Probing the Role of Histidine-372 in Zinc Binding and the Catalytic Mechanism of *Escherichia coli* Alkaline Phosphatase by Site-Specific Mutagenesis†

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ABSTRACT: In the X-ray structure of *Escherichia coli* alkaline phosphatase at 2.0-Å resolution, His-372 was found only 3.8 Å away from the zinc and forms a hydrogen-bonding interaction with Asp-327, a bidentate ligand of the zinc at the M1 site. However, His-372 does not directly interact with the zinc atom at the M1 site. In order to investigate the role of the side chain of His-372 in zinc binding and the catalytic mechanism of *Escherichia coli* alkaline phosphatase, site-directed mutagenesis was used to convert His-372 to alanine. The fact that the His-372→Ala enzyme has similar zinc binding affinity as the wild-type enzyme indicates that His-372 is not involved in zinc binding at the M1 site. However, the altered kinetic behavior of the mutant enzyme compared to the wild-type enzyme suggests that the imidazole ring of His-372 plays an indirect role in the catalytic mechanism of the enzyme. The hydrolysis activity of the His-372→Ala enzyme at pH 8.0 is 10-fold lower than that of the wild-type enzyme. In the presence of a phosphate acceptor at pH 8.0, the mutant enzyme is approximately 80% as active as the wild-type enzyme. Therefore, the His-372→Ala mutation selectively enhances the transphosphorylation activity of the enzyme. The His-372→Ala enzyme also exhibits 4- and 30-fold decreases in K_m as compared to the wild-type enzyme in 0.1 M MOPS buffer and 1.0 M Tris, buffer at pH 8.0, respectively. A change in the rate-determining step at pH 8.0 is also observed for the His-372→Ala enzyme compared with the wild-type enzyme. The presence of a transient burst in the pre-steady-state kinetics of the His-372→Ala enzyme indicates that breaking of the covalent phosphoserine bond is the rate-limiting step in the reaction. The lack of incorporation of [^{32}P]P_i into the His-372→Ala enzyme at pH 8.0 suggests the presence of a stable intermediate during the transition from the enzyme–phosphate covalent complex to the enzyme–phosphate noncovalent complex. Taken together, these kinetic results suggest that the hydroxyl group coordinated to the zinc atom at the M1 site is a weaker nucleophile in the His-372→Ala enzyme than in the wild-type enzyme. Therefore, the interaction between the side chain of His-372 and Asp-327 may be important for stabilizing the zinc hydroxyl which is the nucleophilic group that is responsible for the breakdown of the phosphoserine intermediate.

Alkaline phosphatase isolated from the periplasmic space of *Escherichia coli* (EC 3.1.3.1) catalyzes the nonspecific hydrolysis of phosphomonoesters (Reid & Wilson, 1971). The enzyme can also catalyze a transphosphorylation reaction with the transfer of the phosphate from the phosphomonoester to an alcohol (Dayan & Wilson, 1964; Wilson et al., 1964). In *E. coli*, alkaline phosphatase is a dimeric metalloenzyme, containing two tightly bound zinc atoms and one magnesium atom per monomer. The mechanism of the enzymatic reaction has been thoroughly studied, and a stable phosphoenzyme intermediate with Ser-102 phosphorylated has been isolated (Schwartz & Lipmann, 1961; Engström, 1962; Schwartz et al., 1963). Many kinetic and physicochemical studies suggest that, at acidic pH, the hydrolysis of the covalent phosphoserine intermediate (E–P_i) to form a noncovalent enzyme–phosphate complex (E·P_i) is the rate-limiting step while at alkaline pH the dissociation of the E·P_i complex is the rate-limiting step (Hull et al., 1976; Reid & Wilson, 1971; Gettins & Coleman, 1983a; Gettins et al., 1985; Bloch & Gorby, 1980).

