Rate-Determining Step of *Escherichia coli* Alkaline Phosphatase Altered by the Removal of a Positive Charge at the Active Center†

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ABSTRACT: *Escherichia coli* alkaline phosphatase catalyzes both the nonspecific hydrolysis of phosphomonoesters and a transphosphorylation reaction in which phosphate is transferred to an alcohol via a phosphoseryl intermediate. The rate-determining step for the wild-type enzyme is pH dependent. At alkaline pH, release of the product phosphate from the noncovalent enzyme−phosphate complex determines the reaction rate, whereas at acidic pH hydrolysis of the covalent enzyme−phosphate complex controls the reaction rate. When the lysine at position 328 was substituted with a cysteine (K328C), the rate-determining step at pH 8.0 of the mutant enzyme was altered so that hydrolysis of the covalent intermediate became limiting rather than phosphate release. The transphosphorylation activity of the K328C enzyme was selectively enhanced, while the hydrolysis activity was reduced compared to that of the wild-type enzyme. The ratio of the transphosphorylation to the hydrolysis activities increased 28-fold for the K328C enzyme in comparison with the wild-type enzyme. Several other mutant enzymes for which a positive charge at the active center is removed by site-specific mutagenesis share this characteristic of the K328C enzyme. These results suggest that the positive charge at position 328 is at least partially responsible for maintaining the balance between the hydrolysis and transphosphorylation activities and plays an important role in determining the rate-limiting step of *E. coli* alkaline phosphatase.

Escherichia coli alkaline phosphatase (EC 3.1.3.1) is a homodimeric enzyme with 449 amino acids and three metal ions (two Zn^{2+}, one Mg^{2+}) per monomer (*1*−*3*). It catalyzes the nonspecific hydrolysis of phosphomonoesters to yield inorganic phosphate and the alcohol (*4*). In the presence of a phosphate acceptor, it also exhibits transphosphorylation activity (*4*−*6*). With Tris (1.0 M) as a phosphate acceptor at pH 8.0, the hydrolase and the transphosphorylase activities are approximately equal. The kinetic parameters of the enzyme have been extensively characterized (ref *7* and references therein), and the structure has been determined by X-ray crystallography to 2.0 Å resolution (*8*−*10*). The catalytic reaction proceeds through a covalent enzyme−phosphate intermediate (E−P) which has been isolated (*11*) and has also been observed in the X-ray structure of a mutant enzyme (*12*). Alkaline phosphatase is a highly conserved enzyme; for example, the mammalian enzymes have 25−30% sequence identity with the *E. coli* enzyme. On the basis of this high sequence identity, the amino acid sequence of the mammalian enzyme has been fit successfully to the X-ray structure of the *E. coli* enzyme (*13*), suggesting that all alkaline phosphatases have a similar fundamental three-dimensional structure. Because of the sequence and structural homology between the *E. coli* and mammalian enzymes, the catalytic mechanism of the *E. coli* enzyme is presumed to be similar to that of other alkaline phosphatases.

As proposed by Kim and Wyckoff (*8*), the hydrolysis of phosphomonoesters by *E. coli* alkaline phosphatase occurs in four steps: the formation of the enzyme−substrate complex, the phosphorylation of the enzyme to form the covalent enzyme−phosphate complex (E−P), the hydrolysis of E−P to form the noncovalent enzyme−phosphate complex (E·P), and the release of inorganic phosphate. Previous studies have demonstrated that the rate-determining step for the wild-type enzyme is the hydrolysis of the E−P covalent complex at acidic pH, whereas the release of phosphate from the E·P noncovalent complex controls the reaction rate at alkaline pH (*4*, *14*). The high-resolution X-ray structure of the wild-type enzyme in the presence of phosphate provides a clear picture of the noncovalent E·P complex (*8*), while an X-ray structure of the mutant H331Q enzyme serves as a model for the covalent E−P intermediate (*12*). The hydrolysis of the covalent E−P intermediate to yield the noncovalent E·P complex is proposed to occur through a nucleophilic attack of a water molecule activated by Zn_1 (*8*). In the H331Q structure, this mechanism is fully supported by the observation of a water molecule, which is coordinated by Zn_1 and poised to attack the phosphoseryl intermediate (*12*).

The crystallographic structure of the wild-type E·P complex shows that the phosphate is stabilized by the guanidinium group of Arg-166 and the two Zn^{2+} ions. In addition, there are two water molecules bridging one oxygen of the phosphate to Lys-328 and Asp-153 (*8*). The mutant enzyme with Arg-166 replaced by alanine exhibits significantly reduced phosphate affinity (*15*). The importance of the water bridge for phosphate affinity has been demonstrated by mutating Asp-153 to histidine and Lys-328 to alanine or histidine (*16*, *17*). Since the release of phosphate from E·P is the rate-limiting step for the wild-type enzyme at pH 8.0,

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it was assumed that lowering the phosphate affinity would enable the release of phosphate, thus accelerating the hydrolysis activity. This was indeed consistent with the observed higher activity of the K328A and K328H enzymes at pH higher than 9 in comparison to that of the wild-type enzyme.

