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GMP SYNTHETASE FROM *ESCHERICHIA COLI* B-96

INTERACTIONS WITH SUBSTRATE ANALOGS

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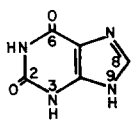
SUMMARY

GMP synthetase was studied with respect to its substrate and inhibitor specificity towards nine xanthosine 5′-phosphate (XMP) and GMP analogs 8-AzaXMP and 6-thioXMP were found to be substrates, but were considerably less efficient than XMP 1-Ribosyloxipurinol 5′-phosphate was aminated with an efficiency of less than 1/18 000 that of XMP The aminated products of the reactions were competitive inhibitors, with respect to XMP

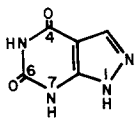
INTRODUCTION

The only known biosynthetic pathway for the formation of guanine occurs at the monophosphate ribonucleotide level via the amination of xanthosine 5′-phosphate (XMP) In view of this, the potential amination of various metabolically produced XMP analogs (see Fig 1 for basic ring structures) to form GMP analogs could be an important reaction The enzyme that catalyzes this reaction, GMP synthetase, (xanthosine 5′-phosphate ammonia ligase (AMP), EC 6 3 4 1), has been purified from calf thymus [1], pigeon liver [2], and bacteria [3, 4] The *Escherichia coli* enzyme can be derepressed [5] and purified to apparent homogenity [4] Studies concerning its physical properties [4] catalytic intermediate [6], and the nature of its inhibition by psicofuranine and decoyenine [5–9] have been reported However, with the exception of a footnote referring to the substrate properties of dXMP [10], no information has been available concerning the interactions of XMP analogs with this enzyme In the present study, a number of XMP analogs and their corresponding GMP analogs were synthesized, a sensitive substrate activity assay was developed, and the interactions of these ribonucleotides with GMP synthetase were investigated

Abbreviations XMP, xanthosine 5′-phosphate, 1-Oxi-5′-*P* (1-ribosyloxipurinol 5′-phosphate), 1-ribosyl-4,6-dihydroxypyrazolo[3,4-d]-pyrimidine 5′-phosphate, 7-Oxi-5′-*P* (7-ribosyloxipurinol 5′-phosphate), 7-ribosyl-4,6-dihydroxypyrazolo[3,4-d]pyrimidine 5′-phosphate, 6-NH_2-1-Alo-5′-*P* (6-NH_2-1-ribosylallopurinol 5′-phosphate), 1-ribosyl-4-hydroxy-6-aminopyrazolo[3,4-d]pyrimidine 5′-phosphate, 3-XMP 3-ribosylxanthosine 5′-phosphate



XANTHINE



OXIPURINOL

(4,6-DIHYDROXYPYRAZOLO[3,4-d]PYRIMIDINE)

Fig 1 Basic ring structures and numbering systems for xanthine and its analogs

MATERIALS AND METHODS

Enzyme purification and assays

GMP synthetase was purified from *E coli* B-96 (ATCC No 13473) according to the method of Sakamoto et al [4] The specific activity was similar to the reported value Assays were performed by monitoring the formation of AMP according to a modification of the coupled enzyme system of Kornberg and Pricer [11] In this assay the production of 1 μmole of GMP is accompanied by the production of 1 μmole of AMP and the concomitant oxidation of two μmoles of NADH ($\Delta\varepsilon_{340\ nm} = 12\ 44$ $mM^{-1}\ cm^{-1}$) For the standard reaction, the cuvettes contained 75 mM Tris–HCl buffer (pH 8 5), 0 125 mM XMP, 1 25 mM ATP, 60 mM $(NH_4)_2SO_4$, 10 mM $MgSO_4$ The following coupling reagents were also present 0 025 mg/ml of myokinase, 0 025 mg/ml of pyruvate kinase, 0 025 mg/ml of lactate dehydrogenase, 0 5 mM phosphoenolpyruvate, 0 2 mM NADH Reaction velocities were monitored on the 0–0 1 absorbance scale of a Gilford recording spectrophotometer at 37 °C Due to the high absorbance of 6-thioXMP and 6-thioGMP at 340 nm, reactions with these compounds were monitored at 360 nm ($\Delta\varepsilon = 8\ 1\ mM^{-1}\ cm^{-1}$) where 6-thioXMP and 6-thioGMP were found to be isosbestic ($\varepsilon = 1\ 1\ mM^{-1}\ cm^{-1}$) Velocities measured at 340 or 360 nm were found to be linear with respect to time (following a brief lag period) and concentration of GMP synthetase Molar velocities determined in this manner agreed within 10% of those determined by the direct spectral assay of Moyed and Magasanik [3] (Assay b), and a radiochemical assay using [8-^{14}C]XMP performed under identical conditions, but omitting the coupling reagents Reactions involving ^{14}C-labeled compounds were terminated with HCl (0 4 M final concn), and the ribonucleotides were hydrolyzed to their corresponding bases by heating the resulting solutions at 100 °C in sealed vials for 1 h The complete hydrolysis of 3-[8-^{14}C]XMP required 2 3 M HCl and heating for 3 h The reaction mixtures were then spotted on Whatman 3 MM paper and chromatographed in an ascending direction in water saturated butanol–NH_4OH (99 1, v/v) for 17 h The spots containing the uv absorbing bases (R_F values xanthine, 0 03, guanine, 0 13, 4,6-dihydroxypyrazolo-[3,4-d]-pyrimidine (oxipurinol), 0 12, 4-hydroxy-6-aminopyrazole[3,4-d]-pyrimidine, 0 23) were cut out and counted for their radioactivity in toluene–Triton-X–Omnifluor (30 10 0 28, v/v/w) scintillation fluid In all assays, the reactions were initiated with GMP synthetase A unit of enzyme is that quantity which can produce 1 μmole of GMP per min Under these standard conditions, 1 unit was previously found to be equal to 2 nmoles of enzyme [6, 7]

