

A Model of the Transition State in the Alkaline Phosphatase Reaction*

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A high resolution crystal structure of *Escherichia coli* alkaline phosphatase in the presence of vanadate has been refined to 1.9 Å resolution. The vanadate ion takes on a trigonal bipyramidal geometry and is covalently bound by the active site serine nucleophile. A coordinated water molecule occupies the axial position opposite the serine nucleophile, whereas the equatorial oxygen atoms of the vanadate ion are stabilized by interactions with both Arg-166 and the zinc metal ions of the active site. This structural complex supports the in-line displacement mechanism of phosphomonoester hydrolysis by alkaline phosphatase and provides a model for the proposed transition state in the enzyme-catalyzed reaction.

Escherichia coli alkaline phosphatase (AP)¹ is a homodimeric metalloenzyme catalyzing the nonspecific hydrolysis of phosphate monoesters into inorganic phosphate and an alcohol. The overall structure is approximately 100 Å × 50 Å × 50 Å with the active sites 30 Å apart from each other on opposite sides of the molecule. Each active site contains two tightly bound zinc ions (Zn₁ and Zn₂) and one magnesium ion. The three closely spaced metal binding sites trace a triangle with the two zinc ions 4 Å apart and the magnesium binding site approximately 5 Å from Zn₂ and 7 Å from Zn₁. The active site pocket shown in Fig. 1 (*top panel*) with bound inorganic phosphate is shallow and, in addition to the three metal ions, is lined by Arg-166 and Ser-102.

Based on the structure of the enzyme with phosphate bound, Kim and Wyckoff (1) have proposed the reaction mechanism shown in Scheme 1. The free enzyme interacts with a phosphomonoester (ROP) to form the Michaelis enzyme-substrate complex (E·ROP). This complex breaks down upon nucleophilic attack of Ser-102 on the phosphate group of the substrate forming the covalent phospho-enzyme intermediate (E-P). This covalent intermediate is subsequently hydrolyzed

into the noncovalent enzyme-phosphate complex (E·P_i). As predicted by the mechanism, the reaction proceeds with overall retention of configuration at the phosphorus center. The two consecutive in-line displacement steps are postulated to have trigonal bipyramidal transition states.

In this study the interaction between vanadate (*i.e.* orthovanadate, VO₄³⁻) and *E. coli* alkaline phosphatase (EC 3.1.3.1) is evaluated by x-ray crystallography. Vanadate, an oxyanion of pentavalent vanadium, readily adopts a five-coordinate geometry resembling the proposed transition state in the enzyme-catalyzed reaction of alkaline phosphatase. In fact, vanadate has been used as a transition state analog in several enzyme-catalyzed reactions including phosphomonoester hydrolysis by rat acid phosphatase and ribonucleotide phosphodiester hydrolysis by ribonuclease A (2, 3). Vanadate is isostructural and isoelectronic with phosphate, a product, and a competitive inhibitor of alkaline phosphatase (4). Unlike the phosphate ion, vanadate can form stable five-coordinate species (4) allowing it to serve as a transition state analog for both the acquisition of phosphate and its release in the catalytic reaction of alkaline phosphatase. This paper describes the five-coordinate trigonal bipyramidal complex of vanadate in the active site of alkaline phosphatase as directly observed in a high resolution x-ray crystal structure refined at 1.9 Å resolution.

EXPERIMENTAL PROCEDURES

E. coli alkaline phosphatase was isolated as described previously (5). *E. coli* SM547 cells transformed with the plasmid pEK48 were used as the host strain for expression of wild-type alkaline phosphatase. The concentration of purified enzyme was determined from absorbance measurements at 280 nm using an extinction coefficient of 0.71 cm² mg⁻¹.

Alkaline phosphatase activity in the presence and absence of ammonium vanadate was measured spectrophotometrically using *p*-nitrophenyl phosphate as the substrate (6). A 10 mM stock solution of ammonium vanadate (NH₄VO₃) was prepared by dissolving the material in hot water followed by pH adjustment to 8.0 upon cooling. The *K_i* value for NH₄VO₃ inhibition of wild-type *E. coli* AP was determined from the double-reciprocal plot shown in Fig. 2.

