Binding of Magnesium in a Mutant *Escherichia coli* Alkaline Phosphatase Changes the Rate-Determining Step in the Reaction Mechanism†

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ABSTRACT: To investigate the role of magnesium at the M3 site in *Escherichia coli* alkaline phosphatase, site-specific mutagenesis was used to substitute Glu-322, a ligand of the Mg^{2+} with either aspartic acid (E322D) or alanine (E322A). The residual Mg^{2+} content of the E322D enzyme is about 16-fold lower than that of the wild-type enzyme, and both mutant enzymes exhibit extremely poor catalytic activity compared to the wild-type enzyme. Mg^{2+} is a strong activator of the E322D enzyme. The hydrolysis activity of the E322D enzyme maximally stimulated by Mg^{2+} is 60% of that of the wild-type enzyme. Under conditions that measure the sum of hydrolysis and transphosphorylation activities, the k_{cat} of the E322D enzyme in the presence of 500 mM Mg^{2+} is 2.6-fold higher than the k_{cat} observed for the wild-type enzyme. Zn^{2+} also activates the E322D enzyme, although it is not as strong an activator as Mg^{2+}. Competition experiments suggest that the activation of the E322D enzyme by Mg^{2+} and Zn^{2+} results from binding of either of these metals to the same site on the enzyme. High concentrations of the substrate *p*-nitrophenyl phosphate inhibit the activity of the E322D enzyme; however, high concentrations of Mg^{2+} can overcome this inhibition. Stopped-flow experiments indicate that the rate-limiting step of the nonstimulated E322D enzyme at pH 8.0 differs from that of the wild-type enzyme and involves the breaking of the covalent bond between the enzyme and phosphate. However, the rate-limiting step exhibited by the Mg^{2+}-stimulated enzyme is identical to that of the wild-type enzyme and involves the release of the noncovalently bound phosphate. Therefore, Mg^{2+} induces a change in the rate-limiting step of the E322D enzyme. These studies suggest that Mg^{2+} at the M3 site of *E. coli* alkaline phosphatase directly influences the catalytic mechanism of the enzyme.

Alkaline phosphatase catalyzes both the nonspecific hydrolysis of phosphate monoesters and a transphosphorylation reaction in which phosphate is transferred to an alcohol (Wilson et al., 1964). The enzyme is maximally active at alkaline pH, and the reaction proceeds through a phosphoenzyme intermediate. Kinetic studies on *Escherichia coli* alkaline phosphatase (EC 3.1.3.1) indicate that the rate-determining step of the enzymatic reaction is pH-dependent; at acidic pH, the hydrolysis of the covalent enzyme–phosphate complex (E–P) is rate-limiting, while under basic conditions the rate-limiting step becomes the release of phosphate from the noncovalent enzyme phosphate–complex (E·P_i) (Reid & Wilson, 1971; Hull et al., 1976; Bloch & Gorby, 1980; Gettins et al., 1985; Kim & Wyckoff, 1991).

E. coli alkaline phosphatase is a dimeric metalloenzyme. In each monomer, there are three distinctive metal binding sites. The isolated native enzyme contains 2–4 mol of zinc and 1–2 mol of magnesium/mol of enzyme dimer (Plocke et al., 1962; Applebury & Coleman, 1969; Anderson et al., 1975; Bosron et al., 1977; Coleman et al., 1983). Considerable variations in the zinc and magnesium content have been observed among enzymes purified by different methods and stored under different conditions. The X-ray crystallographic data indicate that these three metal binding sites, defined as M1, M2, and M3, are located in the active site of the enzyme (Wyckoff et al., 1983; Kim & Wyckoff, 1989, 1991). NMR, equilibrium dialysis, and crystallographic studies suggest that Mn^{2+}, Co^{2+}, Cd^{2+}, and Cu^{2+} can also bind to these metal sites; however, only the Co^{2+} derivative has significant enzymatic activity (Bosron et al., 1977; Spiro, 1983).

Early studies on the reactivation of the apoalkaline phosphatase by metal ions (Plocke et al., 1962; Anderson et al., 1975; Bosron et al., 1977; Simpson & Vallee, 1968; Harris & Coleman, 1968) indicated that at least two zinc atoms per mole of enzyme dimer are required to restore enzymatic activity. NMR and crystallographic studies subsequently suggested that the Zn^{2+} at the M1 site is directly involved in both catalysis and phosphate binding (Gettins & Coleman, 1983b, 1984; Kim & Wyckoff, 1991). The function of the Zn^{2+} atom at the M1 site in catalysis was also investigated by a site-specific mutagenesis study. In particular, Asp-327, a bidentate ligand of the Zn^{2+} at the M1 site, was replaced by alanine and asparagine (Xu & Kantrowitz, 1992). It has been proposed that the Zn^{2+} at the M1 site coordinates a water molecule that exists as hydroxide at high pH. This hydroxide ion then acts as the nucleophile attacking the phosphoserine in the phosphoenzyme intermediate (Coleman & Gettins, 1983; Gettins et al., 1985; Sowadski et al., 1985).

The role of magnesium differs depending upon the source of the alkaline phosphatase. For some alkaline phosphatases, such as enzyme from bovine brain (Brunel & Cathala, 1973), bovine kidney (Cathala & Brunel, 1975), and human liver/bone/kidney (Butterworth, 1968), Mg^{2+} acts as a strong activator. However, Mg^{2+} has no effect or a weak effect on the activity of human placental (Harkness, 1968) and calf intestine alkaline phosphatases (Chappelet-Tordo et al., 1974). The X-ray structure of the *E. coli* enzyme indicates that the binding site of the Mg^{2+} is located in the active site and is 5 and 7 Å away from the two zinc atoms. Mg^{2+} coordination in the enzyme can be described as a slightly disordered octahedron with ligands from the oxygens of Asp-51 and Glu-322, the hydroxyl of Thr-155, and three water molecules. A

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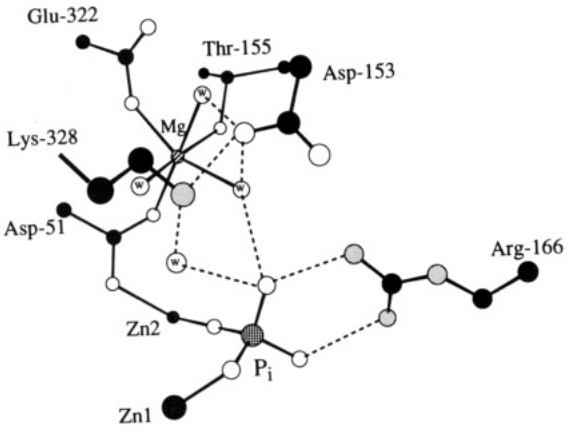

