Ligand-Induced Domain Movement in Pyruvate Kinase: Structure of the Enzyme from Rabbit Muscle with Mg$^{2+}$, K$^+$, and L-Phospholactate at 2.7 Å Resolution$^{1,2}$

Todd M. Larsen, Matthew M. Benning, Gary E. Wesenberg, Ivan Rayment,* and George H. Reed*,$^3$

Institute for Enzyme Research, Graduate School, and *Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53705

Received May 15, 1997, and in revised form June 19, 1997

The structure of rabbit muscle pyruvate kinase crystallized as a complex with Mg$^{2+}$, K$^+$, and L-phospholactate (L-P-lactate) has been solved and refined to 2.7 Å resolution. The crystals, grown from solutions of polyethylene glycol 8000 at pH 7.5, belong to the space group P2$_1$ and have unit cell parameters $a = 144.4$ Å, $b = 112.6$ Å, $c = 171.2$ Å, and $\beta = 93.7^\circ$. The asymmetric unit contains two tetramers. The crystal structure reveals that the eight subunits within the asymmetric unit adopt several different conformations. These conformations are characterized by differences in the relative positions of protein domains A and B, resulting in different degrees of closure of the active site cleft that occupies the interface between these two domains. The global conformational differences may be described as rotations of the B domain with respect to the (βα)_{9}-barrel of the A domain. Carbon atoms of the backbone in domain B rotate $>20^\circ$ from the most open to the most closed subunit. The different conformations among subunits within the asymmetric unit are accompanied by 3–3.8 Å shifts in the position of Mg$^{2+}$ and a significant change in the orientation of the phenyl ring of Phe 243. In all of the subunits, Mg$^{2+}$ coordinates to the protein through the carboxylate side chains of Glu 271 and Asp 295. In the subunit having the most closed conformation, Mg$^{2+}$ also coordinates to the carboxylate oxygen, the bridging ester oxygen, and a nonbridging phosphoryl oxygen of L-P-lactate. Mg$^{2+}$ to L-P-lactate coordination is missing in subunits exhibiting a more open conformation. K$^+$ coordinates to four protein ligands and to a phosphoryl oxygen of the L-P-lactate. The position and liganding of K$^+$ are unaffected by the different conformations of the subunits. The side chain of Arg 72, Mg$^{2+}$, and K$^+$ provides a locus of positive charge for the phosphate moiety of the analog in the closed subunit.

Key Words: pyruvate kinase; L-phospholactate; conformational shifts.

Pyruvate kinase (PK)$^4$ (EC 2.7.1.40) catalyzes the final step of glycolysis—the reaction of P-enolpyruvate, ADP, and a proton to give ATP and pyruvate. PK requires two equivalents of divalent cation (1–3), and the enzyme from most sources exhibits an additional requirement for activation by K$^+$ (4–6). One divalent cation binds to the enzyme as a complex with the nucleotide substrate (ATP or ADP) and the other divalent cation binds to the protein as well as to functional groups from pyruvate (7). The γ-phosphate of ATP bridges the two divalent cations (8, 9). Stereochemical investigations with isotopically chiral forms of [γ-13C]ATPγS suggested that the γ-phosphate group also interacts with additional centers of positive charge (10).

Prevailing evidence indicates that the physiological reaction of PK proceeds in two chemical steps. The first step is phosphoryl transfer from P-enolpyruvate to give ATP and the enolate of pyruvate (11). The second step is the addition of a proton to the 2 si face of the enolate of pyruvate to produce the keto form of the three carbon substrate (12).

PK also catalyzes a variety of side reactions, including the decarboxylation of oxalacetate (13), the enolization of pyruvate (14), an ATP- and bicarbonate-depen-

---

$^1$This research was supported in part by NIH Grants GM35752 (G.H.R.) and AR35186 (I.R.).
$^2$X-ray coordinates for this complex of pyruvate kinase have been deposited in the Brookhaven Protein Data Bank file laqf.
$^3$To whom correspondence should be addressed.

