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THYROID PURINE NUCLEOSIDE PHOSPHORYLASE

II. KINETIC MODEL BY ALTERNATE SUBSTRATE AND INHIBITION STUDIES *

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Summary

Nucleoside analog inhibition studies have been conducted on thyroidal purine nucleoside phosphorylase (purine-nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1) which catalyzed an ordered bi-bi type mechanism where the first substrate is inorganic phosphate and the last product is ribose 1-phosphate. Heterocyclic- and carbohydrate-modified nucleoside inhibitors demonstrate mixed type inhibition suggesting such analogs show an affinity (K_i) for the free enzyme. A kinetic model is proposed which supports the observed inhibition patterns. These studies together with alternate substrate studies indicate that nucleoside binding requires a functional group capable of hydrogen bonding at the 6-position of the purine ring and that the orientation of the bound substrate may be *syn*. Proper geometry of the phosphate is dependent upon the 3'-substituent to be orientated below the furanose ring. The 5'-hydroxyl group is required for substrate activity. The proposed rate limiting step of the phosphorylase mechanism is the enzymatic protonation of the7-N position of the nucleoside.

Introduction

Purine nucleoside phosphorylase (purine-nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1) from the bovine thyroid gland has been partially purified and characterized in this laboratory [1]. The enzyme catalyzes the

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reversible conversion of inosine or guanosine in the presence of inorganic phosphate to the corresponding base and ribose 1-phsophate. The thyroid phosphorylase follows an ordered bi-bi type mechanism [2] and demonstrates the phenomenon of negative cooperativity for inorganic phpsphate [1].

The interest of this laboratory in the thyroid enzyme has been the negative cooperativity characteristic displaced by phosphate and the possible role it may play in regulating purine oxidation to uric acid and hydrogen peroxide. To pursue this further, an extensive study has been undertaken to characterize the active site, the reaction sequence and probe the action of phosphate with the enzyme. The nucleoside specificity and a kinetic model for analog inhibition is presented here.

Base analog inhibition studies on *Escherichia coli* [3] and human erythrocyte [4] purine nucleoside phosphorylase and base specificity of calf spleen [5] or nucleoside specificity of human erythrocyte [6] and bovine thyroid [1] phosphorylase have provided little more than a limited view of the structural constraints of the active site. The work of Krenitsky et al. [4] and others cited by Parks and Agarwal [7] were designed towards the development of chemotherapeutic nucleoside analogs. This study presents information related to the geometry of the enzyme bound nucleoside substrate and insights into the possible reaction mechanism of the phosphorylase on purine nucleosides.

Methods and Materials

Chemicals. The following compounds: inosine, adenosine, ribose, 2′-deoxyinosine, 6-chloropurine riboside, 6-mercaptopurine riboside, 1-methylinosine, 7-methylinosine, 6-methoxypurine riboside, arabinofuranosyl adenine, cordycepin, nebularine (purine riboside), DL-dithiothreitol and two enzymes, adenosine deaminase and milk xanthine oxidase, were purchased from Sigma Chemical Co. Formycin B was obtained from Calbiochem and 5′-deoxyadenosine from P-L Biochemicals. All other reagents were reagent grade.

Chemical deamination with sodium nitrite [8] was performed on 8-azaadenosine and tubercidin (7-deazaadenosine) *. The inosine analog was eluted from a 5 X 40 mm Dowex 50W-X8 column with 10% ethanol and concentrated by lyophilization. Enzymatic deamination was conducted using calf intestinal adenosine deaminase in 50 mM Tris-HCl (pH 7.4) at 25°C. The following inosine analogs were subsequently purified by chromatography on Dowex-50W: xylofuranosyl adenine *, 5′-deoxyxylofuranosyl adenine *, arabinofuranosyl adenine. Descending paper chromatography on Whatman 3 in a *n*-propanol/NH_3/H_2O (3 : 1 : 1) solvent [9] was used to purify 8-aminoadenosine *, 3′-deoxy-3′-aminoadenosine *, cordycepin (3′-dideoxyadenosine) *, 5′-deoxyadenosine, 2′,3′-dideoxyadenosine (obtained from the Drug Development Branch, National Cancer Institute, Bethesda, MD 20014).

The purine nucleoside phosphorylase was purifed 200 fold as described by Moyer and Fischer [1]. Concentrations of all inhibitors and substrates were

* A gift from Dr. R.J. Suhadolnik, Biochemistry Department, Temple University School of Medicine, Philadelphia, PA, U.S.A.

determined spectrophotometrically with established extinction coefficients.

