Mutations at Histidine 412 Alter Zinc Binding and Eliminate Transferase Activity in *Escherichia coli* Alkaline Phosphatase*

(Received for publication, August 2, 1994, and in revised form, October 14, 1994)

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His-412 in wild-type *Escherichia coli* alkaline phosphatase is a direct ligand to one of the two zinc atoms critical for the function of the enzyme. To investigate the function of this residue, site-specific mutagenesis was used to substitute His-412 with asparagine and alanine, generating mutant enzymes H412N and H412A, respectively. Both mutant enzymes show a 5-fold decrease in $k_{cat}$ and 30-fold increase in $K_m$ when compared to the corresponding kinetic parameters for the wild-type enzyme. In contrast to the wild-type enzyme, Tris and ethanolamine inhibit both the mutant enzymes by inhibiting the hydrolysis reaction and not participating in the transferase reaction; furthermore, both mutants have lower zinc and phosphate content than the wild-type enzyme. The addition of Zn$^{2+}$ to the H412N and H412A enzymes restores catalytic activity to within 2-fold of the value for the wild-type enzyme, but more importantly the presence of Zn$^{2+}$ completely restores substrate affinity. The similarity in the kinetic parameters for the H412N and H412A enzymes in the absence and presence of zinc suggests that the asparagine side chain does not play a significant role in coordinating zinc. Furthermore, both the asparagine and alanine substitutions reduce the affinity of the resulting enzymes for zinc. The pH profiles for the two mutant enzymes are different than the pH profile observed for the wild-type enzyme, suggesting that the amino acid substitutions may have altered the $pK_a$ of the zinc coordinated water molecule that is critical in the second step of the mechanism. These data suggest that His-412 does not directly participate in the catalytic mechanism but is mainly involved in zinc binding, and therefore is also indirectly involved in substrate binding and product release.

*Escherichia coli* alkaline phosphatase (EC 3.1.3.1) catalyzes the nonspecific hydrolysis of phosphate monoesters. The reaction proceeds via a phosphoseryl intermediate (Schwartz and Lipmann, 1961). Hydrolysis of the phosphoserine results in the formation of phosphate and the alcohol, while the phosphate can also be transferred to a suitable alcohol such as Tris or ethanolamine via a transphosphorylation reaction (Dayan and Wilson, 1964; Wilson et al., 1964). In the enzyme-catalyzed reaction, the rate-limiting step changes with pH. At acidic pH, the hydrolysis of the covalent enzyme-phosphate complex is rate-limiting while the dissociation of the non-covalent enzyme-phosphate complex is rate-limiting at alkaline pH (Hull et al., 1976).

The alkaline phosphatase from *E. coli* is a homodimer of molecular weight 94,000 and consists of two polypeptide chains of 449 amino acids each. The *E. coli* enzyme contains two tightly bound zinc atoms and one magnesium atom per monomer. The three metal binding sites on each chain are in close proximity to the active site which is located in a groove created by the termination of a number of helices and sheets (Sowadski et al., 1983, 1985; Kim and Wyckoff, 1991).

Previous studies have shown the importance of these metals in both catalysis and structural stabilization of the enzyme. It has been suggested that there are two classes of metals in alkaline phosphatase (Plocek et al., 1982; Simpson and Vallee, 1968; Anderson et al., 1975, 1976). Zinc is absolutely required for activity, while magnesium is not essential for activity but plays an important role, since it enhances the activity of the enzyme in the presence of zinc. The two zinc atoms, 3.9 Å apart, are well positioned to activate Ser-102 and water for the nucleophilic attacks required in the enzymatic reaction. In addition, the two zinc atoms are important for the binding of the product, phosphate, and the phosphate portion of the substrate. At the Zn$^+$ site, a zinc-coordinated hydroxyl or alkoxide may be the nucleophile attacking the phosphoserine to displace Ser-102 in the proposed mechanism. Zn$^+$ also stabilizes the developing negative charge on the alcohol leaving group (Kim and Wyckoff, 1991). Thus, Zn$^+$ is essential for catalysis and plays an important role in substrate and phosphate binding. Zn$^+$ interacts with the hydroxyl of Ser-102 to stabilize the deprotonated form of the serine necessary for the nucleophilic attack on the phosphate. The two-metal-ion mechanism of alkaline phosphatase provides a model for the study of other phosphotransfer reactions, such as the reactions catalyzed by P1 nucleases (Volf beda et al., 1991), phospholipase C (Hugh et al., 1989), the 3'-5' exonuclease activity of DNA polymerase I (Seese and Steitz, 1991; Derbyshire et al., 1988), and the nuclease activity of HIV reverse transcriptase (Jacobo and Arnold, 1991; Anderson and Coleman, 1992; Muller et al., 1993).

