

Structure of the Bis(Mg²⁺)–ATP–Oxalate Complex of the Rabbit Muscle Pyruvate Kinase at 2.1 Å Resolution: ATP Binding over a Barrel^{†,‡}

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ABSTRACT: Pyruvate kinase from rabbit muscle has been cocrystallized as a complex with Mg^{II}ATP, oxalate, Mg²⁺, and either K⁺ or Na⁺. Crystals with either Na⁺ or K⁺ belong to the space group *P*2₁2₁2₁, and the asymmetric units contain two tetramers. The structures were solved by molecular replacement and refined to 2.1 (K⁺) and 2.35 Å (Na⁺) resolution. The structures of the Na⁺ and K⁺ complexes are virtually isomorphous. Each of the eight subunits within the asymmetric unit contains Mg^{II}oxalate as a bidentate complex linked to the protein through coordination of Mg²⁺ to the carboxylates of Glu 271 and Asp 295. Six of the subunits also contain an α,β,γ -tridentate complex of Mg^{II}ATP, and the active-site cleft, located between domains A and B, is closed in these subunits. In the remaining two subunits Mg^{II}ATP is missing, and the active-site cleft is open. Closure of the active-site cleft in the fully liganded subunits includes a rotation of 41° of the B domain relative to the A domain. α -Carbons of residues in the B domain undergo movements of up to 17.8 Å (Lys 124) in the cleft closure. Lys 206, Arg 119, and Asp 177 from the B domain move several angstroms from their positions in the open conformation to contact the Mg^{II}ATP complex in the active site. The γ -phosphate of ATP coordinates to both magnesium ions and to the monovalent cation, K⁺ or Na⁺. A Mg²⁺-coordinated oxygen from the Mg^{II}oxalate complex lies 3.0 Å from P γ of ATP, and this oxygen is positioned for an in-line attack on the phosphorus. The side chains of Lys 269 and Arg 119 are positioned to provide leaving-group activation in the forward and reverse directions. There is no obvious candidate for the acid/base catalyst near the 2-*si* face of the prospective enolate of the normal substrate. A functional group linked through solvent and side-chain hydroxyls may function in a proton relay.

The physiological reaction of pyruvate kinase, the final step in glycolysis, yields ATP and pyruvate from ADP, P-enolpyruvate,¹ and a proton. The initial step in the reaction is believed to be phosphoryl transfer from P-enolpyruvate to Mg^{II}ADP, producing bound Mg^{II}ATP and the enolate of pyruvate (1). The enolate is subsequently protonated on the 2-*si* face prior to release from the active site as the α -keto carboxylate product (2). Ketonization of enolpyruvate supplies the free energy that renders the reaction “metabolically” irreversible. The enzyme catalyzes enolization of pyruvate, the first step in the back reaction, in the presence of metal cofactors and ATP or P_i or other “P_i-like” dianions (3). Despite the unfavorable equilibrium constant, the reverse reaction of pyruvate kinase can be demonstrated (4–6), although the *K*_M for pyruvate is high (~0.01 M).

The weak binding of pyruvate in the presence of ATP, dimerization of pyruvate at high concentrations (7), and an intrinsic, bicarbonate-dependent ATPase activity of the enzyme (8) hamper studies of enzymic complexes of ATP and pyruvate, which require extended incubation periods. In contrast, oxalate, an analogue of the enolate of pyruvate, binds to the enzyme with high affinity in the presence (9) or absence (10) of ATP. The robust complexes of pyruvate kinase, ATP, and oxalate have been exploited in spectroscopic studies, which have revealed information on the interaction of the enzyme and substrates with the 2 equiv of divalent cation which are obligatory cofactors in the reaction (9, 11, 2). Furthermore, synthetic oxalyl phosphate is a substrate for pyruvate kinase (13), and the ATP–oxalate complex represents the product of this reaction. The high affinity of oxalate for the enzyme also excludes bicarbonate-dependent ATPase activity. Properties of the pyruvate kinase complex with ATP and oxalate make it an attractive candidate for high-resolution crystallographic studies.

Previous crystallographic studies of pyruvate kinase have revealed the four domain architecture of each of the four subunits that make up the functional tetramer (14, 15). The N-terminal domain Ile12–Arg 42 consists of a short helix–turn–helix structure. The active site is located in a large cleft formed by the interface between two larger domains, the $\beta_8\alpha_8$ barrel or A domain and the β -barrel of the B domain.

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[‡] X-ray coordinates for these two complexes of pyruvate kinase have been deposited in the Brookhaven Protein Data Bank under file names 1A49 (K⁺) and 1A5U (Na⁺).

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¹ Abbreviations: P-enolpyruvate, phosphoenolpyruvate; ATP γ S, adenosine 5'-*O*-(3-thiotriphosphate); ADP α S, adenosine 5'-*O*-(1-thio-diphosphate); EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid; L-P-lactate, L-2-phospholactate.