Kinetic studies of the K328A and K328H enzymes also indicated that these enzymes have much higher transphosphorylation than hydrolysis activity. This selectively enhanced transphosphorylation activity cannot be explained simply by the easy release of phosphate from the active site. In addition, an instantaneous burst of the alcohol was observed for both mutant enzymes at pH 8.0 in pre-steady-state kinetics experiments, indicating that the rate-determining step for the mutant enzymes may be the hydrolysis of the covalent E−P complex instead of the release of phosphate from the E·P complex (*17*). However, there is no clear explanation for the change in rate-determining step due to the mutation. Furthermore, similar results from pre-steady-state kinetics have been obtained with a double mutant K328H/D153H that has comparable activity to the more active mammalian enzymes (*18*). Therefore, to understand the mechanism for the change in the rate-determining step and the driving force for the higher activity of the Lys-328 mutant enzymes, including the K328H/D153H enzyme, we have investigated further the role Lys-328 in *E. coli* alkaline phosphatase by comparing a series of mutations at position 328 with varying charge, from positive (Lys) to neutral (Ala and His) to negative (Cys).

MATERIALS AND METHODS

Materials. Agar, agarose, ampicillin, chloramphenicol, *p*-nitrophenyl phosphate, sodium dihydrogen phosphate, magnesium chloride, zinc chloride, and zinc sulfate were supplied by Sigma. Tris, enzyme-grade ammonium sulfate, and sucrose were obtained from ICN Biomedicals. Tryptone and yeast extract was purchased from Difco. The ThermoSequenase Version 1.0 DNA sequencing kit was purchased from Amersham Life Sciences Inc. The kit used for site-specific mutagenesis was a product of BioRad. New England Biolabs supplied all restriction enzymes used in the present work. DNA fragments were isolated from agarose gels with the Geneclean II kit from Bio 101 Inc. Oligonucleotides required for the mutagenesis were purchased from Operon Technology. Oligonucleotides used for sequencing were synthesized in the laboratory on an Applied Biosystems 381A DNA synthesizer.

Strains. *E. coli* K12 strain MV1190 [Δ(*lac-proAB*), *supE*, *thi*, Δ(*sri-recA*)306::Tn10(tet^r)/F′ *tra*D36, *pro*AB, *lac*I^q, *lac*ZΔ*M*15] and the phage M13K07 were obtained from J. Messing. The Δ*phoA E. coli* K12 strain SM547 [Δ(*phoA-phoC*), *phoR*, tsx::Tn5, Δ*lac*, *galK*, *galU*, *leu*, str^r] was a gift of H. Inouye. *E. coli* CJ236 [*dut-1*, *ung-1*, *thi-1*, *relA-1*/pCJ105(Cm^r)] was a gift of T. Kunkel.

Site-Specific Mutagenesis. Mutation of Lys-328 to cysteine was accomplished by site-specific mutagenesis using the method of Kunkel (*19*, *20*). The uracil-containing single-stranded DNA template was prepared from phagemid pEK154. A 22-mer oligonucleotide with the codon of Lys-328 changed to that of cysteine was used as the primer. The in vitro synthesized double-stranded DNA plasmids were transformed into MV1190 and grown on YT media plates with 100 μg/mL ampicillin. The plasmid carrying the K328C mutation was selected by sequencing the double-stranded DNA plasmid with ThermoSequenase.

Both the plasmids carrying the K328C and pEK154 mutations were digested with *Sph*I and *Bst*XI. The short DNA fragment containing the K328C mutation and the large fragment from pEK154 were isolated and treated with T4 DNA ligase. The reconstructed plasmid was transformed into MV1190 and the plasmid was sequenced again to exclude nonspecific mutations and confirm the existence of the K328C mutation. The plasmid containing the K328C mutation was named pEK397.

Expression and Purification of Wild-Type and Mutant Alkaline Phosphatase. Both pEK154 and pEK397 were transformed into SM547, a strain with the *phoA* gene deleted. Therefore, the expressed alkaline phosphatase in this plasmid/strain combination is derived exclusively from the *phoA* gene on the plasmid. Purification of the wild-type enzyme followed the protocol described previously (*15*). The method used to purify the K328C enzyme was identical to that used for the wild-type enzyme, except that all the buffers contained 2 mM 2-mercaptoethanol. Enzyme purity was judged by SDS−polyacrylamide gel electrophoresis (*21*). The protein concentration of the wild-type enzyme was determined from the absorbance at 278 nm with an extinction coefficient of 0.71 cm^2/mg (*1*), while the concentration of the K328C enzyme was determined with the Bio-Rad version of Bradford's dye binding assay (*22*) with the wild-type enzyme as the protein standard.

Steady-State Enzyme Kinetics. Steady-state enzyme kinetics were followed spectrophotometrically with *p*-nitrophenyl phosphate as substrate. The release of product, *p*-nitrophenolate, was monitored by measuring the absorbance at 410 nm at 25 °C (*23*). For the experiments at varying [Mg^{2+}], the enzymes, which were stored in buffer containing Mg^{2+}, were dialyzed against buffer lacking Mg^{2+} (0.01 M Tris, 0.1 mM NaH_2PO_4, 0.01 mM $ZnSO_4$, pH 7.4). The reaction buffer was adjusted with 1 M $MgCl_2$ to the final desired Mg^{2+} concentration prior to the addition of enzyme.

Rapid Kinetics. Using the procedure of Xu and Kantrowitz (*17*), pre-steady-state kinetic experiments were performed using a KinTek Inc. stopped-flow spectrophotometer at 25 °C with a dead time of approximately 1 ms. Data were collected at 410 nm directly by a computer via an analog/digital interface. Enzymes were dialyzed against 0.1 M MOPS buffer, pH 8.0, with an ionic strength of 0.83. The enzyme loaded into the syringe had a concentration of 3 mg/mL for wild-type enzyme and 3.5 mg/mL for the K328C enzyme. *p*-Nitrophenyl phosphate was used as substrate and was loaded into the second syringe at a concentration of 0.1 mM in the same buffer used for the enzymes. Equal volumes of enzyme and substrate were introduced into the mixing chamber.

