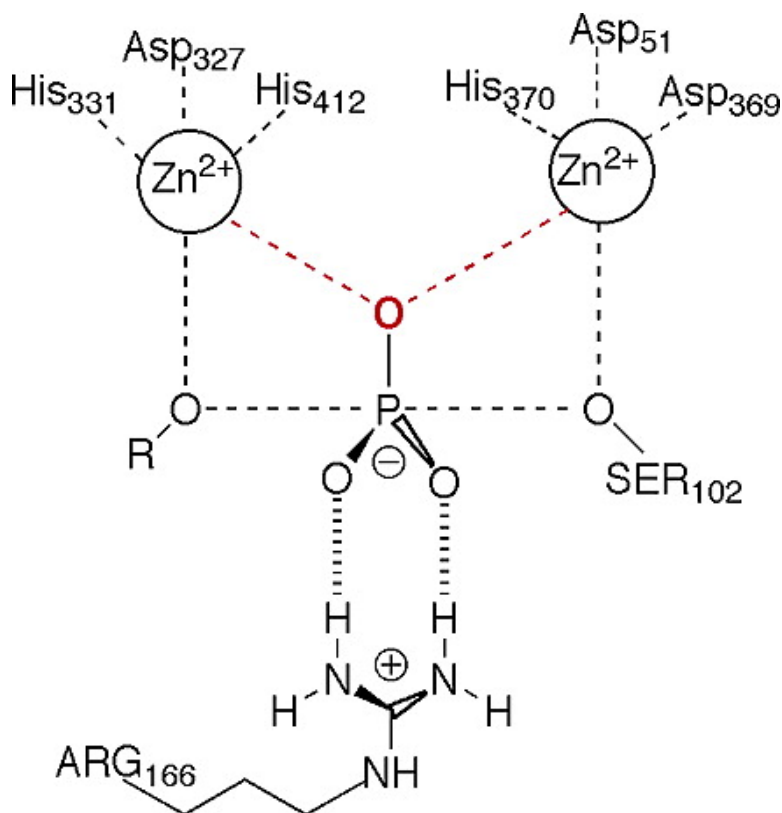## Communication

Alkaline Phosphatase Catalysis Is Ultrasensitive to Charge Sequestered between the Active Site Zinc Ions

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Alkaline Phosphatase Catalysis Is Ultrasensitive to Charge Sequestered between the Active Site Zinc Ions

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Received March 13, 2005; E-mail: herschla@cmgm.stanford.edu

Escherichia coli alkaline phosphatase (AP) employs a binuclear metallocenter of two Zn^{2+} ions, approximately 4 Å apart, to facilitate catalysis of phosphate monoester hydrolysis (Scheme 1).[1,2] One Zn^{2+} ion is proposed to activate the Ser102 nucleophile, and the other Zn^{2+} ion appears to stabilize the charge that develops on the leaving group oxygen in the transition state.[3] Additionally, both metal ions presumably interact in close proximity ($\sim$2 Å) with one of the nonbridging oxygen atoms of the substrate in the transition state for the reaction (Scheme 1B, shown in red).[1b] Many enzymes employ seemingly analogous bimetalloclusters to catalyze a variety of reactions.[4] Indeed, AP has been found to catalyze reactions beyond phosphate monoester hydrolysis. Earlier studies of catalytic promiscuity and the AP catalytic mechanism suggested that substrates with reduced charge on the nonbridging oxygen atoms were less efficiently hydrolyzed relative to dianionic phosphate monoesters.[5] These observations led us to ask whether there is an important catalytic role for the interaction of the nonbridging oxygen atom with the Zn^{2+} ions. The experiments described in this communication were designed to isolate and investigate this interaction.

To determine the energetic properties of the electrostatic interaction between the Zn^{2+} bimetallocluster and the nonbridging oxygen atom, we investigated a series of AP substrates (Table 1) with similar geometries, but systematically varying charge on the nonbridging oxygen atom. To avoid differential effects from interactions of the other nonbridging atoms with Arg166 (Scheme 1B, phosphoryl oxygen atoms facing down), reactions were carried out with an Arg166 mutant (R166S).[5c,6] The AP catalytic proficiency, [CP = $(k_{cat}/K_M)/k_w$], was used in the analysis to normalize for differences in intrinsic reactivity.

