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Evidence for Direct Attack by Hydroxide in Phosphodiester Hydrolysis

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Hydroxide ions can catalyze hydrolysis reactions as either a nucleophile or a general base. Distinguishing between these two mechanisms is important because they represent two possible strategies for enzymatic activation of a water nucleophile.[1] The strong nucleophilic character of hydroxide suggests that it acts directly as a nucleophile, and solvent deuterium isotope effects (^{D_2O}k) for hydroxide-catalyzed reactions initially supported this view.[2] However, ^{18}O isotope effects on the nucleophile ($^{18}k_{nuc}$) and proton inventory studies are interpreted to indicate that hydroxide acts instead as a general base in ester and amide hydrolysis.[3,4] Unlike esters and amides, the nature of the nucleophile in hydroxide-catalyzed phosphodiester hydrolysis has not been explicitly examined, despite the importance of phosphodiester cleavage in biological systems.[5−7] Structure−reactivity and heavy atom isotope effect studies of phosphodiester cleavage reveal a concerted transition state with roughly equal amounts of bond formation to the nucleophile and bond breaking from the leaving group.[6,7] Despite these fundamental studies, relatively little direct information exists regarding the nucleophile for these reactions. We investigated the mechanism of hydroxide catalysis for phosphodiester hydrolysis by examining ^{D_2O}k, ionic strength effects, and $^{18}k_{nuc}$ to probe the hydroxide-catalyzed hydrolysis of thymidine-5′-*p*-nitrophenyl phosphate (T5-PNPP). The results are most consistent with a direct nucleophilic attack by hydroxide, making phosphodiester hydrolysis distinct from both ester and amide hydrolysis.

Initially, ^{D_2O}k was determined for the hydroxide-catalyzed hydrolysis of T5-PNPP.[8] General base catalysis predicts a ^{D_2O}k between 1.5 and 4 due to proton transfer in the transition state.[9] However, hydroxide-catalyzed phosphodiester hydrolysis displays a slightly inverse (0.90) effect (Table 1), suggesting a nucleophilic role for hydroxide.[2,10] Still, the studies on hydroxide-catalyzed ester hydrolysis[3,4] demonstrate that an inverse ^{D_2O}k alone cannot preclude a hydroxide general base.[11]

Phosphodiester cleavage by hydroperoxide displays an α effect[6,12] (and Table 1), demonstrating that hydroperoxide acts directly as a nucleophile. As hydroperoxide and hydroxide are both oxygen anions, it is tempting to speculate that hydroxide also acts as a nucleophile. However, hydroxide may still act as a general base because of its weaker nucleophilicity. This possibility was tested by varying the ionic strength. Because phosphodiesters are negatively charged under neutral and basic conditions, nucleophilic attack by hydroxide and hydroperoxide is inhibited by electrostatic repulsion.[6] The magnitude of this force decreases with the square of the distance, and so it will exert less influence on an anionic general base (acting one bond farther from the negative phosphate center) than on an anionic nucleophile. Therefore, if hydroxide acts as a general base, decreased sensitivity toward ionic strength compared to that of the nucleophilic hydroperoxide should be observed.

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Table 1. Second-Order Rate Constants for Hydroxide- and Hydroperoxide-Catalyzed T5-PNPP Hydrolysis, $I = 0.6$

catalyst	solvent	second-order rate constant (M^{-1} min^{-1})
HO^-	H_2O	$(3.5 \pm 0.1) \times 10^{-5}$
DO^-	D_2O	$(3.9 \pm 0.1) \times 10^{-5}$
HOO^-	H_2O	$(3.3 \pm 0.1) \times 10^{-3}$

Figure 1 depicts the sensitivities of hydroxide-, hydroperoxide-, and quinuclidine (a neutral tertiary amine)-catalyzed T5-PNPP cleavage to ionic strength. The second-order rate constants for catalysis by hydroxide and the hydroperoxide anion both display significant sensitivity to ionic strength. The minimal dependence of the quinuclidine implies that this sensitivity to ionic strength correlates with electrostatic repulsion and not some other aspect of the transition state. The ionic strength dependencies of hydroxide and hydroperoxide catalysis are indistinguishable, suggesting that both anions act by a similar mechanism. The sizable α effect observed in the hydroperoxide reaction is most consistent with a nucleophilic mechanism.

The $^{18}k_{nuc}$ for hydroxide-catalyzed hydrolysis of T5-PNPP also supports a nucleophilic mechanism. Because the equilibrium between water and hydroxide results in a 4.0% enrichment of ^{16}O in the hydrated hydroxide population,[13] an $^{18}k_{nuc}$ with a hydroxide nucleophile will include this $^{18}k_{ionization}$. Any kinetic effect contributed by reaction coordinate motion of the nucleophile would increase the observed effect. In contrast, if a water molecule is the nucleophile, only a kinetic effect would contribute to $^{18}k_{nuc}$. Thus, a small $^{18}k_{nuc}$ would be most consistent for nucleophilic attack by water, and an $^{18}k_{nuc}$ significantly greater than 1.04 would implicate an anionic hydroxide nucleophile. We determined $^{18}k_{nuc}$ for hydroxide-catalyzed phosphodiester hydrolysis by a modified competitive method[14] using ^{18}O-enriched water (∼50%) and mass spectrometry to measure reactant (solvent) and product isotope ratios.