Assays. Kinetic studies were performed using a Perkin-Elmer 124 recording spectrophotometer, water jacket controlled at 30°C. All data was subjected to linear regression analysis for the determination of a slope and an intercept to obtain the kinetic parameters.

Purine nucleoside phosphorylase activity with inosine and the sugar analogs containing hypoxanthine was followed by a coupled enzyme assay with xanthine oxidase [10]. The reaction mixture contained 50 μmol potassium phosphate (pH 7.0), 0.1 μmol dithiothreitol, and 0.035 units of xanthine oxidase in a 1 ml assay volume. The reaction was monitored at 292 nm. 8-Aminoinosine was followed at 300 nm where the change in the absorbance coefficient at pH 7.0 is 186 $M^{-1} \cdot cm^{-1}$ [11].

Phosphorylase activity with substrates which did not produce hypoxanthine was followed by a modified orcinol procedure [12]. After incubation, 0.5 ml reaction mixture was added to an equal volume of cold 0.5 N $HClO_4$ containing a suspension of activated carbon (10 mg/ml or 50 mg/ml) which adsorbed the remaining substrate. Carbon was removed by centrifugation and the supernatant transferred to 1 ml 0.3% $FeCl_3 \cdot 6H_2O$ in concentrated HCl. A 0.10 ml aliquot of the orcinol reagent (300 mg/ml 95% ethanol) was added and the color developed in a boiling water bath for 1 h. After cooling to ambient temperature, the samples were analyzed at 670 nm. A standard curve for ribose prepared by this procedure was linear to 50 μM having an absorbance coefficient of 15 500 $M^{-1} \cdot cm^{-1}$.

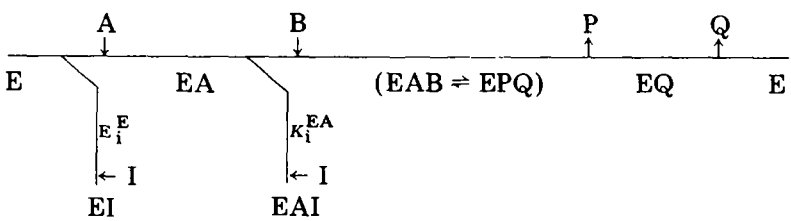
Results

Kinetic model

Thyroidal purine nucleoside phosphorylase catalyzes a sequential ordered bi-bi type mechanism with A and B equivalent to inorganic phosphate and nucleoside substrate, respectively, P is the purine base and ribose 1-phosphate is Q [2]. The Michaelis constants for A and B are 2.07 and 0.0216 mM, respectively, and the dissociation constant (K_{ia}) for A with inosine as the nucleoside substrate is 3.25 mM.

Enzymatic activity with nucleoside analogs which can substitute for substrate were followed as described in Methods and Materials. The K_m and V were extrapolated from double reciprocal plots with inorganic phosphate saturating at 50 mM.

Double reciprocal plots for all nucleoside inhibitors yielded staight lines as did the secondary plots (slope and/or intercept versus I). Replots of the slope versus 1/S obtained from Dixon plots ($1/v$ versus I) were linear but did not extrapolate to zero. Since inhibition by the nucleoside inhibitors cannot be overcome by saturating with substrate it would appear that the inhibitor is interacting with more than one enzyme form. To account for the observed kinetics the following model was developed:



The rate equation derived by the King-Altman method in the absence of product is given in Eqn. 1.

$$\frac{v}{V} = \frac{[A][B]}{K_{ia}K_{mB}\left(1 + \frac{[I]}{K_i^E}\right) + K_{mB}[A]\left(1 + \frac{[I]}{K_i^{EA}}\right) + K_{mA}[B]\left(1 + \frac{[I]}{K_i^E}\right) + [A][B]} \quad (1)$$

$$\frac{1}{V} = \frac{K_{mB}}{V}\frac{1}{[B]}\left(1 + \frac{K_{ia}}{[A]} + \frac{K_{ia}[I]}{K_i^E[A]} + \frac{[I]}{K_i^{EA}}\right) + \frac{1}{V}\left(1 + \frac{K_{mA}}{[A]} + \frac{K_{mA}[I]}{K_i^E[A]}\right) \quad (2)$$

Rearrangement of Eqn. 1 into the double reciprocal format (Eqn. 2) indicates the slope will be affected provided that E, EA, or both enzyme forms have an affinity for the nucleoside inhibitor. The $1/v$-axis intercept will change only when the free enzyme has an affinity for the dead-end inhibitor and the effect cannot be overcome by saturating with the second substrate. An observable change in the intercept would be evident when $K_i^E << K_{mA}$ or if $K_i^E \approx K_{mA}$ when [I]/[A] is statistically significant. Two dissociation constants can be calculated from the data when analyzed using this model for all the nucleoside inhibitors.