FIGURE 1: Region of the active site of *E. coli* alkaline phosphatase near the phosphate and Mg^{2+} binding sites. Mg^{2+} at the M3 site exhibits octahedral coordination. Ligands to the Mg^{2+} include the carboxyls of Glu-322 and Asp-51, the hydroxyl of Thr-155, and three water molecules. One of these water molecules also participates in a water-mediated interaction with the phosphate. Asp-153 forms a salt-link with Lys-328 and participates in the interactions with two water molecules that are ligands to the Mg^{2+}. The phosphate interacts with the guanidinium group of Arg-166 and forms specific interactions with both the zinc at the M1 site (Zn1) and the zinc at the M2 site (Zn2). The carboxylate of Asp-51, which is a ligand to the Mg^{2+}, is also a ligand to the zinc at the M2 site. For clarity, the other ligands to the zinc atoms at the M1 and M2 sites are not shown in this figure. The data used for this figure were provided by Kim and Wyckoff (1991).

water-mediated interaction between the Mg^{2+} and the phosphate is also observed in the X-ray structure (Kim & Wyckoff, 1991) (see Figure 1). In *E. coli* alkaline phosphatase, Mg^{2+} is thought to have a regulatory effect on the activity of the enzyme which contains 2 or 4 mol of zinc per enzyme dimer, and may also be involved in structural stabilization (Anderson et al., 1976). Studies on the function of Asp-153 in *E. coli* alkaline phosphatase have been performed by site-specific mutagenesis (Janeway et al., 1993; Matlin et al., 1992); Asp-153 is hydrogen-bonded to two water molecules that coordinate Mg^{2+} and therefore is an indirect ligand for the Mg^{2+}. The mutant version of the enzyme in which Asp-153 is replaced by histidine (D153H) has a very low residual Mg^{2+} content, and Mg^{2+} strongly enhances the activity of the mutant enzyme. Detailed kinetic studies on the D153H enzyme suggested that both the Mg^{2+}-stimulated and nonstimulated enzymes have a different rate-limiting step than the wild-type enzyme at pH 8.0 (Janeway et al., 1993). These results indicate that the Mg^{2+} in *E. coli* alkaline phosphatase is important both for catalysis and for stabilizing the active conformation of the enzyme. In order to investigate in more detail the function of the Mg^{2+} in catalysis, we have used site-specific mutagenesis to determine the role of Glu-322, a ligand of the Mg^{2+} at the M3 site.

MATERIALS AND METHODS

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.

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

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 from H. Inouye. The strain CJ236 [*dut-1*, *ung-1*, *thi-1*, *relA-1*/pCJ105 (Cm^r)] was a gift from T. Kunkel.

Expression of Wild-Type and Mutant Alkaline Phosphatases. SM547 was used as the host strain for expression of both the wild-type and mutant alkaline phosphatases. This strain has the *phoA* gene deleted from the chromosome as well as a mutation in the *phoR* regulatory gene. Therefore, if a *phoA* containing plasmid is introduced into this strain, the alkaline phosphatase produced will be exclusively from the *phoA* gene on the plasmid.

Purification of the Wild-Type and Mutant Alkaline Phosphatases. The wild-type, the E322D,[1] and the E322A enzymes were isolated from the plasmid/strain combinations pEK48/SM547, pEK174/SM547, and pEK133/SM547, respectively, by the method previously described (Chaidaroglou et al., 1988). Enzyme purity was judged by SDS–polyacrylamide gel electrophoresis, according to the procedure of Laemmli (1970).

Determination of Protein Concentration. The concentration of the wild-type enzyme was determined by absorbance measurements at 278 nm with an extinction coefficient of 0.71 cm^2/mg(Plocke & Vallee, 1962). The concentration of the mutant 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 0.1 M 3-(cyclohexylamino)propanesulfonic acid (CAPS).[2] The ionic strength was held constant at 0.585 with NaCl.

Rapid Kinetic Measurements. Experiments were performed at 25 °C using a KinTek Inc. stopped-flow spectrophotometer 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 the wild-type enzyme or the E322D enzyme with concentrations ranging from 0.02 to 0.45 mg/mL in TMZP buffer. The other syringe contained 1 mM *p*-nitrophenyl phosphate in 0.1 M MOPS,[2] pH 8.2. The

[1] The notation used to name the mutant enzymes is as follows: E322D, the mutant version of alkaline phosphatase in which Glu-322 is replaced by aspartic acid; E322A, the mutant version of alkaline phosphatase in which Glu-322 is replaced by alanine.

[2] Abbreviations: CAPS, 3-(cyclohexylamino)propanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; P_i, inorganic phosphate; TMZP buffer, 10^{-2} M Tris-HCl, 10^{-3} M $MgCl_2$, 10^{-4} M NaH_2PO_4, 3.1 × 10^{-3} M NaN_3, and 10^{-5} M $ZnSO_4$, pH 7.4 (Block & Beckar, 1978).

Table I: Kinetic Parameters of the Wild-Type and Mutant Enzymes in the Absence and Presence of a Phosphate Acceptor[a]

enzyme	k_{cat}[b] (s^{-1})	K_m (μM)	$k_{cat}/K_m \times 10^{-6}$ (M^{-1} s^{-1})	buffer
		(A) At pH 8.0		
wild-type	42	4.0	11	0.01 M Tris[c]
E322D	<0.08	[d]	[d]	0.01 M Tris
E322A	<0.08	[d]	[d]	0.01 M Tris
wild-type	84	13	6.6	1.0 M Tris
E322D	0.9[e]	26	0.02	1.0 M Tris
E322A	0.2	180	0.001	1.0 M Tris
		(B) At pH 10		
wild-type	68	104	0.7	0.1 M CAPS[c]
E322D	0.7[e]	66	0.01	0.1 M CAPS
E322A	1.5	370	0.004	0.1 M CAPS
wild-type	58	107	0.5	1.0 M Tris
E322D	22[e]	28	0.8	1.0 M Tris
E322A	7.4	870	0.001	1.0 M Tris

[a] Assays were performed at 25 °C in the buffers indicated with use of *p*-nitrophenyl phosphate as substrate. [b] The k_{cat} values are calculated from the V_{max} by use of a dimer molecular weight of 94 000. The k_{cat} per active site would be half of the value indicated. [c] The ionic strength of the buffer was adjusted to 0.585 with NaCl. [d] Values could not be determined because of the extremely low activity of these mutant enzymes. [e] Since substrate inhibition is shown, the V_{max} represents the maximal observed specific activity.

