A Water-Mediated Salt Link in the Catalytic Site of *Escherichia coli* Alkaline Phosphatase May Influence Activity†

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ABSTRACT: *Escherichia coli* alkaline phosphatase catalyzes the hydrolysis of a wide variety of phosphomonoesters at similar rates, and the reaction proceeds through a phosphoenzyme intermediate. The active site region is highly conserved between the *E. coli* and mammalian alkaline phosphatases. The three-dimensional structure of the *E. coli* enzyme indicates that Lys-328, which is replaced by histidine in all mammalian alkaline phosphatases, is bridged to the phosphate through a water molecule. This water molecule is also hydrogen bonded to Asp-327, a bidendate ligand of the one of the two zinc atoms. Here we report the use of site-specific mutagenesis to convert Lys-328 to both histidine and alanine. Steady-state kinetic studies above pH 7.0 indicate that both mutant enzymes have altered pH versus activity profiles compared to the profile for the wild-type enzyme. At pH 10.3, in the presence of Tris, the Lys-328 → Ala enzyme is approximately 14-fold more active than the wild-type enzyme. At the same pH in the absence of Tris the Lys-328 → Ala enzyme is still 6-fold more active than the wild-type enzyme. Both mutant enzymes have lower phosphate affinities than the wild-type enzyme at all pH values investigated. Pre-steady-state kinetics at pH 5.5 reveal that the Lys-328 → Ala enzyme behaves very similar to the phosphate-free wild-type enzyme. However, at pH 8.0, as opposed to the wild-type enzyme that does not exhibit a transient phase, the mutant enzyme shows a small transient phase in the pre steady state, suggesting a possible change in the rate-limiting step for this mutant enzyme at this pH. The properties of the mutant enzymes can be rationalized on the basis of a reduction in phosphate affinity. These data also suggest a possible alteration of the pK_a of the zinc-coordinated hydroxyl group.

Escherichia coli alkaline phosphatase (EC 3.1.3.1) catalyzes the hydrolysis of a wide variety of phosphomonoesters at similar rates (Reid & Wilson, 1971) and proceeds through a phosphoenzyme intermediate (Schwartz & Lipmann, 1961; Engström, 1962; Schwartz et al., 1963). In the presence of a phosphate acceptor such as ethanolamine or Tris, the enzyme also catalyzes a transphosphorylation reaction with the transfer of the phosphoryl group to the alcohol (Dayan & Wilson, 1964; Wilson et al., 1964). The rate-determining step of the reaction is pH dependent and at acid pH the hydrolysis of the covalent enzyme–phosphate complex is rate limiting, while under basic conditions the release of phosphate from the noncovalent enzyme–phosphate complex is rate limiting (Hull et. al., 1976; Reid & Wilson, 1971; Gettins & Coleman, 1983; Bloch & Gorby, 1980).

The amino acid sequences of alkaline phosphatase from *E. coli* (Bradshaw et al., 1981; Chang et al., 1986), *Bacillus subtilis* (Hulett et al., 1991), *Saccharomyces cerevisiae*, (Kaneko et al., 1987), rat (Thiede et al., 1988), and human (Berger et al., 1987; Kam et al., 1985; Weiss et al., 1986) have been determined directly or have been deduced from the corresponding DNA sequences. The alkaline phosphatase sequence is highly conserved during evolution. From consideration of only exact amino acid matches, the *E. coli* and mammalian enzymes are 25–30% conserved. The amino acid sequence of the mammalian alkaline phosphatases can be fit to the structure of the *E. coli* enzyme, further supporting the utility of using the *E. coli* enzyme as a model for all the alkaline phosphatases (Kim & Wyckoff, 1989).

Although studies of the *E. coli* enzyme started as early as 1960 and many physicochemical, kinetic [for a review, see Coleman and Gettins (1983)], and structural (Kim & Wyckoff, 1989, 1991; Sowadski et al., 1983, 1985; Wyckoff et al., 1983) properties are known, the exact physiological role and the detailed catalytic mechanism of the enzyme are still unclear. Since alkaline phosphatase from *E. coli* has served as the prototype for all the alkaline phosphatases, especially the mammalian alkaline phosphatases, it has been of great interest to identify and investigate the catalytically important residues and the structure–function relationships in the *E. coli* enzyme. Studies have been greatly facilitated by the determination of the amino acid (Bradshaw et al., 1981) and DNA (Chang et al., 1986) sequences and the solution and refinement of the X-ray structure of the enzyme to 2.0 Å (Kim & Wyckoff, 1989, 1991). The enzyme is comprised of two identical polypeptide chains, each with 449 amino acids, one magnesium atom, and two zinc atoms. The hydroxyl of Ser-102, which is near the binding site of the metals, forms the covalent bond with the phosphate during catalysis (Schwartz & Lipmann, 1961; Engström, 1962; Schwartz et al., 1963). Asp-101 electrostatically interacts with Arg-166, which in turn interacts with the phosphate portion of the substrates (Kim & Wyckoff, 1989, 1991). Previous studies have shown that Arg-166 assists in substrate/phosphate binding but neither the residue itself nor the positive charge is critical for enzymatic activity (Chaidaroglou et al., 1988; Butler-Ransohoff et al., 1988). In addition, Asp-101 has been shown to be unimportant for catalytic activity. In fact, at elevated pH the Asp-101 → Ala enzyme is more active than the wild-type enzyme, although the catalytic efficiency of the mutant enzyme as measured by the k_{cat}/K_m ratio remains unaltered (Chaidaroglou & Kantrowitz, 1989).

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Here we have chosen to investigate the function of the side chain of residue 328. In wild-type *E. coli* alkaline phosphatase, position 328 is occupied by a lysine residue that interacts with the phosphate at the active site via a water-mediated hydrogen bond. This water molecule is also hydrogen bonded to Asp-327, which is a bidentate ligand of the zinc at the M1[1] site. Histidine occupies position 328 in all the mammalian alkaline phosphatases sequenced to date along with the *S. cerevisiae* (Kaneko et al., 1987) enzyme, but in the *B. subtilis* (Hulett et al., 1991) enzyme a tryptophan is found at this position. Here we report the use of site-specific mutagenesis to convert Lys-328 to histidine and alanine. Since position 328 is conserved as a histidine in all the mammalian alkaline phosphatases, the histidine substitution will determine if the enhanced catalytic activity of the mammalian enzyme over the *E. coli* enzyme can be partially or totally attributed to the existence of histidine at this position. Since the replacement of Lys-328 by alanine will completely prevent the water-mediated hydrogen-bonding interaction between the side chain of Lys-328 and the phosphate, this substitution will be used to investigate the importance of this interaction.

