ISSN 0027-1314, Moscow University Chemistry Bulletin, 2008, Vol. 63, No. 2, pp. 114–119. © Allerton Press, Inc., 2008.

Insight into the Mechanism of Domain Movements and Its Role in Functioning of 3-Phosphoglycerate Kinase[1]

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Received November 23, 2007

Abstract—Comprehensive studies with 3-phosphoglycerate kinase revealed the details of transmission of a substrate-triggered conformational effect towards the main molecular hinge at the β-strand L. The unusual kinetic behavior (activation by anions) and flexibility of the phosphate chain of the nucleotide substrate(s) can be related to domain movements. Both phenomena are due to interactions with the catalytic Lys residue, which moves more than 10 Å during domain closure. This movement occurs, in concert with operation of the main hinge, under the simultaneous action of the two substrates.

DOI: 10.3103/S0027131408020156

Conformational flexibility is essential for functioning of all enzymes, the proteins executing and controlling all functions in living organisms. The most spectacular representatives of protein flexibility are the relative movements of the larger structural units, such as modules or domains within multidomain enzymes (cf. reviews [1–3]). The structural principles that govern such movements, as well as the specific role of motions in the biological function, have not been clarified. The mechanism of communication between modules and domains and its control by the bound substrate(s) have to be delineated for an understanding of catalysis.

Domain closure over the active sites of multidomain enzymes can often create an optimal environment for catalysis, orienting the substrate reactive groups properly and/or impeding side reactions. In order to reach general conclusions on the structural and functional aspects of domain closure, individual cases must be investigated. In addition to comparison of the extreme conformations (e.g., [2, 4–6]), simulation of the motion by computer modeling (e.g., [7–9]) and more complex investigations including functional studies (e.g., [10, 11]) are needed.

3-Phosphoglycerate kinase (PGK), a typical hinge-bending enzyme, has been chosen for the present study. It is an essential enzyme for basic carbohydrate metabolism. PGK catalyzes the phosphotransfer from 1,3-bisphosphoglycerate (1,3-BPG) to MgADP and produces 3-phosphoglycerate (3-PG) and MgATP during glycolysis of aerobes, fermentation of anaerobes, and photosynthesis of plants. In addition to its physiological activity, human PGK can phosphorylate the nonnatural *L*-nucleoside analogues that are used in antiviral and anticancer therapies [12, 13]. In this way PGK produces the pharmacologically active triphosphate forms of these compounds and regulates their levels in the cell.

PGK is a monomer with two structural domains of equal sizes. The C domain binds the nucleotide substrate (MgATP or MgADP), while the N domain binds 3-PG or 1,3-BPG (Fig. 1a). Several open [14–20] and closed [21, 22] crystal structures of PGK are known, and one pair of them is illustrated in Fig. 1b. Domain closure was also evidenced by small angle X-ray scattering (SAXS) studies [23, 24]. With its conserved structure, including the well-structured interdomain region, PGK is a suitable model to study the details of domain–domain interplay and its regulation by substrates.

PGK exhibits unusual functional behaviors that may be associated with the domain movements. As shown long ago, PGK is activated by various multivalent physiological anions (such as phosphate, pyrophosphate, or citrate), as well as by an excess of either substrate, 3-PG, or MgATP [25]. Up to now, however, no definitive explanation could be given. We have proposed a model [26] that assumes the existence of a secondary regulatory (activating) site for each substrate or anion in addition to the primary catalytic site. It also explains inhibition at high concentrations of anions. At low concentrations, an anion can bind to the activating site and increase PGK activity, while at high concentrations the anion can replace the substrate at the catalytic site and therefore become inhibitory. Based on these models, we have derived kinetic equations that describe satisfactorily the kinetic data (cf. the curves with wild-type PGK in Figs. 2a and 2b).

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Thus, the kinetic data argue in favor of the existence of a secondary regulatory site, but crystallographic and part of the solution binding data (e.g., [27]) support the existence of only a single site for each of the two substrates. On this basis, we put forward a hypothesis that the regulatory site is formed only during domain closure, i.e., during the catalytic cycle.

Another crucial observation with PGK is the various positions of the bound nucleotide phosphates (or of the analogues) as determined from crystal structures [16, 19, 20, 28]. In the two extreme cases, the nucleotide phosphate either interacts with helix 8 (as for the analogue MgAMP-PCP) or helix 13 (as for MgADP (not shown) or the analogue MnAMP-PNP), while the phosphate chain of MgATP occupies an intermediate position (Fig. 3). The crystallographic *B*-factor values of helix 13 correlate well with these crystallographic observations: helix 13 becomes more ordered due to the interaction with the nucleotide phosphate (Table 1).

