate synthetase at 0.008—0.01 unit/ml. The minimal detectable velocity was 0.1% the velocity observed with IMP as the substrate.

to the reaction. Blank rates, measured with the complete reaction mixture less aspartic acid, were obtained for every analog studied. In all cases these rates were negligible ($\Delta A < 0.0004$ min^{-1}).

Substrates of adenylosuccinate synthetase. The coupled enzyme assay was used to detect the substrate activity of the IMP analogs. If an analog was observed to induce the oxidation of NADH, initial velocity measurements were then carried out over a range of concentrations in order to determine K_m and V values for that analog. When no oxidation of NADH was noted, the concentration of the analog was raised to 1 mM and adenylosuccinate synthetase was raised to a level that would permit the detection of a velocity as low as 0.1% of the velocity that would have been observed with IMP.

This type of study produced a K_m value for IMP of 0.32 ± 0.05 mM which was similar to the value of 0.2 ± 0.1 mM previously determined [3] by the direct spectral assay. Only three of the ten analogs studied were found to be substrates *. Weak substrate inhibition was observed with IMP and 2′-dIMP at concentrations above their K_m values. The high K_m values of β-D-arabinosylIMP and 6-methoxypurine nucleotide discouraged velocity determinations in the concentration ranges where this type of inhibition may be expected. The kinetic constants for the substrates are summarized in Table I.

* A short lived burst of NADH oxidation was initially observed when either 6-chloropurine nucleotide or 6-thioIMP were assayed. The total absorbance change represented between 0.1 and 1% of the concentration of the analog. Since this burst could be reproduced upon the addition of more analog, but not with more adenylosuccinate synthetase, it is suggested that these analogs may contain traces of IMP. High pressure liquid chromatographic (see Materials and Methods) analysis supports this proposal.

Product confirmation. Radiolabeled product formation was used to confirm the results of the above spectral data. The reaction mixtures containing [^{14}C]-aspartic acid and the IMP analog (see Materials and Methods) were incubated until all the GTP and phosphoenolpyruvate were consumed. Samples of the reaction mixtures were then chromatographed by high voltage electrophoresis and the chromatograms were scanned for their radioactivity. Radiolabeled peaks located in the region of adenylosuccinate were observed for reactions containing IMP, 2′-dIMP, β-D-arabinosylIMP or 6-methoxypurine nucleotide. Omission of either the analog or adenylosuccinate synthetase resulted in all the radioactivity remaining at the origin with aspartic acid (see Fig. 2 for examples). Therefore, it is assumed that 2′-dIMP, β-D-arabinosylIMP and 6-methoxypurine nucleotide were converted to 2′-d-adenylosuccinate, β-D-arabinosyladenylosuccinate and adenylosuccinate, respectively.

Inhibitors of adenylosuccinate synthetase. The IMP analogs devoid of sub-

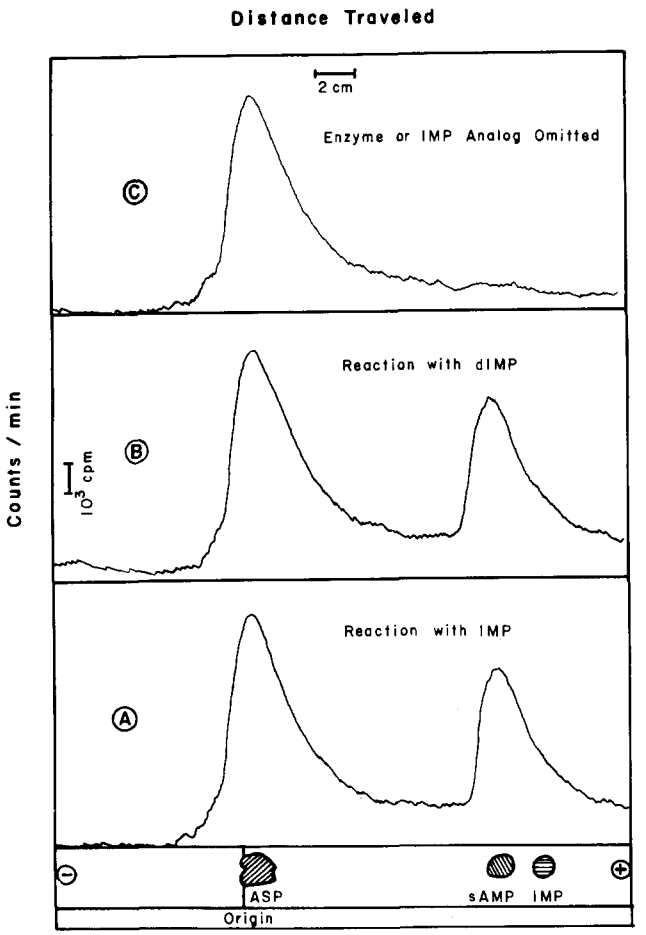

Fig. 2. Radiolabeled confirmation of the aspartylation of IMP and 2′-dIMP by adenylosuccinate synthetase. See text for details.

TABLE II

INHIBITION OF ADENYLOSUCCINATE SYNTHETASE BY ANALOGS OF IMP

Nucleotide	Percent of inhibition at *		Estimated K_i ** (mM)
	0.5 mM	2.0 mM	
6-ThioIMP	43	85	0.4
XMP	33	69	0.7
Allopurinol nucleotide	19	59	1.2
8-AzaIMP	18	42	1.7
6-Methoxypurine nucleotide	6	38	3.5
6-Chloropurine nucleotide	8	32	3.4
3',5'-Cyclic IMP	0	0	—

* Standard reactions were performed in duplicate using the radiochemical assay described in Materials and Methods. The concentration of IMP was 0.15 mM (approximately half its K_m).

