Functional Interrelationships in the Alkaline Phosphatase Superfamily: Phosphodiesterase Activity of *Escherichia coli* Alkaline Phosphatase†

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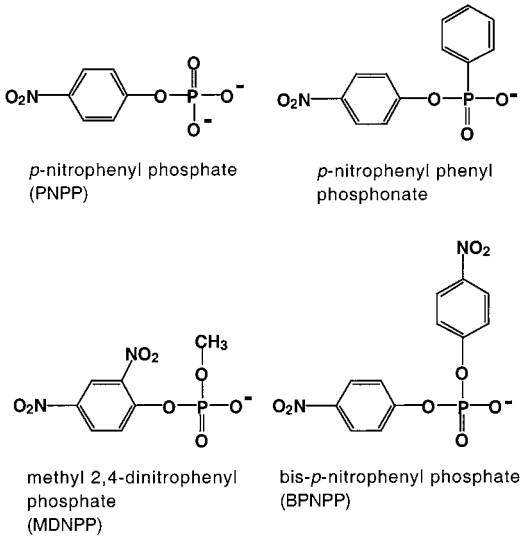
ABSTRACT: *Escherichia coli* alkaline phosphatase (AP) is a proficient phosphomonoesterase with two Zn^{2+} ions in its active site. Sequence homology suggests a distant evolutionary relationship between AP and alkaline phosphodiesterase/nucleotide pyrophosphatase, with conservation of the catalytic metal ions. Furthermore, many other phosphodiesterases, although not evolutionarily related, have a similar active site configuration of divalent metal ions in their active sites. These observations led us to test whether AP could also catalyze the hydrolysis of phosphate diesters. The results described herein demonstrate that AP does have phosphodiesterase activity: the phosphatase and phosphodiesterase activities copurify over several steps; inorganic phosphate, a strong competitive inhibitor of AP, inhibits the phosphodiesterase and phosphatase activities with the same inhibition constant; a point mutation that weakens phosphate binding to AP correspondingly weakens phosphate inhibition of the phosphodiesterase activity; and mutation of active site residues substantially reduces both the mono- and diesterase activities. AP accelerates the rate of phosphate diester hydrolysis by 10^{11}-fold relative to the rate of the uncatalyzed reaction [$(k_{cat}/K_m)/k_w$]. Although this rate enhancement is substantial, it is at least 10^6-fold less than the rate enhancement for AP-catalyzed phosphate monoester hydrolysis. Mutational analysis suggests that common active site features contribute to hydrolysis of both phosphate monoesters and phosphate diesters. However, mutation of the active site arginine to serine, R166S, decreases the monoesterase activity but not the diesterase activity, suggesting that the interaction of this arginine with the nonbridging oxygen(s) of the phosphate monoester substrate provides a substantial amount of the preferential hydrolysis of phosphate monoesters. The observation of phosphodiesterase activity extends the previous observation that AP has a low level of sulfatase activity, further establishing the functional interrelationships among the sulfatases, phosphatases, and phosphodiesterases within the evolutionarily related AP superfamily. The catalytic promiscuity of AP could have facilitated divergent evolution via gene duplication by providing a selective advantage upon which natural selection could have acted.

Two divalent metal ions are a common active site feature found in a number of evolutionarily distinct classes of enzymes, including enzymes that catalyze the hydrolysis of phosphate monoesters, diesters, and triesters (for reviews, see refs *1* and *2*). The structural similarity shared by these active sites raises the possibility that a single active site with two divalent metal ions might be able to catalyze these different classes of reactions. However, the partial negative charge on a phosphoryl oxygen atom of the phosphate monoester dianion is lost upon conversion to a diester monoanion, and the steric bulk of the added esterifying group of the diester could result in steric clashes within the active site (Scheme 1). These differences would be expected to render it difficult for an active site designed to hydrolyze phosphate monoesters to also hydrolyze phosphate diesters. In addition, linear free energy relationships, isotope effects, and results from other physical organic studies indicate that the nonenzymatic hydrolyses of phosphate monoesters and diesters proceed through different transition states (for reviews, see refs *3*−*6*; see also refs *7* and *8*).

Scheme 1



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Nevertheless, there is evidence that members of the alkaline phosphatase (AP)[1] superfamily have diverged from a common ancestor to catalyze hydrolysis of both phosphate monoesters and phosphate diesters, as well as hydrolysis of sulfate esters. Despite a very low degree of sequence homology, alkaline phosphatases and aryl sulfatases have structural homology, suggesting that they are distant evolutionary relatives (*9*, *10*). A subsequent sequence analysis that focused on the metal ligands conserved between alkaline phosphatases and aryl sulfatases identified a larger superfamily of enzymes likely to be related by divergent evolution (*11*). Among these were two genes reported to encode phosphodiesterases, PC-1 (also known as NPP1) and autotaxin (*12*, *13*). These nucleotide pyrophosphatases/phosphodiesterases appear to be capable of catalyzing reactions involving both phosphate monoesters and phosphate diesters (*12*, *14*).

We have previously shown that AP, the best characterized member of the AP superfamily, has a low level of sulfatase activity in addition to its normal phosphomonoesterase activity and have suggested that such promiscuity could facilitate the diversification of enzyme function within an enzyme superfamily (*15*, *16*, and references therein; see also ref *17*). During this study, we sought to extend our understanding of the functional interrelationships among the members of the AP superfamily by investigating whether AP can also catalyze hydrolysis of phosphate diesters. Such experiments can help to elucidate past evolutionary events and present evolutionary potential, and they contribute to our understanding of enzymatic function.

We show that AP exhibits a low level of phosphodiesterase activity, suggesting a possible mechanism for the divergent evolution of phosphodiesterases and phosphomonoesterases in the AP superfamily. Mutation of the active site arginine to serine, R166S, decreases the monoesterase activity but not the diesterase activity, suggesting that the interaction of this arginine with the nonbridging oxygen(s) of the phosphate monoester substrate accounts for much of the preferential hydrolysis of the physiological substrates. Additional mutational analysis suggests that other catalytic features of the AP active site contribute to hydrolysis of both phosphate monoesters and phosphate diesters.

MATERIALS AND METHODS

Materials. Methyl 2,4-dinitrophenyl phosphate (MDNPP) and *p*-nitrophenyl *O*-phosphorothioate were synthesized previously (*18*, *19*). *p*-Nitrophenyl phosphate (PNPP), *p*-nitrophenyl sulfate (PNPS), bis-*p*-nitrophenyl phosphate (BPNPP), and *p*-nitrophenyl phenyl phosphonate were obtained from Sigma; small amounts of contaminating PNPP were removed from BPNPP and *p*-nitrophenyl phenyl phosphonate by incubation with AP. Buffers were prepared at 25 °C, and the reported pH values were not corrected for temperature.

The expression strain SM547 of *Escherichia coli* (ΔPhoA) and plasmids for expression of wild-type (pEK48) and mutant forms of AP (R166S, pEK1152; S102A, pEK305; S102G, pEK307; D327A, pEK167; and H412A, pEK237) were generous gifts from E. Kantrowitz (*20*−*23*). The D327A/H412A mutant was constructed by digestion of plasmids pEK167 and pEK237 with *Eco*NI and *Hin*dIII. The ∼1.5 kb fragment encoding the D327A mutation, from digestion of pEK167, was ligated into the ∼4.5 kb fragment from digestion of pEK237 that contained the H412A mutation, to yield the plasmid pAP327A/412A which encodes the D327A/H412A double mutant. Sequences were confirmed by dideoxy sequencing.

