Altering of the metal specificity of Escherichia coli alkaline phosphatase*

Cheryl L. Wojciechowski and Evan R. Kantrowitz‡

From the Merkert Chemistry Center, Boston College, Chestnut Hill, Massachusetts 02467, USA

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore by hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

∥ Funded by Grant GM42833 from the National Institutes of Health.
‡ To whom all reprint requests should be addressed: Department of Chemistry, Boston College, Merkert Chemistry Center, Chestnut Hill, MA 02467; E-mail: evan.kantrowitz@bc.edu

Running title: E. coli alkaline phosphatase activity with cobalt.

Keywords: Metalloenzyme, enzyme kinetics, isothermal titration calorimetry, metal binding, atomic absorption, site specific mutagenesis
Footnotes

1 *E. coli* alkaline phosphatase residue numbering will be used throughout when referring to specific amino acids.

The abbreviations used are: PNPP, *p*-nitrophenylphosphate; TMZP buffer, 0.01 M Tris-HCl, 0.001 M MgCl₂, 10⁻⁵ M ZnSO₄, 10⁻⁴ M NaH₂PO₄, 0.31 x 10⁻² M NaN₃, pH 7.4.
Summary

Analysis of sequence alignments of alkaline phosphatases revealed a correlation between metal specificity and certain amino acid side chains in the active site that are metal-binding ligands. The Zn$^{2+}$-requiring *E. coli* alkaline phosphatase has an Asp at position 153 and a Lys at position 328. Co$^{2+}$-requiring alkaline phosphatases from *T. maritima* and *B. subtilis* have a His and a Trp at these positions, respectively. The mutations D153H, K328W, and D153H/K328W were induced in *E. coli* alkaline phosphatase to determine if these residues dictate the metal dependence of the enzyme. The wild-type and D153H enzymes showed very little activity in the presence of Co$^{2+}$, but the K328W and especially the D153H/K328W enzymes effectively use Co$^{2+}$ for catalysis. Isothermal titration calorimetry experiments showed that in all cases except for the D153H/K328W enzyme, a possible conformation change occurs upon binding Co$^{2+}$. These data together indicate that the active site of the D153H/K328W enzyme has been altered significantly enough to allow the enzyme to utilize Co$^{2+}$ for catalysis. These studies suggest that the active site residues His and Trp at the *E. coli* enzyme positions 153 and 328, respectively, at least partially dictate the metal specificity of alkaline phosphatase.
Introduction

Alkaline phosphatase (EC 3.1.3.1) is a non-specific phosphomonoesterase found in organisms from all kingdoms of life. The mechanism of the alkaline phosphatase reaction involves the attack of a serine alkoxide on the phosphorus of the substrate to transiently form a covalent enzyme-phosphate complex followed by the hydrolysis of the serine-phosphate (1). Alternatively, the phosphate can be transferred to a phosphate acceptor such as Tris or ethanolamine. Under alkaline conditions, the slow step in the mechanism is the release of inorganic phosphate from the non-covalent enzyme-phosphate complex. Details of the enzymatic mechanism and the involvement of metal ions in catalysis have been determined largely through studies utilizing the *E. coli* enzyme. As a result of these investigations, the specific role of many active site residues has been elucidated (2-9), and through sequence alignment, their function may be extrapolated to alkaline phosphatases from other organisms.

Aligning the sequences from a selection of alkaline phosphatases shows that the enzymes are very well conserved, especially at the active site (Table I). Upon comparing residues within 10 Å of the phosphate position in the active site of the *E. coli* enzyme to the corresponding residues in other alkaline phosphatases, the majority of enzymes show conservation at most of these amino acid positions. Residues that directly interact with the substrate, Ser-102 and Arg-166, are conserved in all cases (Fig. 1). In the *E. coli* enzyme, the active site contains two Zn$^{2+}$ ions and one Mg$^{2+}$ ion. The residues interacting with Zn$_1$ (Asp-327, His-331, and His-412) and the residues interacting with Zn$_2$ (Asp-51, Asp-369,
and His-370) are conserved in all compared sequences. The only variations occur at the 
ono{amino acids} Asp-153 and Lys-328 near the \textit{E. coli} enzyme Mg\textsuperscript{2+} binding site. Invariably, 
the only change observed at \textit{E. coli} position 153 is from an Asp to a His. The most common 
change at \textit{E. coli} alkaline phosphatase position 328 is to a Trp, the exceptions being a His in 
the \textit{P. abyssi} and eukaryotic enzymes.

