The Allosteric Regulation of Pyruvate Kinase
A SITE-DIRECTED MUTAGENESIS STUDY*

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Pyruvate kinase (PK) is critical for the regulation of the glycolytic pathway. The regulatory properties of Escherichia coli were investigated by mutating six charged residues involved in interdomain salt bridges (Arg271, Arg292, Asp297, and Lys413) and in the binding of the allosteric activator (Lys382 and Arg431). Arg271 and Lys413 are located at the interface between A and C domains within one subunit. The R271L and K413Q mutant enzymes exhibit altered kinetic properties. In K413Q, there is partial enzyme activation, whereas R271L is characterized by a bias toward the T-state in the allosteric equilibrium. In the T-state, Arg292 and Asp392 form an intersubunit salt bridge. The mutants R292D and D297R are totally inactive. The crystal structure of R292D reveals that the mutant enzyme retains the T-state quaternary structure. However, the mutation induces a reorganization of the interface with the creation of a network of interactions similar to that observed in the crystal structures of R-state yeast and M1 PK proteins. Furthermore, in the R292D structure, two loops that are part of the active site are disordered. The K382Q and R431E mutations were designed to probe the binding site for fructose 1,6-bisphosphate, the allosteric activator. R431E exhibits only slight changes in the regulatory properties. Conversely, K382Q displays a highly altered responsiveness to the activator, suggesting that Lys382 is involved in both activator binding and allosteric transition mechanism. Taken together, these results support the notion that domain interfaces are critical for the allosteric transition. They couple changes in the tertiary and quaternary structures to alterations in the geometry of the fructose 1,6-bisphosphate and substrate binding sites. These site-directed mutagenesis data are discussed in the light of the molecular basis for the hereditary nonspherocytic hemolytic anemia, which is caused by mutations in human erythrocyte PK gene.

Pyruvate kinase (ATP:pyruvate 2-O-phosphotransferase (EC 2.7.1.40); PK) catalyzes the last step of glycolysis, where the phosphorily group of phosphoenolpyruvate (PEP) is transferred to ADP to form pyruvate and ATP. PK requires monovalent (K+) and divalent cations (Mg2+ or Mn2+) for its activity. The reaction is essentially irreversible under physiological conditions and is critical for the control of the metabolic flux in the second part of glycolysis. Moreover, the substrate PEP and the product pyruvate are involved in a variety of metabolic pathways. Such a central position in the cellular metabolism is reflected in the regulatory properties of PK, which is a typical allosteric protein (1). The activity is controlled by several physiological effectors, including H+, Mg2+, Mn2+, and K+ (2). Furthermore, the enzyme displays sigmoidal kinetics toward the substrate PEP and it is activated by an heterotropic effector whose nature depends on the organism (1, 3). The mammalian isoenzymes R (expressed in erythrocytes), L (in liver), and M2 (in kidney and lung) are regulated by fructose 1,6-bisphosphate (FBP) (4), whereas in trypanosomatid protozoans the allosteric effector is fructose 2,6-bisphosphate (5). Most bacterial PKs are activated by FBP, although in some cases the effector is a monophosphorylated sugar such as ribose 5-phosphate. Thus far, the only known PK displaying hyperbolic kinetics is the mammalian M1 isozyme that is present in muscle, brain, and heart. However, the M1 protein is thought to be a highly specialized descendant of the allosteric PKs, locked in an active R-like conformation (6).

PK has been characterized from a number of prokaryotes and eukaryotes and in most cases has been found to exist as a tetramer of identical subunits, each consisting of approximately 500 residues (Fig. 1A) (1). Crystal structures are available for the enzyme from cat and rabbit muscle (7, 8), yeast (9), Escherichia coli (10), and Leishmania mexicana (11). These proteins share a very similar architecture. Each subunit consists of three domains (Fig. 1B): the A domain with the classic (α/β)n topology, the B domain with a somewhat irregular fold, and the C domain with an α+β organization. The eukaryotic proteins contain an additional small N-terminal domain, which is absent in the E. coli enzyme. The active site is located on the C-terminal side of the A domain (α/β)n barrel, facing the cleft between the A and B domains. The complex between yeast PK and FBP has revealed that the FBP binding site is entirely located in the C domain, at more than 40 Å from the catalytic center (9). Four identical subunits are assembled to form the tetrameric enzyme with 222 symmetry. The subunits mainly interact through the A and C domains located along the molecular twofold axes (Fig. 1A).

