Function of Arginine-166 in the Active Site of Escherichia coli Alkaline Phosphatase[†]

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ABSTRACT: The function of arginine residue 166 in the active site of *Escherichia coli* alkaline phosphatase was investigated by site-directed mutagenesis. Two mutant versions of alkaline phosphatase, with either serine or alanine in the place of arginine at position 166, were generated by using a specially constructed M13 phage carrying the wild-type *phoA* gene. The mutant enzymes with serine and alanine at position 166 have very similar kinetic properties. Under conditions of no external phosphate acceptor, the k_{cat} for the mutant enzymes decreases by approximately 30-fold while the K_m increases by less than 2-fold. When kinetic measurements are carried out in the presence of a phosphate acceptor, 1.0 M Tris, the k_{cat} for the mutant enzymes is reduced by less than 3-fold, while the K_m increases by more than 50-fold. For both mutant enzymes, in either the absence or the presence of a phosphate acceptor, the catalytic efficiency as measured by the k_{cat}/K_m ratio decreases by approximately 50-fold as compared to the wild type. Measurements of the K_i for inorganic phosphate show an increase of approximately 50-fold for both mutants. Phenylglyoxal, which inactivates the wild-type enzyme, does not inactivate the Arg-166 → Ala enzyme. This result indicates that Arg-166 is the same arginine residue that when chemically modified causes loss of activity [Daemen, F. J. M., & Riordan, J. F. (1974) *Biochemistry 13*, 2865–2871]. The data reported here suggest that although Arg-166 is important for activity it is not essential. The analysis of the kinetic data also suggests that the loss of arginine-166 at the active site of alkaline phosphatase has two different effects on the enzyme. First, the binding of the substrate, and phosphate as a competitive inhibitor, is reduced; second, the rate of hydrolysis of the covalent phosphoenzyme may be diminished.

Alkaline phosphatase from *Escherichia coli* (EC 3.1.3.1) is a dimeric metalloenzyme (M_r 94 000) (Bradshaw et al., 1981), containing two tightly bound atoms of zinc and one of magnesium per monomer, which catalyzes the nonspecific hydrolysis of phosphate esters. Alkaline phosphatases in general are present in a broad diversity of eukaryotic and prokaryotic organisms, suggesting that the nonspecific hydrolysis of phosphate esters is functionally important although the exact physiological role of the enzyme is not clear (McComb et al., 1979). In *E. coli*, the synthesis of the enzyme is greatly elevated when cells are grown in media deficient in phosphate (Horiuchi et al., 1959; Torriani, 1960), suggesting that the enzyme functions to provide a source of inorganic phosphate. The alkaline phosphatase reaction proceeds through a phosphoenzyme intermediate. Evidence for this comes from the fact that the enzyme cleaves phosphate esters at identical rates irrespective of the leaving group or its pK_a, and an intermediate with Ser-102 phosphorylated has been isolated (Schwartz & Lipmann, 1961; Engström, 1962; Schwartz et al., 1963). In the presence of a phosphate acceptor such as ethanolamine or tris(hydroxymethyl)aminomethane (Tris),[1] the enzyme also catalyzes a transphosphorylation reaction with the transfer of the phosphate to the alcohol (Dayan & Wilson, 1964; Wilson et al., 1964). In fact, the rate of the reaction is accelerated in the presence of a phosphate acceptor, and, indeed, the standard spectrophotometric assay for the enzyme is carried out in 1 M Tris buffer. A kinetic scheme (Scheme I) has been proposed which takes into account both hydrolysis and transphosphorylation, including the formation of a phosphoenzyme complex, E–P, where S is

Scheme I
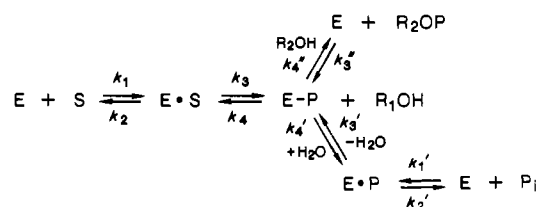
the substrate (R_1OP), R_1OH is the alcohol product, R_2OH is the phosphate acceptor, and R_2OP is the phosphorylated acceptor. The hydrolysis of E–P involves both the formation of a noncovalent enzyme–phosphate complex (E·P) and the dissociation of this complex to free enzyme and inorganic phosphate (P_i) [for a review, see Coleman and Gettins (1983)]. At acidic pH, the hydrolysis of the phosphoenzyme is rate limiting while at alkaline pH the dissociation of the noncovalently bound phosphate from the enzyme is the rate-determining step (Hull et al., 1976).

