Substrate-Induced Double Sided H-bond Network as a Means of Domain Closure in 3-Phosphoglycerate Kinase

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ABSTRACT[†]

Closure of the two domains of 3-phosphoglycerate kinase, upon substrate binding, is essential for the enzyme function. The available crystal structures cannot provide sufficient information about the mechanism of substrate assisted domain closure and about the requirement of only one or both substrates, since lattice forces may hinder the large scale domain movements. In this study the known X-ray data, obtained for the open and closed conformations, were probed by solution small-angle X-ray scattering experiments. The results prove that binding of both substrates is essential for domain closure. Molecular graphical analysis, indeed, reveals formation of a double-sided H-bond network, which affects substantially the shape of the main molecular hinge at β -strand L, under the concerted action of both substrates.

ABBREVIATIONS

PGK: 3-phospho-D-glycerate kinase

or ATP: 3-phospho-D-glycerate 1-phosphotransferase (EC 2.7.2.3),

3-PG: 3-phospho-D-glycerate

1,3-BPG: 1,3-bisphosphoglycerate

AMP-PNP: β,γ -imido-adenosine-5'-triphosphate

AMP-PCP: β,γ -methylene-adenosine-5'-triphosphate

SAXS: small angle X-ray scattering

[†]*Keywords:* Domain movement, Main Hinge, Substrate effects, Phosphoglycerate Kinase, small-angle X-ray scattering

INTRODUCTION

Relative movement of protein domains, as a manifestation of the conformational flexibility (cf. reviews [1-6]) is frequently involved in the mechanism of action of various enzymes, including several members of the kinase family, an ubiquitous group of enzymes [7]. Domain closure over the enzyme active sites often creates an optimal environment for catalysis, reorients the substrate reactive groups and/or impedes side-reactions. The structural principles that govern these domain motions, however, are not always understood. There are attempts of classification of the motions at the level of super secondary structure (e.g. by defining hinges or rotational axis) [2], however, there are not yet many detailed descriptions of these large-scale conformational changes in terms of side-chain and backbone interactions, and the role of substrates in mediating domain motions is poorly understood.

3-Phosphoglycerate kinase (PGK) is a typical hinge-bending enzyme with two structural domains of about equal size [8-10], to which each of the two substrates are bound [11,12]. PGK with its conserved primary structure and tertiary fold, including a well-structured inter-domain region, is a suitable model to study the domain-domain interplay and its regulation by the substrates. A large body of enzymological and structural information accumulated about PGK offers a possibility to get a closer insight into the operation of its assumed molecular hinges at atomic level and into the mechanisms the action of the two substrates.

PGK is an essential enzyme for all living organisms. It catalyses the phospho-transfer from 1,3-bisphosphoglycerate (1,3-BPG) to MgADP and produces 3-phosphoglycerate (3-PG) and MgATP during the carbohydrate metabolism. In addition to its physiological role, human PGK was shown to phosphorylate L-nucleoside analogues, which are potential drugs against viral infection and cancer [13-16].

The C-terminal domain of PGK binds the nucleotide substrates (MgADP or MgATP) [12,17], while the N-terminal domain binds 3-PG [11] or possibly, 1,3-BPG (there is no structural evidence for the binding site of the latter unstable substrate). X-ray crystallographic data on PGK from various sources suggest that domain closure, required by the catalysis, will take place in the presence of *both* bound substrates, i.e. only in the enzyme-substrate ternary complex [18,19]. However, in some cases (e.g. pig muscle PGK), large-scale domain movements are apparently prevented by the crystal lattice forces [20,21], and therefore, additional experiments on solubilized enzyme are required to clarify the role of each substrate in domain closure. Small angle X-ray scattering (SAXS) is an effective method to study the

overall structure of macromolecules and, in particular, to validate the crystal structure in solution (see e.g. [22] for a review). Earlier SAXS experiments with solubilized PGK-s, however, either investigated only the ternary substrate complexes of the pig muscle enzyme [23] or provided ambiguous results concerning the binary complexes of the yeast enzyme [24-26]. In the present work this question is reassessed by a comprehensive SAXS study of various enzyme-substrate complexes of the wild type human PGK. The sequence of the latter enzyme is identical up to 97 % with that of pig muscle PGK. From the total 417 amino acid residues 405 are identical, 10 are strongly similar in their nature and only 2 structurally less important residues are really different in the two sequences. Although X-ray structure has not been determined for human PGK, on this basis highly similar three-dimensional structures can be assumed for the human and pig muscle enzymes. In accordance, the two enzymes are indeed, very similar in both kinetic and physico-chemical aspects [27].