Progress in the knowledge of the allosteric regulation was obtained by comparison of the T-state E. coli PK with the non-allosteric muscle M1 enzyme in the active R-like conformation. The atomic coordinates and structure factors (code 1e0u and r1e0usf (for R271L) and 1e0t and r1e0tsf (for R292D)) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/). To whom correspondence should be addressed: Dept. of Biochemistry, University of Pavia, via Taramelli 3b, 27100 Pavia, Italy. Tel.: 39-382-507320; Fax: 39-382-423108; E-mail: giovale@unipv.it.

The abbreviations used are: PK, pyruvate kinase; PEP, phosphoenolpyruvate; FBP, fructose 1,6-bisphosphate; MES, 4-morpholineethanesulfonic acid.

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mation (10). The study demonstrated that each individual domain approximates a rigid body. On transition from the T- to the R-state, the domains of the functional tetramer modify their relative orientation by up to 29°. These movements are coupled to a conformational change in the active center, which upon transition to the T-state undergoes a distortion of the PEP binding site. The recent structure determination of the yeast R-state (9) and the L. mexicana T-state (11) proteins helped to refine this model, by allowing a better discrimination between the genuine allosteric conformational changes and those due to the inherent structural divergence between the mammalian and bacterial proteins. However, the complete and detailed elucidation of the mechanism for the allosteric transition must await the determination of the structure of the same PK crystallized in both active and inactive conformations.

The “domain rotation model” implies that PK must be equipped with structural elements that enable the coupling of the domain movements to conformational changes in the active site. In this context, the interfaces between domains and subunits are predicted to be critical for the enzyme regulation (3, 6, 9, 12). The intra-subunit interface between the A and C domains may take part in the transmission of the allosteric signal between PEP and FBP binding sites. Likewise, the contact region between twofold related A (A/A' interface along the vertical axis in Fig. 1A) and C domains (along the horizontal axis in Fig. 1A) may fulfil the role of coupling changes in the quaternary structure with modifications of the active center. To test these predictions, we have undertaken a site-directed mutagenesis study on the E. coli FBP-dependent PK. These experiments were guided by the knowledge of the three-dimensional structure of the enzyme crystallized in the T-state. Our approach was to mutate charged amino acid residues involved in inter-domain salt bridges in the A/C and A/A' interfaces and to evaluate the effect of the substitutions on the enzyme activity and regulation. In addition, we replaced charged residues involved in the binding of FBP to elucidate the mechanism of responsiveness to the heterotrophic activator. A total of six mutant proteins were prepared and analyzed by kinetic, thermal inactivation, and crystallographic methods. These data essentially agree with the proposed model for the allosteric transition and highlight the complexity of the enzyme, which has the remarkable ability to transmit a regulatory signal across long distances spanning different domains and subunits.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction and DNA modifying enzymes were purchased from New England Biolabs. FBP, PEP, lactate dehydrogenase, and NADH were from Roche Molecular Biochemicals. Oligonucleotides were synthesized by Life Technologies, Inc. Other chemicals were reagent grade and obtained from Sigma or Aldrich.

**Site-directed Mutagenesis**—For mutagenesis the double-stranded plasmid pGV5A (13), a pBluescript II KS expression vector carrying the pykI gene, was employed. The procedure followed the protocol of Deng and Nickoloff (14), using the commercially available Chameleon™ double-stranded DNA kit (Stratagene). The method described previously (13). All buffers contained 1 mM EDTA and 2 mM -mercaptoethanol. Cells collected by centrifugation were sonicated in a pykI digestion. More than 50% of the plasmids containing the StyI site also contained the desired point mutation, as confirmed by DNA sequencing of the entire pykI gene. Sequencing of double-stranded DNA was performed by the dideoxynucleotide chain-termination method using Thermosequenase™ kit (Amersham Pharmacia Biotech) (15).

**Overexpression and Purification**—E. coli BL21 (DE3) cells transformed with the plasmid pGV5A or its mutant derivatives were grown overnight in a Luria-Bertani medium containing 100 mg/ml ampicillin. The cells were then diluted (1/30 v/v) in a freshly prepared growth medium, and cultures were grown at 37 °C until the optical density at 600 nm reached a value in the range 0.8–1.2. At that point expression was induced by the addition of isopropyl-β-D-thigalactopyranoside to a final concentration of 0.6 mM. After 3 h of induction, the cells were harvested by centrifugation. Recombinant enzyme production was calculated by dividing the total activity of the culture by the specific activity of the purified enzyme after deduction of endogenous PK activity.