The X-ray structure of the *E. coli* enzyme has been solved (Sowadski et al., 1983) and refined to a resolution of 2.8 Å (Sowadski et al., 1985). The Ser-102 which is phosphorylated during catalysis is located near the binding sites of the metals in a pocket which is barely large enough to accommodate inorganic phosphate. Additionally, arsenate, a product analogue and enzyme inhibitor, binds between Ser-102 and the two zinc atoms with the guanidinium group of Arg-166 within hydrogen-bonding distance of the arsenate site (Sowadski et al., 1985).

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; P_i, inorganic phosphate; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; IPTG, isopropyl β-galactoside; MOPS, 3-(N-morpholino)propanesulfonic acid; PNPP, p-nitrophenyl phosphate.
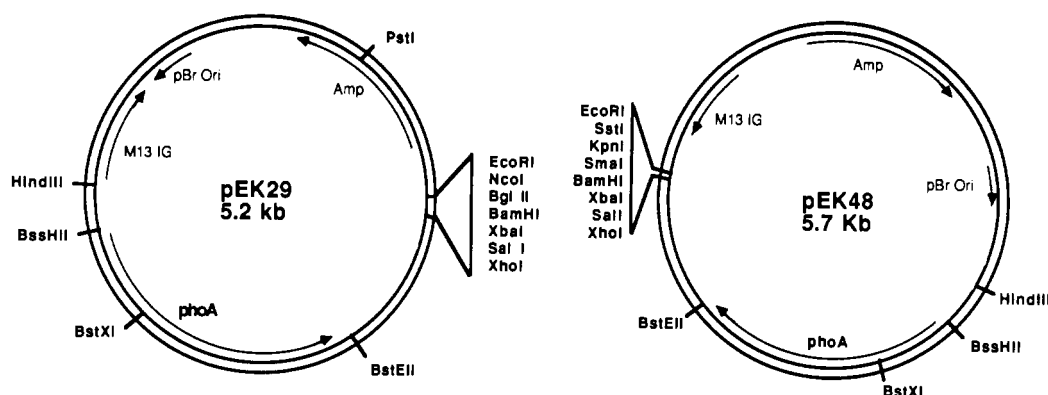
FIGURE 1: Restriction maps of the plasmids pEK29 and pEK48. pEK29 contains the wild-type *phoA* gene inserted into the vector pGC1 (Myers et al., 1985), while pEK48 contains the wild-type *phoA* gene inserted into the vector pUC119. Both plasmids contain the M13 intergenic region (M13 IG), which allows the isolation of single-stranded plasmid after coinfection of a strain containing these plasmids (MV1190) with a helper phage such as M13K07.

Experiments utilizing arginine-specific reagents suggest that there is one arginine residue per monomer, located at the active site, that when modified causes loss of activity, since in the presence of an inhibitor such as phosphate or phenyl phosphonate one less arginine is modified than in the absence of the inhibitor (Daemen & Riordan, 1974). Although the actual residue that remains unmodified in the presence of inhibitor has never been identified, it is reasonable to speculate that it is Arg-166.

The combination of high-resolution X-ray crystallography along with site-directed mutagenesis has provided a new approach for the identification of catalytically crucial residues, the study of structure–function relationships in enzymes, and perhaps the alteration of enzyme specificities and relative activities as well (Leatherbarrow & Fersht, 1986). Here we report the use of site-directed mutagenesis to determine the exact role that Arg-166 plays in the catalytic mechanism of alkaline phosphatase.

MATERIALS AND METHODS

Materials. Agar, agarose, ampicillin, sodium dihydrogen phosphate, Tris, phenylglyoxal, and *p*-nitrophenyl phosphate were purchased from Sigma Chemical Co. Tryptone and yeast extract were obtained from Difco Laboratories. Sucrose and enzyme-grade ammonium sulfate were purchased from ICN Biochemicals. Restriction endonucleases were obtained from either U.S. Biochemicals or New England Biolabs and used according to the supplier's recommendations. T4 DNA ligase, the Klenow fragment of DNA polymerase I, and T4 polynucleotide kinase were products of U.S. Biochemicals. NA45 paper used for the isolation of DNA fragments from agarose gels was purchased from Schleicher & Schuell.

