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Sulfatase Activity of *E. coli* Alkaline Phosphatase Demonstrates a Functional Link to Arylsulfatases, an Evolutionarily Related Enzyme Family

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Recent crystallographic analyses have revealed structural similarity between alkaline phosphatases and arylsulfatases, strongly suggesting that these distinct classes of enzymes are evolutionarily related.[1,2] Superposition of 169 C_α atoms from the central β-sheet of arylsulfatase B and *E. coli* alkaline phosphatase (AP) results in the alignment of the nucleophilic residues, the phosphoryl/sulfuryl moieties at the active site, and active site divalent metal ions (Figure 1).[1] There is no significant overall sequence similarity, suggesting that these enzymes are only distantly related.[1,2] Nevertheless, a recent sequence analysis that focused on conserved metal ligands suggested that AP's and arylsulfatases are related within a larger superfamily.[3]

It was previously reported that AP has no sulfatase activity.[4] However, even a low level of activity could facilitate divergence of an enzyme encoded by a duplicated gene by providing a selective advantage, which would then allow optimization via natural selection. We therefore investigated whether AP exhibits sulfatase activity. The results establish a functional relationship between these two evolutionarily related enzymes. Such relationships can yield insight into past evolutionary pathways and present evolutionary potential.

A highly purified preparation of *E. coli* AP was found to catalyze hydrolysis of *p*-nitrophenyl sulfate (PNPS). Despite the activated leaving group, k_{cat}/K_m is low (0.01 M^{-1} s^{-1}). Nevertheless, this represents considerable catalysis, corresponding to a rate enhancement of $\sim 10^9$ relative to the nonenzymatic attack by water on PNPS.[5]

Several independent lines of evidence strongly suggest that the AP active site is responsible for this sulfatase activity. AP was overexpressed in *E. coli* strain SM547 (phoA$^-$) from plasmid pEK48 and purified from the periplasm by osmotic shock, ammonium sulfate fractionation, heat treatment, and subsequent anion exchange and gel filtration chromatography.[6] The sulfatase activity toward PNPS co-purifies with the phosphatase activity toward *p*-nitrophenyl phosphate (PNPP) upon anion exchange and gel filtration chromatography (Figure 2 and data not shown). No sulfatase activity was detectable in the periplasmic fraction from osmotic shock of *E. coli* lacking the AP-encoding plasmid. Inorganic phosphate, a strong competitive inhibitor of AP, inhibits the phosphatase and sulfatase activities with the same inhibition constant (Figure 3), suggesting that both reactions are catalyzed by the same active site. Finally, k_{cat}/K_m for the phosphatase and sulfatase activities followed the same pH dependence over the experimentally accessible range of pH 7−10.[6]

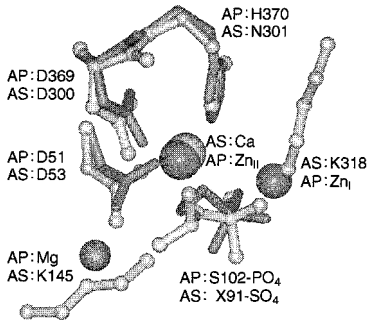

Figure 1. Local superposition of homologous active site residues for AP and arylsulfatase B (AS), adapted from refs 1 and 2. Differences between the Cα positions of D53, X91 (X = formylglycine), and D300 in AS and D51, S102, and D369 in H331Q AP,[21] respectively, were minimized by using Insight. For clarity, only selected active site residues are shown for AP (dark gray, stick representation) and AS (light gray, ball-and-stick representation). The position of the phosphorylated serine of AP (AP: S102-PO_4) coincides with that of the sulfurylated formylglycine of AS (AS: X91-SO_4), Zn_{II} (AP) coincides with Ca (AS), Zn_I (AP) with K318Nξ (AS), Mg (AP) with K145Nξ (AS), and the metal ligand H370 (AP) with the metal ligand N301 (AS). Coordinates were obtained from the PDB (1fsu and 1hjk for AS and H331Q AP, respectively).

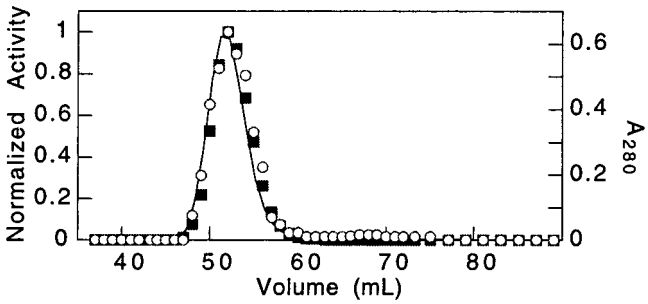

Figure 2. Co-purification of sulfatase and phosphatase activities. Sulfatase activity (PNPS hydrolysis, ○), phosphatase activity (PNPP hydrolysis, ■), and absorbance at 280 nm (line) are shown for gel filtration chromatography (100 mL Superose 12, Pharmacia) of AP. The phosphatase activity was assayed with 100 μM PNPP and the sulfatase activity with 20 mM PNPS. To allow direct comparison, both activities were normalized by dividing the observed activity for each fraction by the activity of the peak fraction.

