

Crystal Structures of *Bacillus stearothermophilus* Adenylate Kinase With Bound Ap₅A, Mg²⁺ Ap₅A, and Mn²⁺ Ap₅A Reveal an Intermediate Lid Position and Six Coordinate Octahedral Geometry for Bound Mg²⁺ and Mn²⁺

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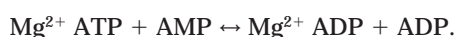
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ABSTRACT Crystal structures of *Bacillus stearothermophilus* adenylate kinase with bound Ap₅A, Mn²⁺ Ap₅A, and Mg²⁺ Ap₅A have been determined by X-ray crystallography to resolutions of 1.6 Å, 1.85 Å, and 1.96 Å, respectively. The protein's lid domain is partially open, being both rotated and translated away from bound Ap₅A. The flexibility of the lid domain in the ternary state and its ability to transfer force directly to the active site is discussed in light of our proposed entropic mechanism for catalytic turnover. The bound Zn²⁺ atom is demonstrably structural in nature, with no contacts other than its ligating cysteine residues within 5 Å. The *B. stearothermophilus* adenylate kinase lid appears to be a truncated zinc finger domain, lacking the DNA binding finger, which we have termed a *zinc knuckle* domain. In the Mg²⁺ Ap₅A and Mn²⁺ Ap₅A structures, Mg²⁺ and Mn²⁺ demonstrate six coordinate octahedral geometry. The interactions of the Mg²⁺-coordinated water molecules with the protein and Ap₅A phosphate chain demonstrate their involvement in catalyzing phosphate transfer. The protein selects for β-γ (preferred by Mg²⁺) rather than α-γ (preferred by Mn²⁺) metal ion coordination by forcing the ATP phosphate chain to have an extended conformation. *Proteins* 32:276–288, 1998. © 1998 Wiley-Liss, Inc.

Key words: adenylate kinase; Mg²⁺ and Mn²⁺ coordination; zinc fingers; entropic substrate release; thermostability

INTRODUCTION

Adenylate kinases are small enzymes whose main function in the cell is to maintain adenylate levels via the following reaction:



Other functions may include mediating cell cycles, binding RNA, and adenosine tetraphosphate production.^{1–4} Adenylate kinases are also of interest because they contain the putative nucleotide triphosphate binding cassette or P loop, thus relating them to a wide variety of NTP binding proteins.^{5–8} Structurally, adenylate kinases are fascinating proteins whose highly flexible nature allows them to adopt a wide range of conformations in the process of binding and releasing substrates.^{9–17} A considerable range of conformations have been seen by X-ray crystallographic means, including states that are open, closed, have the AMP domain closed, and have both domains closed.^{9–15} Some of these domain movements encompass translations of at least 30 Å with concomitant 90° domain rotations.^{18,19} Various liganding states of the fully closed form of the enzyme have been determined by crystallographic means as well, including AMP/ADP, ADP/ADP, AMP/AMPPNP, Ap₅A (diadenosine pentaphosphate, a bisubstrate analog with α, β, γ, δ, and ε phosphates in that order), and, in the yeast enzyme, Mg²⁺ Ap₅A.^{3,11–13,17} The yeast Mg²⁺ Ap₅A structure is unusual in that the Mg²⁺ ion is in the five coordinate state rather than the more common six coordinate form.¹⁷

Recent comparisons of gram-positive adenylate kinases with their gram-negative counterparts have revealed divergence of the gram-positive type via the mutation of the active site lid to include a Zn²⁺ finger-like domain.^{20,21} The bound Zn²⁺ in these enzymes is assumed to play a structural rather than catalytic role and possibly stabilizes this domain more readily than does the network of hydrogen bonds present in the same domain of gram-negative species. The possibility also exists that this motif

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may have similarities to Zn²⁺ finger motifs in DNA/RNA binding proteins.

The *Bacillus stearothermophilus* adenylate kinase is an especially interesting protein as it is from a gram-positive bacteria, has been confirmed as having a bound Zn²⁺, and is from a thermophilic organism. The latter trait has added to our interest in this protein, as the inherent flexibility of adenylate kinases makes them intriguing targets for studying thermostability in proteins, such as adenylate kinases and motor proteins, where it is necessary to achieve a balance between stabilization and mobility/flexibility. Thus, we present the structures of the adenylate kinase from *Bacillus stearothermophilus* complexed with Ap₅A, Mg²⁺ Ap₅A, and Mn²⁺ Ap₅A as determined by X-ray crystallography to resolutions of 1.6 Å, 1.96 Å, and 1.85 Å, respectively.

MATERIALS AND METHODS

Crystallization

B. stearothermophilus adenylate kinase was purchased from Sigma, MO and used without further purification. Crystals were grown at 20°C with 10–30 mg/ml protein in 2.45 M ammonium sulfate/1% PEG 1000 or PEG 750 monomethyl ether in 50 mM HEPES pH 6.8–7.8, with 0.1% sodium azide and 2–5 mM Ap₅A. Crystals could be grown as large as 0.3 mm × 0.3 mm × 0.1 mm. These crystals typically grew in large irregular masses, requiring separation prior to data collection. The crystals are stable in a variety of mounting solutions and show only mild degeneration during data collection.

Crystallization of the *B. stearothermophilus* adenylate kinase from 2.5 M ammonium sulfate makes it impossible to see bound Mg²⁺ in the structure at low concentrations of Mg²⁺ in the mother liquor (such as can be used with PEG as a precipitant) either due to chelation of Mg²⁺ by free sulfate or formation of (NH₃)_nMg²⁺ complexes. We therefore soaked several of our crystals for 1 hour to 2 days in saturated (~2.9 M) magnesium sulfate (buffered in pH 7.0 100 mM HEPES) in hopes that there would be sufficient free Mg²⁺ to be coordinated at high occupancy by the protein/Ap₅A complex. Soaked crystals continue to diffract strongly (initially >2.0 Å) with only a very small increase in mosaicity (increase of 0.1°) but were unstable after 48 hours probably due to alterations in water activity. Mn²⁺ is soluble in high concentrations of ammonium sulfate, and we were able to introduce it into our crystals by soaking overnight in a solution of 3.0 M ammonium sulfate/30 mM manganese 50 mM HEPES, pH 7.0. These crystals were stable for weeks.

Data Collection and Processing

All diffraction data were collected at room temperature on a Rigaku R-AXIS IIC imaging plate detector with X-rays provided by a Siemens rotating anode generator. Data processing was done using DENZO

TABLE I. Data Collection and Refinement Statistics[†]

Space group	P2 ₁
Cell constants (Ap ₅ A)	41.2 Å × 62.3 Å × 41.6 Å, β = 116.7°
Cell constants (Mg ²⁺ Ap ₅ A)	41.2 Å × 62.3 Å × 42.1 Å, β = 117.1°
Cell constants (Mn ²⁺ Ap ₅ A)	41.2 Å × 62.4 Å × 41.7 Å, β = 116.8°
Packing density (% solvent)	43.5
Resolution (Å)	1.6, 1.96, 1.85
Completeness (%)	97, 93.1, 97
Outermost shell completeness (%)	81.5, 90, 87.4
Reflections	23,448, 12,454, 15,235
R _{merge} (%)	9.7, 10.6, 9.6
R (%)	17.3, 16.1, 15.6
R _{free} (%)	21.6, 22.8, 22.0
Water molecules (no.)	268, 203, 202
Average backbone B-factor (Å ²)	14.8, 11.9, 13.7
RMS deviations: bond lengths (Å ²)	0.011, 0.012, 0.010
RMS deviations: bond angles (°)	2.09, 1.99, 1.99

[†]Unless otherwise noted, values are listed for the structures as: Ap₅A, Mg²⁺Ap₅A, and Mn²⁺Ap₅A.

and SCALEPACK.²² A complete data set was collected from a single crystal of the native Ap₅A complex. The model refined against this data set is what is described in the rest of this paper as the Ap₅A-bound structure. Data from the magnesium sulfate soaks were collected from a single crystal after a 1 hour soak. Data from the manganese soaks were also collected from a single crystal, which had been soaked overnight. Data statistics and constants for all three structures are listed in Table I.