Materials and Methods

Materials. Agar, ampicillin, CAPS,[2] MOPS, *p*-nitrophenyl phosphate, and sodium dihydrogen phosphate were purchased from Sigma Chemical Co. Tris, electrophoresis-grade agarose, enzyme-grade ammonium sulfate, and sucrose were purchased from ICN Biomedicals. Tryptone and yeast extract were obtained from Difco Laboratories. All the reagents needed for DNA sequencing were purchased from U.S. Biochemicals. Restriction endonucleases, T4 DNA ligase, the Klenow fragment of DNA polymerase I, and T4 polynucleotide kinase were obtained from either U.S. Biochemicals or New England Biolabs and used according to the supplier's recommendations. DNA fragments were isolated from agarose gels with use of glass beads employing the US Bioclean kit from U.S. Biochemicals. 2,4-Dinitrophenyl phosphate was synthesized by the method of Kirby and Varvoglis (1966) by use of dibenzyl phosphorochloridate as the phosphorylating agent (Atherton et al., 1945).

Strains. The *E. coli* K12 strain MV1190 [Δ(*lac–proAB*), *supE*, *thi*, Δ(*sri–recA*) 306::Tn10(tet^r)/F′ *traD36*, *proAB*, *lacI*^{q}, *lacZ*ΔM15], and the M13 phage M13K07 were obtained from J. Messing. The Δ*phoA E. coli* K12 strain SM547 [Δ(*phoA–proC*), *phoR*, tsx::Tn5, Δ*lac*, *galK*, *galU*, *leu*, str^r] was a gift of H. Inouye. The strain CJ236 [*dut-1*, *ung-1*, *thi*-1, *relA–1*/pCJ105 (Cm^r)] was a gift of T. Kunkel.

Oligonucleotide Synthesis. The oligonucleotides required for the site-specific mutagenesis and the sequencing primers were synthesized on an Applied Biosystems 381A DNA synthesizer and purified by HPLC employing a DuPont Zorbay Oligo ion-exchange column.

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.

Purification of the Wild-Type and Mutant Alkaline Phosphatases. The wild-type, the Lys-328 → His,[3] and the Lys-328 → Ala enzymes were isolated from the plasmid/strain combinations pEK48/SM547, pEK145/SM547, and pEK146/SM547, respectively, by the method previously described (Chaidaroglou et al., 1988); however, this purification scheme was not totally effective for the Lys-328 → Ala enzyme due to the alteration in charge resulting from the amino acid replacement and/or alteration in phosphate affinity. In order to purify this enzyme to homogeneity, gel permeation chromatography was employed after the ion-exchange chromatography step with use of an Ultragel AcA34 (IBF Biotechnics) column (1.5 × 100 cm). The enzyme was eluted with TMZP[4] buffer. The enzyme purity was judged by polyacrylamide gel electrophoresis, according to the procedure of Laemmli (1970).

Determination of Protein Concentration. The concentration of the wild-type enzyme was determined by absorbance measurements at 278 nm with an extinction coefficient of 0.71 cm^2/mg (Plocke & Vallee, 1962). The concentration of the mutant enzyme was determined by the Bio-Rad version of Bradford's dye binding assay (Bradford, 1976) with wild-type alkaline phosphatase as the standard.

Determination of Enzymatic Activity. Alkaline phosphatase activity was measured spectrophotometrically by utilizing *p*-nitrophenyl phosphate as the substrate (Garen & Leventhal, 1960). The release of *p*-nitrophenolate was monitored at 410 nm. The extinction coefficient of *p*-nitrophenolate was determined at each pH value used by measuring the absorbance after complete enzymatic hydrolysis of the substrate. To measure hydrolysis alone either 0.1 M MOPS or 0.1 M CAPS was used. The ionic strength was held constant at 0.585 M, which corresponds to the ionic strength of 1.0 M Tris at pH 8.0.

Determination of Inorganic Phosphate. The phosphate contents of the various preparations of alkaline phosphatase were determined by a modification of the procedure of Chen (1956). Before the phosphate determination, both the wild-type and mutant alkaline phosphatases were dialyzed once against a 500-fold excess of 0.01 M Tris-HCl, 0.1 M NaCl buffer, pH 7.4 for 12–14 h. The phosphate standards, blanks (solution from outside of the dialysis bag), and samples were added to acid-washed tubes and evaporated to dryness at approximately 170 °C. To each tube was added 0.5 mL of 70% perchloric acid, and then the tube was heated at 170 °C until the solution turned clear (approximately 4 h). After the tubes had cooled, 4.3 mL of a freshly prepared solution of 2.3% ascorbic acid and 0.3% ammonium molybdate was added to each. After mixing, the tubes were heated for 5 min at 100 °C and then allowed to cool to room temperature before the absorbance was measured at 660 nm. The absorbance was linear with phosphate concentration between 0 and 60 nmol.

Rapid Kinetic Measurements. Experiments were performed by use of a KinTek Inc. stopped-flow spectrophotometer at 25 °C with a dead time of less than 1 ms. Data was collected at 390 nm directly by computer via an analog/digital interface. One syringe contained either 10.3 μM wild-type enzyme or 7.8 μM Lys-328 → Ala enzyme in 0.01 M Tris, 0.1 M NaCl, pH 7.4. The other syringe contained 0.1 mM 2,4-dinitrophenyl

[1] The metal sites M1, M2, and M3, identified by X-ray crystallography, correspond to the spectroscopically deduced metal sites A, B, and C, respectively (Sowadski et al., 1985).

[2] Abbreviations: CAPS, (cyclohexylamino)propanesulfonic acid; MOPS, 3-(morpholino)propanesulfonic acid; P_i, inorganic phosphate.

