Does the Active Site Arginine Change the Nature of the Transition State for Alkaline Phosphatase-Catalyzed Phosphoryl Transfer?

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Tremendous rate enhancements are a hallmark of biological catalysis.[1] To understand how enzymes achieve such remarkable rate enhancements it is necessary to obtain information about the nature of the transition state for the enzymatic reaction, as catalysis can be defined as stabilization of the transition state relative to the ground state. Phosphoryl transfer reactions constitute the most abundant class of biological reactions and include reactions catalyzed by phosphatases and kinases central to metabolism and signal transduction. Physical organic studies inspired by the seminal work of Westheimer and Bunton have provided extensive evidence of a dissociative transition state for nonenzymatic reaction of phosphomonoesters.[2,3] In this transition state the bond to the leaving group is largely broken and there is little bond formation to the incoming nucleophile (Scheme 1a). In contrast, reactions of phosphotriesters have associative transition states with a large amount of bond formation to the incoming nucleophile and little bond cleavage to the leaving group in the transition state. The transition states for reactions of phosphodiesters are intermediate between phosphomonoesters and phosphotriesters in the degree of associative character.

Consideration of the transition states for these nonenzymatic reactions led to the proposal that enzymes could more readily stabilize the associative transition state than the dissociative one.[3,4] It has been widely suggested that enzymes render phosphoryl transfer transition states more associative by coordination of arginine or other positively charged active site groups to the nonbridging phosphoryl oxygens in the transition state (Scheme 1b).[5] This proposal is wide-reaching as all structurally characterized phosphoryl transfer enzymes have arginine residues and/or other positively charged groups in their active sites that could interact with the phosphoryl group being transferred.
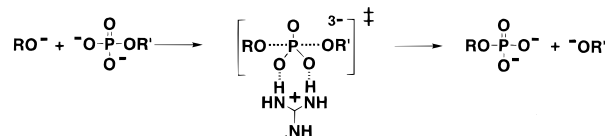
We tested this proposal with *Escherichia coli* alkaline phosphatase (AP). In the crystal structure of AP complexed with vanadate, a transition state analogue, arginine 166 forms hydrogen

Scheme 1

(a) Dissociative transition state for phosphomonoester dianion reaction
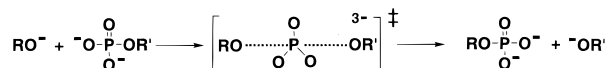
(b) Model for increased associative character from arginine coordination

bonds with two of the vanadate oxygens (Figure 1),[6] supporting previous mechanistic proposals.[7] Kinetic analyses of arginine mutants are consistent with an important functional role of this residue.[8]

To determine the effect of the active site arginine on the transition state for AP-catalyzed phosphoryl transfer, we have employed linear free-energy relationships. Such Brønsted correlations have been instrumental in characterizing transition states for nonenzymatic reactions. Nevertheless, in applying this approach to enzymes, it is a constant concern that specific binding interactions with the different substituents in a series of compounds will obscure the correlation between leaving group basicity and reaction rate, even for a nonspecific enzyme such as AP that has only a shallow binding pocket.[7,9,10] In the current study we compare the dependence of reaction rate on the leaving group properties for the wild-type enzyme and a mutant with the active site arginine removed. As steric interactions of various leaving groups with the active site are expected to be essentially the same for wild-type and mutant enzymes, effects that complicate direct determination of β_{lg} values for enzymatic reactions should largely cancel. Thus, *comparison* of the leaving group dependencies is expected to provide a strong indication of whether the active site arginine causes a substantial change in the nature of the transition state.

To obtain information about the transition state of a reaction, the chemical step must be rate limiting. This has been problematic for AP, as the reactions of aryl phosphates that are commonly employed as substrates are not limited by the chemical step.[10,11] We therefore used a series of alkyl phosphates, as these compounds are inherently less reactive. The large observed variation in k_{cat}/K_m with leaving group pK_a suggests that the chemical step is indeed rate limiting for hydrolysis of alkyl phosphates by both the mutant and wild-type enzymes (see below).

If arginine were to render the transition state more associative, less charge would accumulate on the leaving group oxygen in the transition state so that the value of β_{lg} would be expected to be less negative for wild-type AP relative to that for R166S AP; a shallower slope for wild-type AP would be predicted.

The values of k_{cat}/K_m for R166S AP are decreased ∼10^4-fold relative to wild-type, suggesting that arginine 166 has an important catalytic role. In Figure 2, the data for the wild-type and mutant enzyme are superimposed by plotting them on different scales.

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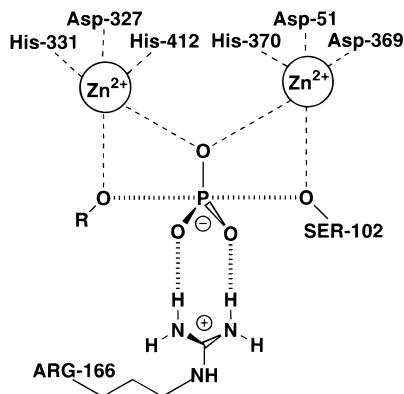
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Figure 1. Transition state model for phosphoryl transfer by AP based upon the structure of AP covalently bound to a pentavalent vanadate ester.[6,7] Arginine 166 makes a bidentate interaction with this transition state analogue.
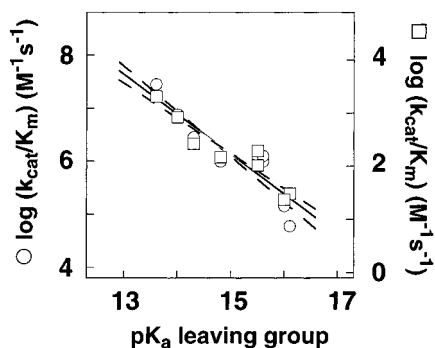

