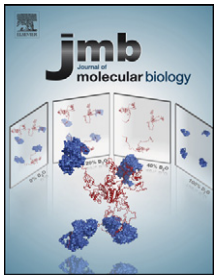

Tungstate as a Transition State Analog for Catalysis by Alkaline Phosphatase

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Abstract

The catalytic mechanisms underlying *Escherichia coli* alkaline phosphatase's (AP) remarkable rate enhancement have been probed extensively. Past work indicated that whereas the serine nucleophile (Ser102) electrostatically repels the product phosphate, another oxyanion, tungstate, binds more strongly in the presence of Ser102. These results predict a covalent bond between the serine nucleophile and tungstate, a model that we test herein. The crystal structure of tungstate-bound alkaline phosphatase provides evidence for a covalent adduct model and further shows that the ligand adopts trigonal bipyramidal geometry, which is infrequently observed for tungstate in small molecules and other active sites but mirrors the geometry of the presumed phosphoryl transfer transition state. The AP active site is known to stabilize another oxyanion, vanadate, in trigonal bipyramidal geometry, but the extent to which binding of either ligand reproduces the energetics of the transition state cannot be deduced from structural inspection alone. To test for transition state analog behavior, we determined the relationship between catalytic activity and affinity for tungstate and vanadate for a series of 20 AP variants. Affinity and activity were highly correlated for tungstate ($r^2 = 0.89$) but not vanadate ($r^2 = 0.23$), indicating that the tungstate•AP complex may better mimic this enzyme's transition state properties. The results herein suggest that tungstate will be a valuable tool for further dissecting AP catalysis and may prove helpful in mechanistic studies of other phosphoryl transfer enzymes.

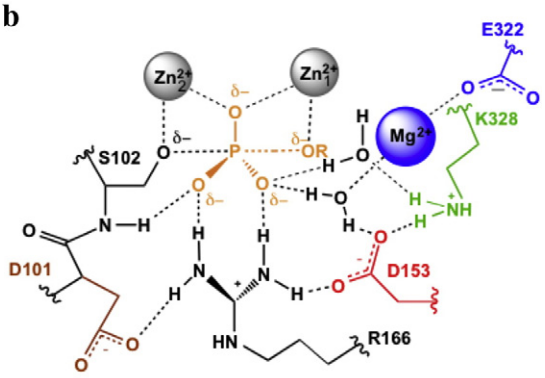
Introduction

Escherichia coli alkaline phosphatase (AP) is a powerful model system for studying general mechanisms by which enzymes accelerate chemical reactions [1,2]. As a nonspecific phosphatase, AP faces the challenge of hydrolyzing diverse phosphate monoesters by interacting exclusively with the transferred phosphoryl group and the leaving group oxygen atom but no other atoms of the leaving group during catalysis (Fig. 1a and b). Despite this, the rate enhancement conferred by AP is up to 10^{27}-fold, one of the largest recorded for enzymes [2]. The AP active site consists of a bimetallic Zn^{2+} core that activates the proximal Ser102 nucleophile and stabilizes charge that develops on the leaving group oxygen atom. Additional stabilization of the phosphoryl transfer transition state is thought to be provided by Arg166 and active site water molecules coordinated to the Mg^{2+} ion and hydrogen bonded to Lys328 (Fig. 1b) [3–5]. The tetrahedral geometry of the substrate is thought to achieve trigonal bipyramidal geometry in the phosphoryl transfer transition state [1].

Prior mechanistic work that probed AP's affinity for differentially protonated phosphate species used tungstate as a control since these anions have different pK_a values. An intriguing observation from this study was that the binding affinity of the two ligands changes distinctly with mutation: specifically, while ablation of active site Arg166 reduces affinity for both anions at pH 8, additional removal of the Ser102 nucleophile increases phosphate affinity by 1200-fold, while it reduces tungstate affinity by 5-fold

a

b



c

	K_d (μM)	
	Phosphate	Tungstate
WT	0.46	0.57
R166S	110	170
S102G/R166S	0.093	890

Fig. 1. Summary of the AP active site. (a) Reaction scheme for the phosphomonoester hydrolysis catalyzed by AP. ROP and E-P represent the phosphate monoester substrate and covalent seryl-phosphate intermediate, respectively. (b) Schematic of the AP active site drawn with partial bonds as in the presumed phosphoryl-transfer transition state. (c) Inhibition constants were calculated for the AP•HPO_4^{2-} and AP•WO_4^{2-} complexes for select AP mutants at pH 8. From Andrews *et al.* [6].

(Fig. 1c) [6]. Stronger tungstate binding in the presence of Ser102 could arise from the formation of a covalent adduct between the serine nucleophile and tungstate, a model that we test herein. Because tungstate in other active sites most frequently adopts tetrahedral [7–14] and more rarely trigonal bipyramidal [7,15] or octahedral geometries [16,17] (Table S1), detailed structural inspection of AP in complex with tungstate would additionally provide insight into the extent to which the AP active site stabilizes tungstate in a geometry that matches the presumed trigonal bipyramidal geometry of the transition state during phosphomonoester hydrolysis (Fig. 1b). Nevertheless, structural mimicry of the presumed transition state geometry does not guarantee that a ligand reproduces the energetics of transition state binding. We have therefore investigated both the structural and energetic properties of tungstate binding to the AP active site and compared tungstate binding to that of vanadate, a putative transition state analog, and phosphate, a known ground state mimic.

Results and Discussion

To test the model that Ser102 forms a covalent adduct with tungstate, we obtained a crystal structure of tungstate-bound AP at 2.0 Å resolution (Table 1). AP is a dimer, and the presence of a ligand in the active site of each monomer was evident from positive density in the difference map that appeared when the structure was modeled as the apo enzyme. We took advantage of density peaks in an anomalous scattering map generated from the same dataset to confirm that the ligand was not phosphate (Fig. S1a), which does not have an absorption edge near the wavelength used for data collection. The anomalous scattering map also confirmed that the Mg^{2+} site was occupied by a Zn^{2+} ion, as residual density in the difference map suggested (Fig. S1a and b). Whereas

Table 1. X-ray crystallographic data collection and refinement statistics

Data Collection	
Space group	$P6_322$
Unit cell axes	
a, *b*, *c* (Å)	161.3, 161.3, 139.4
α, β, γ (°)	90.0, 90.0, 120.0
Wavelength (Å)	0.9795
Resolution range (Å)	52.74-2.03 (2.08-2.03)[a]
R_{merge}	0.059 (0.276)[b]
R_{pim}	0.028 (0.129)
$\langle I \rangle / \langle \sigma I \rangle$	14.1 (3.9)
Completeness (%)	93.6 (93.4) [82.3 (83.9)][c]
Multiplicity	5.6 (5.7) [2.8 (3.2)]
$CC_{1/2}$	0.823 (0.964)
Refinement	
Resolution range (Å)	52.74–2.03
No. of unique reflections	64069 (6309)
No. of atoms	6731
Average B factors (Å^2)	
protein	40.1
solvent	33.4
ligands	34.4
RMSD from standard geometry	
bond lengths (Å)	0.02
bond angles (°)	1.9
Ramachandran statistics	
favored regions	98.1
allowed regions	1.9
outliers (%)	0
PDB code	5C66

[a] Values in parenthesis are for the highest resolution shell.
[b] $R_{merge} = \Sigma | I_{obs} - I_{avg} | / \Sigma I_{obs}$.
[c] Values in brackets are for anomalous reflections.