[3] The notation used to name the mutant enzymes is, for example, the Lys-328 → His enzyme. The wild-type amino acid and location within the peptide chain are indicated to the left of the arrow while the new amino acid is indicated to the right of the arrow.

[4] TMZP buffer: 0.01 M TRIS, 10^{-3} M $MgCl_2$, 10^{-4} M NaH_2PO_4, 3.1 × 10^{-3} M NaN_3, 10^{-5} M $ZnSO_4$, pH 7.4 (Block & Beckar, 1978).

Table I: Kinetic Parameters of the Wild-Type and Mutant Enzymes at pH 8.0[a]

enzyme	k_{cat}[b] (s^{-1})	K_m (μM)	$k_{cat}/K_m \times 10^{-6}$ (M^{-1} s^{-1})	buffer
wild type[c]	80.5	21.1	3.8	1.0 M Tris, pH 8.0
Lys-328 → His	71.4	58	1.2	1.0 M Tris, pH 8.0
Lys-328 → Ala	161	159	1.0	1.0 M Tris, pH 8.0
wild type	44.5	9.4	4.3	0.1 M MOPS, pH 8.0[d]
Lys-328 → His	4.8	1.0	4.8	0.1 M MOPS, pH 8.0
Lys-328 → Ala	9.0	2.5	3.6	0.1 M MOPS, pH 8.0

[a] Assays were performed at 25 °C in the buffer indicated with use of *p*-nitrophenyl phosphate as substrate. [b] The k_{cat} values are calculated from the V_{max} by use of a dimer molecular weight of 94 000 (Bradshaw et al., 1981). The k_{cat} per active site would be half of the value indicated. [c] After purification, the wild-type enzyme used for these studies contained approximately 1.6 mol of phosphate bound per dimer. [d] For the MOPS buffer, the ionic strength was adjusted to 0.585 with NaCl.

phosphate in 0.1 M sodium acetate buffer, pH 5.5, or 0.1 mM *p*-nitrophenyl phosphate in 0.1 M MOPS, pH 8.2. The final pH after mixing equal volumes of enzyme and substrate was either 5.5 or 8.0, respectively.

Results

Construction of the Lys-328 → His and Lys-328 → Ala Alkaline Phosphatases by Site-Specific Mutagenesis. To introduce the histidine and alanine substitutions at position 328 of alkaline phosphatase, the method of Kunkel (Kunkel, 1985; Kunkel et al., 1987) was used by employing a single-stranded copy of the phagemid pEK48 (Chaidaroglou et al., 1988). Infection of the strain CJ236 containing the phagemid pEK48 with the helper phage M13K07 was used to induce the production of the single-stranded copy (Vieira & Messing, 1987). Selection of the mutations was performed directly by dideoxy sequencing (Sanger et al., 1977).

Recloning and Confirmation of the Mutations. After verification of the mutations, a small fragment of the gene was removed with restriction enzymes and inserted into a plasmid that had the corresponding section of the wild-type gene removed. In each case, a *Sph*I–*Bst*XI fragment containing the desired mutation was isolated. In addition, the plasmid pEK48 (Chaidaroglou et al., 1988) was cut with the same two restriction enzymes, and the larger fragment was isolated. This fragment, containing the vector plus the remainder of the *phoA* gene, was combined with the fragment containing the mutation and treated with T4 DNA ligase. The ligation mixture was then transformed into competent MV1190 cells. Single-stranded plasmid DNA was isolated after coinfection with the helper phase M13K07 (Vieira & Messing, 1987), and the mutation was verified a second time by sequencing the *phoA* gene. In this fashion, plasmids pEK145 and pEK146 were isolated, which carried the *phoA* gene for the Lys-328 → His and the Lys-328 → Ala enzymes, respectively.

Kinetics of the Mutant Enzymes at pH 8.0 in the Presence of a Phosphate Acceptor. The k_{cat} of the Lys-328 → Ala enzyme is 2-fold higher than that of the wild-type enzyme (see Table I). With use of *p*-nitrophenyl phosphate as substrate, the k_{cat} for the wild-type alkaline phosphatase under standard assay conditions (1.0 M Tris, pH 8.0) is 81 s^{-1}, while under the same conditions, the k_{cat} of the mutant enzyme is 161 s^{-1}. This noticeable increase in catalytic activity for the Lys-328 → Ala enzyme was accompanied by an increase in the K_m, from 21 μM for the wild-type enzyme to 159 μM for the mutant enzyme.

The substitution of histidine at position 328 also caused an increase in K_m from 21 μM for the wild-type to 58 μM for the Lys-328 → His enzyme at pH 8.0. However, at this pH the k_{cat} values for the Lys-328 → His and the wild-type enzymes are almost identical (see Table I).

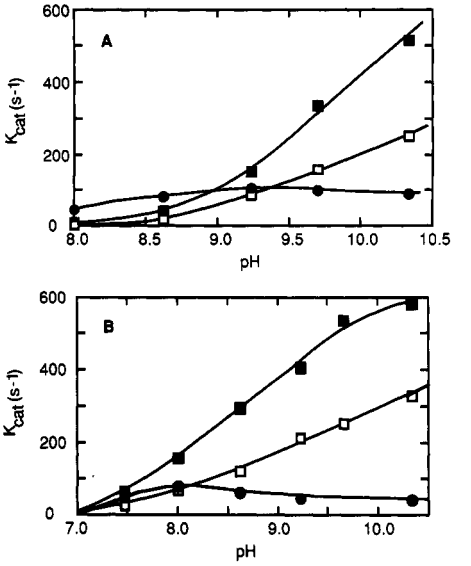

FIGURE 1: The pH dependence of the k_{cat} (s^{-1}) of the wild-type (●), the Lys-328 → Ala (■), and the Lys-328 → His (□) alkaline phosphatases. Reactions were carried out at 25 °C with *p*-nitrophenyl phosphate as substrate. (A) Reactions were performed in the absence of any alternative phosphate acceptor (0.10 M MOPS, pH 7.5–8.7, 0.1 M CAPS, pH 8.7–10.3). (B) Reactions were performed in the presence of a phosphate acceptor (1.0 M Tris). Each point corresponds to the k_{cat} calculated from the V_{max} that was obtained after fitting the data for a complete saturation curve at the indicated pH.