Concentrations of substrate stock solutions were determined by absorbance following complete alkaline hydrolysis (1 M NaOH, 85 °C) using the following extinction coefficients: $\epsilon_{410} = 1.62 \times 10^4$ for *p*-nitrophenolate ion (*24*) and $\epsilon_{360} = 1.47 \times 10^4$ for 2,4-dinitrophenolate ion (*25*).

Alkaline Phosphatase Purification. The purification protocol was modified from that previously described (*20*). *E. coli* strain SM547 harboring pEK48 (wild type) or mutant plasmid was grown to saturation in 1× YT medium, with 100 μg/mL carbenicillin. Due to the long period of growth, a second aliquot of carbenicillin was added after growth had proceeded for 8−12 h. Following osmotic shock and centrifugation, the supernatant was adjusted to 1 mM $MgCl_2$, 100 μM $ZnSO_4$, and 10 mM Tris (pH 7.4), heated rapidly to 80 °C, incubated for 10 min to denature proteins other than AP, and cooled to 0 °C. Two centrifugations (30 min at 20 000 rpm in a JA-20 rotor) were required to remove the precipitated protein. The sample was passed through a 0.45 μm cellulose filter (Lida Manufacturing Corp.), loaded onto an 8 mL source Q column (Pharmacia), washed with 4 column volumes of buffer A [10 mM Tris (pH 7.4), 100 μM $ZnSO_4$, and 1 mM $MgCl_2$], and eluted with a linear gradient of 10 to 120 mM NaCl in buffer A. Following concentration by centrifugation through a filter (50 kDa cutoff, Amicon), the sample was loaded onto a gel filtration column (Superose 12, Pharmacia) and run with 0.1 M NaMOPS (pH 8.0), 1 mM $MgCl_2$, and 100 μM $ZnSO_4$. Peak fractions were pooled and dialyzed extensively against buffer A to remove inorganic phosphate (P_i). In some instances, additional purification of the wild-type enzyme was achieved using phenyl arsenate affinity chromatography (*26*).

Typical yields for an 8 L growth were 10−20 mg of pure protein. A single band was observed by Coomassie blue staining of SDS−polyacrylamide gels, with an estimated purity of >99%. The ratio of phosphomonoesterase activity to phosphodiesterase activity was the same for different enzyme preparations. Protein concentrations were determined by the Bradford assay using BSA as a standard and by absorbance at 278 nm [$\epsilon_{278,AP} = 6.7 \times 10^4$ M^{-1} cm^{-1} (*27*)], which agreed to within 10%. Subsequently, protein concentrations were routinely determined by activity assays using 0.1 mM PNPP, 0.1 M MOPS (pH 8.0), and 0.5 M NaCl [wild-type AP, $k_{cat} = 28$ s^{-1}; R166S AP, $k_{cat} = 1$ s^{-1} (*20*)]. There was no significant change in activity upon storage at 4 °C for at least 1 year.

Kinetic Measurements. Reactions were carried out with 0.1 M NaMOPS (pH 8.0) and 0.5 M NaCl at 25 °C with 1×10^{-10} to 5×10^{-6} M AP, depending on the substrate and enzyme (wild type or mutants). For standard assays, 0.1−1 mM phosphate diester substrate or 0.2−1 μM phosphate

[1] Abbreviations: AP, *E. coli* alkaline phosphatase; BPNPP, bis-*p*-nitrophenyl phosphate; MDNPP, methyl 2,4-dinitrophenyl phosphate; NaCAPS, sodium 3-(cyclohexylamino)-1-propanesulfonate; NaCHES, sodium 2-(cyclohexylamino)ethanesulfonate; NaMOPS, sodium 3-(*N*-morpholino)propanesulfonic acid; P_i, inorganic phosphate; PNPP, *p*-nitrophenyl phosphate.

monoester substrate was used. At protein concentrations of $<1 \times 10^{-9}$ M, zinc sulfate (10 μM) and magnesium chloride (100 μM) were added and were necessary for full activity, presumably because of metal ion dissociation at these low enzyme concentrations. Addition of these metal ions had no significant effect on reactions carried out at higher AP concentrations. Individual reactions (0.8 mL) were carried out in quartz cuvettes, and reaction progress was followed using a Uvikon 9310 UV−vis spectrophotometer. The change in the extinction coefficient at pH 8.0, $\Delta\epsilon_{410}$ for release of *p*-nitrophenolate, is 1.62×10^4 M^{-1} cm^{-1} (*24*), and $\Delta\epsilon_{360}$ for 2,4-dinitrophenolate is 1.47×10^4 M^{-1} cm^{-1} (*25*). For hydrolysis of bis-*p*-nitrophenyl phosphate, $\Delta\epsilon_{410}$ at pH 8.0 is 3.2×10^4 M^{-1} cm^{-1} (twice the magnitude of that of PNPP, because after release of the first *p*-nitrophenolate group to give PNPP the second *p*-nitrophenolate is rapidly cleaved).

Reactions were routinely followed to completion (more than five half-lives), and rate constants were obtained by nonlinear least-squares fits to the data (Kaleidagraph, Synergy Software); however, for the slowest reactions, initial rates were used (up to 5% completion). All fits gave R^2 values of >0.95, and duplicate reactions gave rate constants within 5%. Reaction velocities were proportional to the AP concentration in all cases. The apparent second-order rate constant, k_{cat}/K_m, was determined by varying the concentration of substrate at concentrations sufficiently low such that the reaction velocity was linearly proportional to substrate concentration; typically, the substrate concentration was varied over a 10−100-fold range.

Heat Inactivation of AP. Aliquots of incubation buffer [0.1 M NaMOPS (pH 8.0) and 0.5 M NaCl] were preheated to 90 °C in a water bath. AP was added to a final concentration of 200 nM, rapidly mixed, and returned to the water bath. After incubation for 2−90 min, samples were removed and immediately cooled on ice. After 10 min on ice, the samples were centrifuged in a microcentrifuge to collect condensation and an aliquot was added to a quartz cuvette and equilibrated at 25 °C for 15 min prior to addition of substrate. Reactions were then initiated by adding a small volume of phosphate diester or phosphate monoester substrate. For phosphodiesterase assays, we used 0.1−1 mM MDNPP and 100 nM wild-type or R166S AP, and for phosphomonoesterase assays, we used 0.1 mM PNPP and either 1 nM wild-type AP or 100 nM R166S AP. Initial rates were followed to 1−5% completion, and product formation was linear in all cases ($R^2 \geq 0.95$).

pH Dependence of Phosphodiesterase and Phosphatase Activities. The pH dependence was carried out for the R166S mutant because this enzyme binds P_i much more weakly than the wild-type enzyme, circumventing problems from product inhibition (ref *20* and manuscript in preparation). The buffers used in these experiments (and their pH values at 25 °C) were as follows: NaMOPS (7.0−8.0), NaCHES (8.0−10.0), and NaCAPS (9.4−9.9). The final concentration of buffer was 100 mM, and the ionic strength was held constant at 1 M with NaCl. The k_{cat}/K_m values for PNPP were determined with 0.1−5 μM PNPP and 5−500 nM R166S AP. The k_{cat}/K_m values for MDNPP were determined with 1−10 mM MDNPP and 50−500 nM R166S AP. The concentrations of substrate and enzyme were varied at the upper and lower pH extreme to verify that the apparent rate constant was first-order in the concentration of both substrate and enzyme, corresponding to k_{cat}/K_m. To facilitate comparison of the pH dependence for k_{cat}/K_m with PNPP and MDNPP as substrates, the rate constants at each pH were normalized by dividing by the maximal value of k_{cat}/K_m. An equation for a single ionizable group [k_{cat}/K_m(normalized) $= 1/(1 + pH/pK_a)$] was fit to the data using nonlinear least squares (Kaleidagraph, Synergy Software).