Despite of the well-defined location of molecular hinges responsible for domain movements evidence is still missing for the requirement of both substrates for domain closure. These hinges (as deduced from comparison of the open and closed conformations of various PGK-substrate complexes in the crystal) are located at the N- and the C-terminal ends of the interdomain helix 7 [18,28] as well as in the β -strand L in the interdomain region [29]. It has been demonstrated that the relative position of the two domains is closely correlated with the conformation of β L, which is therefore proposed to be the main hinge [29], as illustrated in Fig. 1, where two open (blue and green) and two closed (orange and red) PGK crystal structures are superimposed according to their C-terminal domain. Molecular contacts have been also identified upon separate binding of each substrate, which describe the possible route of transmission of the substrate-induced structural effects from one domain to the other [30]. Yet, the operation of the main molecular hinge is still has to be elucidated in terms of atomic details and of substrate binding.

In order to answer the question of necessity of binding of a single or both substrates for domain closure and to find satisfactory structural rationale for separate or concerted action of the two substrates, we have carried out simultaneous SAXS measurements in solution and molecular graphical analysis (focused on the main hinge at β L) of the known high resolution crystal structures of various binary and ternary enzyme-substrate complexes. To this end we present here a comprehensive picture about the details of operation of the main molecular hinge of PGK, under the synergistic action of the two substrates.

MATERIALS AND METHODS

Enzyme and chemicals

Wild type human PGK was expressed in *E. coli* BL21-CodonPlus (DE3)-RIL (Stratagene) strain, purified and stored as described previously [27]. Na-salts of 3-PG, ATP and ADP were from Boehringer. The substrates, MgATP and MgADP, were formed by addition of MgCl₂ (Sigma) to ATP or ADP. The dissociation constant of MgATP and MgADP were taken to be 0.1 mM and 0.6 mM, respectively, obtained by averaging the data in the literature [31-35].

SAXS measurements and data processing

Synchrotron radiation X-ray scattering data were collected on the X33 beam line [36,37] at the Hamburg EMBL Outstation (on the DORIS III storage ring, at DESY). Solutions of wild type human PGK, its binary complexes with 3-PG, MgATP or MgADP and the ternary complexes of PGK with 3-PG plus MgATP and PGK with 3-PG and MgADP in 50 mM Tris/HCl buffer, pH 7.5, containing 1 mM dithiothreitol, 1 mM EDTA were measured at protein concentrations in the range from 4.0 to 20 mg/ml using a MAR345 Image Plate at a sample-detector distance of 2.4 m and wavelength $\lambda = 1.5 \text{ \AA}$, covering the momentum transfer range $0.012 < s < 0.45 \text{ \AA}^{-1}$ ($s = 4\pi \sin(\theta)/\lambda$ where 2θ is the scattering angle).

To check for radiation damage two 2-minute exposures were compared; no radiation effects were observed. The data were averaged after normalization to the intensity of the incident beam, corrected for the detector response, and the scattering of the buffer was subtracted. The difference data were extrapolated to zero solute concentration following standard procedures. All data manipulations were performed using the program package PRIMUS [38].

The forward scattering $I(0)$ and the radius of gyration R_g were evaluated using the Guinier approximation [39] assuming that at very small angles ($s < 1.3/R_g$) the intensity is represented as $I(s) = I(0) \exp(-(sR_g)^2/3)$. These parameters were also computed from the entire scattering patterns using the indirect transform package GNOM [40]. The molecular masses of the solutes, evaluated by comparison of the forward scattering with that from reference solutions of bovine serum albumin, were compatible with the monomeric state of the enzyme for all samples.