Wild-type alkaline phosphatase was crystallized by vapor diffusion as described previously (7). Crystals were transferred into a stabilizing buffer containing 65% saturated (NH₄)₂SO₄, 1 mM ZnCl₂, 10 mM MgCl₂, and Tris buffer adjusted to a pH of 7.5. Initially, a high Tris concentration (1 M) was used to complex residual phosphate carried by the enzyme. After three buffer exchanges with high Tris, the crystals were transferred into stabilizing solution containing low Tris (100 mM) and gradually introduced to the vanadate ion (NH₄VO₃). The crystal of the wild-type enzyme was soaked in stabilizing buffer containing a final concentration of 100 μM vanadate (approximately 8 × *K_i*) before mounting in a glass capillary for data collection.

The diffraction data were collected using two area detectors (Area Detector Systems, San Diego, CA), driven by a VAX ALPHA 3300 computer and linked to Rigaku RU-200 rotating-anode generator operated at 50 kV and 150 mA using the Crystallographic Facility in the Chemistry Department of Boston College. Diffraction data for the AP-vanadate complex were collected to 1.90 Å. A total of 93722 unique reflections were obtained from measurements with an average redundancy of 2.8 (Table I). Merging of the reflections was accomplished using the software provided by Area Detector Systems. After correction for Lorentz and polarization effects, a scale factor was calculated for multiple measurements and symmetry-related reflections. For the structure the diffraction data were merged (Table I).

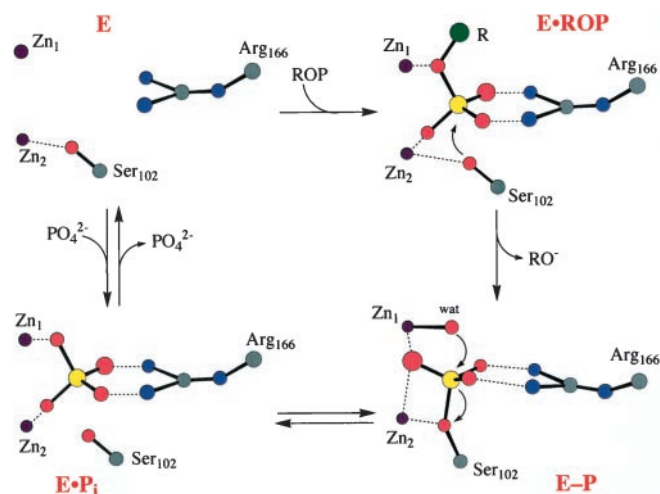
Refinement was first carried out using the wild-type coordinates for *E. coli* alkaline phosphatase as the initial model with inorganic phosphate removed from the active sites. XPLOR V. 3.1 (8) and IMPLORE (Polyvision, Inc. Hopedale, MA) were used to refine the coordinates.

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‡ The atomic coordinates and structure factors (code 1B8J) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

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¹ The abbreviation used is: AP, alkaline phosphatase.



SCHEME 1. Mechanism of the *E. coli* alkaline phosphatase reaction. Mechanism is based on the x-ray crystal structure of the enzyme with inorganic phosphate bound in the active site (1). Hydrogen atoms, the magnesium ion, and the ligands to Zn₁ and Zn₂ are not shown.

Using the initial ($F_o - F_c$) map and VO₄ coordinates obtained from the structure of rat acid phosphatase (Protein Data Bank code 1RPT), vanadate was modeled into electron density in the active sites. In the initial modeling stage, the O_γ Ser-102 was within bonding distance to the vanadium atom, and the three equatorial oxygen atoms were coplanar with the vanadium atom. Water ligands to the metal ions were included in the second refinement cycle. In this cycle, the enzyme-vanadate structure was further refined by positional refinement, temperature factor refinement, and simulated annealing. In the final refinement step, a covalent serine-vanadate group was defined. The refinements were carried out using the Silicon Graphics Indigo II computers at Boston College. An automated water placement feature of IMPLOR was used to add solvent water molecules based on the difference Fourier map ($F_o - F_c$), their distance from surrounding residues, and their temperature factors. Refinement statistics for the AP-vanadate structure are reported in Table I. The coordinates for the AP-vanadate structure have been deposited in the Brookhaven Protein Data Bank as 1B8J.

