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The Substrate-Assisted General Base Catalysis Model for Phosphate Monoester Hydrolysis: Evaluation Using Reactivity Comparisons

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Abstract: Reactions of phosphate monoesters are ubiquitous in biological chemistry. Hence, this class of reactions has been subjected to extensive mechanistic analysis by physical organic chemists seeking to understand the nonenzymatic reactions and to apply this understanding to the corresponding enzymatic reactions. Substrate-assisted general base catalysis of phosphoryl transfer, in which a proton from the nucleophile is transferred to a nonbridging phosphoryl oxygen of the substrate prior to attack, has recently been proposed as a mechanism for both nonenzymatic and enzymatic reactions of phosphate monoester dianions and related compounds, in opposition to the previously accepted mechanism of direct nucleophilic reaction. We have evaluated this new mechanism for the hydrolysis of a phosphate monoester dianion in solution by considering the reactivity of the monoester monoanion that is a reaction intermediate in the proposed proton transfer. The monoanion of the monoester 2,4-dinitrophenyl phosphate ($DNPP^-$) and its diester analogue, methyl 2,4-dinitrophenyl phosphate monoanion ($MDNPP^-$), have similar rate constants for reaction with several nucleophiles ($k_{rel} = k^{DNPP}/k^{MDNPP} \approx 10$). In contrast, the substrate-assisted catalysis proposal requires that the rate constant for reaction of hydroxide ion with $DNPP^-$ be $\sim 10^9$-fold larger than the experimentally determined rate constant for the corresponding reaction of hydroxide ion with $MDNPP^-$. These and additional observations render substrate-assisted general base catalysis an unlikely alternative to the classical mechanism for nonenzymatic phosphoryl transfer.

Introduction

Phosphoryl transfer constitutes the most common class of biological reactions. Enzymes catalyzing this type of reaction include gradient-generating ATPases, energy-trafficking kinases, signal-transducing G proteins, and a host of phosphatases. A substantial amount of data from the field of physical organic chemistry supports a dissociative, metaphosphate-like transition state for the direct reaction of water and other nucleophiles with phosphate monoesters.[1] However, an alternative mechanism, substrate-assisted general base catalysis, has recently been proposed for both nonenzymatic[2] and enzymatic[3] reactions of phosphate monoester dianions and related compounds. This new mechanism, if valid, would fundamentally change the way that we view these reactions and their catalysis by phosphoryl transfer enzymes.

Substrate-assisted general base catalysis differs from the

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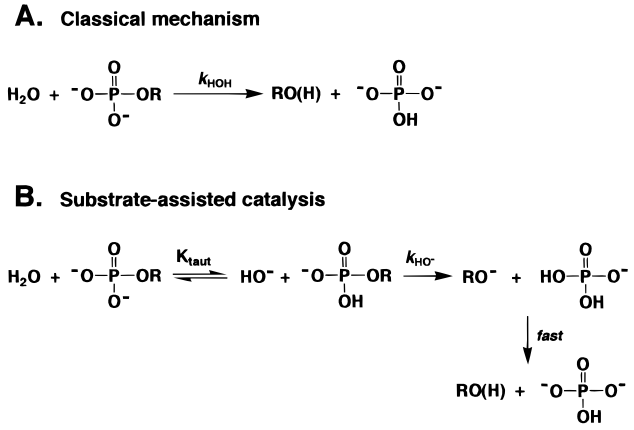
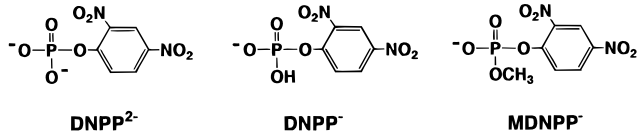


Figure 1. Alternative mechanisms for phosphoryl transfer from a phosphate monoester dianion in aqueous solution. (A) The conventional mechanism consists of direct water attack on the dianion (k_{HOH}). (B) The proposed substrate-assisted catalysis mechanism consists of a preequilibrium proton transfer from water to the dianion (K_{taut}), followed by hydroxide attack on the resulting monoanion (k_{HO^-}). In this mechanism, the proton resides on the transferred phosphoryl group in the transition state (not shown). The nucleophilic hydroxide ion need not be the molecule that donates a proton to the phosphoryl group, provided k_{HO^-} is slow relative to establishing the tautomeric equilibrium. This condition holds for the example described in the text.[26] "R" represents a 2,4-dinitrophenyl group for the reactivity analysis presented in this work (Chart 1).