$^4$Abbreviations used: PK, pyruvate kinase; L-P-lactate, L-phospholactate; P-glycolate, 2-phosphoglycolate; P-enolpyruvate, phospho(enol)pyruvate; PEG, poly(ethylene glycol); Ap5A, diadenosine pentaphosphate.
dent ATPase, an ATP-dependent phosphorylation of α-hydroxy or α-thio carboxylates (15, 16), and an ATP- and bicarbonate-dependent phosphorylations of fluoride (17) and of hydroxylamine (18, 19). These side activities reflect the capacity of the active site to labilize the γ-phosphate of ATP or to stabilize the enolate of pyruvate.

PK from all known sources is a tetramer of MW ~237,000. Mammals express four major isozymes of PK, M1, M2, L, and R. The M1 isozyme of muscle tissue exhibits hyperbolic kinetics under normal assay conditions (5). The M1 isozyme does, however, show cooperative behavior in the presence of the allosteric effector, phenylalanine (20). The other common isozymes of PK, M2, L, and R, exhibit cooperative behavior in the presence of substrates and respond to allosteric effectors, including fructose 1,6-bisphosphate (21).

The first three-dimensional structure of PK reported was that of type M1 from cat muscle (22–24). This structure revealed that each subunit folds into four domains, A, B, C, and N. Domain N (residues 1–42) is a short helix–turn–helix. Domain A (residues 43–115 and 219–387) is a parallel (β/α)₈ barrel. Domain B (residues 116–218) is a nine-stranded β barrel (7). Domain C (residues 388–530) is a collection of five α-helices and five β-sheets. The active site is located in a cleft between domains A and B. The position of the active site in the cleft between domains implies that PK belongs to the group of enzymes that employ interdomain movement as a mechanism for isolating the fully liganded active site from the bulk solvent (25). The topology of the recently refined model for cat PK (26) is identical to that reported for PK from rabbit muscle (7).

The crystal structure of the allosteric type I PK from Escherichia coli in the inactive T-state at 2.5 Å resolution was recently reported (27). The type I isozyme of E. coli is similar to the mammalian isozymes M2, L, and R. This bacterial PK is allosterically activated by fructose 1,6-bisphosphate and inhibited by ATP (28). Each domain of the bacterial PK in the T-state has essentially the same structure as found in the M1 R-state, although the relative orientations of the domains differ (27).

The crystal structure at 2.9 Å resolution (7) of rabbit muscle PK cocrystallized with pyruvate, K⁺, and Mn²⁺ provided additional insight into the roles of various groups in binding of one divalent cation and the monovalent cation. Mn²⁺ coordinates to the protein through the carboxylate side chains of Glu 271 and Asp 295 and to pyruvate through the carbonyl oxygen and the carboxylate oxygen. K⁺, located 5.7 Å from the Mn²⁺, coordinates to four protein ligands: the carbonyl of Thr 113, O' of Ser 76, O³ of Asn 74, and O² of Asp 112. The stereochemistry of the active site indicated that side chains of Arg 72 and Lys 269 were potential sites for binding of the phosphate group of P-enolpyruvate or the γ-phosphate of ATP.

A slow P-enolpyruvate hydratase activity of PK (29), however, presently precludes co-crystallization of a complex with P-enolpyruvate. PK catalyzes the ATP dependent phosphorylation of l-lactate to l-P-lactate (16). l-P-lactate (II) is structurally similar to P-enolpyruvate (I).

Complexes of PK with Mn²⁺ and phosphoesters such as P-lactate and P-glycolate give EPR spectra that are virtually identical to that of Mn²⁺ and P-enolpyruvate (30), and these analogs are therefore considered to be reliable mimics of the substrate. The present paper reports the structure at 2.7 Å resolution of PK complexed with Mg²⁺, K⁺, and l-P-lactate.

**MATERIALS AND METHODS**

Crystalization and data collection. The protocol of Tietz and Ochon (17) was followed to isolate PK from rabbit skeletal muscle. As a final purification step, solutions of the protein were chromatographed over an S-500 gel filtration column (16). The stock solution of the enzyme was stored in 10 mM Hepes, pH 7.0, 0.10 M KCl. Crystals were grown at room temperature by the batch method from 7.5 mg/mL PK, 7.7% PEG 8000, 0.21 M KCl, 5 mM MgCl₂, 5 mM ATP, 4.5 mM l-lactate, 50 mM Hepes pH 7.5, 0.12 mM Asp5a. Single rodlike crystals ~0.5 x 3.0 x 0.2 mm grew within 3 days. The crystals belong to the space group P2₁. The unit cell dimensions are a = 144.4 Å, b = 112.6 Å, c = 171.2 Å, and β = 93.7°. The asymmetric unit contains two tetramers. The combined action of trace contaminations from adenylate kinase and the latent ATPase of PK could deplete the mother liquor of ADP during the period required for crystal growth and data collection. Asp5a was therefore included in the mother liquor to inhibit adenylate kinase. The mother liquor was assayed for ADP by anion-exchange HPLC, and these assays indicated that the concentration of ADP remained ~1.5 mM for more than 14 days.