Table II: Effect of Magnesium Concentration and Ionic Strength on the Wild-Type and E322D Alkaline Phosphatases at pH 8.0 in the Absence and Presence of a Phosphate Acceptor[a]

enzyme	k_{cat}[b] (s^{-1})	K_m (μM)	$k_{cat}/K_m \times 10^{-6}$ (M^{-1} s^{-1})	activation[d]	[$MgCl_2$] (mM)
(A) In the Absence of a Phosphate Acceptor (0.01 M Tris,[c] pH 8.0)					
wild-type	47	9.8	4.8	1.1	100
E322D	5.8	2.3	2.5	>70	100
wild-type	44	7.6	5.8	1.1	500
E322D	28	12	2.5	>350	500
(B) In the Presence of a Phosphate Acceptor (1.0 M Tris, pH 8.0)					
wild-type	101	23	4.3	1.2	100
E322D	289	72	4.0	321	100
wild-type	128	46	2.8	1.5	500
E322D	340	134	2.5	378	500
wild-type	124	31	4.1	1.5	1.5 M NaCl
E322D	0.6	33	0.02	0.6	1.5 M NaCl

[a] Assays were performed at 25 °C in the buffers indicated with use of *p*-nitrophenyl phosphate as substrate. [b] The k_{cat} values are calculated from the V_{max} by use of a dimer molecular weight of 94 000. The k_{cat} per active site would be half of the value indicated. For the E322D enzyme, the substrate inhibition effect was exhibited, and therefore the K_{cat} represents the maximal observed specific activity. [c] The ionic strength of the buffer was adjusted to 0.585 with NaCl. [d] Activation of the enzyme in the presence Mg^{2+} compared to the activity measured in the absence of Mg^{2+} in the same buffer.

concentrations of either Mg^{2+} or Zn^{2+} were adjusted as required. The final pH after mixing equal volumes of enzyme and substrate was 8.0.

Magnesium Determination. The magnesium 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 dialyzed (1000: 1) against 10 mM Tris buffer, pH 7.4, with four or five changes of the buffer.

RESULTS

Construction of the E322D and E322A Alkaline Phosphatases by Site-Specific Mutagenesis. The site-specific mutations were introduced as previously described (Chaidaroglou et al., 1988; Xu & Kantrowitz, 1991). The mutations were then recloned into pEK48 (Chaidaroglou et al., 1988) and reconfirmed by sequencing. The plasmids containing the mutant versions of the *phoA* gene at position 322 were named pEK133 and pEK174 for the alanine and aspartic acid substitutions, respectively.

Steady-State Kinetic Parameters of the Mutant Enzymes Are Very Different from the Wild-Type Enzyme at pH 8.0. The steady-state kinetic parameters of the mutant enzymes were first measured under standard conditions (0.01 or 1.0 M Tris buffer, pH 8.0). The hydrolysis activity of the E332D and E322A enzymes determined in 0.01 M Tris buffer pH 8.0 is more than 500-fold lower than the value for the wild-type enzyme (see Table I). The extremely low activities of these mutant enzymes prevented an accurate determination of the k_{cat} and K_m values.

When the activity is measured in 1.0 M Tris buffer, the rate observed is the sum of transphosphorylation and hydrolysis, since Tris serves as a phosphoryl group acceptor (Wilson et al., 1964; Trentham & Gutfreund, 1968). Under these conditions, the k_{cat} value of the wild-type is 83 s^{-1}, about 2-fold greater than the hydrolysis activity alone. Under the same conditions, the E322A and E322D enzymes exhibited more than 3- and 10-fold increases in activity, respectively, compared to the activity observed in the absence of a phosphate acceptor (see Table I).

The Mutant Enzymes Have a Lower Magnesium Affinity than the Wild-Type Alkaline Phosphatase. Since the carboxyl of Glu-322 is one of the six ligands to the Mg^{2+} bound at the M3 site, it is very likely that the binding affinity of the Mg^{2+} will be decreased in the E322D and E322A enzymes. After being dialyzed against 0.01 M Tris buffer, pH 7.4, the Mg^{2+} contents of the wild-type and E332D enzymes were analyzed by atomic absorption spectrophotometry. The wild-type enzyme contains 1.33 ± 0.16 mol of magnesium/mol of enzyme dimer; however, the E322D enzyme has only 0.08 ± 0.01 mol of magnesium/mol of enzyme dimer.

Magnesium Is a Strong Activator of the E322D Enzyme. Mg^{2+} caused a significant increase in the hydrolysis activity of the E322D enzyme. For example, when the activity of the E332D enzyme was determined in the presence of 500 mM Mg^{2+}, there was more than a 350-fold increase in activity, while for the wild-type enzyme the corresponding increase in activity was very slight (see Table IIA). Furthermore, the Mg^{2+} activation of the E322D enzyme was cooperative with a Hill coefficient of 1.8, and an apparent binding constant of approximately 200 mM (see Figure 2A). In order to rule out the increase in ionic strength by Mg^{2+} as the cause for the cooperative Mg^{2+} activation of the E322D enzyme, the influence of ionic strength on the activity of both the wild-type and mutant enzymes was measured. Under the conditions that determine hydrolysis activity, 0.01M Tris buffer, pH 8.0, with 0.85 M NaCl, and in the presence of 100 mM $MgCl_2$, no change in enzymatic activity was observed for either the mutant or the wild-type enzyme when the ionic strength was further increased by adding 1.2 M NaCl to the above buffer.

The influence of Mg^{2+} on the activity of the mutant and wild-type enzymes was also determined under conditions that measure the sum of the transphosphorylation and hydrolysis activities. In the presence of 100 mM $MgCl_2$, the E322D enzyme shows a 2.8-fold increase in k_{cat} compared to the k_{cat} of the wild-type enzyme under the same conditions (see Table IIB). However, in the presence of 500 mM $MgCl_2$, the k_{cat} of the E322D enzyme increased almost 380-fold (see Table IIB) compared to the k_{cat} obtained in the absence of Mg^{2+}. The activity of the Mg^{2+}-stimulated E322D enzyme is 2.7-fold higher than the k_{cat} of the wild-type enzyme under the

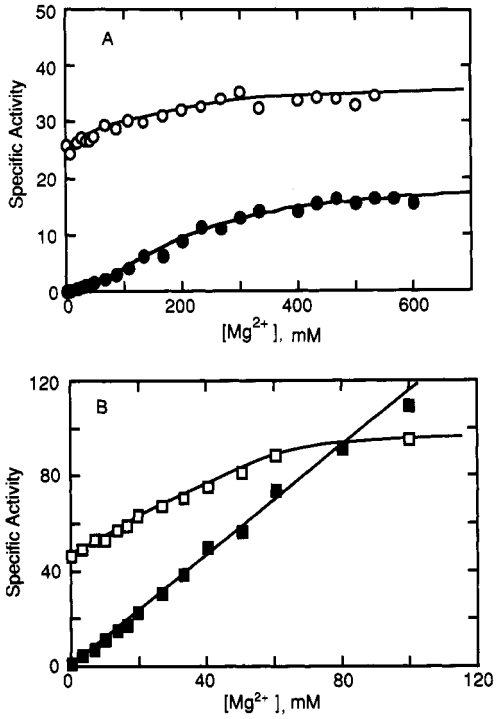

FIGURE 2: Influence of $[Mg^{2+}]$ on the hydrolytic activity. (A) The activities of the wild-type (○) and the E322D enzymes (●) were determined in 0.01 M Tris buffer, pH 8.0, with 0.1 mM *p*-nitrophenyl phosphate as substrate. (B) The activities of the wild-type enzyme (□) and the E322D enzymes (■) were measured in 0.1 M CAPS buffer, pH 10, with 1.5 mM *p*-nitrophenyl phosphate as substrate at 25 °C. Specific activity is reported in micromoles of nitrophenolate formed per minute per milligram of enzyme. The ionic strength of the buffers was adjusted to 0.585 with NaCl.