classical mechanism of direct nucleophilic attack by water (Figure 1A) in that a proton is transferred from the water nucleophile to a nonbridging phosphoryl oxygen of the substrate prior to reaction (Figure 1B). The monoester monoanion intermediate and the hydroxide ion that result from this proton transfer then react to form inorganic phosphate and a dephosphorylated leaving group as products. Central to this proposal is that a proton resides on the transferred phosphoryl group in the transition state. The covalent bond to one of the nonbridging phosphoryl oxygen atoms in the substrate-assisted catalysis mechanism would be expected to result in a transition state with more associative character than the metaphosphate-like transition state posited in the classical mechanism.

We have evaluated the substrate-assisted catalysis mechanism by considering the reactivity of the monoanion intermediate that results from the proposed proton transfer (Figure 1B). A phosphate monoester monoanion and its phosphate diester monoanion analogue have similar measured reactivities for reaction with several nucleophiles, whereas the substrate-assisted catalysis proposal requires that the reaction of hydroxide ion with the monoester monoanion be $\sim 10^9$-fold faster than the corresponding reaction of hydroxide ion with its diester monoanion analogue. These and additional observations cast doubt on substrate-assisted catalysis as an alternative to the classical mechanism of direct nucleophilic attack by water.

Chart 1



Analysis of the Reactivity of Phosphate Ester Monoanions

The mechanistic alternatives presented in Figure 1 can be evaluated for the specific reaction of 2,4-dinitrophenyl phosphate dianion (Chart 1, $DNPP^{2-}$)[4] using existing data. The observed rate constant, k_{obs}, for breakdown of $DNPP^{2-}$ in aqueous solution at 39 °C is 0.011 min^{-1}.[5] The classical view is that this rate constant represents nucleophilic attack by water upon $DNPP^{2-}$, in which case the second-order rate constant is calculated as follows: $k_{HOH}^{DNPP^{2-}} = k_{obs}/[HOH] = (0.011\ min^{-1})/(55\ M) = 2.0 \times 10^{-4}\ M^{-1}\ min^{-1}$ (Figure 1A). In contrast, according to the substrate-assisted catalysis model k_{obs} represents nucleophilic attack by hydroxide upon $DNPP^-$ (Chart 1), and the second-order rate constant for this reaction would be calculated as follows: $k_{HO^-}^{DNPP^-} = k_{obs}/(K_{taut}[HOH]) = (0.011\ min^{-1})/(10^{-11.2} \times 55\ M) = 3.2 \times 10^7\ M^{-1}\ min^{-1}$ (Figure 1B). This expression reflects the requirement for a preequilibrium proton transfer prior to nucleophilic attack by hydroxide upon $DNPP^-$; the pK_a of 15.7 for deprotonation of water[6] and the pK_2 of 4.5 for DNPP[7] give $K_{taut} = 10^{-11.2}$ for the tautomerization of $DNPP^{2-}$ and water to $DNPP^-$ and hydroxide. The unfavorable tautomerization equilibrium corresponds to a free energy barrier[8] of $\sim$16 kcal/mol for proton transfer from the water nucleophile to a phosphoryl oxygen, reflecting the fact that hydroxide ion and $DNPP^-$ are not both the predominant ionic species at any pH.

The two mechanisms for $DNPP^{2-}$ cleavage (Figure 1) would follow the same pH−rate profile and are therefore kinetically indistinguishable.[9] Physical organic chemists have traditionally used a methyl group as a hydrogen substitute to aid in resolving such kinetic ambiguities, as study of the methylated species is not restricted to the narrow pH range that limits study of the ionizable protonated species.[10] Using this approach, the predicted rate constant for reaction of hydroxide and $DNPP^-$, $k_{HO^-}^{DNPP^-} = 3.2 \times 10^7\ M^{-1}\ min^{-1}$ (Figure 2A), can be compared to the measured rate constant for the analogous reaction of hydroxide with the diester monoanion $MDNPP^-$ (Chart 1, Figure 2B), $k_{HO^-}^{MDNPP^-}$.