X-ray data were collected at 4°C with a Siemens X1000D area detector at a crystal-to-detector distance of 20 cm using nickel-filtered copper Kα radiation from a Rigaku RU 200 rotating anode X-ray generator operated at 50 kV x 50 mA. Data were processed with the program XDS (31, 32) and scaled using the program Xcalibur (G. Wesenberg and I. Rayment, unpublished results). The overall R-factor, based on intensity, for the X-ray data set was 7.4% to 2.7 Å resolution. X-ray data collection statistics are given in Table I. The data set was 93% complete to 2.7 Å resolution.

**Molecular replacement.** The three-dimensional structure of the PK–Mg²⁺–K⁺–l-P-lactate complex was solved by molecular replacement using the software package AMORE (33, 34). X-ray coordinates of a tetramer of the PK–Mn²⁺–K⁺–pyruvate complex served as the search model (7). A cross-rotation function calculated with the X-ray data from 20 to 6.0 Å gave eight significant peaks, which were subdivided into two sets of four peaks. Since the search model was a tetramer and there were two tetramers in the asymmetric unit, each set of peaks corresponded to a single solution. One set of angles
from each group was chosen and the translation function was then applied using the two rotation solutions. Rigid body refinement was carried out to further refine the rotation and translation solutions, and the overall R factor dropped from 53.2 to 41.8%. The final Eulerian angles and translation solutions were $\alpha = 287.25^\circ$, $\beta = 119.08^\circ$, $\gamma = 83.42^\circ$, $a = 0.48$, $b = 0.72$, and $c = 0.72$ for solution I and $\alpha = 125.84^\circ$, $\beta = 23.61^\circ$, $\gamma = 23.10^\circ$, $a = 0.02$, $b = 0.00$, and $c = 0.22$ for solution II.

Refinement. After the solutions from molecular replacement were applied, the structure was refined using the least squares refinement package TNT (35). It was apparent after refinement that only subunit 1 had the B domain (residues 116–218) in the same position as in the search model (PK–Mn$^{2+}$–K$^+$–pyruvate). The other seven B domains were removed, and the model was refined. The resulting ($F_o - F_c$) density of the B domain from subunit 2 supplied information such that residues 116–125 could be located. The additional residues were built into the electron density with the molecular modeling program, FRODO (36). The cycle of refinement, calculation of ($F_o - F_c$) density, and addition of 10 to 15 residues was continued until the entire B domain of subunit 2 was complete. This process was applied to determine five of the six remaining B domains. Electron density for the B domain of subunit 5 was almost entirely disordered, and therefore residues 118–210 of that subunit were not included in the model. Following further least-squares refinement, manual adjustments on the model were done with FRODO (36) and O (37). The resulting ($F_o - F_c$) density of the active sites was used to determine the positions of the Mg$^{2+}$, K$^+$, and L-P-lactate. The ($F_o - F_c$) density for the L-P-lactate was complete for subunit 1 at 5 $\sigma$. The other subunits for which the positions of B domains resulted in an open active site cleft had ($F_o - F_c$) density ordered to only 2.5 $\sigma$ for the L-P-lactate. Refinement indicated that the occupancies were less than unity for L-P-lactate in the subunits with open clefts, and occupancies of 0.5 were assigned to these active sites. A difference density corresponding to Mg$^{2+}$ was present to at least 5 $\sigma$ for all subunits except subunit 5. Difference density corresponding to K$^+$ was present to at least 5 $\sigma$ for all subunits except subunit 6. There was no density corresponding to Mg$^{2+}$ADP in any subunit. After further refinement, water molecules were added to the model. Following inclusion of the waters, a final cycle of refinement and manual adjustments was completed to obtain the final model. There are 194 water molecules in the present model. The current R factor for all measured data is 19.6% from 2.7 Å resolution. The RMS deviations from ideal geometry are 0.014 Å for bond lengths, 2.3° for bond angles, and 0.007 Å for dihedral angles. A Ramachandran plot of the main-chain dihedral angles for subunit 1 is shown in Fig. 1. The Ramachandran plot shows the allowed regions for phi and psi values are shown by solid enclosures. Partially allowed regions are enclosed by broken lines. The most severe left-handed outlier is Thr 327. The most severe right-handed outlier is Ser 361.