Table 1: Data Collection Statistics

	Na ⁺	K ⁺
X-ray source ^a	SSRL 7-1 MAR	SSRL 7-1 MAR
no. of crystals used	1	1
maximum resolution (d_{\min} , Å)	2.35	2.1
total reflections	717 630	815 369
independent reflections	193 413	267 793
theoretical no. of reflections	216 128	301 829
% completeness of data	90	89
% completeness of data in highest shell ^b	74	78
R_{merge} (%)	5.3	4.7
R_{merge} (%) in the highest resolution shell ^b	16.4	21.2
average I/σ	22.1	19.8
unit cell parameters (Å)		
<i>a</i>	95.3	95.3
<i>b</i>	216.5	216.5
<i>c</i>	258.6	258.6

^a Wavelength used for data collection was 1.080 Å. ^b The highest-resolution shell is as follows: 2.46–2.35 Å (Na⁺–pyruvate kinase–Mg^{II}oxalate–Mg^{II}ATP) and 2.18–2.10 Å (K⁺–pyruvate kinase–Mg^{II}oxalate–Mg^{II}ATP).

The subunit interface is located in the C-terminal domain, which is a mixture of α and β structure. The A and B domains are connected by a strand that emerges from the A domain at Gly 115 and a second strand that rejoins the A domain at Ala 219. The potential for a ligand-induced closure of the active-site cleft was recognized from the structures of pyruvate kinase–ligand complexes (15, 16).

The primary sequence of pyruvate kinase does not contain motifs that are characteristic of any of the presently known families of nucleotide binding proteins (17, 18). None of the published high-resolution structures of pyruvate kinase has contained a metal–nucleotide complex. Hence, the mode of binding of ATP to pyruvate kinase is of special interest. The bis(Mg²⁺) complexes of pyruvate kinase with ATP, oxalate, and either Na⁺ or K⁺ have been cocrystallized. The crystals diffract to high resolution. The present paper reports the molecular structures of these complexes.

EXPERIMENTAL PROCEDURES

Crystallization and Data Collection. Rabbit muscle pyruvate kinase was isolated following the protocol of Teitz and Ochoa (8). Solutions of the protein were chromatographed over an S-300 gel-filtration column equilibrated with 10 mM Hepes-KOH, pH 7.0, and 0.1 M NaCl or KCl depending on the complex to be studied. Single crystals of pyruvate kinase complexed with Na⁺, oxalate, Mg²⁺, and ATP were grown at room temperature by the batch method from 7.1 mg/mL protein, 7.8% PEG 8000, 0.16 M NaCl, 5 mM MgCl₂, 5 mM ATP, 1 mM oxalate, and 50 mM succinate, pH 6.0. Rodlike crystals $\sim 0.1 \times 0.3 \times 0.6$ mm grew within 3 days. The crystals belong to the space group $P2_12_12_1$. The unit cell dimensions are $a = 95.3$ Å, $b = 216.5$ Å, and $c = 258.6$ Å. The asymmetric unit contains two tetramers.

The protocol for growing crystals of the pyruvate kinase complex with K⁺, oxalate, Mg²⁺, and ATP employed the same conditions as described for the complex of Na⁺, except that KCl replaced NaCl. Crystals of the corresponding complex with K⁺, however, did not grow spontaneously. To obtain these crystals, the crystallization mixture was micro-

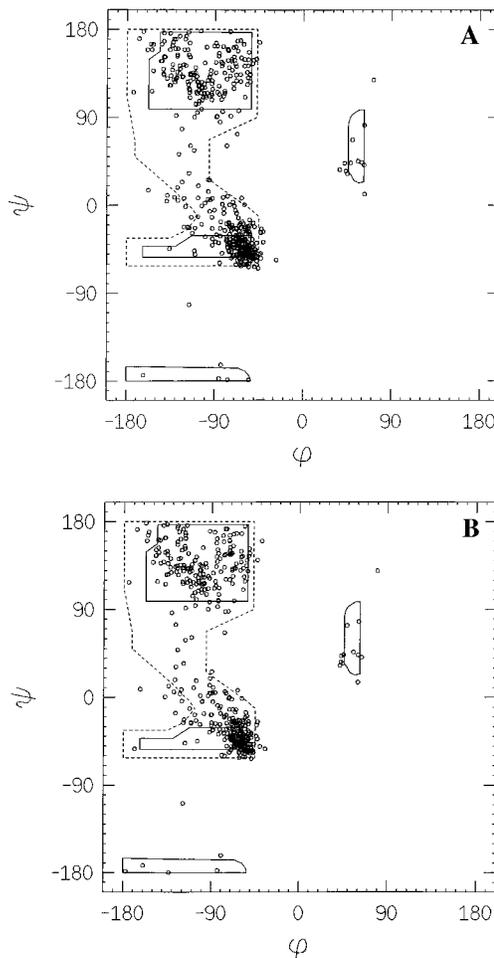


FIGURE 1: Ramachandran plots of the main-chain nonglycinyl dihedral angles of subunit 1 for the Na⁺–pyruvate kinase–Mg^{II}-oxalate–Mg^{II}ATP complex (A) and the K⁺–pyruvate kinase–Mg^{II}-oxalate–Mg^{II}ATP complex (B). Fully allowed ϕ and ψ values are shown by solid enclosures. Partially allowed regions are enclosed by broken lines. For both complexes the most severe left-handed outlier is Thr 327 and the most severe right-handed outlier is Ser 361. Analysis of the Ramachandran plot of the K⁺ complex with the program PROCHECK (45) showed that 90.2% of the residues were in fully allowed regions and 9.4% were in additional allowed regions. The same analysis of the Na⁺ complex revealed that 90.0% of the residues were in fully allowed regions and 9.6% were in additional allowed regions.

seeded from crystals of the Na⁺ complex 2 days after setup. Rodlike crystals grew within 3 days, and the crystals have the same morphology, space group, and cell dimensions as the seed crystals.