The X-ray structure of *E. coli* alkaline phosphatase has been determined and refined to 2.0 Å (Kim & Wyckoff, 1989, 1991). The enzyme is a symmetric dimer and has an α/β topology. The active-site region is located at the carboxyl-terminal end of the β-pleated sheet in a pocket containing Ser-102, Arg-166, and the three metal binding sites. Phosphate is tightly bound in this pocket by the guanidinium group of Arg-166 and the two zinc atoms. In the proposed reaction mechanism of the enzyme, a hydroxyl group coordinated to Zn_1,[1] the zinc atom at the M1[2] metal site, is the nucleophile which attacks the phosphoserine (Coleman & Gettins, 1983; Gettins & Coleman, 1983a; Gettins et al., 1985; Sowadski et al., 1985). The function of Zn_2,[1] which is 3.9 Å away from Zn_1, may involve the activation of Ser-102. Similar two-metal-assisted mechanisms have been proposed for phospholipase C (Hough et al., 1989), the 3′,5′-exonuclease activity of DNA polymerase I, and the RNase H domain of HIV reverse transcriptase (Beese & Thomas, 1991).

In the crystal structure of *E. coli* alkaline phosphatase at 2.8 Å (Sowadski et al., 1985), His-372 was originally believed to be a direct ligand of Zn_1; however, in the structure refined to 2.0 Å (Kim & Wyckoff, 1989, 1991), His-372 was found

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[1] Zn_1 and Zn_2 are the zinc atoms at the M1 and M2 metal sites, respectively.

[2] 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).

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not to be a ligand to the zinc. His-372 is 3.8 Å from the M1 site, and it is hydrogen-bonded to Asp-327 which itself is a bidentate ligand to Zn_1. Site-directed mutagenesis studies indicate that the side chain of Asp-327 is essential for zinc binding. However, the negative charge and at least one carboxyl oxygen of the side chain are not involved in activating or stabilizing the zinc hydroxyl (Xu & Kantrowitz, 1992). In the transphosphorylation reaction, Asp-327 is likely to play an important role by positioning or activating the zinc-coordinated Tris alkoxide. A sequence comparison of alkaline phosphatases from different species shows that His-372 is highly conserved in all mammalian alkaline phosphatases while it is replaced by Thr in the *Bacillus subtilis* enzyme (Hulett et al., 1991). In order to determine the role of His-372 in zinc binding at the M1 site and catalysis, site-directed mutagenesis was used to change it to an alanine.

EXPERIMENTAL PROCEDURES

Materials. Agar, ampicillin, glycerol, *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. Analytical-grade $MgCl_2$ and $ZnSO_4$ were from either Mallinckrodt or Fisher Scientific. 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 using glass beads employing the US Bioclean kit from U.S. Biochemicals. The oligonucleotides required for site-specific mutagenesis were synthesized by Operon Technologies Inc. 2,4-Dinitrophenyl phosphate was synthesized by the method of Kirby and Varvoglis (1966) using 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 *E. coli* strain CJ236 [*dut-1*, *ung-1*, *thi-1*, *relA-1*/pCJ105 (Cm^r)] was a gift of T. Kunkel.

Expression of Wild-Type and Mutant Alkaline Phosphatases. E. coli 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 and the His-372→Ala[3] enzymes were isolated from the plasmid/strain combinations pEK48/SM547 and pEK146/SM547, respectively, as described previously (Chaidaroglou et al., 1988). Enzyme purity was judged by SDS–polyacrylamide gel electrophoresis, according to the procedure of Laemmli (1970).

[3] The notation used to name the mutant enzymes is the His-372→Ala enzyme. The wild-type amino acid and its 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.

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 enzymes was determined by the Bio-Rad version of Bradford's dye binding assay (Bradford, 1976) using wild-type alkaline phosphatase as the standard.

Determination of Enzymatic Activity. Alkaline phosphatase activity was measured spectrophotometrically 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. The buffer systems used were 1.0 M Tris, 0.01 M Tris, and 3 M glycerol in 0.01 M Tris. The ionic strength was held constant at 0.585 M with NaCl.

Rapid Kinetic Measurements. Experiments were performed using a KinTek Inc. stopped-flow spectrophotometer at 25 °C with a dead time of about 1 ms. Data were collected at 410 nm directly by computer via an analog/digital interface. One syringe contained either 10.2 μM wild-type or 8.4 μM His-372→Ala enzyme, in 0.01 M Tris/0.1 M NaCl, pH 7.4. The other syringe contained either 0.1 mM *p*-nitrophenyl phosphate in 0.1 M Tris buffer, pH 8.0, or 1 mM *p*-nitrophenyl phosphate in 0.1 M CAPS buffer, pH 10.0.