Preparation of ribonucleotides

6-ThioXMP was synthesized enzymatically by incubating 6-thioxanthine and

5-phosphoribosyl 1-pyrophosphate with partially purified guanine-xanthine phosphoribosyltransferase from *E coli* B The enzyme was prepared by precipitation from a streptomycin sulfate treated extract with 80% $(NH_4)_2SO_4$ and redissolved in buffer as described elsewhere [12] The product was isolated from the deproteinized and diluted reaction mixture by adsorption onto an ECTEOLA-cellulose column equilibrated with 0 1 mM LiCl containing 0 1 mM dithiothreitol The column was washed with a linear gradient of 0 1–200 mM LiCl containing 0 1 mM dithiothreitol (500 ml gradient/33 ml bed vol) The 6-thioXMP was eluted by a continuation of the 200 mM LiCl The fractions containing the major portion of the uv absorbing (341 nm) material were concentrated by lyophilization and the LiCl extracted with acetone–methanol (9 1, v/v) The isolated ribonucleotide eluted as a single symmetrical peak when subjected to high-pressure liquid chromatography, had a base phosphate ratio of 0 9 1, and was dephosphorylated by 5′-nucleotidase

6-ThioGMP was prepared by two distinct methods The first method was the chemical phosphorylation of 6-thioguanosine and is described elsewhere [13] The other approach utilized the enzymatic synthesis from 6-thioguanine and 5-phosphoribosyl 1-pyrophosphate with an extract of Ehrlich ascites acetone powder according to a previously reported procedure [14] The products from each synthetic procedure were concentrated by lyophilization and applied to a 10 mM β-mercaptoethanol equilibrated BioRad P-2 column and eluted with the same buffer The enzymatically synthesized 6-thioGMP required further purification This was accomplished by thin-layer chromatography on a preparative cellulose plate in *n*-propanol–water (7 3, v/v) containing 10 mM β-mercaptoethanol The product (R_F 0 15) was eluted with water and lyophilized The two preparations were found to be indistinguishable and $> 99\%$ pure as analyzed by high-pressure liquid chromatography The chemically and enzymatically synthesized products had base ribose phosphate ratios of 1 00 1 01 1 01 and 1 00 0 94 0 96, respectively Both products were converted to 6-thioguanosine by 5′-nucleotidase, and chromatographed as one spot on DEAE-cellulose thin-layer plates in 0 2 M ammonium formate (pH 5 0) (R_F 0 33)

1-[6-^{14}C]Oxi-5′-*P* was synthesized by the same procedure used for 6-thioXMP The precursor base, [6-^{14}C]Oxipurinol was enzymatically synthesized [15] from [6-^{14}C]allopurinol that had been chemically synthesized [16] After incubation with the phosphoribosyltransferase, the ribonucleotide product was concentrated by lyophilization and was purified by the same procedure described above for the enzymatically synthesized 6-thioGMP (in the absence of β-mercaptoethanol) The product was found to be 99% radiochemically pure as analyzed by high-pressure liquid chromatography and thin-layer chromatography on DEAE-cellulose in 0 2 M ammonium formate, pH 5 0 (R_F 0 21)