The *E. coli* K12 strains JM101 [Δ(*lac-proAB*), *supE*, *thi*/F′ *traD36*, *proAB*, *lacI*q, *lacZ*ΔM15] and MV1190 [Δ(*lac-proAB*), *supE*, *thi*, Δ(*sri-recA*) 306::Tn10(tetr)/F′ *traD36*, *proAB*, *lacI*q, *lacZ*ΔM15], the plasmid pUC119, and also the M13 phages M13mp19 and M13K07 were obtained from J. Messing. The Δ*phoA* strain SM547 [Δ(*phoA-proC*), *phoR*, tsx::Tn5, Δ*lac*, *galK*, *galU*, *leu*, strr] and the plasmid pHI-1 were a gift of H. Inouye. The strains HB2151 [F′ *ara*, *thi*, Δ(*pro-lac*)/F′ *proAB*, *lacI*q, *lacZ*ΔM15] and HB2154 [F′ *ara*, *thi*, Δ(*pro-lac*), mutL::Tn10(tetr)/F′ *proAB*, *lacI*q, *lacZ*ΔM15] were from G. Winter while the plasmid pGC1 was obtained from R. Myers.

Oligonucleotide Synthesis. The oligonucleotides required for the site-directed mutagenesis were synthesized by using either the liquid-phase phosphotriester method (Gough et al., 1982), starting from protected dimers (P-L Biochemicals), or

an Applied Biosystems 381A DNA synthesizer. Purification was accomplished, after deblocking, by preparative polyacrylamide gel electrophoresis and was checked by reverse-phase HPLC using a Beckman C3 Ultrapore column.

Construction of pEK29. The reaction of the plasmid pHI-1 (Inouye et al., 1981) with the restriction enzymes *Hind*III and *Xho*I produced four fragments including one 2.5-kb fragment containing the wild-type *phoA* gene. This mixture of fragments was combined with the plasmid pGC1 (Myers et al., 1985), which had been previously cut with the same two restriction enzymes, and then treated with T4 DNA ligase at 4 °C overnight. After ligation, the mixture was then transformed into the Δ*phoA* strain SM547. Selection for the desired plasmid was performed on YT medium plates (Miller, 1972) containing 25 μg/mL ampicillin and 40 μg/mL 5-chloro-4-bromo-3-indolyl phosphate. Restriction analysis of plasmid DNA isolated, by the alkaline lysis procedure (Maniatis et al., 1982), from 10 blue colonies was used to identify a plasmid with the correct construction, pEK29 (see Figure 1).

Construction of pEK48. The *Hind*III–*Bam*HI fragment containing the entire *phoA* gene was obtained from plasmid pEK29 and mixed with pUC119 which had been previously cut with the same two enzymes. The mixture was treated with T4 DNA ligase at 4 °C overnight followed by transformation into competent SM547 cells and plated on YT medium plates containing 100 μg/mL ampicillin and 40 μg/mL 5-bromo-4-chloro-3-indolyl phosphate. Ten of the blue colonies were selected, and plasmid DNA was isolated. Each of the plasmid candidates was first checked for proper size and then by restriction analysis. A plasmid was isolated, pEK48, which contained the *phoA* gene in the vector pUC119 (see Figure 1).

Construction of M13phoA. Prior to mutagenesis, the entire *phoA* gene was removed from pEK29 and cloned into M13mp19. To accomplish this, both M13mp19 and pEK29 were cut with *Hind*III and *Bam*HI. The appropriate fragments were isolated after agarose gel electrophoresis, using NA45 paper, followed by treatment with T4 DNA ligase. The ligation mixture was then transformed into competent JM101 cells and overlayed onto YT medium plates containing X-gal and IPTG. Restriction analysis of RF DNA isolated from a number of white plaques was used to verify the construction. In addition, the presence of the *phoA* gene was verified by dideoxy sequencing (Sanger et al., 1977) of a portion of the gene, using single-stranded DNA isolated from this recombinant M13 phage and an oligonucleotide primer specific for the *phoA* gene.

Expression of Wild-Type and Mutant Alkaline Phosphatases. SM547 was used as the host strain for expression of both the wild-type and mutant alkaline phosphatases. This strain has the *phoA* gene deleted from the chromosome as well as a mutation in the *phoR* regulatory gene. Therefore, if a *phoA*-containing plasmid is introduced into this strain, the alkaline phosphatase produced will be exclusively from the *phoA* gene on the plasmid. Wild-type enzyme was prepared from the plasmid/strain combination pEK29/SM547 while the Arg-166 → Ser and Arg-166 → Ala enzymes were prepared from pEK34/SM547 and pEK86/SM547, respectively.