Recombinant wild-type and mutant forms of PK were purified as described previously (13). All buffers contained 1 mM EDTA and 2 mM β-mercaptoethanol. Cells collected by centrifugation were sonicated in a solution consisting of 10 mM Tris, pH 8.5, 100 mM KCl, 10 mM MgCl₂, and 30 μg/ml pancreatic DNase. After DNA digestion, cell debris was removed by centrifugation and the supernatant was loaded onto a DEAE Sephacel (Amersham Pharmacia Biotech) column (4 × 32 cm) equilibrated with 10 mM Tris, pH 8.5, 100 mM KCl. The protein was eluted with 1200 ml of a linear gradient of KCl (0.1–0.5 M KCl in 10 mM Tris, pH 8.5) and subjected to ammonium sulfate precipitation (70% saturation). Next, it was applied to a Octyl-Sepharose CL-4B (Amersham Pharmacia Biotech) column (4 × 20 cm) equilibrated with 50 mM Tris, pH 7.5, 100 mM KCl, and 1.7 mM ammonium sulfate. Elution was performed with 1000 ml of a linear gradient of decreasing ammonium sulfate (1.17–0 M) and increasing ethylene glycol (0–50% v/v) concentrations. The enzyme preparation was divided into two aliquots, and each of them was applied to a Sephacryl S-200 (Amersham Pharmacia Biotech) column (1.9 × 100 cm) equilibrated in 10 mM Tris, pH 7.5. The protein was eluted with the equilibration buffer. Protein concentration was determined according to Lowry (16), using bovine serum albumin as standard.

**TABLE I**

| **R271L** | **R292D** |
|----------------|-----------|
| Resolution (Å) | 15–2.8 | 15–2.8 |
| Unique reflections | 151954 | 132307 |
| Completenss of data (%) | 97.7 (86.1) | 96.3 (90.5) |
| Multiplicity | 2.7 (2.0) | 2.4 (2.1) |
| Intensities (/σ²) | 6.5 (3.1) | 4.9 (3.6) |
| Rsym (%) | 9.0 (20.1) | 9.5 (16.8) |
| Cell dimensions (Å) | 74.01, 129.59, 241.77 | 74.47, 129.34, 240.37 |
| Number of protein atoms | 13780 | 13228 |
| Number of water atoms | 200 | 137 |
| r.m.s.d. from ideality^a | 0.019 | 0.016 |
| Bond angles (°) | 2.062 | 2.943 |
| Trigonal groups (Å) | 0.009 | 0.009 |
| Planar groups (Å) | 0.009 | 0.009 |
| Ramachandran plot (%^b) | 89.3/10.3/0.4/0 | 88/11.6/0.4/0 |

^a The values relating to the highest resolution shell are given in parentheses.

^b The root mean square deviations (r.m.s.d.) were calculated with the program Refmac (19).

The level of expression of most of the mutants was comparable to that of the recombinant wild-type enzyme (30–100 mg/liter of culture). The endogenous activity of the control cells transformed with pBluescript II was reduced by 95% when compared to that of the recombinant enzyme after deduction of endogenous PK activity.

The selection primer used for changing the vector unique restriction site AflIII into StyI had the following sequence: CCAGGAAAAAGGCTCCGATGCTAATCAATAGGCCCT.

Regulation of Pyruvate Kinase
Regulation of Pyruvate Kinase

The regulation of Pyruvate Kinase (PK) activity was determined at 25 °C by the lactate dehydrogenase-coupled spectrophotometric assay (18). The standard reaction mixture contained: 10 mM Hepes, pH 7.5, 10 mM MgCl₂, 50 mM KCl, 2 mM PEP, 2 mM FBP, 2 mM ADP, 0.12 mM NADH, and 22 units of crystalline lactate dehydrogenase in a final volume of 1 ml. The reaction was started by adding enzyme solution. All measurements were performed in triplicate, and the plot of Lineweaver-Burk was used to determine Vₘₐₓ and apparent Kₘ values.