6-NH_2-1-Alo-5′-*P* was enzymatically synthesized by the same procedure used for 6-thioXMP The product was then chromatographed on a ECTEOLA-cellulose column in a manner similar to that for 6-thioXMP with the exception that dithiothreitol was absent and the ribonucleotide eluted during the LiCl gradient This column was repeated two additional times using a 0 1–100 mM linear gradient (400 ml gradient/82 ml bed volume) The last ion-exchange chromatography step was followed by gel filtration with a water equilibrated BioRad P-2 column The product was concentrated to a small volume by lyophilization and collected as a barium salt by treatment with 1 vol of 2 0 M $BaClO_4$ and 2 vol of 95% ethanol High-pressure liquid

chromatography of the ribonucleotide resulted in a single symmetrical peak

8-AzaXMP was enzymatically synthesized from 8-azaxanthine and 5-phosphoribosyl 1-pyrophosphate using partially purified xanthine phosphorybosyltransferase from *Streptococcus faecalis* The enzyme was purified according to a reported procedure [17] through the first $(NH_4)_2SO_4$ precipitation The product was isolated from the deproteinized and diluted reaction mixture by batch adsorption to BioRad AG-1-X8 (chloride form) anion exchange resin equilibrated with 0 1 mM LiCl The gel was poured into a column and eluted with a linear gradient of 0 1–1000 mM LiCl (200 ml gradient/approx 3 ml bed volume) The fractions containing the 8-azaXMP (ultraviolet absorbing material) were lyophilized and the LiCl extracted with acetone–methanol (9 1, v/v) The product was then chromatographed on a DEAE-cellulose (Whatman DE-52) column equilibrated with 3 mM ammonium formate (pH 4 4), eluting with a linear gradient from 3 to 300 mM of the same salt (600 ml gradient/20 ml bed volume) The ribonucleotide was concentrated and the salt concentration decreased by repeated lyophilization and the product chromatographed on a water-equilibrated BioRad P-2 column, eluting with water The final product was found to be $> 99\%$ pure as analyzed by high-pressure chromatography and its base ribose phosphate ratio was 1 00 1 01 0 99

[8-^{14}C]XMP was synthesized in the same manner as 8-azaXMP using [8-^{14}C]-xanthine (Schwartz/Mann) The product was isolated with the thin-layer chromatographic procedure described above for the enzymatically synthesized 6-thioGMP The product chromatographed on thin-layer cellulose plates in 5% Na_2HPO_4–isoamylalcohol (2 1, v/v) as one spot, which contained all the radioactivity, (R_F 0 84) The labeled XMP could be completely converted to [8-^{14}C]GMP by GMP synthetase

1-Oxi-5′-*P*, 7-Oxi-5′-*P* and 3-[8-^{14}C]XMP were synthesized and purified according to a previously reported procedure [18]

Molarity of ribonucleotides

Ribonucleotide concentrations were determined spectrally from published extinction coefficients [18–22]

High-pressure liquid chromatography

Ribonucleotides were analyzed with a Varian LCS-1000 equipped with a P4-38 anion exchange column, eluting with an ammonium acetate gradient from 0 175 M (pH 5 5) to 5 M (pH 4 7) The details of this procedure are described elsewhere [23]

Other materials

XMP, ATP, GMP NADH, NAD, and AMP were purchased from P L Biochemicals, cyclic XMP and 5′-nucleotidase *(Crotalus adamanteus)* from Sigma, phosphoenolpyruvate, myokinase (No 15355), pyruvate kinase (No 15744), and lactate dehydrogenase (No 15372) from Boehringer Mannheim Corp

RESULTS

Substrates

The kinetic parameters derived from data obtained with the coupled spectral assay are presented in Table I The K_m value of 0 058 mM for XMP was reproduced

TABLE I

INTERACTIONS OF RIBONUCLEOTIDES WITH GMP SYNTHETASE

V and K_m values were determined from computer analyzed Lineweaver–Burk graphical presentations of data obtained from the standard coupled assay (described in the text) varying the concentration of the analog The concentration of GMP synthetase was 6 nM for the reactions with XMP, 26 nM for 8-azaXMP 130 nM for 6-thioXMP, and 300 nM (spectral) and 927 nM (radiolabeled) for 1-Oxi-5′-P Where no velocity was detected, the analog was at a concentration of 1–2 mM under conditions where a lower limit of 3 pmoles/min could be detected and 23 000–120 000 pmoles/min of XMP would have been aminated

Compound	V (turnover/min)	K_m (mM)	K_i* (mM)	V/K_m	Product**	K_i* (mM)
XMP	725	0 058	—	12 500	GMP	0 33
8-AzaXMP	92	0 35	—	263	8-AzaGMP	0 40
6-ThioXMP	60	0 42	0 38	143	6-ThioGMP	2-3
1-Oxi-5′-P	1 3	2	3	0 7	6-NH_2-1-Alo-5′-P	—
3-XMP	No velocity detected	—	>10			
7-Oxi-5′-P	No velocity detected	—	>5			
Cyclic XMP	No velocity detected	—	>5			