This study focuses on the highly conserved residue His-412 (Weiss et al., 1986; Kaneko et al., 1987; Thiede et al., 1988; Hulett et al., 1991), which is a ligand to Zn$^+$ (see Fig. 1). In wild-type *E. coli* alkaline phosphatase, His-412 interacts directly with Zn$^+$ through its imidazole nitrogen (Kim and Wyckoff, 1991). To determine the importance of His-412 in *E. coli* alkaline phosphatase for zinc binding and the catalytic mechanism, site-specific mutagenesis was used to replace it with asparagine or alanine.

**EXPERIMENTAL PROCEDURES**

**Materials**

Agar, agarose, ampicillin, chloramphenicol, CAPS, MOPS, p-nitrophenyl phosphate, sodium dihydrogen phosphate, magnesium chloride,

$^{1}$ Zn$^+$ and Zn$^{2+}$ are the two zinc sites in each monomer of the enzyme. These correspond to the M1 and M2 sites identified by x-ray crystallography, while the Mg site corresponds to the M3 site.

$^{2}$ The abbreviations used are: CAPS, cyclohexylamino)propanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; HPLC, high pressure liquid chromatography.
and zinc chloride were purchased from Sigma. Tris, ethanolamine, enzyme-grade ammonium sulfate, and sucrose were supplied by ICN Biochemicals. Trypsin and yeast extract were from Difco. All of the reagents needed for DNA sequencing were obtained from U.S. Biochemical Corp. Restriction endonucleases, T4 DNA ligase, the Klenow fragment of DNA polymerase I, and T4 polynucleotide kinase were from either U. S. Biochemical Corp. or New England Biolabs and used according to the supplier's recommendations. DNA fragments were isolated from agarose gels with the GeneClean II kit from BIO 101 Inc.

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 Zorbax Oligo ion-exchange column.

Methods

Construction of the H412N and the H412A Alkaline Phosphatase by Site-specific Mutagenesis—The oligonucleotides containing the desired mutant sequences were synthesized and purified by HPLC. The DNA template used for the mutagenesis (Kunkel, 1986; Kunkel et al., 1987), a uracil-containing single-stranded DNA, was obtained by infection of strain CJ236 containing the phagemid pEK48 (Chaidaroglou et al., 1988) with the helper phage M13K07 (Vieira and Messing, 1987; Xu and Kantrowitz, 1991). To perform the site-specific mutagenesis, the mutagenic oligonucleotide was annealed to the single-stranded DNA template, then the complementary DNA strand was synthesized with the Klenow fragment of DNA polymerase I. T4 DNA ligase was also included in the reaction mixture to seal the nick in the resulting double-stranded DNA. The reaction mixture was then transformed into E. coli strain MV1190. A number of candidates were sequenced (Sanger et al., 1977) in order to select for introduction of the mutation. After a mutant candidate was identified, a small DNA fragment containing the mutation was inserted into pEK48 which had the corresponding section of the wild-type gene removed (Xu and Kantrowitz, 1991). The mutation was verified a second time by directly sequencing the inserted fragment.

Expression of the Wild-type and Mutant Alkaline Phosphatases—Both the wild-type and mutant alkaline phosphatases were expressed in the host strain SM474 which has the phoA gene deleted from the chromosome as well as a mutation in the ploR regulatory gene. If a phoA-containing plasmid is introduced into this strain, only the alkaline phosphatase encoded by the phoA gene on the plasmid will be expressed. The wild-type, the H412N, and the H412A enzymes were prepared from the plasmid/strain combinations pEK48/SM474, pEK202/SM474, and pEK237/SM474, respectively.

Assays were performed at 25 °C with p-nitrophenyl phosphate as substrate.

| Enzyme | Moles Zn²⁺/mole enzyme | kₐₑₚ⁻⁻⁺ | Kₐₑₚ⁻⁻⁺ | kₐₑₙ/kₐₑₚ × 10⁻⁶ |
|--------|------------------------|----------|----------|-------------------|
| Wild-type | 3.3 (±0.4) | 31 (±1) | 7 (±2) | 4.4 |
| H412N | 1.0 (±0.3) | 7 (±0.5) | 210 (±5) | 0.033 |
| H412A | 0.9 (±0.2) | 6 (±1) | 220 (±10) | 0.027 |

* Both the H412N and the H412A enzymes as well as the wild-type enzyme were dialyzed versus TMZP buffer without zinc before use.