![Ramachandran plot](https://example.com/ramachandran.png)

**FIG. 1.** Ramachandran plot of the main chain nonglycyl dihedral angles of subunit 1 for rabbit muscle pyruvate kinase. Fully allowed phi and psi values are shown by solid enclosures. Partially allowed regions are enclosed by broken lines. The most severe left-handed outlier is Thr 327. The most severe right-handed outlier is Ser 361.
all subunits have well-defined side chains. The overall chain topology of each subunit is identical to that described by Larsen et al. (7). The conformation of subunit 1 is identical to that previously described for the complex of pyruvate.

The most striking feature of the structure is the diversity of subunit conformations within the asymmetric unit. The active site cleft of subunit 1 is in a more closed conformation than any of the other subunits (Fig. 3). The different positions of the B domains relative to their respective A domains can be described by rotation of the B domain about a unique axis. The openings of the active site clefts, relative to that of subunit 1, in the other subunits are characterized by the following rotations: subunit 2, 23.5°; subunit 3, 23.5°; subunit 4, 22.0°; subunit 6, 19.9°; subunit 7, 21.5°; and subunit 8, 24.3°. Pro 116 is in the region linking the B domain to the A domain, and the confor-
nation of this residue is a local indicator for relative movement of the two domains. The $\phi, \psi$ values for Pro 116 are $-61, 60$ for the closed conformation, whereas the $\phi, \psi$ values for Pro 116 range from $-60^\circ, 130^\circ$ to $-65^\circ, 150^\circ$ for the more open conformations.

**Active Site of the Subunit with the Closed Cleft**

The active site lies in the cleft between domains A and B (23). Difference ($F_o - F_c$) electron density for L-P-lactate from the active site of subunit 1 is shown in Fig. 2 (bottom). A ball-and-stick model of L-P-lactate is shown in the density. The difference density was generated by excluding L-P-lactate from the refinement and phase calculation. L-P-lactate was modeled into the density with a carboxylate oxygen and bridging ester oxygen coordinated to Mg$^{2+}$ in an orientation similar to that observed for pyruvate (7). L-P-lactate also has a third coordination site to the divalent cation—a peripheral oxygen of the phosphate moiety. Mg$^{2+}$ coordinates to the protein through the carboxylate side chains of Glu 271 and Asp 295. The active site of subunit 1 is shown in Figure 4 (top).

Difference density (data not shown) confirms coordination of K$^+$ (the carbonyl oxygen of Thr 113, the hydroxyl oxygen of Ser 76, the carboxamide oxygen of Asn 74, and a carboxylate oxygen of Asp 112) observed previously for the complex of PK with K$^+$, Mn$^{2+}$, and pyruvate (7). K$^+$ also coordinates to a phosphoryl oxygen of the L-P-lactate. Thus, the phosphate group of the L-P-lactate is a bridging ligand of Mg$^{2+}$ and K$^+$ (Fig. 4, top). The distance between Mg$^{2+}$ and K$^+$ is 5.7 Å—the same distance found in the complex of PK–Mn$^{2+}$–K$^+$-pyruvate (7). Potassium ions have been located in coordination to ADP and P$_i$ in the active site of molecular chaperone Hsc70 (39). As predicted from the probable orientation of pyruvate (7), the side chain of Arg 72 lies close to the phosphate moiety of L-P-lactate. The hydroxyl of Thr 327 is proximal to the methyl group of L-P-lactate. The side chains of Arg 72 and Thr 327 would lie in the vicinity of the 2 si face of the double bond of a P-enolpyruvate bound in a manner analogous to L-P-lactate.