Molecular Replacement

The Ap₅A-bound form of the *B. stearothermophilus* adenylate kinase was determined initially. The structure of the yeast adenylate kinase/Ap₅A complex was used as an initial basis for molecular replacement trials.¹⁷ The amino acids of this structure were modified to correspond to the sequence of *B. stearothermophilus* adenylate kinase using MIDAS.²³ The bound Zn²⁺ was modeled using CHAIN.²⁴ The bound Ap₅A molecule was left in the same position it occupied in the yeast structure. Water molecules and bound Mg²⁺ were removed from the model. This model was used as the probe molecule for rotation and translation functions. Molecular replacement and refinement were done using X-PLOR.²⁵ Both the rotation and translation functions yielded single peaks significantly above background, which were used to place the molecule in the cell.

Refinement

Initial refinement consisted of rounds of simulated annealing (from 2,000 K to 300 K), conjugate gradient minimization, and manual rebuilding. This process stalled at an R of 26.9% and an R^{free} of 39.9%, whereupon it was determined (based on $2F_o - F_c$ and $F_o - F_c$ electron density maps) that the lid was in a different position from our model. Therefore, the lid was refit to a position ~ 5 Å farther away from the binding site based on the highest peak in the $F_o - F_c$ difference map, which was assumed to be the position of the Zn^{2+} atom. From this point, refinement continued with cycles of Powell minimization, temperature factor fitting, manual rebuilding, and solvent addition, which generated a final structure with an R of 17.3% and an R^{free} of 21.6% with 268 solvent molecules at a resolution of 1.6 Å (Table I). This structure contains an alternate conformation for the δ phosphate of Ap_5A .

The data from the magnesium sulfate soaked crystals were refined based on the final structure of the Ap_5A ($\text{Mg}^{2+}/\text{Mn}^{2+}$ -free) structure, minus solvent molecules and the alternate conformation of the δ phosphate (Table I). The initial Ap_5A -bound model was debiased versus the Mg^{2+} Ap_5A data. An $F_o - F_c$ difference map made after the first refinement cycle revealed five high sigma peaks in the Mg^{2+} coordination site (between Asp84 and the β and γ phosphates) corresponding to bound Mg^{2+} and four coordinated waters. The Mg^{2+} and its four coordinated water molecules have low temperature factors and occupancies of 100%. No restraints were used in the refinement of either the Mg^{2+} ion or its coordinated waters.

As in the Mg^{2+} Ap_5A structure, the data from the manganese soaked crystals was refined using the final structure from the Ap_5A ($\text{Mg}^{2+}/\text{Mn}^{2+}$ -free) structure, minus solvent molecules and the alternate conformation of the δ phosphate. Once again, we could clearly see five peaks in a difference map that correspond to Mn^{2+} and its four coordinated waters. This structure was refined identically to the Mg^{2+} Ap_5A structure, including initial debiasing. As in the Mg^{2+} Ap_5A structure, the Mn^{2+} atom and its associated water molecules all refined to have low temperature factors and occupancies of 100%.

All structures have been deposited at the Protein Data Bank under access codes 1zin, 1zio, and 1zip for Ap_5A , Mg^{2+} Ap_5A , and Mn^{2+} Ap_5A structures, respectively.

RESULTS

Position of the Lid Domain

The conformation of *B. stearothermophilus* adenylate kinase in the crystal is similar to that seen in prior structures of the fully closed state of large form adenylate kinases, except that the globular lid domain (residues 128–159) does not fully close over the

bound inhibitor (Fig. 1a).^{11,12,17,26} Compared with the structure of the complex of yeast adenylate kinase with Ap_5A (Fig. 1b), the lid of the *B. stearothermophilus* adenylate kinase is rotated $\sim 20^\circ$ near the C_α of Arg127 and $\sim 55^\circ$ near the C_α of Arg160, resulting in a twist and a hinge bending motion. The lid is approximately 5 Å more open than seen in other structures.^{11,17} This twist is similar to that seen in the open conformation of unligated *E. coli* adenylate kinase and in some of our molecular dynamics simulations.^{3,15,16} That this conformation is not due to the bound Zn^{2+} atom can be seen on comparison with the yeast structure, which reveals that the entire lid domain has moved as a rigid body (Fig. 1b).

The lid domain has a number of packing contacts within the crystal, which may explain its conformation (Table II). Almost half of the direct intermolecular protein-protein hydrogen bonds in the crystal occur between the lid domain and the core and AMP binding domains of neighboring molecules. These contacts and the interaction of Arg160 with the alternate conformation of the δ phosphate of Ap_5A are assumed to contribute to stabilizing this conformation in the crystal.

Ap_5A Binding

Ap_5A is bound in the same general conformation (occupying both AMP/ADP and Mg^{2+} ADP/ATP binding sites) in our structures, as has been seen in other complexes of adenylate kinases with this inhibitor (Table III, and see Fig. 3).^{11,17,26,27} As in the *E. coli* enzyme complex, *B. stearothermophilus* adenylate kinase binds Ap_5A with the δ phosphate (PD) in two separate conformations, one pointing away ($\sim 70\%$ occupancy) and one pointing toward ($\sim 30\%$ occupancy) the opening of the active site (Asp84 is defined as being at the opening of the active site). In the less occupied state (pointing toward the opening of the active site), the δ phosphate hydrogen bonds/forms a salt bridge to Arg160, which is thought to stabilize one side of the transferred phosphate's pentacoordinate intermediate. Otherwise, Arg160 only weakly interacts (at 3.4 Å) with the Ap_5A phosphate chain. Interestingly, there is no evidence for an alternate conformation of Arg160, which appears to be rigidly held in place in the active site (averaged temperature factor for side chain atoms 13.6 Å²). The Mg^{2+} Ap_5A and Mn^{2+} Ap_5A complexes lack the alternate conformation of the δ phosphate, probably due to steric constraints imposed by the bound cation and its coordinating waters.