Kinetics in the Absence of an Alternative Phosphate Acceptor at pH 8.0. In 1.0 M Tris buffer the rate observed is the sum of transphosphorylation and hydrolysis, since Tris serves as a phosphoryl group acceptor (Wilson et al., 1964; Trentham & Gutfreund, 1968). To determine solely the hydrolytic activity of the mutant enzymes, assays were also performed in a buffer system that could not serve as an alternative nucleophile (0.1 M MOPS).

In 0.1 M MOPS buffer, pH 8.0, the k_{cat} values for both mutant enzymes are reduced significantly compared to that of the wild type (see Table I). At pH 8.0, the Lys-328 → Ala and Lys-328 → His enzymes exhibit 5-fold and 9.3-fold reductions in activity, respectively. The k_{cat}/K_m ratio for each of the mutant enzymes in 0.1 M MOPS, pH 8.0, is almost identical with the corresponding value for the wild-type enzyme, since the reduction in k_{cat} is compensated for by decreases of 3.8- and 9.4-fold in the K_m of the Lys-328 → Ala and Lys-328 → His enzymes, respectively (see Table I). Therefore, despite the lower catalytic activity of the mutant enzymes each is still as effective a catalyst as the wild-type enzyme.

pH Dependence of Mutant Enzyme Activity in the Absence of a Phosphate Acceptor. For wild-type alkaline phosphatase in the absence of a phosphate acceptor, the k_{cat} increased up to approximately pH 9.0. This increase is primarily due to the change in the rate-determining step from hydrolysis of the covalent enzyme–phosphate complex to dissociation of phosphate from the noncovalent enzyme–phosphate complex. At basic pH values the variation in activity is much different for both the Lys-328 → His and Lys-328 → Ala enzymes as compared to that of wild-tye enzyme (Figure 1A). In both cases, as the pH increases the k_{cat} increases up to pH 10.3, which was the highest pH at which data was collected. The Lys-328 → Ala enzyme was more active than the Lys-328 → His enzyme at all pH values. At pH 10.3, the activities of

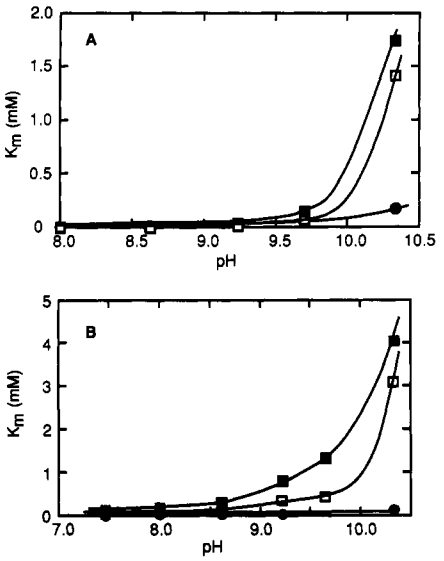

FIGURE 2: The pH dependence of the K_m of the wild-type (●), the Lys-328 → Ala (■), and the Lys-328 → His (□) alkaline phosphatases. Reactions were carried out at 25 °C with *p*-nitrophenyl phosphate as substrate. (A) Reactions were performed in the absence of any alternative phosphate acceptor (0.10 M MOPS, pH 7.5–8.7, 0.10 M CAPS, pH 8.7–10.3). (B) Reactions were performed in the presence of a phosphate acceptor (1.0 M Tris).

the Lys-328 → His and Lys-328 → Ala enzymes were 2.8-fold and 5.8-fold higher, respectively, than that of the wild-type enzyme under identical conditions.

As seen in Figure 2, the K_m of the mutant enzymes increases dramatically as the pH increases. This trend is observed for the wild-type enzyme but is much more striking for the Lys328 → His and Lys328 → Ala enzymes.

pH Dependence of Mutant Enzyme Activity in the Presence of a Phosphate Acceptor. When steady-state kinetic assays are performed in 1.0 M Tris buffer, the catalytic activity of the alkaline phosphatase is the sum of both the transphosphorylation and hydrolysis reactions. Under such conditions, the pH activity profile peaks at pH 8.0 (Wilson et al., 1964; Trentham & Gutfreund, 1968). The pH dependence of the transferase activity measured by ^{31}P NMR in the presence of 1.0 M Tris shows a bell-shaped pH dependence with a maximum activity at pH 7.5 (Gettins et al., 1985). However, the pH versus activity curve of the mutant enzymes is more similar to that observed in the absence of a phosphate acceptor (see Figure 1B). As the pH increases, the activity of the mutant enzyme increases. At pH 10.3, which was the highest pH used in these experiments, the Lys-328 → His and Lys-328 → Ala enzymes exhibit 7.8-fold and 13.8-fold higher activity, respectively, than the wild-type enzyme. Since both mutant enzymes exhibit a more significant increase in K_m than k_{cat} at each pH investigated (see Figure 2B), the mutant enzymes actually exhibit a lower k_{cat}/K_m ratio than the wild-type enzyme at the same pH, and thus exhibit lower activity at low substrate concentrations.

The Mutant Enzymes Have a Reduced Affinity for Inorganic Phosphate. To investigate the ability and degree of phosphate binding to the two mutant enzymes, the K_i of phosphate was determined. Since inorganic phosphate is not only a competitive inhibitor of alkaline phosphatase but also a product of the reaction, a competitive product inhibition assay (Segel, 1975) was used to measure the K_i of phosphate for both the enzymes (see Figure 3). At pH 8.0, the decrease in the K_m of *p*-nitrophenyl phosphate prevents an accurate determination of the K_i of phosphate for the Lys-328 → His enzyme. Like the wild-type enzyme, the K_i of inorganic

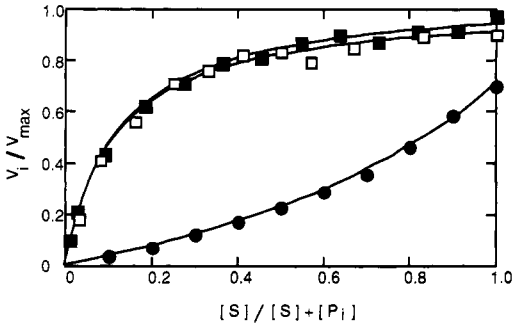

FIGURE 3: Phosphate inhibition of the wild-type (●), the Lys-328 → Ala (■), and the Lys-328 → His (□) alkaline phosphatases at pH 8.9. Reactions were carried out at 25 °C with *p*-nitrophenyl phosphate as substrate in 0.1 M CAPS buffer. The ionic strength of the buffer was adjusted to 0.585 with sodium chloride. The concentrations of *p*-nitrophenyl phosphate and inorganic phosphate were both varied; however, the sum of the two concentrations was held constant at $10K_m$. Analysis of the experimental data was according to the method of Segel (1975) for a system in which a product is also an inhibitor.