The radii of gyration and the scattering patterns from the crystallographic models of wild type PGK and its complexes were computed using the program CRY SOL [41]. Given the atomic coordinates, the program fits the experimental intensity by adjusting the excluded volume of the particle and the contrast of the hydration layer to minimize the discrepancy, defined by χ^2 :

$$\chi^2 = \frac{1}{N-1} \sum_j \left[\frac{I_{\text{exp}}(s_j) - cI_{\text{calc}}(s_j)}{\sigma(s_j)} \right]^2 \quad (1)$$

where N is the number of experimental points, c is a scaling factor, $I_{\text{exp}}(s_j)$ and $I_{\text{calc}}(s_j)$ are the experimentally determined and calculated intensities, respectively; and $\sigma(s_j)$ the experimental error at the momentum transfer s_j .

Molecular graphical analysis

For visualizing and analysing the molecular details of the 10 investigated PGK structures the Insight II (Biosym/MSI, San Diego, CA) software was used. The pdb codes of the protein co-ordinates used in structural comparisons are as follows: **1PHP** (*B. stearothermophilus* PGK*MgADP [12]), **1VJC** (pig muscle PGK*MgATP [17]), **1HDI** (pig muscle PGK*3PG*MgADP[29]), **1KF0** (pig muscle PGK*3PG*MgAMP-PCP [42]), **13PK** (*T. brucei* PGK*3PG*MgADP [43]), **1VPE** (*T. maritima* PGK*3PG*MgAMP-PNP [19]) and **3PGK** (substrate-free yeast PGK [10]. The pdb co-ordinates of pig muscle PGK*3PG binary [11], pig muscle PGK*3PG*MnAMP-PNP [44] and of the substrate-free pig muscle PGK [42]) were obtained from the authors. For the graphical comparison a structure-based sequence alignment, prepared by us previously [29,30], was used. The molecular contacts including and proximal to β -strand L were enumerated, within the protein and between the protein and the substrates, for all the investigated structures. Following common practice, only distances below 3.50 Å were attributed to atomic interactions.

RESULTS and DISCUSSION

Substrate-Caused Conformational Changes of Human PGK as Detected by SAXS

The scattering curves of human PGK in the absence of substrates and of the binary complexes with 3-PG, MgATP or MgADP, as well as of the ternary complexes with 3-PG plus MgATP (the functional complex) and with 3-PG plus MgADP (a non-functional complex) are presented in Fig. 2A. The experimentally determined radii of gyration R_g are summarized in Table 1. A significant decrease of R_g is detected in the presence of both substrates, similar to previous observations with the pig muscle PGK [23]. This decrease is compatible with the change expected upon domain closure, as seen from the R_g values computed from the crystal structures (Table 1). In contrast, none of the binary complexes exhibited significant changes of the R_g compared to the substrate-free protein.

To validate the available crystal structures further, the computed scattering profiles were systematically screened against the experimental scattering data and the discrepancy between the theoretical and measured curves are summarized in Table 1. For the substrate-free-enzyme and all binary complexes, the best fits (presented in Fig. 2A) were obtained for the crystal structures, representing open domain conformation. This is expressed numerically by the discrepancy χ^2 values between the experimental scattering data and the corresponding crystallographic models (Table 1). There is only one apparent exception for the MgADP binary complex, where the *Bacillus stearothermophilus* (Bs)⁺ PGK [12] model does not yield the best fit to the data. The reason is most probably the extra loop between G128 and K145, which is present in pig (and certainly in human), but missing in Bs PGK. The scattering data of the MgADP binary complex, however, correlate rather well with all of the three other open crystal structure models. These results clearly demonstrate that all these binary complexes exhibit an open domain conformation, both in crystal and in solution. Since in solution no conformational restriction by the crystal lattice forces exists, the open structures observed in the crystals of 3-PG binary [11], of MgATP binary [17] complexes of pig muscle PGK and of the MgADP binary complex of Bs PGK [12] are not artefacts, and can be used in structural comparisons (see below).