## Purification of the Wild-type and Mutant Alkaline Phosphatases—The same purification procedure was used for both the wild-type and mutant enzymes. The bacterial cells were isolated after growth to stationary phase in YT medium (8 g of tryptone, 5 g of yeast extract, and 5 g of sodium chloride/liter). The periplasmic proteins including alkaline phosphatase were released from the cells by osmotic shock at 4 °C and then precipitated with ammonium sulfate at 85% saturation. After recovery, the ammonium sulfate precipitate was dissolved in TMZP buffer (0.01 M Tris, 0.001 M MgCl₂, 10⁻⁷ M ZnSO₄, 10⁻³ M NaH₂PO₄, 0.31 × 10⁻⁷ M NaCl, pH 7.4) (Bloch and Becker, 1978), followed by extensive dialysis in the same buffer. The enzyme solution was purified by ion-exchange chromatography employing Q-Sepharose Fast Flow (Chaidaroglou et al., 1988) and further purified by size exclusion chromatography, if necessary. SDS-gel electrophoresis was used to judge the purity of the enzyme (Laemmli, 1970).

## Determination of Protein Concentration—The wild-type enzyme concentration was measured by its absorbance at 280 nm with an extinction coefficient of 0.71 cm²/mg (Plocek et al., 1962). The concentration of the mutant enzyme was determined using the Bio-Rad version of Bradford’s (1976) dye binding assay or the Lowry et al. (1951) method with the wild-type enzyme as the standard.

## Measurement of Inorganic Phosphate—The phosphate content of various preparations of alkaline phosphatase was determined by the procedure of Chen et al. (1956) with modifications (Xu and Kantrowitz, 1991). Both the wild-type and mutant enzymes were dialyzed against a 500-fold excess of 0.05 M Tris buffer at pH 7.4 in the absence or presence of 0.5 mM ZnCl₂ for 12–14 h before the phosphate determination.

## Zinc Determination—The zinc content of the wild-type and the mutant enzymes was determined with a Perkin-Elmer 3100 atomic absorption spectrophotometer using the stabilized temperature platform furnace technique. Before the measurements, the enzymes were extensively dialyzed against TMZP buffer without zinc.

## Steady-state Kinetics—The velocity of the enzyme-catalyzed reaction was measured spectrophotometrically at 410 nm using p-nitrophenol phosphate as substrate at 25 °C by monitoring the release of p-nitrophenolate (Garen and 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. The sum of hydrolysis and transferase activities was determined using 0.01 M Tris-or 0.1 M MOPS as the reaction buffer, while the hydrolysis activity alone was determined using 0.01 M Tris-HCl, 0.1 M CAPS, or 0.1 M MOPS buffer depending upon the pH. The ionic strength of these buffers was held constant at 0.5 with NaCl.

## RESULTS

Steady-state Kinetics of the Wild-type and Mutant Enzymes at pH 8.0 in the Absence of a Phosphate Acceptor—The initial kinetic analyses of the H412N and H412A enzymes were performed using 0.01 M Tris or 0.1 M MOPS as the reaction buffer. With either of these buffers only the hydrolysis activity of the enzyme is measured. Both mutant enzymes showed a 5-fold decrease in kₐₑₚ and 30-fold increase in Kₐₑ compared to the corresponding values for the wild-type enzyme (Table I). The
The active site of E. coli alkaline phosphatase. Shown are Zn
$\text{His}_331\text{His}_412$
phosphate (P$^\text{i}$) and their ligands. Zn$^2+$ interacts with an imidazole nitrogen of His-412 and His-331, one of the phosphate oxygens, and the carboxylate oxygen of Asp-327. Also shown is Ser-102 which is phosphorylated during the reaction. Arg-166 which interacts with the phosphate, and three water molecules that are ligands to the Mg$^{2+}$. For clarity, these water molecules are not labeled.

substantial decrease in the $k_{\text{cat}}/K_M$ ratio for both mutant enzymes indicates that the replacement of His-412 results in the formation of an enzyme with reduced catalytic efficiency.