RESULTS

Vanadate in its monomeric and oxidized form (VO₄³⁻) is a strong competitive inhibitor of wild-type AP (4, 9). In agreement with the previous experiments, kinetic data obtained for the wild-type enzyme in the presence of vanadate follow a competitive trend. From the double-reciprocal data shown in Fig. 2, an inhibition constant of 12 μM was determined for vanadate. Vanadate binds as tightly to AP as inorganic phosphate having a reported K_i value of 10 μM (10). A higher molecular weight vanadate species not accounted for in the determination could raise the observed K_i value.

In the x-ray crystal structure of the complex refined at 1.9 Å resolution, the vanadium ion is clearly bound in the active site of AP with five-coordinate geometry. The omit map (Fig. 1, bottom panel) shows the trigonal bipyramidal structure of vanadate when bound in its complex with AP. A summary of the important bond angles and bond lengths in the AP-vanadate complex is provided in Table II.

In the AP-vanadate complex, Ser-102 and a Zn₁-coordinated water molecule occupy the axial positions. The axial groups are separated by 172 and 169° in active sites A and B, respectively. Based upon bond lengths, Ser-102 is covalently bound to the vanadium center in both active sites of AP (1.72 Å in subunit A and 1.70 Å in subunit B). The covalent bond distance of this axial interaction is equivalent to the three covalent equatorial vanadium-oxygen bonds. In contrast, the interaction between the vanadium atom and the oxygen of the axial hydroxyl ligand is slightly longer (1.92 Å in subunit A and 1.88 Å in subunit B).

TABLE I
Data collection and refinement summary of the AP-vanadate complex

Space group	I ₂₂₂
d_{\min} (Å)	1.9
Reflections	
Total	266977
Unique	93722
Completeness (%)	94.6
Average redundancy	2.8
Unit cell (Å) ^a	$a = 195.41$ $b = 167.81$ $c = 76.52$
Final R_{merge} ^b	0.064
Working R -factor	
Beginning	0.348
End	0.177
Free R -factor	
Beginning	0.348
End	0.196
Water molecules	939
Root mean square deviations	
Bond length	0.0191
Bond angles (degrees)	2.1
Improper angles (degrees)	2.0
Dihedral angles (degrees)	24.7

^a Unit cell dimensions of the wild-type enzyme: $a = 195.3$ Å, $b = 167.4$ Å, and $c = 76.2$ Å.

$$^b R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_{\text{mean}} - I_i|}{\sum_{hkl} \sum_i I_i}$$

In both sites, the bond length is approximately 0.15–0.2 Å longer than covalent interactions of vanadium with the O_γ of Ser-102 and the three equatorial oxygen atoms (average 1.75 Å). The close proximity of this oxygen ligand to the vanadium atom indicates significant bond formation at this axial position. In comparison, the distance between the Zn₁ ligand and the oxygen atom of the coordinated hydroxyl group is a much longer interaction (2.41 Å in active site A and 2.39 Å in active site B). The equatorial plane of the complex is defined by the three oxygen atoms spaced 120° apart. The guanidinium group of Arg-166 forms two strong hydrogen bonds to two equatorial oxygen atoms of vanadate. The third oxygen atom of vanadate in the equatorial plane appears to be positioned to form equally strong interactions with both zinc ions.

DISCUSSION

Several mechanistic proposals have been advanced based upon details in the 2.0 Å structure of the wild-type enzyme with inorganic phosphate (1). Although the phosphate ion is tetrahedral, the spatial arrangement of contacts to the zinc ions and Arg-166 is consistent with an in-line mechanism involving a five-coordinate species. The observed orientation of phosphate appears to place Ser-102 in an expected axial position for nucleophilic attack at the phosphorus center. Zn₂ is proposed to coordinate the hydroxyl group of Ser-102 activating the O_γ oxygen for nucleophilic attack. A metal-oxygen interaction between Zn₁ and the phosphate is approximately opposite the Ser-102 nucleophile and could occupy the other axial position. The position of Zn₁ is consistent with its dual role in the proposed mechanism of AP to coordinate the negatively charged leaving group of the substrate (RO⁻) in the first displacement step and then activate an incoming nucleophile in the second displacement step by lowering the pK_a of a coordinated water molecule. The remaining interactions of phosphate with Zn₂ and the guanidinium group of Arg-166 bisect the axial plane defined by Ser-102 and Zn₁. Although the trigonal interaction is not planar, such an arrangement could conceivably form the equatorial plane in a five-coordinate species.