same conditions. NaCl was used to determine whether the ionic strength was responsible for the dramatic increase in activity observed for the E322D enzyme under these conditions. For the wild-type enzyme, when the activity is measured in the presence of 1.5 M NaCl, which has the same ionic strength as 500 mM $MgCl_2$, the k_{cat} value is about the same as the k_{cat} value obtained with 500 mM $MgCl_2$ (see Table IIB). However, a dramatic difference is observed for the k_{cat} value of the E322D enzyme measured under these two conditions. Also seen in Table II, the K_m values of both the wild-type and E322D enzymes increase in the presence of $MgCl_2$.

Zinc Can Also Activate the E322D Enzyme, but to a Lesser Extent than Magnesium. In the presence of 1 mM Zn^{2+}, the hydrolysis activity of the E322D enzyme increases more than 45-fold compared to the activity measured in the absence of Zn^{2+} (see Table IIIA). When the hydrolysis activity of the E322D enzyme was measured as a function of Zn^{2+} concentration, the enzyme activity increased. At saturating Zn^{2+} (2.3 mM), the activity was more than 50-fold higher than in the absence of Zn^{2+}. The apparent binding constant for the Zn^{2+} activation of the E322D enzyme was approximately 1.0 mM (see Figure 3A).

The influence of Zn^{2+} was also determined under conditions that measure the hydrolysis plus transphosphorylation activities. The k_{cat} value of the E322D enzyme increases from 0.9 s^{-1} (see Table I) with no additional Zn^{2+} to 11.1 and 15.8 s^{-1} with 5 and 10 mM Zn^{2+} (see Table IIIB), respectively.

Do Mg^{2+} and Zn^{2+} Bind to the Same Site on the E322D Enzyme? This question was investigated by determining if there is competition between Mg^{2+} and Zn^{2+} at the M3 site of the E322D enzyme. As seen in Figure 3A, in the absence of magnesium, a simple Zn^{2+} activation curve is observed

Table III: Zinc Concentration Effect on the Wild-Type and E322D Alkaline Phosphatases at pH 8.0 in the Absence and Presence of a Phosphate Acceptor

enzyme	k_{cat}[b] (s^{-1})	K_m (μM)	$k_{cat}/K_m \times 10^{-6}$ (M^{-1} s^{-1})	activation[b]	[$ZnSO_4$] (mM)
(A) In the Absence of a Phosphate Acceptor (0.01 M Tris,[c] pH 8.0)					
wild-type	38	5.4	7.1	0.9	0.5
E322D	2.0	2.4	0.8	>25	0.5
wild-type	31	2.0	15	0.7	1
E322D	3.9	5.8	0.7	>45	1
(B) In the Presence of a Phosphate Acceptor (1.0 M Tris, pH 8.0)					
wild-type	97	30	3.2	1.2	5
E322D	11	98	0.1	12	5
wild-type	90	19	4.7	1.1	10
E322D	16	89	0.2	17	10

[a] Assays were performed at 25 °C in the buffers indicated with use of *p*-nitrophenyl phosphate as substrate. [b] The k_{cat} values are calculated from the V_{max} by use of a dimer molecular weight of 94 000. The k_{cat} per active site would be half of the value indicated. [c] The ionic strength of the buffer was adjusted to 0.585 with NaCl. [d] Activation of the enzyme by Zn^{2+} compared to the activity measured without Zn^{2+} in the same buffer.

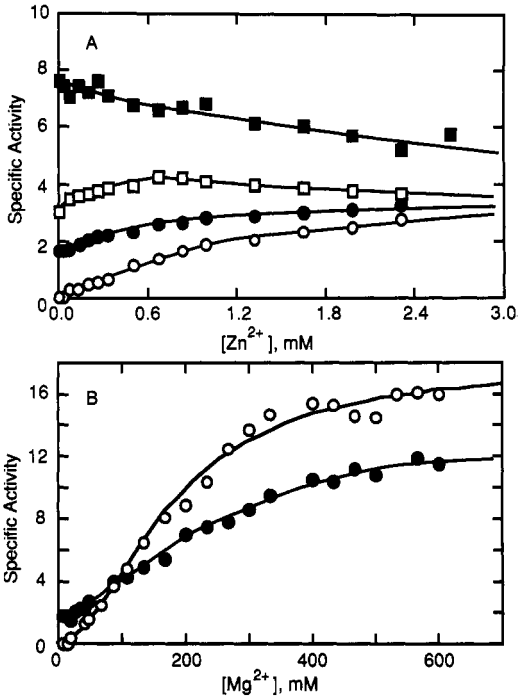

FIGURE 3: Determination of the competition between Mg^{2+} and Zn^{2+}. (A) Experiments were performed at various Zn^{2+} concentrationswith no additional Mg^{2+} (○), 50 mM Mg^{2+} (●), 100 mM Mg^{2+} (□), and 200 mM Mg^{2+} (■). (B) Experiments were performed at various Mg^{2+} concentrations with no additional Zn^{2+} (○) or 0.5 mM Zn^{2+}(●). The reactions were performed at 25 °C in 0.01 M Tris, pH 8.0, buffer with 0.05 mM *p*-nitrophenyl phosphate as subtrate in the reactions. Specific activity is reported in units of micromoles of *p*-nitrophenolate formed per minute per milligram of enzyme.

with a maximal activity of 3 μmol mg^{-1} min^{-1}. The Zn^{2+} activation effect was then determined in the presence of 50 mM Mg^{2+}. With no Zn^{2+} present, the enzyme showed a specific activity of 1.7 μmol mg^{-1} min^{-1}, which increased gradually to 3 μmol mg^{-1} min^{-1} as the Zn^{2+} concentration was increased. When the Mg^{2+} concentration was kept constant at 100 mM, the activity of the E322D enzyme was first activated by Zn^{2+} to a maximum of 4 μmol mg^{-1} min^{-1}, and then decreased as the Zn^{2+} concentration was increased further. When the Mg^{2+} concentration was held constant at 200 mM, increasing concentrations of Zn^{2+} only caused inhibition of the enzyme.