Concerning the functional MgATP*3-PG ternary complex of human PGK, the present SAXS-based analysis provided unequivocal result in favour of domain closure showing the best fit to the completely closed *Trypanosoma brucei* (Tb) PGK [43] crystal structure (Table 1). This is in good agreement with the earlier SAXS results with yeast [24] and pig muscle [23] PGK-s. In case of MgADP*3-PG ternary complex of human PGK, however, the best fit

was obtained to the largely (but not completely) closed (cf. Fig. 1) *Thermotoga maritima* (Tm) PGK [19] structure (Table 1). This means that MgADP and 3-PG do close the domains in the unproductive ternary complex, but not as much as MgATP and 3-PG in the functioning complex. The characteristic features of the scattering profiles of the ternary complexes (curves (5) and (6) in Fig. 2A) are the less pronounced shoulder at $s=0.18 \text{ \AA}^{-1}$ and the more pronounced maximum at $s = 0.25\text{-}0.30 \text{ \AA}^{-1}$. These alterations in the scattering profile upon domain closure are clearly visible in Fig. 2B, where the scattering patterns of the MgATP*3-PG ternary complex (5) and the open form of unligated PGK (1) are directly compared. These features of the ternary complexes are much better represented by the closed Tm PGK and Tb PGK models than by any of the open crystallographic models.

It is also notable that the experimental data from the human PGK ternary complexes are incompatible with the theoretical curves (not illustrated) calculated from the open crystal structures of MgADP*3-PG [29], MnAMP-PNP*3-PG [44] and MgAMP-PCP*3PG [42] ternary complexes of pig muscle PGK. These results also imply that these open structures are crystallographic artefacts, as it was also proposed by analysing the nature of the lattice forces operating in these crystals [21]. Such kind of lattice forces are absent in the crystals of the closed ternary complexes of Tb [43] and of Tm [19] PGK-s, and, indeed, as shown above, the solution scattering curves from human PGK ternary complexes correlate well with the curves computed from the closed structures of Tb and Tm PGK-s (Table 1 and Figure 2).

In summary, with the exception of pig muscle PGK ternary complexes, SAXS and crystallographic data correlate well, supporting the view that simultaneous binding of both substrates to PGK are required for complete domain closure. The present analysis, using a systematic screening of the entire computed scattering patterns against the experimental data, is much more conclusive than previous SAXS studies, based solely on the values of the radii of gyration (which might be disturbed *e.g.* by aggregation or concentration effects).

Structural effects of the two substrates on the main hinge: a double switch mechanism of domain closure

To further compare the consorted effect of both substrates with their separate effects, molecular contacts of the main hinge region (β -strand L) of PGK, as determined in 10

*[‡] Numbering of residues throughout the text refers to pig muscle PGK sequence, unless quoted differently, as *e.g.* Bs(*Bacillus stearothermophilus*), Tm(*Thermotoga maritima*) and Tb(*Trypanosoma brucei*).

different crystal structures, including the substrate-free enzyme and its binary and ternary complexes with substrates (or analogues) were analysed. Although each static structure reflects time and space average of numerous dynamic structures, the crystal structures of the various enzyme-substrate complexes can be considered as representatives of various stages of substrate-caused conformational changes on the route from open to closed domain conformations. Our comparison is based on this rationale. The inter-atomic distances in the most relevant structures are listed in Table 2.

As expected, the crucial C-terminal part of β -strand L and the connecting loop towards helix 14, *i.e.* the part, which conformation is characteristically changed between the open and domain-closed structures (Fig. 1), is a highly conserved region. It includes the sequence Ser 392-Thr 393-Gly 394-Gly 395-Gly 396-Ala 397-Ser 398 (mammalian PGK numbering). Among these residues even the less conserved Ser 398 is identical at least up to 80 % among the 168 PGK sequences available in ExPASy Molecular Biology Server. From Table 2, it is evident, that independent of the presence of substrates, the functional OH-groups of Ser 392/Bs370/Tm373/Tb395, Thr 393/Bs371/Tm374/Tb396 and Ser 398/Bs376/Tm379/Tb401 form 3 invariable H-bonding interactions with the peptide N- and O-atoms of this region, existing in all the known PGK crystal structures (also in those structures not listed in Table 2). These H-bonds, therefore, contribute to the basic folding feature of this region, which is independent of substrate binding or the conformational states of the protein molecule, but which may serve as a prerequisite for the operation of the hinge. The usual H-bonds between the peptide atoms of parallel parts of the β -strands K and L (e.g. between Ile 370/Bs348/Tm351/Tb373:O and Ser 392/Bs370/Tm373/Tb395:N) may have similar roles. A part of the invariant H-bonds (dashed black lines) is shown in Fig. 3.