Detection of the E−P Covalent Intermediate by ^{32}P Labeling. Wild-type alkaline phosphatase has been shown to incorporate ^{32}P-labeled inorganic phosphate at acidic pH but not at alkaline pH (*4*, *24*). The mutant enzymes were tested to determine whether they incorporated ^{32}P-labeled inorganic phosphate at both pH 5.5 and 7.5, using the wild-type enzyme as the control. Enzymes were dialyzed against 0.1 M sodium acetate pH 5.5 or against 0.1 M MOPS buffer

Table 1: Summary of Kinetic Parameters for the Wild-Type and K328C Enzymes at pH 8.0[a]

enzyme	k_{cat}[b] (s^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})	buffer
wild-type	36.0 ± 1.4	3.7 ± 0.3	9.73	0.1 M MOPS
K328C	3.2 ± 0.2	0.54 ± 0.01	5.85	0.1 M MOPS
wild-type	82.6 ± 0.1	25.6 ± 0.9	3.23	1.0 M Tris
K328C	123.2 ± 0.4	367 ± 52	0.34	1.0 M Tris

[a] Assays were performed at 25 °C with *p*-nitrophenyl phosphate as substrate in the buffer indicated. The ionic strength of 0.1 M MOPS buffer, pH 8.0, was adjusted to be identical to the ionic strength of 1.0 M Tris buffer at pH 8.0 with NaCl. [b] The k_{cat} values are calculated from V_{max} by using a dimer molecular weight of 94 000. The k_{cat} per active site would be half of the value indicated.

pH 7.5. The enzymes were then diluted to 0.625 mg/mL. ^{32}P-labeling solution was prepared by adding ^{32}P-labeled orthophosphate to 0.03 M nonradioactive NaH_2PO_4 to 1 μCi/μL. ^{32}P-labeling solution (50 μL) was mixed with enzyme (100 μL), and the mixture was allowed to stand at room temperature for 30 min. The protein was then precipitated by addition of 150 μL of 6 N perchloric acid followed by 1.5 mL of 5% trichloroacetic acid. After centrifugation, the precipitate was washed twice with 1 mL of H_2O and then dissolved in 25 μL of SDS sample buffer. Samples were resolved on a 10% SDS polyacrylamide gel (*21*). After being stained with coomassie blue with the image recorded, the gel was dried prior to autoradiography using a PhosphorImager 425 from Molecular Dynamics.

RESULTS

Steady-State Kinetics of the Mutant and Wild-type Enzymes at pH 8.0. Steady-state kinetic experiments were carried out in either the presence or the absence of a phosphate acceptor. Since Tris serves as a phosphoryl group acceptor (*6*, *25*), the rate observed in 1.0 M Tris buffer is the sum of the transphosphorylation and hydrolysis reactions. In contrast, the rate observed in 0.1 M MOPS buffer (with the ionic strength adjusted to be equivalent to 1.0 M Tris buffer) represents only the hydrolysis activity.

The kinetic parameters of the wild-type and the K328C enzymes are summarized in Table 1. In the presence of phosphate acceptor and with *p*-nitrophenyl phosphate as substrate, the k_{cat} and K_m values for the wild-type enzyme were 82.6 s^{-1} and 25.6 μM. Under the same conditions, the k_{cat} and K_m values for the K328C enzyme were 123.2 s^{-1} and 367 μM. While the k_{cat} value of the K328C enzyme did show a small increase compared to the wild-type enzyme, the K_m of the K328C enzyme was more than 10-fold higher than that of the wild-type enzyme. When activity measurements were carried out in the absence of phosphate acceptor (0.1 M MOPS buffer), the k_{cat} value of the wild-type enzyme was 36.0 s^{-1}, approximately half the k_{cat} value obtained in the presence of phosphate acceptor, and the K_m value was 3.7 μM. However, the k_{cat} value for the K328C enzyme under these conditions was only 3.2 s^{-1} , which is 11-fold lower than the wild-type enzyme, while the K_m of the K328C enzyme decreased to 0.54 μM.

pH Profiles of the Mutant and Wild-type Enzymes. The pH profiles of the K328C in the absence and presence of a phosphate acceptor are shown in Figure 1. For comparison the data for the wild-type and K328A (*17*) enzymes are also