Tris Affects the Steady-state Kinetics of the Wild-type and Mutant Enzymes at pH 8.0—When the activity of alkaline phosphatase is determined in 1.0 mM Tris buffer at pH 8.6, the rate observed is the sum of the transphosphorylation and hydrolysis reactions, since Tris can serve as a phosphoryl group acceptor. Therefore, it was expected that the activity in 1.0 mM Tris buffer should be the same or greater than the activity measured in the absence of a phosphate acceptor. However, this was not the case for the H412N or the H412A enzymes. Both mutant enzymes had extremely low activities in the presence of a high concentration of Tris (1.0 mM), in fact much lower than the values in the absence of a phosphate acceptor (Table I). The $k_{\text{cat}}$ of the H412N and the H412A enzymes in 1.0 mM Tris were reduced more than 40,000-fold and 10$^5$-fold, respectively, compared to the wild-type value. Under these conditions, the $K_M$ values for the H412N and the H412A enzymes were increased approximately 20- and 100-fold, respectively.

Kinetic parameters were also measured as a function of Tris concentration. For both mutant enzymes, Tris inhibits the activity throughout the range of concentrations employed (0.05–1.0 M). Above 0.75 M Tris, the enzymes have extremely low activities. In order to determine whether high concentrations of Tris were chelating Zn$^{2+}$ and thereby reducing activity, the mutant enzymes were first dialyzed in 1.0 M Tris buffer, then dialyzed into TMZP buffer without Zn$^{2+}$, before being assayed in 0.01 M Tris buffer. The activity observed was the same as that of the control enzyme that had not been first dialyzed into 1.0 M Tris buffer. Experiments were also carried out with another phosphoryl acceptor, ethanolamine. Results were analogous to those obtained with Tris. In the presence of ethanolamine, the activities of both mutant enzymes were lower than that observed in the absence of a phosphate acceptor (data not shown); however, the activities were much higher in the presence of ethanolamine than in the presence of Tris. In 1.0 mM ethanolamine, the $k_{\text{cat}}$ of the H412N enzyme was reduced 15-fold, while the $K_M$ was increased 35-fold compared to the wild-type values.

The Mutant Enzymes Have a Lower Residual Zinc Content than the Wild-type Enzyme—Since His-412 is a direct ligand to Zn$^2+$, (Fig. 1), the replacement of His-412 by asparagine or alanine would be expected to alter the zinc affinity of the resulting enzymes. Therefore, the residual zinc content of the mutant enzymes was measured and compared to the value for the wild-type enzyme determined under identical conditions. After being dialyzed against buffer without zinc, the zinc content of the wild-type, H412N, and H412A enzymes was analyzed by atomic absorption spectrophotometry. The wild-type enzyme contained 3.3 ± 0.4 mol of zinc/mol of enzyme dimer; however, the H412N and H412A enzymes contained only 1.0 ± 0.3 and 0.9 ± 0.2 mol of zinc per mol of enzyme dimer, respectively.

Effects of Zinc on the Steady-state Kinetics of the Wild-type and Mutant Enzymes at pH 8.0 in the Absence and Presence of a Phosphate Acceptor—Since the replacement of His-412 by asparagine or alanine reduces the zinc affinity of the resulting enzymes, the dependence of the activity on zinc concentration was determined. The activity of both the H412N and the H412A enzymes increased with increasing zinc concentration (Fig. 2). The influence of Zn$^{2+}$ on the specific activities of the wild-type (○), the H412N (●), and the H412A (□) enzymes in the absence of a phosphate acceptor. All of the enzymes were dialyzed in TMZP buffer without zinc before use. The activity was determined at 25 °C by using 0.5 mM p-nitrophenyl phosphate as substrate at pH 8.0.

FIG. 2. The influence of Zn$^{2+}$ on the specific activities of the wild-type (○), the H412N (●), and the H412A (□) enzymes in the absence of a phosphate acceptor. The specific activities were determined as a function of zinc concentration (0.2 mM, 1.0 mM, and 2.0 mM). The zinc concentration was increased at each point in the absence of a phosphate acceptor (data not shown). The activity of the H412N enzyme increased 35-fold while the activity of the H412A enzyme increased 30-fold. The activity of the H412N enzyme increased 20-fold while the activity of the H412A enzyme increased 10-fold. The activity of the H412N enzyme increased 5-fold while the activity of the H412A enzyme increased 4-fold. The activity of the H412N enzyme increased 3-fold while the activity of the H412A enzyme increased 2-fold. The activity of the H412N enzyme increased 1-fold while the activity of the H412A enzyme increased 0.5-fold.

