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ВЫЯСНЕНИЕ МЕХАНИЗМА ДВИЖЕНИЯ ДОМЕНОВ И ЕГО РОЛЬ В ФУНКЦИОНИРОВАНИИ 3-ФОСФОГЛИЦЕРАТ КИНАЗЫ

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Всестороннее изучение 3-фосфоглицерат киназы позволило выявить детали переноса субстрата путем управляемого конформационного эффекта по направлению к главной молекулярной петле β-слоя L. Необычное кинетическое поведение (активация анионами) и подвижность фосфатной цепи нуклеотидных субстратов могут быть ответственны за движение доменов. Оба явления обусловлены взаимодействием с остатком Lys каталитического центра, который сдвигается более чем на 10 при закрытии доменов. Это движение осуществляется синхронно с движением главной петли при одновременном действии двух субстратов.

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 controlling by the bound substrate(s) have to be delineated for understanding catalysis.

Domain closure over the active sites of multidomain enzymes can often create an optimal environment for catalysis, orients the substrate reactive groups properly and/or impedes side-reactions. In order to reach general conclusions on the structural functional aspects of domain closure, individual cases has to be investigated. Besides comparison of the extreme conformations (e.g. [2,4-6]), simulation of the motion by computer modelling (e.g. [7-9]) more complex investigation 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 essential enzyme for the basic carbohydrate metabolism. PGK catalyses the phospho-transfer 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 in photosynthesis of plants. In addition to its physiological activity, human PGK can phosphorylate the non-natural L-nucleoside analogues which are used in antiviral and anticancer therapies [12,13]. By this way PGK produces the pharmacologically active triphosphate forms of these compounds and regulates their levels in the cell.

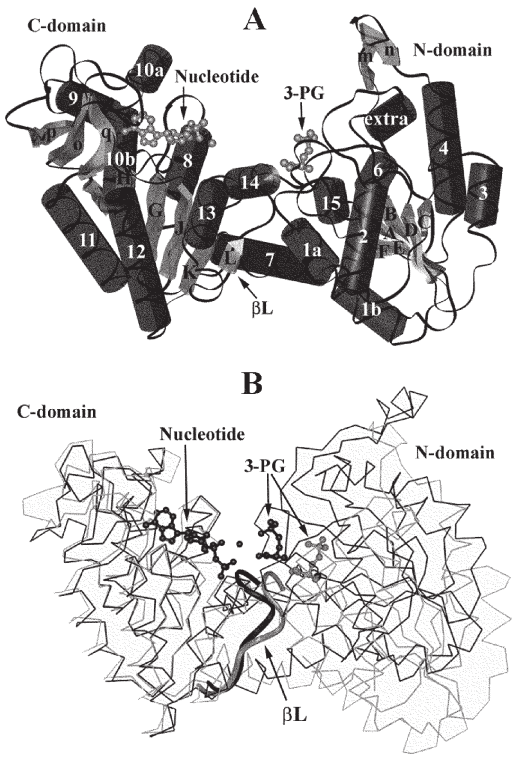

Fig. 1. Schematic representation of PGK three-dimensional structure (A) and the conformational changes during domain closure (B). Arrangement and labelling of the secondary structural elements are shown in (A) as determined in the crystal structure of 3-PG*MgADP ternary complex of pig muscle PGK [18]. The open (grey) and closed (black) structures of 3-PG binary complex of pig muscle [17] and of 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 colours. The bound substrates are indicated by ball and stick models in both figures

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 studies [23, 24]. With its conserved structure, including the well-structured inter-domain region, PGK is a suitable model to study the details of domain-domain interplay and its regulation by the substrates.

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

Thus, the kinetic data argue in favour of the existence of a secondary regulatory site, but crystallographic and part of the solution binding data (e.g. [27]) support the

