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Use of site-directed mutagenesis to elucidate the role of arginine-166 in the catalytic mechanism of alkaline phosphatase

(active site mutants/phosphoryl transfer)

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ABSTRACT **The guanidinium group of arginine-166 has been postulated to act as an electrophilic species during phosphorylation of alkaline phosphatase. Its role could be either to stabilize the developing negative charge on the oxygen of the leaving group or the pentacoordinate transition state or to help bind the $-PO_3^{2-}$ group. We have produced via site-directed mutagenesis two *Escherichia coli* alkaline phosphatase mutants (lysine-166 and glutamine-166) to test whether the guanidinium group plays a critical role in catalysis. Comparative kinetic characterization of the lysine-166 and glutamine-166 mutants indicates that the charge at residue 166 is not required for the hydrolysis of phosphate monoesters. Small decreases in k_{cat} are observed for both the lysine and glutamine mutants, relative to the wild-type enzyme, but the value for the uncharged glutamine mutant is only one-third that of lysine. Thus, the stabilizing effect of the positively charged guanidinium group does not appear to play a major role in the rate-limiting step for substrate hydrolysis. A significant effect on the K_m value is seen only for the glutamine mutant.**

The catalytic mechanism of alkaline phosphatases has been investigated in many chemical, kinetic (1, 2), and structural (3) studies. Nevertheless, the role of specific residues in the mechanisms used by this and other enzymes to catalyze phosphoryl transfer are not fully understood. The widely accepted kinetic scheme for the action of *Escherichia coli* alkaline phosphatase has four steps encompassing an essential phosphoryl enzyme intermediate (Eq. 1).

$$E + ROP_i \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot ROP_i \underset{k_{-2}[ROH]}{\overset{k_2}{\rightleftharpoons}} E - P_i \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E\cdot HOP_i \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + HOP_i \quad [1]$$

The phosphoryl group ($-P_i = -PO_3^{2-}$) is attached covalently to serine-102 in the active site. Subsequently, water or an alternative nucleophilic acceptor dephosphorylates the phosphoryl enzyme. One of the striking features of alkaline phosphatase is the lack of sensitivity of the k_{cat}/K_m values for monophosphate ester hydrolysis toward the leaving group of the substrate. It has been proposed that the very small change in effective charge on the leaving group's oxygen in the course of the reaction is consistent with substantial electrophilic interaction of groups in the enzyme with this atom (4). The role of this electrophile could be filled by either a zinc ion or by the side chain of arginine-166. The former possibility appears more likely based on the crystal structure (3). Also, the effects of divalent metal ions on the rate and transition-state structure in the nonenzymatic solvolysis of 4-nitrophenyl phosphate are consistent with the proposal that the electrostatic interaction of the metal ion with the leaving group stabilizes the development of electron density on the leaving group in the transition state (5). The guanidinium group of arginine-166, on the other hand, could stabilize the pentacoordinate transition state (Fig. 1) (3, 4).

Site-directed mutagenesis, combined with kinetic studies, provides a method for assessing the contribution of particular interactions to catalysis and to binding (6). Studies of amino acid residues thought to be directly involved in substrate binding and/or catalysis have burgeoned within the past 5 years (7). In experiments with rat carboxypeptidase A, for example, the substitution of phenylalanine for tyrosine-248, a residue that had been considered a potential proton donor in the catalytic action of the enzyme for peptide substrates, resulted in a mutant enzyme that was fully competent at hydrolyzing a peptide substrate (8). We have used site-directed mutagenesis to incorporate separately the codon for lysine and glutamine in place of arginine-166 in *E. coli* alkaline phosphatase. Substitution by lysine was chosen as a conservative change that would maintain (at pH <10) the charge and the hydrogen bonding ability of the wild-type arginine. By changing to glutamine, we have tried to conserve hydrogen bonding ability while deleting the charge and thus any electrophilic functionality at residue 166. Since the guanidinium group of arginine-166 is within hydrogen-bonding distance of aspartic acid-101, it is possible that a hydrogen bond between residue 166 and aspartic acid-101 is important in keeping serine-102 in the right orientation for nucleophilic attack on phosphorus (3). We thought it appropriate to characterize the function of the charge while maintaining the hydrogen-bonding ability at residue 166. Although the side chains of lysine and glutamine are shorter than arginine, modelling studies with an Evans and Sutherland PS-300 graphics system indicate that they could be within hydrogen-bonding distance of aspartic acid-101.

Our finding that arginine-166 can be replaced by lysine and glutamine without substantial loss of phosphatase activity suggests that neither this specific residue nor the positive charge is required for catalysis of substrate hydrolysis by the enzyme.