The transfer, freezing procedure, and data collection described below were applied to crystals of the complexes with either Na⁺ or K⁺. The crystals were transferred from the crystallization medium into a synthetic mother liquor containing 20% PEG 8000, 0.3 M salt (NaCl or KCl), 5 mM Mg²⁺, 2mM oxalate, and 5 mM ATP in 50 mM succinate, pH 6.0 (solution A), for transport. Prior to freezing, each crystal was equilibrated with the cryoprotectant ethylene glycol. The equilibration was carried out by serial transfers of each crystal to solution A containing increasing concentrations of ethylene glycol. Initially, each crystal was moved to solution A that contained 10% ethylene glycol and allowed to equilibrate for 2 min. The crystal was transferred two additional times, first to solution A with 15% ethylene glycol

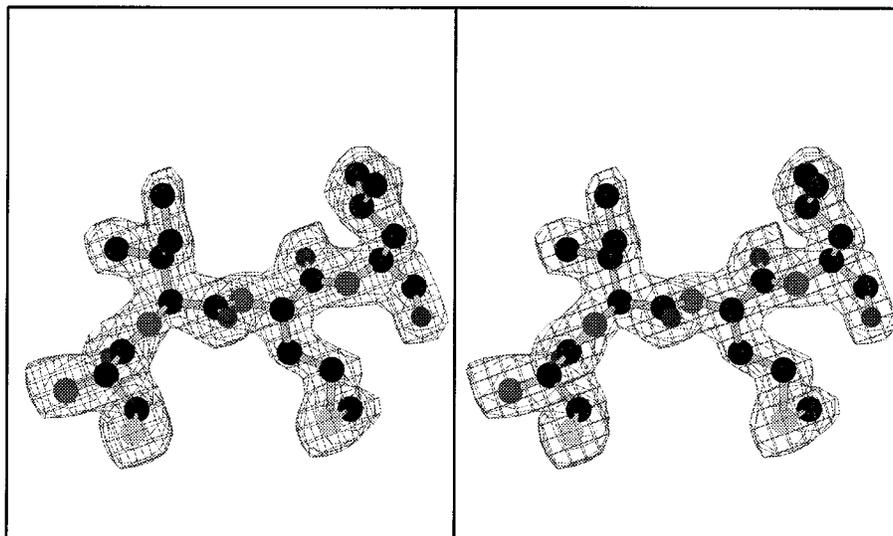


FIGURE 2: Representative portion from $2F_o - F_c$ electron density map of the K^+ -pyruvate kinase- Mg^{II} oxalate- Mg^{II} ATP complex calculated with the final refined phases. Electron density for residues $^{357}C1ML$ of subunit 1 was contoured at 1σ . The program MOLDED (A. Fisher, unpublished results) was used to prepare this figure.

for 2 min and then to solution A with 20% ethylene glycol. The crystal was picked up within 20 s after addition to the 20% ethylene glycol solution in a loop of surgical suture and flash-frozen in a stream of N_2 at $-160^\circ C$ (19, 20). X-ray data were collected with a MAR detector from one frozen crystal of each complex on beamline 7-1 at Stanford Synchrotron Radiation Laboratory. Data collection statistics are summarized in Table 1.

Molecular Replacement. The three-dimensional structure of Na^+ -pyruvate kinase- Mg^{II} oxalate- Mg^{II} ATP was solved by molecular replacement using the software package AMORE (21, 22). X-ray coordinates of a tetramer of pyruvate kinase- Mg^{2+} -L-phospholactate (16) served as the search model. The rotation function revealed a symmetry-unique peak at a level of 8.7σ . A second symmetry-unique peak was present at a level of 7.6σ . The next largest peak in the map appeared at a level of 3σ . The translation function was applied to the two solutions. Rigid-body refinement was executed to refine the rotation and translation solutions, and the overall R -factor dropped from 50.7% to 41.4%. The final Eulerian angles and translation solutions were $\alpha = 167.1^\circ$, $\beta = 18.86^\circ$, $\gamma = 256.8^\circ$, $a = 0.44$, $b = 0.15$, and $c = 0.09$ for solution I and $\alpha = 46.84^\circ$, $\beta = 114.4^\circ$, $\gamma = 107.2^\circ$, $a = 0.48$, $b = 0.40$, and $c = 0.40$ for solution II.

Refinement. The structure of Na^+ -pyruvate kinase- Mg^{II} oxalate- Mg^{II} ATP was refined to 2.35 \AA with the least-squares refinement program TNT (23), resulting in a drop in the crystallographic R -factor from 41.4% to 34.6%. Initial examination of the map using the molecular modeling program FRODO (24) revealed that all eight B domains were in conformations differing from those of the search model. Hence, all B domains were removed from the model, and a round of refinement was performed. The resulting $F_o - F_c$ density for the B domains of all subunits was well defined to 3σ . The $F_o - F_c$ density was used to build in the residues of the B domains using FRODO. Another cycle of refinement, following addition of all eight B domains reduced the R -factor to 27.9%. Further manual adjustments in the model were made with FRODO and O (25). Regions of density