Base analogs of inosine

The K_m, V, and the dissociation constants for the analogs are given in Table I. Of the compounds surveyed, only purine riboside was neither a substrate nor an inhibitor. It would then appear that a substituent at the 6-position is instrumental in the binding of the nucleoside. The 6-sulfhydryl analog is a substrate with a K_m approximately equal to that observed with inosine. Adenosine which can effectively bind in the presence of phosphate is not a substrate but does act as a competitive inhibitor. The 6-methoxy and 6-chloro nucleoside analogs are inhibitors which have poor affinities for either enzyme

TABLE I

BASE ANALOGS

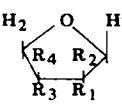
Values were determined for base analogs linked $\beta(1' - 9)$ to ribose as described in Methods and Materials. The units for the Michaelis constant (K_m), the dissociation constant for the free enzyme (E) form (K_i^ξ); and the dissociation constants fro the enzyme-phosphate (EA) form $K_i^{\xi A}$ are in μM. The V is the maximum velocity relative to that observed for inosine as substrate.

Base	K_m	V	K_i^ξ	$K_i^{\xi A}$
Hypoxanthine	16.4	1.00		
Purine	Not Substrate or Inhibitor			
6-Mercaptopurine	35	0.84		
6-Methoxypurine			14 000	15 000
6-Chloropurine	—	—	2 200	7 850
Adenine	—	—		480
1-Methylhypoxanthine	1 530	0.85		
7-Deazahypoxanthine	—	—	360	72
8-Azahypoxanthine	—	—	213	250
9-Deazahypoxanthine	—	—		47
7-*N*-Methylhypoxanthine	340	12.0		
8-Aminohypoxanthine	118	0.26		

TABLE II

SUGAR ANALOGS

Values were determined as described in Methods and Materials. The units for the Michaels constant (K_m), the dissociation constant for the free enzyme (E) form (K_I^E), and the dissociation constant for the enzyme-phosphate (EA) form K_I^{EA} are in μM. The V is the maximum velocity relative to that observed for inosine as substrate.



Sugar	R_1	R_2	R_3	R_4	R_5	K_m	V	K_I^E	K_I^{EA}
Ribose	OH	H	OH	H	OH	16.6	1.00		
2'Deoxyribose	H	H	OH	H	OH	23.7	0.85		
3'-Deoxyribose	OH	H	H	H	OH	516	0.054		
2', 3'-Dideoxyribose	H	H	H	H	OH	570	0.079		
5'-Deoxyribose	OH	H	OH	H	H	—	—	309	103
Arabinose	H	OH	OH	H	OH	506	0.018		
Xylose	OH	H	H	OH	OH	—	—	315	1740
5'-Deoxyxylose	OH	H	H	OH	H	—	—	1680	1570
3'-Deoxy-3'-aminoribose	OH	H	NH_2	H	OH	263	0.73		

form. The enzyme also demonstrates a poor affinity for the keto form of inosine (1-methylinosine) but readily converts it to product once bound.

Alterations of the purine imidazole ring did not dramatically affect the ability of the enzyme to bind an inosine analog relative to the pyrimidine ring modified analogs (Table I). 7-Deazainosine, 8-azainosine, and 9-deazainosine (formycin B) did not serve as substrates. The EA form preferentially binds the 7- and 9-substituted analogs, however, no selectivity is shown towards the 8-substituted inhibitor. Methylation of the N-7 of inosine induces an electron deficient imidazole ring which facilitates a more rapid transformation of the substrate/unit of enzyme. Addition of an electron-donating amine to C-8 yields a substrate with a reduced relative V.

Pentose analogs of inosine

The influence of the pentose on the specificity of the enzyme can be seen in Table II. The 2'-hydroxyl has little effect on the K_m and maximum velocity of the enzyme with the analog. Removal of the 3'-hydroxyl, however, does modify the K_m for the enzyme and reduces the catalytic rate. Substituting an amino group for the 3'-hydroxyl maintains the substrate activity of the analog comparable to that of inosine. No substrate activity was observed for 5'-deoxyinosine which has a good affinity for both enzyme forms.

Substitutions above the plane of the furanose ring shows that the arabinosyl nucleoside serves as substrate but exhibits a reduced V relative to that observed for 2'-deoxyinosine or inosine. The xylose nucleoside analog is an inhibitor which preferentially binds to the E form of the enzyme. The 5'-deoxyxylose nucleoside is a low affinity inhibitor which shows no preference for either enzyme form.