A few of the alkaline phosphatases from organisms listed in Table I have been 
analyzed as to their metal requirements for catalysis. With the exception of the Mg\textsuperscript{2+} 
requirement of the enzyme from TAB5 (10), these characterized enzymes require Co\textsuperscript{2+}, not 
Zn\textsuperscript{2+} for activity (11,12). Among the Co\textsuperscript{2+}-requiring enzymes, \textit{T. maritima} alkaline 
phosphatase and the, \textit{B. subtilis phoAIII} and \textit{phoAIV} gene products have His and Trp at \textit{E. coli} 
positions 153 and 328 respectively. These data taken together suggest that either the 
presence of a Trp at \textit{E. coli} alkaline phosphatase position 328 alone, or in combination with a 
His at position 153 is required for the enzyme to utilize Co\textsuperscript{2+} for catalysis. To test this 
proposal, we have constructed the mutations D153H and K328W individually and in 
combination in the Zn\textsuperscript{2+}-requiring \textit{E. coli} enzyme to determine if the alterations result in an 
enzyme that can utilize Co\textsuperscript{2+} for phosphate ester hydrolysis.
EXPERIMENTAL PROCEDURES

Materials — Agar, sodium chloride, ampicillin, chloride metal salts, EDTA and PNPP were purchased from Sigma Chemical Company (St. Louis, Missouri). Tris and ammonium sulfate were purchased from ICN Biomedicals (Costa Mesa, California). Bacto tryptone and yeast extract were obtained from Difco Laboratories (Detroit, Michigan). Mutagenesis was performed with the QuickChange site-directed mutagenesis kit from Stratagene (La Jolla, California). Kits for isolation and purification of DNA fragments from agarose gels and for plasmid purification were from Qiagen (Valencia, California). Electrophoresis-grade agarose, Bio-Rad protein assay solution, Chelex 100 resin, and Source 15Q strong anion exchange resin were from Bio-Rad Laboratories (Hercules, California). Phenyl Sepharose hydrophobic interaction resin was purchased from Amersham Pharmacia Biotech (Piscataway, New Jersey).

Strains and plasmids — The ΔphoA E. coli K12 strain SM547 [Δ(phoA-proC), phoR, tsx::Tn5, Δlac, galK, galU, leu, strR] was used for the expression of the wild-type and mutant enzymes. The wild-type alkaline phosphatase and the D153H, K328W, D153H/K328W and K328A enzymes (13) were expressed as the plasmid/strain combinations, pEK154/SM547, pEK529/SM547, pEK547/SM547, pEK529/SM547, pEK548/SM547 and pEK146/SM547 respectively.

Inducing alkaline phosphatase mutations — The mutations D153H, K328W and the double mutation D153H/K328W were induced using the procedures outlined by Stratagene in the QuickChange mutagenesis kit protocol. The entire gene was sequenced to ensure the correct mutation was present and to confirm that no other mutations were induced.
Growth, expression and purification of wild-type and mutant alkaline phosphatases —

Wild-type and mutant enzymes were isolated and purified as previously described (2) with one additional purification step. Forty percent ammonium sulfate was added to enzyme solutions and the sample was then applied to a Phenyl Sepharose column (1 x 28 cm). The enzyme was eluted with a gradient from 40% to 0% ammonium sulfate in TMZP buffer over 5 column volumes. Enzyme purity was assessed at each step by SDS polyacrylamide gel electrophoresis. Protein concentration was determined by the Bio-Rad protein assay using wild-type alkaline phosphatase as the standard.

Determining alkaline phosphatase activity — Phosphatase activity was measured spectrophotometrically using PNPP as a substrate by monitoring the release of p-nitrophenolate at 410 nm (14). Standard assays were performed by incubating 1.5 mL buffered PNPP solutions at 25º C then starting the reaction with 25 µl enzyme solution. The sum of the hydrolysis and transphosphorylation rates were measured by using 1.0 M Tris-HCl as the phosphate acceptor. A unit is defined as 1 µmol PNPP hydrolyzed per minute.

Preparation of metal-free apo-alkaline phosphatase — Alkaline phosphatase was rendered metal-free by using a procedure adapted from Dirnbach et al. (15). A 5-10 mL enzyme solution of no more than 1.5 mg/mL protein was dialyzed to 50 mM Tris-HCl, pH 8.0 initially to remove extraneous metals and phosphate. The enzyme solution was then dialyzed twice against 2 M (NH₄)₂SO₄, 1 mM EDTA pH 9.0. Ammonium sulfate was removed by dialysis to 10 mM Tris-HCl pH 9.0, 1 mM EDTA. Finally, enzyme solutions were dialyzed to 100 mM Tris-HCl pH 8.0 to remove EDTA and any remaining ammonium sulfate. All buffers were treated with equilibrated Chelex resin, and all glassware was washed...
with 20% nitric acid, followed by three rinses with deionized water. Metal-free enzyme solutions were confirmed by lack of enzymatic activity and by atomic absorption.