Table IV: Metal and Ionic Strength Effect on the Wild-Type, E322D, and E322A Alkaline Phosphatases at pH 10 in the Absence and Presence of a Phosphate Acceptor[a]

enzyme	k_{cat}[b] (s^{-1})	K_m (μM)	k_{cat}/K_m $\times 10^{-6}$ ($M^{-1}\ s^{-1}$)	activation[d]	$MgCl_2$, $ZnSO_4$, or NaCl
(A) In the Absence of a Phosphate Acceptor (0.1 M CAPS,[c] pH 10)					
wild-type	151	200	0.8	2.2	50 mM $MgCl_2$
E322D	68	122	0.6	97	50 mM $MgCl_2$
E322A	7.5	4190	0.002	5	50 mM $MgCl_2$
wild-type	85	127	0.7	1.2	0.1 mm $ZnSO_4$
E322D	1.7	443	0.004	2.4	0.1 mM $ZnSO_4$
E322A	8.0	3380	0.002	5	0.1 mM $ZnSO_4$
(B) In the Presence of a Phosphate Acceptor (1.0 M Tris, pH 10)					
wild-type	135	202	0.7	2.3	100 mM $MgCl_2$
E322D	569	1070	0.5	26	100 mM $MgCl_2$
E322A	8.8	11370	0.001	1.2	100 mM $MgCl_2$
wild-type	53	89	0.6	0.9	1 mM $ZnSO_4$
E322D	2.5	94	0.03	0.1	1 mM $ZnSO_4$
E322A	<0.1				1 mM $ZnSO_4$
wild-type	86	232	0.4	1.5	300 mM NaCl
E322D	4.0	2863	0.001	0.2	300 mM NaCl
E322A	<0.3			<0.05	300 mM NaCl

[a] Assays were performed at 25 °C in the buffers indicated with use of *p*-nitrophenyl phosphate as substrate. [b] The k_{cat} values are calculated from the V_{max} by use of a dimer molecular weight of 94 000. The k_{cat} per active site would be half of the value indicated. [c] The ionic strength of the buffer was adjusted to 0.585 with NaCl. [d] Activity of the enzyme with Mg^{2+} or Zn^{2+} present compared to the activity in the absence of metals.

The competitive binding between Zn^{2+} and Mg^{2+} was also tested by keeping the Zn^{2+} concentration constant and varying the Mg^{2+} concentration. As seen in Figure 3B, in the absence of Zn^{2+}, the specific activity of E322D enzyme increases cooperatively (see Figure 2A also), with increasing concentrations of Mg^{2+} to a maximum of 16 μmol mg^{-1} min^{-1}. When the Zn^{2+} concentration was kept constant at 0.5 mM, and the concentration of Mg^{2+} was increased from 0 to 600 mM, the maximum activity observed, about 11 μmol mg^{-1} min^{-1}, was lower than that observed with no Zn^{2+} present.

pH Effect on the Activity of the Mutant Enzymes in the Absence and Presence of a Phosphate Acceptor. Since the hydrolysis activity of the wild-type alkaline phosphatase has an alkaline pH optimum, the activities of the E322A and E322D enzymes were also determined at pH 10 in the absence of a phosphate acceptor. The k_{cat} of hydrolysis of the E322D enzyme increased from less than 0.08 s^{-1} at pH 8.0 (see Table I) to 0.7 s^{-1} at pH 10 (see Table I). An even larger increase in activity was observed for the E322A enzyme between pH 8 and 10, from less than 0.08 s^{-1} (see Table I) to 1.5 s^{-1} (see Table I).

The effects of Mg^{2+} and Zn^{2+} on the hydrolysis activities of the wild-type and mutant enzymes were also determined at pH 10. In the presence of 50 mM Mg^{2+}, the activity of the wild-type enzyme increases 2.2-fold (see Table IVA). A more significant change was observed for the E322D and E322A enzymes in the presence of Mg^{2+}, with an almost 100-fold and a 5-fold increase in their values, respectively (see Table IVA). Zn^{2+} also affects the activities of all three enzymes at pH 10. However, more significant changes are observed for the two mutant enzymes. In the presence of 0.1 mM $ZnSO_4$, 2.4- and 5-fold enhancements in the enzymatic activity were observed for the E322D and E322A enzymes, respectively (see Table IVA).

The activities of the enzymes were also measured in 1.0 M Tris at pH 10. The activity of the wild-type enzyme in 1.0 M Tris, pH 10, is lower than the activity determined in 0.1 M CAPS, pH 10 (see Table I), because at alkaline pH Tris is a poor phosphate acceptor for the wild-type enzyme. In 1.0 M Tris buffer, the addition of 100 mM Mg^{2+} results in an increase in activity of the wild-type enzyme from 58 s^{-1} (see Table I) to 135 s^{-1} (see Table IVB). The influence of ionic strength was determined by including 300 mM NaCl in the 1.0 M Tris buffer. Under these conditions, a k_{cat} of 86 s^{-1} was obtained, indicating that Mg^{2+} can activate the wild-type enzyme at pH 10 in 1.0 M Tris buffer. On the other hand, there is a slight decrease in the activity of wild-type enzyme when 1 mM Zn^{2+} was present in the buffer.

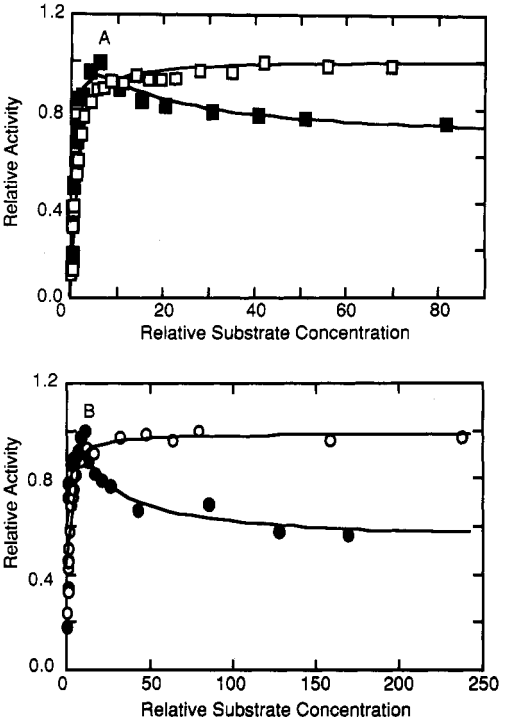

FIGURE 4: Influence of high concentrations of *p*-nitrophenyl phosphate on enzyme activity at pH 8.0. (A) The saturation curves were determined in 0.01 M Tris buffer with 50 mM $MgCl_2$, pH 8.0, with either the wild-type (□) or the E322D (■) enzymes. The ionic strength of the buffer was adjusted to 0.585 with NaCl. (B) The saturation curves were determined in 1.0 M Tris, pH 8.0, buffer with either the wild-type (○) or the E322D (●) enzymes. Substrate concentrations plotted in the figure are normalized to the K_m values calculated from the saturation curves.

