Magnesium in the Active Site of *Escherichia coli* Alkaline Phosphatase Is Important for both Structural Stabilization and Catalysis†

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ABSTRACT: Site-specific mutagenesis was used to explore the roles of the side chains of residues Lys-328 and Asp-153 in *Escherichia coli* alkaline phosphatase. The D153H enzyme exhibits a 3.5-fold decrease in activity at pH 8.0 compared to that of the wild-type enzyme, while a double mutant D153H/K328H exhibits a 16-fold decrease in activity under these conditions. However, the K_m values for both enzymes, employing the substrate *p*-nitrophenyl phosphate, are lower than the value for the wild-type enzyme. The K_i for phosphate, which is pH- and Mg^{2+}-dependent, is decreased for the D153H enzyme and increased for the D153H/K328H enzyme. Relative to the wild-type enzyme, both mutant enzymes bind Mg^{2+} more weakly and undergo a time-dependent activation induced by Mg^{2+}. The half-time of the activation process is independent of the Mg^{2+} concentration, indicating that the activation most probably involves a conformational change. The pH versus activity profiles of both enzymes are altered relative to that of the wild-type enzyme and exhibit greatly enhanced activity, relative to that of the wild-type enzyme, at high pH values. The pre-steady-state kinetics for the D153H and D153H/K328H enzymes exhibit a transient burst of product formation at pH 8.0, under conditions at which the wild-type enzyme exhibits no transient burst, indicating that at pH 8.0 the hydrolysis of the covalent enzyme–phosphate complex is rate-determining and not the release of phosphate from the noncovalent enzyme–phosphate complex as is observed for the wild-type enzyme. Therefore, these mutations are directly influencing catalysis. The introduction of either the D153H or the D153H/K328H mutations reduces the heat stability of the enzyme, whereas the K328H mutation alone exhibits the same heat stability as the wild-type enzyme. Energy minimization and Langevin molecular dynamics calculations suggest that the enhanced phosphate affinity of the D153H enzyme is due to the repositioning of Lys-328 so that it can directly interact with the phosphate and thereby stabilize its binding to the enzyme. These data also indicate that although Asp-153 is not a direct ligand to Mg^{2+} in the wild-type enzyme, it plays a role in stabilizing the binding of Mg^{2+} in the M3 site and indicates that the Mg^{2+} at the M3 site is important for catalysis by stabilizing the active conformation of the enzyme. The introduction of histidine residues at both position 153 and 328 results in an enzyme that is remarkably similar to mammalian alkaline phosphatases in terms of pH versus activity profile, heat stability, maximal activity, and stimulation by Mg^{2+}, suggesting that these two mutations are responsible for many of the differences in properties between the *E. coli* and mammalian alkaline phosphatases.

Alkaline phosphatase (EC 3.1.3.1) is a nonspecific phosphomonoesterase that functions through a phosphoseryl intermediate (Schwartz & Lipmann, 1961) to produce inorganic phosphate and an alcohol. In the presence of a phosphate acceptor such as ethanolamine or Tris, the enzyme catalyzes a transphosphorylation reaction with the transfer of the phosphoryl group to the alcohol (Dayan & Wilson, 1964; Wilson et al., 1964). The catalytic mechanism has been the subject of numerous kinetic (Coleman & Gettins, 1983) and structural studies (Kim & Wyckoff, 1989; Kim & Wyckoff, 1991). The rate-determining step of the mechanism is pH dependent; at acidic pH the hydrolysis of the covalent enzyme–phosphate complex (E–P) is rate-limiting, while under basic conditions the rate-limiting step becomes the release of phosphate from the noncovalent enzyme–phosphate complex (E·P_i) (Hull et al., 1976; Reid & Wilson, 1971; Gettins & Coleman, 1983; Bloch & Gorby, 1980).

The amino acid sequences of alkaline phosphatase from *Escherichia 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 sequence of alkaline phosphatase has been highly conserved during evolution with a 25–30% homology between the *E. coli* and the mammalian enzymes. The amino acid sequence of the mammalian alkaline phosphatases has been fit to the structure of the *E. coli* enzyme, further supporting the utility of using the *E. coli* enzyme as a model for all alkaline phosphatases (Kim & Wyckoff, 1989).

Alkaline phosphatase is a dimeric metalloenzyme that contains two atoms of zinc and one atom of magnesium per monomer. The X-ray structure of the *E. coli* enzyme, first solved by Sowadski et al. (1983, 1985) has been reported to a resolution of 2.0 Å, with inorganic phosphate complexed to the protein, and a detailed mechanism has been proposed on the basis of the crystal structure (Kim & Wyckoff, 1991). Ser-102, which is phosphorylated during the reaction, resides in a small pocket near the zinc and magnesium binding sites. When bound, inorganic phosphate essentially fills the volume of the pocket. Consistent with the nonspecificity of the enzyme, there is no apparent binding site for the R group of the phosphate monoester. The catalytic zinc atoms, which are 3.9 Å apart, are well positioned to activate Ser-102 and water for the nucleophilic attacks required in the enzymatic reaction.

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Although magnesium, which is 5 and 7 Å from the two zinc atoms, is thought to be important for the function of alkaline phosphatase, its exact role is uncertain (Anderson et al., 1975; Bosron et al., 1977).

The role of specific amino acids in the catalytic mechanism has been investigated by site-specific mutagenesis. Surprisingly, when Ser-102, which is phosphorylated during the reaction, is replaced by cysteine (Butler-Ransohoff et al., 1988a) and by leucine and alanine (Butler-Ransohoff et al., 1992) the enzymatic activity is reduced but not totally abolished. When Arg-166, which directly interacts with the phosphate, is replaced by alanine or serine (Chaidaroglou & Kantrowitz, 1989) and glutamine (Butler-Ransohoff et al., 1988b), there is a reduction in activity, but much less than would be expected for a residue critical in catalysis. The replacement of Asp-101 by alanine (Chaidaroglou & Kantrowitz, 1989) and replacement of some conserved residues within the active site of *E. coli* alkaline phosphatase (Mandecki et al., 1991) resulted in increased activity at alkaline pH probably due to increased accessibility of the substrate and reduced phosphate affinity.

In the present study, site-specific mutagenesis is used to explore the role of the side chain of residues Lys-328 and Asp-153 in the *E. coli* enzyme. In the more active mammalian alkaline phosphatases, these positions are occupied by histidine residues. Histidine substitutions at these positions, in the *E. coli* enzyme, will determine whether the enhanced catalytic activity of the mammalian enzymes over that of the *E. coli* enzyme can be partially or totally attributed to the existence of these histidines. Since previous studies (Xu & Kantrowitz, 1991) have shown the importance of water-mediated interactions between the side chain of Lys-328 and the phosphate, this study will also determine the importance of the water-mediated interactions between both Asp-153 and the Mg^{2+} and Asp-153 and the phosphate.