Purification of the Wild-Type and Mutant Alkaline Phosphatases. As a first step in the extraction of alkaline phosphatase, the bacterial cells were grown to stationary phase in YT medium containing 150 μg/mL ampicillin. The cells were harvested, and the cell pellet was washed and osmotically shocked according to the method of Brockman and Heppel (1968). The periplasmic proteins were precipitated from the cold water wash with gradual addition of ammonium sulfate to 85% saturation. The precipitate was pelleted by centrifugation at 8000g for 25 min and then resuspended in a small volume of TMZP buffer (0.01 M Tris-HCl, 10^{-3} M MgCl$_2$, 10^{-4} M NaH$_2$PO$_4$, 3.1 × 10^{-3} M NaN$_3$, and 10^{-5} M ZnSO$_4$, pH 7.4) (Block & Beckar, 1978). This suspension was then exhaustively dialyzed against TMZP buffer before purification by ion-exchange chromatography employing Q-Sepharose Fast Flow (Pharmacia) resin. The enzyme was eluted from the column by utilizing a linear gradient of 0–0.1 M NaCl in TMZP buffer. The fractions containing essentially pure alkaline phosphatase were identified by electrophoresis on a 10% polyacrylamide denaturing gel and then pooled and dialyzed against TMZP buffer followed by storage at 4 °C.

Polyacrylamide Gel Electrophoresis. Electrophoretic analyses were performed in denaturing polyacrylamide gels containing 10% acrylamide according to the procedure of Laemmli (1970).

Determination of Protein Concentration. Concentrations of the wild-type and mutant enzymes were determined either by the method of Lowry et al. (1951) or by the Bio-Rad version of Bradford's dye binding assay (Bradford, 1976) using bovine serum albumin as the standard.

Assay. Alkaline phosphatase activity was measured spectrophotometrically utilizing *p*-nitrophenyl phosphate as the substrate (Garen & Levinthal, 1960). The release of *p*-nitrophenolate was monitored at 410 nm, and the velocity was calculated by utilizing a molar absorptivity of 1.62 × 10^4 M^{-1}·cm^{-1} (Halford, 1971). All assays were carried out at 25 °C either in 1.0 M Tris-HCl, pH 8.0, buffer (high Tris), in 0.01 M Tris-HCl/0.5 M NaCl, pH 8.0, buffer (low Tris), or in 0.1 M MOPS buffer, pH 8.0 (MOPS buffer).

RESULTS

Construction of the Arg-166 → Ser Alkaline Phosphatase by Site-Directed Mutagenesis. The introduction of serine at position 166 of alkaline phosphatase was accomplished by site-directed mutagenesis utilizing the procedure of Zoller and Smith (1982) as modified by Norris et al. (1983). A 17-mer oligonucleotide was synthesized to introduce the specific C to A base change necessary for the substitution. This oligonucleotide would also introduce a new *Xho*I site if the mutagenesis was successful. Before synthesis, the sequence of the oligonucleotide was compared to the sequence of the entire *phoA* gene to determine if there were any secondary hybridization sites. Although the computer search indicated no significant secondary sites, after synthesis the oligonucleotide was tested to ensure that no secondary priming sites existed

by using the synthetic oligonucleotide as a primer in Sanger sequencing (Sanger et al., 1977). Excellent sequence was obtained indicating that the oligonucleotide annealed to only the desired site. Following primer extension, ligation, and transformation, screening of possible mutants was performed by restriction analysis of M13 RF DNA using *Xho*I. The sequence of the putative mutants was confirmed by dideoxy sequencing. Approximately 16% of the candidates were identified as having the desired single nucleotide change.

Construction of the Arg-166 → Ala Alkaline Phosphatase by Site-Directed Mutagenesis. The replacement of arginine by alanine at position 166 was accomplished by site-directed mutagenesis using the method of Zoller and Smith (1982) with one modification. After the fill-out and ligation steps, the M13 RF was transformed into strain HB2154. HB2154 is defective in DNA repair, and therefore mismatch repair is prevented (Carter et al., 1985). In order to avoid the introduction of undesirable mutations, the exposure of the M13 RF to the repair-defective background was reduced by plating the transformation mixture onto a lawn of HB2151, a repair positive version of HB2154. Following primer extension, ligation, and transformation, 48 transformants were screened by dot blot hybridization, and 10 of them were selected as putative mutants. Single-stranded DNA from these candidates was isolated, and the region around position 166 was sequenced by the dideoxy method (Sanger et al., 1977). Of the 10 candidates, 5 were identified as having the desired nucleotide change.