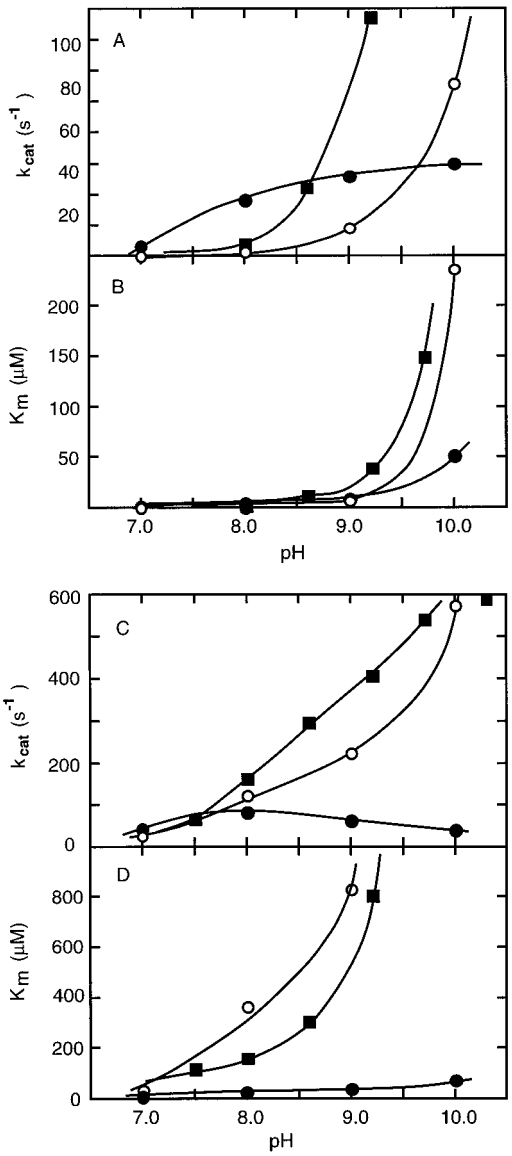

FIGURE 1: pH dependence of k_{cat} (A, C) and K_m (B, D) for the wild-type (●), K328C (○), and K328A (■) enzymes. In panels A and B the reactions were performed in the absence of a phosphate acceptor. The reaction buffer was 0.1 M MOPS for pH values below 9.0 and 0.1 M CAPS for pH values of 9.0 and above. The ionic strength of the buffers were adjusted to be identical to the 1.0 M Tris buffer with NaCl. In panels C and D the reactions were performed in the presence of a phosphate acceptor (1.0 M Tris). All reactions were carried out at 25 °C with *p*-nitrophenyl phosphate as substrate. The previously published data for the K328A enzyme are shown here for comparison (*17*).

shown. At pH values below approximately 9.5, the k_{cat} values of the K328C enzyme are lower than those of the wild-type enzyme and the K_m values of the K328C enzyme are either lower than or similar to the wild-type enzyme. However, both the k_{cat} and K_m values for the K328C enzyme increased dramatically at pH values above 9.5. The k_{cat} for the K328C enzyme was twice that of the wild-type enzyme at pH 10. Although the k_{cat} and K_m values for the K328C enzyme cannot be determined accurately at pH values above 10, both values increased with pH. For the wild-type enzyme above pH 10, the k_{cat} remained relatively constant, while a small increase in the K_m was observed with increasing pH. Both in the absence and presence of a phosphate acceptor, the pH profiles for the K328C enzyme are remarkable similar

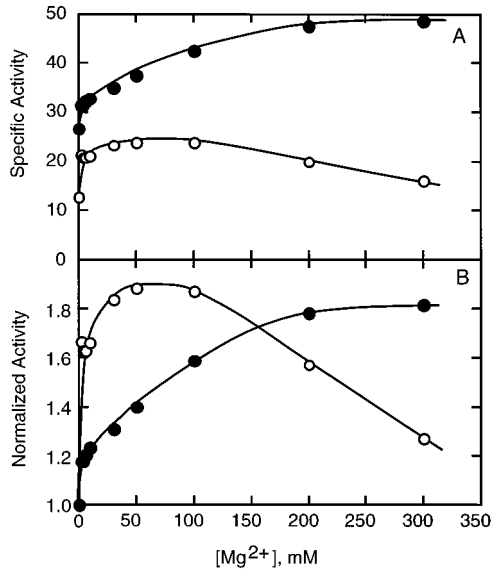

FIGURE 2: Influence of $[Mg^{2+}]$ on the hydrolytic activity. (A) The specific activity of the wild-type (●) and the K328C (○) enzymes is plotted as a function of $[Mg^{2+}]$. (B) Data in (A) are replotted as normalized activity. The specific activity at zero $[Mg^{2+}]$ was set to one for the wild-type (●) and the K328C (○) enzymes. Reactions were performed in 0.1 M CAPS buffer, pH 9.0 with 1 mM *p*-nitrophenyl phosphate as substrate at 25 °C. The ionic strength of the buffer was adjusted to be identical to 1.0 M Tris buffer with NaCl. Specific activity is reported in micromoles of *p*-nitrophenolate formed per minute per milligram of enzyme.

to that previously observed for the K328A (see Figure 1) and K328H (*17*) enzymes.

Steady-State Kinetics of the Mutant and Wild-Type Enzymes in the Presence of Magnesium. Since Lys-328 serves as a second-sphere ligand of Mg^{2+} as observed in the X-ray structure of the wild-type enzyme (*8*), we studied the influence of Mg^{2+} concentration on the specific activity of both the wild-type and K328C enzymes. The pH profiles showed that the mutant enzyme had higher k_{cat} and K_m values above approximately pH 9.0. To determine precisely the dependence of enzyme activity on Mg^{2+}, 0.1 M CAPS, pH 9.0, was selected as the buffer for more extensive experiments. Under these conditions, the enzyme exhibits a relatively high activity and a reasonable K_m. The ionic strength of the buffer was adjusted to be equivalent to the ionic strength of 1.0 M Tris buffer at pH 8.0. As seen in Figure 2A, both the wild-type and K328C enzymes can be activated by the addition of Mg^{2+}; however, the Mg^{2+} concentration required for maximal activity of the K328C enzyme was significantly lower than that for the wild-type enzyme.

At Mg^{2+} concentrations of approximately 2 mM, the specific activity of the K328C enzyme reaches 50% of the maximum activity, while for the wild-type enzyme, 50 mM Mg^{2+} is required to reach half of its maximal activity (Figure 2B). In addition, the specific activity of the K328C enzyme was inhibited slightly at Mg^{2+} concentrations higher than 100 mM, whereas this effect was not observed for the wild-type enzyme, within the concentration range tested. High concentrations of Mg^{2+} did not inhibit the activity of the K328C enzyme in the presence of 1 mM $ZnCl_2$ (data not shown).