Figure 2. Leaving group dependence of R166S and wild-type AP for hydrolysis of primary alkyl phosphates. As the values of k_{cat}/K_m for R166S AP (□) are decreased ~10^4-fold relative to the wild-type enzyme (○), the two sets of data are plotted on different scales to facilitate comparison of the leaving group dependencies. The alcohol leaving groups and pK_a values are as follows: propanol (16.1), ethanol (16.0), methanol (15.5), allyl alcohol (15.5), 2-methoxyethanol (14.8), 2-fluoroethanol (14.3), 3-cyanoethanol (14.0), and propargyl alcohol (13.6). The solid line represents the best least-squares fit to both mutant and wild-type data sets, with a slope (β_{lg}) of -0.75 ± 0.1. Independent fits to the wild-type and mutant data sets give $\beta_{lg} = -0.85 \pm 0.1$ and -0.66 ± 0.1, respectively (dashed lines). The errors in individual k_{cat}/K_m values (≤15% for 6−12 independent determinations) are smaller than the symbols. The alkyl phosphates were synthesized with ^{32}P labels to allow detection of reaction products at the low concentrations required to avoid inhibition by the inorganic phosphate product ($K_i = 1$ µM).[16] Reactions were carried out at 25 °C with 0.1 M NaMOPS, pH 8.0, 0.5 M NaCl, 100 µM ZnSO$_4$, and 1 mM MgCl$_2$. The inorganic phosphate product was separated from alkyl phosphate substrates by polyacrylamide gel electrophoresis, and relative amounts were quantitated with a phosphorimager.[17] Reactions were followed for >7 half-lives, and rate constants were obtained from nonlinear least-squares fits. As expected for subsaturating substrate, the observed rate constants were independent of the concentration of alkyl phosphate (0.001−30 nM) and linearly dependent on enzyme concentration (0.01−10 nM wild-type and 0.1−10 µM R166S AP). Enzymes were purified as previously described.[18]

Despite the ~10^4-fold effect of the R166S mutation, the data for both enzymes fit a single linear correlation with $\beta_{lg} = -0.75 \pm 0.1$. Individual fits give values of β_{lg} that are within error, or

perhaps slightly steeper for wild-type AP. The small difference in the individual fits could be due to experimental error, changes in steric effects for R166S relative to wild-type, or a change in solvation of the transition state when the arginine is mutated.[12]

Linear free-energy relationships with aryl phosphate monoesters and diesters provide a crude estimate for how much the value of β_{lg} would be expected to change if the active site arginine changed the transition state from a dissociative transition state to a more associative, diester-like transition state. Using the concept of effective charge, a change in the value of β_{lg} of +0.47 is estimated for a more associative transition state, in contrast to the observed change of -0.1 ± 0.1 in Figure 2.[13]

In summary, the active site arginine of alkaline phosphatase does not substantially change the leaving group dependence, suggesting that this residue provides little or no increase in the associative character of the transition state. These results are complementary to those obtained with kinetic isotope effects in a recent study of the *Yersinia* protein tyrosine phosphatase.[15] Together, these results test the proposal that positively charged groups change the nature of the transition state for phosphoryl transfer, strongly suggesting that an interaction of arginine with the nonbridging phosphoryl oxygens does not necessarily render the transition state for phosphoryl transfer more associative.

(12) As k_{cat}/K_m includes a binding step in addition to the chemical step, effects of arginine on binding could also contribute to the observed value of β_{lg}. The positively charged side chain would be expected to favor binding of substrates with more negative charges on the nonbridging phosphoryl oxygens. Such an effect, if significant, would render β_{lg} less negative. Thus, a less negative β_{lg} for wild-type AP could result from either a change to a more associative transition state, as introduced in the text, or an effect on binding. The observation that β_{lg} does not change suggests the absence of a significant effect on binding or transition state structure.

(13) The charge that develops on a leaving group atom in a transition state has been described by an effective charge, defined by the ratio β_{lg}/β_{eq} (β_{eq} is the slope of the dependence of log K_{eq}, the equilibrium constant for the overall reaction, on the pK_a of the leaving group: Williams, A. *Acc. Chem. Res.* **1984**, *17*, 425. Jencks, W. P. *Catalysis in Chemistry and Enzymology*; Dover: New York, 1987). For hydrolysis of aryl phosphomonoester dianions, β_{lg}/β_{eq} is 0.91 ($-1.23/-1.35 = 0.91$; ref 14 and Kirby and Varvoglis: Kirby, A. J.; Varvoglis, A. G. *J. Am. Chem. Soc.* **1967**, *89*, 415). For hydrolysis of aryl phosphodiesters the effective charge obtained from β_{lg}/β_{eq} is 0.56 ($-0.97/-1.74$; ref 14 and Kirby and Younas: Kirby, A. J.; Younas, M. *J. Chem. Soc. (B)* **1970**, 510). Thus, a decrease in effective charge of 0.35 is predicted for reaction of a monoester via a diester-like transition state ($[\beta_{lg}/\beta_{eq}]_{diester} - [\beta_{lg}/\beta_{eq}]_{monoester} = 0.56-0.91 = -0.35$). This change in effective charge can be converted to a change in β_{lg} by normalizing for $\bar{\beta}_{eq}$, giving a predicted change in β_{lg} of +0.47 for conversion to a more associative, diester-like reaction $\{([\beta_{lg}/\beta_{eq}]_{diester} - [\beta_{lg}/\beta_{eq}]_{monoester}) \times \beta_{eq,monoester} = (-0.35) \times (-1.35) = +0.47\}$. An even larger change in β_{lg} would be expected for reaction via a triester-like transition state.

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