The conformation of the *B. stearothermophilus* lid domain is not likely to be a result of the alternate position of the nonphysiological δ phosphate of Ap_5A found in our structure, since the fully closed form of adenylate kinase has been seen in both *E. coli* and yeast adenylate kinase complexes with Ap_5A , and in the *E. coli* case with an alternate conformation of the δ phosphate.^{11,17} In addition, crystals of our Mn^{2+}

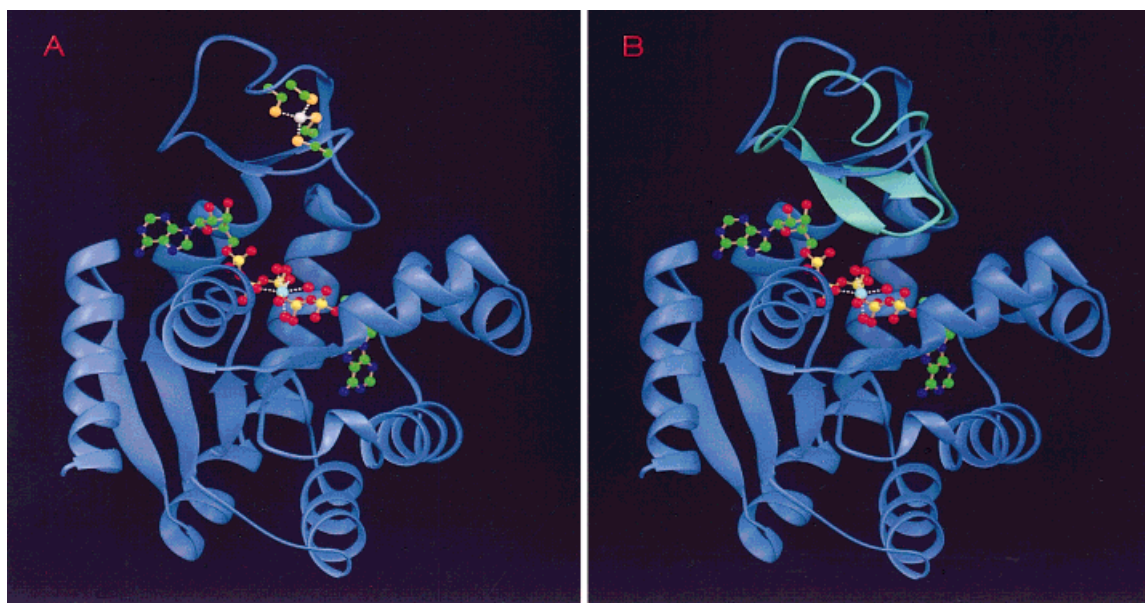


Fig. 1. Structural overview and demonstration of the intermediate position of the lid domain. **A:** The structure of *Bacillus stearothermophilus* adenylate kinase with bound Mg²⁺Ap₅A is shown. The bound Ap₅A molecule spans the active site of the enzyme underneath the lid domain. The bound Zn²⁺ atom and its four coordinating cysteine residues (Cys130, Cys133, Cys150, and Cys153) are shown in the enzyme's lid domain. Mg²⁺ is shown in light blue, and coordinated water molecules are shown as red spheres. Unlike the structure of yeast adenylate kinase with bound Mg²⁺ Ap₅A, the *B. stearothermophilus* structure demonstrates four Mg²⁺-coordinating water molecules rather than three.¹⁷ The fourth

coordinated water molecule is farthest to the right. Coordination bonds are shown as dotted lines of white spheres. **B:** The lid domain from the complex of yeast adenylate kinase with Mg²⁺ Ap₅A (shown in green) is shown overlapped with the *B. stearothermophilus* structure to demonstrate the relative lid position of the *B. stearothermophilus* structure.¹⁷ (The zinc coordination site of the *B. stearothermophilus* lid is not shown to improve clarity in the figure.) The lid domain in the *B. stearothermophilus* structure has rotated as a rigid body 5 Å away from the active site. Overlap of the two structures was based on all C_α atoms for both structures. Color figures were produced with RIBBONS and Pixar's RenderMan.⁴³

TABLE II. Electrostatic Packing Interactions[†]

Residue (asymmetric unit)	Distance (Å)	Residue and symmetry
Tyr52 OH	2.7	Glu210 OE1
Asp54 O	2.8	Lys74 NZ, sym 2
Glu122 OE1	3.2	Arg209 NH2, sym 2
Asn132 OD2	3.1	Arg193 NE, sym 1
Ile140 O	2.9	Met1 N, sym 2
Asp151 O	3.1	Asp61 N, sym 1
Lys152 O	3.0	Glu62 N, sym 1
Glu156 OE1	3.0	Asn194 ND2, sym 1
Glu165 OE1	2.7	Thr43 OG1, sym 2
Glu165 OE2	3.1	Leu45 N, sym 2
Ap ₅ A N1A	2.9	Asn79 OD1, sym 2

[†]A cutoff of 3.3 Å was used to determine interactions. Residues in the first column refer to the asymmetric unit (x, y, z), while those in the third column refer to the symmetry interaction. Sym 1 refers to molecules in the symmetry 1 position (x, y, z) in neighboring cells. Sym 2 refers to molecules in the symmetry 2 position (-x, y + 1/2, -z) in the same or neighboring cells. These values are from the Ap₅A structure and are equivalent to those of the Mg²⁺ Ap₅A and Mn²⁺ Ap₅A structures.

Ap₅A complex lack the alternate conformation of the δ phosphate entirely and yet are stable indefinitely.

The side chains of the other four active site arginines (36, 88, 127, and 171) demonstrate well-

defined positions and form hydrogen bonds/salt bridges to the bound Ap₅A molecule. Of these, Arg88 deviates most from the "standard" adenylate kinase arginine arrangement by having a novel hydrogen bond between its head group NH1 atom and the OD1 atom of Asn175. This hydrogen bond orients the head group of Arg88 in a similar manner to aspartates 162 and 163 in positioning arginines 127 and 160 to form the roof of the binding pocket and close the lid. cursory sequence comparison suggests that the equivalent to Asn175 may be present in other adenylate kinases, including the closely related *Bacillus subtilis* enzyme.

The Zinc Finger Lid

Glaser and coworkers determined that a Zn²⁺ atom is bound in the lid domain of *B. stearothermophilus* adenylate kinase.²⁰ The bound Zn²⁺ atom is very obvious in the lid domain of our structure and is tetrahedrally ligated to Cys130, Cys133, Cys150, and Cys153 at distances of ~2.3 Å (Fig. 2a). No other residues besides the four bound cysteines are close enough to interact directly with the zinc atom. In fact, there are no other side chains, besides the four ligated cysteines, within 5 Å of the bound zinc atom. The overall structure of the lid domain is otherwise

TABLE III. Interactions of Ap₅A With the Protein[†]

Ap ₅ A atom	Distance (Å)	Atom	Residue
O1PE	3.0, 3.2, 3.4	NH1 and NH2, NH2	Arg36, Arg160
O1PD	3.0, 3.0	NH1, NH2	Arg88
O2PD (alt)	2.8, (2.4)	NH2 (NH1)	Arg171 (Arg160)
O1PG	2.8, 2.8	NH2, NH2	Arg127, Arg171
O2PG	3.1, 3.4	NH1, NH1	Arg127, Arg160
O1PB	3.0, 3.0, and 3.2	N, N and NZ	Gly12, Lys13
O2PB	2.9	N	Gly14
O3PB	3.3	N	Gly10
O1PA	2.9, 2.7	N, OG1	Thr15
O2PA	2.9	NH1	Arg127
O2RA	2.4	NE2	His142
O3RA	3.2	O	Tyr137
O2RB	2.7	O	Asp57
N6A	3.0	O	Gln199
N1B	3.0	NE2	Gln92
N3B	3.1	N	Val59
N6B	3.0, 3.0	O, OE1	Gly85, Gln92
N7B	2.9, 3.3	OG1, NH2	Thr31, Arg88

[†]These values are from the 1.6 Å resolution Ap₅A-bound (Mg²⁺-free) structure and are generally equivalent to those of the Mg²⁺ Ap₅A structure, except where Mg²⁺ interacts directly with the phosphate chain. A hydrogen bond cutoff of 3.3 Å was used, with interactions of Arg160 with the phosphate chain as the exception. Interactions with water molecules are not listed. The bracketed values in the O2PD row refer to the alternate conformation of the δ phosphate, which occurs in approximately 30% of bound Ap₅A molecules in this structure.