Affinity of K328C and Wild-Type Enzymes for Inorganic Phosphate. The product inorganic phosphate is also a competitive inhibitor of alkaline phosphatase. At pH 8.0, the K_i determined for the K328C enzyme was 160 μM, which is approximately 20-fold higher than the K_i of phosphate for the wild-type enzyme (7.7 μM) determined under the same conditions (Table 2). These data suggest that the phosphate affinity of the mutant enzyme is significantly lower than that of the wild-type enzyme.

Table 2: Comparison of Transphosphorylation/Hydrolysis to the K_i of Phosphate for the Wild-Type and Mutant Enzymes[a]

enzyme	$k_{cat(tran)}/k_{cat(hydro)}$	K_i of phosphate (μM)
wild-type	1.3	7.7 (0.1 M MOPS, pH 8.0)
K328C	37	160 (0.1 M MOPS, pH 8.0)
K328A[b]	17	87 (0.1 M MOPS, pH 8.0)
K328H[b]	14	N/A
R166A[c]	37	665 (0.01 M Tris, pH8.0)

[a] The $k_{cat(tran)}$ values were calculated by subtracting $k_{cat(hydro)}$ from the k_{cat} values of the sum of hydrolysis and transphosphorylation activities. The latter were calculated from the V_{max} determined under the conditions of 1.0 M Tris, pH 8.0, with *p*-nitrophenyl phosphate as substrate at 25 °C. The $k_{cat(hydro)}$ was calculated from the V_{max} determined at 25 °C with *p*-nitrophenyl phosphate as substrate and 0.1 M MOPS as reaction buffer. The reaction buffer was adjusted to pH 8.0 with HCl and the ionic strength adjusted to be identical to 1.0 M Tris buffer at pH 8.0 with NaCl. The molecular weight used for the calculation of k_{cat} was 94 000. [b] Data were obtained from the work of Xu and Kantrowitz (*17*). The K_i of phosphate for K328H could not be determined because of its extremely low K_m at pH 8.0. [c] Data from the work of Chaidaroglou et al. (*15*).

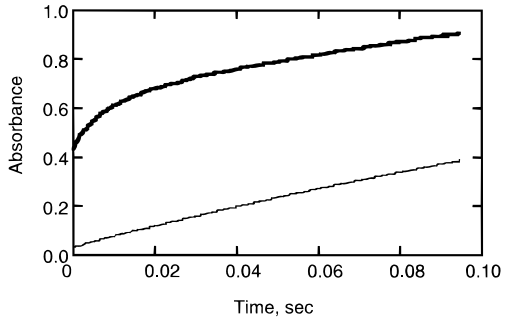

FIGURE 3: Pre-steady-state kinetics of the wild-type (thin line) and the K328C enzyme (heavy line) at pH 8.0. Reactions were carried in 0.1 M MOPS buffer at 25 °C with 10^{-4} M *p*-nitrophenyl phosphate in 0.1 M MOPS buffer, pH 8.0, as substrate.

Pre-Steady-State Kinetics of Wild-Type and Mutant Enzymes at pH 8.0. Pre-steady-state kinetics of the wild-type enzyme has been used extensively to investigate alkaline phosphatase. At pH 5.5, the hydrolysis of phosphomonoesters by the wild-type enzyme containing residual phosphate yields a biphasic curve with a transient burst of alcohol product followed by a linear steady-state phase, while, at alkaline pH, only a linear phase is observed (*26*−*28*). The stopped-flow traces for the K328C and the wild-type enzymes at pH 8.0 are shown in Figure 3. As previously reported the wild-type enzyme only shows a linear increase in absorbance with time (Figure 3). However, the K328C enzyme shows an instantaneous burst of *p*-nitrophenolate followed by a linear phase (Figure 3), indicating that, under these conditions, the rate-determining step for the K328C enzyme has changed from the release of phosphate from the noncovalent E·P complex to the breakdown of covalent E−P complex.

Comparison of the Ratio of Transphosphorylation/Hydrolysis between the Wild-Type and K328C Enzymes. The k_{cat} value calculated from the V_{max} measured in MOPS buffer

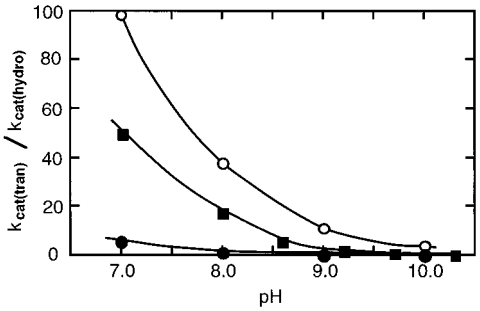

FIGURE 4: Variation of the $k_{cat(tran)}/k_{cat(hydro)}$ ratio as a function of pH for the wild-type (●), K328C (○), and K328A (■) enzymes. Data used to calculate the $k_{cat(tran)}/k_{cat(hydro)}$ ratio were from Figure 1.

represents the hydrolysis activity ($k_{cat(hydro)}$), and the k_{cat} value calculated from the V_{max} measured in 1.0 M Tris buffer represents the sum of the hydrolysis and transphosphorylation activities ($k_{cat(hydro+tran)}$). The $k_{cat(tran)}$ can therefore be estimated by subtracting the $k_{cat(hydro)}$ from $k_{cat(hydro+tran)}$, which allows the $k_{cat(tran)}/k_{cat(hydro)}$ ratio to be calculated. As seen in Table 2, the mutant enzymes have significantly higher transphosphorylation activity and much lower hydrolytic activity than the wild-type enzyme, as shown by a $k_{cat(tran)}/k_{cat(hydro)}$ ratio greater than 1. For the K328C enzyme, the $k_{cat(tran)}/k_{cat(hydro)}$ ratio is 37 compared to approximately 1 for the wild-type enzyme. Other mutant enzymes, such as K328H, K328A, and R166A, also have $k_{cat(tran)}/k_{cat(hydro)}$ ratios significantly higher than that observed for the wild-type enzyme (see Table 2). All the enzymes listed in Table 2 also have higher K_i values for inorganic phosphate than the wild-type enzyme.