Solution experiments, however, do not always correlate with the crystallographic data. Similar behaviors of MgATP and its analogues have been detected in both isothermal titration (ITC) and differential scanning (DSC) microcalorimetric experiments, in contrast to the crystallographic data. ITC experiments showed stronger binding of MgADP with large binding enthalpy on the one hand and weaker binding of MgATP (or its analogue MgAMP-PNP) with large binding entropy on the other hand. The large entropy value is consistent with the flexibility of MgATP phosphates [20] (Table 1). DSC experiments also revealed a much stronger stabilization effect of MgADP on PGK conformation as compared to MgATP, while the two structural analogues of ATP, AMP-PNP and AMP-PCP, behaved similarly to ATP [20, 29] (Table 1).

Thus, the binding modes of MgATP and its analogues, AMP-PNP and AMP-PCP, are similar in solution but different in the crystal. This contradiction has led to a second hypothesis: in solution, the flexible phosphate chain of MgATP may fluctuate between the termini of helices 8 and 13 and thereby it may assist in the relative movement of these helices during domain closure. The large binding entropy of MgATP as determined in the ITC experiments is consistent with this hypothesis. In the crystal, however, the phosphates are fixed in one or the other position, depending on the crystallization conditions.

There is a completely conserved Lys 215 in helix 8, the side chain of which can interact with MgATP. In this way, MgATP may promote movement of helix 8 closer to helix 13 during domain closure. It is also remarkable that this side chain moves a distance of at least 10 Å during domain closure.

In both hypotheses, the peculiar behaviors of PGK (activation by anions and variations in the nucleotide binding modes) were related to the occurrence of domain closure.

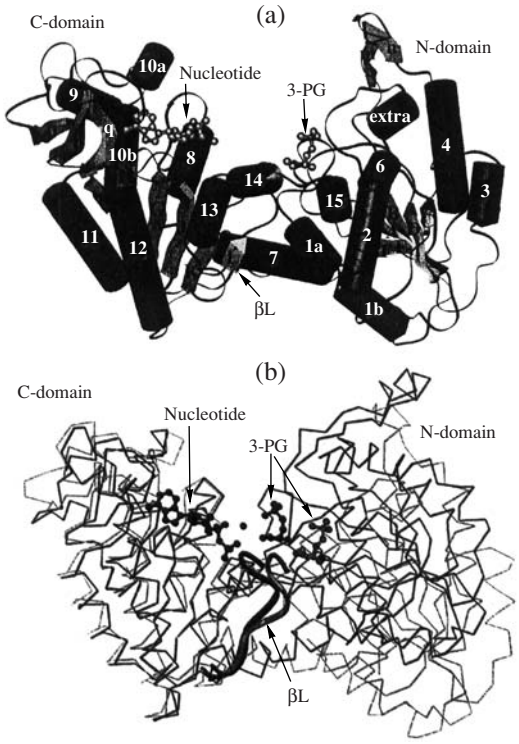

Fig. 1. Schematic representation of PGK three-dimensional structure (a) and the conformational changes during domain closure (b). The arrangement and labeling of the secondary structural elements are shown in (a) as determined in the crystal structure of the 3-PG*MgADP ternary complex of pig muscle PGK [18]. The open (gray) and closed (black) structures of the 3-PG binary complex of pig muscle [17] and of the 3-PG*MgADP ternary complex of *Trypanosoma brucei* [21] PGKs, respectively, are superimposed according to the backbone atoms of the core b-strands of the C-terminal domain in (b). The positions of bL are highlighted by ribbons of the respective colors. The bound substrates are indicated by ball and stick models in both figures.

The hypotheses were tested by several approaches: (i) modeling of anion binding to PGK, (ii) site-directed mutagenesis of Lys 215, and (iii) graphical analysis of various PGK crystal structures.

Due to difficulties in experimental detection of a secondary (possibly weak) anionic site, we modeled binding of anions into the PGK molecule. Remarkable docking results were obtained only in case of the closed PGK crystal structure [30], supporting our hypothesis that the activating anion site is formed upon domain closure. Fig. 4 illustrates the binding of an activating phosphate ion outside the closed active site. The anionic site is constituted by participation of Arg 65 (N domain) and Lys 215 (C domain), respectively.

It is remarkable that the docking suggested interaction of the anion with the same Lys 215, which may also temporally interact with the nucleotide phosphate.