To characterize the sulfatase activity and compare this activity to the phosphatase activity, several previously described mutants of AP were overexpressed, purified, and assayed for sulfatase activity. The mutant S102C changes the identity of the nucleophile and greatly reduces the level of phosphatase activity;[7,8] R166S removes an active site residue that interacts with the negatively charged oxygens of phosphomonoester substrates;[9] and D327A removes a bidentate ligand of Zn_I, disrupting the metal ion binding site.[10] Each of these mutations substantially reduces both the phosphatase and sulfatase activity, with effects of 10^2−10^6-fold

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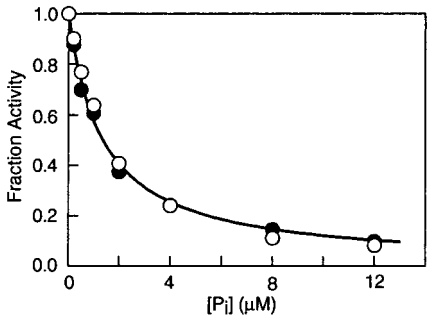

Figure 3. Coincident inhibition of phosphatase and sulfatase activities of AP by inorganic phosphate (P_i). Inhibition of phosphatase activity (PNPP hydrolysis, ○) and sulfatase activity (PNPS hydrolysis, ●) of AP. For comparison, activity was normalized by dividing the observed rate constant in the presence of inhibitor by the rate constant in the absence of inhibitor. The line represents a nonlinear least-squares fit to the combined data for competitive inhibition of both activities and gave an inhibition constant of $K_i = 1.3 \pm 0.1$ μM. Individual fits to the phosphatase and sulfatase data gave values of K_i that are the same within error (1.3 ± 0.1 and 1.4 ± 0.1 μM, respectively).

Table 1. Sulfatase and Phosphatase Activity of Alkaline Phosphatase Mutants[a]

	k_{cat}/K_m (M^{-1} s^{-1})	
	PNPP	PNPS
wild-type	3.0×10^7 [b]	1×10^{-2}
R166S	1.0×10^5 [c]	$\leq 1 \times 10^{-4}$
S102C	7.0×10^2 [d]	$\leq 1 \times 10^{-4}$
D327A	3.0×10^0 [e]	$\leq 1 \times 10^{-4}$

[a] 0.1 M NaMOPS, pH 8.0, 0.5 M NaCl at 25 °C; the apparent second-order rate constant, k_{cat}/K_m, is reported per active site. Concentration of AP was varied between 1×10^{-5} and 10 μM for the phosphatase assays and 0.5 and 50 μM for the sulfatase assays. These rate constants were independent of enzyme and substrate concentrations; each was varied 5- to 50-fold. AP retained full activity during the course of the assays (up to 72 h).[6] [b] Within error of the previously reported value of 4×10^7 M^{-1} s^{-1}.[20] [c] Within error of the previously reported value of 6×10^4 M^{-1} s^{-1}.[9] [d] The same as a recently published value;[7] earlier reports gave a range of values for $(k_{cat}/K_m)^{PNPP}$.[8] [e] Within 4-fold of the previously reported value of 0.8 M^{-1} s^{-1}.[10]

and $>10^2$-fold, respectively (Table 1). These results further substantiate that AP is a sulfatase and suggest that common active site features, the serine nucleophile, the active site arginine, and Zn_I contribute to both the sulfatase and phosphatase activities. Nevertheless, there is no evidence for a physiological role of AP as a sulfatase. Rather, active site features that contribute to catalysis of both reactions may have played a role in the evolutionary diversification of AP's and arylsulfatases.[1,2,3]

The difference in the observed transition state stabilization for phosphate monoester hydrolysis and sulfate ester hydrolysis by AP corresponds to ~12 kcal/mol of discrimination,[6] in accord with the enzyme's biological role as a phosphate scavenger. In light of this large energetic difference and the evidence for an evolutionary relationship between AP and arylsulfatases, how could enhanced sulfatase activity develop from the low level exhibited by AP? Sulfate and phosphate esters have similar bond lengths and geometries,[11] and linear free energy relationships suggest that their reactions in solution proceed via similar dissociative transition states.[12] However, sulfate ester monoanions have less overall charge than phosphate monoester dianions. Consistent with this charge difference, AP has two Zn^{2+} ions and a Mg^{2+} ion in close proximity to the bound phosphoryl group,[13] whereas arylsulfatases have only a single active site divalent metal ion.[1,2] The apparent use of electrostatic interactions to localize dianionic phosphate esters to AP is consistent with its physiological role as a nonspecific phosphatase. In contrast, these sulfatases appear to act on specific physiological substrates.[14] Evolution of binding interactions could have aided the optimization as well as the substrate specialization of the arylsulfatases.

The ability of enzymes to accept alternative substrates has long been recognized, and several elegant structural and functional studies have suggested that mechanistic features of enzymatic catalysis can be adapted to catalyze different types of reactions during the course of evolution.[15] AP provides an example of an enzyme that can catalyze two different reactions, even though it is likely that AP is under direct selective pressure only for its phosphatase activity. Similarly, adenylate kinase, which is structurally related to estrogen sulfotransferase,[16] has been reported to catalyze sulfuryl transfer.[17,18] Interestingly, human alkaline nucleotide phosphodiesterase is a member of the AP superfamily,[3] and experiments analogous to those described herein have shown that AP also has a low level of phosphodiesterase activity (unpublished results). Such catalytic promiscuity provides one factor, of many, that influence the probabilistic course of natural selection. A duplicated gene encoding an enzyme that has an ability to catalyze a different reaction would have a head start toward being captured by adaptive evolution and thereby fixed in the genome.[19]

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Supporting Information Available: Descriptions of enzyme purification, enzymatic assays, and pH−rate profiles (4 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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