There is, however, no guarantee that the methyl group of $MDNPP^-$ will be a valid substitute for the hydrogen of $DNPP^-$. It is therefore necessary to first obtain an independent calibration of how faithfully the methyl group mimics the hydrogen atom. The reactivities of $DNPP^-$ and $MDNPP^-$ with a series of nucleophiles provide such a calibration. $DNPP^-$ and $MDNPP^-$

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(4) Abbreviations: DNPP, 2,4-dinitrophenyl phosphate; MDNPP, methyl 2,4-dinitrophenyl phosphate; BDNPP, bis-2,4-dinitrophenyl phosphate; *p*NPP, *p*-nitrophenyl phosphate; *p*NPPS, *p*-nitrophenyl phosphorothioate.

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(8) $\Delta G = -RT \ln K_{taut}$ (39 °C).

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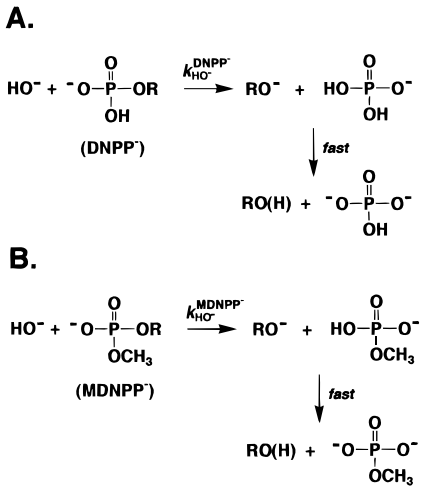


Figure 2. The analogous reactions of hydroxide with (A) the monoester monoanion DNPP$^-$ and (B) the diester monoanion MDNPP$^-$. "R" represents a 2,4-dinitrophenyl group (Chart 1).

Table 1. The Reactivity of Phosphate Ester Monoanions[a]

nucleophile	k_{rel} (measured)[b]	k_{rel} (predicted)
H_2O	20	–
nicotinamide	8	–
3-cyanopyridine	3	–
F^-	4	–
HO^-	ND[c]	1 000 000 000

[a] At 39 °C, $I = 1.0$. [b] $k_{rel} = k_{nuc}^{DNPP^-}/k_{nuc}^{MDNPP^-}$, the ratio of the second-order rate constant for the reaction of a nucleophile with DNPP$^-$ and the second-order rate constant for the reaction of the same nucleophile with MDNPP$^-$. Values of $k_{nuc}^{DNPP^-}$ are from ref 22, and values of $k_{nuc}^{MDNPP^-}$ are from the following: Kirby, A. J.; Younas, M. *J. Chem. Soc. (B)* **1970**, 1165−1172. [c] Cannot be determined experimentally for the reasons described in the text.

have rate constants that are within 3−20-fold of one another for reactions with several nucleophiles (Table 1), supporting the treatment of these compounds as molecular analogues.[11]

A value of $k_{HO^-}^{MDNPP^-}$ of 2.8×10^{-2} M^{-1} min^{-1} has been experimentally determined for the reaction of hydroxide with MDNPP$^-$ (Figure 2B).[12] This rate constant is greatly exceeded by the value of $k_{HO^-}^{DNPP^-}$ of 3.2×10^{7} M^{-1} min^{-1} calculated above assuming substrate-assisted catalysis for the reaction of DNPP^{2-} (Figure 2A). Thus, a relative rate constant of 10^9 is predicted from the hypothetical and experimental second-order rate constants for reactions of DNPP$^-$ and MDNPP$^-$ with hydroxide, respectively (Table 1, k_{rel}). This enormous value of k_{rel} for hydroxide ion required for substrate-assisted catalysis, contrasted with the measured k_{rel} values of 3−20 obtained for reactions with the other nucleophiles, renders this mechanistic alternative highly unlikely.

Additional Observations

The reactivity analysis performed above strongly suggests that the substrate-assisted catalysis mechanism is not general

(11) Additional support for analogous reactions of DNPP$^-$ and MDNPP$^-$ includes their similar β_{nuc} values, β_{lg} values, and deuterium isotope effects: reactions of monoanions with substituted pyridines give similar β_{nuc} values of 0.56 for DNPP$^-$ and BDNPP$^-$ (ref 22) and a β_{nuc} value of 0.38 for MDNPP$^-$ (Kirby, A. J.; Younas, M. *J. Chem. Soc. (B)* **1970**, 1165−1172); linear free energy relationships give $\beta_{lg} = -1.06$ for reactions of aryl methyl diester monoanions, including MDNPP$^-$, with pyridine (Kirby, A. J.; Younas, M. *J. Chem. Soc. (B)* **1970**, 1165−1172), and $\beta_{lg} = -1.03$ for reactions of aryl monoester monoanions, including DNPP$^-$, with the substituted pyridine nicotinamide (ref 22); similar deuterium isotope effects of 1.45 (ref 5) and 1.45−1.55 (ref 12) are observed for hydrolysis of DNPP$^-$ and BDNPP$^-$, respectively.