corresponding to Mg^{II} oxalate and to Na^+ were clearly present in all eight subunits. Density corresponding to Mg^{II} ATP was clearly present in subunits 1 and 3–7. Oxalate, ATP, and the metal ions were included in the model at this point. Water molecules were added to the model after another round of least-squares refinement and manual adjustment. The positions of the ordered water molecules were determined using the peak search algorithm in the TNT package. Following completion of manual adjustments and inclusion of waters, the model was refined, excluding the metal ions, oxalate, and ATP. The resulting $F_o - F_c$ electron density confirmed the positions of the metal ions, oxalate, and ATP, which were returned to the model for a final round of refinement. The current R -factor including all data to 2.35 \AA resolution is 19.0%. The present model with two tetramers includes 1810 water molecules (temperature factor < 85). The rms deviations from “ideal” geometry are 0.013 \AA for bond lengths, 2.1° for bond angles, and 0.007 \AA for coplanar groups.

Initial phases for the complex of K^+ -pyruvate kinase- Mg^{II} oxalate- Mg^{II} ATP were calculated from the refined coordinates of the Na^+ -pyruvate kinase- Mg^{II} oxalate- Mg^{II} ATP structure, leaving out the metal ions, ATP, oxalate, and water molecules. Crystallographic least-squares refinement using the program TNT reduced the R -factor to 29.4% for all data to 2.1 \AA . Manual adjustments in the model were made with the program O. Metal ions, ATP, and oxalate were then included in the model. Water molecules were added to the model after a cycle of refinement and manual adjustments. Strategies for further refinement of the model were the same as described for the corresponding model of the complex with Na^+ . The current R -factor including all data to 2.1 \AA resolution is 19.8%. The present model with two tetramers includes 1923 water molecules (temperature factor < 85). The rms deviations from “ideal” geometry are 0.022 \AA for bond lengths, 2.0° for bond angles, and 0.012 \AA for coplanar groups. Ramachandran plots for subunit 1 main-chain nonglycyl dihedral angles of the complexes of Na^+ and K^+ are shown in Figure 1, panels A and B, respectively.