Preparation of ^{32}P-Labeled Alkaline Phosphatase. The wild-type and His-372→Ala enzymes were first dialyzed against 0.1 M sodium acetate, pH 5.5, or 0.1 M Tris, pH 8.0, and then 100 μL of each enzyme (0.25 mg) was mixed with 10 mM inorganic phosphate containing 50 μCi of [^{32}P]-orthophosphate in either 0.1 M sodium acetate, pH 5.5, or 0.1 M Tris, pH 8.0, buffer in a total volume of 150 μL. After a 30-min incubation at room temperature, 150 μL of 6 N perchloric acid was added to the reaction mixture, followed immediately by 1.5 mL of H_2O to precipitate the proteins. The solution was centrifuged, and the protein precipitate was washed twice with 2 mL of 5% trichloroacetic acid (Reid et al., 1969). The samples were resolved on a 10% SDS–polyacrylamide gel, the protein bands were visualized by Coomassie blue staining, and the gel was autoradiographed after it was dried.

RESULTS

Construction of the His-372→Ala Alkaline Phosphatase by Site-Specific Mutagenesis. The mutational change at position 327 of alkaline phosphatase was introduced by the method of Kunkel (Kunkel, 1985; Kunkel et al., 1987) as previously modified (Xu & Kantrowitz, 1991). Selection of the mutation was performed directly by sequencing, and the mutation was then recloned and confirmed by the procedure described previously (Chaidaroglou et al., 1988; Xu & Kantrowitz, 1991). In this fashion, the plasmid pEK146, which carries the mutant version of the *phoA* gene, was constructed and isolated.

The His-372→Ala Alkaline Phosphatase Has a 10-Fold Lower Hydrolysis Activity and Enhanced Transphosphorylation Activity Compared to the Wild-Type Enzyme. The activity of the His-372→Ala enzyme was first determined in 0.1 M MOPS buffer at pH 8.0. Since there is no phosphate acceptor present in this buffer, the activity is due solely to the hydrolysis activity of the enzyme. Under these conditions, the k_{cat} value of the mutant enzyme is 4.8 s^{-1}, 10-fold lower than the wild-type enzyme which has a k_{cat} of 44.5 s^{-1} (see Table 1A). Since alkaline phosphatase has both hydrolysis

Table 1: 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 ($M^{-1}\cdot s^{-1}$)	buffer
(A) In the Absence of a Phosphate Acceptor				
wild-type	44.5	9.4	4.3×10^6	0.1 M MOPS, pH 8.0[c]
His-372→Ala	4.8	0.3	1.6×10^7	0.1 M MOPS, pH 8.0
(B) In the Presence of a Phosphate Acceptor				
wild-type	80.5	21.1	3.8×10^6	1.0 M Tris, pH 8.0
His-372→Ala	63.4	4.7	1.3×10^6	1.0 M Tris, pH 8.0

[a] Assays were performed at 25 °C in the buffer indicated using *p*-nitrophenyl phosphate as substrate. [b] The k_{cat} values are calculated from the V_{max} using a dimer molecular weight of 94 000 (Bradshaw et al., 1981). The k_{cat} per active site would be half the value indicated. [c] For the MOPS buffer, the ionic strength was adjusted to 0.585 with NaCl.

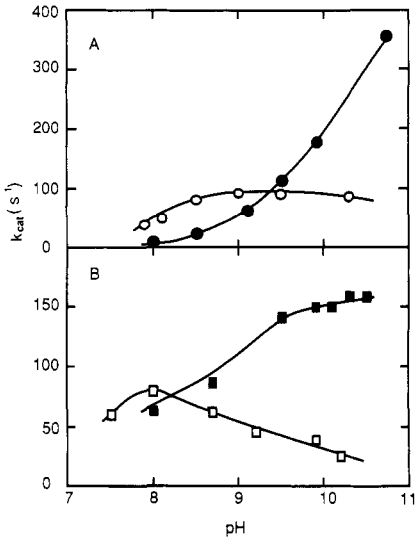

FIGURE 1: pH dependence of the k_{cat} (s^{-1}) of the wild-type and His-372→Ala alkaline phosphatases. Reactions were carried out at 25 °C with *p*-nitrophenyl phosphate as substrate. (A) Reactions were performed in the absence of any alternative phosphate acceptor (0.1 M MOPS, pH 7.5–8.7, and 0.1 M CAPS, pH 8.7–10.3) with the wild-type (○) and His-372→Ala (●) enzymes. (B) Reactions were performed in the presence of a phosphate acceptor (1.0 M Tris) with the wild-type (□) and His-372→Ala (■) enzymes. 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.

and transphosphorylation activities in the presence of a phosphate acceptor, the activity measured in 1.0 M Tris buffer should be the sum of the hydrolysis and transphosphorylation activities of the enzyme. When the activity was determined in 1.0 M Tris buffer at pH 8.0, the mutant enzyme exhibited 80% of the activity of the wild-type enzyme (see Table 1B). Therefore, the His-372→Ala mutation selectively enhances transphosphorylation activity as well as causing a 10-fold decrease in hydrolysis activity compared to the wild-type enzyme.