Previously, second-order rate constants k_{cat}/K_M for R166S AP-catalyzed hydrolysis were obtained for a phosphate monoester and a phosphate diester.[5b,c] In this work, we determined rate constants for R166S AP-catalyzed hydrolysis of a sulfate monoester, an additional phosphate diester, and a phosphate triester. We further took advantage of the perturbation in nonbridging oxygen charge that arises from the presence of a nonbridging sulfur atom in phosphorothioate esters[7] and determined the value of k_{cat}/K_M for R166S AP-catalyzed hydrolysis of a phosphorothioate monoester and a phosphorothioate diester. In these compounds, an oxygen atom is presumed to take the position between the Zn^{2+} ions, rather than the sulfur atom, due to the inability of the larger sulfur atom to fit within the steric confines between the Zn^{2+} ions.[8]

The results are summarized in Table 1, and the log of the catalytic proficiency is plotted against the nonbridging oxygen atom charge in Figure 1.[9] Remarkably, the data are well fit by a single linear correlation over a range of more than 8 orders of magnitude of the AP catalytic proficiency ($R^2 = 0.98$). The slope of the line is

Scheme 1 Nonenzymatic (A) and AP-Catalyzed (B) Hydrolysis of Phosphate Monoesters
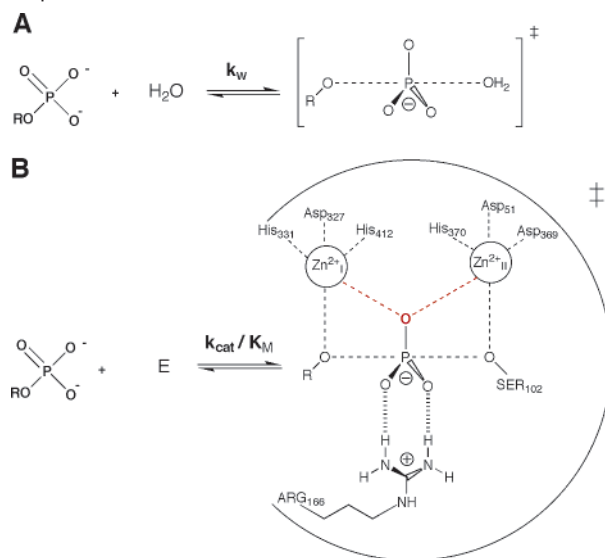

Table 1. AP Substrates, Their Nonbridging Oxygen Atom Ground-State Charges,[9] and the Respective R166S AP Catalytic Proficiencies [CP = $(k_{cat}/K_M)/k_w$][a]

AP substrate	oxygen charge[b]	catalytic proficiency[c]
p-nitrophenyl phosphate (pNPP)	−0.67	$10^{15.5}$
p-nitrophenyl phosphorothioate (pNPPS)	−0.62	$10^{13.1}$
bis(*p*-nitrophenyl) phosphate (bpNPP)	−0.50	$10^{10.8}$
methyl, *p*-nitrophenyl phosphate (MpNPP)	−0.50	$10^{11.5}$
methyl, *p*-nitrophenyl phosphorothioate (MpNPPS)	−0.45	$10^{10.7}$
p-nitrophenyl sulfate (pNPS)	−0.33	$10^{7.2}$
diethyl, *p*-nitrophenyl phosphate (dEpNPP)	0	$<10^{5}$[d]

[a] MpNPP and MpNPPS were synthesized as previously described,[10] and their purity was confirmed via NMR and total hydrolysis. Other substrates were purchased from Sigma. [b] See Supporting Information for estimation of the nonbridging oxygen atom charge. [c] k_{cat}/K_M, the second-order rate constant for the enzymatic reaction, was taken from literature for pNPP[3b] and bpNPP[3c] and determined for the other substrates as described in the Supporting Information. k_w, the second-order rate constant in water, was determined for pNPS and obtained from literature values for the other substrates, as described in the Supporting Information. [d] See Supporting Information.

extraordinarily steep (31 $\pm$ 2 kcal/mol per unit charge), indicating that the AP active site greatly discriminates between differentially charged substrates. We suggest that this discrimination arises via an electrostatic interaction with the Zn^{2+} bimetallocluster, as described below.

As Figure 1 represents a correlation and, as such, cannot prove a cause–effect relationship, we considered whether other differences between the compounds in this series might account for the observed trend. Total substrate charge, P–O (nonbridging) bond length, and substrate solution transition state character each correlates poorly

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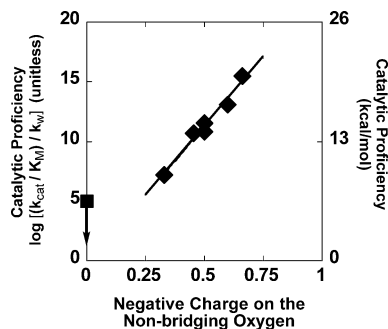[||] Department of Chemistry, Stanford University.