EXPERIMENTAL PROCEDURES

Materials

Agar, ampicillin, CAPS,[1] MOPS, *p*-nitrophenyl phosphate, sodium dihydrogen phosphate, magnesium, and zinc chloride 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 the use of glass beads employing the Geneclean II kit from Bio 101.

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-directed mutagenesis and the sequencing primers were synthesized on an Applied Biosystems 381A DNA synthesizer and purified by HPLC employing a Du Pont Zorbay Oligo ion-exchange column.

Methods

Expression of Wild-Type and Mutant Alkaline Phosphatases. SM547 was used as the host strain for expression of 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 come exclusively from the *phoA* gene on the plasmid.

Purification of the Wild-Type and Mutant Alkaline Phosphatases. The wild-type, K328H, D153H, and D153H/K328H enzymes were isolated from the plasmid/strain combinations pEK48/SM547, pEK145/SM547, pEK175/SM547, and pEK190/SM547, respectively, by the method previously described (Chaidaroglou et al., 1988) with minor modifications for the K328H enzyme as reported (Xu & Kantrowitz, 1991). The enzyme purity was judged by SDS–polyacrylamide gel electrophoresis according to the procedure of Laemmli (1970).

Determination of the 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 enzymes 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. Phosphatase activity was measured spectrophotometrically by utilizing *p*-nitrophenyl phosphate as the substrate at 25 °C by monitoring the release of *p*-nitrophenolate at 410 nm (Garen & Leventhal, 1960). The extinction coefficient of *p*-nitrophenolate was determined at each pH value used by measuring the absorbance after complete hydrolysis of a known amount of substrate. In order to measure the hydrolytic activity either 0.1 M MOPS, 0.1 M CAPS, or 0.01 M Tris-HCl[2] was used and the ionic strength was held constant at 0.5 M with NaCl. In order to measure the sum of the hydrolytic and the transferase activities, 1 M Tris was used as the phosphate acceptor.

Determination of Inorganic Phosphate. The phosphate content of the various preparations of alkaline phosphatase was determined by the procedure of Chen and Toribara (1956) as previously modified (Xu & Kantrowitz, 1991). Before the phosphate determination, both the wild-type and mutant enzymes were dialyzed once versus a 500-fold excess volume of 0.01 M Tris-HCl, 0.1 M NaCl, pH 7.4 for 12–14 hr.

Rapid Kinetic Measurements. Experiments were performed with a KinTeK Inc. stopped-flow spectrophotometer at 25 °C with a dead-time of approximately 1 ms. Data were collected at 410 nm directly by computer via a National Instruments analog/digital interface. One syringe contained approximately 5.0 μM enzyme in 0.05 M Tris buffer at pH 8.0, with or without Mg^{2+} as indicated. If the reaction was carried out in the presence of Mg^{2+}, the enzyme was preincubated in buffer containing the same Mg^{2+} concentration for at least

[1] Abbreviations: CAPS, (cyclohexylamino)propanesulfonic acid; MOPS, 3-(morpholino)propanesulfonic acid; TMZP buffer, 0.01 M Tris, 1 mM $MgCl_2$, 0.1 mM NaH_2PO_4, 3.1 mM NaN_3, 10^{-5} M $ZnSO_4$, pH 7.4 (Bloch & Bickar, 1978); K328H, the mutant alkaline phosphatase in which Lys-328 is replaced by histidine; D153H, the mutant alkaline phosphatase in which Asp-153 is replaced by histidine; K328H/D153H, the double mutant alkaline phosphatase in which Asp-153 and Lys-328 are both replaced by histidine residues.

[2] At this concentration of Tris, negligible transferase activity is observed.

1 h prior to the experiment. The other syringe contained 1 mM *p*-nitrophenyl phosphate in the same buffer at pH 8.0, with or without Mg^{2+} as indicated.

Magnesium Determination. The magnesium content of the wild-type and mutant enzymes was determined with a Perkin Elmer 3100 Atomic Absorption Spectrophotometer using the stabilized temperature platform furnace technique. Before the measurements, the enzyme was dialyzed against 10 mM Tris buffer, pH 7.4, with four or five buffer changes (1000:1).

Molecular Dynamics. Molecular dynamics (Langevin) were calculated at 0.001-ps intervals for 2.5 ps after 50 cycles of Powell energy minimization using the program XPLOR (Molecular Simulations, Inc., Waltham MA) running on an IBM RISC/6000 computer. A trajectory was generated from data saved at 0.05-ps intervals. Finally, an average structure was calculated from the last 2 ps of the trajectory data. For the mutant enzymes, the amino acid replacement was built into the structure by deletion of the original amino acid followed by insertion of the new amino acid using the visualization program QUANTA (Molecular Simulations, Inc.). An energetically favorable conformation for the new amino acid was manually selected prior to the energy minimization and molecular dynamics. The trajectories as well as the average data sets were examined using the program QUANTA.

RESULTS

Construction of the D153H Alkaline Phosphatase by Site-Specific Mutagenesis. To introduce the histidine at position 153 of alkaline phosphatase, the method of Zoller and Smith (1982) was utilized, with the modifications previously described (Carter et al., 1985; Ladjimi et al., 1988). The template for mutagenesis was single-stranded DNA isolated from a recombinant M13mp19 phage, which had the structural gene for alkaline phosphatase, *phoA*, inserted (Chaidaroglou et al., 1988). Selection of the mutation was accomplished directly by dideoxy sequencing (Sanger et al., 1977) with single-stranded DNA isolated from a number of candidates.

Recloning and Confirmation of the Mutation. After verification of the mutation, a small fragment of the gene was removed with the restriction enzymes *Bst*EII and *Bss*HII and then inserted into the plasmid pEK48 that had the corresponding section of the wild-type gene removed (Chaidaroglou et al., 1988). The larger 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 at 15 °C overnight. The ligation mixture was transformed into competent MV1190 cells. Verification of the plasmid construction was accomplished by restriction analysis. The mutation was verified a second time by dideoxy sequencing with single-stranded DNA isolated after coinfection with the helper phage M13K07 (Vieira & Messing, 1987). In this fashion, the plasmid pEK175 was isolated.

