

Operational Control of Stereoselectivity during the Enzymatic Hydrolysis of Racemic Organophosphorus Compounds

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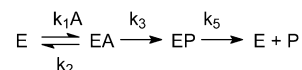
The bacterial phosphotriesterase (PTE) has proven to be a remarkable catalyst for the enzymatic hydrolysis of organophosphate nerve agents.¹ Typical turnover numbers for the best substrates can exceed 10^4 s^{-1} , while the associated values for $k_{\text{cat}}/K_{\text{m}}$ approach the diffusion limit of $10^8 \text{ M}^{-1} \text{ s}^{-1}$.² High-resolution X-ray crystal structures have shown that the hydrolytic water molecule is activated for nucleophilic attack via complexation to a binuclear metal center within the active site of the protein.³ The native enzyme contains Zn^{2+} , but high activity can also be obtained upon substitution with Co^{2+} , Mn^{2+} , Ni^{2+} , or Cd^{2+} . The overall stereochemical course of the hydrolysis reaction occurs with net inversion of configuration at the phosphorus center.⁴ Therefore, the overall chemical reaction mechanism occurs in a single step that does not involve the participation of a covalent phosphorylated intermediate.

The native enzyme has been shown to exhibit a modest degree of stereoselectivity during the hydrolysis of racemic pairs of chiral organophosphate substrates.^{4,5} For example, with a small library of disubstituted *p*-nitrophenyl phosphate substrates, the S_{P} -enantiomer is hydrolyzed 1–90 times faster than the corresponding R_{P} -enantiomer. This relationship holds for all combinations of methyl-, ethyl-, isopropyl-, and phenyl-substituents attached to the phosphorus center. Mutagenesis experiments have demonstrated that the inherent stereoselectivity within the wild-type PTE is dictated to a significant extent by the relative sizes of the subsites that accommodate the binding of organophosphate substrates within the active site.⁶ This latter property has been exploited to the point where rationally constructed mutant variants of PTE have been constructed in which the stereoselectivity has been enhanced, relaxed, or reversed.⁶ This mutant library has been utilized to kinetically resolve racemic mixtures of chiral organophosphates for the isolation of both enantiomers on a preparative scale with substantial chiral purity.⁷

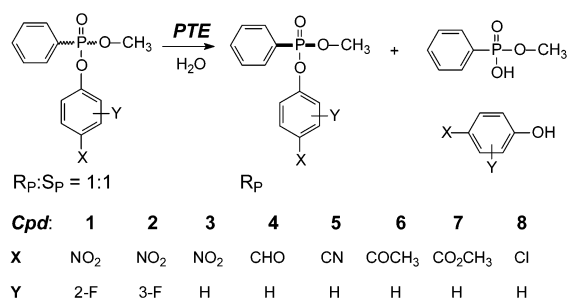
A simplified kinetic mechanism for the hydrolysis of organophosphorus compounds by PTE is presented in Scheme 1, where the three successive steps represent substrate binding, chemical hydrolysis, and product dissociation, respectively. Stereoselectivity is dictated by the relative values of $k_{\text{cat}}/K_{\text{m}}$ for the two enantiomers. For this kinetic mechanism, the specificity constant is given by the relationship $k_1 k_3 / (k_2 + k_3)$. For PTE, it is unclear which of these three rate constants dominates the net expression of stereoselectivity for a given pair of enantiomers. However, the observed stereoselectivity can potentially be manipulated by altering the relative magnitudes for the diffusional (k_1 and k_2) and chemical components (k_3) to $k_{\text{cat}}/K_{\text{m}}$. In principle, the chemical step (k_3) can be made entirely rate limiting by experimentally altering the degree of difficulty for P–O bond cleavage.⁸ The strength of the P–O bond can be titrated by altering the $\text{p}K_{\text{a}}$ value of the leaving group phenol.