** Calculated from the equation:

$$\text{percent inhibition} = \frac{100\,[I]}{[I] + K_i\left(1 + \frac{[\text{IMP}]}{K_m}\right)}$$

assuming that the analogs were competitive inhibitors with respect to IMP.

strate activity with adenylosuccinate synthetase were tested for their ability to inhibit this enzyme. The results are summarized in Table II. Clearly, none of these compounds were strong inhibitors and only 6-thioIMP and XMP had K_i values that were of comparable magnitude to the K_m of IMP.

In contrast to the IMP-analogs, the GMP analogs were considerably stronger inhibitors and therefore warranted a more detailed investigation. GMP was found to be a competitive inhibitor with respect to GTP and to have a K_i of 0.035 ± 0.005 mM. This value is very similar to the K_m value of GTP determined in this study (0.024 ± 0.005 mM) and that previously reported (0.01 ± 0.005 mM) [3]. The results of the inhibitor studies with the analogs of GMP are summarized in Table III.

TABLE III

INHIBITION OF ADENYLOSUCCINATE SYNTHETASE BY ANALOGS OF GMP

With the exception of GMP K_i values were determined from Dixon plots [10] performed with the concentration of GTP fixed at 0.029 and 0.043 mM. The coupled enzyme assay was used (see Materials and Methods).

Nucleotide	Nature of binding with respect to GTP	K_i (mM)
GMP	competitive	0.035
8-AzaGMP	competitive	0.03
7-Deaza-8-azaGMP	competitive	0.03
2'-dGMP	competitive	0.05
β-D-ArabinosylGMP	competitive	0.08
6-ThioGMP	non-competitive *	0.2
3',5'-Cyclic GMP	not determined	1—5

* 6-ThioGMP was also a non-competitive inhibitor with respect to IMP. In both cases the K_i was approx. 0.2 mM and the lines were curvilinear upwards.

Discussion

Earlier reports of the substrate specificities of IMP dehydrogenase [1] and GMP synthetase [2] from mammalian sources have discussed the likelihood by which an IMP analog would be converted to a XMP analog and subsequently to a GMP analog. This discussion can now be expanded to include the efficiency with which these IMP analogs can be converted to form precursors of AMP.

This composite of enzyme studies demonstrates that β-D-arabinosylIMP is an efficient substrate of IMP dehydrogenase [1] as well as adenylosuccinate synthetase. Furthermore, β-D-arabinosylXMP shows similar substrate efficiency with GMP synthetase [2]. A consistency is thus noted between these data and the earlier claim that both β-D-arabinosylGMP and β-D-arabinosylAMP are metabolically produced in mouse liver pretreated with hypoxanthine arabinoside [11].

Similar results are also observed with 2′-dIMP which can be converted to either 2′-dGMP [1,2] or 2′-d-adenylosuccinate with the highest substrate efficiency of all the analogs studied. However, the physiological significance of the 2′-deoxynucleotide monophosphates is presently unclear.

The 6-thio and 8-aza analogs of IMP are substrates for IMP dehydrogenase [1] and in turn their corresponding XMP analogs are substrates for GMP synthetase [2], but neither 6-thioIMP nor 8-azaIMP are substrates for adenylosuccinate synthetase. The inability of adenylosuccinate synthetase to convert 6-thioIMP to a physiological purine suggests that this enzyme does not contribute to the previously reported in vivo dethiation of 6-mercaptopurine [12].

The finding that 8-azaIMP is not a substrate of adenylosuccinate synthetase is inconsistent with the claim that 8-azaAMP is metabolicially produced from 8-azainosine in both H.Ep. No 2 and Ca 755 cells [13]. This discrepancy could possibly be explained if adenylosuccinate synthetase from these cell lines has a different substrate specificity than that from the rabbit muscle.

Metabolic studies have shown that in addition to 6-thioIMP, 6-methylthiopurine nucleotide is also formed from 6-mercaptopurine [14]. This nucleotide was neither a substrate for IMP dehydrogenase [1] nor adenylosuccinate synthetase, but is converted to di- and tri-phosphate nucleotides in human tissue [15,16]. Allopurinol nucleotide is similar to 6-methylthiopurine nucleotide in that it also is devoid of substrate activity with both IMP dehydrogenase [1] and adenylosuccinate synthetase.

Analogs of IMP which were devoid of substrate activity with adenylosuccinate synthetase were also unimpressive inhibitors. 6-ThioIMP, the best inhibitor of this category was found to have an approximate K_i of 0.4 mM which is identical to the value reported for the inhibition of adenylosuccinate synthetase from Ehrlich ascites cells [17]. However, since treatment of animals with 6-mercaptopurine produces concentrations of 6-thioIMP that are less than 0.4 mM, it is believed that the pharmacological action of 6-mercaptopurine is not related to its ability to inhibit this enzyme [18].

On the other hand, nucleotides binding to the GTP site were potentially effective inhibitors. In addition to GMP, which was previously shown to inhibit adenylosuccinate synthetase from other mammalian sources [19,20], a number of its analogs have very similar affinity for the GTP binding site (K_i range from

30 to 80 μM). It appears that the basic requirement is that the analog contains amino and hydroxyl substituents in positions that correspond to those substituents on GMP and that the phosphate group is not cyclized.

Although the IMP binding site has a considerably narrower specificity than the GTP site, three analogs of IMP were substrates of this enzyme. Studies are presently in progress to determine whether these condensed intermediates are substrates for adenylosuccinate lyase, the enzyme that converts adenylosuccinate to AMP.

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