Construction of the D153H/K328H Alkaline Phosphatase. The DNA corresponding to the D153H/K328H double mutation was constructed by combining DNA fragments that carried the individual mutations. A 472-bp fragment containing the D153H mutation was obtained by treatment of the mutant M13RF with the restriction enzymes *Bss*HII and *Nco*I. This fragment was inserted into the phagemid pEK145 (Xu & Kantrowitz, 1991) that had the corresponding *Bss*HII/*Nco*I fragment removed but still contained the K328H mutation. After the construction was checked by restriction site analysis, verification that the phagemid contained both mutations was carried out by dideoxy sequencing (Sanger et al., 1977) employing single-stranded DNA isolated after coinfection with the helper phage M13K07 (Vieira & Messing, 1987). In this fashion, the phagemid pEK190 was isolated.

Table I: Kinetic Parameters of the Wild-Type and Mutants 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	89 (±9)	21 (±3)	4.2	1.0 M Tris
D153H	57 (±6)	20 (±1)	2.9	1.0 M Tris
K328H[c]	71 (±3)	60 (±2)	1.2	1.0 M Tris
D153H/K328H	20 (±5)	65 (±7)	0.3	1.0 M Tris
wild type	38 (±0.5)	7 (±2)	5.5	0.1 M MOPS[c]
D153H	11 (±2)	2.1 (±0.3)	5.0	0.1 M MOPS
K328H[c]	4.8 (±0.2)	1.0 (±0.1)	4.8	0.1 M MOPS
D153H/K328H	2.4 (±0.3)	1.5 (±0.4)	1.6	0.1 M MOPS

[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] For the reactions in 0.1 MOPS buffer, the ionic strength was adjusted to 0.5 M with NaCl.

Steady-State Kinetics of the Mutant Enzymes at pH 8.0. A summary of the kinetic parameters for the wild-type and the mutant enzymes is given in Table I. In 1.0 M Tris buffer, the rate observed is the sum of transphosphorylation and hydrolysis reactions, since Tris serves as a phosphoryl group acceptor (Wilson et al., 1964; Trentham & Gutfreund, 1968). Under these conditions, the k_{cat} value for the wild-type enzyme is 89 s^{-1} and the *p*-nitrophenyl phosphate K_m is 21 μM. The k_{cat} values for the K328H and the D153H enzymes are only slightly lower than that observed for the wild-type enzyme. In the case of the D153H/K328H enzyme, the K_{cat} drops approximately 4.5-fold to 20 s^{-1} and the K_m for *p*-nitrophenyl phosphate increases to 65 μM, resulting in a 14-fold decrease in the catalytic efficiency as expressed by the k_{cat}/K_m ratio.

When assayed in 0.1 M MOPS or 0.01 M Tris buffers (i.e., in the absence of a phosphate acceptor), only the hydrolytic activity of the enzyme is measured. Under these conditions, the K328H and the D153H enzymes have lower k_{cat} values than the wild-type enzyme. However, the k_{cat}/K_m ratio of these enzymes remains similar to that of the wild-type enzyme because of a corresponding decrease in their respective K_m values. The hydrolytic activity of the D153H/K328H enzyme is reduced by 16-fold with a corresponding decrease of 3.4-fold in the k_{cat}/K_m ratio (see Table I).

The Mutant Enzymes Contain Less Residual Magnesium Than the Wild-Type Enzyme. In the structure of the wild-type alkaline phosphatase (Kim & Wyckoff, 1989, 1991), Asp-153 forms a salt link with Lys-328 and also interacts with two water molecules that themselves are ligands of the magnesium. Since the replacement of Asp-153 by His may destabilize the magnesium coordination, the magnesium content of the mutant enzymes was determined by atomic absorption spectrophotometry. As indicated in Table II, the magnesium content of the wild-type enzyme was 1.33 ± 0.16 mol of magnesium/mole of enzyme dimer. This drops to 0.9 ± 0.05 mol of magnesium/mole of the K328H enzyme and to 0.13 ± 0.02 and 0.24 ± 0.03 mol of magnesium/mole of the D153H and the D153H/K328H enzymes, respectively. Therefore, the water-mediated interaction between Asp-153 and the magnesium is extremely important for the stability of the complex between the wild-type enzyme and the magnesium.

Mg^{2+} Stimulation of the D153H and D153H/K328H Enzymes. In order to determine if the lower activity of the mutant enzymes is related to their lower magnesium content,

Table II: Magnesium and Phosphate Content of the Wild-Type and Mutant Enzymes[a]

enzyme	mol of Mg^{2+}/ mol of enzyme	mol of P_i/ mol of enzyme
wild type	1.33 (±0.16)	1.6 (±0.1)
D153H	0.13 (±0.02)	1.6 (±0.1)
K328H[c]	0.90 (±0.05)	<0.2[b]
D153H/K328H	0.24 (±0.03)	1.7 (±0.1)

[a] See the Methods section for details of the magnesium and phosphate analyses of the wild-type and mutant enzymes. [b] The extremely low phosphate content of this enzyme prevented an accurate determination.

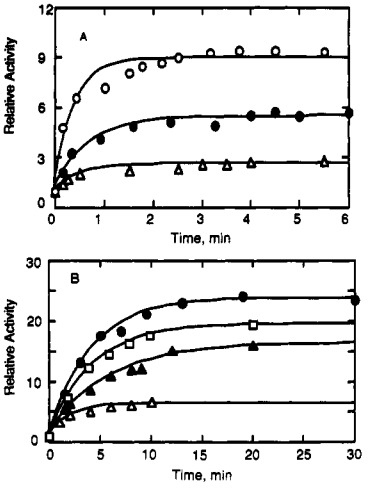

FIGURE 1: Kinetics of Mg^{2+} stimulation of the D153H and D153H/K328H enzymes. The enzymes (5 nM) were incubated at 25 °C in 0.05 M Tris-HCl, pH 8.5, 0.4 M NaCl, in the presence of the indicated concentration of Mg^{2+}. At the specified times, an aliquot of the reaction mixture was removed and the hydrolytic activity was measured in the same buffer at 25 °C, at a saturating concentration of *p*-nitrophenyl phosphate (5 mM) as substrate. The formation of *p*-nitrophenolate was monitored at 410 nm. (A) The activation of the D153H enzyme by 25 mM Mg^{2+} (△), 50 mM Mg^{2+} (●), and 75 mM Mg^{2+} (○); (B) the activation of the D153H/K328H enzyme by 1 mM Mg^{2+} (△), 5 mM Mg^{2+} (▲), 10 mM Mg^{2+} (□), and 50 mM Mg^{2+} (●).

activity measurements were also performed in the presence of magnesium. In the case of the D153H and D153H/K328H enzymes, the addition of magnesium to the assay resulted in nonlinear initial rates, with a lag phase before the maximal rate was observed. However, when these two mutant enzymes were incubated in a buffer containing 10 mM Mg^{2+} prior to the determination of the activity, the lag phase was eliminated.