The His-372→Ala enzyme also exhibits 30- and 4-fold decreases in the K_m in 0.1 M MOPS buffer and in 1.0 M Tris buffer at pH 8.0, respectively, as compared to the wild-type enzyme. Since the k_{cat}/K_m values of the His-372→Ala and wild-type enzymes are comparable, the His-372→Ala enzyme is still as effective a catalyst as the wild-type enzyme both in the presence and in the absence of a phosphate acceptor.

The pH Dependence of the His-372→Ala Enzyme Activity in the Absence and Presence of a Phosphate Acceptor Is Different from the Wild-Type Enzyme. For the wild-type enzyme in the absence of a phosphate acceptor, the k_{cat} value

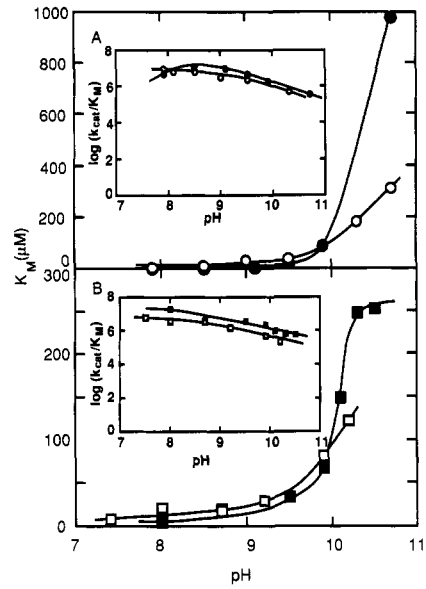

FIGURE 2: pH dependence of the K_m of the wild-type and His-372→Ala alkaline phosphatases. Reactions were carried out at 25 °C with *p*-nitrophenyl phosphate as substrate. (A) Reactions were performed in the absence of any alternative phosphate acceptor (0.10 M MOPS, pH 7.5–8.7, and 0.10 M CAPS, pH 8.7–10.3) with the wild-type (○) and His-372→Ala (●) enzymes. Inset: influence of pH on the k_{cat}/K_m of the wild-type (○) and His-372→Ala (●) enzymes. (B) Reactions were performed in the presence of a phosphate acceptor (1.0 M Tris) with the wild-type (□) and His-372→Ala (■) enzymes. Inset: influence of pH on the k_{cat}/K_m of the wild-type (□) and His-372→Ala (■) enzymes.

reaches a maximum at pH 9.0 (see Figure 1A). The pH dependence of the hydrolysis activity of the wild-type enzyme is related to the change in the rate-determining step from hydrolysis of the covalent enzyme–phosphate complex to the dissociation of the phosphate from the noncovalent enzyme–phosphate complex. In the case of the His-372→Ala enzyme, the hydrolysis activity increases up to pH 10.7, the highest pH at which the activity was determined (see Figure 1A). Although the hydrolysis activity of the mutant enzyme is lower than that of the wild-type enzyme at pH values below 9.3, it becomes higher than the wild-type enzyme at pH values over 9.3. The K_m of the His-372→Ala enzyme also increases more significantly than the wild-type enzyme at high pH (see Figure 2A). Moreover, the optimal pH for the k_{cat}/K_m ratio is shifted to more alkaline pH for the His-372→Ala enzyme compared to the wild-type enzyme (see Figure 2A, inset).