METHODS

Bacterial Strains and Media. *E. coli* strain AW1043 (Δ*lac galU galK* Δ(*leu-ara*) *phoA-E15 proC*::Tn*5*) was used as the host cell for all experiments except site-directed mutagenesis, in which the male derivative AW1043F'AMP [Δ*lac galU galK* Δ(*leu-ara*) *phoA-E15 proC*::Tn*5* F'Amp] (9, 10) was used. Standard LB medium (11) was used for general propagation of cells and for mutagenesis. For enzyme production and isolation, the host cells were grown in low phosphate-containing (0.1 mM KH_2PO_4) Mops medium (12) maintained at pH 7.8 ± 0.3. All media contained ampicillin (250 μg/ml) and kanamycin (50 μg/ml).

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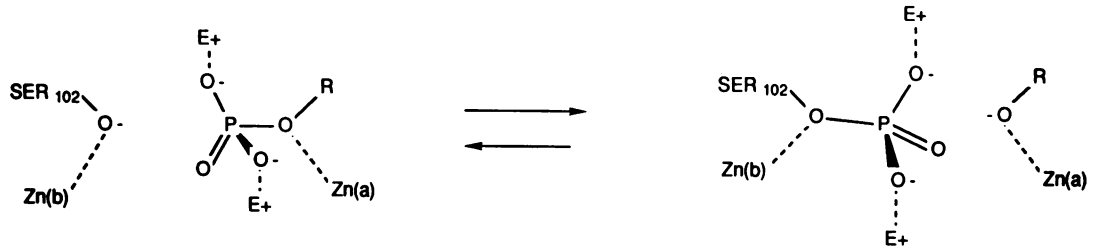

FIG. 1. Metal ion stabilization of the leaving group during enzyme phosphorylation. E+, positively charged side chain of the enzyme; R, any alkyl or aryl group; Zn(a) and Zn(b), two different enzyme-bound Zn atoms.

Enzymes. Restriction enzymes, T4 DNA ligase, and DNA polymerase (Klenow) were from Boehringer Mannheim.

Mutagenesis. The template for mutagenesis was M13mp19 into which the structural gene for alkaline phosphatase (*phoA*) (9) had been cloned (10). Oligonucleotides designed to produce the desired mutations (CGC → AAG for lysine-166; CGC → CAG for glutamine-166) were synthesized by the phosphoramidite method (13) and purified by ion-exchange chromatography on a Pharmacia FPLC system. To facilitate mutant screening, the primer used to make lysine alkaline phosphatase was also designed to produce a silent codon change for threonine-164 (ACC → ACT) and consequently to create an *Asu* II site not present in the wild-type sequence. For mutagenesis, the double primer method of Zoller and Smith (14) was used to construct heteroduplex DNA. Mutant plasmids were selected after two rounds of transformation and identified by colony screening with the ^{32}P-labelled oligomer encoding the mutant sequence. A 2.5-kilobase DNA fragment containing the mutant *phoA* gene was then subcloned into pBR322 between the *Bam*HI and *Hin*dIII sites. The presence of the desired mutation was verified by direct DNA sequencing (15) and for lysine-166 also by the concurrent appearance of *Asu* II sensitivity. In addition, the pertinent active-site region was resequenced using the DNA isolated from the same cells used for enzyme production.

Purification of the Mutant Enzymes. All procedures were done at 4°C. About 4 g (wet weight) of *E. coli* was harvested from 1 liter of culture grown to 2 OD_{660} units in shaker flasks. The cells were washed (100 ml, 10 mM Tris·HCl), treated with cold 20% sucrose (80-ml solution) and then with cold distilled water (80 ml). The osmotic shock releases the periplasmic content into the cold water, which is quickly adjusted to 10 mM Tris·HCl, pH 8/1 mM $MgCl_2$/10 μM $ZnCl_2$. The periplasmic fraction was then passed through an Affi-Gel Blue (Bio-Rad) column. The eluate was further purified by ion-exchange chromatography on DEAE-cellulose [15–200 mM NaCl gradient in 10 mM Tris·HCl (pH 8.0)]. The peak containing the phosphatase activity was dialyzed against 10 mM Tris·HCl (pH 8) and loaded onto another DEAE-cellulose column (same gradient and conditions). Fractions with specific activity no smaller than 95% of the maximum were pooled and stored at 4°C without change in activity for up to at least 8 weeks.

The electrophoretic mobility of both mutants was identical to that of wild-type alkaline phosphatase on reducing sodium dodecyl sulfate/polyacrylamide gel electrophoresis. The final volume was 20 ml and the concentration was 0.84 mg/ml. Protein concentration was measured from the absorbance at 278 nm ($E^{1\%}_{278}$ = 0.71) (16).