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

enzyme	K_i[b] (pH 8.0) (μM)	K_i[b] (pH 8.9) (μM)	K_i[b] (pH 10.8) (μM)
wild type	9.6	32	518
Lys-328 → Ala	87	2233	6550
Lys-328 → His	[c]	224	3190

[a] Assays were performed at 25 °C in 0.1 M MOPS buffer with use of p-nitrophenyl phosphate as substrate. In each case the ionic strength was kept constant at 0.585 with NaCl. [b] The K_i values were determined by the method of Segel (1975). The data were fit to the theoretical equation by nonlinear least-squares analysis. [c] The K_i for the Lys-328 → His enzyme could not be determined because of its extremely low K_m at this pH.

phosphate for the mutant enzymes is sensitive to pH over the range between 8.0 and 10.8 (see Table II). However, the K_i of phosphate for either of the mutant enzymes is about 9–70-fold higher than the value observed for the wild-type enzyme under the same pH conditions.

Bloch and Schlesinger (1973) have reported that, depending upon the purification, wild-type alkaline phosphatase retains phosphate tightly bound to the active site amounting to between 0.5 and 1.0 mol of phosphate/mol of active site. Since the Lys-328 → Ala and the Lys-328 → His enzymes have an increased K_i for phosphate, and therefore reduced phosphate affinity, we wanted to determined the phosphate content of the mutant enzymes after purification. Since our purification is different from that used by Bloch and Schlesinger (1973), the phosphate content of the wild-type alkaline phosphatase was also determined. The phosphate analysis of the wild-type enzyme revealed the presence of 1.6 ± 0.2 mol of phosphate/mol of enzyme, consistent with the endogenous phosphate content of the wild-type enzyme (Bloch & Schlesinger, 1973). However, both mutant enzymes show at least a 10-fold reduction in the phosphate content.

Pre-Steady-State Kinetic Characterization of the Wild-Type and the Mutant Enzymes. The pre-steady-state kinetics of wild-type alkaline phosphatase have been investigated in detail (Ko & Kézdy, 1967; Bloch & Schlesinger, 1973; Bloch & Gorby, 1980; Bale et al., 1979). At pH 5.5, the hydrolysis reaction proceeds in two steps: a pre-steady-state burst followed by a linear steady-state phase. However, the endogenous phosphate content of wild-type alkaline phosphatase influences the transient kinetics. When the enzyme has about 1.0 mol of phosphate/active site, a transient burst followed by the linear steady-state release of product is observed in the stopped

Table III: Pre-Steady-State Kinetics of the Wild-Type and the Lys-328 → Ala Enzymes[a]

enzyme[d]	k_t^c (s^{-1}) ($[P_i] \sim 10^{-5}$ M)[e]	k_t^c (s^{-1}) ($[P_i] = 10^{-3}$ M or 10^{-2} M)[f]
A. pH 5.5, [2,4-Dinitrophenyl Phosphate][b] = 10^{-4} M		
wild type	26.6 (0.26)[g]	7.9 (0.35)
Lys-328 → Ala	37.9 (0.21)	19.4 (0.68)
B. pH 8.0, [*p*-Nitrophenyl Phosphate][h] = 10^{-4} M		
Lys-328 → Ala	240.7 (24.54)	

[a] All the experiments were performed at 25 ± 1 °C. [b] 2,4-Dinitrophenyl phosphate was dissolved in 0.1 M sodium acetate buffer, pH 5.5. [c] Rate constant of the transient phase of the pre-steady-state kinetic curve. [d] Stock enzyme concentrations before 1/1 dilution with substrate in the stopped flow were 9–11 μM in 0.01 M Tris-HCl, pH 7.4, buffer. [e] The trace amount of phosphate comes from the TMZP buffer. [f] The stopped-flow experiments for the wild-type and Lys-328 → Ala enzymes were performed in the presence of 10^{-3} M and 10^{-2} M phosphate, respectively. A higher concentration of phosphate was used for the mutant enzyme due to its 10-fold lower affinity. [g] The values in parentheses are the standard deviations of at least five runs. [h] *p*-Nitrophenyl phosphate was dissolved in 0.1 M MOPS buffer, pH 8.2.

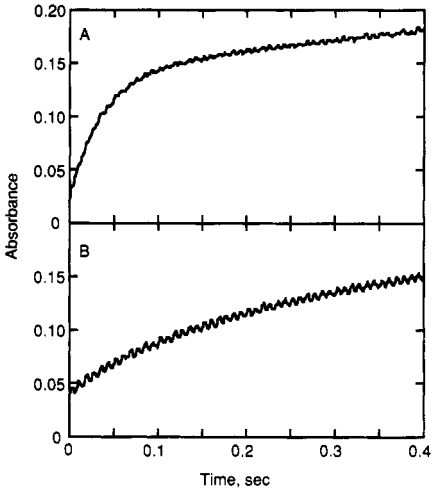

FIGURE 4: Rapid kinetics for the wild-type enzyme at pH 5.5. Reactions were carried out at 25 ± 1 °C with 10^{-4} M 2,4-dinitrophenyl phosphate as substrate. The data were collect at 390 nm. (A) Reactions were performed in the absence of phosphate (phosphate concentration was about 10^{-5} M). (B) Reactions were performed in the presence of 10^{-3} M phosphate.

flow. But when the enzyme is purged of phosphate, a large instantaneous burst is observed within the dead time of the stopped flow and the amplitude of the transient burst becomes much smaller compared to that observed for the phosphate-containing enzyme (Bloch & Schlesinger, 1973). At pH 8.0, neither an instantaneous nor a transient burst is observed in the pre-steady-state kinetics for the phosphate-containing enzyme. However, an instantaneous burst was observed for phosphate-free enzyme.