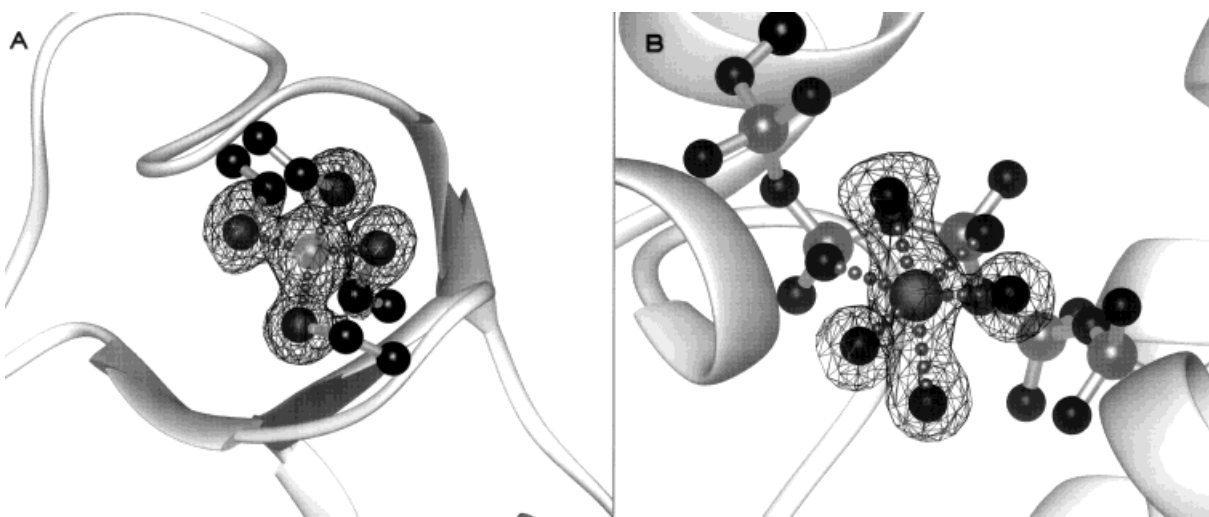


Fig. 2. Electron density for bound Zn²⁺ and Mg²⁺. **A:** Isosurface rendered 3σ electron density from F_o-F_c omit maps for the lid domain's zinc coordination site. **B:** Electron density for the

magnesium complex. In A, the Zn²⁺ and its four liganding sulfurs were omitted from map calculation, while in B, the Mg²⁺ and its four liganding waters were omitted.

unchanged from that seen in other large-form adenylate kinases.

Mg²⁺ and Mn²⁺ Coordination

Soaking experiments with magnesium and manganese proved successful, resulting in full occupation of the known metal coordination site in both cases (the coordination site is formed in the forward direction

primarily by Asp84 and the phosphate chain; Figs. 2b, 4, Table IV).

The *B. stearothermophilus* adenylate kinase/Ap₅A complex demonstrates hexacoordination (six ligands) for both bound Mg²⁺ and Mn²⁺, with four water (numbers 300–303) and two phosphate oxygen (O2PB and O2PG) ligands. The sixth ligand in the *B. stearothermophilus* structures, which is not seen in the yeast adenylate kinase/Mg²⁺ Ap₅A structure,¹⁷ is

TABLE IV. Interactions of the Mg²⁺ and Mn²⁺ Coordination Complexes

Mg ²⁺ /Mn ²⁺	Distance (Å)	Interacting atom	Residue
Mg ²⁺	2.2, 2.2, 2.2, 2.1, 1.9, 2.2	O2PG, O2PB, OH2, OH2, OH2, OH2	Ap ₅ A and H ₂ O 300, 301, 302, 303
H ₂ O 300	2.8, 3.1, 2.9, 3.0	OD2, NH2, O1PE, O2PG	Asp33, Arg36, Ap ₅ A
H ₂ O 301	3.2, 2.5	N, OD2	Gly14, Asp84
H ₂ O 302	3.1, 2.8, 2.9	O2PA, O2PB, O2PG	Ap ₅ A
H ₂ O 303	2.9, 3.0	OD1, O2PE	Asp84, Ap ₅ A
Mn ²⁺	2.3, 2.3, 2.2, 2.1, 2.1, 2.1 Å	O2PG, O2PB, OH2, OH2, OH2, OH2	Ap ₅ A and H ₂ O 300, 301, 302, 303
H ₂ O 300	3.4, 2.9, 2.8, 3.1	OD2, NH2, O1PE, O2PG	Asp33, Arg36, Ap ₅ A
H ₂ O 301	3.5, 2.6	N, OD2	Gly14, Asp84
H ₂ O 302	3.3, 3.0, 3.2	O2PA, O2PB, O2PG	Ap ₅ A
H ₂ O 303	3.1, 3.0	OD1, O2PE	Asp84, Ap ₅ A

water molecule 300, which completes the square plane of coordination (with water 302, O2PB and O2PG).¹³ Water molecules 300 and 303 (the axial ligand hydrogen bonded to Asp84) hydrogen bond to the O1 and O2 ϵ phosphate oxygens of Ap₅A (equivalent to the α phosphate of AMP). Water molecule 300 also hydrogen bonds to Asp33 and Arg36 (2.8 and 3.1 Å, respectively). Water molecule 302 (the axial ligand opposite to water 303) coordinates the remainder of the phosphate chain, interacting with the O2 atoms of the α , β , and γ phosphates at hydrogen bonding distances. Water molecule 301, which is closest to the opening of the active site, is bound by Asp84 and also hydrogen bonds to the peptide nitrogen of Gly14.

Comparison of bound and unbound forms reveals that both the protein and the phosphate chain undergo subtle changes on binding Mg²⁺ or Mn²⁺ (Fig. 5). In general, there is evidence for a tightening of the binding site, both in terms of the protein and the phosphate chain, around bound Mg²⁺/Mn²⁺. For the protein, these motions involve repositioning of Asp33, Arg36, and Asp84 to hydrogen bond to the coordinated waters of the divalent cation. In terms of the phosphate chain, binding of the ion causes a pinching of the ATP-equivalent region of the Ap₅A phosphate chain by directly bonding to the O2PB (β phosphate) and O2PG (γ phosphate) phosphate oxygens. Binding of the metal ion also causes movement of the ϵ phosphate (AMP α phosphate equivalent) toward the γ phosphate, based on interactions of the ion-bound water molecules 300 and 303 with the ϵ phosphate.