In the H331Q mutant alkaline phosphatase (12), the stability

FIG. 1. *Top panel*, stereoview of the noncovalent E-P_i complex. Shown are Zn₁ and Zn₂ with side chain ligands, phosphate (labeled PO₄), Ser-102 nucleophile, and Arg-166. Coordinates for the wild-type enzyme with phosphate bound were taken from Protein Data Bank file 1ALK. *Middle panel*, stereoview of the covalent E-P intermediate. The covalent E-P intermediate (Ser102-PO₃) is captured in the H331Q structure. The activated water molecule (red sphere labeled Wat) is coordinated to Zn₁ (2.2 Å). Coordinates for the H331Q structure were taken from Protein Data Bank file 1HJK. *Bottom panel*, stereoview of vanadate bound in the active site of *E. coli* alkaline phosphatase. The omit electron density map is drawn in stereo at a contour level of 3.0σ. The vanadate ion is bound to the nucleophilic serine residue (Ser102-VO₄) in a position intermediate between those of the covalent E-P intermediate and the noncovalent E-P_i complex. The free axial oxygen atom of the vanadate ion is 2.4 Å from the Zn₁ site. This figure was drawn with the program SETOR (11).

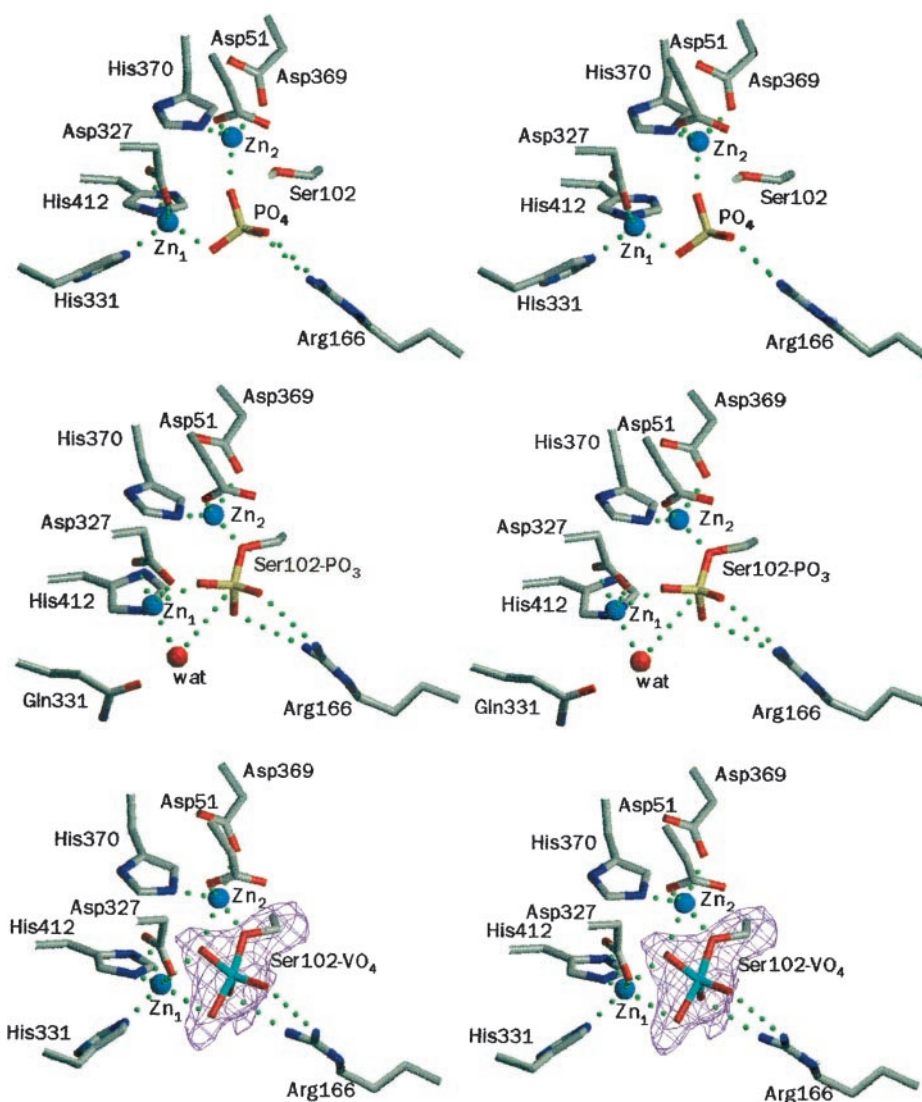


TABLE II
Bond distances and bond angles for AP-vanadate complex

Enzyme-vanadate interaction	Bond distance (subunit A/subunit B)
	Å
Axial interactions	
O _γ (Ser-102) . . . VO ₄ (vanadium atom)	1.72/1.70
H ₂ O (axial water) . . . VO ₄ (vanadium atom)	1.92/1.88
Equatorial interactions ^a	
Zn ₁ . . . O _A -VO ₄	2.06/2.08
Zn ₂ . . . O _A -VO ₄	2.18/2.14
NH ₂ (Arg-166) . . . O _B -VO ₄	3.03/2.96
NH ₂ (Arg-166) . . . O _C -VO ₄	2.74/2.84
	Bond angle (subunit A/subunit B)
	degrees
Axial interactions	
H ₂ O (axial water) . . . O _γ (Ser-102) . . . V (vanadium atom)	172/169
Equatorial interactions ^a	