A summary of the transient rate constants obtained from the stopped-flow experiments for the wild-type and the Lys-328 → Ala enzymes is given in Table III. Representative stopped-flow traces for the wild-type enzyme are shown in Figure 4. The transient burst observed at pH 5.5 for the wild-type enzyme not purged of phosphate (Figure 4A) is completely eliminated at pH 8.0 (Figure 6A). In agreement with Bloch and Schlesinger (1973), the addition of 10^{-3} M inorganic phosphate at pH 5.5 dramatically increases the half-time of the burst phase (Figure 4B and Table III) and decreases the burst amplitude (Figure 4B). At pH 8.0 the steady-state rate of substrate hydrolysis is dramatically decreased by the addition of 10^{-3} M phosphate (Figure 6A).

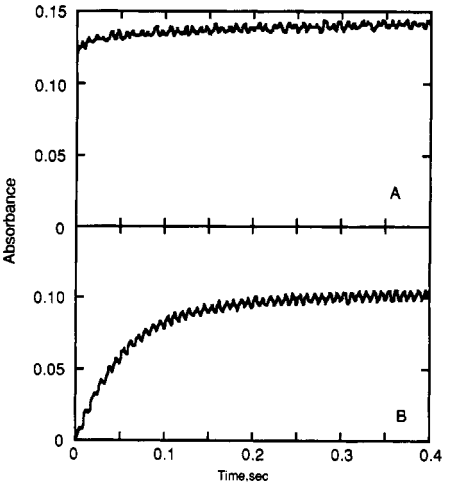

FIGURE 5: Rapid kinetics for the Lys-328 → Ala enzyme at pH 5.5. Reactions were carried out at 25 ± 1 °C with 10^{-4} M 2,4-dinitrophenyl phosphate dissolved in 0.1 M sodium acetate, pH 5.5, as substrate. The data were collected at 390 nm. (A) Reactions were performed in the absence of phosphate (phosphate concentration was about 10^{-5} M) or (B) in the presence of 10^{-2} M phosphate.

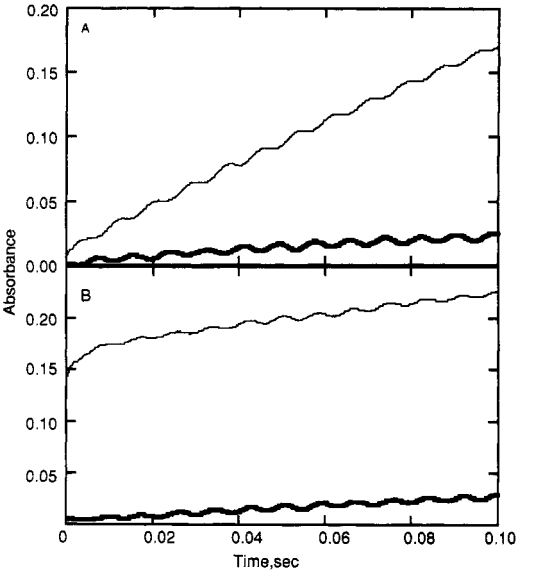

FIGURE 6: Rapid kinetics for the wild-type (A) and Lys-328 → Ala enzymes (B) at pH 8.0. Reactions were carried out at 25 ± 1 °C with 10^{-4} M *p*-nitrophenyl phosphate dissolved in 0.1 M MOPS, pH 8.2, as substrate. The data were collected at 410 nm. Reactions were performed in the absence of phosphate (phosphate concentration was about 10^{-5} M) for the lighter curve and in the presence of 10^{-3} M phosphate for the darker curve for wild-type enzyme (A) and 10^{-2} M for Lys-328 → Ala (B).

For the Lys-328 → Ala enzyme the results are much different. At pH 5.5, the mutant enzyme exhibits a very small transient burst but shows a large instantaneous increase in absorbance after mixing that occurs faster than the dead time of the stopped flow (Figure 5A). Since the Lys-328 → Ala enzyme has at least 10-fold decreased affinity for phosphate compared to that of the wild-type enzyme, the concentration of the phosphate used to study the phosphate effect on the mutant enzyme was 10-fold higher than that used for the wild-type enzyme. In the presence of 10^{-2} M phosphate, the Lys-328 → Ala enzyme exhibits a significant transient burst with a decreased transient rate constant (Figure 5B and Table III). As opposed to the wild-type enzyme, the Lys-328 → Ala enzyme exhibits a small transient burst but a large instantaneous burst at pH 8.0 that is very similar to that observed at pH 5.5 (see Figure 6B). When phosphate is added to the

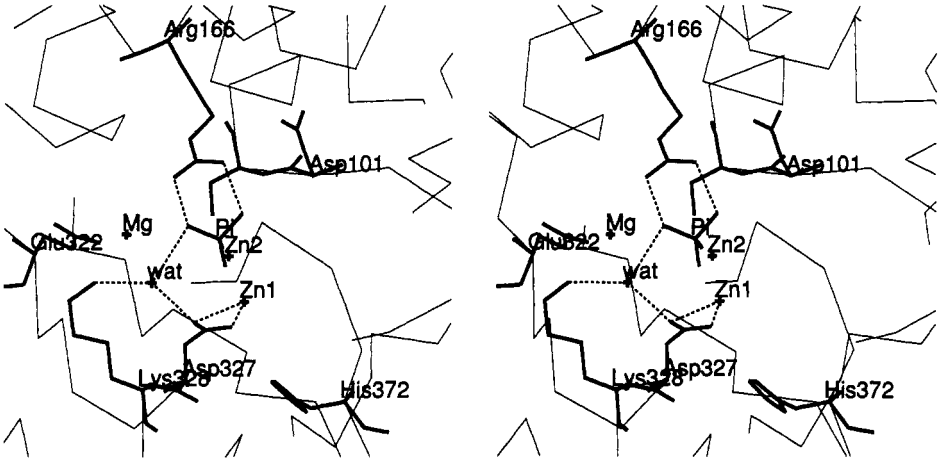

FIGURE 7: Stereo view of the active site region of *E. coli* alkaline phosphatase (Kim & Wyckoff, 1991). The Ser-102 that is phosphorylated during the reaction and the Asp-101 that forms a salt link with Arg-166 are shown, along with the phosphate bound in the active site. The phosphate also interacts with Arg-166 and a water molecule that is hydrogen bonded to the side chain of Lys-328. Also shown is the interaction between this water moelcule and the side chain of Asp-327 that is a bidentate ligand of the zinc at the M1 site (Zn1). The other zinc (Zn2) is at the M2 site while magnesium is at the M3 site. A portion of the α-carbon trace of the polypeptide chain is shown lighter than the side chains, phosphate, and metals.

enzyme before mixing with the substrate, neither burst phase is observed (see Figure 6B).