This bound anion may regulate domain closure and opening. In order to test the role of Lys 215, we carried out site-directed mutagenesis. It was changed either to

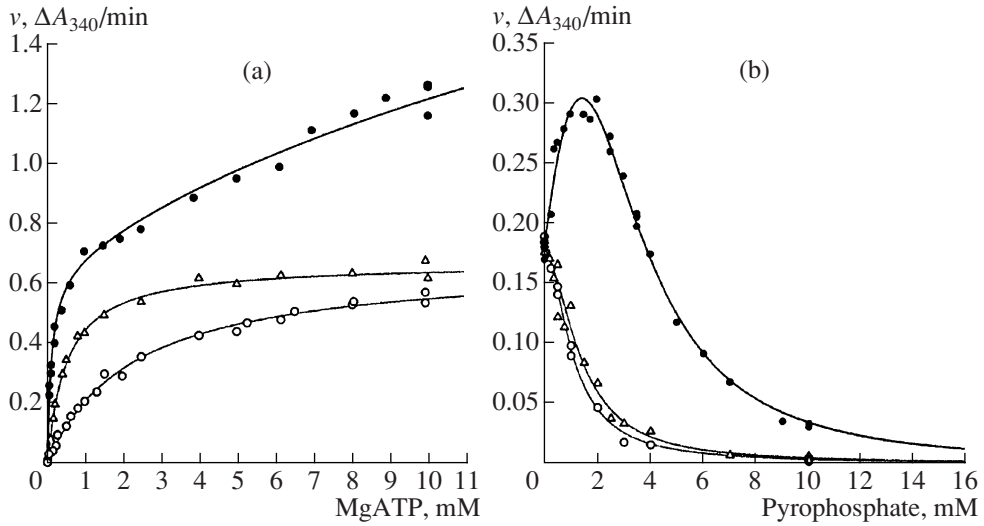

Fig. 2. Substrate saturation kinetic curves (a) and the anion activation–inhibition kinetics (b) of wild-type and mutant human PGKs [30]. Activity of 9 nM wild-type (*1*) and 4 mM K215A (*2*) and 0.8 mM K215R (*3*) mutant PGKs were measured as a function of MgATP concentration in the presence of 10 mM 3-PG (a) and as a function of pyrophosphate concentration in the presence of 0.5 mM 3-PG and 0.5 mM MgATP (b). The time courses of the PGK-catalyzed reaction were recorded spectrophotometrically at 340 nm by using a coupled assay system. The continuous lines represent the best fits of the experimental points in (a) and (b) to Eqs. (1) and (2), respectively, given in [26, 30]:

$$v = v_S\frac{[S]}{K^S_{cat}+[S]} + v_S(a-1)\frac{[S]}{K^S_{cat}+[S]}\frac{[S]}{K^S_{act}+[S]}, \quad (1)$$

where v_S stands for the activity at saturation of the catalytic site when no activation by an excess of substrate (S) occurs; the activation factor a can be varied between 0 and 1; K^S_{cat} and K^S_{act} are the dissociation constants of substrates for the catalytic and activating sites, respectively; and

$$v = v_0 + v_0(a-1)\frac{[A]}{K^A_{act}+[A]} - \left\{v_0 + v_0(a-1)\frac{[A]}{K^A_{act}+[A]}\right\}\frac{[A]^n}{K^A_{inh}+[A]^n}, \quad (2)$$

where v_0 stands for the activity in the absence of bound anion (a) at fixed substrate concentrations, K^A_{act} and K^A_{inh} are the apparent dissociation constants for the sites of activation and inhibition by anions, and n is the number of the inhibiting anion sites per PGK molecule.

Ala or to Arg and the results confirmed this assumption. The substrate saturation curves are changed into hyperbolic ones (Fig. 2a) that lack activation at a high excess of the substrates, and the mutants cannot be activated by anions, just inhibited (Fig. 2b) [30].

Thus, modeling and mutagenesis studies have proved the assumed relationship between anion activation and domain closure.

The kinetic and binding constants (Table 2) provided information about the binding mode of nucleotides. Increasing the K_m value for both mutants proved the interaction of Lys 215 with the transferring phospho group of ATP in the functioning ternary complex. Weakening of MgATP binding (an increase in K_d) even in the absence of the other substrate indicates an initial interaction of Lys 215 with the γ-phosphate of MgATP already in the open conformation of the nonfunctioning binary complex.

Therefore, it follows that, during domain closure, Lys 215 possibly moves together with the transferring nucleotide γ-phosphate; meanwhile this transferring phosphate itself is being positioned for the catalysis. At the same time, MgATP can assist in movement of helix 8 during domain closure. All of these ideas are in line with the hypothesis that mobility of the nucleotide phosphate is related to the domain closure.