The activity of the His-372→Ala enzyme as a function of pH is also different from the wild-type enzyme in the presence of a phosphate acceptor (see Figure 1B). For the wild-type enzyme, the optimal pH for the activity in the presence of a phosphate acceptor is approximately pH 8.0. However, the activity of the His-372→Ala enzyme increases up to pH 10.7, the highest pH at which the activity was determined. Similar to the wild-type enzyme, the K_m of the His-372→Ala enzyme increases as the pH increases up to pH 10.3, and shows a higher value than the wild-type enzyme above pH 10 (see Figure 2B). In the presence of a phosphate acceptor, the pH effect on the k_{cat}/K_m ratio for the His-372→Ala enzyme is similar to that of the wild-type enzyme; however, the His-372→Ala enzyme shows slightly enhanced values at all the pH values investigated.

Pre-Steady-State Kinetic Analysis of the Wild-Type and His-372→Ala Alkaline Phosphatases at pH 8.0 and 10.0.

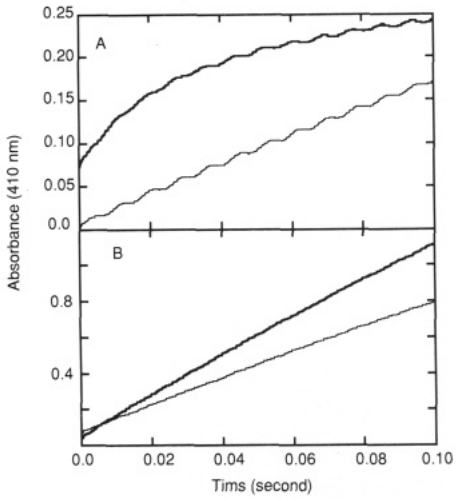

FIGURE 3: Pre-steady-state kinetics of the wild-type (thinner line) and His-372→Ala (thicker line) enzymes at 25 °C. The reactions were carried out at (A) pH 8.0 with 0.1 mM *p*-nitrophenyl phosphate as substrate or at (B) pH 10.0 with 1 mM *p*-nitrophenyl phosphate as substrate. See Experimental Procedures for details.

For the wild-type enzyme, a transient burst[4] of the alcohol product is observed at pH 5.5, indicating that the hydrolysis of the E–P_i intermediate is the rate-limiting step. However, at pH 8.0, only a linear steady-state phase is observed for wild-type enzyme that contains 1 mol of noncovalently bound phosphate per active site. At pH 8.0, the slow release of this noncovalently bound phosphate from the active site becomes the rate-limiting step. Stopped-flow experiments were used to study the kinetics of the His-372→Ala enzyme at pH 8.0 and pH 10.0. By contrast to the wild-type enzyme, there was a transient burst phase of the alcohol product in the pre-steady-state kinetics of the mutant enzyme at pH 8.0. An instantaneous burst phase of product, which is completed within the dead time of the stopped-flow apparatus (about 1–2 ms), was also observed before the transient burst phase (see Figure 3A). In contrast, when the stopped-flow experiments were performed on the wild-type and the His-372→Ala enzymes at pH 10.0, a transient burst was not observed for either enzyme (see Figure 3B).

The His-372→Ala Enzyme Has the Same Zinc and Phosphate Binding Affinity as the Wild-Type Enzyme at pH 8.0. In order to investigate whether the side chain of His-372 is important for the binding of zinc at the M1 site, the effect of increasing the Zn^{2+} concentration on the hydrolysis activity of the His-372→Ala enzyme was measured. As shown in Figure 4, Zn^{2+} has a similar effect on the activity of the mutant enzyme as it does on the activity of the wild-type enzyme at pH 8.0.

The phosphate binding affinity of the His-372→Ala enzyme was determined by measuring the phosphate inhibition constant (K_i) at pH 8.9. In 0.1 M MOPS buffer at pH 8.9, the K_i for the His-372→Ala enzyme is 29 μM. Under the same conditions, the K_i value for the wild-type enzyme is 32 μM.

The Wild-Type and His-372→Ala Enzymes Incorporate Inorganic Phosphate at pH 5.5, but Not at pH 8.0. Wild-type alkaline phosphatase has been shown to incorporate inorganic phosphate at acid pH (Schwartz & Lipmann, 1961; Engström, 1962; Milstein, 1964; Pigretti & Milstein, 1965; Reid et al., 1969). When the enzyme is incubated with ^{32}P-labeled P_i at acidic pH, a phosphorylated serine is obtained. However, the incorporation decreases as the pH increases. Furthermore, ^{31}P NMR studies indicate that at acidic pH, the enzyme exists as E–P_i, and as the pH is increased, E·P_i becomes the dominant form of the enzyme. The labeling of the wild-type and His-372→Ala enzymes with ^{32}P-labeled P_i was performed at both pH 5.5 and pH 8.0. As shown in Figure 5, both the wild-type and His-372→Ala enzymes can incorporate inorganic phosphate at pH 5.5 but not at pH 8.0.