Discussion

Alternate substrate and inhibition studies have been employed in developing the conformational and configurational constraints of the nucleoside in the active site. These studies have also provided an insight into the possible enzymatic mechanism of the substitution reaction.

Nucleoside binding appears to result from hydrogen bonding at the 1- and 6-positions of the purine ring with the enzyme. Analogs (6-methoxy and 6-chloronucleosides) incapable of strong hydrogen bonding demonstrate a poor affinity for the enzyme. The conformation of the nucleoside may by *syn* since the orientation of the hydroxyl group above the plane of the furanose ring as in arabinofuranosyl hypoxanthine decreases the ability of the analog to bind and effectively reduces conversion of substrate to product.

Further evidence to support the *syn* conformation of the enzyme bound nucleoside comes from the kinetics observed for 5′-deoxyinosine. The presence of a hydroxyl at the 5′-position is required for the substitution reaction to proceed even though the 5′-deoxy analog will strongly associate with the enzyme and act as a good inhibitor.

Molecular orbital calculations of purine nucleosides indicate that ribosyl purine nucleosides strongly prefer the *syn* conformation due to the presence of the intramolecular hydrogen bond between 5′-hydroxyl and the N-3 position of the base [13]. In contrast to the above, proton magnetic resonance spectroscopy studies of arabinosyl purine nucleosides in solution indicate the *anti* conformation to predominate [14]. Conformational energy maps indicate that the energy changes between the *syn* and *anti* forms of the purine nucleosides are not great [15], and therefore it is conceivable that interaction with an enzyme may constrain the nucleoside in a conformation other than that predominating in the free state in solution.

Hydrogen bonding between the 5′-hydroxyl and N-3 of the purine nucleoside in the *syn* conformation provides a means to reduce the electron density of the electron rich pyrimidine ring and thus participates in accelerating the rate of both the hydrolysis in dilute acid and enzymatic phosphate substitution reaction.

Thyroidal purine nucleoside phosphorylase follows an ordered bi-bi type mechanism which appears to be concerted since no kinetic evidence or enzyme-substrate intermediate exists to suggest otherwise. Phosphate binds first in the active site. The nucleoside in the *syn* conformation must bind so as to orientate the inorganic phosphate below the furanose ring in close proximity to the 1′-carbon. Although the presence or absence of the 2′-hydroxyl of the ribose does not modify the enzymatic activity, the 3′-hydroxyl strongly influences the substitution reaction. With the location of the 3′-hydroxyl relatively far from the 1′-carbon, its participation may be in a 2-point orientation of the phosphate. Binding of the first substrate may result from charge neutralization of the phosphate oxygens with an appropriate group on the enzyme. The proton of the phosphate may then hydrogen bonded with the electrons of the 3′-hydroxyl of the bound nucleoside, structurally orientating the electrons of the phosphoryl group towards the 1′-carbon and resulting in the formation of α-D-ribose 1-phosphate.

The use of non-enzymatic acid hydrolysis of purine nucleosides as a kinetic model has pointed out two factors which accelerate the rate of hydrolysis: (1) Methylation or protonation of the N-7 may facilitate hydrolysis by producing a general electron deficiency within the imidazole ring and a general excess of electrons in the pyrimidine ring [16]; (2) Fixing the purine nucleoside in the *syn* conformation by substituting bulky substituents at the C-8 position will also accelerate hydrolysis during dilute acid treatment [17].

Introduction of a proton by the enzyme to the N-7 position could produce a sufficient electron sink to draw electrons from the C-N glycosidic bond for initiation of the reaction. The kinetics observed for 7-methylinosine as substrate seem to bear out the protonation hypothesis. The reaction rate with the 7-methyl analog is 12 times faster than that observed when inosine is the substrate. It appears that protonation at N-7 of the substrate by the enzyme or the subsequent redistribution of the electron density is rate limiting for inosine as substrate because prior modification of the substrate by methylation increases the reaction rate.

The ability of the substituent at the 6-position to undergo keto-enol tautomerization appears to also influence the reaction mechanism. Adenosine which binds to the enzyme presumably by hydrogen bonding cannot tautomerize and does not serve as a substrate. Inosine, guanosine, xanthosine [1] and 6-mercaptopurine riboside which can tautomerize do function as substrates. 1-Methylinosine, which is maintained as the 6-keto form of inosine, is a substrate with a reaction rate similar to that observed with inosine. The necessity of the substrate to be able to tautomerize to the keto form is additional evidence that the electron density of the pyrimidine ring must be further dispersed before the substitution reaction may proceed.

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