DISCUSSION

The presence of a bound zinc atom and sequence similarity to DNA binding zinc finger motifs in the lid domain of gram-positive adenylate kinases has led us to examine the structure of the *B. stearothermophilus* protein. Our structure shows that the bound Zn²⁺ is structural rather than catalytic in nature. The four cysteine zinc finger of the *B. stearothermophilus* lid replaces the intricate network of hydrogen bonds that holds the lid together in

the structures of gram-negative bacteria and all of the eukaryotic large-form adenylate kinases determined to date (Fig. 6). Since this feature occurs in nearly all gram-positive bacteria whose sequences are known (including mesophilic, thermophilic, and hyperthermophilic species), it is unlikely that this is an artifact of *B. stearothermophilus* thermostability.²⁸

As noted by Gilles et al.,²⁸ it is tempting to suggest that the zinc finger lid of gram-positive adenylate kinases may have a purpose (such as binding DNA) other than simply stabilizing this domain. A recent report of in vitro binding of RNA by a *Nicotiana tabacum* adenylate kinase further supports the speculation that the structural similarity between the adenylate kinases and zinc finger DNA binding proteins may not be based solely on the need to bind the Zn²⁺ atom.⁴ Following this line of reasoning, we have sought to compare the enzyme's lid domain with other zinc finger containing proteins. A somewhat cursory investigation of zinc finger containing proteins in the Protein Data Bank reveals that a variety of these molecules have weak topological similarity in the region of the bound zinc to the stacked loop design of the adenylate kinase lid (PDB entries 1tfi, 1chc, 1hvn, 1paa, 1pyi, 1rgd, 1zaa). The zinc finger domains of these structures all have similar "sandwiches" made of two reverse turns between which the Zn²⁺ is bound by histidine and/or cysteine residues. The stacked reverse turns allow the zinc binding residues to be placed at the correct angles and distances to ensure that the tetrahedral geometry of the bound Zn²⁺ is accommodated.

Among the structures in the Protein Data Bank that we have examined, the non-sequence-specific transcriptional elongation factor SII nucleic acid binding domain (also known as TFIIS; PDB entry 1tfi) seems to share the most topological and structural similarity to the adenylate kinase lid (Fig. 6c).^{29,30} In this molecule the Zn²⁺ atom is bound by four cysteine residues, which are located on a stacked loop assembly very similar to the *B. stearothermophilus* lid. With the exception of the elongation of one loop, the structure of the peptide backbone of the

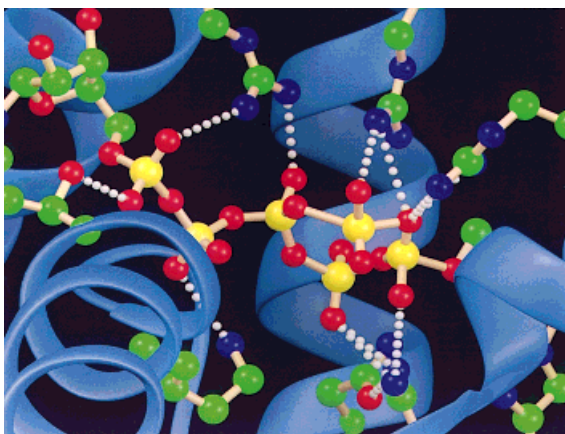


Fig. 3. Interactions between Ap_5A and the protein. The hydrogen bonding network of the protein to bound Ap_5A is shown (from the Mg^{2+} -free structure). Hydrogen bonds are shown as dotted lines of white spheres. Arginines 36, 88, 127, 160, Lys13, Thr15, and Asn175 are shown. Naming of the phosphates of Ap_5A increases in alphabetical order from left (α) to right (ϵ). Arginines in the top of the figure are Arg127, Arg160, and Arg36, from left to right. Arg88 is in the bottom right of the figure, while behind and hydrogen bonding to Arg88 is Asn175. Thr15 and Lys13 are on the middle and lower left of the figure hydrogen bonding to the α and β phosphate of Ap_5A , respectively. Both conformations of the δ phosphate are shown, one of which hydrogen bonds to Arg160. The lower occupancy position is closer to the top of the figure. This conformation does not occur in the presence of bound Mg^{2+} or Mn^{2+} .

TFIIS domain shows general topological similarity to the large form adenylate kinase lid domain. The TFIIS domain also utilizes four cysteines to bind zinc, as does the *B. stearotherophilus* adenylate kinase. However, the elongated loop, which is present in the TFIIS domain but not in the adenylate kinase lid, is probably the “zinc finger” actually responsible for DNA binding. In the *B. stearotherophilus* case, this finger is truncated to only a “knuckle.”

Because of the lack of the “finger” part of the zinc finger-like lid domains of adenylate kinases, we feel it is unlikely that this structural motif of the lid domain plays a role in directly binding DNA/RNA. It is possible, however, that the similarities between the adenylate kinase lid and TFIIS domain are an example of convergent evolution required to create a specific topological fold.

The sequence of the adenylate kinase from the closely related gram-positive bacteria *B. subtilis* (76% identity to *B. stearotherophilus*) has been determined and indicates that one of the cysteines of this protein's zinc finger domain, Cys153, has mutated to Asp²¹. We have modeled this and several other mutations made by Gilles et al.²⁸ in this region using the present structure to determine their effects on the binding of the Zn^{2+} atom. What we have found is that Cys153 is capable of swinging out toward the solvent, and thus this position is capable of accommodating histidine and aspartate residues while retain-

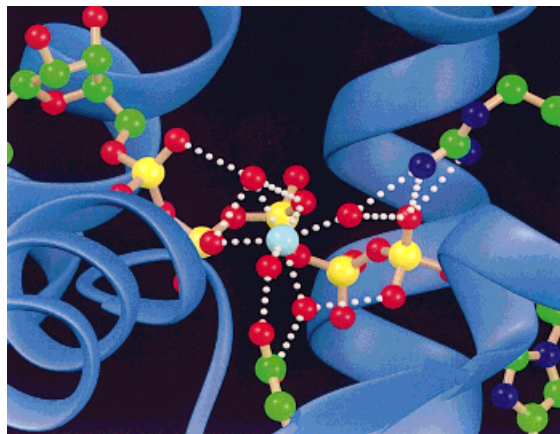


Fig. 4. Interactions of Mg^{2+} with Ap_5A and the protein. The coordination sphere of Mg^{2+} (turquoise) and hydrogen bonding interactions of this complex with Ap_5A and the protein are shown. Hydrogen bonds and Mg^{2+} coordination bonds are shown as dotted lines of white spheres, while water ligands are shown as red spheres. Arg36 is depicted on the right and Asp84 is shown at the bottom of the figure. Water molecule 300 (the fourth coordinating water) is on the right, water molecule 301 is in the center, water molecule 302 is at the top, and water molecule 303 is at the bottom of the image. The $\text{Mg}^{2+}(\text{H}_2\text{O})_4$ complex bridges the γ and ϵ phosphates by way of coordination and hydrogen bonding, stabilizing the route the transferred phosphate would take.

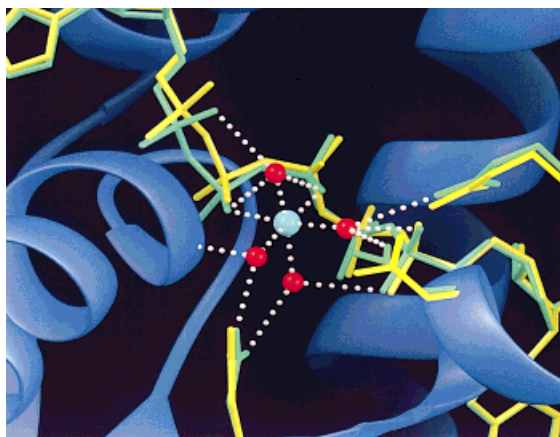


Fig. 5. Conformation changes of Ap_5A and active site residues upon Mg^{2+} binding. The Mg^{2+} -bound conformation is shown in green while the Mg^{2+} -free state is shown in yellow. Variations in the conformation of Ap_5A and the protein are identical in both Mg^{2+} and Mn^{2+} bound structures. Asp33, Arg36, and Asp84 demonstrate the largest conformational changes among the residues in the active site upon binding of Mg^{2+} or Mn^{2+} .