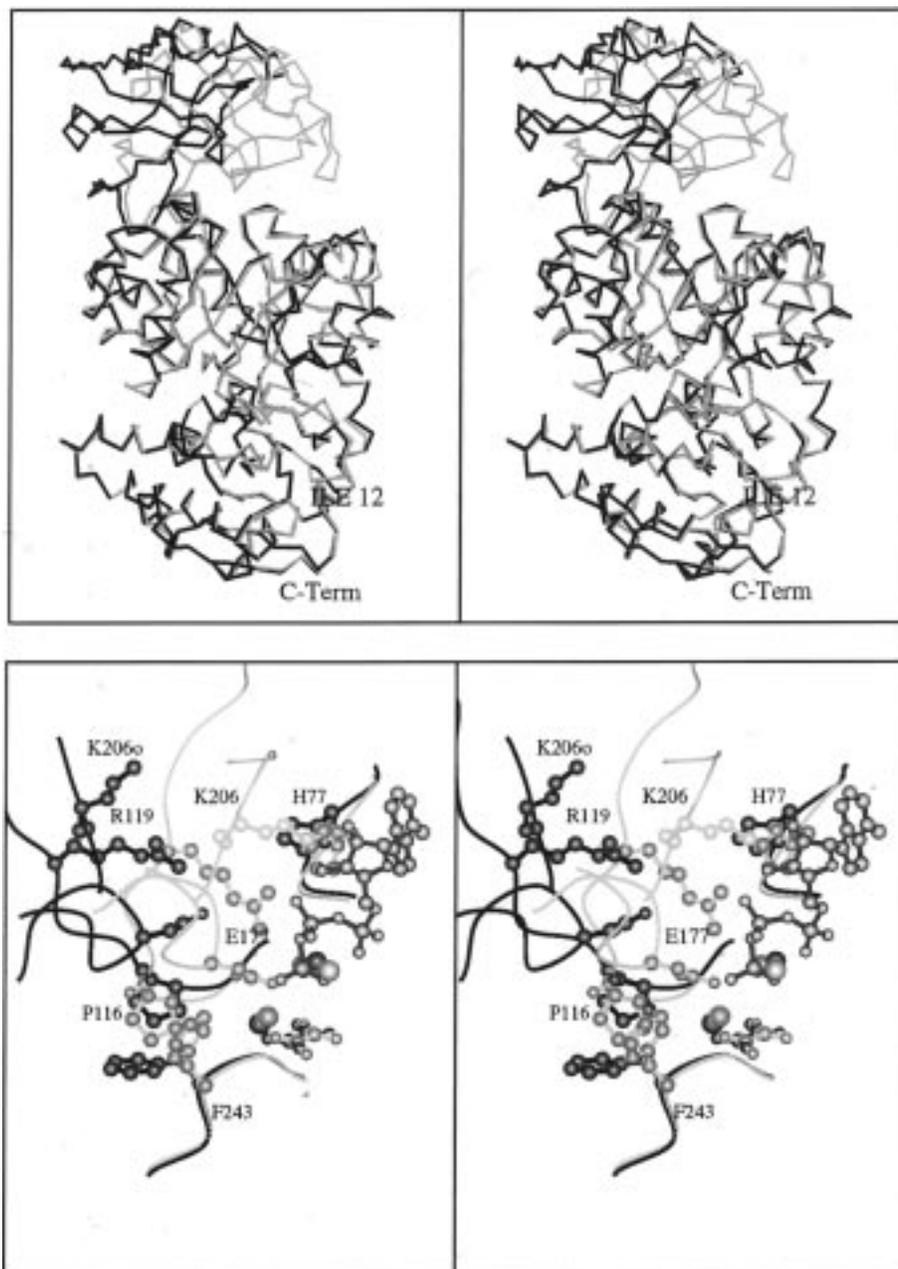


FIGURE 3: (a, top) Overlay of the α -carbons of subunits 5 and 8. Subunit 5, shown in gold, has a closed active-site cleft. Subunit 8, shown in green, has an open active-site cleft. (b, bottom) Overlay of the active site of subunits 5 and 8. Subunit 5 is shown in gold and subunit 8 is shown in green. A ball-and-stick model of $\text{Mg}^{\text{II}}\text{ATP}$ present in subunit 5 is shown in red/metallic brown. The green and gold spheres behind the γ -phosphate of ATP represent K^+ in the open and closed clefts, respectively. Lys 206 in the open conformation (green) is labeled K206o. The program MOLSCRIPT (46) was used to prepare this figure.

RESULTS AND DISCUSSION

Only subtle differences were observed in the structures of the complexes with K^+ and Na^+ . Figure 2 illustrates representative electron density calculated from the coefficients $2F_o - F_c$ for the complex with K^+ . All residues in each of the eight subunits of both complexes (with the exception of some surface residues and residues 1–11) have well-defined side chains. The overall chain topology of each subunit is identical to that described previously (15).

A distinguishing characteristic of the structure is the variety of subunit conformations within the asymmetric unit. Changes in the positions of the B domains (residues 116–218), relative to the remainder of the protein, account for the various conformations. Movement of the B domain can be

described as a pure rotation of the α -carbons about a unique axis. The angle of rotation determines the volume of the active-site cleft in the interface between the A and B domains. The active-site cleft is closed in subunits 1, 3–5, and 7. The cleft of subunit 6 is slightly opened, with the B domain having an angle of rotation of 11° relative to the closed subunits. The clefts of subunits 2 and 8 are open, and in these subunits the B domain exhibits an angle of rotation of 41° relative to the closed subunits (Figure 3a). The subunits that have the closed conformation (1 and 3–7) all have $\text{Mg}^{\text{II}}\text{ATP}$ bound in their active sites; whereas the subunits that have the open conformation (2 and 8) do not have $\text{Mg}^{\text{II}}\text{ATP}$ in their active sites. Pro 116, located in the region linking subunits A and B, is an indicator for the cleft