O _A . . . V (vanadium atom) . . . O _B	119/116
O _A . . . V (vanadium atom) . . . O _C	120/122
O _B . . . V (vanadium atom) . . . O _C	121/122

^a Equatorial oxygen atoms are distinguished by the subscripts A, B, and C.

of the noncovalent E-P_i complex is shifted in favor of the covalent phospho-enzyme intermediate E-P at pH 7.5 allowing the phosphoserine catalytic intermediate to be captured in a crystal structure of the mutant. In the 2.3 Å structure of the H331Q enzyme (12), the O_γ oxygen of Ser-102 is within bonding distance of the phosphorus atom, and continuous electron density joins the two

groups. A water molecule coordinated to Zn₁ is clearly observed in the x-ray crystal structure. This water molecule directly opposite Ser-102 is ideally positioned for a nucleophilic attack on the phosphoserine intermediate (Fig. 1, *middle panel*). Comparison of the E-P intermediate and the E-P_i complex clearly shows the inversion of configuration about the tetrahedral phosphorus

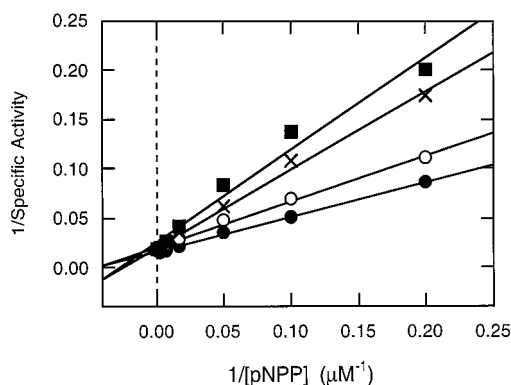


FIG. 2. Double-reciprocal plot of the activity of *E. coli* alkaline phosphatase in the presence of increasing concentrations of vanadate: ●, no NH_4VO_3 ; ○, 5 μM NH_4VO_3 ; ×, 10 μM NH_4VO_3 ; and ■, 20 μM NH_4VO_3 . From the kinetic data a K_i of 12 μM was calculated for vanadate inhibition of AP. Assays were carried out at 25 °C in 1.0 M Tris-HCl, pH 8.0. The formation of *p*-nitrophenolate was monitored at 410 nm, and the specific activity is reported in units of $\mu\text{mol/min/mg}$.

atom of the phosphate group. The x-ray crystal structure of alkaline phosphatase in the presence of vanadate provides a way to visualize the five-coordinate transition state postulated in the E-P to E-P_i conversion.