ing tetrahedral coordination of the zinc atom. Position 130 (Cys130), on the other hand, cannot accommodate histidine or aspartate residues due to steric constraints within the interior of the lid. To accommodate histidine at this position, the lid would have to reorganize significantly, which would probably destroy its ability to coordinate the zinc tetrahedrally. When Gilles et al.²⁸ made the Cys130 to His mutant of the *B. stearotherophilus* adenylate ki-

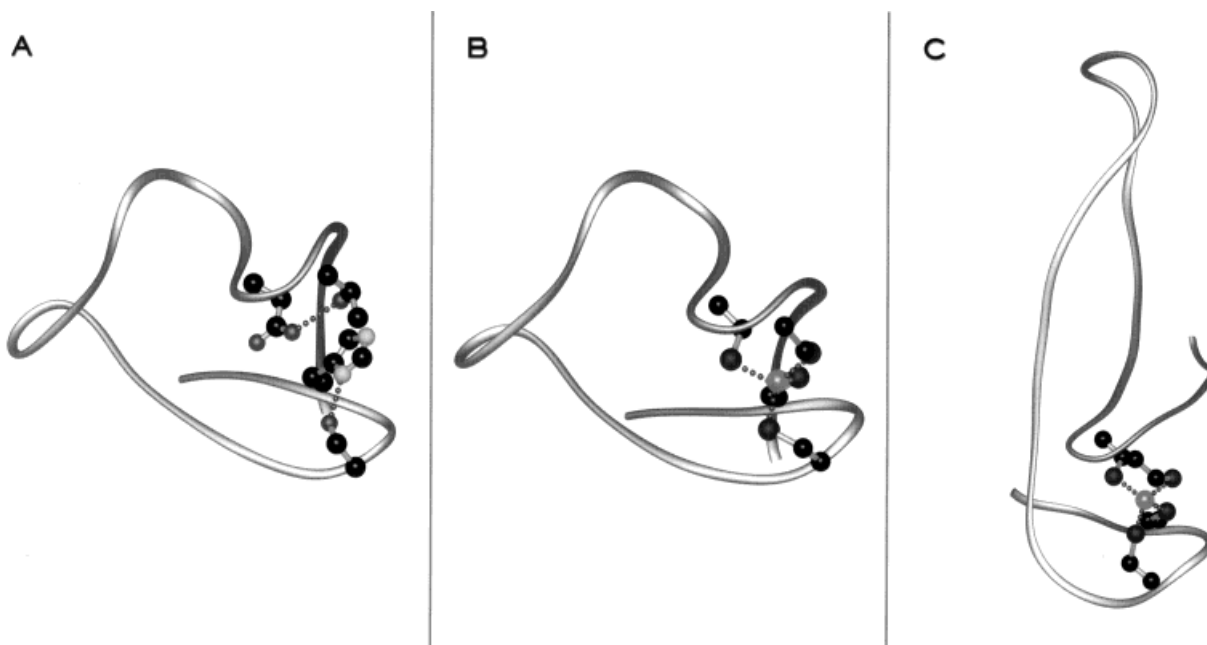


Fig. 6. Similarities between the lid domains of gram-negative and gram-positive adenylate kinases and the non-sequence-specific transcriptional elongation factor SII nucleic-acid binding domain (TFIIS). **A:** The lid (residues 123–156) of the gram-negative *E. coli* adenylate kinase. **B:** The lid (residues 127–160) of the *Bacillus stearothermophilus* adenylate kinase. **C:** The TFIIS DNA binding domain. The *E. coli* lid varies from the *B. stearother-*

mophilus lid in that a hydrogen bonding network has replaced the zinc coordination assembly. The TFIIS domain shows similarity to the *B. stearothermophilus* lid both in the conformation of its zinc coordination domain and in the topology of the fold, accepting that one loop has been greatly elongated (top loop in C; compare with the top leftmost loops in A and B). This loop is probably the means by which the TFIIS domain interacts with DNA.

nase, they found that it only weakly bound the zinc ion, supporting this conclusion.

It should be noted that an attempt to model the bound zinc atom in an adenylate kinase lid has been recently published.³¹

Position of the Lid Domain

The partially open conformation of the lid domain seen in the present structure is intermediate between the fully open and closed conformations seen in other adenylate kinases, despite the fully bound state of the protein. This is probably due to crystal packing forces locking the lid in this particular conformation. A recent comparison of Ap₅U with ADP/[U/C]MP and ADP/[U/C]MP/[Be/A1]F_n complexes of *D. discoideum* UMP/CMP kinase suggests, alternatively, that the lid moves away from the active site 13° in the presence of the bisubstrate analog.³² However, the same cannot be said of the comparison of *E. coli* adenylate kinase Ap₅A and AMP/AMPPNP complexes, which demonstrate essentially identical conformations of the lid, and thus the *D. discoideum* UMP/CMP kinase hypothesis may not explain the conformation of the *B. stearothermophilus* structure.^{11,12} Regardless of its origin, the conformation of the lid domain in the *B. stearothermophilus* structure implies a certain degree of flexibility of this domain in the fully ligated state and that motion

of the lid is translated directly to the active site via Arg160 and Arg127.

The displacement of the bridging arginine (e.g., Arg160) away from the phosphate chain due to the lid conformation is not unique to the present structure and has recently been seen in the structure of the *D. discoideum* UMP/CMP kinase with bound Ap₅U.³³ The structure of the *D. discoideum*/Ap₅U complex shows the lid flap to be displaced from the binding site, increasing the distance of the bridging arginine (Arg137) to the ϵ phosphate to 3.9 Å. This protein is essentially structurally identical to lidless (e.g., small form) adenylate kinases, with only minor differences due to the necessity of binding uridylyate as the acceptor of the transferred phosphate. We therefore feel that this movement is equivalent to that seen in the present structure.

Our focus on this movement is due primarily to the fact that the bridging arginine, Arg160, is absolutely required for catalysis, and any movement of it away from the binding site is likely to result in an interruption of the reaction. Studies by Yan and coworkers³⁴ have shown that mutation of this residue to lysine or methionine results in a decrease in k_{cat} by a factor of 10⁴. Based on its location in the active site and catalytic necessity, we assume that this residue plays a critical role in stabilizing the pentacoordinate intermediate of the reaction, and that mutation