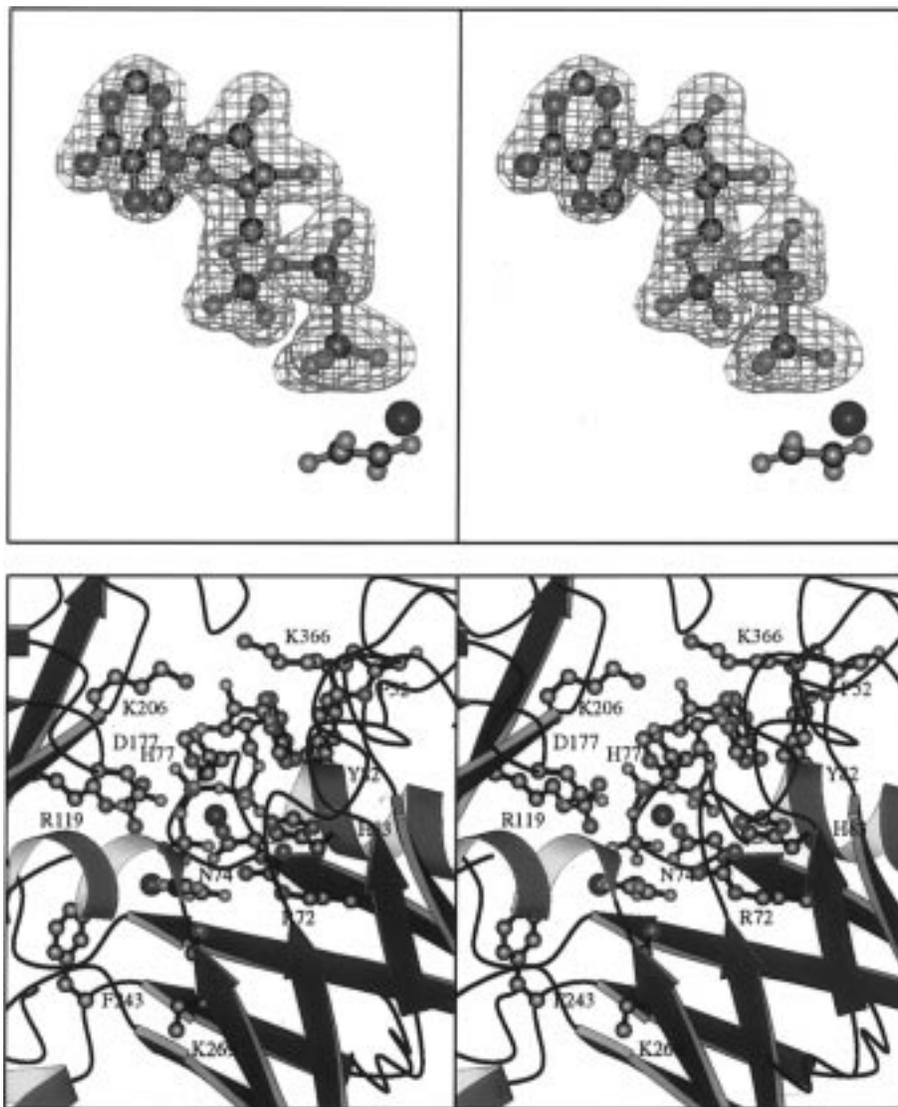


FIGURE 4: (a, top) $F_0 - F_c$ electron density map contoured at 4σ for ATP of the K^+ -pyruvate kinase- Mg^{II} oxalate- Mg^{II} ATP complex (subunit 5). A ball-and-stick model of ATP is shown in the density. The Mg^{II} oxalate complex is also shown as a ball-and-stick model. The program MOLDED (A. Fisher, unpublished results) was used to prepare this panel. (b, bottom) Ribbon representation of the closed active site of the K^+ -pyruvate kinase- Mg^{II} oxalate- Mg^{II} ATP complex (subunit 5). Significant residues are represented by ball-and-stick models. For simplicity, water molecules were not included. Color codes: green, Mg^{2+} ; violet, K^+ ; purple, phosphorus; red, oxygen; blue, nitrogen; gray, carbon. The program MOLSCRIPT (46) was used to prepare this panel.

closure. The ϕ , ψ values for Pro 116 range from -62° , 58° to -67° , 68° for the closed conformations and from -59° , 142° to -65° , 152° for the open conformations (see Table 2). The orientation of the phenyl ring of Phe 243 from the A domain flips up toward the active site in the closed clefts (Figure 3b). The flip of the Phe ring avoids a close contact with Pro 116 in the closed conformation.

Active Site of the Closed Subunits. Difference electron density ($F_0 - F_c$) of ATP from the active site of subunit 5 of the complex with K^+ is shown in Figure 4a. The difference density of Figure 4a was generated by excluding ATP from the refinement and phase calculation. The density exhibits well-defined lobes for the phosphate and ribose oxygens and for the exocyclic amino nitrogen of adenine. A ball-and-stick model of ATP is shown in the density. Figure 4b shows the active site of the closed subunit for the K^+ complex.

Metal Ion Coordination in the Closed Subunits. Coordination of the inorganic cations in the subunits with ATP is

Table 2: Unit Cell Contents and Domain Orientations within Subunits

subunit	ϕ , ψ values of Pro 116 (deg)	active site contains Mg^{II} ATP	rotation angle, θ , of the B domain relative to the A domain ^a (deg)
1	-67, 68	+	0
2	-59, 152	-	41
3	-62, 66	+	1
4	-68, 68	+	1
5	-68, 59	+	0
6	-65, 68	+	11
7	-65, 58	+	1
8	-65, 142	-	41
1 (L-P-lac) ^b	-61, 60		21
8 (L-P-lac) ^b	-65, 150		45

^a Referenced to subunit 1, where $\theta = 0^\circ$. ^b Angles correspond to ϕ, ψ values of Pro 116 and domain rotations in subunits 1 and 8 of the complex of pyruvate kinase, L-P-lactate, Mg^{2+} , and K^+ (16).

summarized in Figure 5a. The Mg^{II} oxalate complex is bound to the protein through coordination of Mg^{2+} to the carboxyl-