Figure 1. Correlation of the R166S AP catalytic proficiency with the charge on the nonbridging oxygen atom.[9] Data from Table 1. The square denotes a limit for dEpNPP and was not included in the fit.

with AP catalytic proficiency, as shown in the Supporting Information. These poor alternate correlations support our focus on the importance of the nonbridging oxygen charge (Figure 1) and the electrostatic model described below.

Literature values for charge perturbation effects are significantly smaller than the slope of the correlation of Figure 1. Studies aiming to investigate charge effects in other proteins, while minimizing geometrical and other changes, on metal ion binding,[12] transition state analogue binding,[13] protein stability,[14] and catalysis[15] report electrostatic effects in these systems of 0.5 to 5 kcal/mol per unit charge. Although much larger energetic effects have been obtained in some computational studies, such as in the preferential binding of Gln[16] and Asp[17] to their respective amino acyl-tRNA synthetases, over Glu (14 kcal/mol) and Asn (15 ± 3 kcal/mol), respectively, other studies have suggested smaller effects.[18] Indeed, the enormity of the dependence of the AP catalytic proficiency on the oxygen atom charge, observed in Figure 1, appears to be unprecedented.

We suggest the following model to account for the high sensitivity of catalytic proficiency to charge on the nonbridging oxygen atom. There is electrostatic repulsion between the two Zn^{2+} ions bound only 4 Å apart, and this close spacing within the active site may further render it difficult to effectively solvate the metallocluster; AP presumably uses some of its folding energy and the preference of Zn^{2+} for nitrogen rather than oxygen ligands to localize the Zn^{2+} ions.[11] In the ground state for AP-catalyzed reactions, water molecules occupy the active site and solvate the Zn^{2+} ions. The substrate's proposed transition state geometry positions the nonbridging oxygen atom to achieve optimal "solvation" in the center of the Zn^{2+} ion metallocluster, with the Zn^{2+} ions simultaneously stabilizing the leaving group and the anionic nucleophile, as described above and shown in Scheme 1B. The solvation by the nonbridging oxygen atom can ameliorate electrostatic repulsion between the Zn^{2+} ions, but the effectiveness of this interaction depends on the charge density of the solvating oxygen atom. In principle, the solvation by this oxygen atom in the transition state could be energetically favorable or unfavorable relative to the solvation by water in the free enzyme ground state. Regardless, a strong dependence of catalytic proficiency on the charge of the nonbridging oxygen atom is predicted.

Given the large sensitivity of the AP active site to the nonbridging oxygen atom charge, it will be of interest to explore the sensitivity to electrostatic interactions of other known bimetalloenzymes from the AP superfamily, especially those optimally catalyzing reactions of substrates with less charge. These data will be critical in relating the electrostatic environment of enzymes to their catalytic power and specificity.

Although it is tempting to describe increased electrostatic sensitivity, such as that observed in the AP active site, in terms of a reduced dielectric constant relative to water, the bulk concept of

a dielectric is not applicable to idiosyncratic environments of atomic scale such as enzyme active sites.[19] Instead, we must find alternative descriptions for these environments. Determination of the local sensitivity to subtle electrostatic perturbations provides one such powerful means of probing local electrostatic potential.