Discussion

In order to determine the functional importance of residues in the active site of *E. coli* alkaline phosphatase, we have used site-specific mutagenesis to replace Lys-328 with either an alanine or a histidine residue. The X-ray structure of the wild-type enzyme (Kim & Wyckoff, 1989, 1991) shows that Lys-328 is located on the surface of the protein near the active site region. The side chain of Lys-328 is hydrogen bonded to a water molecule, which is further hydrogen bonded to an oxygen of the phosphate. This water molecule also forms a hydrogen bond with Asp-327. The carboxyl of the side chain of Asp-327 interacts in a bidentate fashion with the zinc at the M1 site. Asp-327 also forms a link with His-372, which is near the zinc at the M1 site (see Figure 7). His-372 is conserved in *E. coli* and all mammalian alkaline phosphatases but is replaced by threonine in the *B. subtilis* (Hulett et al., 1991) and *S. cerevisiae* (Kaneko et al., 1987) enzymes. In the more active mammalian enzymes, Lys-328 is replaced by histidine. The replacement of Lys-328 by histidine still leaves the possibility of an interaction with the phosphate via a water molecule; however, the alanine substitution would prevent this interaction completely. Since the *E. coli* Lys-328 → His enzyme has enhanced activity at high pH, the substitution of histidine at this position in the mammalian enzymes may be partially responsible for the enhanced activity of these enzymes.

The Mutant Enzymes Have Reduced Rates of Hydrolysis along with Enhanced Rates of Transphosphorylation. At pH 8.0 in the absence of a phosphate acceptor, both mutant enzymes exhibit significant decreases in catalytic activity compared to that of the wild-type enzyme corresponding to approximately 9.2-fold for the Lys-328 → His enzyme and 5-fold for the Lys-328 → Ala enzyme. Yet, in the presence of a phosphate acceptor the Lys-328 → His enzyme has about the same activity as the wild-type enzyme while the Lys-328 → Ala enzyme is 2-fold more active. Since the latter experiment measures the sum of both hydrolysis and transphosphorylation, the mutations must result in inhibition of hydrolysis activity along with a significant enhancement of the transphosphorylation activity amounting to approximately 2-fold for the Lys-328 → His enzyme and 4-fold for the Lys-328 → Ala enzyme.

Both Mutant Enzymes Exhibit Reduced Affinity for Phosphate and Substrates. The fact the substrates of alkaline phosphatase contain a phosphate group and the product is phosphate suggests that a single mutant may alter the affinity of the enzyme for both substrate and product simultaneously. The measurement of the K_i for phosphate indicates that both mutant enzymes have significantly reduced affinity for phosphate. For the mutant as well as the wild-type enzymes, the catalytic step is slow compared to substrate binding, which leads to the conclusion that the increase in the K_m of the substrate *p*-nitrophenyl phosphate for the mutant enzymes is a reflection of weaker binding of this substrate. Additional support for the weakened binding of phosphate also comes from the direct analysis of the residual phosphate associated with the enzyme after purification. The approximately 10-fold reduction in residual phosphate content of both mutant enzymes again indicates the weakened binding of phosphate as a result of the Lys-328 → His and Lys-328 → Ala substitutions.

The Reduced Phosphate Affinity of the Mutant Enzymes Accounts for Many of the Altered Properties of the Mutant Enzymes at pH 5.5. As was shown by Bloch and Gorby (1980), the reaction sufficient to explain the observed kinetic properties of *E. coli* alkaline phosphatase is

$$E \underset{k_{-1}}{\overset{S,\ k_1}{\rightleftharpoons}} E{\cdot}S \xrightarrow{k_2} E{-}P_2 \underset{k_{-3}}{\overset{H_2O,\ k_3}{\rightleftharpoons}} E{\cdot}P_2 \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E$$

where P_1 is the alcohol product and P_2 is inorganic phosphate. For the wild-type enzyme under acid conditions and excess substrate over enzyme, k_3 is rate controlling. Since k_2 is much faster than the dead time of stopped flow, an instantaneous rather than a transient burst is observed for the enzyme lacking phosphate (Bloch & Schlesinger, 1973). The wild-type enzyme containing residual phosphate at pH 5.5 shows a transient burst indicative of a slow step after the release of the *p*-dinitrophenol, while the transient burst is almost undetectable in the case of the Lys-328 → Ala enzyme. However, this mutant enzyme shows a large instantaneous burst within the dead time of the stopped flow. The pre-steady-state kinetic curve of the Lys-328 → Ala enzyme is similar to that observed for the wild-type enzyme in the absence of phosphate (Bloch & Schlesinger, 1973). When phosphate is added to the Lys-328 → Ala enzyme, the transient burst is observed (Figure 5B) but the

instantaneous burst disappears, suggesting that the lack of the transient burst and the appearance of a large instantaneous burst for the Lys-328 → Ala enzyme are simply due to the fact that the wild-type enzyme has bound approximately 0.8 mol of phosphate/active site initially and therefore exists mainly in the E·P_i form, while the Lys-328 → Ala enzyme has bound less than 0.1 mol of phosphate/active site. In the stopped-flow experiments the mutant enzyme is therefore in a form that has almost no phosphate bound.