Assays. The velocity of enzyme-catalyzed substrate hydrolysis was followed spectrophotometrically (for 4-nitrophenolate, $\varepsilon_{410} = 1.85 \cdot 10^4\ M^{-1} \cdot cm^{-1}$, pKa = 7.16); (for phenol, ε_{268} = 505 $M^{-1} \cdot cm^{-1}$ at pH 8.0). Extinction coefficients were confirmed at different pH values after complete hydrolysis.

All measurements were at 25.0 ± 0.1°C in 50 mM Tris·HCl (unless otherwise specified) and at constant ionic strength (200 mM) adjusted with NaCl. Initial rates were determined graphically from the first 5% of the reaction at substrate concentrations bracketing the apparent K_m for each pH value. Values of k_{cat} and K_m were obtained from the Lineweaver–Burk plots of six to eight points. Product inhibition was not considered in our analysis, since only initial rates were used in the calculations.

RESULTS AND DISCUSSION

Both mutants lysine-166 (R166K) and glutamine-166 (R166Q) are effective catalysts in the hydrolysis of some phosphate monoesters. Table 1 compares the values of k_{cat} and K_m for the mutant alkaline phosphatases and the wild type. With 4-nitrophenyl phosphate, the k_{cat} for the lysine-166 mutant is lower by a factor of 4 and the k_{cat} of the glutamine-166 mutant is lower by a factor of 14 relative to the value for the wild-type enzyme. While these are significant differences that reflect the perturbation of the active site by these amino acid substitutions, it is remarkable that the k_{cat} value for the mutant lacking the positive charge in the active site (R166Q) is smaller by just a factor of 3 than the value for the mutant that conserves the charge (R166K). However, the relative effect of the glutamine compared to the lysine mutation on the K_m value is more pronounced; while the lysine-166 mutant has almost the same value as the wild type, there is a >10-fold increase for the glutamine-166 mutant.

Other important features of the wild-type enzyme are conserved in both mutants. For each mutant, the k_{cat} values for two different substrates are very similar, showing the same lack of dependence on the substrate leaving group that has been so thoroughly documented for the wild-type enzyme (4). For each enzyme, though, there is a marked increase in the K_m value for phenyl phosphate relative to that for 4-nitrophenyl phosphate, in contrast to the reported (4) measurements for the wild-type enzyme. Based on the Michaelis–Menten parameters at pH 8 and 25°C with two substrates of widely different pK values, a Bronsted plot of log (k_{cat}/K_m) vs. the pK of the leaving group would give a slope (β_{LG}) of −0.36 for both mutants. Admittedly, only two substrates were examined and one cannot place a great deal of confidence in the value of this slope. Clearly, however, the change in the effective charge on the oxygen of the leaving group during enzyme phosphorylation is small.

The effect of pH on k_{cat}/K_m (Fig. 2) and K_m (Fig. 3) over

Table 1. Kinetic parameters for the action of wild-type (arginine-166), lysine-166, and glutamine-166 alkaline phosphatases on two aryl phosphates (pH 8.0, 50 mM Tris, ionic strength kept constant with NaCl at 200 mM)

	4-Nitrophenyl phosphate			Phenyl phosphate		
Enzyme	k_{cat}, s^{-1}	K_m, μM	k_{cat}/K_m, $M^{-1} \cdot s^{-1}$	k_{cat}, s^{-1}	K_m, μM	k_{cat}/K_m, $M^{-1} \cdot s^{-1}$
Wild type	19.0	5.5	3.5×10^6			
R166K	4.4	5.2	8.4×10^5	6.8	81.1	8.4×10^4
R166Q	1.4	65.9	2.1×10^4	1.1	575.0	1.9×10^3

Values for the reaction of the wild-type enzyme with phenyl phosphate are provided in ref. 4.

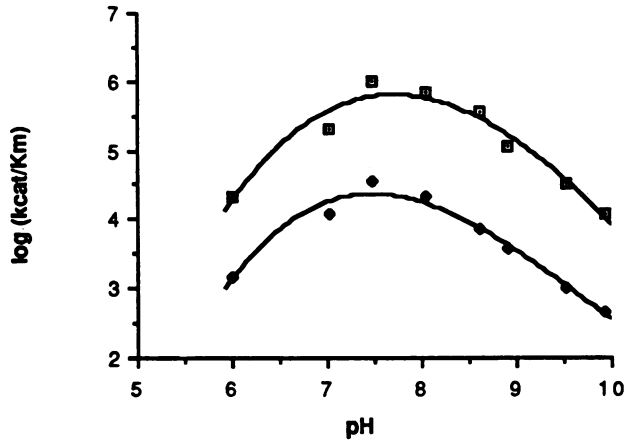

FIG. 2. The pH dependence of $\log(k_{cat}/K_m)$ for the hydrolysis of 4-nitrophenyl phosphate catalyzed by the mutant alkaline phosphatases. Enzyme assays were in 50 mM Tris buffer at constant ionic strength (200 mM) at 25.0 ± 0.1°C. ⊡, Lysine-166 alkaline phosphatase; ■, glutamine-166 alkaline phosphatase.

the pH range measured is similar to that observed for the wild-type enzyme (17). The profiles of the mutant enzymes do not show shifts in their pH dependence. When compared with the pH profiles of the wild type (17), the only difference observed is the slightly narrowed optimal pH range.