The time-dependent activation of the D153H and D153H/K328H enzymes by Mg^{2+} is shown in Figure 1. The activation of both of these enzymes is a first-order process and therefore independent of the Mg^{2+} concentration. The half-time for the activation at 25 °C is approximately 0.25 and 3 min for the D153H and D153H/K328H enzymes, respectively. Although the half-time of the activation process is independent of the Mg^{2+} concentration, the extent of the activation is dependent upon the Mg^{2+} concentration. Once activated by Mg^{2+}, the enzymes do not lose activity even after 24 h (data not shown).

Steady-State Kinetics of the Mutant and Wild-Type Enzymes in the Presence of Magnesium. The kinetics of the enzymes were also determined after preincubation with magnesium.[3] Under these conditions, the hydrolytic activity of the wild-type and the K328H enzymes is not affected by additional Mg^{2+} up to 100 mM (see Figure 2). However,

[3] The storage buffer for the enzyme contains 1 mM Mg^{2+}.

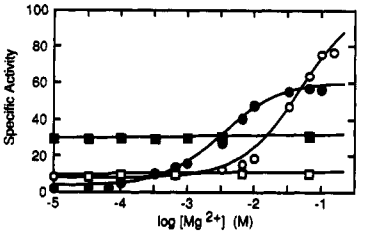

FIGURE 2: Influence of Mg^{2+} on the activity of the wild-type and mutant enzymes. The enzymes (10–25 nM) were preincubated at 25 °C for 2 h in the presence of increasing concentrations of Mg^{2+}, in 50 mM Tris-HCl, 0.4 M NaCl, pH 8.5. The hydrolytic activity was then measured at a saturating concentration of *p*-nitrophenyl phosphate (5 mM) at 25 °C in the same buffer at the Mg^{2+} concentration indicated for the wild-type (■), the K328H (□), the D153H (○), and the D153H/K328H (●) enzymes. The specific activity is reported in units of micromoles per minute per milligram.

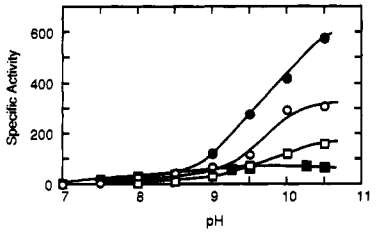

FIGURE 3: pH versus activity profiles for the Mg^{2+}-stimulated wild-type (■), K328H (□), D153H (○), and D153H/K328H (●) enzymes. All the enzymes were preincubated with 50 mM Mg^{2+}, except for the D153H enzyme, which was preincubated in 100 mM Mg^{2+}, in 0.05 M Tris-HCl buffer, 0.4 M NaCl, pH 8.5, at 25 °C for 2 h prior to measurement of activity in the presence of 10 mM Mg^{2+}. The Mg^{2+} was reduced to 10 mM to prevent precipitation at high pH values. The specific activity for the hydrolytic reaction was determined using 10 mM *p*-nitrophenyl phosphate in the following buffers: 0.05 M Tris-HCl, pH 7.0–9.0, and 0.1 M CAPS, pH 9.5–10.5. The ionic strength was maintained at 0.5 M with NaCl. The specific activity is reported in units of micromoles per minute per milligram.

both the D153H and D153H/K328H enzymes are activated by Mg^{2+}. The Mg^{2+} dissociation constants calculated from these data are 3.4 mM for the D153H/K328H enzyme and approximately 10-fold larger for the D153H enzyme.[4] The maximal stimulation of the D153H and D153H/K328H enzymes by magnesium is approximately 10-fold and 20-fold, respectively, compared to the activities of these enzymes in the absence of added Mg^{2+}.

pH Profiles of the Mutant and the Wild-Type Alkaline Phosphatases. The pH profiles of hydrolytic activity, in the presence of Mg^{2+}, are shown in Figure 3 for the D153H, K328H, and D153H/K328H enzymes. The highest specific activities for the D153H, K328H, and D153H/K328H enzymes are observed at pH values greater than 10. At pH 10.5, the D153H/K328H enzyme is 10-fold more active than the wild-type enzyme, the D153H enzyme is 5.5-fold more active, while the K328H enzyme is 2.5-fold more active than the wild-type enzyme.

At pH 10.5, the Mg^{2+}-stimulated D153H/K328H enzyme is 35-fold more active than the nonstimulated enzyme. At pH values above 10 in the absence or presence of Mg^{2+}, the K_m values for both the D153H and the D153H/K328H enzymes abruptly increase (data not shown), resulting in a decrease in the k_{cat}/K_m ratio.

Steady-State Kinetics of the Mutant Enzymes at pH 10.0. The kinetic parameters for the wild-type, the D153H, and the

[4] The low solubility of Mg^{2+} under these conditions prevents the accurate determination of the dissociation constant of Mg^{2+} for the D153H enzyme.

Table III: Kinetic Parameters of the Wild-Type and Mutant Enzymes at pH 10.0[a]

enzyme	k_{cat}[b] (s^{-1})	K_m (μM)	k_{cat}/K_m ($\times 10^{-6}$) ($M^{-1}\ s^{-1}$)	Mg^{2+} (mM)
wild type	82 (±7)	106 (±7)	0.77	1
wild type	86 (±4)	86 (±5)	1.0	10
D153H/K328H	17 (±1)	407 (±14)	0.04	<0.1
D153H/K328H	262 (±10)	405 (±20)	0.65	1[c]
D153H/K328H	508 (±44)	608 (±65)	0.84	10[c]
D153H	48 (±7)	477 (±52)	0.10	1[c]
D153H	292 (±4)	545 (±65)	0.64	10[c]

[a] Assays were performed at 25 °C in 0.1 M CAPS buffer at pH 10.0 (ionic strength adjusted to 0.5 M with NaCl) with *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] The enzymes were preincubated for 2 h at 25 °C in 10 mM Mg^{2+} in 0.1 M CAPS buffer, pH 10.0, before the assays were carried out.