FIG. 1. The active site of E. coli alkaline phosphatase. Shown are Zn$^2+$, Zn$^{2+}$, Mg$^{2+}$, phosphate (P$^\text{i}$) and their ligands. Zn$^2+$ interacts with an imidazole nitrogen of His-412 and His-331, one of the phosphate oxygens, and the carboxylate oxygen of Asp-327. Also shown is Ser-102 which is phosphorylated during the reaction. Arg-166 which interacts with the phosphate, and three water molecules that are ligands to the Mg$^{2+}$. For clarity, these water molecules are not labeled.

atomic absorption spectrophotometry. The wild-type enzyme contained 3.3 ± 0.4 mol of zinc/mol of enzyme dimer; however, the H412N and H412A enzymes contained only 1.0 ± 0.3 and 0.9 ± 0.2 mol of zinc per mol of enzyme dimer, respectively.

Effects of Zinc on the Steady-state Kinetics of the Wild-type and Mutant Enzymes at pH 8.0 in the Absence and Presence of a Phosphate Acceptor—Since the replacement of His-412 by asparagine or alanine reduces the zinc affinity of the resulting enzymes, the dependence of the activity on zinc concentration was determined. The activity of both the H412N and the H412A enzymes increased with increasing zinc concentration (Fig. 2). The influence of Zn$^{2+}$ on both the $K_M$ and $k_{\text{cat}}$ for the H412N enzyme was also measured. Zn$^{2+}$ increases the catalytic efficiency of the enzymes in that the $k_{\text{cat}}$ increases while the $K_M$ decreases as the Zn$^{2+}$ concentration is increased (Fig. 3). At the optimal zinc concentration (0.2 mM), the $k_{\text{cat}}$ and $K_M$ values were within 2-fold of the corresponding values for the wild-type enzyme (Table I). In the presence of 1.0 mM Tris and 0.2 mM Zn$^{2+}$, the $k_{\text{cat}}$ increased, and the $K_M$ decreased dramatically for both mutant enzymes, compared to the corresponding values in the absence of Zn$^{2+}$. In the presence of 1.0 mM ethanolamine and 0.2 mM Zn$^{2+}$, the $K_M$ decreased and the $k_{\text{cat}}$ increased. Experiments were also performed with the H412N enzyme which had been preincubated with Zn$^{2+}$ before reaction. Preincubation of the mutant enzyme with Zn$^{2+}$ did not alter the activity, when compared to samples that had not been preincubated.
H412N enzyme.

In the absence of a phosphate acceptor (0.01 M Tris buffer) at pH 8.0, with p-nitrophenyl phosphate as substrate. The enzymes were treated as reported in the legend to Fig. 2.

Phosphate inhibition-Inorganic phosphate is a product of the wild-type enzyme as compared to the wild-type enzyme; however, the phosphate affinity increased in the presence of ZnO

The inhibition constant of both mutant enzymes could not be determined because of the difficulty in measuring these very low activities. The K_ values were determined by the method of Segel (1975) which takes into account the fact that the product of the reaction is also an inhibitor. The theoretical equation was fit to the data by nonlinear least-squares.

Comparison of the pH Profile of the Wild-type and Mutant Enzymes in the Absence of a Phosphate Acceptor—Kinetic parameters for the mutant enzymes were determined in the absence of a phosphate acceptor and in the presence of 0.02 mM ZnCl_2. This Zn^2+ concentration was selected to avoid precipitation of Zn(OH)_2 at high pH values. For both the H412N and the H412A enzymes, the k_cat versus pH profile is different from that observed for the wild-type enzyme; both mutant enzymes exhibited a bell-shaped curve with maximal activity between pH 8.0 and 9.5.

Phosphate Inhibition—Inorganic phosphate is a product of the alkaline phosphatase reaction and is also a competitive inhibitor. To measure the apparent strength of phosphate binding to the wild-type and mutant enzymes, the phosphate inhibition constant K_i was determined at pH 8.0 (Segel, 1975) in 0.01 M Tris buffer in the absence and presence of 0.2 mM ZnCl_2.