Density corresponding to the other product of the reaction of Mg$^{2+}$ATP and L-lactate, Mg$^2+$ADP, was not
FIG. 4. (Top) Stereoview of the active site of the l-P-lactate complex in subunit 1 (closed subunit). The Mg$^{2+}$ to ligand distances are l-P-lactate carboxylate O, 2.0 Å; l-P-lactate bridging ester O, 2.1 Å; l-P-lactate phosphate O, 2.0 Å; E271 carboxylate O, 2.0 Å; D295 carboxylate O, 2.1 Å. (Bottom) Stereoview of the active site of the l-P-lactate complex in subunit 2 (open subunit). The orange lines show the coordination for the magnesium ion and for the potassium ion. The dark purple lines represent the backbone trace.

present in any of the eight active sites. HPLC assays confirmed the presence of ample concentrations of ADP in the mother liquor for time periods exceeding that necessary for crystal growth and data collection. The mother liquor also contained sufficient Mg$^{2+}$ to satisfy the stoichiometric requirement for two magnesium ions per subunit. The absence of Mg$^{2+}$ADP in the crystals remains somewhat mysterious. Assays of PK in the presence of the precipitant, PEG, revealed no detectable influence of PEG on activity. The inhibition constant of the complex of l-P-lactate with PK is ~0.4 mM (40). The low occupancy of l-P-lactate in seven of the eight subunits in the asymmetric unit and the array of conformations adopted by the subunits suggest that crystal forces interfere with changes in structure that accompany binding of l-P-lactate.

Active Site of the Subunits with Open Clefts

Difference density contoured at 2.5 $\sigma$ was used to determine the position of the l-P-lactate in the seven subunits for which the active site cleft was open. Refinement revealed that the l-P-lactate sites were not fully occupied for the open subunits, and occupancies of 0.5 were assigned. The active site of subunit 2 is shown in Fig. 4 (bottom). As in subunit 1, Mg$^{2+}$ coordi-
nates to the protein through the carboxylate side chains of Glu 271 and Asp 295. The positions of Mg\(^{2+}\) and its carboxylate ligands from Glu 271 and Asp 295 are altered relative to those in subunit 1 such that Mg\(^{2+}\) no longer coordinates to L-P-lactate in the subunits that have open active site clefts. Liganding of K\(^+\) is the same as that in subunit 1, and coordination of the phosphate moiety of L-P-lactate to K\(^+\) is the sole binding determinant for the analog in the subunits with open clefts. The temperature factor of K\(^+\) in the subunits with open clefts increases by a factor of >2. Distances between Mg\(^{2+}\) and K\(^+\) vary from 5.7 to 7.1 Å among the open-cleft subunits.

**Comparison of Open versus Closed B Domains**

As noted above, only seven of the eight B domains are visible in the map. Among these seven visible domains, differences in position represented by rotations greater than 20° with respect to the A domain are observed. Figure 5 (top) shows a superposition of the active sites of subunit 1 and subunit 2. The position of Mg\(^{2+}\) is shifted by 3–3.8 Å from the closed conformation to the open conformation, and the carboxylate side chains (Glu 271 and Asp 295) which anchor Mg\(^{2+}\) in the active site rotate in the two different conformations. The orientation of the phenyl ring of Phe 243 also changes.
between the closed and open conformations. Despite the proximity of K+ to the conformationally sensitive linker region between domains A and B (Fig. 5, bottom), the position of K+ is not influenced significantly by the conformational changes.

Conclusions

The asymmetric unit of crystals of PK crystallized in the presence of Mg2+, K+, ADP, and L-P-lactate provides several views of the active site cleft between domains A and B. Different subunits within the asymmetric unit adopt different conformations that result in different degrees of closure of the cleft. The ligand, L-P-lactate, coordinates to Mg2+ in the subunit wherein the active site cleft is in the more closed form. K+ coordinates to the phosphate moiety of L-P-lactate in all of the subunits. Opening of the active site cleft alters the orientation of the carboxylate ligands from Glu 271 and Asp 295 such that Mg2+ moves ~3.8 Å from its position in the closed subunit. The present structure highlights the conformational flexibility of the subunits of PK. The salient features of subunit 1 in the present structure are confirmed in a 2.1 Å structure of the complex of PK with ATP and oxalate (T. M. Larsen, M. M. Benning, I. Rayment, and G. H. Reed, unpublished results).

REFERENCES