P_i Inhibition. The concentration of sodium P_i was varied at constant enzyme and substrate concentrations. Subsaturating substrate was used so that the inhibition constant was equal to the dissociation constant for binding of P_i. For wild-type AP phosphomonoesterase activity, 0.2 μM *p*-nitrophenyl *O*-phosphorothioate was used as the substrate ($\Delta Abs_{410} = 0.003$); this low substrate concentration was required to avoid product inhibition. *p*-Nitrophenyl *O*-phosphorothioate was a convenient substrate, because the lower catalytic activity of AP toward this substrate allowed both monoesterase and diesterase activities to be assayed at the same enzyme concentration, and in some cases in the same cuvette. Control reactions with a lower concentration of wild-type AP and 0.2 μM PNPP yielded the same inhibition constant, within 10%, as observed with *p*-nitrophenyl *O*-phosphorothioate. Inhibition of phosphodiesterase activity was determined with 0.1 mM MDNPP under the standard assay conditions. Rate constants were determined from initial rates in time courses with $\leq$0.2 μM product formed to avoid product inhibition. For R166S AP, 1 μM PNPP and 1 mM MDNPP were used under the standard assay conditions for phosphomonoesterase and phosphodiesterase assays, respectively. Higher substrate concentrations could be used with the mutant enzyme, because P_i binding is weaker (*20*).

We considered the following alternative as a potential explanation for the apparent phosphodiesterase activity of AP. If a phosphodiesterase contaminant preferentially released the methoxy substituent of MDNPP rather than the 2,4-dinitrophenolate substituent, then the release of 2,4-dinitrophenolate from MDNPP would require AP, even if AP had no phosphodiesterase activity. If this scenario occurred, then both enzymes would be required to observe the phosphodiesterase activity in the colorometric assay employed and inhibition of this observed activity could simply reflect inhibition of AP. This alternative explanation for the ability of P_i to inhibit the phosphodiesterase activity was eliminated as follows. First, this alternative reaction scheme was modeled using Kinsim (*28*) with the known rate constants for AP catalysis of phosphate monoesters. The scheme predicts a lag phase in the release of the dinitrophenolate ion in the presence of high concentrations of the AP inhibitor, P_i, as the putative reaction intermediate, 2,4-dinitrophenyl phosphate, would need to accumulate before it could effectively compete with the P_i for cleavage by AP. However, no lag was detected. A second, independent line of evidence also indicates that this model does not hold. *p*-Nitrophenyl phenyl phosphonate (Scheme 1), which has a P−C bond that would prevent hydrolysis of its second substituent, was found to react at a rate similar to that of bis-*p*-nitrophenyl phosphate (BPNPP, Table 2). This activity is also inhibited by P_i with the same K_i as for the reactions of phosphate monoesters for both wild-type and R166S AP (data not shown).

Table 1: Phosphomonoesterase and Phosphodiesterase Activities of Alkaline Phosphatase Mutants[a]

	k_{cat}/K_m (M^{-1} s^{-1})	
	PNPP	MDNPP
wild type	3×10^7	1×10^2
D327A/H412A	$\leq 3 \times 10^2$ [b]	$\leq 2 \times 10^0$ [c]
S102A	$\leq 5 \times 10^2$ [b]	$\leq 3 \times 10^{-2}$ [c]
S102G	$\leq 2 \times 10^0$ [b]	$\leq 3 \times 10^{-3}$ [d]

[a] In 0.1 M NaMOPS (pH 8.0) and 0.5 M NaCl at 25 °C; the apparent second-order rate constant, k_{cat}/K_m, is reported per active site. [b] The phosphomonoesterase activity was linearly dependent upon the concentration of enzyme added, and the values of k_{cat}/K_m were calculated from the concentration of AP. However, K_m values for PNPP were in the low micromolar range for each of these mutants, similar to that of the wild type (data not shown). It is therefore possible that low levels of wild-type enzyme are responsible for the observed activity. Such concentrations of AP could arise from translation errors that are on the order of 1 in 10^5-10^7. Inhibition of the phosphatase activity of D327A/H412A revealed tight binding of P_i, consistent with the presence of wild-type contamination (data not shown). The limits for k_{cat}/K_m with S102A and S102G AP are similar to the values that were reported previously (*21*). [c] The observed phosphodiesterase activity of these mutants was linearly dependent upon the concentration of enzyme, but we could not establish whether these low levels of activity were due to AP or to the presence of contaminants. Therefore, the values of k_{cat}/K_m that were calculated from the observed phosphodiesterase activity are upper limits for the activity of these AP mutants. [d] No phosphodiesterase activity could be detected with this mutant, whereas a k_{cat}/K_m value of 3×10^{-3} M^{-1} s^{-1} would have given a 2-fold rate increase at the highest enzyme concentration that was employed.

Table 2: Phosphodiesterase Activity of Wild-Type and R166S Alkaline Phosphatase[a]

phosphate diester substrate	k_{cat}/K_m (M^{-1} s^{-1})		k_{rel}
	wild type	R166S	wild type/ R166S
methyl 2,4-dinitrophenyl phosphate	130	45	3
bis-*p*-nitrophenyl phosphate	0.050	0.050	1
p-nitrophenyl phenyl phosphonate	0.030	0.060	0.5

[a] In 0.1 M NaMOPS (pH 8.0) and 0.5 M NaCl at 25 °C. The P_i inhibition experiments (see Figure 3) were repeated with each phosphate diester substrate to confirm that AP was responsible for the observed phosphodiesterase activity (data not shown), as a phosphodiesterase contaminant could have had different activity with phosphate diesters having different substituents.

RESULTS

As AP appears to be evolutionarily related to several phosphodiesterases and has active site features similar to those of other phosphodiesterases, we sought to determine whether AP has activity toward phosphate diester substrates. Assay of a commercial preparation of *E. coli* AP with a phosphate diester substrate (MDNPP, Scheme 1) revealed a low level of phosphodiesterase activity. If it is assumed that the activity came from AP, this gave an apparent second-order rate constant, k_{cat}/K_m, of 130 M^{-1} s^{-1}. However, *E. coli* AP has previously been reported to lack phosphodiesterase activity (*29, 30*), and the low observed activity could have been provided by a minor contaminant. It has been suggested that some enzymes exhibit both phosphomonoesterase and phosphodiesterase activity, although it is often difficult to exclude the possibility of a contaminating enzyme (*29, 31−36*). Below we outline several experiments which provide strong evidence for a phosphodiesterase activity of AP.