The trigonal bipyramidal complex of vanadate in the active site of AP provides details on the formation and breakdown of the axial coordination of the phosphorus atom as the covalently bound species is converted into inorganic phosphate. In both active sites, the AP-vanadate complex is more closely associated to the Ser-102 nucleophile (Table II). In fact, Ser-102 is rotated toward vanadate assuming the same orientation observed in the covalent phosphoserine intermediate of the E-P complex (Fig. 1, middle panel). The O γ oxygen of Ser-102 moves approximately 0.8 Å to form the transition state from the noncovalent complex based on the respective structures of AP with vanadate and phosphate. In the AP-vanadate complex, the longer interaction between the coordinated hydroxyl occupying the second axial position and vanadium indicates a weaker complex in this direction despite the constraints used in the refinement (Table II). The strength of this axial interaction is most likely influenced by Zn₁. The weak but evident interaction between Zn₁ and this axial oxygen atom is consistent with the role of this metal ion in activating a water molecule for attack on the phosphoserine intermediate. In fact, the axial hydroxyl group of vanadate is nearly superimposable with a phosphate oxygen atom in the structure of the noncovalent E-P_i complex. In contrast, the covalent E-P intermediate of the H331Q structure shows the potential nucleophilic water molecule fully coordinated to Zn₁ (2.2 Å) and approximately 3.6 Å away from the collapsed phosphorus center. The pathway of the nucleophilic water molecule can be traced from the three structures (Fig. 1). Initially, it is fully bound by Zn₁ (2.2 Å) in the E-P intermediate. In the AP-vanadate structure the Zn₁-OH distance increases (2.4 Å) with significant bonding between this oxygen atom and the vanadium center. In the E-P_i complex, the oxygen atom is covalently attached to the phosphorus atom to form inorganic phosphate. From examining these three x-ray crystal structures, the axial bonding properties of the AP-vanadate complex are clearly intermediate between those of the E-P intermediate and the E-P_i complex.

The motions required for the formation and breakdown of the transition state are minimal on the part of the enzyme. Comparison of the AP-vanadate complex with the noncovalent E-P_i complex shows that active site residues move only slightly to accommodate the transition state. To setup for nucleophilic attack, the side chain hydroxyl of Ser-102 rotates approximately 0.8 Å into the active site toward vanadate. In the E-P_i complex, the side chain is rotated away from the ion. Arg-166, on the other hand, is almost in the same position in both the ground state structure represented by the E-P_i complex and the transition state structure represented by the AP-vanadate complex. Once the covalent E-P intermediate is formed, the Arg-166 side chain shifts slightly back (~0.5 Å) into the active site to maintain its interactions with the oxygen atoms of the covalently bound phosphate. Because very little change takes place in the enzyme, the similar binding constants observed for phosphate and vanadate can be expected despite the fact that the latter is a transition state analog. The structure of the AP-vanadate complex shows that only minor changes in the positions of Ser-102 and Arg-166 are required in the catalytic reaction and the active site arrangement is optimized for stabilization of a trigonal bipyramidal species.

The bond distances of the vanadate ion, although not identical to those of phosphate, provide an accurate picture of the crucial structural elements involved in transition state stabilization. Furthermore, the increased bond distances of about 0.2 Å (13) in the vanadate ion compared with the phosphate ion simulate the bond formation and breakage taking place in the transition state of the phosphatase reaction lending further support to the importance of this model study. From the AP-vanadate crystal structure, both zinc ions and Arg-166 clearly play an important role in transition state stabilization. Zn₁ stabilizes the five-coordinate intermediate while also activating a water molecule for its hydrolysis. The structure of the AP-vanadate complex is the third and final structure in a set depicting the conversion of the phospho-enzyme intermediate into the noncovalent enzyme-phosphate complex. The structure of the AP-vanadate complex serves as a direct and convincing model for the five-coordinate transition state in the catalytic reaction of AP and supports the mechanism proposed by Kim and Wyckoff (1) based on the x-ray crystal structure of AP with inorganic phosphate.

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