At pH 8.0 the Rate-Determining Steps of the Mutant and Wild-Type Enzymes Are Different. For the wild-type enzyme, k_3 varies directly with pH (Hull et al., 1976; Bloch & Gorby, 1980). At pH values greater than 7, k_3 is no longer rate controlling since it is much enhanced compared with its value at pH 5.5, and therefore no transient burst is observed for the wild-type enzyme (see Figure 6A) (Aldridge et al., 1964; Fernley & Walker, 1966). For the Lys-328 → Ala enzyme, a large instantaneous burst and a small transient burst are observed at pH 8.0 (see Figure 6B and Table III), suggesting that the rate-limiting step for the mutant enzyme at pH 8.0 is still k_3. One possible explanation for this is that the Lys-328 → Ala mutation may alter the pK_a of the proposed hydroxyl group that is coordinated to the zinc and therefore the pH dependence of k_3 (Coleman & Gettins, 1983; Gettins et al., 1985). With addition of inorganic phosphate, the wild-type enzyme shows a reduction in the steady-state rate; however, for the Lys-328 → Ala enzyme both the instantaneous and transient bursts disappear (see Figure 6B). The phosphate influence on the pre-steady-state kinetics of mutant enzyme at pH 8.0 may be explained by both a reduction in k_3 and a reduction in the affinity of the enzyme for phosphate.

Relationship between the Properties of the Mutant Enzymes and the Structure of the Wild-Type Enzyme. The crystal structure of *E. coli* alkaline phosphatase shows that the side chain of Lys-328 interacts with the phosphate in the active site through a water-mediated salt link. However, in the Lys-328 → Ala enzyme this interaction is completely lost. Since the results presented in this paper indicate that the Lys-328 → Ala enzyme has a decreased affinity for phosphate, it is reasonable to propose that the hydrogen-bonding interaction mediated by this water molecule with the phosphate is important for the binding of both the substrate and the product inorganic phosphate.

In a previously proposed mechanism (Coleman & Gettins, 1983; Gettins et al., 1985; Sowadski et al., 1985) for the dephosphorylation of E–P, a Zn-coordinated hydroxyl group acts as the nucleophile attacking the phosphoserine residue. This step is rate limiting for the wild-type enzyme at acidic pH. For the Lys-328 → Ala enzyme, the pre-steady-state kinetic data at pH 8.0 indicate that the rate-limiting step is the dephosphorylation of the covalently bound phosphate and it is not the dissociation of the noncovalently bound phosphate that is the rate-limiting step for the wild-type enzyme at alkaline pH. This alteration in the rate-limiting step for the mutant enzyme can be partially explained by an enhanced dissociation rate of the noncovalently bound phosphate from the enzyme. However, the fact that the Lys-328 → Ala enzyme has a 5-fold decrease in hydrolysis activity as compared to that of the wild-type enzyme at pH 8.0 (see Table I) cannot be explained by just the change in the rate-limiting step. Therefore, it is likely that there are other possible alterations in this mutant enzyme. The X-ray structure of the wild-type enzyme indicates that the water molecule that is hydrogen bonded to the phosphate and the side chain of Lys-328 may also form a hydrogen-bonding interaction with Asp-327, which is a bidentate ligand of zinc at the M1 site (Kim & Wyckoff, 1991). In the case of the Lys-328 → Ala enzyme, these interactions may be disrupted, resulting in a change in the pK_a of the hydroxyl coordinated to the zinc and reducing the k_3 in the kinetic scheme mentioned above. Additional kinetic and crystallographic studies are planned for these mutant enzymes to further our understanding of the catalytic mechanism of alkaline phosphatase.

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Site-Directed Mutagenesis of *Escherichia coli* Aspartate Aminotransferase: Role of Tyr70 in the Catalytic Processes†

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ABSTRACT: Site-directed mutagenesis of Tyr70 in the active site of *Escherichia coli* aspartate aminotransferase (AspAT) followed by kinetic studies has elucidated the roles of the hydroxyl group and benzene ring of Tyr70. X-ray crystallographic analysis showed that replacement of Tyr70 by Phe did not alter the active-site conformation of the enzyme. Comparison of the kinetic parameters of the four half-transamination reactions (the pyridoxal 5′-phosphate form of the enzyme with L-aspartate or L-glutamate and the pyridoxamine 5′-phosphate form with oxalacetate or 2-oxoglutarate) between the wild-type and [Tyr70 → Phe]AspATs showed that the mutation increases the energy level of the transition state by 2 kcal·mol^{-1} for all the four substrates, suggesting some contribution of the hydroxyl group of Tyr70 to the transition state. When Phe70 was further replaced by Ser, the energy level of the transition state for L-glutamate or 2-oxoglutarate, but not for L-aspartate or oxalacetate, was further increased by 2-3 kcal·mol^{-1}, suggesting that the presence of a benzene ring at position 70 is essential for recognizing the L-glutamate–2-oxoglutarate pair as substrates.

Aspartate aminotransferase [L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1] (AspAT)[1] catalyzes the reversible transamination reaction

L-aspartate + 2-oxoglutarate ⇌ oxalacetate + L-glutamate (1)

via the "ping-pong bi-bi" mechanism (Velick & Vavra, 1962; Kiick & Cook, 1983; Jenkins & Fonda, 1985).

Recent crystallographic studies of *Escherichia coli* AspAT at 2.5-Å resolution (Kamitori et al., 1990) have revealed that the spatial structure of the *E. coli* enzyme is virtually identical with those of the animal isozymes (Ford et al., 1980; Borisov et al., 1980; Harutyunyan et al., 1982; Arnone et al., 1985a) and that most of the active-site residues are conserved and located at corresponding positions. We have examined some of the active-site residues of the *E. coli* enzyme, including Lys258,[2] Tyr225, Arg292, Arg386, and Trp140, for their

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[1] Abbreviations: Asp, L-aspartate; 2-CH_3-Asp, 2-methyl-DL-aspartate; AspAT, aspartate aminotransferase; Y70X AspAT, AspAT of which Tyr70 is replaced with residue X; Bicine, *N,N*-bis(2-hydroxyethyl)-glycine: CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; EL, pyridoxal 5′-phosphate form of AspAT; EM, pyridoxamine 5′-phosphate form of AspAT; Glu, L-glutamate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; OA, oxalacetate; 2OG, 2-oxoglutarate; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate.

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