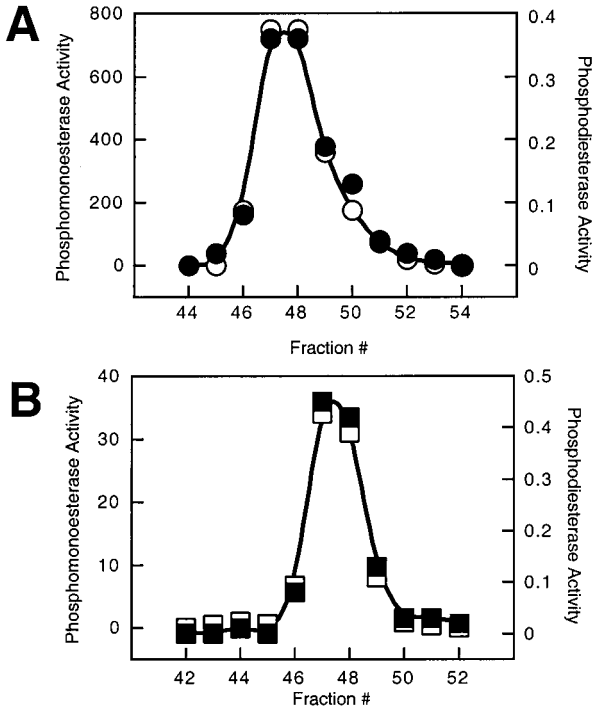

FIGURE 1: Copurification of phosphomonoesterase and phosphodiesterase activities. Activity profiles from Superose 12 gel filtration chromatography for wild-type AP (A) and R166S AP (B). Phosphomonoesterase activity [PNPP hydrolysis (○ and □)] and phosphodiesterase activity [MDNPP hydrolysis (● and ■)] are given in units of micromolar product formed per minute per milliliter. Activity assays were performed under the standard assay conditions (Materials and Methods) with 1 mM PNPP or 1 mM MDNPP.

Copurification of Phosphomonoesterase and Phosphodiesterase Activities. AP was overexpressed in an *E. coli* strain deficient for AP, and several purification steps were carried out in an attempt to separate the phosphodiesterase activity from the phosphomonoesterase activity of AP (see Materials and Methods). Osmotic shock was used to enrich for AP, which is a periplasmic protein. Following osmotic shock, two peaks of phosphodiesterase activity were separated by anion exchange chromatography. One of the phosphodiesterase peaks coincided with the AP elution peak, and upon subsequent anion exchange and gel filtration chromatography, only a single phosphodiesterase peak was observed. This phosphodiesterase activity profile could be superimposed with that for the phosphomonoesterase activity (Figure 1A and data not shown), consistent with a single protein exhibiting both activities.

Although copurification of the two catalytic activities suggested that AP is responsible for both activities, it remained possible that these purification techniques failed to resolve two distinct enzymes or that a phosphodiesterase remained associated with AP. We therefore sought to compare the physical properties of the enzyme(s) catalyzing these two reactions.

Heat Inactivation of AP. Inactivation of AP at 90 °C for varying times was followed by assaying for phosphomonoesterase activity [Figure 2A (○)]. Aliquots from this sample were also used to follow phosphodiesterase activity. The phosphodiesterase activity displayed an identical inactivation profile (●), suggesting that both activities arise from AP. A model in which a distinct phosphodiesterase associated with AP is heat denatured immediately following denatur-

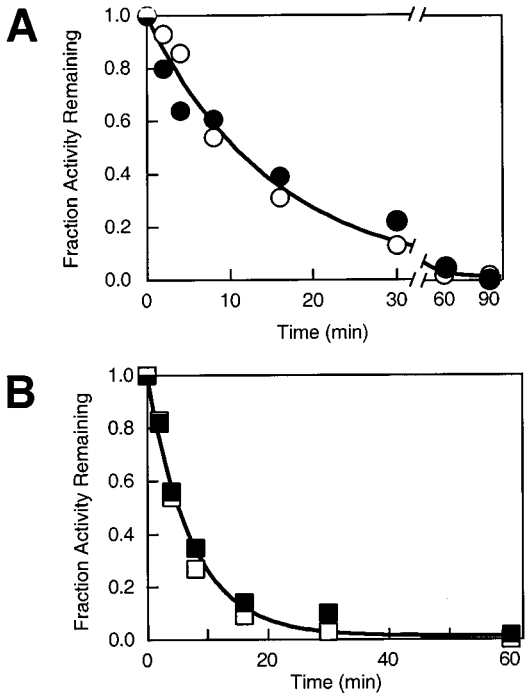

FIGURE 2: Simultaneous heat inactivation of phosphomonoesterase and phosphodiesterase activities. Inactivation of phosphomonoesterase activity [PNPP hydrolysis (○ and □)] and phosphodiesterase activity [MDNPP hydrolysis (● and ■)] are shown for wild-type AP (A) and R166S AP (B). To facilitate comparison, activity remaining was normalized by dividing the observed activity by the amount of activity prior to incubation at 90 °C. The curves represent nonlinear least-squares fits of the data for both substrates to a first-order process and give rate constants of 0.067 ± 0.007 min^{-1} for the wild type (A; $R^2 = 0.99$) and 0.15 ± 0.01 min^{-1} for R166S AP (B; $R^2 = 0.99$). The values obtained from the combined fits to the loss of phosphomonoesterase and phosphodiesterase activity were the same, within error, as the values from the individual fits (0.070 ± 0.008 and 0.061 ± 0.010 min^{-1} for wild-type AP and 0.15 ± 0.01 and 0.14 ± 0.01 min^{-1} for R166S AP).

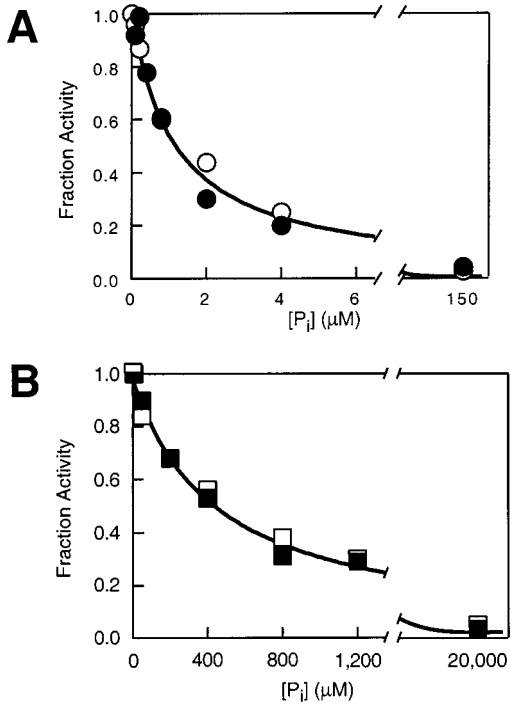

FIGURE 3: Identical inhibition of the phosphomonoesterase and phosphodiesterase activities by inorganic phosphate. Inhibition of phosphomonoesterase activity [*p*-nitrophenyl *O*-phosphorothioate hydrolysis (○ and □)] and phosphodiesterase activity [MDNPP hydrolysis (● and ■)] for wild-type AP (A) and R166S AP (B). For comparison, activity was normalized by dividing the observed rate constant in the presence of inhibitor by the rate constant in the absence of inhibitor. The lines represent nonlinear least-squares fits to the combined data for competitive inhibition of phosphomonoesterase and phosphodiesterase activities and give a K_i of 1.1 ± 0.1 μM for the wild type ($R^2 = 0.99$) and a K_i of 460 ± 40 μM for R166S ($R^2 = 0.99$). Individual fits of the phosphomonoesterase and phosphodiesterase data give values of K_i that are the same, within error (1.2 ± 0.1 and 1.0 ± 0.2 μM for wild-type AP and 460 ± 40 and 430 ± 30 μM for R166S AP).

ation of AP cannot be eliminated by these data, but is eliminated by the results described below.