(12) Kirby, A. J.; Younas, M. *J. Chem. Soc. (B)* **1970**, 510−513.

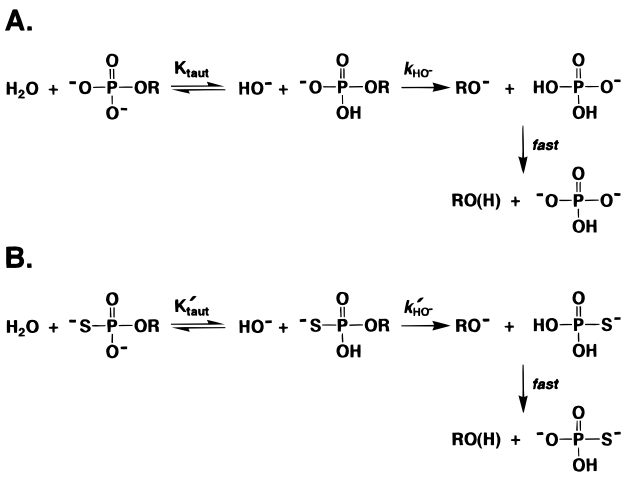
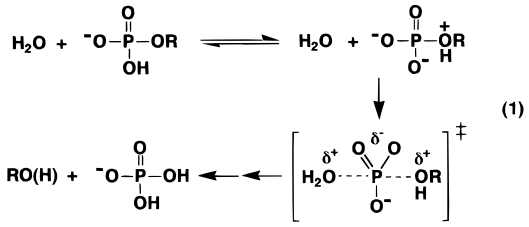


Figure 3. The substrate-assisted catalysis mechanism as it applies to hydrolysis of phosphorothioate dianions. "R" represents a *p*-nitrophenyl group for the purposes of the comparison in the text (Chart 2). For both *p*NPP^{2-} (A) and *p*NPPS^{2-} (B), the proposed mechanism consists of a preequilibrium proton transfer from water to the dianion (K_{taut} or K'_{taut}), followed by hydroxide attack on the resulting monoanion (k_{HO^-} or k'_{HO^-}). *p*NPPS species are depicted with negative charge localized on sulfur.[27]

to phosphoryl transfer. Could substrate-assisted catalysis nevertheless apply to phosphate monoesters with leaving groups that are less activated than the 2,4-dinitrophenol leaving group of DNPP, the phosphate monoester analyzed above? Unfortunately, reactivity comparisons between the mono- and diester monoanion analogues of compounds with less activated leaving groups, equivalent to the comparisons described above for DNPP$^-$ and MDNPP$^-$, are not possible. This is because monoester monoanions with less activated leaving groups react substantially faster than their diester counterparts.[5,12,13] According to the classical mechanism, this rate discrepancy results because the phosphoryl proton can transfer to the leaving group to stabilize the transition state for the monoester monoanion reaction (eq 1).[5,14] Because of this large rate difference and the



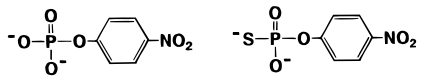
possibility of proton transfer, a methyl group is not an appropriate substitute for hydrogen and therefore cannot be used to aid in resolving the kinetic ambiguity of Figure 1 for these compounds. Despite this limitation, several independent observations can be used to evaluate the substrate-assisted catalysis mechanism for additional phosphoryl transfer reactions.

The reactivity of the phosphate monoester *p*NPP^{2-} can be compared to the reactivity of its corresponding phosphorothioate, *p*NPPS^{2-} (Chart 2).[4] Previous studies suggest that these

(13) Bunton, C. A.; Llewellyn, D. R.; Oldham, K. G.; Vernon, C. A. *J. Chem. Soc.* **1958**, 3574−3587.

(14) The mechanistic similarity for the reactions of DNPP$^-$ and MDNPP$^-$ (Table 1, footnote 11), despite the potential proton transfer to the leaving group in the monoester reaction, presumably arises because the low pK_a of the 2,4-dinitrophenol leaving group renders bond scission without proton transfer favorable.