Kinetic Analyses—Enzymatic activity was assayed at 25 °C using various concentrations of PEP, ADP, and FBP under conditions identical to those above except for substrates and effectors. Kinetic parameters were determined as follows: for PEP at fixed concentration of 2 mM ADP in the absence and in the presence of 2 mM FBP; for ADP at 2 mM PEP and 2 mM FBP; for FBP at 1 mM PEP and 2 mM ADP. In all cases, the enzyme activity was assayed at different concentrations of substrate or effector. All measurements were performed in triplicate, and the plot of Lineweaver-Burk was used to determine Vₘₐₓ and apparent Kₘ values. The Hill plot was used to determine the apparent S₀.₅ (the substrate concentration giving one-half of Vₘₐₓ) and nₜ₇ (Hill coefficient) values.

Thermal Stability Assay—Thermal stability studies were carried out at 55 °C in the absence and in the presence of 2 mM FBP. The enzyme (100–200 mg/ml) was incubated in 10 mM Tris, pH 7.5, 50 mM KCl, 1 mM EDTA, 2 mM β-mercaptoethanol, and 2 mg/ml bovine serum albumin. Samples were removed at intervals and rapidly cooled in ice, and the enzyme activity measured as described previously.

Structure Determination—Crystals of the R271L and R292D mutants were obtained using the hanging drop vapor diffusion method at conditions (12% w/v polyethylene glycol 4000, 20 mM KCl, 20 mM MgSO₄, 50 mM MES, pH 6.5) identical to those used for the wild-type enzyme (10). The absence from the crystallization medium of PEP, FBP, or other activating molecules is consistent with the protein being crystallized in the inactive T-state. The diffraction data for the R271L and R292D mutants were collected at the x-ray diffraction beam-line of Elettra (Trieste, Italy) at 100 K. Before freezing, the crystals were exposed for a few seconds to a solution containing 15% w/v polyethylene glycol 400, 25% v/v polyethylene glycol 400, 20 mM KCl, 20 mM MgSO₄, and 50 mM MES, pH 6.5. The data were processed using MOSFLM (written by A. G. Leslie) and programs of the CCP4 (19) suite. Crystals of the mutants are isomorphous to those of the wild-type protein and belong to space group P2₁2₁2₁. Crystallographic refinement was performed with the program REFMAC (20), whereas model building was done using the program O (21). Tight non-crystallographic restraints were applied throughout the refinement following the same protocol used for the native structure (10). The free R-factor was calculated employing the same reflections used for the free R-factor calculations in the refinement of the wild-type protein. Table I gives a summary of the data collection and refinement statistics. Analysis of the model was done with the O (21), Procheck (22) and programs of the CCP4 suite (19). The drawings were generated with Molscript (23).

RESULTS

Site-directed mutagenesis was employed to investigate the allosteric regulation of E. coli PK. The residues subjected to mutagenesis (Fig. 1B) are located in the intrasubunit interface between the A and C domains (A/C interface, R271L and K413Q), in the interface between the A domains of twofold related subunits (AA’ interface, R292D and D297R), and in the FBP-binding site (K382Q and R431E). The mutant enzymes were overexpressed in E. coli cells and purified to homogeneity. Their kinetic (Fig. 2 and Tables II and III) and thermal inac-

![FIG. 1](http://www.jbc.org/)

**FIG. 1.** A, schematic representation of the E. coli PK crystallized in the inactive T-state. The A/A’ interface (see text) is formed by subunits related by the molecular twofold axis running in the vertical direction. The Ca atoms of Phe⁴⁴⁵ and Leu⁴⁵² are connected by a dashed line because residues 346–351 are disordered. B, the E. coli PK subunit. The Ca atoms of the mutated residues are outlined by gray spheres. The orientation is the same as in A. Throughout the paper, the secondary structure elements are named according to the domain where they occur and the type (α-helix or β-strand) and are numbered sequentially (see Ref. 10).
tivation (Table IV) properties were characterized as described under "Experimental Procedures." Moreover, two mutants, R271L and R431E, were analyzed by x-ray crystallography. The A/C Interface: R271L and K413Q—An intriguing feature of the PK structure is the large 40-Å separation between the FBP binding site, located in the C domain (9), and the