For the E322D and E322A enzymes, k_{cat} values of 22.0 and 7.4 s^{-1}, respectively, were obtained in 1.0 M Tris, pH 10, approximately 30-fold and 5-fold higher than the activity determined in 0.1 M CAPS at pH 10. Therefore, Tris is still a very effective phosphate acceptor for these mutant enzymes. A 26-fold increase in the activity was observed for the E322D enzyme when 100 mM $MgCl_2$ was added to the 1.0 M Tris buffer at pH 10. The k_{cat} of the E322D enzyme, 569.4 s^{-1} (see Table IVB), is 4-fold higher than the k_{cat} of the wild-type enzyme determined under the same conditions. No significant increase in the activity was observed for the E322A enzyme when 100 mM $MgCl_2$ was added to the assay buffer. 1 mM Zn^{2+} inhibits the activity of the E322D enzyme by almost 10-fold (see Table IVB).

High Concentrations of p-Nitrophenyl Phosphate Inhibit the Activity of the E322D Enzyme. As shown in Figure 4, the E322D enzyme exhibits substrate inhibition at pH 8.0 both in 0.01 M Tris buffer with 50 mM $MgCl_2$ (see Figure 4A) and in 1.0 M Tris buffer (see Figure 4B). The extent of the inhibition is stronger in the 1.0 M Tris than in the 0.01 M Tris buffer. High concentrations of *p*-nitrophenyl phosphate have no inhibitory effect on the activity of the wild-type enzyme under either set of conditions. For the E322D enzyme,

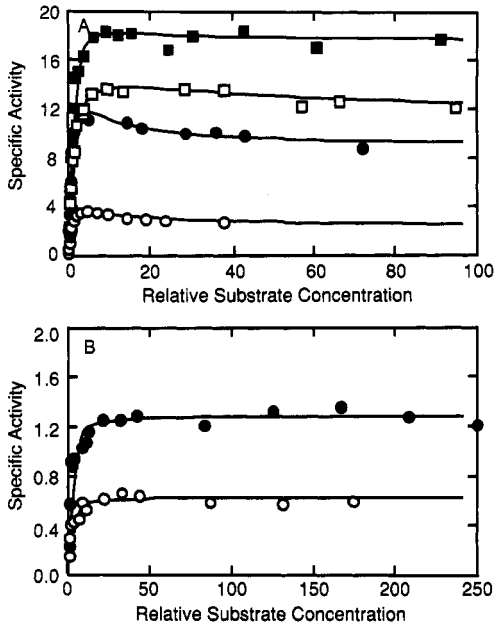

FIGURE 5: Influence of Mg^{2+} or Zn^{2+} on the enzyme activity and substrate inhibition of the E322D enzyme with *p*-nitrophenyl phosphate as substrate. (A) The reactions were performed in 0.01 M Tris buffer, pH 8.0, with 100 (○), 300 (●), 400 (□), and 500 mM (■) Mg^{2+}. (B) The reactons were performed in 0.01 M Tris buffer, pH 8.0, with 0.25 (○) or 0.5 mM (●) Zn^{2+}. Substrate concentrations plotted in the figure are normalized to the K_m values calculated from the saturation curves.

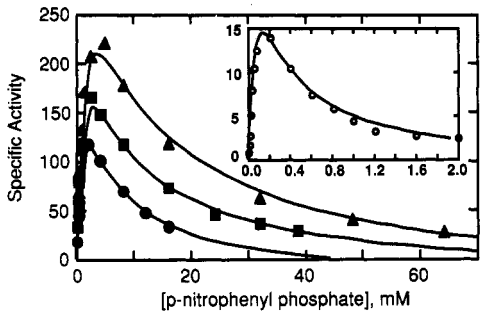

FIGURE 6: Substrate saturation curves of the E322D enzyme in 1.0 M Tris buffer, pH 10, with no additional Mg^{2+} (insert), 15 mM Mg^{2+} (●), 30 mM Mg^{2+} (■), or 50 mM Mg^{2+} (▲). The reactions were performed with *p*-nitrophenyl phosphate as substrate at 25 °C. Specific activity is reported in units of micromoles of *p*-nitrophenolate formed per minute per milligram of enzyme.

the substrate inhibition is weaker at higher concentrations of Mg^{2+} (see Figure 5A). Thus, the inhibitory effect of high concentrations of *p*-nitrophenyl phosphate on the activity of the E322D enzyme can be overcome by high concentrations of Mg^{2+}. High concentrations of *p*-nitrophenyl phosphate have no inhibitory effect on the E322D enzyme when either 0.25 mM or 0.5 mM $ZnSO_4$ was included in the 0.01 M Tris buffer at pH 8.0 (see Figure 5B).

At pH 10, in 1.0 M Tris buffer, the E322D enzyme exhibits strong substrate inhibition (see Figure 6). Because of the poor solubility of Mg^{2+} at pH 10, it was impossible to determine whether Mg^{2+} could overcome this substrate inhibition. Weak substrate inhibition was also observed in 0.1 M CAPS buffer, pH 10 (data not shown).

The Phosphate Binding Affinity of the E322D Enzyme Is Reduced at both pH 8 and pH 10. Inorganic phosphate is a competitive inhibitor of wild-type alkaline phosphatase. In order to investigate the possible change in phosphate binding to the E322D enzyme, the K_i of phosphate was determined at pH 8.0 and pH 10. The K_i of phosphate for the E322D enzyme

Table V: Inhibition of the Wild-Type and E322D Enzymes by Inorganic Phosphate at pH 8.0 and 10[a]

enzyme	K_i (μM)	$[Mg^{2+}]$ (mM)	buffer
wild-type	44	500	0.01 M Tris,[b] pH 8.0
E322D	158	500	0.01 M Tris, pH 8.0
wild-type	60	5	0.1 M CAPS,[b] pH 10
E322D	182	5	0.1 M CAPS, pH 10

[a] Inhibition assays were carried out with *p*-nitrophenol phosphate as substrate at 25 °C. [b] The ionic strength of the buffer was adjusted to 0.585 with NaCl.