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

enzyme	K_i[b] (μM)	Mg^{2+} (mM)	buffer
wild type	33 (±0.2)	1	0.1 M CAPS[c]
wild type	45 (±1)	10	0.05 M Tris[c,d]
D153H/K328H	140 (±3)	1	0.1 M CAPS
D153H/K328H	1300 (±26)	10	0.05 M Tris
D153H	19 (±1)	1	0.1 M CAPS
D153H	33 (±0.4)	10	0.05 M Tris
K328H	241 (±7)	1	0.1 M CAPS
K328H	328 (±16)	10	0.05 M Tris

[a] Assays were performed at 25 °C in the buffers indicated, at pH 9.5, with use of *p*-nitrophenyl phosphate as substrate. The D153H and D153H/K328H enzymes were preincubated for 2 h at 25 °C at a Mg^{2+} concentration 5–10-fold higher than the concentration used in the assay. [b] The K_i values were determined by the method of Segel which takes into account the fact that the product of the reaction is also an inhibitor (Segel, 1975). The data were fit to the theoretical equation by nonlinear least-squares. [c] In each case the ionic strength was kept constant at 0.5 M with NaCl. [d] The 0.05 M Tris buffer was used to avoid precipitation of Mg^{2+}. During the course of the reaction the pH remained constant.

D153H/K328H enzymes at pH 10.0 are given in Table III. The activity of the D153H enzyme in 10 mM Mg^{2+} is increased approximately 6-fold over that measured in 1 mM Mg^{2+}. In contrast to the results at pH 8.5, at pH 10.5 the D153H/K328H enzyme is more active than the D153H enzyme (see Figure 2). The K_m values for *p*-nitrophenyl phosphate are not significantly altered by the Mg^{2+} activation of the D153H or the K328H/D153H enzymes up to pH 10.0 (Table III).

Affinity of the Mutant Enzymes for Inorganic Phosphate. It has been reported (Xu & Kantrowitz, 1991) that the K328H enzyme, at high pH, has increased hydrolytic activity due to a reduced affinity for phosphate. Therefore, the affinity of inorganic phosphate for the wild-type and mutant enzymes was determined (see Table IV). At pH 9.5, the K_i of the D153H enzyme is 19 μM in 1.0 mM Mg^{2+} and 33 μM in 10 mM Mg^{2+}, a reduction of approximately two-thirds compared to the values for the wild-type enzyme. In contrast, the K328H and the D153H/K328H enzymes exhibit substantially increased values of the K_i in both 1 mM and 10 mM Mg^{2+}. Furthermore, the K_i values increase significantly at the higher Mg^{2+} concentrations (see Table IV). For example, the K_i of the D153H/K328H enzyme is 30-fold larger than the value for the wild-type enzyme in the presence of 10 mM Mg^{2+}.

The Mutant and Wild-Type Enzymes Contain Residual Phosphate after Purification. Bloch and Schlesinger (1973) have reported that, depending upon the purification, wild-type alkaline phosphatase retains phosphate bound to the active site amounting to between 1.0 and 2.0 mol of phosphate/mole of enzyme. Since the residual phosphate content of the enzyme influences the pre-steady-state kinetics (Bloch & Schlesinger, 1973), it was important to determine the residual phosphate content of the mutant enzymes. As seen in Table II, the residual phosphate content of the wild-type, the D153H, the K328H (Xu & Kantrowitz, 1991), and the D153H/K328H enzymes were 1.6 ± 0.1, 1.6 ± 0.1, <0.2, and 1.7 ± 0.1 mol of phosphate/mole of enzyme, respectively.

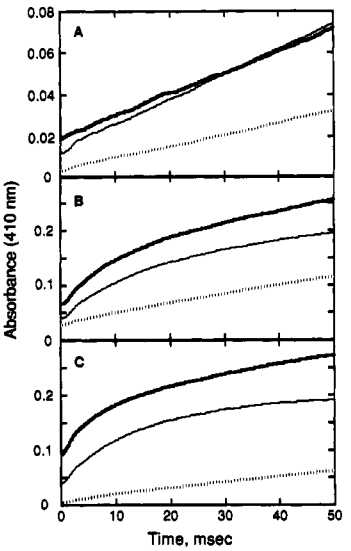

FIGURE 4: Pre-steady-state kinetics of the wild-type and mutant enzymes at pH 8.0. Reactions were carried out in 0.05 M Tris buffer at 25 °C with 1 mM *p*-nitrophenyl phosphate as substrate, and absorbance changes were measured at 410 nm. For reactions in the presence of Mg^{2+}, the wild-type or mutant enzymes were preincubated for at least 1 h in 0.05 M Tris buffer containing the indicated amount of Mg^{2+}. (A) The wild-type enzyme with <0.1 mM Mg^{2+} (lighter line), with 50 mM Mg^{2+} (darker line), and with 50 mM Mg^{2+} plus 1 mM phosphate (dotted line). (B) The D153H enzyme with <0.1 mM Mg^{2+} (lighter line), with 50 mM Mg^{2+} (darker line), and with 50 mM Mg^{2+} plus 1 mM phosphate (dotted line). (C) The D153H/K328H enzyme with <0.1 mM Mg^{2+} (lighter line), with 10 mM Mg^{2+} (darker line), and with 10 mM Mg^{2+} plus 1 mM phosphate (dotted line). For reactions with no additional phosphate, the phosphate concentration was approximately 10^{-5} M.

Pre-Steady-State Kinetics of the Wild-Type and Mutant Enzymes. For wild-type alkaline phosphatase, stopped-flow kinetics have been used extensively to investigate the catalytic mechanism of the enzyme (Ko & Kézdy, 1967; Bloch & Schlesinger, 1973; Bloch & Gorby, 1980; Bale et al., 1980). At pH 5.5, employing enzyme containing residual phosphate, a transient burst of alcohol production is observed followed by a linear steady-state phase. However, at pH 8.0, no transient burst of alcohol production is observed preceding the linear steady-state phase, indicating that the rate-determining step has changed from the breakdown of the covalent E–P complex at pH 5.5 to the release of phosphate from the noncovalent E·P_i complex at pH 8.0. Both the D153H and the D153H/K328H enzymes exhibit pre-steady-state kinetics that are different from those of the wild-type at pH 8.0. Representative stopped-flow traces for the wild-type, the D153H, and the D153H/K328H enzymes are shown in Figure 4.

At pH 8.0, for the wild-type enzyme there is a very small instantaneous burst[5] followed by a linear steady-state phase

[5] Instantaneous burst refers to the absorbance increase due to the *p*-nitrophenolate produced within the dead-time of the stopped-flow apparatus, while the transient burst refers to the exponential absorbance increase due to the *p*-nitrophenolate produced slower than the dead-time of the apparatus.