Metals were then added to apo-enzyme solutions of approximately 0.1 mg/mL by incubating with 10 mM metal salt solutions at 25°C for 12 hours. These mixtures were then used directly for activity measurements.

**Metal determination** — The metal content of the wild-type and mutant enzymes was determined using a Perkin Elmer 3100 Atomic Absorption Spectrophotometer. Prior to measurements, enzyme preparations were dialyzed to a 4000-fold excess of metal-free 10 mM Tris-HCl pH 8.0 for 12 hours. Enzyme solutions of approximately 4 mg/mL were aspirated into the flame at a rate of 1 mL/min and compared with solutions of metal salt standards at known concentrations.

**Isothermal titration calorimetry** — All thermodynamic data were collected on a VP-ITC titration microcalorimeter (MicroCal, Inc., Northampton, Massachusetts). Proteins were purified, concentrated and treated to remove metals. Protein solutions were then diluted to 0.019 - 0.01 mM in metal-free buffer. Metal solutions were in the range of 0.75 to 6 mM, depending on the amount needed for enzyme saturation. All solutions were prepared in 0.1 M Tris-HCl pH 8.5, and degassed prior to use. The reaction cell of 1.34 mL was completely filled with protein solution, and the reference cell was filled with deionized water. Proteins were titrated with 17 to 130-fold excess of metal ligand. All titrations took place at 25°C, with 30 injections of 10 µl with a 20 second duration, followed by 4-5 minute intervals between injections. Titrations of both ZnCl₂ and CoCl₂ into buffer produced negligible enthalpy changes, and were disregarded. Data analysis was carried out with the ORIGIN
software from MicroCal.
RESULTS

Steady state kinetics of the wild-type and mutant alkaline phosphatases — Wild-type E. coli alkaline phosphatase is optimally active in the presence of Zn$^{2+}$ and Mg$^{2+}$ at pH 8.0. Therefore, the wild-type and mutant enzymes were initially compared under these conditions (Table II). For these experiments, the enzymes were not treated to remove metals, and no metals were added other than the Zn$^{2+}$ and Mg$^{2+}$ included in the purification buffer.

The enzymes were characterized in conditions where the sum of the hydrolysis and transphosphorylation rates are observed (1). Under these conditions, the mutant enzymes catalyze the reaction at a much slower rate than the wild-type enzyme, the slowest being the D153H/K328W enzyme with a $k_{cat}$ of 8.0 s$^{-1}$. The PNPP $K_m$ for the wild-type and D153H enzymes are comparable, but the $K_m$ values for both the K328W and D153H/K328W enzymes are increased significantly.

Apo-enzyme activity and metal content — The apo-D153H, apo-K328W and apo-D153H/K328W enzymes had no detectable activity, and the apo-wild-type enzyme retained less than 0.05% of full activity. Based upon atomic absorption measurements, the enzyme preparations had a zinc content of <0.006 zinc atoms per active site.

Enzymatic activity in the presence of metals — The wild-type and mutant enzymes were treated to remove all metals, then incubated with a variety of metal salts and analyzed to determine the activity. The only metal ions that restored significant activity to the enzymes
were Zn$^{2+}$, Mg$^{2+}$, Co$^{2+}$ and Ca$^{2+}$. Other metal ions tested were Ni$^{2+}$, K$^+$, Na$^+$, Cu$^{2+}$, and Mn$^{2+}$. The wild-type and D153H enzymes were most active in the presence of Zn$^{2+}$ and Mg$^{2+}$. The K328W enzyme attained the highest activity in the presence of Zn$^{2+}$ and Co$^{2+}$ alone, or these in combination with Mg$^{2+}$. The D153H/K328W enzyme showed approximately equal activity in Co$^{2+}$ and Zn$^{2+}$, but was most active in combinations of these metals with Mg$^{2+}$. Based upon these results, the enzymes were further tested with Zn$^{2+}$, Mg$^{2+}$ and Co$^{2+}$.