**TABLE II**

| Kinetic parameters of the wild-type and mutant E. coli pyruvate kinases |
|---|
| Results are means ± standard errors for three determinations. |

|                      | PEP | ADP |
|----------------------|-----|-----|
|                      | FBP (−) | 2 mM FBP | FBP (−) | 2 mM FBP |
|                      | $K_{m}$ | $S_{0.5}$ | $n_{H}$ | $K_{cat}$ | $S_{0.5}$ | $n_{H}$ | $K_{cat}$ | $S_{0.5}$ | $n_{H}$ |
| Wild type            | 123 ± 8 | 3.63 ± 0.4 | 3.2 ± 0.06 | 33.9 | 160 ± 8 | 0.08 ± 0.01 | 1.0 ± 0.02 | 2000.0 | 132 ± 7 | 0.30 ± 0.02 | 440.0 |
| R271L                | 126 ± 9 | 5.21 ± 0.4 | 2.8 ± 0.09 | 24.2 | 130 ± 7 | 0.12 ± 0.01 | 1.6 ± 0.06 | 1083.3 | 142 ± 7 | 0.28 ± 0.03 | 507.1 |
| R292D                 | 42 ± 3  | 7.36 ± 0.8 | 2.8 ± 0.15 | 5.7 | 111 ± 6 | 1.10 ± 0.11 | 1.9 ± 0.03 | 100.9 | 110 ± 5  | 0.28 ± 0.05 | 392.9 |
| R292D                 | 158 ± 9 | 2.01 ± 0.2 | 1.7 ± 0.08 | 78.6 | 188 ± 9 | 0.15 ± 0.02 | 0.7 ± 0.01 | 1253.3 | 147 ± 8  | 0.32 ± 0.02 | 459.4 |
| R431E                 | 109 ± 8 | 1.90 ± 0.2 | 3.7 ± 0.12 | 57.4 | 109 ± 5 | 0.05 ± 0.01 | 1.0 ± 0.05 | 2180.0 | 103 ± 5  | 0.35 ± 0.02 | 294.3 |

* Kinetic parameters for PEP were obtained by fitting data to the Hill equation.

**Fig. 2.** PEP titration curves for the wild-type PK and the mutants R271L, K382Q, K413Q, and R431E. Enzyme activity was assayed at 25 °C and pH 7.5, as described under "Experimental Procedures." ADP was present at the concentration of 2 mM. In A, the titrations were performed in the absence of FBP, whereas, in B, they were carried out in the presence of 2 mM activator: wild-type, ◆; R271L, ●; K382Q, ○; K413Q, ▲; R431E, ▼.
Regulation of Pyruvate Kinase

R271L is the second mutant designed to probe the role of the A/C interface in the PK regulation. Arg 271 lies on a loop connecting helix Cα3 and strand CB2 of the C domain (Fig. 1B). Its side chain amino group makes a salt bridge with Glu356, a side chain belonging to the C domain (Fig. 1B). In the T-state, this residue forms an interdomain salt bridge with Glu356, a side chain belonging to the C domain (Fig. 3A). Arg271 was mutated to leucine and the mutant analyzed by X-ray crystallography in its inactive T-form at 2.8 Å resolution. 

The mutants R292D and D297R are inactive. Incubation at 55 °C leads to a 50% reduction of the enzyme activity.

Wild type 127 ± 6 0.045 ± 0.005 1.96 ± 0.004 2822.2
R271L 148 ± 7 0.206 ± 0.014 1.91 ± 0.082 718.4
R292D λ
D297R 0.038 ± 0.005 1.00 ± 0.024 3815.8
K382Q 103 ± 4 0.025 ± 0.002 1.31 ± 0.100 4120.0

The mutants R292D and D297R are inactive.

No significant variation of activity after 60 min of incubation.

"The contact distances indicated by dashed lines are in angstroms (Å)."
the PEP saturation curve in the absence of the allosteric activator (Fig. 2A). Moreover, the Hill coefficient for PEP in the presence of FBP is 1.6, rather than the value of 1.0 measured for the wild-type protein (Table II). Apparently, the mutation leads to a reduced responsiveness to FBP and to a bias the allosteric equilibrium toward the T-state, reflected in the shift of the saturation curve for PEP. The involvement of Arg271 in the allosteric transition is further supported by the heat inactivation properties exhibited by the mutant. In the absence of FBP (e.g. T-state), the thermal inactivation is faster in the R271L protein than in the wild type, whereas, in the presence of the activator (R-state), the activity of the mutated protein displays a slightly slower decay (Table IV). In other words, the mutation makes the T-state more susceptible to heat inactivation in contrast to the R-state, which becomes more resistant.