Supporting Information Available: Determination of nonbridging oxygen atom charge and R166S AP catalytic proficiencies and discussion of alternate correlations. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Kim, E. E.; Wyckoff, H. W. *J. Mol. Biol.* **1991**, *218*, 449. (b) Holtz, K. M.; Stec, B.; Kantrowitz, E. R. *J. Biol. Chem.* **1999**, *274*, 8351. (c) Coleman, J. E. *Annu. Rev. Biophys. Biomol. Struct.* **1992**, *21*, 441.
(2) In addition to the two Zn^{2+} metal ions, there is a Mg^{2+} ion in close proximity, which, although it does not interact directly with the transition state, may contribute to the electrostatic potential of the active site.
(3) O'Brien, P. J.; Herschlag, D. *Biochemistry* **2002**, *41*, 3207.
(4) For reviews, see: (a) Strater, N.; Lipscomb, W. N.; Klabunde, T.; Krebs, B. *Angew. Chem., Int. Ed.* **1996**, *35*, 2024. (b) Wilcox, D. E. *Chem. Rev.* **1996**, *96*, 2435.
(5) (a) Hollfelder, F.; Herschlag, D. *Biochemistry* **1995**, *34*, 12255. (b) O'Brien, P. J.; Herschlag, D. *J. Am. Chem. Soc.* **1998**, *120*, 12369. (c) O'Brien, P. J.; Herschlag, D. *Biochemistry* **2001**, *40*, 5691. (d) Nikolic-Hughes, I.; Rees, D. C.; Herschlag, D. *J. Am. Chem. Soc.* **2004**, *126*, 11814.
(6) (a) Butler-Ransohoff, J. E.; Kendall, D. A.; Kaiser, E. T. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 4276. (b) Chaidaroglou, A.; Brezinski, D. J.; Middleton, S. A.; Kantrowitz, E. R. *Biochemistry* **1988**, *27*, 8338.
(7) (a) Frey, P. A.; Sammons, R. D. *Science* **1985**, *228*, 541. (b) Liang, C.; Allen, L. C. *J. Am. Chem. Soc.* **1987**, *109*, 6449.
(8) Brautigam, C. A.; Steitz, T. A. *J. Mol. Biol.* **1998**, *277*, 363.
(9) We used the ground-state charge on the nonbridging oxygen atom to establish the correlation of Figure 1. Ideally, one would want to use the transition-state charge, as catalytic proficiency reflects transition state and not ground-state binding. Although much information is available to estimate bridging oxygen charges in the transition state for both solution and enzymatic reactions, the charge distribution between the phosphorus atom and the nonbridging oxygen atoms is not known. See: (a) Rajca, A.; Rice, J.; Streitweiser, A.; Schaefer, H. *J. Am. Chem. Soc.* **1987**, *109*, 4189. (b) Horn, H.; Alhrichs, R. *J. Am. Chem. Soc.* **1990**, *112*, 2121.
(10) Herschlag, D.; Piccirilli, J. A.; Cech, T. R. *Biochemistry* **1991**, *30*, 4844.
(11) Compare: Christensen, T.; Gooden, D. M.; Kung, J. E.; Toone, E. J. *J. Am. Chem. Soc.* **2003**, *125*, 7357.
(12) (a) Needham, J. V.; Chen, T. Y.; Falke, J. J. *Biochemistry* **1993**, *32*, 3363. (b) Renner, M.; Danielson, M. A.; Falke, J. J. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 6493. (c) McCall, K. A.; Fierke, C. A. *Biochemistry* **2004**, *43*, 3979.
(13) Jiang, Y. L.; Drohat, A. C.; Ichikawa, Y.; Stivers, J. T. *Biochemistry* **2003**, *42*, 1922.
(14) Irun, M. P.; Maldonado, S.; Sancho, J. *Prot. Eng.* **2001**, *14*, 173.
(15) Metanis, N.; Brik, A.; Dawson, P. E.; Keinan, E. *J. Am. Chem. Soc.* **2004**, *126*, 12726.
(16) Green, D. G.; Tidor, B. *J. Mol. Biol.* **2004**, *342*, 435.
(17) (a) Simonson, T.; Archontis, G.; Karplus, M. *J. Phys. Chem. B* **1997**, *101*, 8349. (b) Archontis, G.; Simonson, T.; Moras, D.; Karplus, M. *J. Mol. Biol.* **1998**, *275*, 823. (c) Archontis, G.; Simonson, T.; Karplus, M. *J. Mol. Biol.* **2001**, *306*, 307.
(18) See, for example: (a) Antosiewicz, I.; McCammon, A.; Wlodek, S. T.; Gilson, M. K. *Biochemistry* **1995**, *34*, 4211. (b) Dong, F.; Vijayakumar, M.; Zhou, H.-X. *Biophys. J.* **2003**, *85*, 49. (c) Luisi, D. L.; Snow, C. D.; Lin, J.-J.; Hendsch, Z. S.; Tidor, B.; Raleigh, D. P. *Biochemistry* **2003**, *42*, 7050. See also: (d) Qiang, C.; Karplus, M. *J. Am. Chem. Soc.* **2001**, *123*, 2284. (e) Jiang, Y. L.; Drohat, A. C.; Ichikawa, Y.; Stivers, J. T. *J. Biol. Chem.* **2002**, *277*, 15385. (f) Mandal, A.; Hilvert, D. *J. Am. Chem. Soc.* **2003**, *125*, 5598. (g) Kangas, E.; Tidor, B. *J. Phys. Chem. B* **2001**, *105*, 880.
(19) (a) Gilson, M. K.; Honig, B. H. *Nature* **1987**, *330*, 84. (b) Warshel, A.; Russell, S. T. *Q. Rev. Biophys.* **1984**, *17*, 283. (c) Lee, L. P.; Tidor, B. *Nat. Struct. Biol.* **2001**, *8*, 73. (d) Schaefer, M.; Karplus, M. *J. Phys. Chem.* **1996**, *100*, 1578. (e) Cheng, H.; Nikolic-Hughes, I.; Wang, J. H.; Deng, H.; O'Brien, P. J.; Wu, L.; Zhang, Z.-Y.; Herschlag, D.; Callender, R. *J. Am. Chem. Soc.* **2002**, *124*, 11295.

JA051603J