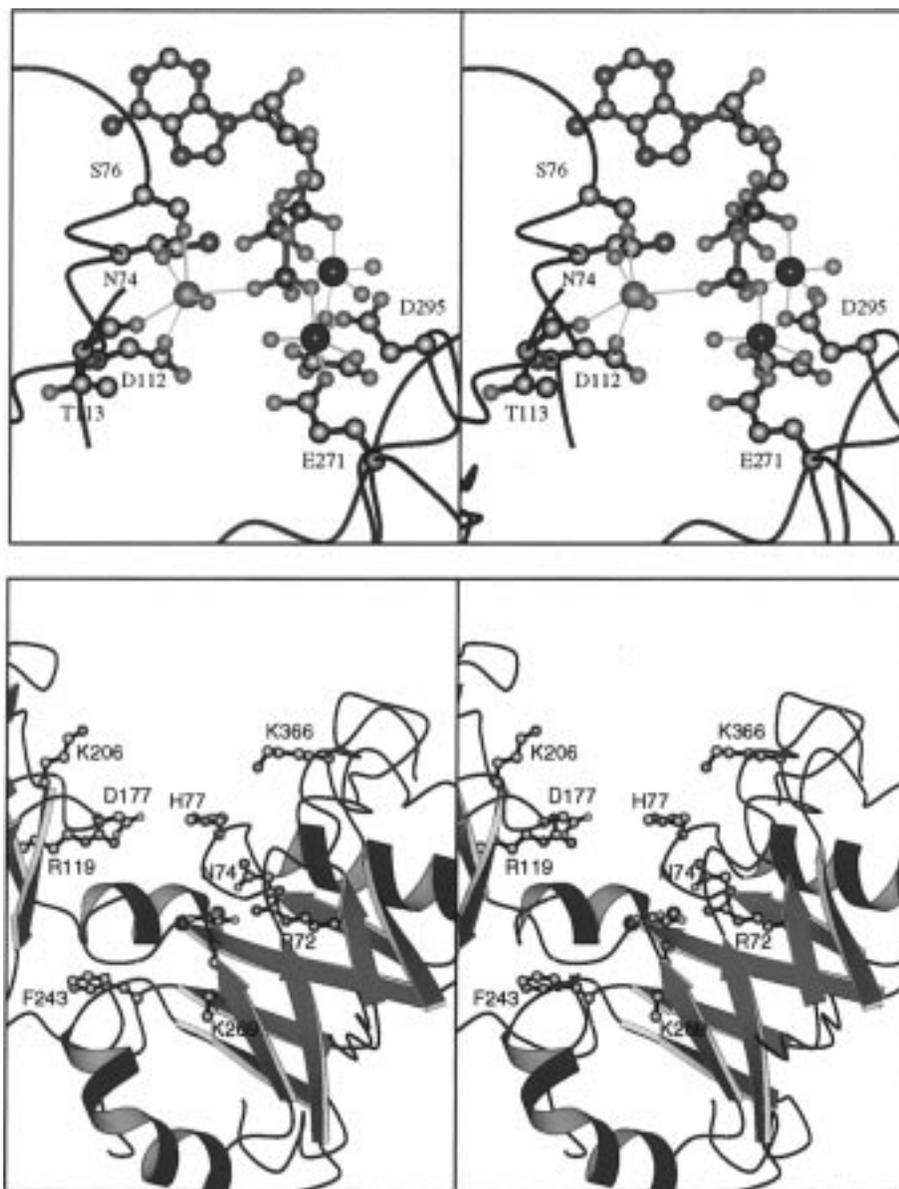


FIGURE 5: (a, top) Ball-and-stick model of the K^+ -pyruvate kinase- Mg^{II} oxalate- Mg^{II} ATP complex closed active site (subunit 5). The coordination of the inorganic cations is shown by the orange lines. The Mg^{2+} to ligand distances range from 2 to 2.3 Å and the K^+ to ligand distances range from 2.6 to 3.0 Å. (b, bottom) Ribbon representation of the K^+ -pyruvate kinase- Mg^{II} oxalate- Mg^{II} ATP complex open active site (subunit 8). Ball-and-stick models represent important residues. For simplicity, water molecules are not included. The program MOLSCRIPT (46) was used to prepare this figure.

ate side chains of Glu 271 and Asp 295. The carboxylate ligands to the metal ion, Mg^{2+} or Mn^{2+} , are present in the structures of complexes of the enzyme with L-phospholactate and pyruvate, respectively (15, 16). The Mg^{II} oxalate complex is bidentate, and the other ligands in the octahedral coordination sphere of Mg^{2+} are an oxygen from the γ -phosphate of ATP and a water molecule. The coordination scheme of Mg^{2+} at this site is identical to that determined by EPR spectroscopy for Mn^{2+} in metal hybrid complexes of pyruvate kinase with Mg^{II} ATP and oxalate (9).

The triphosphate chain of ATP is "curled" due to its α,β,γ -tridentate coordination to Mg^{2+} . The stereochemical configuration of the Mg^{II} ATP complex is consistent with the results from studies with isomers of exchange-inert Rh^{III} -ATP complexes (26) and with the stereoselectivity observed for nucleoside phosphorothioate substrates (27). The octahedral coordination sphere of this second Mg^{2+} is completed by three water molecules. The stereochemistry of the bis-

(Mg^{2+}) coordination to the γ -phosphate is identical to that observed in complexes of Mn^{2+} and Mg^{2+} with epimers of [γ - ^{17}O]ATP γ S (12). As found in two other complexes of pyruvate kinase (15, 16), the monovalent cation, K^+ or Na^+ , coordinates to four protein ligands: the carbonyl of Thr 113; the hydroxyl of Ser 76, the carboxylate of Asp 112, and the carboxamide oxygen of Asn 74. The monovalent cation also coordinates to an oxygen from the γ -phosphate of ATP. A sixth ligand to the monovalent cation, a water molecule, is discernible in all of the subunits that contain ATP except for subunit 1 in the complex of K^+ and subunit 3 in the complex of Na^+ .

In the subunits containing ATP, each of the three peripheral oxygens of the γ -phosphate moiety coordinates to one of the three inorganic cations in the active site. The γ -phosphate bridge between the two divalent cations was detected earlier in EPR studies of the ATP/oxalate complex (9). The possibility that the monovalent cation coordinated