[4] Transient burst represents the exponential burst appearance of the 2,4-dinitrophenolate in the stopped-flow experiments.

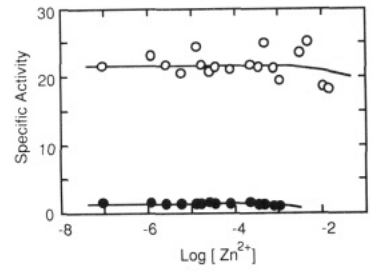

FIGURE 4: Influence of zinc concentration on the activity of the wild-type (○) and His-372→Ala enzymes (●) in 0.01 M Tris, pH 8.0 buffer. Reactions were carried out at 25 °C with 10 mM *p*-nitrophenyl phosphate as substrate at pH 8.0, and the formation of *p*-nitrophenolate was monitored at 410 nm. Specific activity is reported in units of micromoles per minute per milligram of protein.

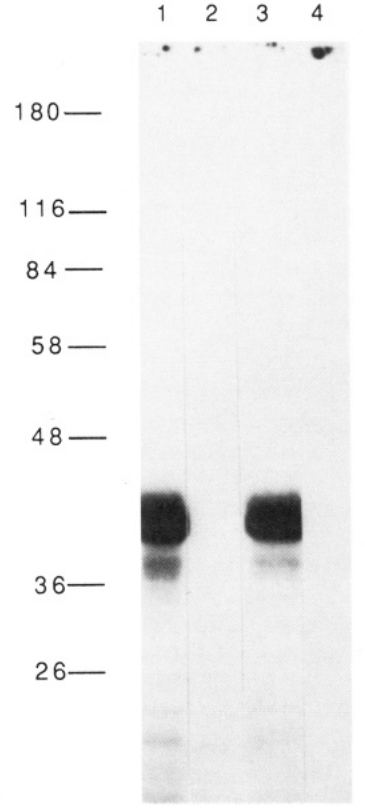

FIGURE 5: Autoradiograph of SDS–polyacrylamide gel electrophoresis of ^{32}P-labeled alkaline phosphatase. Lanes 1 and 3 are the wild-type and His-372→Ala enzymes, respectively, labeled at pH 5.5 with [^{32}P]P_i. Lanes 2 and 4 are the wild-type and His-372→Ala enzymes, respectively, labeled at pH 8.0 with [^{32}P]P_i. Molecular masses of marker proteins (in kilodaltons) are indicated at the left. The molecular weights of the prestained marker proteins can only be used as an estimate of the molecular weight of the protein.

DISCUSSION

E. coli alkaline phosphatase has proven to be a useful model system to study the role of metals in metalloenzymes. Extensive evidence obtained by various biochemical, physicochemical, and mutagenesis studies indicated that zinc is

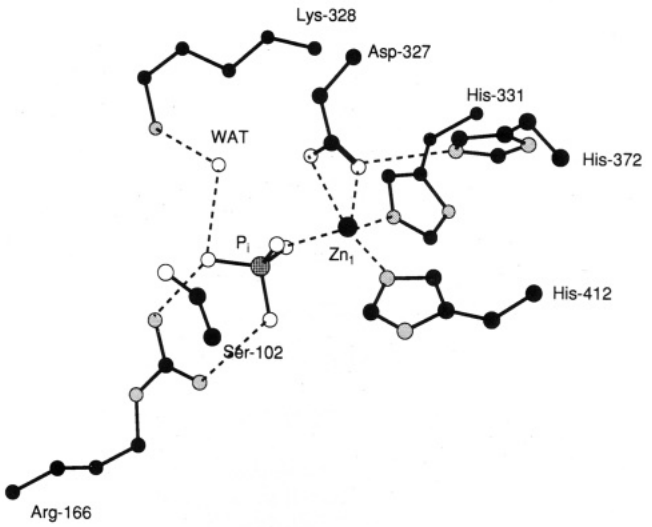

FIGURE 6: Zn_1 binding site of *E. coli* alkaline phosphatase. His-372 is only 3.8 Å away from the zinc atom and interacts with Asp-327 through a hydrogen-bonding interaction. Zn_1 interacts with an imidazole nitrogen of His-331 and His-412, one of the phosphate oxygens, and both of the carboxylate oxygens of Asp-327. The phosphate interacts with Arg-166, Zn_1 and Zn_2 (not shown), and a water molecule (WAT) that is hydrogen-bonded to the side chain of Lys-328. Also shown is Ser-102 which is phosphorylated during the reaction.