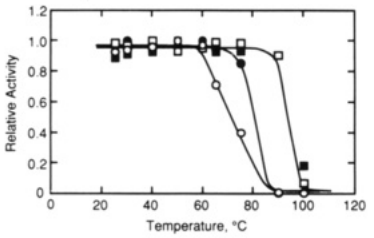

FIGURE 5: Heat stability of the wild-type (■), the K328H (□), the D153H (○), and the D153H/K328H (●) enzymes. The enzyme was diluted 100-fold into TMZP buffer with the Mg^{2+} concentration increased to 100 mM. Following a minimum 2 h preincubation, the enzyme sample was heated at the indicated temperature for 15 min. The samples were then quenched on ice and the activity was measured in 0.01 M Tris, 100 mM Mg^{2+}, pH 8.0, at a saturating concentration of *p*-nitrophenyl phosphate (10 mM).

(Bloch & Schlesinger, 1973). The results are dramatically different for the K328H, the D153H, and the D153H/K328H enzymes. For the K328H enzyme, a large instantaneous burst is observed followed by a transient phase which is in turn followed by a steady-state phase (Xu & Kantrowitz, 1991). For the D153H and the D153H/K328H enzymes, there is an instantaneous burst of reduced magnitude, compared to the K328H enzyme, followed by a transient phase, and finally a steady-state phase. The pre-steady-state kinetics of the wild-type and the K328H enzymes are not significantly influenced by the addition of Mg^{2+}; however, for the D153H and D153H/K328H enzymes the addition of Mg^{2+} results in a larger instantaneous burst, as well as alterations in the rate constants of the transient and steady-state phases of the reaction (see Figure 4). For the D153H and D153H/K328H enzymes, the addition of 1 mM phosphate results in almost complete elimination of both the instantaneous and transient phases of the reaction.

Thermal Stability of the Mutant Enzymes. In order to determine whether the mutations had altered the thermal stability of the enzyme, the denaturation temperature of each was measured after a 2-h preincubation in TMZP buffer with the Mg^{2+} concentration increased to 100 mM. Under these conditions, the wild-type and the K328H enzymes must be heated at 96 °C for 15 min in order to be half-inactivated. This compares to a 15-min incubation at 72 °C to half-inactivate the D153H enzyme and 83 °C to half-inactivate the D153H/K328H enzyme under comparable conditions (see Figure 5).

Molecular Dynamics. As seen in Table IV, the D153H enzyme has enhanced affinity for phosphate while the opposite is true for the D153H/K328H enzyme. When Lys-328 was replaced by either His or Ala, the phosphate affinity was also dramatically decreased, suggesting that the loss of the water-mediated interaction between Lys-328 and the phosphate was important for the binding of phosphate (Xu & Kantrowitz, 1991). Since the carboxylate of Asp-153 also interacts with the phosphate via a water-mediated interaction, it was unexpected that the D153H enzyme would exhibit enhanced affinity for phosphate. In order to probe the consequences of the replacement of Asp-153 by His, molecular dynamics simulations were carried out on the coordinates of the D153H enzyme generated by building histidine into the structure at position 153 followed by energy minimization. As seen in Figure 6, the energy minimization and molecular dynamics calculations suggest that the replacement of histidine at position 153 has an additional effect besides the loss of the water-mediated interaction between the carboxylate of Asp-153 and the phosphate. These calculations indicate that the

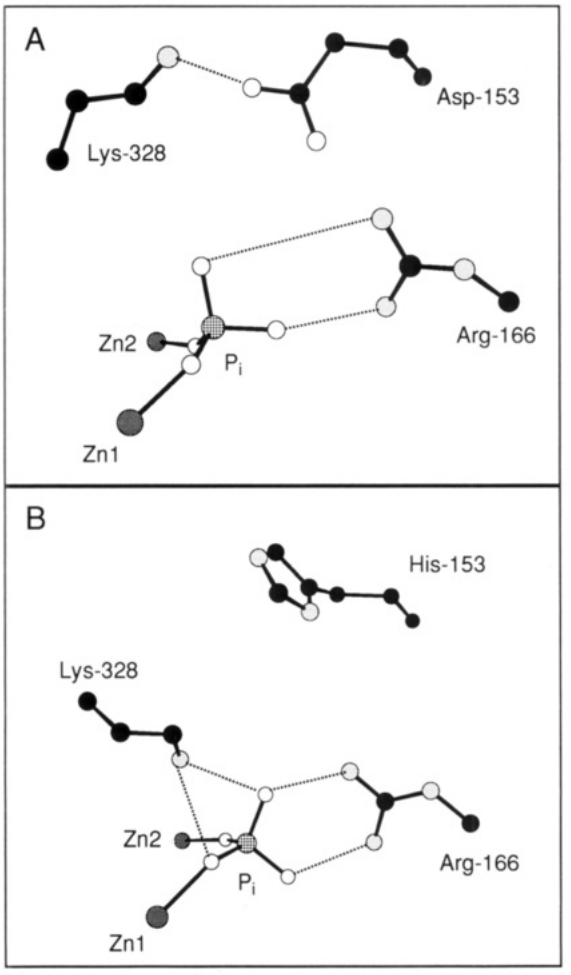

FIGURE 6: Energy minimization and Langevin molecular dynamics calculations on the wild-type (A) and the D153H (B) enzymes. Shown are the average positions of Lys-328, Arg-166, P_i, the two zinc atoms, the M1 (Zn1) and M2 (Zn2) sites, and the residue at 153 calculated from the last 2 ps of the molecular dynamics runs (see the Methods Section for details). The salt link between Lys-328 and Asp-153 (A), observed in the wild-type X-ray structure (Kim & Wyckoff, 1991), is eliminated in the D153H enzyme (B). Without Asp-153 to restrain the position of Lys-328 in the D153H enzyme, the Lys-328 can reorient and directly interact with the phosphate. These calculations help to explain the enhanced phosphate affinity of the D153H enzyme.

position of Lys-328 can shift in the D153H enzyme. In the wild-type structure, Lys-328 forms a salt link with Asp-153; however, in the D153H enzyme this interaction cannot form, allowing Lys-328 to reorient and take up a position directly interacting with the phosphate (see Figure 6B). This direct interaction between Lys-328 and the phosphate, in the D153H enzyme, can explain the enhanced phosphate affinity of the mutant enzyme as well as the lower K_m of this enzyme at pH 8.0 compared to that of the wild-type enzyme.