We have evidence that the mechanism followed by the mutant alkaline phosphatases lysine-166 and glutamine-166 is the same as in the wild-type enzyme. This evidence is provided by preliminary studies of the pre-steady-state kinetic behavior of both mutants. Upon mixing excess 2,4-dinitrophenyl phosphate (53.0 μM) at pH 5.5 (0.1 M acetate buffer) with the mutant enzyme (5.3 μM) at pH 7.8 (0.005 M *N*-methyl morpholine/HCl) in a Durrum–Gibson stopped-flow spectrophotometer, we observed at 400 nm an initial rapid liberation of 2,4-dinitrophenolate ion for ≈100 ms with the lysine-166 mutant (R166K) and for about 120 ms with the glutamine-166 mutant (R166Q), followed by a slow, zero-order hydrolysis of the substrate. This is consistent with the accumulation of a phosphorylated enzyme intermediate, as reported for the wild type (18). The transient formation of a phosphorylated intermediate in the catalytic mechanism of the mutants is also evident by the effect of Tris during catalytic turnover (Table 2): a significant increase in the k_{cat} value with increasing Tris concentration for both mutants is

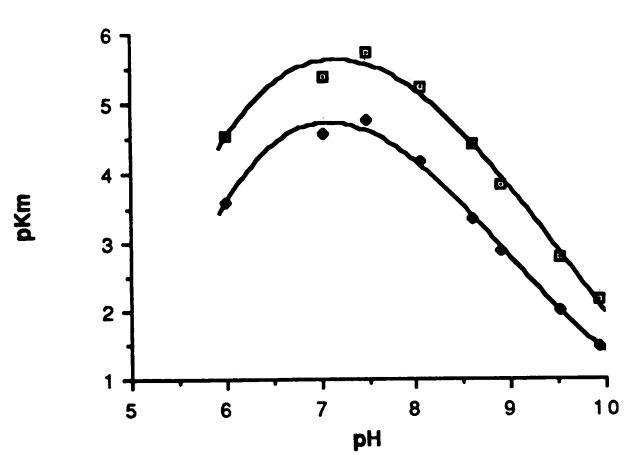

FIG. 3. The pH dependence of $-\log(K_m)$ for the hydrolysis of 4-nitrophenyl phosphate catalyzed by the mutant alkaline phosphatases. Enzyme assays were in 50 mM Tris buffer at constant ionic strength (200 mM) at 25.0 ± 0.1°C. ⊡, Lysine-166 alkaline phosphatase; ■, glutamine-166 alkaline phosphatase.

Table 2. Effect of Tris concentration on kinetic parameters of lysine-166 and glutamine-166 alkaline phosphatases (4-nitrophenyl phosphate, 25°C, ionic strength constant at 200 mM)

	1 M Tris		0.05 M Tris	
Enzyme	k_{cat}, s^{-1}	K_m, μM	k_{cat}, s^{-1}	K_m, μM
R166K	11.7	74	4.4	5.2
R166Q	15.9	2690	1.4	65.9

in accord with the expected dependence of enzyme activity on buffer concentration. The nucleophilic Tris acts as an alternative phosphoryl group acceptor for the phosphorylated enzyme intermediates of both mutants as well as of the wild type and thus enhances the observed k_{cat} values, consistent with the hypothesis that dephosphorylation of the intermediate is rate limiting.

Based on the experiments described above, we conclude that the catalytic efficiency of alkaline phosphatase does not require the presence of a positive charge at residue-166 and that this residue is not the electrophile required to explain the observed small change in effective charge on the leaving oxygen of the phosphate ester (4). This role is most likely fulfilled by one of the active-site zinc atoms. We have shown that for alkaline phosphatase it is feasible to change a potential active-site electrophile and still observe high catalytic efficiency. Combined with the results of our work showing that the active site nucleophile serine-102 (19) is also replaceable (although with more significant mechanistic consequences), it could be that the coordinative environment of the active-site zinc atoms is the crucial feature in nature's design of this phosphate hydrolase.

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