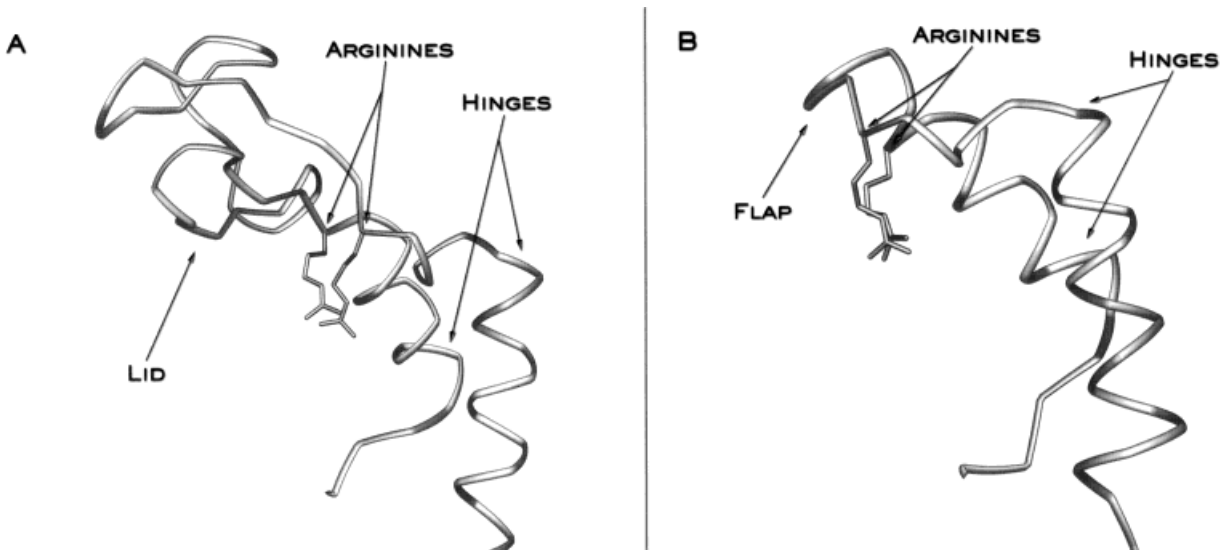


Fig. 7. Illustration of the mechanical structure of large and small form nucleotide kinase lid domains. **A:** The *B. stearothermophilus* lid assembly. **B:** The yeast uridylate kinase (PDB entry 1ukz) lid assembly. Generalized hinge points, catalytic arginine residues, and lid/flap domains are indicated. The location of the arginine residues relative to both lid/flap domains and hinge points causes them to be conformationally sensitive to fluctuations in the

position of the lid/flap domain, as is demonstrated in the present structure. In our model, the small size of the lid flap in small form enzymes better conveys entropic solvent forces to the catalytic arginines than in the large form enzymes. This increased sensitivity is thus the cause of the higher turnover rate in small form nucleotide kinases relative to the large forms.

to any other residue prevents this from occurring at significant rates. Therefore, the movement of this residue out of the active site as a result of small lid displacements, as seen in the *D. discoideum* and present structures, suggests a putative role for lid motions to interrupt phosphate transfer and trigger enzyme turnover via the movement of Arg160 (and probably Arg127) out of the active site.

Model of Lid Mediated Catalysis and Product Release

To give more support to the above argument, we are putting forth a mechanistic model for correlating lid or flap (in the case of small form adenylate kinases) motions with catalytic turnover. The primary grounds for this model are in the structural location of the Mg^{2+} ADP/ Mg^{2+} ATP binding and bridging arginines (Arg127 and Arg160 in the present structure) between the lid/flap hinges and the lid/flap itself (Fig. 7). Essentially, this places the residues responsible for pentacoordinate intermediate stabilization and binding of Mg^{2+} ADP/ Mg^{2+} ATP along a lever arm, whose mobile end (the lid/flap) sits in the energy bath of the solvent. The addition of random solvent derived forces to the end of this lever arm (again, the lid/flap), in the direction away from the active site, also applies a force on these two arginines. The result of these forces is to move the two arginines away from the active site while at the same time increasing the exposure of the phosphate chains to the solvent. This has the dual effect of both interrupting the transition state and increasing the

likelihood of product/reactant escape due to increased solvation of the binding site. In the *D. discoideum* and present structures, crystal packing (or other) forces mimic the effect of entropic solvation forces on the lid/flap, resulting in stabilization of an intermediate conformation.

Also notable in the present and *D. discoideum* structures is that the force created by lid displacement seems to be unequally distributed between the lid domain arginines, as can be seen by the lack of perturbation in the binding of Arg127 (Arg131 in *D. discoideum*) to the phosphate chain. This may occur because the lid/flap domain has a counterclockwise rotation (when looking "down" on the active site and relative to the main mass of the protein) in going from closed to open conformations, which has its major pivot point nearer to the Mg^{2+} ADP/ Mg^{2+} ATP binding arginine (Arg127) than to the bridging arginine (Arg160). A similar conformation is seen in the unbound structure of *E. coli* adenylate kinase,¹⁵ and we have also seen very similar motions in our molecular dynamics simulations, correlating the validity of this as a movement towards the open form.^{3,16} The effect of this asymmetric rotation is to produce larger displacements for the bridging arginine (e.g., Arg160) compared with the Mg^{2+} ADP/ Mg^{2+} ATP binding arginine (e.g., Arg127) as a result of lid motion. Thus, the phosphate bridging arginine (Arg160) may be a sensitive "on/off" switch, capable of interrupting the phosphate transfer reaction as a result of lid motions.

The electrostatic attraction of the Mg²⁺ADP/Mg²⁺ATP binding and bridging arginines for the phosphate chains undoubtedly balances or overcomes the input of force from the solvent to these residues most of the time, making lid opening/catalytic interruption a relatively rare event (on short time scales). This balance is probably due to the enzyme fine tuning the solvent's force input via the size of the flap/lid. This conclusion is supported by the fact that the small variety of adenylate kinases, which only have a 6 residue flap in place of the 30+ residue lid, have a reaction rate six times that of the large form species despite having identical active sites.³⁵ The small flap of these enzymes has a faster "diffusion time" (due to its smaller radius of gyration and mass) and hence it allows more direct transfer of entropic energy from the solvent to the Mg²⁺ADP/Mg²⁺ATP binding and bridging arginines, thus increasing enzyme turnover rate.

Product Release

Binding of both substrates (AMP/ADP in one site and Mg²⁺ATP/Mg²⁺ADP in the other) to the protein is not dependent on lid closure. This has been shown by kinetic studies in which mutation of either of the lid/flap arginines decreases k_{cat} greatly, but has negligible effects on binding or dissociation of either ligand.^{34,36} The random Bi-Bi catalytic mechanism of the enzyme supports the idea that both binding sites have similar accessibility to the diffusing substrate and that they also have similar access to the solvent during product release.³⁷ For this to be the case, both binding sites must be equally solvated after product formation.

Solvation of both binding sites in the postcatalytic state can only occur if the lid domain changes position to allow influx of solvent molecules into the active site. Once active site solvation occurs, there is an approximately equal chance of either ligand escaping from its binding site to the solvent. Preliminary high temperature molecular dynamics studies have suggested to us that product escape is by "backing" out of the pocket, adenine first, and that this can occur at either binding site with equal likelihood once the lid reaches a certain degree of openness.³

Based on the presence of ordered solvent, it is clear in the present structure that the partially open position of the lid domain has increased access of bulk solvent to the active site relative to fully closed complexes.^{11,12,17} In our model of product release, this serves to hydrate both the phosphate chains of the products/reactants and the polar residues of the active site and lid. Bulk water, which is excluded from active site during the reaction, can then interrupt the binding of the phosphate chains by replacing hydrogen bonds to the protein with hydrogen bonds to water molecules. The fact that we were able to crystallize this complex may indicate that the

conformation of the lid domain is not sufficiently open at room temperature to allow enough solvation of the binding site for product release to occur readily.

Mg²⁺ and the Ternary Complex

The Mg²⁺Ap₅A complex which we present here varies somewhat from what has been reported for the yeast enzyme (Fig. 4, Table IV).¹⁷ The *B. stearothermophilus* adenylate kinase complex has a fourth coordinated water molecule and the Mg²⁺-coordinated water molecules demonstrate tighter and more extensive interactions with the protein and Ap₅A than has been seen in the yeast complex (analysis based on hydrogen bond lengths in the PDB entry 2aky.full). Analysis of our structure has shown that the coordinated water molecules of the Mg²⁺ ion not only distribute charge but also orient the phosphate chains for nucleophilic attack.