The next question was how the results are related with operation of the main molecular hinge. We proposed earlier the existence of this hinge at the β-strand L on the basis of comparison of two open and two closed crystal structures [18]. As shown in Fig. 1b, the β-strand L is located between the two domains. The question arose of whether the nucleotide phosphates, in addition to interacting with helices 8 and 13 (as described above), can affect the conformation of the main hinge. Another relevant question is whether the

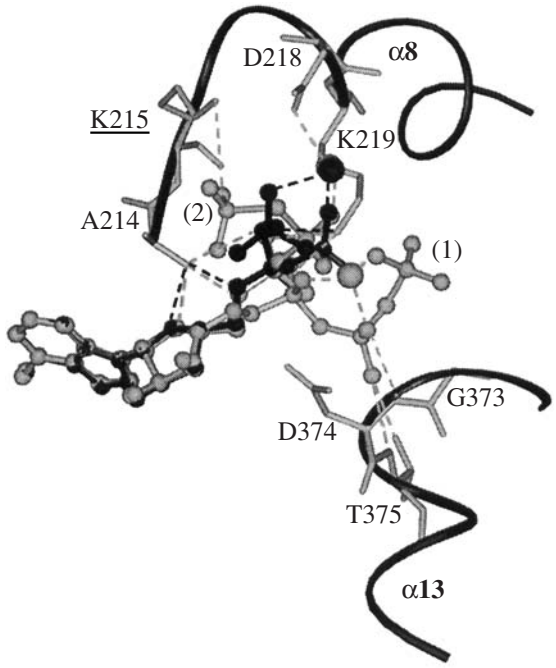

Fig. 3. Various positions of phosphates of the nucleotides bound to PGK in the crystal structures. The surroundings of the bound nucleotides (ball and stick models) are shown as determined in the MgATP (black) binary complex of pig muscle PGK [20]. The nearby helices (8 and 13) are shown as black ribbons, while the interacting side chains are illustrated by gray stick models. MnAMP-PNP (gray (*1*)) and MgAMP-PCP (gray (*2*)) are taken from separate crystal structures of [28] and [19], respectively, and superimposed according to all atoms of the adenine and ribose rings.

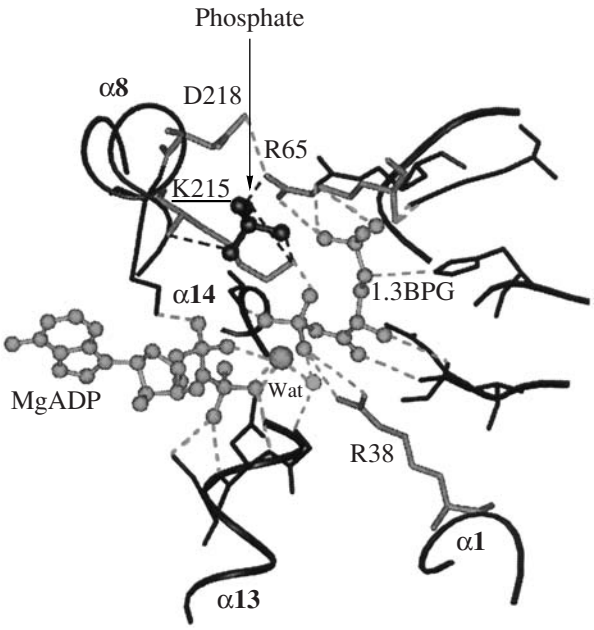

Fig. 4. The possible binding mode of the activating phosphate ion to PGK. A phosphate ion (black ball and stick model) was modeled into the closed conformation of the *Trypanosoma brucei* PGK crystal structure [21] by docking [30]. Secondary structural elements (black ribbons) and part of the side chains (stick models) at the active site are illustrated. The side chains important for activity or activation by anions are colored gray. The bound substrates MgADP and 1,3-BPG are shown as gray ball and stick models. The position of 1,3-BPG was suggested by docking into the place of 3-PG, bound originally in the crystal structure.

simultaneous binding of both substrates is required for closing the hinge. The necessity of both substrates was observed from the crystal structures, which exhibit a closed domain conformation only in case of the ternary enzyme–substrate complexes containing both bound substrates, and it was also confirmed by our SAXS experiments with solubilized PGK [24].