Table VI: Kinetic Parameters Observed at pH 8.0 in the Stopped-Flow Experiments[a] for the E322D Enzyme[b]

$[Mg^{2+}]$ or $[Zn^{2+}]$ in both syringes[c] (mM)	burst rate constant (s^{-1})	sp act. in steady-state phase (μmol^{-1} mg^{-1} min^{-1})
(A) In the Presence of Magnesium		
0[c]	6.3	0.07
1	3.3	1.6
10	2.8	2.0
30	1.1	15
50	1.0	21
100	0	56
(B) In the Presence of Zinc		
0.05	3.6	3.9
0.1	5.0	4.9

[a] Assays were performed at 25 °C on the stopped-flow spectrophotometer with 1 mM *p*-nitrophenyl phosphate dissolved in 0.1 M MOPS, pH 8.2, as substrate. [b] E322D enzyme was dialyzed against TMZP buffer extensively before use. [c] E322D enzyme was dialyzed against 0.01 M Tris, 10^{-5} M $ZnSO_4$, and 10^{-4} M NaH_2PO_4, pH 7.4, buffer extensively before use. [d] The concentration of magnesium or zinc in both the enzyme and the substrate solutions was adjusted to various levels as indicated before the reaction by the addition of magnesium chloride or zinc sulfate.

was about 3-fold higher than the wild-type enzyme under both pH conditions (see Table V).

The Pre-Steady-State Kinetic Behavior of the E322D Enzyme Is Very Different from the Wild-Type Enzyme. The catalytic mechanism of the E322D enzyme was also investigated by stopped-flow kinetics. For the wild-type enzyme, the rate-limiting step at pH 8.0 is mainly the release of the noncovalently bound phosphate from the active site. Since native wild-type alkaline phosphatase contains 2 mol of phosphate per dimer, only a linear steady-state phase is observed in the pre-steady-state.[3] However, for the E322D enzyme, biphasic kinetics are observed in the presence of phosphate, with an exponential burst phase followed by a linear steady-state phase.

When the stopped-flow experiment was performed on the E322D enzyme at increasing Mg^{2+} concentrations, the specific activity in the steady-state phase increased. Also observed was a gradual decrease in the rate constant of the burst phase with increasing concentrations of Mg^{2+} (see Table VIA). In the presence of 100 mM Mg^{2+}, the rate constant of the burst phase becomes almost zero, and only a linear steady-state phase is observed; 100 mM Mg^{2+} did not have a significant effect on the pre-steady state kinetics of the wild-type enzyme (data not shown).

Biphasic kinetics were also observed for the E322D enzyme when the stopped-flow experiments were performed at increasing concentrations of Zn^{2+}. However, the rate constant of the burst phase increased as the concentration of Zn^{2+} increased from 0.05 to 1 mM (see Table VIB).

[3] For phosphate-free wild-type alkaline phosphatase, an instantaneous burst phase is observed within the dead time of the stopped-flow (Bloch & Schlesinger, 1973).

DISCUSSION

Alkaline phosphatase isolated from the periplasmic space of *E. coli* by osmotic shock contains between 1 and 2 mol of magnesium/mol of enzyme dimer (Anderson et al., 1975; Janeway et al., 1993). Although magnesium alone cannot activate apoalkaline phosphatase, it has been shown that magnesium can modulate the mode of Zn^{2+} binding, and increase the activity of the enzyme containing either two or four zinc atoms per dimer by 4.4-fold and 1.2-fold, respectively (Anderson et al., 1975, 1976).

In order to investigate the function of the magnesium in alkaline phosphatase directly, Glu-322 was changed to either aspartic acid or alanine by site-specific mutagenesis. The X-ray structure of *E. coli* alkaline phosphatase (Kim & Wyckoff, 1989, 1991) shows that the carboxyl oxygen of Glu-322 is one of the six ligands to the Mg^{2+} at the M3 site (see Figure 1). In the case of the E322D enzyme, the octahedral coordination sphere of the magnesium can still remain intact; however, the magnesium binding affinity might be weakened, and the orientation of the water molecules that bind to the magnesium atom might be altered. In the E322A enzyme, the octahedral coordination sphere would be abolished, resulting in very weak binding of the Mg^{2+} to the enzyme.

The Mutant Enzymes Have Significantly Reduced Magnesium Affinity and Catalytic Activity Compared to the Wild-Type Enzyme. The 16-fold decrease in the magnesium content of the E322D enzyme compared to the wild-type enzyme indicates that this mutant enzyme has a much lower magnesium affinity than the wild-type enzyme. Both of the mutant enzymes also show extremely poor catalytic activity—the hydrolysis activity is reduced 500-fold compared to the wild-type enzyme at pH 8.0. The k_{cat} value of both mutant enzymes is much lower than the value for the wild-type enzyme that contains only four zinc atoms per enzyme dimer. These results along with those on the D153H enzyme suggest that Mg^{2+} at the M3 site has a stronger influence on the catalytic activity of *E. coli* alkaline phosphatase than had been previously thought.

Both Magnesium and Zinc Can Activate the E322D Enzyme, Suggesting That the Extremely Low Catalytic Activity of This Mutant Enzyme Is Due to the Lack of Metal at the M3 Site. At pH 8.0, the hydrolysis activity of the E322D enzyme increases to about half of the wild-type value in the presence of 500 mM Mg^{2+} (see Table IIA). At this concentration of Mg^{2+}, the mutant enzyme becomes more active than the wild-type enzyme, under conditions that measure the sum of the hydrolysis and transphosphorylation activities (see Table IIB). Therefore, Mg^{2+} must also activate the transphosphorylation activity of the E322D enzyme.

Zinc can also activate both the hydrolysis and transphosphorylation activities of the E322D enzyme at pH 8.0; however, the activation effect of Zn^{2+} on the activity of the mutant enzyme is less than that of Mg^{2+} (see Table III). Competition experiments between Mg^{2+} and Zn^{2+} suggest that both metals bind to the same site on the E322D enzyme. Although the E322D enzyme has a reduced Mg^{2+} affinity at the M3 site, and a more than 500-fold lower activity than the wild-type enzyme, this mutant enzyme can still bind Mg^{2+} and become almost as active as the wild-type enzyme. The fact that Zn^{2+} inhibits the Mg^{2+}-activated E322D enzyme also argues against the possible change in the Zn^{2+} binding affinity to the M1 or M2 site in the mutant enzyme. If the E322D enzyme has a weaker Zn^{2+} affinity at the M1 or M2 site due to the change at the M3 site, then the full activation of the E322D enzyme would require both Mg^{2+} and Zn^{2+}. The cooperative Mg^{2+} activation of the E322D enzyme suggests the possible linkage between the magnesium binding site and the phosphate/substrate binding site on one monomer or between the two magnesium binding sites on the dimer.

The E322D enzyme can also bind Zn^{2+} at the M3 site, but Zn^{2+} can restore only 15% of the activity of the wild-type enzyme, and, therefore, Zn^{2+} is only able to partially substitute for Mg^{2+} at the M3 site. The lack of cooperativity in the activation by Zn^{2+} suggests that the enzyme has different binding properties for Zn^{2+} than for Mg^{2+} at the M3 site.