Using the pH versus activity data in the absence and presence of a phosphate acceptor (Figure 1), the influence of pH on the $k_{cat(tran)}/k_{cat(hydro)}$ ratio could be evaluated. As seen in Figure 4, for the wild-type as well as the K328A and K328C enzymes, the $k_{cat(tran)}/k_{cat(hydro)}$ ratio is highest at pH 7 and decreases steadily as the pH is increased. The K328C and K328A enzymes have similar profiles with significantly higher $k_{cat(tran)}/k_{cat(hydro)}$ ratios at all pH values measured. The similarity of the results for the K328A and K328C enzymes as a function of pH suggest that if any alteration in ionization of the cysteine is occurring over this range, it is not causing significant alterations to the kinetic characteristics of the enzyme.

Detection of the E−P Covalent Intermediate of Mutant Enzymes at Alkaline pH. It has been shown for the wild-type enzyme that the equilibrium between E−P and E·P favors E−P at pH values lower than 7.0, while, above pH 7.0, the equilibrium is shifted toward E·P (for a review, see ref *7*). The E−P covalent intermediate can be stabilized by acidification and can be detected by the incorporation of ^{32}P-labeled inorganic phosphate (*4*, *24*). For the wild-type enzyme, the enzyme is labeled in this manner at pH 5.5 but not at pH 8.0.

In our experiments, we prepared samples both at pH 7.5 and 5.5. After resolution of the samples by SDS gel electrophoresis, the gels were stained with coomassie blue, which showed that the quantities of protein loaded were almost identical for each sample both at pH 5.5 and 7.5 (Figure 5, row A). Furthermore, the autoradiograph of the same gel for the wild-type enzyme showed the band at pH 5.5 to be very strong, whereas it was extremely weak at pH

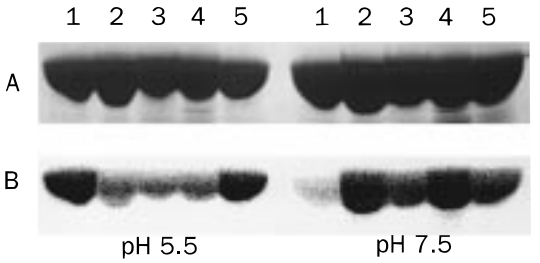

FIGURE 5: Detection of the phosphoseryl intermediate using ^{32}P-labeled phosphate. (A) SDS polyacrylamide gel electrophoresis (SDS−PAGE) of the wild-type and mutant enzymes. (B) Autoradiograph of the SDS−PAGE shown in (A): lane 1, the wild-type enzyme; lane 2, the K328A enzyme; lane 3, the K328C enzyme; lane 4, the K328H enzyme; lane 5, the R166A enzyme. The experiment was repeated at pH 5.5 and pH 7.5.

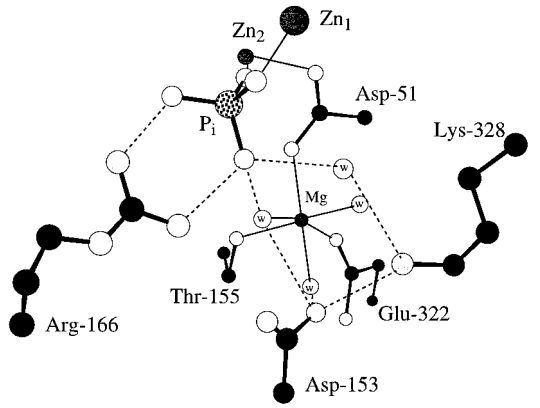

FIGURE 6: Region of the active site of *E. coli* alkaline phosphatase near the phosphate and Lys-328 (*8*). The phosphate interacts with the guanidinium group of Arg-166 and forms specific interactions with two zinc ions at the M1 site (Zn_1) and M2 site (Zn_2). Lys-328 and Asp-153 interact with the phosphate through a water molecule. Asp-153 forms a salt-link with Lys-328 and participates in the interactions with two water molecules that are ligands to the magnesium ion at the M3 site. For clarity, the ligands to Zn_1 and Zn_2 are not shown.

7.5. However, for the K328A, K328C, and K328H as well as R166A enzymes, the autoradiograph showed strong density bands at both pH 5.5 and pH 7.5 (Figure 5, row B). In addition, the density at pH 7.5 was even stronger than the density at pH 5.5 for the K328A, K328C, and K328H enzymes.

