

Activating the Phosphate Nucleophile at the Catalytic Site of Purine Nucleoside Phosphorylase: A Vibrational Spectroscopic Study

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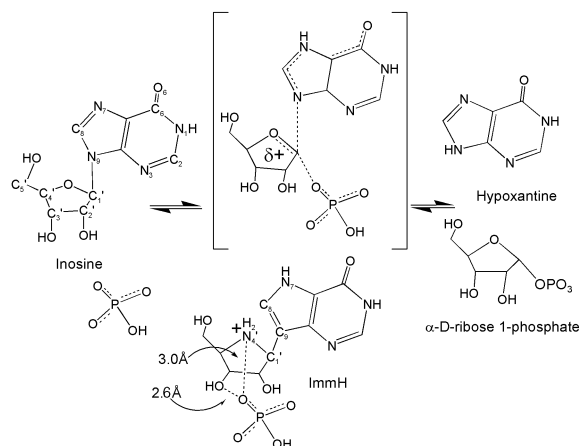
The transition state for human purine nucleoside phosphorylase (PNP) involves a fully dissociative ribosyl cation with a negligible bond formation between C1' and the attacking phosphate (bond order < 0.02).^{1,2} The transition-state inhibitor immucillin-H (ImmH) was developed to resemble the transition state of bovine PNP and binds to this enzyme with a K_i^* of 23 pM, $\sim 10^6$ -fold tighter (~ 8 kcal/mol) than inosine.²⁻⁴ However, this is a small fraction of the rate enhancement energy (~ 18 kcal/mol) for PNP catalysis.⁵ The transition-state structure of human PNP has a more dissociative structure than found in bovine PNP, and ImmH binds with a K_i^* of 56 pM.²

The electronic state of enzyme-bound phosphate nucleophiles has not been described. Here we use isotope-edited vibrational spectroscopy on a transition-state analogue complex of human PNP, PNP•ImmH•PO₄, to investigate how PNP activates the phosphate nucleophile near the transition state (Scheme 1). Understanding the transition-state structure of PNP is crucial to understanding its catalytic mechanism. Also, human PNP is an important target with regard to T-cell cancers and immunodeficiency diseases.⁵ The rational design of inhibitors against PNP relies on transition-state knowledge. High-resolution molecular structure, with a bond length accuracy of ± 0.004 Å for phosphates,⁶ can be obtained via difference Raman and FTIR spectroscopies complemented by vibrational analysis based on *ab initio* calculations. Our results show that the dianionic phosphate in the complex is forced into a unique bonding arrangement in which one of the P=O bonds is greatly polarized by enzyme active site interactions, such that it resembles a P=C bond that is about one-quarter of the way toward forming a bridging P-O-C single P-O bond.

Figure 1 shows the ¹⁸O isotope edited difference FTIR and Raman spectra of HPO₄²⁻ in solution and for the PNP•ImmH•PO₄ complex with human PNP. For HPO₄²⁻ in water, the three P=O stretches are coupled to form a symmetric stretch at 990 cm⁻¹, which is active in both IR and Raman (Figure 1a, left and right). A two-fold degenerate IR-active asymmetric stretch mode appears at 1084 cm⁻¹ (Figure 1a). Since the P-OH stretch is weak in Raman and water absorbs strongly below 900 cm⁻¹ in the IR spectrum, additional FTIR experiments on phosphate in D₂O revealed that the P-OD stretch mode for HPO₄²⁻ is at 834 cm⁻¹ and it shifts slightly to 846 cm⁻¹ in the PNP•ImmH•PO₄ complex (data not shown).

The three bands observed in the difference IR spectra of the PNP•ImmH•PO₄ complex shown in Figure 1b (left), at 970, 1016, and 1119 cm⁻¹ for ¹⁶O-labeled phosphate and at 934, 991, and 1084 cm⁻¹ for ¹⁸O phosphate, arise from P=O stretches of the PO₄ moiety and lie in the frequency range consistent with dianionic phosphate (and not for H₂PO₄⁻). These three bands are highly unusual and indicate a highly distorted phosphate moiety in PNP•ImmH•PO₄. As shown below, they can be assigned to an uncoupled P=O stretch mode and a coupled symmetric-asymmetric

Scheme 1. PNP-Catalyzed Phosphorolysis of Inosine and the Interaction between Phosphate and Transition-State Analogue Inhibitor ImmH in the PNP•ImmH•PO₄ Complex^a



^a Note the oxocarbenium ion nature of the transition state.