*Kinetic characterization at optimum metal content and pH* — Each of the enzymes was characterized kinetically at the pH and metal conditions for optimum activity (Table III). The wild-type and D153H enzymes were analyzed in the presence of Zn$^{2+}$ and Mg$^{2+}$, at pH 8.0 and pH 10.0, respectively. Both the K328W and D153H/K328W enzymes were active in the presence of Co$^{2+}$ and Mg$^{2+}$, and Zn$^{2+}$ and Mg$^{2+}$, so kinetic analyses were performed with both sets of metals. Kinetic parameters were determined with apo-enzymes that had specific metals reintroduced. The wild-type alkaline phosphatase showed only slightly different kinetic values from those obtained with the unaltered enzyme. The D153H enzyme has a lower $k_{cat}$ than the wild-type enzyme under these conditions, and also shows an increased $K_m$. The Co$^{2+}$, Mg$^{2+}$ K328W enzyme has the highest activity at pH 10.0, with a $k_{cat}$ of 31.2 s$^{-1}$ compared to a $k_{cat}$ of 167.5 s$^{-1}$ for the Zn$^{2+}$ Mg$^{2+}$ K328W enzyme. For the D153H/K328W enzyme, the $K_m$ values are comparable to the K328W enzyme. However, the
D153H/K328W enzyme has a $k_{cat}$ five-fold higher in the presence of $\text{Co}^{2+}$ ($43.7 \text{ s}^{-1}$) as compared to the $k_{cat}$ in the presence of $\text{Zn}^{2+}$ ($8.9 \text{ s}^{-1}$). These enzymes were also analyzed under hydrolysis only conditions. In all cases, both the $k_{cat}$ and $K_m$ decreased, resulting in more efficient but slower enzymes (data not shown).

Importance of a Trp at E. coli alkaline phosphatase position 328 for activity in the presence of cobalt — To determine if the presence of a Trp is necessary for the enzyme to utilize $\text{Co}^{2+}$ as well as $\text{Zn}^{2+}$, or if the absence of a Lys allows the enzyme to utilize $\text{Co}^{2+}$, the K328A enzyme (9) was purified and assessed as to the metals that are required for highest activity. The kinetic measurements were performed under conditions measuring the sum of the hydrolysis and transphosphorylation rates with 1 mM PNPP, using apo-K328A enzyme that had metals added individually or in combination. $\text{Zn}^{2+}$ alone yielded the highest specific activity ($45.4 \pm 2.5 \text{ U/mg}$). Also rendering the enzyme active were the combinations $\text{Zn}^{2+}$ and $\text{Mg}^{2+}$, ($32.9 \pm 2.7 \text{ U/mg}$), and $\text{Co}^{2+}$ and $\text{Mg}^{2+}$ ($11.8 \pm 0.2 \text{ U/mg}$). The presence of $\text{Co}^{2+}$ and $\text{Mg}^{2+}$ also yielded activity, $7.5 \pm 0.9 \text{ U/mg}$ and $1.4 \pm 0.2 \text{ U/mg}$ respectively.

Metal content of wild-type, K328W and D153H/K328W enzymes — Atomic absorption measurements of the wild-type, K328W and D153H/K328W enzymes were performed to determine the cobalt content of each enzyme. Enzyme solutions were incubated with CoCl$_2$, dialyzed, then tested to determine residual cobalt content. The wild-type enzyme had 1.3 cobalt ions per active site. The K328W enzyme was very similar, with 1.4 cobalt ions per active site. The D153H/K328W enzyme also had 1.3 cobalt ions per active site.
site. Activity measurements showed that after dialysis, the mutant enzymes retained 70% of the original activity.

Isothermal titration calorimetry — Isothermal titration calorimetry was performed on the apo-wild-type, the apo-K328W and the apo-D153H/K328W enzymes to investigate the nature of Zn\(^{2+}\) and Co\(^{2+}\) binding. Due to the mix of endothermic and exothermic reactions shown in many of the isotherms, the data were difficult to fit. Therefore, thermodynamic parameters were not calculated. However, observed interactions and approximate dissociation constants were used to analyze metal binding.

The apo-wild-type enzyme exhibits at least two Zn\(^{2+}\) binding sites with differing affinities, the binding at each site results in an exothermic reaction (Fig. 2). The model for two classes of binding sites was the best fit to the data. The higher affinity sites have a \(K_d\) of 10-20 nM, and the weaker affinity site has a \(K_d\) of 600-750 nM. When Co\(^{2+}\) is added to the apo-wild-type enzyme, the reaction is mainly exothermic. However, in the mid-range of the titration energy changes consisting of two phases, an exothermic process with a fast relaxation rate followed by an endothermic process with a slower relaxation rate are observed (Fig. 2).