DISCUSSION

A characteristic feature of mammalian alkaline phosphatases is their lower thermal stability and significantly enhanced catalytic activity compared to those of the *E. coli* enzyme. A comparison of the amino acid sequence of the *E. coli* enzyme with a variety of mammalian alkaline phosphatases reveals that there are a number of amino acid substitutions in the active site area (Thiede et al., 1988; Berger et al., 1987; Kam et al., 1985; Weiss et al., 1986). In particular, Asp-153 and Lys-328 in the *E. coli* enzyme are both His in the mammalian enzymes. In the *E. coli* enzyme, Lys-328 and

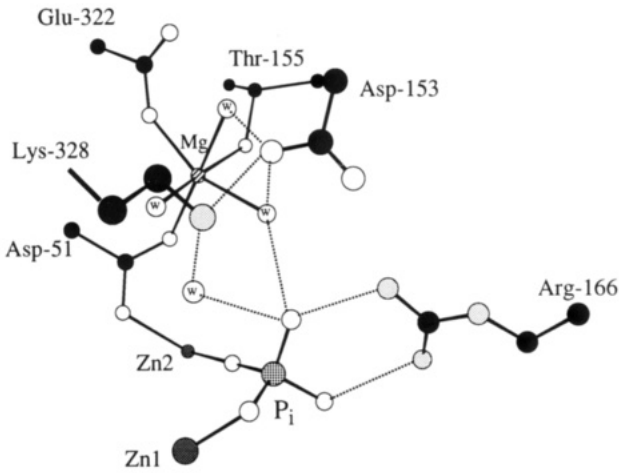

FIGURE 7: Region of the active site of *E. coli* alkaline phosphatase near the phosphate and Mg^{2+} binding sites. Mg^{2+} at the M3 site exhibits octahedral coordination. Ligands to the Mg^{2+} include the carboxylates of Glu-322, Asp-51, the hydroxyl of Thr-155, and three water molecules (encircled letter w). Asp-153 forms a salt link with Lys-328 and participates in interactions with two of the water molecules that are ligands to the Mg^{2+}. One of these water molecules also participates in a water-mediated interaction with the phosphate. Besides the salt link to Asp-153, Lys-328 also interacts with the phosphate via a water-mediated interaction. The phosphate interacts with the guanidinium group of Arg-166 and forms specific interactions with both the zinc at the M1 site (Zn1) and the zinc at the M2 site (Zn2). The carboxylate of Asp-51, which is a ligand to the Mg^{2+}, is also a ligand to the zinc at the M2 site. For clarity, the other ligands to the zinc atoms at the M1 and M2 sites are not shown. The data used for this figure were provided by Kim and Wyckoff (1991).

Asp-153 interact to form a salt link and are both involved in water-mediated interactions (Kim & Wyckoff, 1989, 1991). Asp-153 interacts with two water molecules that are ligands to the Mg^{2+}, while Lys-328 interacts with the phosphate via a water-mediated interaction (see Figure 7). Our previous studies (Xu & Kantrowitz, 1991) have shown that the interaction between Lys-328 and the phosphate via this water-mediated interaction is important for catalysis. The release of phosphate from the noncovalent enzyme–phosphate complex, which is the slow step in the mechanism at alkaline pH, is increased dramatically when the water-mediated interaction between Lys-328 and the phosphate is eliminated (Xu & Kantrowitz, 1991). Here, we continue the examination of the water-mediated interactions in the active site of alkaline phosphatase and comparison between the *E. coli* and mammalian enzymes, by investigation of the D153H enzyme as well as an enzyme that contains both the K328H and D153H mutations.

Replacement of Asp-153 by Histidine Results in Diminished Affinity of the Enzyme for Mg^{2+}. The substitution of Asp-153 with histidine in *E. coli* alkaline phosphatase results in an enzyme that requires magnesium in order to exhibit maximal activity. Not only does the addition of Mg^{2+} restore activity, but also the activity of the Mg^{2+}-activated D153H enzyme is substantially higher than that of the wild-type enzyme at elevated pH values.[6] The Mg^{2+} activation of the enzyme with histidines at both 153 and 328 must also be primarily due to the replacement of Asp-153 since the K328H enzyme does not show any Mg^{2+} activation (see Figure 2) (Xu & Kantrowitz, 1991).

The D153H and D153H/K328H enzymes require preincubation with Mg^{2+} for maximal activity. Because the activation of these enzymes by Mg^{2+} is slow (see Figure 1) and the rate of activation is independent of the Mg^{2+} concentration, the activation process most probably involves a conformational change. The new structural state, stabilized by Mg^{2+}, is more active than the native state of the mutant enzymes and remains activated as long as Mg^{2+} is present. The activation by Mg^{2+} is reversible, and after the removal of the Mg^{2+} the enzyme can be reactivated.

The magnesium effect of the enzymes containing the D153H mutation is not unexpected. Mg^{2+} at the M3[7] site is coordinated to Asp-51, Thr-155, Glu-322, and three water molecules. Asp-153 is not a direct ligand to the Mg^{2+}, but an oxygen of its carboxyl group is hydrogen-bonded to two water molecules that are in turn coordinated to the Mg^{2+} (Kim & Wyckoff, 1989, 1991). Furthermore, one of the water molecules coordinated to Asp-153 and the Mg^{2+} is also hydrogen-bonded to the phosphate (see Figure 7). The replacement of Asp-153 by histidine in both the D153H and the K328H/D153H enzymes will result in the loss of the hydrogen bonds between the carboxylate of the side chain of residue 153 and the two water molecules coordinated to the Mg^{2+}. The loss of these interactions could destabilize Mg^{2+} binding and decrease the affinity of the mutant enzymes for Mg^{2+}. In fact, both the D153H and the D153H/K328H enzymes exhibit substantially reduced binding affinity for Mg^{2+} compared to that of the wild-type enzyme. The requirement of these mutant enzymes for Mg^{2+} indicates that Asp-153 plays an important role in stabilizing Mg^{2+} binding at the M3 site. Furthermore, the low activity of these enzymes in the absence of Mg^{2+} and the conformational change stabilized upon the addition of Mg^{2+} suggest that Mg^{2+} plays an important role in the structural stabilization of the enzyme in its catalytically active form. These data also suggest that the role of Mg^{2+} in the M3 site of *E. coli* alkaline phosphatase is more important than has been previously suggested (Anderson et al., 1975; Bosron et al., 1977).