In the absence of Zn^2+, phosphate bound weakly to the H412N enzyme as compared to the wild-type enzyme; however, the phosphate affinity increased in the presence of Zn^2+. For the H412A enzyme, the phosphate K_i was also measured as a function of pH (Table III). The mutants behave similarly to the wild-type enzyme, in that the K_i of inorganic phosphate is sensitive to pH, increasing with increasing pH.

Assays were carried out at 25 °C with p-nitrophenyl phosphate as substrate at pH 8.0. 0.2 mM ZnCl_2 was added to all reactions. The pH dependence of the k_cat (A) and the K_i (B) for the wild-type (●), the H412N (○), and the H412A (□) enzymes. All reactions were carried out in the absence of an alternative phosphate acceptor. The buffers used were 0.10 M MOPS, pH 7-8, 0.10 M CAPS, pH 9-10.5. All the enzymes were dialyzed in TMZP buffer without zinc. Zinc concentration in the reaction buffer is 0.02 mM.

Assays were carried out at 25 °C with p-nitrophenyl phosphate as substrate. At pH 8.0, 0.01 M Tris was used as reaction buffer. At pH 9.5, 10, and 10.5, 0.1 M CAPS buffer was used. The ionic strength of the buffers was kept constant at 0.5 with NaCl. ZnCl_2 was added to the reaction buffer as indicated.

Comparison of the pH Profile of the Wild-type and Mutant Enzymes in the Absence of a Phosphate Acceptor—Kinetic parameters for the mutant enzymes were determined in the absence of a phosphate acceptor and in the presence of 0.02 mM ZnCl_2. This Zn^2+ concentration was selected to avoid precipitation of Zn(OH)_2 at high pH values. For both the H412N and the H412A enzymes, the k_cat versus pH profile is different from that observed for the wild-type enzyme; both mutant enzymes exhibited a bell-shaped curve with maximal activity between pH 8.0 and 9.5.

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The inhibition constant of both mutant enzymes could not be determined because of the difficulty in measuring these very low activities.

Determination of Inorganic Phosphate Content—The residual phosphate content of alkaline phosphatase can vary depending upon the purification procedure and on the affinity of the enzyme for phosphate. The phosphate content of the wild-type and the H412N enzymes were measured in the absence and presence of Zn^2+. The wild-type enzyme had 1.93 ± 0.04 mol of phosphate/mol of enzyme dimer in the absence of zinc, and 2.03 ± 0.05 mol of phosphate/mol of enzyme dimer in the presence of 0.5 mM ZnCl_2. In agreement with previous reports, the
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wild-type enzyme retains approximately 2 phosphates per molecule (Bloch and Schlesinger, 1973). For the H412N enzyme in the absence of zinc, the phosphate content was 0.44 ± 0.02 mol of phosphate/mol of enzyme dimer. In the presence of 0.5 mM ZnCl₂, the phosphate content increased to 1.52 ± 0.02 mol of phosphate/mol of enzyme dimer.

**Discussion**

In the catalytic mechanism of *E. coli* alkaline phosphatase, Zn₂⁺ plays a critical role in binding the substrate, in stabilizing the developing negative charge on the leaving group, in altering the pHₖ₆ of the zinc-coordinated water that is the nucleophile in the second half of the reaction, and in releasing the phosphatase product (Kim and Wyckoff, 1991; Anderson et al., 1975; Dayan and Wilson, 1964; Schwartz and Lipmann, 1961). The amino acids coordinated to Zn₂⁺, are His-412, His-331, and Asp-327, which are conserved in all alkaline phosphatases that have been sequenced to date (Bradshaw et al., 1981; Weiss et al., 1986; Kaneko et al., 1987; Thiede et al., 1988; Hulett et al., 1991). In order to probe the role of Zn₂⁺ in the mechanism of alkaline phosphatase and to determine the importance of the residues in the coordination sphere of Zn₂⁺, we have used site-specific mutagenesis to replace His-412 by either asparagine or alanine.

**His-412 Is Important but Not Critical for the Binding of Zinc**—For both the H412N and H412A enzymes the binding of zinc is significantly reduced. In contrast to the wild-type enzyme, dialysis removes sufficient zinc to reduce the activity of both mutant enzymes; however, even after dialysis these enzymes retain substantial catalytic activity (see Table I). These mutant enzymes not only exhibit reduced catalytic activity, but also increased Kₗ values. The increased Kₗ values are most likely linked to the reduced zinc affinity of both enzymes, since the x-ray structure of the wild-type enzyme suggests that both zinc atoms are directly involved in substrate binding (Kim and Wyckoff, 1991).