For the apo-K328W enzyme, Zn\(^{2+}\) binding is exothermic for the tight binding site, and endothermic for the weaker binding site (Fig. 3). Dissociation constants of 100-200 nM, and 200-300 nM were obtained from fitting the model for two classes of binding sites to the data. The binding of Co\(^{2+}\) to the apo-K328W enzyme has more endothermic character than the binding of Zn\(^{2+}\) to the apo-K328W enzyme, with endothermic and exothermic processes
overlapping in the second half of the titration (Fig. 3). The model for two classes of binding sites was fit to the data, and resulted in $K_d$ values of 2.2-2.8 µM, and 8.0-8.5 µM, respectively.

The Zn$^{2+}$ titration of the apo-D153H/K328W enzyme also exhibits two phases, one purely exothermic and the second, similar in magnitude to the first, mainly endothermic (Fig. 4). In mid-titration, the energy changes were first endothermic with a fast relaxation rate then exothermic, with a slower relaxation rate. The model for two classes of binding sites was fit to these data, and resulted in dissociation constants of 100-150 nM and 200-260 nM respectively. For the apo-D153H/K328W enzyme, the binding of Co$^{2+}$ is significantly different as compared to the binding of Zn$^{2+}$ in that Co$^{2+}$ binds to the apo-D153H/K328W enzyme with almost no endothermic character (Fig. 4). The model for two classes of binding sites was also the best fit to these data, with dissociation constants of 3.8-4.5 µM, and 8.0-8.3 µM, respectively.
A comparison of several alkaline phosphatase amino acid sequences reveals a trend linking the nature of certain active site residues, and the metal required for catalysis (Table I). In the *E. coli* enzyme, residues 153 and 328 are Asp and Lys respectively. This enzyme requires two Zn$^{2+}$ ions and one Mg$^{2+}$ ion per active site for activity (Fig. 1) (16). Removing these metals and replacing them with either Co$^{2+}$ alone or Co$^{2+}$ in combination with Mg$^{2+}$ results in greatly diminished activity. Alternatively, enzymes with a His and a Trp at *E. coli* positions 153 and 328 respectively have higher activity with Co$^{2+}$, as compared to Zn$^{2+}$ (11,12). Specifically, the *T. maritima* enzyme, which contains the His, Trp combination, has been reported to be most active in the presence of Co$^{2+}$, or Co$^{2+}$ and Mg$^{2+}$ combinations, and is even inhibited by high concentrations of Zn$^{2+}$ (11).

The roles of the Asp-153 and Lys-328 residues in wild-type and mutant versions of *E. coli* alkaline phosphatase have been elucidated through kinetic and crystallographic studies. Asp-153 binds the Mg through two water molecules, and forms a salt-bridge to Lys-328 in the wild-type *E. coli* enzyme (8,16). Lys-328 binds the phosphate through a water molecule (9). Through these interactions, Asp-153 and Lys-328 participate in the charge and structure of the active site (Fig. 1).

Zinc and cobalt share some similar properties, but exhibit differences that might allow enzymes to discriminate between the two. Cobalt is slightly smaller than zinc, having an ionic radius of 1.25 Å as compared to a zinc ionic radius of 1.38 Å. There is also a slight
difference in pKₐ values; cobalt has a pKₐ of 9.6 as opposed to a zinc pKₐ of 9.0 (17).

Although both metals can adopt either a tetrahedral or octahedral configuration, zinc more readily adopts a tetrahedral configuration, and cobalt more readily adopts an octahedral configuration (18).

**Catalysis** — The wild-type enzyme only shows significant activity with Zn²⁺; with the combination of Zn²⁺ and Mg²⁺ yielding the highest activity. Incubating the apo-enzyme with Co²⁺ alone or Co²⁺ in combination with Mg²⁺ yields an enzyme with at best 10% of the activity seen in the Zn²⁺-Mg²⁺-wild-type enzyme. The D153H enzyme has optimal activity with the same metals as the wild-type enzyme, but requires more Mg²⁺ for maximal activity, as was shown in previous studies (5). Even though this indirect ligand to the Mg site has been altered, the D153H enzyme still remains a Zn²⁺-Mg²⁺ requiring enzyme. The single change from an Asp to a His at position 153 alone does not allow the *E. coli* enzyme to utilize Co²⁺.