In our Mg²⁺Ap₅A complex (Fig. 4), the right-planar and bottom-axial Mg²⁺ coordinated water molecules (300 [the fourth coordinating water] and 303) orient two of the three oxygens of the ϵ phosphate (equivalent to the α phosphate of AMP) placing the third phosphate oxygen (O3PE) in position to attack the γ phosphate in the other adenylate binding site. The rightmost water molecule, number 300, also hydrogen bonds to the NH₂ atom of Arg36 and the OD2 atom of Asp33, providing a link to the protein scaffold of the AMP/ADP binding site. The top-axial Mg²⁺ coordinated water molecule, number 302, is within hydrogen bonding distance of the α , β , and γ phosphates of Ap₅A (equivalent to the entire ATP phosphate chain), which, in addition to direct Mg²⁺ coordination of the phosphate chain, helps to orient the γ phosphate in the optimal position to be attacked. Unlike the other three coordinated water molecules, the back-planar water molecule, number 301, has no interactions with the phosphate chain. However, it has a strong bond (2.5 Å) with Asp84 and a weak hydrogen bond to the peptide chain of the glycine loop at Gly14.

From these observations, it is possible to see how Mg²⁺ binding activates catalysis in the *B. stearothermophilus* enzyme. Two of the four coordinated water molecules act to bridge the gap between phosphate chains, linking together and presumably stabilizing the motions of the two phosphate chains in the active site. This assembles a catalytic complex in which the O3 phosphate oxygen of AMP is free to attack the Mg²⁺ polarized γ phosphate of ATP. Once phosphate transfer occurs, the interactions between water molecules 300 and 303 (right-planar and bottom-axial) with the new ADP molecule's α phosphate and residues Asp33 and Arg36 provide a new scaffolding for the Mg²⁺ coordination complex and a starting point for the back reaction. Water molecule 302 (top-axial) may then act to facilitate the back reaction by hydrogen bonding back to the terminal

phosphate of the ADP in the Mg^{2+} ADP/ Mg^{2+} ATP binding site.

Binding of Mn^{2+}

In the body, Mn^{2+} is a potent mutagen, due to its ability to alter the reaction of DNA polymerases.³⁸ This activity is probably due to the preference of the Mn^{2+} ion for α - γ phosphate coordination, as opposed to Mg^{2+} , which prefers β - γ coordination. In adenylate kinases, however, Mn^{2+} binds as the less preferred β - γ chelate in a manner identical to Mg^{2+} , as has been shown in our Mn^{2+} Ap_5A structure and in a yeast adenylate kinase structure.²⁶ From examining our structure, we can see that in order to have α - γ coordination, Mn^{2+} would need to bind approximately in the location of water molecule 302 (the same as in the Mg^{2+} Ap_5A structure). However, there is an obvious problem with Mn^{2+} binding in this location: the α and γ phosphate oxygens to which it would coordinate are too distant (at 2.9 and 3.1 Å) for coordination, which typically occurs at distances of ~ 2.2 Å (Table IV). In order for α - γ coordination to be successful, the phosphate chain must wrap around the Mn^{2+} ion such that the γ phosphate moves toward the front of the binding site, closer to the Mn^{2+} ion. For mechanical reasons this is not possible with bound Ap_5A , although it might be possible with bound ATP. Overall, though, it appears that the protein prevents α - γ coordination of metal ions by forcing the phosphate chain to adopt an extended conformation when bound. This may be true of many of the proteins that contain the glycine loop nucleotide-binding structure and transfer the γ phosphate of ATP.⁵⁻⁸

Thermostability

The factors that contribute to the thermostability of a protein are known to be very subtle.³⁹ In general, it appears that nature utilizes a variety of factors to achieve this, including hydrophobic packing density, surface area to volume, hydrogen bonding, and salt bridging. Of these, hydrophobic packing density is probably the most important, although the others undoubtedly contribute. To determine the factors that have led to the increased thermostability (T_m 74.5°C) of the *B. stearothermophilus* adenylate kinase, we decided to compare it at the sequence level with the closely related (76% identity, Zn^{2+} binding, and gram positive) and mesophilic *B. subtilis* enzyme.^{20,21}

Comparison of the sequences of the *B. subtilis* and *B. stearothermophilus* adenylate kinases can give some general information on possible trends toward increasing thermostability. In terms of nonpolar interactions, there is no obvious shift toward hydrophobic residue shrinkage, growth, altered branching, or switching to hydrophilicity in the thermophilic enzyme. However, between the two structures there is a net gain of four methionines for the *B.*

stearothermophilus structure, which, in three of four cases, are mutated from isoleucines. This may be an indication that the more flexible methionine side chain can fill some internal cavities better than the β branched isoleucine side chain, thus allowing for tighter hydrophobic packing. Another trend seen is mutation of valine to isoleucine, which occurs again in three of the four cases where nonpolar residues increase in size. This also points toward increasing packing density by filling small cavities (where β branching is acceptable) in the interior of the protein with the extra δ methyl group of isoleucine.

Changes in polar interactions are difficult to predict in the absence of direct structural comparison due to the flexibility of most polar residues. However, arginines have been indicated to contribute to thermostability based on their ability to form more extended hydrogen bonding networks than any other residue, and we have therefore examined their contributions to the stability of the present structure.⁴⁰⁻⁴² Eight residues in the *B. subtilis* adenylate kinase change to arginines in the *B. stearothermophilus* protein, five of which are from lysine. Of these, arginines 55, 105, and 209 form some sort of hydrogen bond with neighboring side chains in our structure, with Arg105 forming three hydrogen bonds simultaneously with Asn2, Cys77, and Gln78.

Of the remaining three mutations to arginine, two form salt bridges with glutamates. In the *B. stearothermophilus* enzyme, Arg116 is mutated from aspartate and forms a strong salt bridge to Glu198, which has itself mutated from glutamine. This is likely to be a structurally significant interaction as it bridges two distant regions of polypeptide, may not exist in the *B. subtilis* enzyme, and therefore could add to thermostability. Arg131 in the lid domain is mutated from serine and forms a salt bridge to Glu156. Again, this is an example of a polar interaction between distant sections of polypeptide, in this case the top and bottom flaps at the "front" of the lid domain. Without site-specific mutation studies, however, it is difficult to determine conclusively the effect of any of these interspecies mutations on thermostability in the *B. stearothermophilus* structure.

CONCLUSIONS

The structure of the adenylate kinase from *B. stearothermophilus* has been determined in the Ap_5A , Mg^{2+} Ap_5A , and Mn^{2+} Ap_5A ligation states to high resolution. The enzyme's zinc finger-like motif has little impact on the structure of the lid domain but can be seen to have some homology to the DNA binding zinc finger domains of other proteins. However, the adenylate kinase lid is a truncation of typical DNA binding zinc finger domains, leaving it essentially a "zinc knuckle" domain. The lid domain of the *B. stearothermophilus* enzyme is in an intermediate conformation, despite the fully ligated state of the protein. The perturbation of the active site, due

to the lid's conformation, suggests that the lid domain may entropically regulate both termination of catalysis and product release. Bound Mg²⁺/Mn²⁺ in the *B. stearothermophilus* structure exists in the hexacoordinate state, having four water and two phosphate ligands, with a large number of protein and phosphate chain contacts that position the active site for catalysis. Finally, the extended state of the phosphate chain, at least in the case of Ap₅A, prevents Mn²⁺ from assuming its preferred α - γ coordination state, instead forcing it to assume the β - γ state typical of Mg²⁺.

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