* Competitive with respect to XMP Analogs marked "> 10" at a concentration of 2 mM inhibited the amination of XMP (at 0 05 mM) by less than 10% Those marked "> 5" were assayed at 1 mM

** The products were analyzed by high-pressure liquid chromatography (see Materials and Methods) The comparison of the retention times of the products of the reaction mixtures with the retention times of authentic compounds under conditions where individual peaks for all substrate and product nucleotides could be clearly distinguished served to verify the formation of these compounds

within 5% in three separate experiments The K_m value for XMP previously reported under these conditions was 0 034 mM [10] 8-AzaXMP and 6-thioXMP were also found to be substrates With 6-thioXMP, the lag period, which is typically brief for the other substrates (seconds), was considerably extended (approx 2 min) However, a double reciprocal plot of the velocities recorded after the lag period versus the concentration of 6-thioXMP resulted in a straight line Substrate activity for 1-Oxi-5′-*P* was detected only when this compound and the enzyme were incubated at high concentrations Under these conditions a very slow blank rate (1-Oxi-5′-*P* omitted) was also present and was therefore subtracted from the rate in the presence of the analog At low concentrations of 1-Oxi-5′-*P*, the blank rate and the catalytic rates were of a similar order of magnitude The Lineweaver–Burk plot determined from these data was curvilinear upward at concentrations of 1-Oxi-5′-*P* below 1 0 mM Since the rates with 1-Oxi-5′-*P* were so slow that the lower limits of the spectral assay were being approached, a confirmatory ^{14}C assay was used The incubation of 1-[6-^{14}C]Oxi-5′-*P* with high concentrations of enzyme for 30 and 60 min at 37 °C under standard conditions resulted in rates that were linear with time and were similar to those determined by the spectral assays The K_m and V values were determined from the linear region of the plot No substrate activity was detected for 3-XMP (radiochemical assay) 7-Oxi-5′-*P*, or cyclic XMP

Inhibitors

The aminated products of the reactions were identified and tested for their

ability to inhibit the amination of XMP (Table I) All of the aminated products tested appeared to be competitive inhibitors with respect to XMP 6-ThioGMP, prepared either by chemical or by enzymatic synthesis, showed very little inhibition at 1 0 mM and interfered with the spectral assay (360 nm) above this concentration Therefore, its inhibition constant was determined radiochemically

The two slow substrates, 6-thioXMP and 1-Oxi-5′-*P*, were examined as alternate-substrate inhibitors Inhibition constants (competitive with XMP) for these compounds are presented in Table I

DISCUSSION

The data clearly show that the substrate specificity of GMP synthetase is not strictly limited to XMP The V/K_m ratio (Table I) is a good indication of how efficiently the amination reaction can occur Relative to XMP, 8-azaXMP and 6-thioXMP are poorer substrates Although it has been previously demonstrated that the incubation of 6-mercaptopurine with Adenocarcinoma 755 tumor cells [23], or *E coli* bacterial cells* can give rise to 6-thioGMP, this is the first direct evidence that GMP synthetase may be responsible for the biosynthesis of this compound 1-Oxi-5′-*P*, with a maximal V/K_m ratio less than unity, has extremely poor substrate activity, and could only be aminated under forced conditions This observation is consistent with the finding that 6-NH_2-1-Alo-5′-*P* could not be detected in rat tissue extracts following the administration of large doses of radiolabeled allopurinol or oxipurinol under conditions where the two oxipurinol ribonucleotides were detected [24]

The finding that the K_m values of 6-thioXMP and 1-Oxi-5′-*P* were determined to be approximately equal to their respective K_i values suggest that, in this case, the K_m approximates a binding constant Furthermore, since high V values correlate positively with low K_m values, the reactivity of substrate may be related to its ability to bind to the enzyme This hypothesis is supported by the finding that the ability of XMP and dXMP to induce a conformative response in the enzyme is related to their K_m values [10]

It is of interest that no general relationship can be established between the binding constant of the substrates (K_m or K_i) and that of their corresponding aminated products (K_i) While 8-azaGMP bound to the enzyme approximately as efficiently as 8-azaXMP, GMP and 6-thioGMP bound about 10-fold less efficiently than their precursors

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* Radiolabeled 6-thioguanine has been identified in acid hydrolysates of nucleic acids isolated from *E coli* cells preincubated with [6-^{35}S]mercaptopurine (Zimmerman, T P and Chu, L -C unpublished observation)

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