The crystal structure shows that the mutation effectively removes an interdomain salt bridge, thus providing a rationale for the T-state reduced stability (Fig. 3, A and B). On the other hand, on transition to the R-state, the conformation and local environment of Arg271 may change significantly so that the salt bridge involving Arg271 and Glu356 may not be present in the R-form. Thus, the difference between the thermal inactivation properties of the T- and R-states may result from different conformations and/or environments of Arg271 side chain in the active and inactive states.

The A/A′ Interface: R292D and D297R—Comparison between the T-state E. coli PK structure (10) and the R-state conformations of the rabbit M1 isoenzyme (8) and yeast PK in complex with FBP (9) provided strong evidence for a pivotal role of the residues at the interface between the A domains of twofold related subunits (A/A′ interface). In this region of the protein are located Arg292 and Asp297, two residues that are strictly conserved among all known PK sequences (1, 9, 11). In the T-state, Arg292 and Asp297 form an intersubunit salt bridge (Fig. 4A). On transition to the R-state, the subunit and domain rotations are associated to a reorganization of the interface area with Arg292 rotating away in the direction of the protein surface. Such a rearrangement would be coupled to a movement of loop 6 of the A domain (αβ)8 barrel, allowing the active center to attain the conformation that is competent in PEP binding (10).

Two mutant enzymes were generated, R292D and D297R (Fig. 1B), with the purpose of destabilizing the T-state conformation, possibly inducing a bias in the allosteric equilibrium toward the R-state. The two mutants turned out to be totally inactive. R292D was crystallized in conditions identical to those used for the T-state wild-type protein and its three-dimensional structure solved at 2.8-Å resolution (Table I). The overall structure of the R292D is very similar to that of the wild type (root mean square deviation of 0.39 Å for 1784 equivalent Co pairs of the tetramer). This fact is of particular significance: it shows that E. coli PK has the remarkable ability to tolerate a charge reversal for a residue located at the heart of the subunit interface. A local rearrangement of a few side chains in the A/A′ contact region is at the basis of this feature. In the mutant, the side chain of Asp297 points toward the surface concomitant to the shift of Arg244, which moves closer to Asp297, so that these two charged residues form an intrasubunit salt bridge (Fig. 4B). Remarkably, such a network of interactions is very similar to that observed in the R-state conformation of the yeast and M1 PK proteins (8, 9). In these structures, Arg297 and Arg244 (E. coli PK numbering) interact with each other, while the Arg residue at position 292 is oriented toward the surface in the same way as Asp297 in the R292D mutant. The R292D mutation seems to elicit a local conformational change similar to that occurring in the allosteric transition. Such a hybrid situation of a local R-like conformation present in a T-state enzyme is reminiscent of what has been observed in the crystal structure of L. mexicana PK (11). This protein was crystallized in the T-state with eight crystallographically independent subunits in the asymmetric unit. Remarkably, the conformation of the residue homologous to Arg292 differs among the eight independent monomers, varying from the R-state conformer (Arg292 pointing toward the surface) to the T-state conformation (Arg292 engaged in intersubunit salt bridge with Asp297). Thus, both L. mexicana PK and the R292D mutant are able to retain the T-state quaternary and ternary structure despite the Arg292 and surrounding residues being engaged in a sort of “R-type organization.”

In addition to the reorganization in the A/A′ interface, the R292D protein displays another change with respect to the wild-type protein. In all four crystallographically independent subunits, residues 282–289 and 315–320 could not be located in the electron density map, reflecting a disordered conformation (Fig. 5). These amino acids form loops 7 and 8 of the A domain (αβ)8 barrel and are integral part of the active center. Inspection of the wild-type structure shows that the guanidinium group of Arg244 is H-bonded (2.9 Å) to the carbonyl oxygen of Met282, the first residue of loop 7. Furthermore, also the side chain of Arg292 is weakly interacting (3.4 Å) with the Met282 main chain oxygen. Such H-bonds are lost in the mutants due
of the 6-phosphate binding sites, respectively. We performed site-directed mutagenesis studies targeting the A/C interface. In particular, given their strategic location at the heart of the intersubunit interface, Arg292 and neighbor- ing residues are able to couple the subunit rotations occurring in the T ↔ R transition to the alterations in the active site geometry. However, the crystal structure also shows that R292D retains the T-state quaternary structure. This implies that a local rearrangement in the A/A interface is not sufficient to trigger the full transition to the R-state, despite being instrumental to the transmission of the allosteric signal. Apparently, only the binding of either PEP and/or FBP is able to elicit all the conformational changes that result in the attainment of the active R-state conformation.