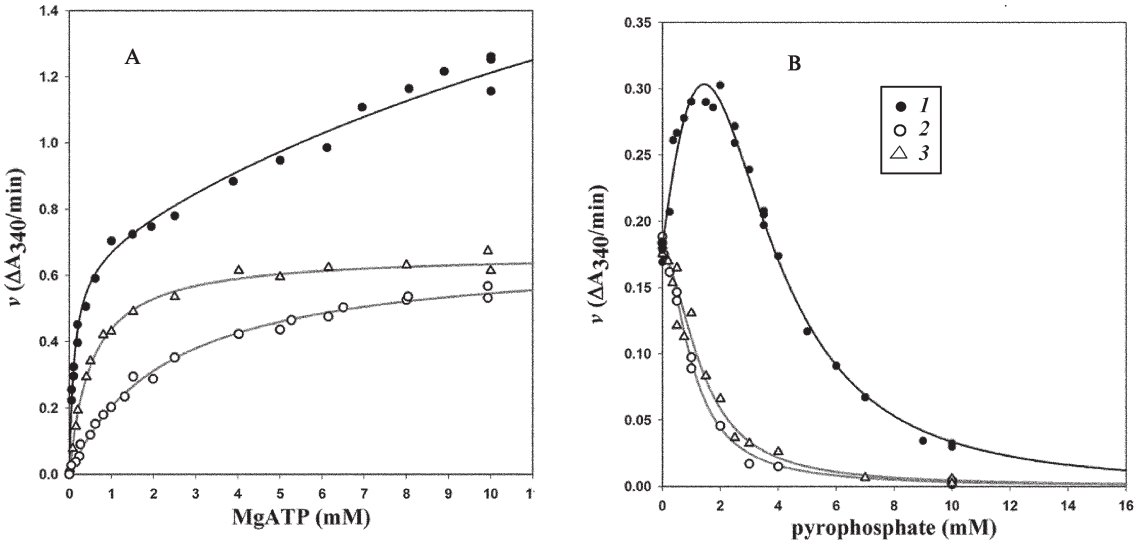

Fig. 2. Substrate saturation kinetic curves (A) and the anion activation-inhibition kinetics (B) of wild type and mutant human PGKs (ref. [30]). Activity of 9 nM wild type (*1*), 4 mM K215A (*2*) and 0,8 mM K215R (*3*) mutant PGKs were measured as a function of MgATP concentration in the presence of 10 mM 3-PG (A) and as a function of pyrophosphate concentration in the presence of 0.5 mM 3-PG and 0.5mM MgATP (B). The time courses of the PGK catalysed 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]:

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

where v_s stands for the activity at saturation of the catalytic site when no activation by the 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

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

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

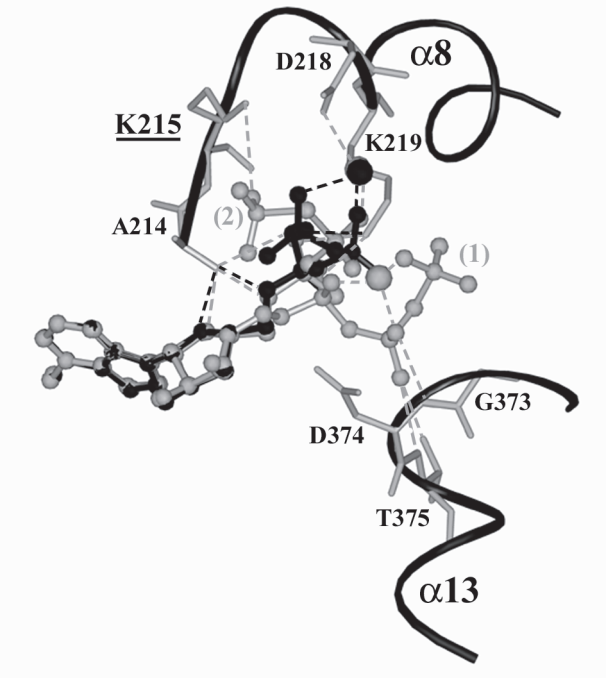

Fig.3. Various positions of phosphates of the nucleotides bound to PGK in the crystal structures. The surrounding of the bound nucleotides (ball and stick models) is shown as determined in the MgATP (black) binary complex of pig muscle PGK [20]. The nearby helices (8 and 13) are shown as black ribbons,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

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 interact 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 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 behaviours 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 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 much stronger stabilisation 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 crystallisation conditions.

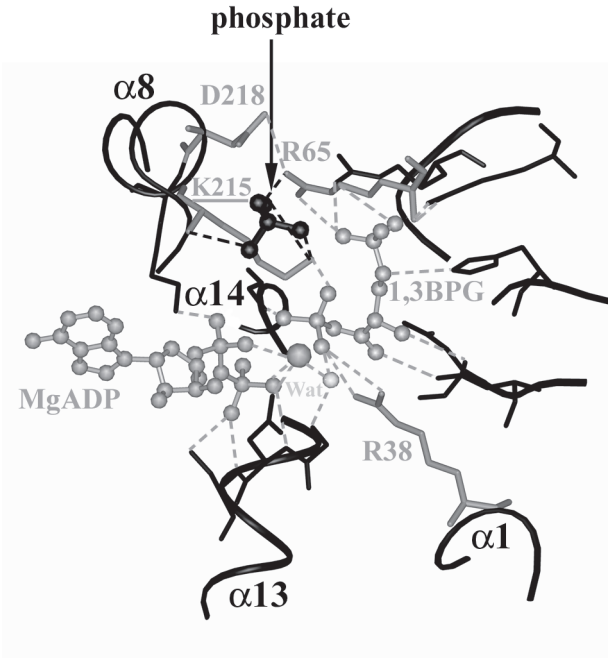

Fig. 4. The possible binding mode of the activating phosphate ion to PGK. A phosphate ion (black ball and stick model) was modelled into the closed conformation of *Trypanosoma brucei* PGK crystal structure [21] by docking [30]. Secondary structural elements (black ribbons) and part of the side chains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

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)	$T{\cdot}\Delta S$ (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 the differential scanning calorimetric (DSC) experiments were taken from references [20] and [29], respectively.

