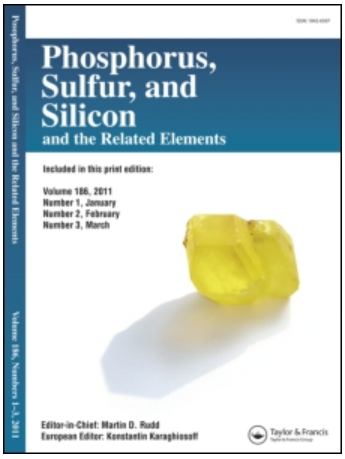

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Mechanisms of phosphate transfer on the purine salvage pathway

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MECHANISMS OF PHOSPHATE TRANSFER ON THE PURINE SALVAGE PATHWAY

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Abstract α-D-Ribofuranosyl-1,2-cyclic monophosphate and 5-phospho-α-D-ribofuranosyl-1,2-cyclic monophosphate were synthesized in good yields. The five-membered ring cyclic phosphates have ^{31}P chemical shifts similar to those found for such structures, presumably reflecting the smaller O-P-O bond angle, compared to that in six-membered ring phosphates. The rate of OH^- catalyzed ring opening was similar to that reported for ethylene phosphate, indicating relief of ring strain during hydrolysis. α-D-Ribofuranosyl-1,2-cyclic monophosphate was found to irreversibly inactivate purine nucleoside phosphorylase (EC 2.4.2.1) at its catalytic center.

INTRODUCTION

The purine salvage pathway converts the nucleosides guanosine or inosine to the corresponding 5'-nucleotides according to Eqs (1-4).

$$\text{Guanosine} + P_i \underset{\text{Phosphorylase, PNP}}{\overset{\text{Purine Nucleoside}}{\rightleftharpoons}} \text{Guanine} + \alpha\text{-D-Ribofuranosyl-1-phosphate (R-1-P)} \quad (1)$$

$$\alpha\text{-D-Ribofuranosyl-1-phosphate} \xrightarrow{\text{Phosphopentomutase}} \text{D-Ribose-5-phosphate} \quad (2)$$

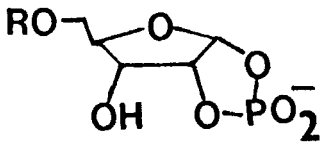
$$\text{D-Ribose-5-phosphate} + \text{ATP} \xrightarrow[\text{Synthase}]{\text{PRPP}} \text{5-Phospho-}\alpha\text{-D-ribofuranosyl-1-pyrophosphate (PRPP)} \quad (3)$$

$$\text{PRPP} + \text{Guanine} \xrightarrow[\text{Phosphoribosyltransferase}]{\text{Guanine (H)}} \text{5'-GMP} + PP_i \quad (4)$$

We have a continuing interest in the elucidation of the chemical mechanism of these enzymes. The development of specific, potent inhibitors for this pathway has potential pharmacological applications. Recently, we reported the synthesis and testing of the first irreversible inactivator, an Arg directed affinity

label, for PNP.[1]

We had found earlier that, in addition to Arg (presumably an anion recognition site),[1,2] His[3], Tyr[3] and Cys[3] are probably also near the catalytic center of PNP. It occurred to us that the α-1,2-cyclic monophosphate analogues of R-1-P (α-D-Ribofuranosyl-1,2-cyclic monophosphate, R-1,2-cP), and of PRPP (5-Phospho-α-D-ribofuranosyl-1,2-cyclic monophosphate, 5PR-1,2-cP) may act as potent electrophiles, and inhibit the enzymes depicted in Eqs (1)



R = H; R-1,2-cP

R = PO_3; 5PR-1,2-cP

and (4), respectively. This expectation is based on the facts that five-membered ring cyclic phosphates are thermodynamically [4] and kinetically[5] unstable towards hydrolysis. We here report that R-1,2-cP is indeed a catalytic center directed, irreversible inactivator of PNP. In addition, we have employed PNP from E. coli[6] to drive the equilibration depicted in Eq (1). The $K_{synthesis}$ can then be measured, by employing ^{31}P NMR, to enable determination of the relative glycosyl C-N bond strengths of purine nucleosides.

RIBOFURANOSYL-1,2-CYCLIC MONOPHOSPHATES: SYNTHESIS, ^1H AND ^{31}P NMR SPECTROSCOPY

R-1,2-cP, 5PR-1,2-cP, α-D-glucopyranosyl-1,2-cyclic monophosphate (G-1,2-cP) and 6-phospho-α-D-glucopyranosyl-1,2-cyclic monophosphate (6PG-1,2-cP) were all synthesized by dicyclohexylcarbodiimide catalyzed ring closures of R-1-P,[8] PRPP,[9] α-D-glucopyranosyl-1-phosphate[10] and 6-phospho-α-D-glucopyranosyl-1-phosphate, respectively. Four thin layer chromatographic systems were employed to monitor the reaction progress and to provide preliminary structure proof (according to R_f values).