Recloning and Confirmation of the Arg-166 → Ser Mutation. The construction of a plasmid containing the Arg-166 → Ser mutation was accomplished by cutting both plasmid pEK29 and the mutant M13 RF with the restriction enzymes *Bss*HII and *Bst*EII. Digestion with these enzymes produced a fragment of the *phoA* gene containing the 166 site. The strategy was to replace the fragment from the wild-type gene in pEK29 with the mutant fragment from the mutant M13 RF DNA. To ensure that the fragment from the wild-type gene was not able to religate, the pEK29 backbone was removed from an agarose gel after electrophoresis using NA45 paper. Transformation was performed into competent SM547 cells, and selection for the correct construction was augmented by restriction analysis using *Xho*I which would cut at the mutation site. A plasmid was isolated, pEK34, which contained the desired mutation.

Recloning and Confirmation of the Arg-166 → Ala Mutation. In this case, a *Bss*HII–*Bst*XI fragment of 441 base pairs containing the Arg-166 → Ala mutation was isolated from the purified M13 RF after agarose gel electrophoresis using NA45 paper. In addition, the plasmid pEK48 was cut with the same two restriction enzymes, and the larger fragment was isolated in a similar fashion. This fragment, containing the vector pUC119 along with the remainder of the *phoA* gene, was combined with the fragment of the mutant RF and the mixture treated with T4 DNA ligase at 4 °C overnight followed by transformation into competent SM547 cells. Plasmid DNA was isolated from several candidates and checked by restriction analysis. A plasmid was isolated, pEK86, which carried the desired mutation.

Single-stranded DNA was isolated from plasmid pEK86 in MV1190 after coinfection with M13K07. The entire *Bss*HII–*Bst*XI fragment used in the construction of pEK86 was sequenced to ensure that no other mutations had occurred during the mutagenesis. Dideoxy sequencing was carried out by using three oligonucleotide primers spaced along the fragment. Analysis of the sequence data revealed no mutations
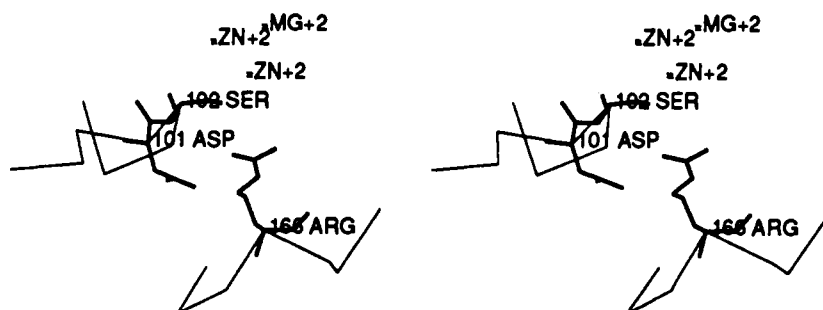
FIGURE 2: Stereoview of the active-site region of *E. coli* alkaline phosphatase. The coordinates used for this figure were provided by H. Wyckoff. The serine which is phosphorylated (Ser-102) is shown along with Arg-166 and Asp-101 which form a salt link. The zinc and the magnesium atoms at the active site are also shown.

other than the desired change.

Mutations at Position 166 Do Not Cause Loss of Activity. The chemical modification results of Daemen and Riordan (1974) on *E. coli* alkaline phosphatase suggested that there was one catalytically essential arginine residue per active site involved in the binding of the negatively charged phosphate group of the substrate. As seen in Figure 2, an examination of the X-ray structure of the enzyme (Sowadski et al., 1985) reveals that Arg-166 is probably a catalytically important residue. Arg-166 is positioned near the metals, near the serine which is phosphorylated (Ser-102), and interacts with arsenate. Therefore, it seems probable that Arg-166 is the arginine residue implicated in the chemical modification experiments. In order to investigate the function of Arg-166, two substitutions were made at this position by site-directed mutagenesis. The catalytic ability of the mutant enzymes was first tested in vivo by using strain SM547. A *phoA*-containing plasmid in this strain will overexpress alkaline phosphatase even in rich medium which contains high levels of P_i. The presence or absence of activity can be checked by plating the strain on rich plates in the presence of 5-chloro-4-bromo-3-indolyl phosphate and observing whether the resulting colonies have a blue color. When plasmids pEK34 and pEK86, which carry the *phoA* genes with the Arg-166 → Ser and Arg-166 → Ala mutations, respectively, were introduced into strain SM547 and plated on YT medium plates containing ampicillin and 5-chloro-4-bromo-3-indolyl phosphate, blue colonies were observed indicating phosphatase activity. Furthermore, the relative color of the colonies suggested that both mutant enzymes still possessed significant catalytic activity in vivo.