DISCUSSION

In the structure of the wild-type enzyme, Lys-328 forms a salt link with Asp-153 which participates in interactions with two water molecules that are ligands of the Mg^{2+} in the active site. More importantly, Lys-328 forms a water-mediated interaction with the phosphate in the active site (Figure 6). Previously, Lys-328 has been substituted by alanine and histidine (*17*). The K328A and K328H enzymes exhibited a decrease in hydrolysis activity along with an enhancement in transphosphorylation activity. Pre-steady-state kinetics also suggested a change in the rate-determining step for these mutant enzymes from the release of phosphate from the noncovalent E·P complex to the breakdown of the covalent E−P complex both in the presence and absence of excess Mg^{2+} (*16*, *17*). The structure of the K328H enzyme has been solved and indeed lacks the water-mediated interaction with phosphate, which is seen in the wild-type

structure (*18*). These missing wild-type interactions between the phosphate and Lys-328 can explain the fact that the K328H and the K328A enzymes have lower affinity for phosphate than the wild-type enzyme. The increased K_i observed for these mutant enzymes at position 328 was expected to favor the release of phosphate from the active site and therefore to accelerate the hydrolysis activity, since the rate-determining step of the wild-type enzyme is the release of the phosphate from noncovalent E·P complex. An increase in k_{cat} above the wild-type was actually observed only at pH values above 9. However, at pH 8 reduced hydrolysis activity was observed along with selectively enhanced transphosphorylation activity.

To understand these properties of the mutant enzymes at position 328 and to elucidate the role of Lys-328 in the wild-type enzyme, we prepared a Lys-328 to cysteine mutation in this work. Unlike alanine and histidine, which remain neutral under alkaline conditions, cysteine can be deprotonated to form a negatively charged species. In fact, within the highly positive charged active site of alkaline phosphatase, containing three metal dications and Arg-166, the cysteine will most likely be negatively charged over the entire pH range investigated here. Therefore, a charge transition series at position 328, from positive (Lys) to neutral (Ala and His) to negative (Cys), may serve to elucidate more clearly the role of Lys-328 in the wild-type enzyme.

K328C Mutation Selectively Enhances the Transphosphorylation Activity and Reduces Hydrolysis Activity. In a manner analogous to the K328A and K328H enzymes (*17*), the catalytic activity of the K328C enzyme also showed a significant decrease at pH 8.0 in the absence of a phosphate acceptor; however, in the presence of a phosphate acceptor (1.0 M Tris, pH 8.0) the activity was about 50% higher than that of the wild-type enzyme. The ratio of transphosphorylation to hydrolysis activities is compared in Table 2. The selective enhancement of transphosphorylation activity for the K328C enzyme is significantly greater than it is for either the K328A or K328H enzymes.

K328C Enzyme has Reduced Phosphate Affinity and Requires Higher Concentrations of Magnesium for Activity. As for the K328A and K328H enzymes, the K328C enzyme has a higher K_i value for inorganic phosphate than the wild-type enzyme. The weaker phosphate affinity can also be explained by the absence of the water-mediated interaction from Lys-328 to phosphate as observed in the structure of the K328H enzyme (*18*). The K328C enzyme requires a higher concentration of magnesium than the wild-type enzyme to obtain maximal activity as demonstrated in Figure 2. The higher magnesium requirement of the K328C enzyme may be due to the more negatively charged environment provided by the cysteine for the binding of the magnesium ion.

Rate-Determining Step of the Mutant Enzymes Is Different from the Wild-Type Enzyme at pH 8.0. In the pre-steady-state kinetics experiment of the wild-type enzyme at pH 8.0 phosphate release is rate-determining; there is no burst of alcohol product in the presence of residual phosphate. The initial burst can only be observed for the wild-type enzyme at acidic pH in which the rate-determining step is the hydrolysis of E−P. In contrast to the wild-type enzyme, we observed an instantaneous burst of alcohol product at pH 8.0 for the K328C enzyme (Figure 3) just as for the K328A and K328H enzymes (*17*), suggesting that the rate-determining step for the K328 mutant enzymes is the breakdown of the E−P complex.

The E−P complex can be directly visualized by SDS gel electrophoresis by the incorporation of ^{32}P-labeled inorganic phosphate into the protein. One covalent complex predicted by pre-steady-state kinetics and ^{32}P-labeling has recently been resolved in a crystal structure (*12*). The stopped-flow result is further supported by the direct visualization of the covalent E−P complex by the use of ^{32}P-labeled inorganic phosphate (Figure 5). In Figure 5, we observed that all three enzymes with mutations at position 328 have somewhat weaker ^{32}P incorporation at pH 5.5 than the wild-type enzyme. One possible reason for the weaker incorporation is a shift in the E + P ⇌ E−P equilibrium for the K328 mutant enzymes as compared to the wild-type enzyme.

Removal of the Positive Charge around the Active Site May Influence the pK_a of the Nucleophile Water so That a Neutral Nucleophile Is Preferred as a Phosphate Acceptor. Substituting alanine, histidine, and cysteine for Lys-328 results in three mutant enzymes with similar kinetic properties. A common feature of these three amino acids is the absence of the positive charge of Lys-328. The observed change in the rate-determining step for all three mutant enzymes (K328A, K328H, and K328C) suggests that the positive charge of Lys-328 is important for determining the rate-limiting step in the enzyme catalyzed reaction.

Arg-166 has been substituted by alanine previously (*15*). The R166A enzyme offers a useful control, since in this enzyme a positively charged side chain has been changed to a neutral side chain at the active site. For this mutant enzyme, the ratio of transphosphorylation to hydrolysis activity is 37 compared to 1.3 for the wild-type enzyme (Table 2). In addition, the R166A enzyme has a higher phosphate K_i value than the wild-type enzyme. In the ^{32}P incorporation experiment, higher density was also observed in the autoradiograph band for the R166A enzyme at pH 7.5 (Figure 5, row B), clearly indicating that the rate-determining step of the R166A enzyme was also altered to the breakdown of the covalent E−P complex rather than the release of phosphate from the noncovalent E·P complex. The behavior of the R166A enzyme further suggests that not only the positive charge of Lys-328 but also other positive charges at the active site are important for determining the rate-limiting step of *E. coli* alkaline phosphatase.