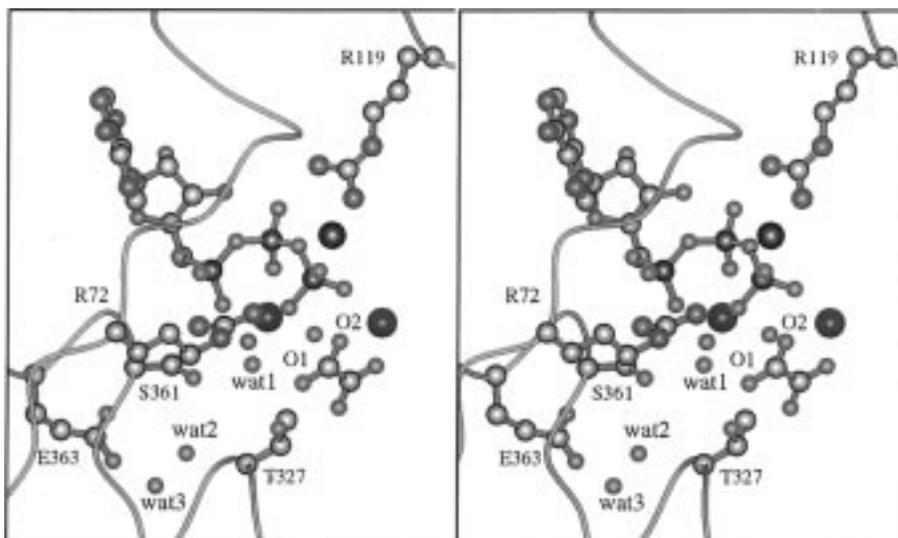


FIGURE 7: Stereoview of the K^+ -pyruvate kinase- Mg^{II} oxalate- Mg^{II} ATP complex closed active site (subunit 5). Ball-and-stick models of residues having potential mechanistic roles are shown. Green spheres represent Mg^{2+} , and the dark blue sphere represents K^+ . The program MOLSCRIPT (46) was used to prepare this figure.

scopic observations on Mn^{2+} complexes of pyruvate kinase with P-enolpyruvate are compatible with a strained, four-member chelate ring interaction between Mn^{2+} and the substrate (36). The ϵ -amino group of Lys 269 is also close to the “nucleophilic” oxygen of oxalate and could participate in leaving-group activation as well as promote enolization of a bound pyruvate. Hence, there is potential for leaving-group activation in both directions of the reaction.

The identity of the group or groups responsible for general acid/base catalysis in the reaction remains somewhat elusive. The ϵ -amino group of Lys 269 has previously been mentioned as a candidate for the acid/base catalyst (6, 14). The side chain of Lys 269, however lies on the *2-re* face of the prospective enolate (15, 16) and this position is incompatible with the known stereochemistry of protonation (2). The proximity of the ϵ -amino of Lys 269 to the “nucleophilic” oxygen of oxalate (O2, Figure 7) indicates that this group may participate in stabilizing the enolate and in leaving-group activation.

A water molecule that is in the coordination sphere of Mg^{2+} of the Mg^{II} ATP (wat1, Figure 7) occupies the strategic position above the prospective *2-si* face of the enolate of pyruvate (O1, Figure 7). Previous studies of the exchange of the methyl protons of pyruvate with solvent promoted by pyruvate kinase and metal ATP complexes indicated an electrophilic role of the divalent metal ions (37). Coordination of a divalent cation to pyruvate (15) is expected to promote enolization. Subsequent experiments with Cr^{III} ATP as the activator of exchange, however, also implicated the nucleotide-bound metal ion in activation of the acid/base catalyst (38). Enthusiasm for assignment of the Mg^{2+} -bound water as the catalyst is, however, restrained by the observation that $Co^{III}(NH_3)_4$ ATP also promotes the exchange reaction at a rate only 3-fold lower than that of Mg^{II} ATP (39). Although the amine ligand in the $Co^{III}(NH_3)_4$ ATP that would occupy the position analogous to that of the “catalytic water” ligand is in a *trans* position and exchanges with solvent rapidly above pH 5 (40), it is unlikely that a $Co^{III}-NH_2^-$ species participates in the enzyme-catalyzed exchange reac-

tion owing to the low acidity of the amine ligand (41). It is possible that an alternative pathway for proton transfer becomes viable in the $Co^{III}(NH_3)_4$ ATP complex. For example, in the exchange reaction of pyruvate promoted by phosphate and “phosphate-like anions” (3), the reaction rate correlates with the pK_a of the anion. The nucleotide-bound metal and its waters of hydration are presumably missing in the phosphate-activated exchange.

More recent 3H -trapping experiments (42, 43) and measurements of solvent 2H isotope effects (43) suggested the possibility of a proton relay in the acid/base chemistry of pyruvate kinase. A proton relay might explain the absence of a clear candidate for the general acid/base catalyst proximate to the *2-si* face of the prospective enolate. The side chains of the highly conserved residues, Ser 361, Thr 327, and Arg 72, project into the region above the *2-si* face of the prospective enolate. The functional groups of these side chains could participate in a proton relay leading to a general acid/base catalyst somewhat removed from the site of proton addition and removal. Two water molecules (wat2 and wat3, Figure 7) “connect” the hydroxyls of Ser 361 and Thr 327 to the carboxyl(ate) of Glu 363, another highly conserved residue (Figure 7). The “buried” side chain of Glu 363 does not lie close to a source of positive charge and might function as the acid/base catalyst through a relay of bound water and the hydroxyls of Ser 361 or Thr 327. The water molecules and hydroxyl side chains could conserve a pool of 3H in the presence of a bound metal P-enolpyruvate complex and thus allow 3H trapping in the chase phase of the experiments (43). Alternatively, the guanidinium moiety of Arg 72 might constitute a small reservoir of relatively slowly exchanging 3H (44) that eventually gets trapped in pyruvate in the chase phase of the trapping experiments. Experiments with site-specific mutant forms of pyruvate kinase should assist in resolving these issues.

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