The D153H Enzyme Has Enhanced Affinity for Phosphate. In the case of the K328H enzyme, the replacement of Lys-328 by histidine results in an enzyme with diminished affinity for phosphate but almost identical affinity for the substrate (Xu & Kantrowitz, 1991). Furthermore, the enhanced activity of the K328H enzyme suggested that the loss of the water-mediated interaction between Lys-328 and the phosphate was only important in phosphate release and was therefore responsible for the enhanced activity of this enzyme. Since there is an analogous water-mediated interaction between Asp-153 and the phosphate (see Figure 7), the replacement of Asp-153 by His was expected to decrease the affinity of the enzyme for phosphate resulting in a more active enzyme. However, the D153H enzyme exhibits enhanced phosphate affinity compared to that of the wild-type enzyme, although the D153H/K328H enzyme exhibits even weaker phosphate affinity than the K328H enzyme in the presence of Mg^{2+}. Although the enhanced phosphate affinity could be due to a more positive electrostatic field in the active site area due to the replacement of Asp-153 by histidine, energy minimization and Langevin molecular dynamics calculations on the D153H enzyme showed that the side chain of Lys-328 can take up a new conformation in the mutant enzyme. When Lys-328 is not positionally restricted by the salt link to Asp-153, it can

[6] The D153H enzyme cannot be fully activated by Mg^{2+} between pH 7 and 9 because the experiments cannot be performed at high enough concentrations of Mg^{2+}. As the pH increases, the binding of Mg^{2+} improves so that above pH 9 the enzyme can be saturated by Mg^{2+}.

[7] 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., 1983).

reorient itself to directly interact with the phosphate (see Figure 6); this result can explain the enhanced phosphate affinity of the D153H enzyme. Furthermore, these calculations clarify why the D153H/K328H enzyme has even worse affinity for phosphate than either of the single mutants—both water-mediated interactions between the enzyme and the phosphate are eliminated and the direct interaction of Lys-328 to the phosphate, in the D153H enzyme, cannot form.

The Rate-Determining Step for the Mutant Enzymes Is Different Than for the Wild-Type Enzyme at Alkaline pH. At pH 5.5 for enzyme containing residual phosphate, the hydrolysis reaction proceeds in three steps: an instantaneous burst phase followed by a transient phase which in turn is followed by a linear steady-state phase. However, at pH 8.0 the transient phase is not observed (Bloch & Schlesinger, 1973). At both pH values, the small instantaneous burst is due to the fraction of the initial enzyme that does not contain residual phosphate. The elimination of the transient phase at pH 8.0 is indicative of a change in the rate-determining step of the reaction. At pH 5.5, the rate-determining step is the release of phosphate from the covalent E–P complex, while at pH 8.0, the rate-determining step is the release of phosphate from the noncovalent E·P_i complex (Hull et al., 1976; Reid & Wilson, 1971; Gettins & Coleman, 1983· Bloch & Gorby, 1980).

For the K328H enzyme at pH 8.0, the pre-steady-state kinetics are different from what is observed for the wild-type enzyme (Xu & Kantrowitz, 1991). This enzyme exhibits a large instantaneous burst followed by a small transient phase before the steady-state phase. The very low phosphate content of the K328H enzyme explains the large instantaneous burst; however, the small transient phase observed at pH 8.0 is indicative of the hydrolysis of the E–P complex becoming partially rate-limiting.

For the D153H and the D153H/K328H enzymes at pH 8.0, the pre-steady-state kinetics are dramatically different from what is observed for the wild-type enzyme and are dependent upon the Mg^{2+} concentration. Both the D153H and D153H/K328H enzymes exhibit an instantaneous burst phase followed by a transient phase which, in turn, is followed by a linear steady-state phase. The appearance of the transient phase at pH 8.0 indicates that the hydrolysis of the E–P complex is the rate-determining step for these mutant enzymes rather than release of phosphate. In the case of the D153H and the D153H/K328H enzymes, the addition of Mg^{2+} has only a small effect on the transient rate constant but causes a significant increase in the steady-state phase which can be directly correlated with the increase in the steady-state rates of these enzymes in the presence of Mg^{2+}. Finally, both of these mutant enzymes exhibit larger instantaneous bursts in the presence of Mg^{2+} suggesting that the presence of Mg^{2+} influences the amount of residual phosphate bound to these enzymes. The increase in the K_i of phosphate for both of these mutant enzymes in the presence of Mg^{2+} supports this conclusion. Finally, the shift in the rate-determining step at pH 8.0, from the release of phosphate from the E·P_i complex for the wild-type enzyme to the hydrolysis of the E–P complex for the D153H and D153H/K328H enzymes, indicates that the Asp-153 and Lys-328 side chains directly influence catalysis.

The D153H and the D153H/K328H Enzymes Are Analogous to the Mammalian Enzymes That Have Histidine Residues at Positions 153 and 328. Cathala et al. (1975) reported that the bovine kidney alkaline phosphatase is stimulated by the addition of Mg^{2+} and that the stimulation is pH dependent. Furthermore, the bovine enzyme exhibits a pH versus activity profile unlike that of the wild-type *E. coli* enzyme with optimal activity at pH greater than 10, and the bovine enzyme is substantially more active than the *E. coli* enzyme at any pH. These data for the bovine enzyme parallel closely the results we have reported here for the D153H and the D153H/K328H enzymes. Since positions 328 and 153 are occupied by histidines in the mammalian enzymes, many of the altered characteristics of the mammalian enzymes may be due to the existence of histidines at these two positions, since most of the other residues in the catalytic site are conserved between the *E. coli* and the mammalian enzymes.

Function of Asp-153 and Lys-328 in E. coli Alkaline Phosphatase. On the basis of the mutagenesis experiments reported here and previous studies involving substitutions at position 328 (Xu & Kantrowitz, 1991), the function of Asp-153 and Lys-328 in *E. coli* alkaline phosphatase is becoming more evident. The primary function of Lys-328 is to stabilize the binding of phosphate via a water-mediated interaction. This interaction is not present until after the substrate binds; therefore, Lys-328 is not involved in substrate binding. The removal of Lys-328 does alter catalysis, since the hydrolysis of the E–P complex becomes partially rate-limiting at pH 8.0. The exact mode by which Lys-328 alters the hydrolsyis rate is unclear; however, the loss of Lys-328 would alter the hydrogen-bonding network and the electrostatic field of the active site, which could alter the protonation of the hydroxyl coordinated to Zn^{2+} at the M1 site and thereby alter catalysis.

Asp-153 takes on a similar role as does Lys-328. It is also involved in a water-mediated interaction with the phosphate. Therefore, all the arguments presented above would hold for Asp-153 as well. In addition, the interaction of the carboxylate of Asp-153 to the Mg^{2+} via two water molecules is also important for Mg^{2+} binding. In fact, without Mg^{2+} bound at the M3 site, the enzyme does not exist in the high-activity high-affinity form. Asp-153 also stabilizes the position of Lys-328, preventing Lys-328 from directly interacting with the phosphate. These data also suggest that the Mg^{2+} has a more important role in alkaline phosphatase than had been previously realized. Finally, positions 153 and 328 are responsible for many of the observed differences between the *E. coli* and mammalian alkaline phosphatases.

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