The two mutants targeting the A/C interface display altered kinetic and regulatory properties. In the K413Q protein there is partial enzyme activation, in contrast to the stabilization of the inactive T-state conformation, which characterizes the R271L variant. Additionally, thermal inactivation studies show that the interface amino acids greatly contribute to the enzyme stability. In particular, the data on the R271L protein indicate that this contribution may vary in the T- and R-states.

**DISCUSSION**

The control of PK activity is of paramount importance for the cell, given the central role of this enzyme in the cellular metabolism. Comparisons between the available structures of PK molecules from different species agree in indicating that the allosteric transition involves mutual rotations of both the domains within each subunit and the subunits within the tetramer. A key prediction of this model is that residues at the domain interfaces would have the critical function to relay the allosteric signal from and to the catalytic and regulatory sites.

The mutants R292D and D297R affecting the A/A interface are totally inactive. The crystal structure of R292D reveals that the mutation induces a local reorganization of a few interface residues. Remarkably, this turns out to be coupled to the disordering of loop 7, which are part of the active site. This observation is of particular significance; it proves the existence of a direct link between the catalytic center and the residues at the A/A interface. In particular, given their strategic location at the heart of the intersubunit interface, Arg292 and neighboring residues are able to couple the subunit rotations occurring in the T ↔ R transition to the alterations in the active site geometry.
depending on the specific interactions established by the residue in two forms. Such a differential effect on stability may affect the allosteric equilibrium, thereby representing an additional factor that influences the enzyme regulatory properties.

The K413Q and R271L mutants do not exhibit significant alterations in the kinetic parameters for ADP binding (Table II). This is consistent with the idea that the two mutants do not alter the active site geometry. Rather, the mutations appear to genuinely affect the enzyme allosterically. From this point of view, the mutagenesis data on the E. coli protein agree remarkably well with studies performed on Bacillus stearothermophilus PK (24, 25). In this enzyme, Trp433, which is homologous to Lys413 of E. coli PK, was mutated to Tyr, producing a protein with altered regulatory properties that are qualitatively similar to those of the K413Q E. coli mutant. Moreover, the effects of the W433Y mutation in the B. stearothermophilus enzyme were reversed by introducing a second mutation affecting another residue (Glu356, homologous to Asp307 in E. coli PK) of the A/C interface. Clearly, the theme emerging from these mutagenesis studies on the two prokaryotic enzymes is that the A/C interface is a critical component for the enzyme regulation.

The R431E and K382Q mutations were designed to probe the FBP binding site in the E. coli protein. The K382Q mutant exhibits a huge change in the regulatory properties. This suggests that effector binding in E. coli PK will be generally similar to that observed in the crystal structure of the yeast enzyme (9), despite a relatively low level of sequence homology in the region forming the regulatory site. This observation points to the more general concept that eukaryotic and prokaryotic PK proteins are likely to share the essential features in their regulatory apparatus. The best evidence for this notion is given by the mutations in the human erythrocyte PK that cause the hereditary nonspherocytic hemolytic anemia. More than 100 pathologival mutations have been identified (26). Most of them cluster in a few well defined regions, which include the domain interfaces and the FBP binding sites (3, 26). For instance, the R479H mutation (27) in PK affects a residue that is homologous to Lys382 of the E. coli protein. In the latter, the K382Q substitution severely affects the protein functionality, providing a rationale for the pathological effect of the R479H mutation. Likewise, the R510Q (28, 29) substitution in the human PK affects a residue homologous to Lys413 of the E. coli protein. The properties of the E. coli K413Q variant indicate that the human R510Q mutation will have an adverse effect on both stability and regulation, ultimately affecting the correct physiological function of the enzyme.

The portrait emerging from site-directed mutagenesis, crystallography, and genetics of PK is that of a very complex protein. The architecture of PK consists of an assembly of domains and subunits in which allosteric and catalytic sites are able to communicate with each other across relatively long distances. Various protein regions, including domain interfaces and flexible domain linkers, couple changes in the tertiary and quaternary structures to alterations in the geometry of the active and allosteric sites. The full understanding of such sophisticated machinery represents a fascinating subject awaiting future studies.

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