Inhibition of Phosphomonoesterase and Phosphodiesterase Activities. Inhibition provided an independent test of whether AP has phosphodiesterase activity. The inhibition constant for competitive inhibition of AP phosphomonoesterase activity by P_i was observed to be 1.1 ± 0.1 μM [Figure 3A (○)], similar to that measured previously (*15*, *37*, *38*). P_i was also found to be a competitive inhibitor of the phosphodiesterase activity (●). The phosphodiesterase and phosphomonoesterase activities were inhibited by P_i with the same K_i, providing further evidence that both reactions are catalyzed by AP.

A Mutation in AP Changes the Behavior of the Phosphodiesterase. A change in the phosphodiesterase behavior resulting from a point mutation in AP would provide strong evidence that AP is indeed responsible for the diesterase activity. An active site mutant of AP, R166S, was therefore purified and its phosphodiesterase activity characterized. As for the wild type, phosphodiesterase activity copurified with R166S AP through ion exchange and gel filtration chromatography (Figure 1B and data not shown). Both the phosphomonoesterase and phosphodiesterase activities displayed identical heat inactivation profiles (Figure 2B), as also observed for wild-type AP.

The phosphomonoesterase activity of R166S AP was inhibited weakly by P_i, with a K_i value of 460 ± 40 μM [Figure 3B (□)], in agreement with previous results [K_i = 420 ± 30 μM (*20*)]. The same inhibition constant was observed for P_i inhibition of the phosphodiesterase activity [Figure 3B (■)]. The change in the inhibition constant for the phosphodiesterase activity from 1 to 460 μM in response to mutation at the active site of AP provides compelling evidence that AP is responsible for the phosphodiesterase activity.

Mutational Analysis of the Phosphodiesterase Activity. Phosphatase and phosphodiesterase activities of several mutants of AP were examined to test whether similar catalytic mechanisms are used in the two reactions. Extensive studies of AP have established that AP requires two Zn^{2+} ions for hydrolysis of phosphate monoesters (for reviews, see refs *39*−*41*), with Zn_{II} stabilizing the alkoxide of the Ser102 nucleophile (Figure 4). Mutations of Ser102 lead to drastically reduced phosphatase activity (*21*, *42*, *43*). The other zinc ion, Zn_I, is thought to stabilize the leaving group in the first step of the reaction, formation of the phosphoserine intermediate, and to activate a water molecule as the Zn·hydroxide for subsequent hydrolysis of the covalent intermediate (Figure 4). Mutations that disrupt this metal ion binding site have also been shown to greatly reduce phos-

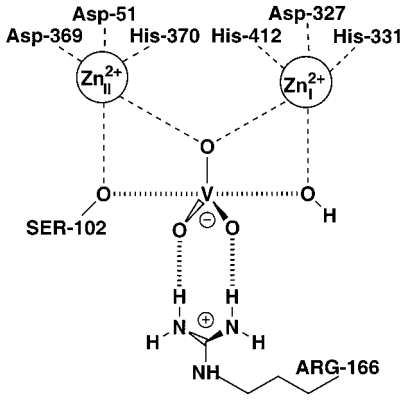

FIGURE 4: Active site of *E. coli* AP. The structure of AP covalently bound to vanadate, a transition state analogue for phosphoryl transfer, was determined by Holtz et al. (*65*). The Zn^{2+} ions are ~4 Å apart, and their proximity to Ser102 and the vanadyl oxygen atoms suggests there are electrostatic interactions at these positions. Arg166 makes a bidentate hydrogen bonding interaction to the vanadyl oxygens and presumably makes a similar interaction with the phosphoryl oxygen atoms in the transition state for reaction of phosphate monoesters (*41*, *65*).

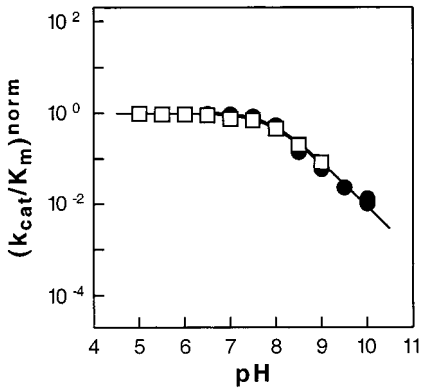

FIGURE 5: Identical pH−rate profiles for the phosphomonoesterase [PNPP (●)] and phosphodiesterase [MDNPP (□)] activities of R166S AP. To facilitate comparison of the two activities, the value of k_{cat}/K_m was normalized by dividing by the maximal observed value of k_{cat}/K_m. A nonlinear least-squares fit of the equation for a single ionization to each of the pH−rate profiles yielded pK_a values of 7.92 ± 0.1 and 7.95 ± 0.1 for the monoesterase and diesterase activities, respectively.

phatase activity (*22*, *23*). If the phosphodiesterase reaction follows the same mechanism as the phosphatase reaction, mutations that remove the serine or disrupt the metal ion binding site should eliminate or severely reduce the phosphodiesterase activity. We therefore expressed and purified the S102A, S102G, and D327A/H412A AP mutants and characterized their phosphodiesterase activities.

As expected, each of the mutants examined had greatly reduced phosphatase activity (Table 1). The phosphodiesterase activity was also decreased by at least 10^2-fold for D327A/H412A and by at least 10^3-fold for mutations of S102, suggesting that both Zn_I and Ser102 play important catalytic roles in the phosphodiesterase reaction. In each case, the reported values of k_{cat}/K_m were limits determined by enzyme purity and/or assay limits. The crystal structures of S102A and S102G do not reveal any global structural rearrangements (*21*), and circular dichroism spectroscopy suggests that there are no differences in the secondary structures of R166S, D327A/H412A, and wild-type AP (data not shown). The large deleterious effect observed upon mutation of S102 to alanine or glycine suggests that S102 is directly involved in catalyzing the hydrolysis of phosphate diesters, as it is in the reaction of phosphate monoesters.

pH Dependence for Phosphodiesterase and Phosphomonoesterase Activities. As an additional probe of the mechanism for AP-catalyzed phosphate diester hydrolysis, we compared its pH dependence with that for hydrolysis of phosphate monoesters. Figure 5 shows the pH dependence for k_{cat}/K_m with PNPP and MDNPP with R166S AP. The same pK_a of 8.0 ± 0.1 is observed for the two activities, supporting the notion that both reactions are occurring at the same active site. This inactivating pK_a may be due to ionization of a zinc-coordinated water molecule in the free enzyme, to form Zn·OH and block the binding of substrate (manuscript in preparation). The observation of the same pK_a for hydrolysis of a phosphate diester and a phosphate monoester provides further evidence that both reactions occur at the same active site.

Wild-Type and R166S AP Activity with Phosphate Diester Substrates. Reactions of several different phosphate diester substrates with wild-type and R166S AP were investigated, and the k_{cat}/K_m values are shown in Table 2. Both wild-type and R166S AP are more active for cleavage of methyl 2,4-dinitrophenyl phosphate (MDNPP) than for cleavage of substrates with a *p*-nitrophenolate leaving group, as expected because the lower pK_a of the 2,4-dinitrophenolate leaving group renders MDNPP a more activated compound. *p*-Nitrophenyl phenyl phosphonate, a phosphate diester analogue, is also a substrate for both wild-type and R166S AP with reactivity similar to that for bis-*p*-nitrophenyl phosphate (BPNPP, Table 2).