The addition of zinc to the H412N and H412A enzymes restores catalytic activity to within a factor of two of the value for the wild-type enzyme, but more importantly, Zn²⁺ completely restores substrate affinity (see Table IIA). In the case of the H412N enzyme, the addition of zinc also restores the affinity of the mutant enzyme for phosphate to the same level as the wild-type enzyme. In fact, the residual phosphate content in the presence of zinc is similar for the wild-type and H412N enzymes. The restoration of activity to the H412N and H412A enzymes upon addition of zinc indicates that His-412 is not essential for catalysis and is not absolutely required for zinc binding. Furthermore, the fact that the kinetic parameters for the H412N and H412A enzymes are very similar in the absence and presence of zinc suggests that the asparagine side chain does not play a significant role in coordinating the zinc at the Zn₂⁺ site.

**Tris Inhibits the Activity of the H412N and the H412A Enzymes**—In 1.0 M Tris buffer, the activity of both mutant enzymes is extremely low, much lower than that measured in 0.01 M Tris. In these two mutant enzymes, Tris does not function as a phosphoryl acceptor in the transferase reaction and in addition inhibits the hydrolysis reaction. One possible explanation for the Tris inhibition of these two mutant enzymes is that Tris chelates zinc, thereby reducing activity. Experiments with enzymes first dialyzed in high Tris buffer suggest that this is not the case. Another possible explanation is that the binding of Tris competes with zinc at the Zn₂⁺ site, thereby diminishing both the hydrolysis and transferase activities. Experimental evidence supports this proposal. In fact, the Tris inhibition can be overcome by the addition of zinc. Furthermore, the zinc concentration for optimal activity in 1.0 M Tris buffer is much higher (5 mM) than the zinc concentration required for optimal activity in 0.01 M Tris (0.2 mM). Ethanolamine, another phosphoryl acceptor, also inhibits the activity of the two mutant enzymes, and this inhibition is also reversed by addition of zinc. The inhibition caused by 1.0 M ethanolamine is much less than that caused by 1.0 M Tris. When the inhibition caused by Tris or ethanolamine is overcome by the additional zinc, the higher activity observed also suggests that Tris and ethanolamine can then bind to the mutant enzymes for transphosphorylation to occur as they do in the wild-type enzyme.

**The Ionic Environment Around the Zn₂⁺ Site Has Been Affected**—The Zn₂⁺ binding site of *E. coli* alkaline phosphatase directly interacts with the hydroxyl group which is the nucleophile that attacks the covalent phospho-enzyme intermediate (Kim and Wyckoff, 1991). Both the asparagine and alanine substitutions reduce the affinity of the resulting enzymes for Zn₂⁺. The pH profiles for these two mutant enzymes are different than the pH profile observed for the wild-type enzyme. For both mutant enzymes the optimal pH for activity shifts to lower pH. For the H412N enzyme, the optimal pH is approximately 9.5, and for the H412A enzyme it is approximately pH 9. Under the same conditions, the activity of the wild-type enzyme increases with pH up to pH 10.5. Since the coordination around Zn₂⁺ is changed, the ionic environment around the Zn₂⁺ site would be altered. This would result in a change in the pHₖ₆ of the zinc coordinated water molecule and would in turn alter the pH profile of the mutant enzyme.

**The Role of His-412 in Catalysis of Alkaline Phosphatase**—Activation of the H412N and the H412A enzymes by zinc to levels almost as high as shown by the wild-type enzyme implies that His-412 is not involved in facilitating catalysis by positioning or deprotonating the zinc coordinated water molecule that acts as the nucleophile attacking the phosphoserine. Rather His-412 is involved directly in zinc binding at the Zn₂⁺ site and indirectly involved in substrate binding and phosphate release, via its role in coordination of Zn₂⁺. The fact that the two mutant enzymes have similar properties implies that the asparagine cannot functionally replace histidine. Further studies with the H412N enzyme, such as x-ray crystallography will more precisely determine how the asparagine introduced at the His-412 site is positioned to influence catalysis. These studies on the substitutions at the His-412 site have provided more insight on the function of the metals, and the correlation between the transferase and hydrolysis reactions in *E. coli* alkaline phosphatase.

**Acknowledgments**—We thank D. Robinson for his assistance with the atomic absorption experiments, and to X. Xu for her assistance.