The K328W enzyme, unlike the wild-type and the D153H enzymes can catalyze the dephosphorylation of PNPP with either Co²⁺ or Zn²⁺. However, the reaction is not as efficient with Co²⁺ as with Zn²⁺, and the Kₘ is greatly increased for the Co²⁺-K328W enzyme. The poor Kₘ value might be due to a loss of interactions with the substrate, or with the Asp-153. In previous crystallographic studies on the K328H enzyme, interactions between the phosphate and the residue at position 328 were compromised (9). Also, the change from a positive Lys to a hydrophobic Trp is drastic, and must change the electrostatics
and perhaps the geometry of the active site.

The D153H/K328W enzyme shows equal activity with Zn$^{2+}$ and Co$^{2+}$ under many of the conditions tested. However, under optimal conditions, the D153H/K328W enzyme is a better catalyst with Co$^{2+}$ than with Zn$^{2+}$. Like the K328W enzyme, the $K_m$ value is very poor. For the reasons listed above, interactions with the phosphate could be compromised by the changes.

*Metal binding* — The interaction of the apo-wild-type enzyme with Zn$^{2+}$ shows at least two, possibly three, classes of Zn$^{2+}$ binding sites with different affinities (Fig. 2). When Zn$^{2+}$ binds to the apo-wild-type enzyme, all interactions are exothermic in nature, suggesting highly favorable bond formations, with little reorganization at the active site (19). When this enzyme is titrated with Co$^{2+}$, there is some endothermic character to the metal-protein interactions at the mid-point in the titration. Also, the wild-type enzyme has a much lower affinity for Co$^{2+}$. These results indicate that Co$^{2+}$ is a poor ligand for the wild-type enzyme, and that the Co$^{2+}$ binding occurs in a different manner than Zn$^{2+}$ binding.

Initially, Zn$^{2+}$ binds exothermically to high affinity sites on the apo-K328W enzyme, indicating a process both entropically and enthalpically driven. At the weaker binding site, the binding changes to mostly endothermic, showing that the later binding process is driven mainly entropically (Fig. 3) (20). The entropic contribution to metal binding could be due to the loss of water from the Zn$^{2+}$ coordination sphere, or more likely, the release of structured water molecules from surrounding polar or non polar residues due to a rearrangement (21).
The binding of Co$^{2+}$ to the high affinity sites on the apo-K328W enzyme is exothermic. While the binding of Co$^{2+}$ to the low affinity sites is a mixture of exothermic with a rapid relaxation rate, followed by endothermic with a much slower relaxation rate (Fig. 3). The more pronounced endothermic character suggests that a reorganization of the binding site may be necessary to bind Co$^{2+}$ to the apo-K328W enzyme, and may explain the lower affinity of the apo-K328W enzyme for Co$^{2+}$ as compared to the affinity of the apo-wild-type enzyme for Co$^{2+}$. However, a rearrangement could result in an active site conformation better able to utilize Co$^{2+}$ for catalysis.

The apo-D153H/K328W enzyme initially binds Zn$^{2+}$ exothermically, then in the mid-range of the titration, metal binding has a large endothermic contribution (Fig. 4). Again, the large endothermic part could represent a change in Zn$^{2+}$ solvation or an active site reorganization upon binding. In this case, the endothermic character is much more pronounced, indicating that a large change is necessary to accommodate Zn$^{2+}$ at the active site. When the apo-D153H/K328W enzyme is titrated with Co$^{2+}$, there is very little endothermic character to the binding of the metal ion. Even though the binding constants are poor for the Co$^{2+}$-D153H/K328W enzyme, the geometry of the active site may be more suitable for Co$^{2+}$.

**Conclusions** — These data indicate that very small differences in the active site of *E. coli* alkaline phosphatase are necessary for the enzyme to utilize Co$^{2+}$ for catalysis instead of
Zn\textsuperscript{2+}. Although the K\textsubscript{m} values for the K328W and D153H/K328W enzymes are greatly increased, the k\textsubscript{cat} values indicate that catalysis does occur in these enzymes to a much greater extent than in the wild-type enzyme. Isothermal titration data also suggests that in the D153H/K328W enzyme there is much less rearrangement when Co\textsuperscript{2+} binds to the enzyme, as is the case for Zn\textsuperscript{2+} binding the wild-type enzyme. For Co\textsuperscript{2+} to be most effective in the \textit{E. coli} alkaline phosphatase active site, both a His and a Trp must be in positions 153 and 328, respectively. In order to better understand the structural basis of the isothermal titration calorimetry data, crystallographic studies are in progress to determine the alteration of the metal sites upon Co\textsuperscript{2+} or Zn\textsuperscript{2+} binding to the D153H/K328W enzyme.
ACKNOWLEDGEMENTS