Both Mutations Have a Larger Effect on K_m than V_{max}. Initial kinetic characterization of the purified mutants, under standard assay conditions (high Tris), confirmed that both mutant enzymes retain significant activity (see Figure 3). Compared to a maximal velocity of 39.8 μmol·min^{-1}·mg^{-1} for the wild-type enzyme, the maximal velocities of the mutants were 16.1 μmol·min^{-1}·mg^{-1} for the Arg-166 → Ser and 15.8 μmol·min^{-1}·mg^{-1} for the Arg-166 → Ala enzymes (see Table I). On the other hand, both mutant enzymes exhibit a significant increase in the K_m for *p*-nitrophenyl phosphate. For the wild-type enzyme, the K_m for this substrate is 12.7 μM while it is approximately 640 and 1620 μM for the Arg-166 → Ser and Arg-166 → Ala enzymes, respectively (see Table I).

Tris Affects both the K_m and the V_{max}. The normal assay for alkaline phosphatase is carried out in 1.0 M Tris buffer since Tris is known to enhance the activity of the enzyme by acting as a phosphate acceptor in the transphosphorylation reaction. In either a low-Tris buffer (0.01 M Tris/0.5 M NaCl, pH 8.0) or a MOPS buffer (0.1 M MOPS, pH 8.0; data not shown), there is a much larger alteration in V_{max} than K_m
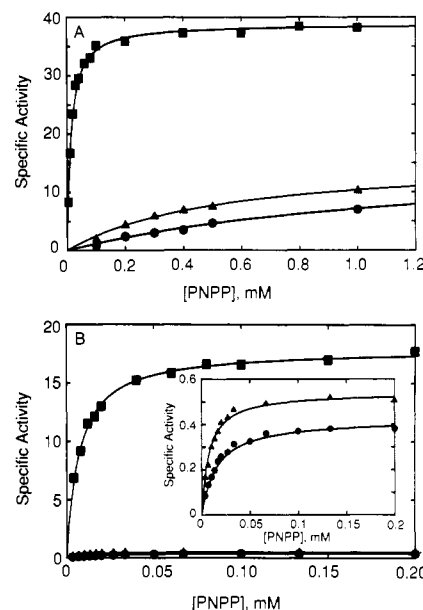

FIGURE 3: Steady-state kinetics of wild-type alkaline phosphatase (■) and the Arg-166 → Ser (▲) and Arg-166 → Ala (●) enzymes. Reactions were carried out at 25 °C in either high-Tris buffer (1.0 M Tris-HCl, pH 8.0) (A) or low-Tris buffer (0.01 M Tris-HCl/0.5 M NaCl, pH 8.0) (B). The formation of *p*-nitrophenolate was monitored at 410 nm, and the specific activity is reported in units of micromoles per minute per milligram.

Table I: Kinetic Parameters of the Wild-Type and Mutant Enzymes

enzyme	k_{cat}[a] (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1}·s^{-1})	buffer
wild type	13.6 (2.9)	7.4 (0.8)	1.8 × 10^6	low Tris[b]
Arg-166 → Ser	0.44 (0.02)	7.6 (0.7)	5.8 × 10^4	low Tris[b]
Arg-166 → Ala	0.33 (0.02)	13.8 (1.8)	2.4 × 10^4	low Tris[b]
wild type	31.2 (2.0)[d]	12.7 (3.2)	2.5 × 10^6	high Tris[c]
Arg-166 → Ser	12.6 (1.4)	640 (60)	2.0 × 10^4	high Tris[c]
Arg-166 → Ala	12.4 (1.1)	1620 (200)	0.8 × 10^4	high Tris[c]