The AP phosphomonoesterase and phosphodiesterase activities and rate enhancements were compared for a phosphate monoester (PNPP) and a phosphate diester (BPNPP) with the same leaving group (Table 3). These compounds have different nonenzymatic reactivities, so catalytic proficiencies were calculated to allow comparison of the efficiency of AP as a phosphodiesterase to its efficiency as a phosphomonoesterase. The catalytic proficiency is defined as the ratio of the apparent second-order rate constant (k_{cat}/K_m) divided by the second-order rate constant (k_w) for the uncatalyzed hydrolysis of the same compound [catalytic proficiency = $(k_{cat}/K_m)/k_w$ (*44*)]. AP has a catalytic proficiency of 10^{11} for hydrolysis of BPNPP, which approaches the catalytic proficiencies of several enzymes for their physiological substrates (*44*). Nevertheless, the catalytic proficiency of 10^{11} for the phosphate diester substrate is 10^6-fold lower than the catalytic proficiency of 10^{17} for the phosphate monoester substrate (Table 3).

The active site arginine of AP has been implicated in binding to the nonbridging phosphoryl oxygens of the substrate (refs *20* and *45*−*48* and unpublished results). Consistent with previous observations (*20*, *46*, *48*), mutation of arginine to serine (R166S) decreased the monoesterase activity substantially (~300-fold, Table 3).[2] This arginine was predicted to interfere with the ability of AP to act as a phosphodiesterase, because of potential electrostatic and steric clashes with bound phosphate diester substrates. However, the R166S mutant did not have reduced phosphodiesterase activity with several substrates (Table 2). This result suggests that there is not a significant energetic barrier

Table 3: Catalytic Proficiency of Alkaline Phosphatase toward Phosphate Monoester and Phosphate Diester Substrates

	PNPP (monoester)			BPNPP (diester)		
	k_{cat}/K_m[a] (M^{-1} s^{-1})	k_w[b] (M^{-1} s^{-1})	catalytic proficiency[c]	k_{cat}/K_m[a] (M^{-1} s^{-1})	k_w[b] (M^{-1} s^{-1})	catalytic proficiency[c]
wild type	3×10^7	5×10^{-11} [d]	6×10^{17}	5×10^{-2}	2×10^{-13} [e]	3×10^{11}
R166S	1×10^5	5×10^{-11} [d]	2×10^{15}	5×10^{-2}	2×10^{-13} [e]	3×10^{11}
ratio	300			1		

[a] Enzymatic data were collected at pH 8.0 and 25 °C and are reported per active site. [b] k_w is the second-order rate constant for nonenzymatic hydrolysis. [c] Catalytic proficiency is defined as the ratio $(k_{cat}/K_m)/k_w$ and is unitless. [d] From ref *75*; the value at 39 °C was corrected to 25 °C using the reported temperature dependence. [e] From ref *76*; the value at 80 °C was corrected to 25 °C using the reported temperature dependence.

for rotation of the arginine side chain out of the active site. This movement may be facilitated by the high charge density of the active site and location of this residue at the surface of the protein without an extensive network of interactions that position it (*49*).

DISCUSSION

A low level of promiscuous activity toward an alternative reaction could facilitate the evolution of new enzymes after gene duplication by providing a selective advantage (*16*, *50*−*55*). It is therefore of interest to determine the ability of enzymes to catalyze alternative reactions to gain insight into past evolutionary events and present evolutionary potential. The AP superfamily is only one of many examples of diverse enzyme superfamilies that appear to have diverged from a common ancestor to catalyze a variety of chemical transformations (*11*; for reviews, see refs *17* and *56*−*58*). AP exhibits a low level of sulfatase activity in addition to its proficient phosphatase activity (*15*). This provides functional support for the proposed evolutionary relationship between arylsulfatases and alkaline phosphatases (*9*, *10*). More recent observation of sequence homology between AP and nucleotide pyrophosphatases/phosphodiesterases (*11*, *14*) prompted us to examine whether AP can also catalyze this additional reaction.

The experiments described herein demonstrate that *E. coli* AP catalyzes phosphate diester hydrolysis. The phosphomonoesterase and phosphodiesterase activities copurify; both activities exhibit the same pH dependence, and P_i is a competitive inhibitor for both activities, inhibiting each with the same inhibition constant. Furthermore, a point mutation that weakens P_i binding to AP weakens P_i inhibition of the phosphodiesterase activity to the same extent. The rate enhancement of AP as a phosphodiesterase is substantial, 10^{11}-fold relative to the nonenzymatic reaction (Table 3). On one hand, this efficient catalysis was unexpected because phosphate diesters differ sterically and electrostatically from phosphate monoesters, the physiological substrates for AP, and because bonding and charge distribution in the transition state for hydrolysis of phosphate monoesters and diesters also differ (*3*, *6*). On the other hand, many phosphodiesterases have two divalent metal ions that are bound in a manner similar to that of the metal ions of AP, including phospholipase C, P1 nuclease, and DNA pol I exonuclease (for reviews, see refs *1* and *2*). Furthermore, the distantly related nucleotide pyrophosphatases/phosphodiesterases catalyze reactions with both mono- and disubstituted phosphate esters (*12*, *14*). The two Zn^{2+} ions in the AP active site could accelerate the hydrolysis of both phosphate monoesters and diesters via common catalytic strategies, such as activation and positioning of the nucleophile and stabilization of the leaving group (see refs *3*, *49*, and *59*−*64* and references therein). The large deleterious effects observed for the phosphodiesterase activity upon mutation of the Zn_I site and Ser102 suggest that similar catalytic features are involved in the hydrolysis of phosphate monoesters and diesters.

Comparison of Phosphodiesterase and Phosphomonoesterase Activities of AP. Although the rate enhancement of 10^{11}-fold for AP-catalyzed hydrolysis of phosphate diesters is substantial, k_{cat}/K_m is small relative to k_{cat}/K_m for hydrolysis of phosphate monoesters and the phosphomonoesterase rate enhancement is $\geq 10^6$-fold larger than the phosphodiesterase rate enhancement (Table 3). This raises the question of why AP is a more efficient catalyst of phosphate monoester hydrolysis than of phosphate diester hydrolysis.

Consideration of linear free energy relationships in nonenzymatic reactions of phosphate mono- and diesters suggests that the catalysis expected simply from nucleophilic activation by a Zn^{2+} ion and leaving group stabilization by another Zn^{2+} ion are similar for both reactions. In contrast, mutation of R166 to serine reduces the phosphomonoesterase activity ~300-fold, without adversely affecting the phosphodiesterase activity (Table 3).[2] R166 appears to interact with the nonbridging phosphoryl oxygen atoms of a phosphate monoester (*65*) and could stimulate the phosphomonoesterase reaction by helping to bind and position the substrate, by stabilizing charge rearrangement in the transition state, and/or by desolvating the phosphoryl group (*49*, *65*−*67*). This interaction does not contribute to the reaction of phosphate diesters (Tables 2 and 3), presumably because the introduction of steric bulk upon esterification of an oxygen of the transferred phosphoryl group prevents this interaction with the phosphate diester substrate. Nevertheless, the presence of this residue does not hinder the phosphodiesterase reaction. Rotation of the arginine side chain out of the active site without a significant energetic barrier could account for this absence of an inhibitory effect (see the Results). Thus, interaction with R166 accounts for a considerable amount of the greater efficiency of phosphate monoester hydrolysis.