**References**

Anderson, R. A., Boorman, W. F., Kennedy, P. S., and Vallee, B. L. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 2989-2993
Anderson, R. A., Kennedy, P. S., and Vallee, B. L. (1976) Biochemistry 15, 3710-3716
Anderson, S. P., and Coleman, J. E. (1992) Biochemistry 31, 8221-8228
Benz, L. S., and Steitz, T. A. (1991) EMBO J. 10, 25-33
Bloch, W., and Bicker, D. (1978) J. Biol. Chem. 253, 6211-6217
Bloch, W., and Schlesinger, M. J. (1975) J. Biol. Chem. 249, 3754-3760
Bradford, M. M. (1976) Anal. Biochem. 68, 248-254
Bradshaw, R. A., Cancado, F., Ericsson, L. H., Newman, P. A., Piccoli, S. P., Schlesinger, K., and Walsh, K. A. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 2473-2477
Chaidaroglou, A., Brezinski, J. D., Middleton, S. A., and Kantrowitz, E. R. (1988) Biochemistry 27, 8388-8434
Chen, P. S., Torbaya, T. Y., and Warner, H. (1956) Anal. Chem. 28, 1756-1758
Dayan, J., and Wilson, I. B. (1984) Biochim. Biophys. Acta 81, 620-623
Derbyshire, V., Freemont, P. S., Sanderson, M. R., Bee, L., Fridman, J. M., Joyce, C. M., and Steitz, T. A. (1988) Science 240, 196-201
Garen, A., and Levinthal, C. (1966) Biochem. Biophys. Acta 30, 470-480
Hough, E., Hansen, L. K., Birkoors, B., Juyge, K., Hansen, S., Hordvik, A., Little, C., Dodson, E. J., and Derewenda, S. (1989) Nature 338, 387-390
Hulett, F. M., Kim, E. E., Bookstein, C., Rapo, N. V., Edward, C. W., and Wyckoff, H. W. (1991) J. Biol. Chem. 266(6), 1077-1084
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Hull, W. E., Halford, S. E., Gutfreund, H., and Sykes, B. D. (1976) *Biochemistry* 15, 1547–1561
Jacobo, M. A., and Arnold, E. (1991) *Biochemistry* 30, 6351–6361
Kaneko, Y., Hayashi, N., Tobe, A., Sanno, I., and Oshima, Y. (1997) *Gene* (Amst.) 56, 137–146
Kim, E. H., and Wyckoff, H. W. (1991) *J. Mol. Biol.* 218, 449–464
Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 82, 488–492
Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol.* 154, 367–382
Laemmli, U. K. (1970) *Nature* 227, 680–685
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
Muller, B., Immendorfer, U., and Divita, G. (1999) *Biochemistry* 32, 7966–7971
Plocke, D. J., Levinthal, C., and Vallee, B. L. (1962) *Biochemistry* 1, 373
Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* 74, 5463–5467
Schwartz, J. H., and Lipmann, F. (1961) *Proc. Natl. Acad. Sci. U. S. A.* 47, 1966–2005
Segel, I. H. (1975) *Enzyme Kinetics*, pp. 120–125, Wiley, New York
Simpson, R. T., and Vallee, B. L. (1995) *Biochemistry* 34, 4345–4349
Sowadski, J. M., Handschumacher, M. D., Murthy, H. M. K., Kundrot, C., and Wyckoff, H. W. (1983) *J. Mol. Biol.* 170, 575–581
Sowadski, J. M., Handschumacher, M. D., Murthy, H. M. K., Foster, B. A., and Wyckoff, H. W. (1985) *J. Mol. Biol.* 186, 417–433
Thiede, M. A., Yoon, K., Gelab, E. E., Noda, M., and Rodan, G. A. (1988) *Proc. Natl. Acad. Sci. U. S. A.* 85, 319–323
Vieira, J., and Messing, J. (1987) *Methods Enzymol.* 153, 3–11
Volbeda, A., Lahm, A., Sakiyama, F., and Suck, D. (1991) *EMBO J.* 10, 1607–1611
Weiss, M. J., Heathorn, P. S., Lafferty, M. A., Slaughter, C., Raducha, M., and Harris, H. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 89, 7182–7186
Wilson, I. B., Dayan, J., and Cyr, K. (1964) *J. Biol. Chem.* 239, 4182–4185
Xu, X., and Kantrowitz, E. R. (1991) *Biochemistry* 30, 7789–7796