[a] The k_{cat} values are calculated per active site from the V_{max} using a dimer molecular weight of 94 000 (Bradshaw et al., 1981). [b] 0.01 M Tris-HCl/0.5 M NaCl buffer, pH 8.0. [c] 1.0 M Tris-HCl buffer, pH 8.0. [d] The values reported are the average of at least four independent determinations with standard deviations in parentheses.

for both mutant enzymes as compared to the wild type under high-Tris conditions (see Figure 3). For example, the V_{max} for the wild-type enzyme is reduced less than 3-fold in either the low-Tris or the MOPS buffers, as compared to the high-Tris buffer, while the V_{max} is reduced by approximately 30-fold for both mutant enzymes (see Table I). For the wild-type enzyme, the K_m decreases less than 2-fold in the low-Tris buffer but approximately 85-fold for the Arg-166 → Ser enzyme and almost 120-fold for the Arg-166 → Ala enzyme.

Table II: Inhibition of the Wild-Type and Mutant Enzymes by Phosphate[a]

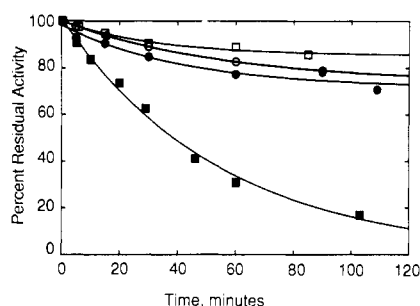| enzyme	K_i (μM)	$(K_i)_{mutant}/(K_i)_{wild\ type}$
wild type	14.3	
Arg-166 → Ser	417	29
Arg-166 → Ala	665	47

[a] 0.01 M Tris-HCl/0.5 M NaCl buffer, pH 8.0.

FIGURE 4: Reaction of the wild-type and Arg-166 → Ala alkaline phosphatases with phenylglyoxal. The reaction was carried out at 25 °C in 125 mM sodium bicarbonate buffer, pH 7.5, at 20 mM phenylglyoxal. When phosphate was present, it was at 15 mM (approximately $1000K_i$ for the wild type and $20K_i$ for the Arg-166 → Ala enzyme). The activity was monitored spectrophotometrically in the high-Tris buffer system: wild-type alkaline phosphatase alone (■) and in the presence of phosphate (□); Arg-166 → Ala alkaline phosphatase alone (●) and in the presence of phosphate (○).

k_{cat}/K_m *for the Mutants Is Reduced Substantially.* As seen in Table I, the introduction of either the serine or the alanine at position 166 of alkaline phosphatase causes more than a 100-fold reduction in k_{cat}/K_m. Under low-Tris conditions (Table I), both mutant enzymes also exhibit substantial reductions in k_{cat}/K_m compared to the wild-type enzyme; however, the reductions in k_{cat}/K_m are not as large as those observed under high-Tris conditions.

Phosphate Binds More Weakly to the Mutants than the Wild Type. In order to further assess the differences between the mutant and wild-type enzymes, the ability of phosphate to act as a competitive inhibitor of the reaction was measured for the wild-type and mutant enzymes. As seen in Table II, the K_i for inorganic phosphate increases 30–50-fold for the Arg-166 → Ser and Arg-166 → Ala enzymes.

Phenylglyoxal Does Not Inactivate the Mutant Enzymes. Daemen and Riordan (1974) showed that phenylglyoxal or 2,3-butanedione inactivates wild-type alkaline phosphatase and that inhibitors protect the enzyme against inactivation. Furthermore, they correlated the loss of activity with one arginine residue per monomer. If the arginine protected from inactivation is Arg-166, then the mutant enzymes should not be inactivated by phenylglyoxal or 2,3-butanedione. The reaction of both the wild-type and the Arg-166 → Ala enzymes with phenylglyoxal, in the presence and absence of 15 mM phosphate, is shown in Figure 4. For the wild-type enzyme, the rate of phenylglyoxal inactivation is dramatically reduced in the presence of a high concentration of phosphate. However, for the Arg-166 → Ala enzyme, the inactivation is not affected by phosphate, and furthermore, the rate of inactivation of the mutant is nearly identical with that of the wild-type enzyme in the presence of phosphate.