Nevertheless, R166S AP remains a more efficient phosphomonoesterase than a phosphodiesterase by at least 10^4-fold (Table 3). The simplest model to account for this difference is that the greater negative charge of the phosphate monoester relative to the phosphate diester allows more favorable electrostatic interactions with the Zn^{2+} ions in the

[2] It should be noted that the chemical step is not rate-limiting for k_{cat}/K_m with PNPP as a substrate for the wild-type enzyme (*7*, *19*, *77*). Thus, 300-fold represents a lower limit for the effect of the R166S mutation on the chemical step. The R166S mutation reduces k_{cat}/K_m for alkyl phosphates, for which the chemical step limits reaction of both the wild type and mutant by ~10^4-fold (ref *47* and unpublished results).

AP active site. Natural phosphodiesterases may instead use binding interactions with the additional substituent on the transferred phosphoryl group to ensure specificity and to enhance the rate of the reaction (*5*, *14*).

Other Examples of Promiscuous Phosphodiesterase Activity. Binuclear metal ion centers appear to be particularly well suited for catalyzing phosphoryl transfer, as there are many examples of enzymes that utilize this catalytic motif and many of these act on phosphate diester substrates (*1*, *2*). Recent findings that other two metal ion catalysts have promiscuous phosphodiesterase activity provide additional evidence for the inherent catalytic potential of binuclear clusters for catalyzing phosphate diester hydrolysis. The bacterial phosphotriesterase (*68*), an aminopeptidase from *Streptomyces* (*69*), and now alkaline phosphatase have each been shown to provide substantial rate enhancements for cleavage of a phosphate diester bond [$(k_{cat}/K_m)/k_w = 10^{10}-10^{15}$]. As each of these enzymes catalyzes different chemical transformations in their physiological roles and has independent evolutionary histories, this suggests that two metal ions positioned ~4 Å apart are especially conducive to catalysis of phosphate diester hydrolysis. This observation has been further developed with model binuclear metal ion complexes, some of which provide substantial catalysis of phosphate diester hydrolysis (e.g., refs *70*−*72*). Each of these observations points to an inherent propensity of two metal ion centers for catalyzing hydrolysis of phosphate diesters.

Nevertheless, despite large rate enhancements for reactions of phosphate diesters, both AP and the phosphotriesterase provide much larger rate enhancements for their physiological reactions and their rate enhancements of phosphate diester hydrolysis are considerably smaller than those of natural phosphodiesterases (*44*, *73*). Given that two divalent metal ions situated ~4 Å apart are the central feature of these phosphomonoesterases, phosphodiesterases, and phosphotriesterases, it is interesting to consider how individual two-metal ion centers might be tuned toward the reactions of mono-, di-, and trisubstituted phosphoryl compounds. As discussed above, the active site arginine of AP represents a single residue that contributes substantially to the preferential hydrolysis of phosphate monoesters over phosphate diesters. And for the phosphotriesterase it has been suggested that hydrophobic binding pockets favor the reaction of phosphate triesters over that of phosphate diesters. Additional, and perhaps more subtle, molecular mechanisms for specificity await further characterization of these active sites with respect to the different types of phosphoryl transfer reactions. It will be interesting to learn how reaction specificity might be changed over the course of evolution, as has apparently taken place in the AP superfamily with the divergence of phosphomonoesterases and phosphodiesterases.

Catalytic Promiscuity and Divergent Evolution. *E. coli* AP exhibits catalytic promiscuity, catalyzing hydrolysis of phosphate diesters and sulfate esters in addition to its physiological role in the hydrolysis of phosphate monoesters. These promiscuous activities suggest a possible pathway for the evolutionary diversification of the AP superfamily. Following gene duplication, an ancestral alkaline phosphatase with promiscuous activity would have had a higher probability of being optimized for one of its promiscuous activities relative to any other gene product without a low level of activity for the new reaction (*16*). This is because random drift will cause an accumulation of mutations in duplicated genes, many of which will be deleterious to structure and function, and the majority of duplicated genes will be lost (*53*). If, however, the gene is placed under selective pressure for the gene product's ability to catalyze an alternative reaction, then natural selection can bias the evolution toward a more efficient catalyst of the new reaction. Although catalytic promiscuity is expected to increase the probability for evolution of a new activity, promiscuous activity is only one of many factors that determine evolutionary potential. For example, there must be a pathway of sequential mutations, each of which confers a selectable advantage, for evolution to progress from an efficient catalyst of one reaction to an efficient catalyst of a distinct reaction (*74*). Understanding the malleability of enzyme active sites (i.e., the relative ease by which an enzyme can be optimized for a different reaction) and the consequences of the mutagenic changes that arise through the course of evolution present exciting and fundamental challenges for the future.

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REFERENCES

1. Strater, N., Lipscomb, W. N., Klabunde, T., and Krebs, B. (1996) *Angew. Chem., Int. Ed. 35*, 2024−2055.
2. Wilcox, D. E. (1996) *Chem. Rev. 96*, 2435−2458.
3. Benkovic, S. J., and Schray, K. J. (1978) in *Transition States of Biochemical Processes* (Gandour, R. D., and Schowen, R. L., Eds.) pp 493−527, Plenum, New York.
4. Cleland, W. W., and Hengge, A. C. (1995) *FASEB J. 9*, 1585−1594.
5. Herschlag, D., and Jencks, W. P. (1990) *Biochemistry 29*, 5172−5179.
6. Thatcher, G. R. J., and Kluger, R. (1989) *Adv. Phys. Org. Chem. 25*, 99−265.
7. Hengge, A. C., Edens, W. A., and Elsing, H. (1994) *J. Am. Chem. Soc. 116*, 5045−5049.
8. Hengge, A. C., Tobin, A. E., and Cleland, W. W. (1995) *J. Am. Chem. Soc. 117*, 5919−5926.
9. Bond, C. S., Clements, P. R., Ashby, S. J., Collyer, C. A., Harrop, S. J., Hopwood, J. J., and Guss, J. M. (1997) *Structure 5*, 277−289.
10. Lukatela, G., Krauss, N., Theis, K., Selmer, T., Gieselmann, V., von Figura, K., and Saenger, W. (1998) *Biochemistry 37*, 3654−3664.
11. Galperin, M. Y., Bairoch, A., and Koonin, E. V. (1998) *Protein Sci. 7*, 1829−1835.
12. Clair, T., Lee, H. Y., Liotta, L. A., and Stracke, M. L. (1997) *J. Biol. Chem. 272*, 996−1001.
13. Oda, Y., Kuo, M.-D., Huang, S. S., and Huang, J. S. (1993) *J. Biol. Chem. 268*, 27318−27326.
14. Gijsbers, B., Ceulemans, H., Stalmans, W., and Bollen, M. (2001) *J. Biol. Chem. 276*, 1361−1368.
15. O'Brien, P. J., and Herschlag, D. (1998) *J. Am. Chem. Soc. 120*, 12369−12370.
16. O'Brien, P. J., and Herschlag, D. (1999) *Chem. Biol. 6*, R91−R105.
17. Gerlt, J. A., and Babbitt, P. C. (2000) *Gen. Biol. 1*, 1−10.
18. Herschlag, D., Piccirilli, J. A., and Cech, T. R. (1991) *Biochemistry 30*, 4844−4854.
19. Hollfelder, F., and Herschlag, D. (1995) *Biochemistry 34*, 12255−12264.
20. Chaidaroglou, A., Brezinski, D. J., Middleton, S. A., and Kantrowitz, E. R. (1988) *Biochemistry 27*, 8338−8343.