81 MHz ^{31}P NMR and 200 MHz ^1H NMR spectra were recorded for all products. The most significant result of the ^{31}P studies is the unusual chemical shift that is characteristic of five-membered ring cyclic phosphates, and, very likely, of a 95-98° O-P-O bond angle.[11] The chemical shifts and multiplicities at pH 8.0, 25°C, (monoanionic cyclic diesters), in the presence of 10 mM EGTA are presented below (+ sign represents downfield, a minus upfield from internal HPO_4^{2-}).

Compound	δ_P(ppm, multiplicity)-cyclic P	δ_P other P
R-1,2-cP	17.0 (d of d)	-
5PR-1,2-cP	17.0 (d of d)	1.3 (t)
G-1,2-cP	8.2 (d of d)	-
6PG-1,2-cP	8.0 (d of d)	2.0 (t)
2',3'-cyclic AMP	17.5 (d of d)	-
3',5'-cyclic AMP	-4.0 (d of t)	-

The appearance of the C1-H (anomeric) and C2-H resonances clearly locate the position of the cyclic phosphate ($J_{H-C1-O-P}$ = 17 Hz, $J_{H-C1-C2-H}$ = 4 Hz) at C1 and C2.

HYDROXIDE ION CATALYZED RING OPENING OF R-1,2-cP

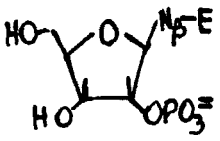
Based on approximately ten million-fold rate acceleration of OH^- catalyzed hydrolysis of ethylene phosphate (a five-membered ring phosphate) compared to an acyclic analogue,[5] it was important to compare the rate of alkaline hydrolysis of R-1,2-cP with that for ethylene phosphate. At 35°C, 0.1 N KOH, 10 mM EGTA, ^{31}P NMR was employed to determine $t_{1/2}$ = 2.7 hours for hydrolytic conversion of R-1,2-cP into ribose-2-phosphate and R-1-P in the ratio of 80% to 20%. Hydroxide ion attack at P is very likely. We conclude that the five membered ring cyclic phosphates here reported are highly strained, as is ethylene phosphate. The enormous rate acceleration in the cyclic phosphate compared to the acyclic analogue is due to relief of ring strain in going to the transition state.

IRREVERSIBLE INACTIVATION OF PNP BY R-1,2-cP

When incubated at 40°C, pH 7.6, 50 mM PIPES buffer, 1 to 4 mM R-1,2-cP caused a time-dependent inactivation of PNP (calf-spleen, Sigma, St. Louis). Plots of log (activity remaining) vs. time were strictly linear to at least 95% inactivation, thus demonstrating irreversible modification of the enzyme. During the time course of inactivation the ^{31}P NMR spectrum of the R-1,2-cP remained unchanged, i.e. R-1,2-cP was not being turned over. Following the standard treatment,[12] a K_i of 1-2 mM was estimated. The presence of 50 mM P_i or of 2 mM formycin B (a known competitive inhibitor of PNP) reduced the first order rate constant for inactivation by 88% and 90%, respectively. This affirms that the inactivation is taking place at the catalytic center. In addition to proving an excellent probe for the catalytic center of PNP, this observed inactivation also suggests a test of a hypothesis by Spector,[13] stating that all enzyme catalyzed reactions proceed by covalent intermediates. The reactions depicted in Eqs (1) and (4) proceed by inversion at the anomeric carbon. Such an inversion could be the result of either a single S_N2 displacement or of any odd number of inversions, e.g.:

$$N_\alpha\text{-E-}N_\beta \xrightarrow{B_\beta\text{-R}} \text{R-}N_\alpha\text{-E-}N_\beta \rightarrow N_\alpha\text{-E-}N_\beta\text{-R} \xrightarrow{P_i} N_\alpha\text{-E-}N_\beta + \text{R-1-P} \qquad (5)$$

where B_β represents the nucleic base always entering and leaving on the β-side, R is ribofuranosyl bonded to B_β or E-N_α or E-N_β, the enzymic nucleophiles specific to the α and β sides of the ribofuranosyl moiety, respectively. According to this hypothesis, R-1,2-cP would react stereospecifically with E-N_β and lead to an



anomeric carbon linked β-2-phosphoribofuranosyl-enzyme complex. This constitutes a viable and testable working hypothesis for the mode of inactivation of PNP by R-1,2-cP.

DETERMINATION OF $K_{SYNTHESIS}$ FOR PURINE NUCLEOSIDES

The K (toward nucleoside synthesis) depicted by the equilibration in Eq (1) can be determined by ^{31}P NMR.[7] The broad specificity of PNP isolated from E. coli[6] allows one to determine such K's for a wide variation in nucleoside structure (both in the base and sugar portions). A comparison of K's for two nucleosides that differ only in the base or only in the sugar structure will provide [log (K/K') = ΔΔG], as a first approximation the relative glycosyl C-N bond strengths. We have so far found that $K_{syntheses}$ can differ by at least a factor of ten. This technique is unique in enabling one to estimate relative C-N glycosyl bond strengths in related nucleosides.

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