For the initial phase of this investigation, eight pairs of racemic organophosphonate diesters were synthesized (see Supporting Information) where the leaving group phenols had $\text{p}K_{\text{a}}$ values that ranged

Scheme 1



Scheme 2



from 5.5 to 9.4 (Scheme 2).⁹ Each of these racemates was subjected to hydrolysis by the Co-substituted wild-type PTE and the G60A mutant enzyme at 25 °C and pH 9.0.² The time courses for the hydrolysis reactions were followed spectrophotometrically, and the data were fit to single or double exponential decay equations. The time courses were repeated at multiple enzyme concentrations to obtain relative rate constants for both phases when the individual enantiomers were hydrolyzed at substantially different rates. The first-order rate constants from these fits were divided by the total enzyme concentration to obtain an approximation of $k_{\text{cat}}/K_{\text{m}}$ for each enantiomer. The configuration of the faster-hydrolyzed isomer was assigned in each case to be the S_{P} -enantiomer by a direct correlation to previous results obtained with a thiophosphonate diester analogue.⁴

The relative rate constants for the hydrolysis of each enantiomer for the eight pairs of racemic organophosphonates were plotted versus the $\text{p}K_{\text{a}}$ of the leaving group phenol, and the correlation is presented in Figure 1A. For the S_{P} -isomers within this series of compounds, the Brønsted plot demonstrates that there is a change in the rate-limiting step for $k_{\text{cat}}/K_{\text{m}}$ as the $\text{p}K_{\text{a}}$ of the leaving group increases. For those compounds where the $\text{p}K_{\text{a}}$ is less than ~ 8.5 , $k_{\text{cat}}/K_{\text{m}}$ is dictated by k_1 ($k_3 > k_2$), whereas P–O bond cleavage dominates for those compounds where the $\text{p}K_{\text{a}}$ is ≥ 8.5 ($k_2 > k_3$). In contrast, for the R_{P} -isomers, there is no apparent break in the Brønsted plot, and thus the rate constant for P–O bond cleavage dominates for all substrates tested. Therefore, the stereoselectivity during the hydrolysis of chiral organophosphorus compounds can be experimentally manipulated by simple alteration of the $\text{p}K_{\text{a}}$ of the leaving group. For racemate **1** there is no stereoselectivity, whereas with racemate **7** a stereoselectivity of $\sim 5000:1$ is obtained. The change in stereoselectivity as a function of the leaving group $\text{p}K_{\text{a}}$ is presented in Figure 1B.

These results clearly demonstrate that a significant improvement in the ability to construct and isolate chiral phosphorus centers has been achieved. To appraise the practical aspects of this methodol-

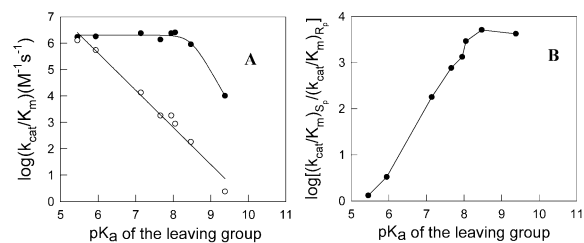


Figure 1. (A) Brønsted plots for the hydrolysis of S_P - (●) and R_P -enantiomers (○) for compounds 1–8 with wild-type PTE. (B) Stereoselectivity for the enzymatic hydrolysis of compounds 1–8, plotted as the ratio of k_{cat}/K_m as a function of the pK_a of the leaving group phenol.