There is a completely conserved Lys 215 in helix 8, side chain of which can interact with MgATP. By 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 at least 10 Å distance during domain closure.

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

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

Due to difficulties in experimental detection of a secondary (possibly weak) anionic site, we modelled binding of anions into 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 of the closed active site. The anionic site is constituted by participation of Arg 65 (N-domain) and of 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 have carried out site-directed mutagenesis. It was changed either to Ala or to Arg and the results confirmed this assumption. The substrate saturation curves are changed into hyperbolic ones (Fig. 2, *a*) which lack activation at high excess of the substrates; and the mutants cannot be activated, just inhibited by anions (Fig. 2, *b*) [30].

Thus, modelling and mutagenesis studies have approved the assumed relationship between anion-activation and domain closure.

About the binding mode of nucleotides the kinetic and binding constants (Table 2) provided information. Increasing the K_m value for both mutants approved the interaction of Lys 215 with the transferring phosphogroup of ATP in the functioning ternary complex. Weakening of MgATP binding (increase of 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 non-functioning binary complex.

It follows, therefore, 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 are in line with the hypoth

Table 2

Effect of mutations of Lys 215 on the functional properties of PGK*

PGK	k_{cat} (1/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 ref. [30] and were determined in enzyme kinetic experiments similar to the one shown in Fig. 2, *a*.

esis 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, β-strand L is located between the two domains. The question arose whether the nucleotide phosphates, in addition to their interaction with the helices 8 and 13 (shown above), can affect the conformation of the main hinge? Another relevant question whether the simultaneous binding of both substrates is required for closing the hinge? The necessity of both substrates came from the crystal structures which exhibit 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 the solubilized PGK [24].

In order to describe the operation of the molecular hinge at βL at atomic level, we have analysed 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 grey atomic contacts, that are formed already in the respective binary complexes (Fig. 5). The grey contacts 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 β-strand L is substantially changed due to the formation of these new H-bonds. This is the clue for domain closure. By this way βL acts as a double molecular switch, controlled by the concerted action of both substrates. Naturally, the closed conformation is stabilised by other contacts, too, not shown here, including the contact with Lys 215.

In summary, the catalytic cycle of PGK briefly consist 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 contributions of Lys 215 (C-domain) and

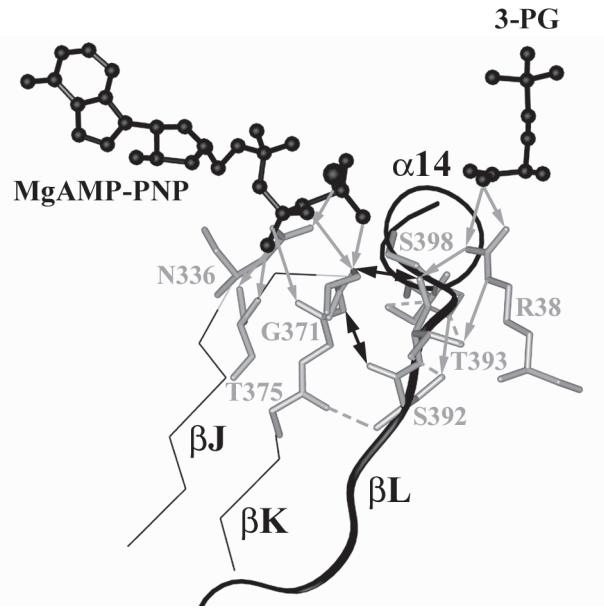

Fig.5. Mechanism of operation of the main hinge at bL. Molecular contacts in the surroundings of b strand L (highlighted by black ribbon) of the closed conformation of MgAMP-PNP*3-PG ternary complex of *Thermotoga maritima* PGK [22] are determined by molecular graphical analysis [29]. Black ball and 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 coloured as grey. Black double-sided arrows indicate the H-bonds formed only upon domain closure in the ternary complex

Arg 65 (N-domain). The activating anion can bind here and stabilises 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 consequent domain opening and dissociation of the products.

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INSIGHT INTO THE MECHANISM OF DOMAIN MOVEMENTS AND ITS ROLE IN FUNCTIONING OF 3-PHOSPHOGLYCERATE KINASE

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Comprehensive studies with 3-phosphoglycerate kinase revealed the details of transmission of substrate triggered conformational effect towards the main molecular hinge at the b-strand L. The unusual kinetic behaviour (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 the interactions with the catalytic Lys residue, which moves more than 10 E during domain closure. This movement occurs, in concert with operation of the main hinge, under the simultaneous action of the two substrates.