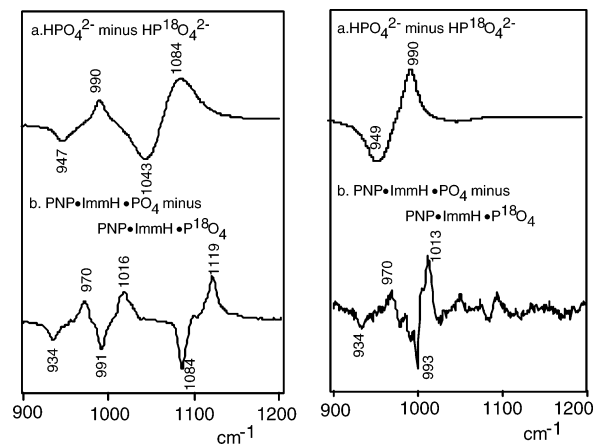


Figure 1. Difference FTIR (left panel) and difference Raman (right panel) spectra between P¹⁶O₄ and P¹⁸O₄ (a) in aqueous solution at pH 9.5 (dianionic) and (b) in the PNP•ImmH•PO₄ complex (4 mM:4 mM:4 mM, pH 7.2). The positive bands are from P¹⁶O₄ and the negative bands are from P¹⁸O₄. The resolutions for IR and Raman spectra were 2 and 8 cm⁻¹, respectively.

P=O stretch pair. One of the P=O bonds is strongly polarized, causing its stretch motion to be decoupled from the stretches of the other two P=O bonds. The uncoupled P=O stretch is active in both IR and Raman spectra, while the other two P=O stretches are coupled to form an IR-active asymmetric stretch and an IR- and Raman-active symmetric stretch.

For P=O bonds of phosphates, very accurate empirically derived relationships for bond length and bond order are known as a function of vibrational frequency.⁶ The error in the bond length/

Table 1. Derived P—O Bond Length ($R_{\text{P-O}}$) and Bond Order ($S_{\text{P-O}}$)^a from Observed P—O Stretch Frequencies

species	P—O stretch (cm ⁻¹)		$R_{\text{P-O}}$ (Å)/ $S_{\text{P-O}}$ (vu)
	ν_s	ν_a	
HPO ₄ ²⁻	990	1084	1.520/1.314
case I	970	1119	1.522/1.308
	1016		1.530/1.277
case II	1016	1119	1.516/1.329
	970		1.543/1.231

^a $R_{\text{P-O}} = [0.2838 \ln(224500/\nu)]$ and $S_{\text{P-O}} = [0.175 \ln(224500/\nu)]^{-4.29}$, where ν is the average frequency of the phosphate stretches, defined as $\nu^2 = (\nu_s^2 + n_a \nu_a^2)/(n_a + 1)$, where ν_s and ν_a are the symmetric and asymmetric modes for these bonds, respectively, n_a is the degeneracy of ν_a .^{6,7}

frequency relationship in determining $R_{\text{P-O}}$ is estimated to be about ± 0.004 Å, and bond orders (in valence units) to ± 0.04 vu.^{6,7}

The bond lengths of the P—O bonds for HPO₄²⁻ in solution and in the PNP·ImmH·PO₄ complex can be calculated from the bond length/frequency relationship (Table 1). Two cases are considered: in case I, the 970 cm⁻¹ band is assumed to be the symmetric stretch mode of the two coupled P—O bonds, while in case II, the 1016 cm⁻¹ band is taken to be this symmetric stretch. In case I, the bond length of the coupled P—O bonds is 1.522 Å, similar to the uncoupled P—O bond length of 1.530 Å. For case II, the bond length of the coupled P—O bonds is 1.516 Å, which is significantly different from the uncoupled P—O bond length at 1.543 Å. Symmetry arguments suggest that one P—O bond motion will decouple from the other two P—O bond motions only when this bond is significantly different. Hence, case II of Table 1 is most consistent for the PNP·ImmH·PO₄ complex.