essential for the catalytic activity of alkaline phosphatase (Plocke & Vallee, 1962; Plocke et al., 1962; Simpson & Vallee, 1968; Harris & Coleman, 1968; Anderson et al., 1975, 1976; Gettins & Coleman, 1983b, 1984b; Kim & Wyckoff, 1991; Xu & Kantrowitz, 1992; Mangani, 1992). In *E. coli* alkaline phosphatase, Zn_1 at the M1 site in the E·P_i complex exhibits penta coordination. The ligands to the zinc are imidazole nitrogens of His-331 and His-412, both carboxyl oxygens of Asp-327, and one of the phosphate oxygens (Kim & Wyckoff, 1989, 1991) (see Figure 6). In the proposed reaction mechanism of the enzyme, a water molecule, activated by Zn_1, is the nucleophile which attacks the phosphoserine. Evidence from ^{35}Cl NMR (Gettins & Coleman, 1984a), EXAFS spectroscopy (Mangani, 1992), and X-ray crystallography studies (Kim & Wyckoff, 1991) suggests the presence of a solvent molecule as an additional ligand to Zn_1. Although His-372 is not a direct ligand of the zinc atom at the M1 site, it is only 3.8 Å away and interacts with one of the carboxyl oxygens of Asp-327, which itself is a bidentate ligand of Zn_1 (Kim & Wyckoff, 1991).

In this study, we demonstrate that replacing His-372 with alanine results in a mutant enzyme with altered kinetic behavior compared to the wild-type enzyme. The fact that the mutant enzyme has the same zinc binding affinity as the wild-type enzyme (see Figure 4) indicates that the altered kinetic behavior of the His-372→Ala enzyme is not the result of weaker zinc binding. This result is consistent with the crystal structure of the wild-type enzyme at 2.0 Å which indicates that the imidazole ring of His-372 is not involved in the binding of Zn_1 (Kim & Wyckoff, 1989, 1991). Although the His-372→Ala enzyme shows a 10-fold decrease in hydrolysis activity compared to the wild-type enzyme at pH 8.0, the transphosphorylation activity is enhanced in the mutant enzyme (see Table 1). Furthermore, the pH profiles of the k_{cat} are very different for the wild-type and His-372→Ala enzymes (see Figure 1A,B). In the presence and absence of a phosphate acceptor, the activity of the His-372→Ala enzyme is optimal above pH 10.3, while the pH optima of the wild-type enzyme are approximately pH 8 and 9.5, respectively. The biphasic appearance of nitrophenolate at pH 8.0 in the pre-steady-state kinetics of the His-372→Ala enzyme (see Figure 3A) suggests that this mutant enzyme has a different rate-determining step than that of the wild-type enzyme. Since, for the wild-type enzyme at pH 8.0, the slow step of the reaction is the release of the phosphate from the E·P_i complex, and the purified wild-type enzyme contains about 1 mol of endogenous phosphate per active site, only a linear steady-state phase is observed in the pre-steady-state kinetics of the enzyme (Bloch & Schlesinger, 1973; Hull et al., 1976; Gettins & Coleman, 1983b; Xu & Kantrowitz, 1991). The presence of a transient burst of nitrophenolate in the pre-steady-state kinetics of the mutant enzyme suggests that the rate-determining step for the mutant enzyme is located in a step before the release of the noncovalently bound phosphate. One possibility is that the rate-determining step of the His-372→Ala enzyme at pH 8.0 is the same as the rate-determining step of the wild-type enzyme at pH 5.5, that is, the breaking of the covalent bond between the enzyme and phosphate (Fernley & Walker, 1966; Ko & Kézdy, 1967; Chlebowski & Coleman, 1972; Bloch & Schlesinger, 1973; Bloch & Gorby, 1980; Xu & Kantrowitz, 1991, 1992). The fact that similar biphasic kinetics are observed in the pre-steady-state kinetics of the wild-type enzyme at pH 5.5 supports this hypothesis.