Product thymidine-5′-monophosphate (TMP)[15] $^{16}O/^{18}O$ ratios were determined using electrospray ionization quadrupole mass spectrometry (ESI-MS). Previous studies have used whole molecule ESI-MS to determine kinetic isotope effects to within 0.3%, acceptable precision for drawing qualitative conclusions.[16] Single-ion monitoring was used to measure the ion intensities at the M ($m/z = 321$), M + 1 (322), M + 2 (323), and M + 4 (325) peaks. The M + 4 intensity was used to correct the M + 2 peak for the natural abundance of ^{18}O at the other eight oxygen atoms in TMP, and the product $^{16}O/^{18}O$ ratio was computed by calculating M/(M + 2). Accuracy was determined by comparing the observed (M + 1)/M ratio with the expected value assuming natural abundance for ^{13}C, ^{15}N, ^{17}O, and D. The observed values were all within 0.002 of the expected ratio of 0.120.

Solvent $^{16}O/^{18}O$ ratios were determined by diluting an aliquot of the reaction with water of known ^{18}O content. The dilution was measured by mass, and a correction was included to account for

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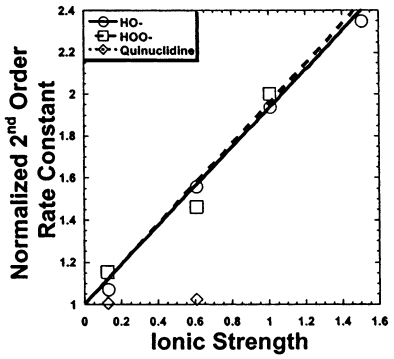

Figure 1. Dependence of the second-order rate constants of hydroxide-, hydroperoxide-, and quinuclidine-catalyzed T5-PNPP hydrolysis on ionic strength. The second-order rate constants are normalized to the extrapolated value at zero ionic strength. Data are fit to linear equations with slopes of 0.93 and 0.95 for hydroxide and hydroperoxide, respectively.

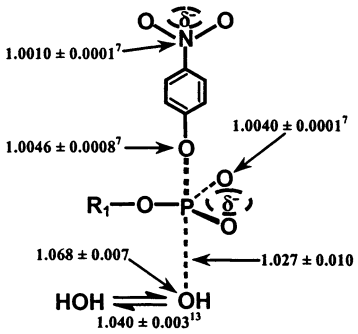

Figure 2. Summary of the heavy atom isotope effects in hydroxide-catalyzed phosphodiester hydrolysis with a *p*-nitrophenolate leaving group. The short, fat dashed line to the leaving group indicates strong bond order in the transition state, whereas the long, thin dashed line to the nucleophile indicates weak bond order.

the contribution of the solutes to the mass of the solution. The $^{16}O/^{18}O$ ratio of the diluted sample was measured by exchange with CO_2 and subsequent analysis of the CO_2 by an isotope ratio mass spectrometer (IRMS).[3,17] The ^{18}O mole fraction of the reactant was then calculated from the following equation:

$$f_r = \frac{m_f f_f / M_f - m_d f_d / M_d}{m_r / M_r}$$

where f_r is the ^{18}O mole fraction for the reactant, m_f, M_f, and f_f are the mass, average molecular weight, and ^{18}O mole fraction of the final diluted mixture, m_d, M_d, and f_d are the mass, average molecular weight, and ^{18}O mole fraction of the diluent, and m_r and M_r are the mass and average molecular weight of the reaction aliquot. The reactant $^{16}O/^{18}O$ ratio was calculated directly from the mole fraction.

We determined the $^{18}k_{nuc}$ for hydroxide-catalyzed T5-PNPP hydrolysis to be 1.068 ± 0.007[18] (Figure 2). This value is too large to be accounted for solely by a kinetic effect. Results were essentially identical for reactions incubated 2, 3, or 4 days, eliminating the possibility that exchange with atmospheric water contributes to the large $^{18}k_{nuc}$. Assuming a 1.040 equilibrium isotope effect, the kinetic component ($^{18}k_{kinetic}$) is 1.027 ± 0.010. This value represents a large but reasonable kinetic isotope effect for nucleophilic attack on a phosphoryl center. Therefore, the observed $^{18}k_{nuc}$ is most consistent with direct attack by hydroxide.

The $^{18}k_{kinetic}$ of 1.027 ± 0.010 also provides some qualitative information on the transition state. Large kinetic isotope effects on the nucleophile indicate that the normal reaction coordinate effect dominates over the inverse effect on bond formation,[19] and therefore suggest low bond order to the nucleophile in the transition state and/or substantial reaction coordinate motion. This conclusion is consistent with previous leaving group and nonbridging heavy atom isotope effect studies (Figure 2).[7b,c] Here, small ^{18}O and effects on the *p*-nitrophenyl leaving group and nonbridging oxygens were interpreted to indicate that little change in bond order occurred in the transition state. These isotope effects predict that little bond order to the nucleophile would occur in the transition state, consistent with the large $^{18}k_{kinetic}$. Additionally, the significant difference in mass between the hydroxide and the phosphoryl group predicts significant reaction coordinate motion for the nucleophile. This motion is also consistent with a large $^{18}k_{kinetic}$. The data together suggest direct nucleophilic attack by hydroxide with an early, reactant-like, transition state.

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Supporting Information Available: Procedure for $^{18}k_{nuc}$ determination, and TMP chromatogram and mass spectrum (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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