In order to describe the operation of the molecular hinge at βL at the atomic level, we analyzed the contacts of the conserved side chains and backbone atoms in various open and closed crystal structures. Only two additional backbone H bonds (black arrows) are formed at the hinge in the closed conformation, in addition to the gray atomic contacts, which are formed already in the respective binary complexes (Fig. 5). The gray con-

Table 1. Binding characteristics of the nucleotide ligands to PGK*

Ligand	*B* factor (helix 13)	ITC binding study			DSC experiment	
		K_d, mM	ΔH, cal/mol	$T\Delta S$, cal/mol	T_m, °C	ΔT_m, °C
No	53.04	–	–	–	53.0	–
MgAMP-PCP	55.86	1.07 ± 0.25	–	–	55.1	2.1
MgATP	38.87	0.26 ± 0.154	–1360 ± 280	3300 ± 80	54.9	1.9
MgAMP-PNP	27.09	0.35 ± 0.10	–1150 ± 145	3630 ± 160	55.0	2.0
MgADP	19.54	0.054 ± 0.008	–3180 ± 50	2530 ± 30	57.1	4.1

* The average crystallographic *B*-factor values for the backbone atoms of helix 13 were derived from the crystal structures of the substrate-free enzyme [14] and of the complexes of PGK with 3-PG*MgAMP-PCP [19], with MgATP [20], with 3-PG*MgAMP-PNP [22], and with MgADP [16]. The results of isothermal calorimetric (ITC) titrations and of differential scanning calorimetric (DSC) experiments were taken from references [20] and [29], respectively.

Table 2. Effect of mutations of Lys 215 on the functional properties of PGK*

PGK	k_{cat}, l/min	MgATP		3-PG	
		K_m, mM	K_d, mM	K_m, mM	K_d, mM
Wild-type	50000 ± 3000	0.11 ± 0.03	0.33 ± 0.15	0.05 ± 0.01	0.035 ± 0.008
K215R	105 ± 9	0.57 ± 0.05	0.83 ± 0.18	0.35 ± 0.02	0.037 ± 0.009
K215A	29 ± 3	2.47 ± 0.2	1.45 ± 0.20	0.41 ± 0.04	0.039 ± 0.010

* The data were taken from [30] and were determined in enzyme kinetic experiments similar to the one shown in Fig. 2a.

tacts describe the route of transmission of substrate-triggered conformational changes from one domain to the other. The two new H bonds complete a special H-bonding network including βL. The separate binary complexes are open structures, but under the concerted action of the two substrates, the shape of the β-strand L is substantially changed due to the formation of these new H bonds. This is the key for domain closure. In this way, βL acts as a double molecular switch, controlled by the concerted action of both substrates. Naturally, the closed conformation is stabilized by other contacts as well, not shown here, including the contact with Lys 215.

In summary, the catalytic cycle of PGK briefly consists of the following events. Upon binding of both substrates, under their concerted action, the shape of βL is substantially changed, and this leads to closure of the two domains. Simultaneously, an anion-binding site is formed by the contributions of Lys 215 (C domain) and Arg 65 (N domain). The activating anion can bind here and stabilizes the closed active site, in which the reactive groups are precisely oriented and the phospho group is transferred between the two substrates. Anions may also accelerate the subsequent domain opening and dissociation of the products.

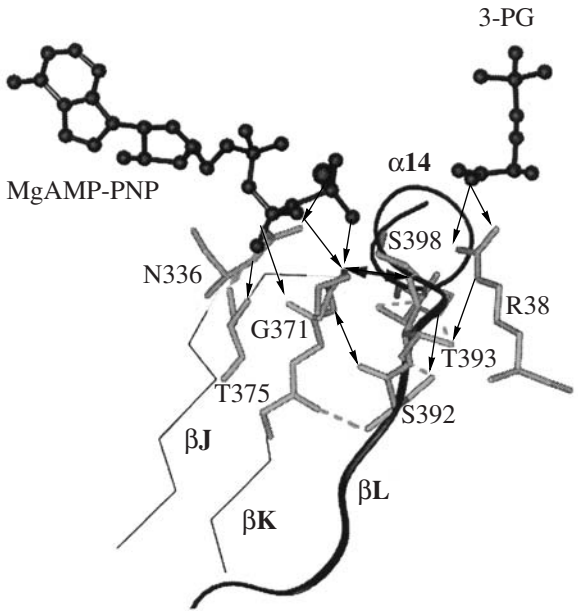

Fig. 5. Mechanism of operation of the main hinge at bL. Molecular contacts in the surroundings of the b-strand L (highlighted by a black ribbon) of the closed conformation of the MgAMP-PNP*3-PG ternary complex of *Thermotoga maritima* PGK [22] are determined by molecular graphical analysis [29]. Black ball and stick models show the bound substrate and analogue. The contacts formed upon separate binding of 3-PG or MgAMP-PNP in the respective binary complexes are colored gray. Black double-sided arrows indicate the H bonds formed only upon domain closure in the ternary complex.

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