[^{32}P]P_i labeling experiments were used to test for the presence of a covalent enzyme–phosphate intermediate. The fact that the wild-type enzyme incorporates inorganic phosphate at pH 5.5 (see Figure 5) is evidence for the presence of a stable enzyme–phosphate covalent intermediate at acidic pH. When the [^{32}P]P_i labeling experiments were performed on both the wild-type and His-372→Ala enzymes at both pH 5.5 and pH 8.0, the mutant enzyme behaved similarly to the wild-type enzyme under both pH conditions. For the His-372→Ala enzyme at pH 8.0, the E–P_i intermediate is not as stable as that of the wild type at pH 5.5. However, the presence of the transient burst in the pre-steady-state kinetics of the mutant enzyme indicates that the slow step of the reaction is located in the step involving the hydrolysis of E–P_i. Therefore, the mutant enzyme may be trapped in an intermediate state during the transition from the E–P_i form to the E·P_i form. These hypotheses are supported by the crystallographic results on the Asp-153→His enzyme (Murphy et al., 1993). Although the pre-steady-state kinetic data on this mutant enzyme indicate the presence of a transient burst at pH 8.0 (Janeway et al., 1993), the X-ray crystallographic study on the enzyme at pH 7.5 suggests that the mutant enzyme exists as the E·P_i complex. However, detailed analysis of the structure of the Asp-153→His E·P_i complex indicates that it is different from the wild-type E·P_i complex crystallized under the same conditions. The orientation of the phosphate in the mutant enzyme actually resembles the structure of the covalent intermediate or the structure of the pentacovalent transition state (Murphy et al., 1993).

When the pre-steady-state kinetics of the wild-type and His-372→Ala enzymes were measured at pH 10.0, neither of the enzymes showed a transient burst phase in their pre-steady-state kinetics. These results indicate that, as is the case for the wild-type enzyme, the rate-determining step for the His-372→Ala enzyme is pH-dependent and, at pH 10, the release of the noncovalently bound phosphate from the active site is the slow step of the reaction.

A possible answer to the question of how His-372 is involved in the catalytic mechanism of alkaline phosphatase comes from both the previous mutagenesis studies on Asp-327 (Xu & Kantrowitz, 1992) and the results reported here. The

hydrogen-bonding interaction between His-372 and Asp-327 may be important for neutralizing the negative charge on Asp-327, and therefore may make the hydroxyl coordinated to the zinc more stable. In the His-372→Ala enzyme, Asp-327 donates more electrons to Zn_1 due to the lack of the hydrogen-bonding interaction with His-372. This would result in an increase in the pK_a of the zinc hydroxyl or make it a less effective nucleophile, which in turn would slow the breakdown of the phosphoserine intermediate. Additional evidence for the above hypothesis is provided by the results on the Asp-327→Asn enzyme (Xu & Kantrowitz, 1992). The change of Asp-327, the only negatively charged amino acid ligand of Zn_1, to an asparagine created a mutant enzyme which has higher hydrolysis activity at pH 8.0 in the presence of 0.1 mM Zn^{2+} compared to the wild-type enzyme under the same conditions. Furthermore, the optimal pH of the activity of this mutant enzyme is about 1 pH unit lower than that of the wild-type enzyme, and only a linear steady-state-phase was observed in the pre-steady-state kinetics at pH 8.0 (Xu & Kantrowitz, 1992). Therefore, despite the fact that the negative charge on Asp-327 is important for the high-affinity binding of the zinc atom at the M1 site, it is also destabilizing the hydroxyl group coordinated to the zinc. The change of Asp-327 to a neutral asparagine makes the zinc hydroxyl a stronger nucleophile. However, this comes at the expense of lower zinc binding affinity.

The mutagenesis studies on His-372 reported here support the conclusion of Kim and Wyckoff (1989, 1991) that His-372 is not a direct ligand of Zn_1 in *E. coli* alkaline phosphatase. However, the interaction between the imidazole ring of His-372 and the carboxyl oxygen of Asp-327 may be important for the stability of the hydroxyl group coordinated to Zn_1. Therefore, the mutation of His-372 to an alanine introduces significant changes in the kinetic behavior of the enzyme including alterations in both the rate-determining step at pH 8.0 and the pH–activity profile of the enzyme.

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