Chart 2



*p*NPP^{2-} *p*NPPS^{2-}

analogues react via similar mechanisms,[15] and the possibility that both are hydrolyzed via the substrate-assisted catalysis mechanism is shown in Figure 3. K'_{taut} for proton transfer from water to *p*NPPS^{2-} is ~20-fold smaller than K_{taut} for the corresponding proton transfer from water to *p*NPP^{2-}, based on the 1.3 pK_a unit difference between the pK_2 values of *p*NPPS and *p*NPP.[15b] The value of k'_{HO^-} is expected to be ~10-fold smaller than the value of k_{HO^-}, based on thio effects ranging from 4 to 11 for phosphate diester monoanions,[16] which are analogous to the monoester anion intermediates of the substrate-assisted catalysis model (Figure 3). Thus, the substrate-assisted catalysis mechanism predicts that the *p*NPPS^{2-} reaction will proceed ~200-fold slower than the *p*NPP^{2-} reaction.[17] However, the observed hydrolysis of *p*NPPS^{2-} is ~10-fold *faster* than that of *p*NPP^{2-}.[15,18] Likewise, the ~10-fold faster hydrolysis of ethyl phosphorothioate dianion than ethyl phosphate dianion[19] is inconsistent with predictions from the substrate-assisted catalysis mechanism.[20]

Results from linear free energy relationships are also difficult to account for in the context of substrate-assisted catalysis. Water, which has a transferable proton, does not deviate from anionic oxygen nucleophiles, which are unable to transfer a proton to the substrate, in β_{nuc} or β_{lg} correlations for attack on phosphorylated pyridines.[21] Thus, no special mechanism is required to describe the reaction of water with these phosphorylated compounds. Further, linear free energy relationships yield similar β_{lg} values of −1.2 for attack by water[5] and −(0.9−1.0) for attack by tertiary amines,[22] which lack a transferable proton, in reactions of aryl phosphate dianions, providing no indication that a unique substrate-assisted catalysis mechanism is operative for reactions of water.

Concluding Remarks

Although the data from physical organic chemistry that we have revisited herein do not disprove substrate-assisted general base catalysis, they indicate that the proposed mechanism requires an enormous second-order rate constant for the reaction of hydroxide ion with a phosphate monoester, rendering it an unlikely alternative to the classical mechanism. A recent theoretical treatment reanalyzing the substrate-assisted catalysis mechanism also concluded that this mechanism is unlikely.[23]

It is important to recognize that results from physical organic chemistry, including the reactivity comparisons described herein, are generally correlative in nature and therefore cannot prove or disprove a given transition state structure. Nevertheless, there is direct physical evidence in support of dissociative reactions of phosphate monoester dianions. Although inversion of configuration is observed for reactions of phosphate monoester dianions in protic solvents,[24] suggesting that a free metaphosphate intermediate does not accumulate in these reactions, phosphoryl transfer to *tert*-butyl alcohol and thiophosphoryl transfer to water and ethanol results in racemization of products,[18,25] suggesting that metaphosphate and thiometaphosphate intermediates are formed in these reactions. The physical evidence for formation of metaphosphate and thiometaphosphate in these closely related reactions, combined with full consideration of earlier linear free energy relationships and other reactivity comparisons,[1] bolsters the classical view that reactions of phosphate monoester dianions in aqueous solution proceed through dissociative, metaphosphate-like transition states.

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(17) $k_{obs}^{pNPPS}/k_{obs}^{pNPP} = (K'_{taut}k'_{HO^-})/(K_{taut}k_{HO^-})$.

(18) Domanico, P.; Mizrahi, V.; Benkovic, S. J. In *Mechanisms of Enzymatic Reactions: Stereochemistry*; Frey, P. A., Ed.; Elsevier: New York, 1986; pp 127−138.

(19) O'Brien, P. J.; Wolfenden, R.; Herschlag, D. Unpublished results.

(20) It is important to note that the hydrolysis of ethyl phosphate dianion could still occur via the alkyl phosphate monoanion and hydroxide ion in a fashion consistent with the classical mechanism (eq 1). Such a pathway would be favored if the advantage from proton transfer to the leaving group and the modest increase in reactivity for hydroxide ion relative to water compensated for the lower concentrations of these species relative to the alkyl phosphate dianion and water. This possibility is currently under investigation. Regardless, the observed thio effect suggests that the proton does not remain on the transferred phosphoryl group in the transition state, as postulated in the substrate-assisted catalysis mechanism.

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