We thank Professor J. E. Billo, R. R. Boulanger and J. P. Cardia, for helpful discussions. In addition, we are grateful to Dr. K. Metz for instruction on atomic absorption, and Dr. P. Phelan for instruction on isothermal titration calorimetry. We also thank Dr. J. Sun for the use of the VP-ITC and advice regarding the experiments.
REFERENCES


Brooks/Cole Publishing Company, Belmont, CA


Hickey, E. K., Peterson, J. D., Nelson, W. C., Ketchum, K. A., McDonald, L.,
Utterback, T. R., Malek, J. A., Linher, K. D., Garrett, M. M., Stewart, A. M., Cotton,
M. D., Pratt, M. S., Phillips, C. A., Richardson, D., Heidelberg, J., Sutton, G. G.,
159, 47-58
TABLE I

A Comparison of Amino Acids at the Positions that Correspond to Metal Ligands in E. coli

Alkaline Phosphatase.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Identity to E. coli enzyme</th>
<th>Residues near the Mg binding site</th>
<th>Metal</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>100 %</td>
<td>D T E K</td>
<td>Zn, Mg</td>
<td>(16)</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>29.9%</td>
<td>H T E W</td>
<td>NDb</td>
<td>(22)</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>29.5%</td>
<td>H T E W</td>
<td>ND</td>
<td>AAG33874c</td>
</tr>
<tr>
<td>B. subtilis phoAIV</td>
<td>29.4%</td>
<td>H T E W</td>
<td>Co</td>
<td>(12)</td>
</tr>
<tr>
<td>S. aureus</td>
<td>29.3%</td>
<td>D T E K</td>
<td>ND</td>
<td>(23)</td>
</tr>
<tr>
<td>B. halodurans</td>
<td>28.3%</td>
<td>H T E W</td>
<td>ND</td>
<td>(24)</td>
</tr>
<tr>
<td>T. maritima</td>
<td>27.6%</td>
<td>H T E W</td>
<td>Co, Mg</td>
<td>(25)</td>
</tr>
<tr>
<td>TAB5</td>
<td>27.6%</td>
<td>H T E W</td>
<td>Mg</td>
<td>(10)</td>
</tr>
<tr>
<td>B. subtilis phoAIII</td>
<td>27.4%</td>
<td>H T E W</td>
<td>Co</td>
<td>(12)</td>
</tr>
<tr>
<td>H. sapiens</td>
<td>26.5%</td>
<td>H T E H</td>
<td>Zn, Mg</td>
<td>XM044139.3c</td>
</tr>
<tr>
<td>D. melanogaster</td>
<td>24.8%</td>
<td>H T E H</td>
<td>ND</td>
<td>AE003565.2c</td>
</tr>
<tr>
<td>P. abyssi</td>
<td>24.4%</td>
<td>H T E H</td>
<td>Mg, Co</td>
<td>(26)</td>
</tr>
<tr>
<td>Vibrio sp.</td>
<td>23.2%</td>
<td>H T E W</td>
<td>ND</td>
<td>(27)</td>
</tr>
<tr>
<td>B. licheniformis</td>
<td>22.9%</td>
<td>H T E W</td>
<td>ND</td>
<td>(28)</td>
</tr>
</tbody>
</table>

a Sequences were aligned using ClustalW

b ND, not determined

c NCBI accession number
### TABLE II

**Kinetic Parameters of the Wild-type and Mutant Alkaline Phosphatases at pH 8.0, in the Presence of Zn$^{2+}$ and Mg$^{2+}$**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{\text{cat}}$ $^b$</th>
<th>$K_m$</th>
<th>$k_{\text{cat}}/K_m$ (x 10^{-6})</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>$175.5 \pm 5.0$</td>
<td>$34.9 \pm 5.1$</td>
<td>5.0</td>
<td>$1.0$ M Tris</td>
</tr>
<tr>
<td>D153H</td>
<td>$36.7 \pm 1.0$</td>
<td>$33.3 \pm 3.9$</td>
<td>1.1</td>
<td>$1.0$ M Tris</td>
</tr>
<tr>
<td>K328W</td>
<td>$40.6 \pm 1.2$</td>
<td>$310.2 \pm 29.2$</td>
<td>0.1</td>
<td>$1.0$ M Tris</td>
</tr>
<tr>
<td>D153H/K328W</td>
<td>$8.0 \pm 0.2$</td>
<td>$198.5 \pm 22.6$</td>
<td>0.04</td>
<td>$1.0$ M Tris</td>
</tr>
</tbody>
</table>

$^a$ Enzymes were prepared in TMZP buffer, and kinetic constants were determined at pH 8.0, 25°C. Assays were performed in the buffer indicated with PNPP as substrate.