A possible mechanistic explanation for the alteration in the rate-limiting step is that the positive charge is involved in helping to lower the pK_a of the water molecule stabilized by Zn_1, which acts as the nucleophilic hydroxide group. When the positive charge of Lys-328 or Arg-166 is removed by site-specific mutagenesis, the hydrolysis activity is lowered because of the reduced concentration of the nucleophilic hydroxide group, while the E−P complex will have a greater probability to be attacked by a phosphate acceptor. This may explain the enhancement of hydrolysis activity with increasing pH, since the concentration of the nucleophilic hydroxide will be higher at higher pH.

This hypothesis is further supported by the contrasting steady-state kinetic character of a mutant enzyme D153H, which has a negative charge, removed from the active site (*16*). As expected, this mutant enzyme shows higher phosphate affinity. The turnover number determined in the

presence of phosphate acceptor (1.0 M Tris) is approximately half that of the wild-type enzyme but almost identical to wild-type enzyme in the absence of a phosphate acceptor (*16*). This result demonstrates that the transphosphorylation activity of D153H was reduced in comparison to the wild-type enzyme, while hydrolysis activity remained the same as for the wild-type enzyme. This example further suggests that the electrostatic environment is very important in determining the rate-limiting step of *E. coli* alkaline phosphatase.

In conclusion, Lys-328 is very important for the catalytic reaction of *E. coli* alkaline phosphatase; however, its participation is indirect. This side chain stabilizes the phosphate at the active site through a water-mediated salt link. Its positive charge, as discussed in the present work, likely participates in lowering the pK_a of the nucleophilic water molecule and thereby determining the rate-limiting step of the wild-type enzyme. These studies of mutant versions of *E. coli* alkaline phosphatase with alterations at Lys-328 probe the role of charged amino acid residues located near the active site on the catalytic mechanism of the enzyme.

REFERENCES

1. Plocke, D. J., and Vallee, B. L. (1962) *Biochemistry 1*, 1039−1043.
2. Anderson, R. A., Bosron, W. F., Kennedy, F. S., and Vallee, B. L. (1975) *Proc. Nat. Acad. Sci. U.S.A. 72*, 2989−2993.
3. Bradshaw, R. A., Cancedda, 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*, 3473−3477.
4. Reid, T. W., and Wilson, I. B. (1971) *Enzymes (3rd Ed.) 4*, 373−415.
5. Dayan, J., and Wilson, I. B. (1964) *Biochim. Biophys. Acta 81*, 620−623.
6. Wilson, I. B., Dayan, J., and Cyr, K. (1964) *J. Biol. Chem. 239*, 4182−4185.
7. Coleman, J. E. (1992) *Annu. Rev. Biophys. Biomol. Struct. 21*, 441−483.
8. Kim, E. E., and Wyckoff, H. W. (1991) *J. Mol. Biol. 218*, 449−464.
9. Sowadski, J. M., Handschumacher, M. D., Murthy, H. M. K., Kundrot, C., and Wyckoff, H. W. (1983) *J. Mol. Biol. 170*, 575−581.
10. Sowadski, J. M., Handschumacher, M. D., Murthy, H. M. K., Foster, B. A., and Wyckoff, H. W. (1985) *J. Mol. Biol. 186*, 417−433.
11. Schwartz, J. H., and Lipmann, F. (1961) *Proc. Natl. Acad. Sci. U.S.A. 47*, 1996−2005.
12. Murphy, J. E., Stec, B., Ma, L., and Kantrowitz, E. R. (1997) *Nature Struct. Biol. 4*, 618−621.
13. Kim, E. E., and Wyckoff, H. W. (1989) *Clin. Chim. Acta 186*, 175−188.
14. Hull, W. E., Halford, S. E., Gutfreund, H., and Sykes, B. D. (1976) *Biochemistry 15*, 1547−1561.
15. Chaidaroglou, A., Brezinski, J. D., Middleton, S. A., and Kantrowitz, E. R. (1988) *Biochemistry 27*, 8338−8343.
16. Janeway, C. M. L., Xu, X., Murphy, J. E., Chaidaroglou, A., and Kantrowitz, E. R. (1993) *Biochemistry 32*, 1601−1609.
17. Xu, X., and Kantrowitz, E. R. (1991) *Biochemistry 30*, 7789−7796.
18. Murphy, J. E., Tibbitts, T. T., and Kantrowitz, E. R. (1995) *J. Mol. Biol. 253*, 604−617.
19. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.
20. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol. 154*, 367−382.
21. Laemmli, U. K. (1970) *Nature (London) 227*, 680−685.
22. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
23. Garen, A., and Levinthal, C. (1960) *Biochim. Biophys. Acta 38*, 470−483.
24. Reid, T. W., Pavlic, M., Sullivan, D. J., and Wilson, I. B. (1969) *Biochemistry 8*, 3184−8.
25. Trentham, D. R., and Gutfreund, H. (1968) *Biochem. J. 106*, 455−460.
26. Bloch, W., and Schlesinger, M. J. (1973) *J. Biol. Chem. 248*, 5794−5805.
27. Bloch, W., and Gorby, M. S. (1980) *Biochemistry 19*, 5008−5018.
28. Bale, J. R., Huang, C. Y., and Chock, P. B. (1980) *J. Biol. Chem. 255*, 8431−8436.

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