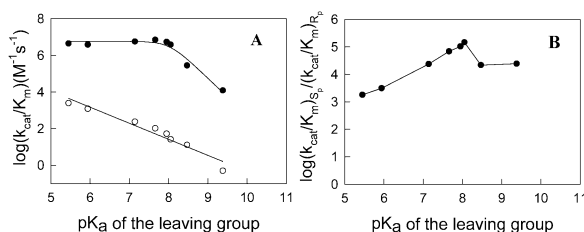


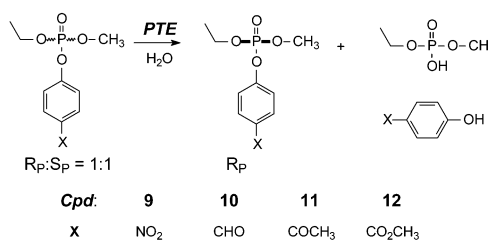
Figure 2. (A) Brønsted plots for the hydrolysis of S_P - (●) and R_P -enantiomers (○) for compounds 1–8 with G60A mutant enzyme. (B) Stereoselectivity for the enzymatic hydrolysis of compounds 1–8, plotted as the ratio of k_{cat}/K_m as a function of the pK_a of the leaving group phenol.

ogy, the R_P -enantiomer of **6** was isolated on a preparative scale. Wild-type PTE (0.023 mg in 0.032 mL of HEPES buffer) was added to a solution of racemic **6** (1.084 g) in CH_3CN (100 mL) and water (250 mL). The hydrolysis reaction was monitored by following the change in absorbance at 322 nm. The initial phase of the reaction was complete in 90 min, and the reaction was quenched by the addition of 200 mL of $CHCl_3$. Chromatography on silica gel provided the R_P -enantiomer of **6** in 98% isolated yield. The enantiomeric excess (ee) was quantitated by chiral electrophoresis and found to be greater than 99%.¹⁰

A comparable Brønsted analysis using the G60A mutant of PTE was conducted to ascertain the general applicability of stereoselective control during the hydrolysis of racemic organophosphorus mixtures. The G60A mutant of PTE reduces the dimensions of the active site by a single methylene group.^{5b} This mutant has been previously shown to enhance the stereoselectivity during the hydrolysis of organophosphate racemates by substantially reducing the overall rate of hydrolysis of the slower enantiomer.^{5,6} The overall effect of changing the pK_a of the leaving group on k_{cat}/K_m for the series of racemic mixtures 1–8 is presented in Figure 2A. The general trends are similar to those observed for the wild-type enzyme. For the S_P -enantiomer, there is a change in the rate-limiting step as the pK_a of the leaving group exceeds 8. However, the R_P -enantiomers are all limited by P–O bond cleavage and are significantly slower substrates relative to their S_P -counterparts. There is a stereoselective advantage of approximately 3 orders of magnitude for those substrates with leaving groups of very low pK_a that rises in excess of 100 000:1 when the pK_a approaches 8 (Figure 2B). For hydrolysis of the R_P -enantiomers with either the wild-type or the G60A mutant, the absolute value of the β -value is smaller than that for the S_P -enantiomers. This observation suggests that there is less net negative charge in the transition state for the R_P -enantiomers.

The most difficult kinetic resolution attempted thus far with this system has involved the stereochemical discrimination between

Scheme 3



methoxy and ethoxy substituents attached to the phosphorus core. With *p*-nitrophenol as the leaving group, no selectivity was observed with the wild-type enzyme, and a modest stereoselectivity was observed with the G60A mutant.⁶ Racemic mixtures of **9**–**12** were prepared,¹¹ and the time courses for hydrolysis catalyzed by wild-type PTE and the mutant G60A were obtained spectrophotometrically (Scheme 3). No stereoselectivity was observed with the wild-type enzyme for any of the leaving groups tested. However, with the G60A mutant, the stereoselectivity increased from an initial value of 11 for compound **9** to ratios of 27, 58, and 55 for compounds **10**, **11**, and **12**, respectively.

In this Communication, we have demonstrated that the stereoselectivity of the native phosphotriesterase can be systematically manipulated by alteration of the pK_a value of the leaving group phenol. For the wild-type enzyme, the stereoselectivity has been enhanced in excess of 3 orders of magnitude. We are unaware of previous reports that document the control of enzyme stereoselectivity via systematic alteration in the reaction coordinate profile. This result enhances the utility of this enzyme toward the preparation of chiral phosphorus building blocks that are difficult to obtain by other means.

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Supporting Information Available: Experimental details (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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