$^b$ The $k_{\text{cat}}$ values were calculated from $V_{\text{max}}$ values obtained by using a dimer molecular mass of 94,000 Da.
### TABLE III

**Kinetic Parameters of the Wild-type and Mutant Alkaline Phosphatases at Their Optimal Conditions**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{\text{cat}}$ $^{b}$ (s$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$k_{\text{cat}}/K_m$ (x 10$^{-6}$) (M$^{-1}$ s$^{-1}$)</th>
<th>Metal</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>167.5 ± 6.0</td>
<td>32.7 ± 5.3</td>
<td>5.1</td>
<td>Zn, Mg</td>
<td>8.0</td>
</tr>
<tr>
<td>Wild-type$^{c}$</td>
<td>0.3 ± 0.5</td>
<td>272 ± 29</td>
<td>0.001</td>
<td>Co</td>
<td>10.0</td>
</tr>
<tr>
<td>D153H</td>
<td>47.4 ± 2.7</td>
<td>730 ± 90</td>
<td>0.06</td>
<td>Zn, Mg</td>
<td>10.0</td>
</tr>
<tr>
<td>K328W</td>
<td>167.5 ± 15.6</td>
<td>2900 ± 232</td>
<td>0.05</td>
<td>Zn, Mg</td>
<td>10.0</td>
</tr>
<tr>
<td>K328W</td>
<td>31.2 ± 4.5</td>
<td>4000 ± 300</td>
<td>0.008</td>
<td>Co, Mg</td>
<td>10.0</td>
</tr>
<tr>
<td>D153H/K328W</td>
<td>8.9 ± 0.5</td>
<td>1600 ± 116</td>
<td>0.005</td>
<td>Zn, Mg</td>
<td>10.0</td>
</tr>
<tr>
<td>D153H/K328W</td>
<td>43.7 ± 1.3</td>
<td>1940 ± 140</td>
<td>0.02</td>
<td>Co, Mg</td>
<td>10.0</td>
</tr>
</tbody>
</table>

---

$^{a}$ Reactions were performed in 1.0 M Tris-HCl at the indicated pH at 25°C.

$^{b}$ The $k_{\text{cat}}$ values were calculated from $V_{\text{max}}$ values obtained by using a dimer molecular mass of 94,000 Da.

$^{c}$ The data for the wild-type enzyme in the presence of cobalt is provide for comparison.
FIG. LEGENDS

FIG. 1. **Wild-type* E. coli alkaline phosphatase active site showing the residues Asp-153 and Lys-328 and their interactions.** Coordinates were taken from the protein data bank file 1ED8 (29), and drawn using the program SETOR (30). Water molecules are shown as small spheres, and metal ions are shown as large spheres as indicated.

FIG. 2. **Isothermal titration calorimetric analyses of Zn\(^{2+}\) (top panel) and Co\(^{2+}\) (bottom panel) binding to wild-type alkaline phosphatase.** An exothermic interaction is shown by a negative energy change and an endothermic interaction is shown by a positive energy change. See Experimental Procedures for details. Note that in the midsection of the titration shown in the bottom panel, the endothermic process has a longer relaxation time than the exothermic process.

FIG. 3. **Isothermal titration calorimetry analyses of Zn\(^{2+}\) (top panel) and Co\(^{2+}\) (bottom panel) binding to K328W alkaline phosphatase, as described in Fig. 2.** Note that in the second half of the titration shown in the bottom panel, the endothermic process has a longer relaxation time than the exothermic process.

FIG. 4. **Isothermal titration calorimetry analyses of Zn\(^{2+}\) (top panel) and Co\(^{2+}\) (bottom panel) binding to D153E/K328W alkaline phosphatase, as described in Fig. 2.** Note that in the second half of the titration shown in the top panel, the exothermic process has a longer relaxation time than the endothermic process.
Altering of the metal specificity of Escherichia coli alkaline phosphatase
Cheryl L. Wojciechowski and Evan R. Kantrowitz

J. Biol. Chem. published online October 23, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M209326200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2002/10/23/jbc.M209326200.citation.full.html#ref-list-1