The Pre-Steady-State Kinetic Behavior of the E322D Enzyme Can Be Explained by a Model Based on Weakened Magnesium Affinity and a Change in the Rate-Limiting Step upon the Binding of Mg^{2+}. For the wild-type enzyme, previous studies indicated that the rate-limiting step of the reaction is pH-dependent (Hull et al., 1976; Reid & Wilson, 1971; Gettins & Coleman, 1983b; Bloch & Gorby, 1980). At acidic pH, the rate-limiting step is the breaking of the covalent bond between the enzyme and phosphate, and, therefore, biphasic pre-steady-state kinetics are observed. However, at high pH, the dissociation of the noncovalently bound phosphate becomes the rate-limiting step for the wild-type alkaline phosphatase. Since the wild-type enzyme usually contains approximately 2 mol of phosphate per dimer, at pH 8.0, only a linear steady-state phase is observed in the pre-steady-state phase of the reaction (Bloch & Schlesinger, 1973; Hull et al., 1976; Gettins & Coleman, 1983a). The pH-dependency of the rate-limiting step for the wild-type enzyme can be related to the pK_a of the Zn^{2+}-coordinated water which is the nucleophile attacking the phosphoserine.

However, at pH 8.0, in the absence of Mg^{2+}, the E322D enzyme exhibits a biphasic curve which is composed of an exponential burst phase followed by a linear steady-state phase. These data indicate that the E322D enzyme has a different rate-limiting step than the wild-type enzyme at pH 8.0. The exponential phase observed in the pre-steady-state of the mutant enzyme disappears gradually when the Mg^{2+} concentration is increased from 0 to 100 mM. This is caused by the gradual increase in the rate of the linear steady-state phase and the gradual decrease in the rate of the exponential phase as the concentration of Mg^{2+} is increased (see Table VI). In the presence of 100 mM Mg^{2+}, the rate constant of the exponential phase becomes so small that only a linear steady-state phase is observed. These results suggest that a change in the rate-limiting step occurs when the E322D enzyme has Mg^{2+} bound at the M3 site.

On the basis of the pre-steady-state kinetic behavior of the E322D enzyme, a different rate-limiting step is proposed for the Mg^{2+}-free and Mg^{2+}-containing E322D enzymes. The biphasic pre-steady-state kinetics suggest that breaking of the covalent bond between the enzyme and the phosphate is the rate-limiting step when the E322D enzyme exists in the Mg^{2+}-free form. The linear pre-steady-state kinetic behavior of the E322D enzyme at high Mg^{2+} concentrations suggests that the release of the noncovalently bound phosphate becomes the rate-limiting step for the Mg^{2+}-bound enzyme.

As mentioned above, the change from biphasic kinetics to single linear phase kinetics for the E322D enzyme, when the Mg^{2+} concentration is increased from 0 to 100 mM, was accompanied by an increase in the specific activity in the linear steady-state phase and a decrease in the rate constant of the exponential phase. The increase in the specific activity of the linear steady-state phase with high concentrations of Mg^{2+} is consistent with the steady-state kinetic data on the enzyme (see Figure 2). These data indicate that the rate-

limiting step of the E322D enzyme containing Mg^{2+} is not only different from the Mg^{2+}-free enzyme but also much faster than that of the Mg^{2+}-free enzyme. In this fashion, Mg^{2+} can activate the mutant enzyme.

The above kinetic model alone cannot account for the change in the rate constant of the exponential phase at different Mg^{2+} concentrations (see Table VI), since this model suggests that the rate constant of the exponential phase should be independent of the Mg^{2+} concentration, and equivalent to the rate constant obtained for the Mg^{2+}-free enzyme. However, the observed substrate inhibition can account for this discrepancy.

As shown in Figure 4, substrate inhibition is observed for the E322D enzyme, but not for the wild-type enzyme. This result suggests that high concentrations of the substrate *p*-nitrophenyl phosphate can pull Mg^{2+} away from the mutant enzyme,[4] since the mutant enzyme has a very low affinity for Mg^{2+}. The fact that high concentrations of Mg^{2+} can overcome the substrate inhibition strongly supports this proposal (see Figure 5A). When the mutant enzyme is mixed with substrate at low Mg^{2+} concentrations, the concentration of Mg^{2+}-bound enzyme decreases since high concentrations of substrate pull Mg^{2+} away from the mutant enzyme. Therefore, the rate constant of the exponential phase of the reaction observed in the stopped-flow experiments is influenced by the Mg^{2+} concentration. So in the presence of Mg^{2+}, the rate constant of the burst phase is actually affected by two factors: the rate of breaking of the covalent bond between the enzyme and the phosphate and the rate of the inactivation process. Although Zn^{2+} can partially substitute for Mg^{2+} at the M3 site, the pre-steady-state kinetic data indicate that Zn^{2+} cannot alter the rate-limiting step of the E322D enzyme.

Magnesium in the Active Site of E. coli Alkaline Phosphatase Has a Direct Effect on the Catalytic Mechanism of the Enzyme. In the previously proposed reaction mechanism for *E. coli* alkaline phosphatase (Coleman, 1992; Kim & Wyckoff, 1991), Mg^{2+} at the M3 site was thought not to be close enough to the substrate binding site to participate directly in the hydrolysis mechanism, although the Mg^{2+} might make a contribution to the positive electrostatic field around the active site. However, EPR studies on the wild-type enzyme demonstrate that binding of the Mg^{2+} at the M3 site affects the coordination geometry of Co^{2+} bound to the M1 site (Anderson et al., 1975, 1976). Studies on the D153H enzyme (Janeway et al., 1993) indicated the importance of the Mg^{2+} in the catalytic mechanism; however, the fact that the rate-limiting step of the Mg^{2+}-stimulated D153H enzyme is still different from the wild-type enzyme suggests that the lack of metal at the M3 site alone cannot account for all the differences in the kinetic mechanism between the D153H and wild-type enzymes. The slow Mg^{2+} activation process observed in the D153H enzyme suggested a conformational change upon Mg^{2+} binding. Therefore, Janeway et al. (1993) suggested that the role of Mg^{2+} in catalysis is to stabilize an active conformation of the D153H enzyme. However, Mg^{2+} activation of the E322D enzyme is nearly instantaneous. Therefore, there is no slow conformational change involved in the Mg^{2+} activation process. The pre-steady-state kinetics indicate that the rate-limiting step in the catalysis of the Mg^{2+}-free enzyme differs from that of the Mg^{2+}-stimulated enzyme. Moreover, the Mg^{2+} stimulated enzyme has the same rate-limiting step as the wild-type enzyme. These results suggest that Mg^{2+} at the M3 site has a direct effect on catalysis in this mutant enzyme, and this effect is most likely involved in the step in the mechanism in which the covalent bond between the enzyme and the phosphate is broken. However, it is not clear whether or not a fast conformational change is involved in the Mg^{2+} activation of the E322D enzyme. Further structural studies including X-ray crystallography on this mutant enzyme should help to answer this question.

[4] ^{31}P NMR experiments verify an interaction between *p*-nitrophenyl phosphate and Mg^{2+} (data not shown).

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