The idea that decoupling of the P—O stretch motions requires strong interactions is tested quantitatively by normal-mode analysis using ab initio calculations on dianionic phosphate with or without external hydrogen bonding/electrostatic interactions on one of the P—O bonds. The details of the calculations are presented in the Supporting Information. The calculations indicate that one strong hydrogen bond with an O—O distance of 2.5 Å can significantly change the asymmetric P—O stretch but not the symmetric P—O stretch. Similar results are obtained for electrostatic interaction of a cation (charge +1) 3.0 Å from the P—O oxygen or two hydrogen bonds with bonding distances of 2.5 and 2.8 Å to one P—O oxygen. In these calculations, the polarized P—O bond is 0.02–0.03 Å longer than the other two P—O bonds. When the interactions on one of the P—O oxygens include both a 2.5 Å hydrogen-bonding and a 3.0 Å ion-pair interaction, the stretch motion of this P—O bond becomes decoupled from the other two P—O bond stretches, and its frequency becomes lower than that of the symmetric stretch in dianionic phosphate. Here, the bond length of the polarized P—O bond is 0.04–0.05 Å longer than the others. This is in good agreement with case II of Table 1, where it is found that one P—O bond is 0.027 Å longer than the others.

Crystal structures of PNP·ImmH·PO₄ complexes reveal that at least two hydrogen bonds on each P—O bond hold PO₄ tightly in the catalytic site.^{4,8} The narrowness of the bound PO₄ asymmetric vibrational bands also indicates a limitation in conformational flexibility. One of the P—O oxygens also forms a ~ 3.0 Å electrostatic interaction with N4' of ImmH,^{8,9} which is doubly protonated and carries a positive charge.¹⁰ On the basis of the combined experimental and computational results, we conclude this

P—O bond to be 0.027 Å longer than the other two P—O bonds, or 0.023 Å longer than the P—O bond of dianionic phosphate in water. This oxygen has been proposed to be the nucleophile for the PNP reaction.^{8,9} The bond order to this attacking P—O bond in the complex is decreased by ~ 0.08 compared to the P—O bonds in dianionic phosphate (Table 1). Since the total bond order of the oxygen is expected to be a constant, 2.0, this change of the P—O bond requires compensation by the formation of additional bonding to this oxygen, presumably from the hydrogen on the N4' in this complex or from the C1' in the actual transition state.

The bond lengths for a bridging P—O—(C) bond of dianionic phosphate in a number of small-molecule crystal structures range from 1.6 to 1.64 Å, while the bond lengths of P—O bonds vary from 1.50 to 1.53 Å.¹¹ Thus, the 0.023 Å increase in the P—O bond is 20–25% of the bond length change from a P—O bond to a bridging P—O—C bond. This change is expected at the transition state before phosphate forms a covalent bond to C1' of ribose in the reaction catalyzed by PNP.

Human PNP forms a dissociative transition state with fully developed carbocation character at C1' of the ribose ring.² Our results suggest that, during catalysis, the active site hydrogen bonds hold PO₄ tightly in place while the combination of the positive charge on C1' of ribose and H-bonds to the nucleophilic P—O bond oxygen are responsible for the polarization of the P—O bond to form a highly reactive oxygen nucleophile. The reaction coordinate is completed by migration of the C1' carbon cation toward the activated and immobilized phosphate group.⁸ This unusual bond distortion of bound phosphate in the PNP·ImmH·PO₄ complex supports the proposal that this complex captures a unique point on the reaction coordinate with features of the PNP transition state.

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Supporting Information Available: Details of normal-mode analysis based on ab initio calculations of HPO₄²⁻ hydrogen-bonded complexes with water and/or complexed with cations. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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