DISCUSSION

Arg-166 Is Important for Activity of Alkaline Phosphatase but Not Essential. Under standard assay conditions (high Tris), when Arg-166 in the active site of *E. coli* alkaline phosphatase is replaced by either serine or alanine, only relatively small changes in k_{cat} are observed, although much more

significant changes in K_m and the k_{cat}/K_m ratio occur. Under low-Tris conditions, where the transphosphorylation reaction is negligible, the k_{cat} for the mutant enzymes is reduced by approximately 30-fold. Preliminary experiments have shown that the reduction in enzymatic activity of the two mutant enzymes, compared to the wild-type, is not significantly dependent upon pH over the range 7.5–10.5. These kinetic results indicate that a positive charge at position 166 is not essential for the catalytic activity of alkaline phosphatase.

Upon chemical modification of a single arginine residue per monomer, enzyme activity is eliminated (Daemen & Riordan, 1974). The results presented here indicate either that the arginine residue implicated by these experiments is not Arg-166 or that the bulky nature of the complex between Arg-166 and the arginine reagents 2,3-butanedione or phenylglyoxal sterically hinders catalysis. The X-ray structure of alkaline phosphatase, which shows that Arg-166 is in the active site in close proximity to the Ser-102 and zinc atoms that are required for catalysis, suggests that Arg-166 is the most likely candidate for the residue modified (see Figure 2). The inability of the Arg-166 → Ala enzyme to be inactivated by phenylglyoxal suggests very strongly that Arg-166 is the residue altered in the chemical modification experiments. However, our experiments establish that, although Arg-166 may be important for activity, it is not essential.

Substrate Binding to the Mutant Enzymes Must Be Altered. The inhibition of the wild-type and mutant enzymes by phosphate was measured to directly determine whether the affinity of the mutant enzymes for phosphate has been altered by the mutations. As seen in Table II, both mutant forms of alkaline phosphatase have substantially reduced affinity for P_i. A reduction in affinity for P_i without a concomitant reduction in substrate affinity although possible seems unlikely.

A Decrease in Substrate Affinity Alone Cannot Explain All the Experimental Data. If the elimination of the positively charged Arg-166 results in only a reduction in substrate affinity, what effect would this have on the kinetic parameters? For the wild-type enzyme under low-Tris conditions, k_{cat}/K_m would decrease as substrate affinity decreased (Levine et al., 1969). Actually, the decrease in k_{cat}/K_m for the mutant enzymes, relative to the wild type, correlates well with their increased K_i for phosphate which suggests that the alteration in substrate affinity is primarily responsible for the decrease in k_{cat}/K_m. However, a decrease in substrate affinity alone cannot explain the substantial decreases in k_{cat} observed for the mutant enzymes under low-Tris concentrations.

At least for the wild-type enzyme in the presence of a phosphate acceptor, K_m should increase if substrate affinity is reduced given that k_2 is substantially larger than k_3 (Hull et al., 1976). This is in agreement with the K_m data for the mutant enzymes under high-Tris concentrations (see Table I). If substrate affinity is not altered substantially by the mutations, then as the concentration of phosphate acceptor (i.e., Tris) increases the k_{cat} for the wild type and mutants should become equal. As seen in Table I, under high-Tris conditions the differences in k_{cat} between the wild type and mutants are small. These data suggest that k_3 may have decreased slightly by the elimination of Arg-166. One possible explanation for the large reduction in k_{cat} of the mutant enzymes in the absence of Tris, relative to the small change in the presence of Tris, is either a decrease in k_4' or an increase in k_4.

Function of Arg-166. The measured increase in the inhibition constant of P_i for the mutant enzymes suggests that Arg-166 assists in substrate/phosphate binding. Since Arg-166

not only is involved in the initial binding of the substrate to form the E·S complex but also is involved in the release of phosphate from the noncovalent E·P complex, the loss of Arg-166 may destabilize the E–P and/or E·P complexes. Hull et al. (1976) have shown that, at pH 8.0, the release of P_i from the noncovalent E·P complex after hydrolysis of the phosphoenzyme complex (E–P) is the rate-determining step, not the hydrolysis of the E–P itself. For the mutant enzymes, a reduction in substrate/phosphate affinity alone should therefore result in an enzyme with improved, or unchanged, catalytic ability at pH 8.0. Since this is not the case, the mutations have altered additional steps in the mechanism, one of which may be hydrolysis of the phosphoenzyme intermediate, making the mutant enzymes less efficient catalysts. Experiments are currently in progress to better quantitate the effects of these two amino acid substitutions on *E. coli* alkaline phosphatase and to determine which steps in the mechanism are influenced by these substitutions.

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