21. Stec, B., Hehir, M. J., Brennan, C., Nolte, M., and Kantrowitz, E. R. (1998) *J. Mol. Biol. 277*, 647−662.
22. Xu, X., and Kantrowitz, E. R. (1992) *J. Biol. Chem. 267*, 16244−16251.
23. Ma, L., and Kantrowitz, E. R. (1994) *J. Biol. Chem. 269*, 31614−31619.
24. Applebury, M. L., Johnson, B. P., and Coleman, J. E. (1970) *J. Biol. Chem. 245*, 4968−4976.
25. Kirby, A. J., and Varvoglis, A. G. (1967) *J. Am. Chem. Soc. 89*, 415−423.
26. Brenna, O., Perrella, M., Pace, M., and Pietta, P. G. (1975) *Biochem. J. 151*, 291−296.
27. Plocke, D. J., Levinthal, C., and Vallee, B. L. (1962) *Biochemistry 1*, 373−378.
28. Barshop, B. A., Wrenn, R. F., and Frieden, C. (1983) *Anal. Biochem. 130*, 134−145.
29. Smile, D. H., Donohue, M., Yeh, M., Kenkel, T., and Trela, J. M. (1976) *J. Biol. Chem. 252*, 3399−3401.
30. Heppel, L. A., Harkness, D. R., and Hilmoe, R. J. (1962) *J. Biol. Chem. 237*, 841−846.
31. Rezende, A. A., Pizauro, J. M., Ciancaglini, P., and Leone, F. A. (1994) *Biochem. J. 301*, 517−522.
32. Yamane, K., and Maruo, B. (1978) *J. Bacteriol. 134*, 100−107.
33. Zaug, A. J., and Cech, T. R. (1986) *Biochemistry 25*, 4478−4482.
34. Fujimoto, M., Kuninaka, A., and Yoshino, H. (1974) *Agric. Biol. Chem. 38*, 785−790.
35. Polya, G. M., Brownlee, A. G., and Hynes, M. J. (1975) *J. Bacteriol. 124*, 693−703.
36. Richardson, C. C., and Kornberg, A. (1964) *J. Biol. Chem. 239*, 242−250.
37. Snyder, S. L., and Wilson, I. B. (1972) *Biochemistry 11*, 1616−1623.
38. Lazdunski, C., and Lazdunski, M. (1969) *Eur. J. Biochem. 7*, 294−300.
39. Coleman, J. E. (1992) *Annu. Rev. Biophys. Biomol. Struct. 21*, 441−483.
40. Reid, T. W., and Wilson, I. B. (1971) in *The Enzymes* (Boyer, P. D., Ed.) pp 373−415, Academic Press, New York.
41. Holtz, K. M., and Kantrowitz, E. R. (1999) *FEBS Lett. 462*, 7−11.
42. Ghosh, S. S., Block, S. C., Rokita, S. E., and Kaiser, E. T. (1986) *Science 231*, 145−148.
43. Han, R., and Coleman, J. E. (1995) *Biochemistry 34*, 4238−4245.
44. Radzicka, A., and Wolfenden, R. (1995) *Science 267*, 90−93.
45. Kim, E. E., and Wyckoff, H. W. (1989) *Clin. Chim. Acta 186*, 175−188.
46. Butler-Ransohoff, J. E., Kendall, D. A., and Kaiser, E. T. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4276−4278.
47. O'Brien, P. J., and Herschlag, D. (1999) *J. Am. Chem. Soc. 121*, 11022−11023.
48. Holtz, K. M., Catrina, I. E., Hengge, A. C., and Kantrowitz, E. R. (2000) *Biochemistry 39*, 9451−9458.
49. Kim, E. E., and Wyckoff, H. W. (1991) *J. Mol. Biol. 218*, 449−464.
50. Jensen, R. A. (1976) *Annu. Rev. Microbiol. 30*, 409−425.
51. Lewis, E. B. (1951) *Cold Spring Harbor Symp. Quant. Biol. 16*, 159−163.
52. Ohno, S. (1970) *Evolution by gene duplication*, George Allen and Unwin, London.
53. Hughes, A. L. (1994) *Proc. R. Soc. London, Ser. B 256*, 119−124.
54. Parsot, C. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5207−5210.
55. Piatigorsky, J., and Wistow, G. (1991) *Science 252*, 1078−1079.
56. Babbitt, P., Hasson, M. S., Wedekind, J. E., Palmer, D. R. J., Barrett, W. C., Reed, G. H., Rayment, I., Ringe, D., Kenyon, G. L., and Gerlt, J. A. (1996) *Biochemistry 35*, 16489−16501.
57. Babbitt, P. C., and Gerlt, J. A. (1997) *J. Biol. Chem. 272*, 30591−30594.
58. Gerlt, J. A., and Babbitt, P. C. (1998) *Curr. Opin. Chem. Biol. 2*, 607−612.
59. Steitz, T. A., and Steitz, J. A. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6498−6502.
60. Cooperman, B. S. (1976) in *Metal Ions in Biological Systems* (Sigel, H., Ed.) pp 79−125, Marcel Dekker, New York.
61. Curley, J. F., Joyce, C. M., and Piccirilli, J. A. (1997) *J. Am. Chem. Soc. 119*, 12691−12692.
62. Hendry, P., and Sargeson, A. M. (1990) in *Progress in Inorganic Chemistry: Bioorganic Chemistry* (Lippard, S. J., Ed.) pp 201−258, John Wiley & Sons, New York.
63. Herschlag, D., and Jencks, W. P. (1990) *J. Am. Chem. Soc. 112*, 1942−1950.
64. Knowles, J. R. (1980) *Annu. Rev. Biochem. 49*, 877−919.
65. Holtz, K. M., Stec, B., and Kantrowitz, E. R. (1999) *J. Biol. Chem. 274*, 8351−8354.
66. Murphy, J. E., Stec, B., Ma, L., and Kantrowitz, E. R. (1997) *Nat. Struct. Biol. 4*, 618−622.
67. Abell, K. W. Y., and Kirby, A. J. (1986) *Tetrahedron Lett. 27*, 1085−1088.
68. Shim, H., Hong, S., and Raushel, F. M. (1998) *J. Biol. Chem. 273*, 17445−17450.
69. Park, H. I., and Ming, L.-J. (1999) *Angew. Chem., Int. Ed. 38*, 2914−2916.
70. Williams, N. H., Cheung, W., and Chin, J. (1998) *J. Am. Chem. Soc. 120*, 8079−8087.
71. Tsubouchi, A., and Bruice, T. C. (1994) *J. Am. Chem. Soc. 116*, 11614−11615.
72. Molenveld, P., Kapsabelis, S., Engbersen, J. F. J., and Reinhoudt, D. R. (1997) *J. Am. Chem. Soc. 119*, 2948−2949.
73. Serpersu, E. H., Shortle, D., and Mildvan, A. S. (1986) *Biochemistry 25* (1), 68−77.
74. Schultes, E. A., and Bartel, D. P. (2000) *Science 289*, 448−452.
75. Kirby, A. J., and Jencks, W. P. (1965) *J. Am. Chem. Soc. 87*, 3209−3216.
76. Chin, J., Banaszczyk, M., Jubian, V., and Zou, X. (1989) *J. Am. Chem. Soc. 111*, 186−190.
77. Simopoulos, T. T., and Jencks, W. P. (1994) *Biochemistry 33*, 10375−10380.

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