For the present analysis, however, the most interesting atomic contacts are formed exclusively in the specific enzyme-substrate complexes, especially in the closed ternary complex structures, in addition to the contacts already present in the respective binary complexes. Several PGK ternary complex crystal structures are available from various sources [18,19,29,42,44], but some of them are open structures [18,19,29,42,44]. As shown by the above SAXS analysis, these open structures must be crystallographic artefacts. Therefore, in the present analysis these open structures were considered as binary complexes. There are only two structures that exhibit really closed conformations, namely, the abortive ternary complex of Tb PGK with 3-PG and MgADP [18] and the ternary complex of Tm PGK with 3-PG and MgAMP-PNP (an unreactive analogue of MgATP) [19]. The atomic contacts

in the main hinge β -strand L, exclusively characteristic of the closed conformations of Tb and Tm PGK-s (2.5 and 2.0 Å resolutions, respectively) are selected on the basis of comparison to the open structures of the two available binary complexes with 3-PG (2.0 Å resolution, pig muscle PGK, [11]) and with MgADP (1.6 Å resolution, Bs PGK, [12]). The structure-based sequence alignment was performed to compare of PGK-s from various sources. The contacts characteristic of each binary complex and of the closed conformations are listed in Table 2 and depicted in Fig. 3.

It is shown that, in addition to the permanent invariant H-bonds (dashed black lines), further contacts are formed in and around β -strand L upon binding of either 3-PG (Fig. 3A) or MgADP (Fig. 3B) in the open conformations of their binary complexes (blue and green colours, respectively). As noted earlier [11,30], upon binding of 3-PG a new contact is formed between one of the two NH atoms of Arg 38/Bs36/Tm36/Tb39 (the residue involved in electrostatic interaction with the carboxylate of 3-PG) and the peptide O-atom of Thr 393/Bs371/Tm374/Tb396. This important new interaction connects the N- and C-terminal domains. As an extension of this H-bond network, a new strong H-bond is formed between the OH-group of Ser 392/Bs370/Tm373/Tb395 and the peptide N-atom of Gly 394/Bs372/Tm375/Tb397. These H-bonds are characteristic of all the known PGK crystal structures that bind 3-PG, independent of their conformational (open or closed) states. There is another noticeable H-bond between the peptide O-atom of Gly 371/Bs349/Tm352/Tb374 and the OH-group of Ser 398/Bs376/Tm379/Tb401, but it can only be observed in the 3-PG binary complex [11] among the 3-PG binding structures. Thus, this H-bond on itself may not be relevant for the domain closure, but it still indicates a close distance between the N-termini of helices 13 and 14, which is not the case for the substrate-free enzyme. Movement of helix 14 upon 3-PG binding, indeed, was reported [11,18].

In the MgADP-bound structure (Fig. 3B) the β -phosphate O-atoms of the nucleotide are linked through well-formed H-bonds to the OH-group of Thr/Ser 375/Bs353/Tm356/Tb378, the residue located in helix 13 (sequentially preceding β -strand L), as well as to the peptide N-atoms of the same residue and of Gly 373/Bs351/Tm354/Tb376. There are also further contacts of MgADP with helix 13, noted elsewhere [12]. Furthermore, due to the simultaneous interaction of the β -phosphate of MgADP with the conserved Asn 336/Bs316/Tm317/Tb338 from the β -strand J, this strand is fixed together with the connected β -strand K and with the sequentially following helix 13 within the C-domain upon the nucleotide binding. As a consequence, Asn 336/Bs316/Tm317/Tb338 directly interacts with

the peptide O-atom of Gly 371/Bs349/Tm352/Tb374 at the N-terminus of helix 13. These new interactions, also characteristics of other PGK complexes, in which the bound nucleotide interacts with helix 13 and thereby inducing a typical conformational change at the N-terminus of this helix. Due to this change, the N-atom of the sequentially next Gly 372/Bs350/Tm353/Tb375 is now directed towards the β -strand L, but no detectable interaction is yet formed with it. Thus, in contrast to the 3-PG binary complex, no definitive new contact is formed with β -strand L in the binary complex with the nucleotide. In spite of this, a new strong H-bond is observed at the end of β -strand L between the OH-groups of the serines 392/Bs370/Tm373/Tb395 and 398/Bs376/Tm379/Tb401. This bond can stabilize a certain conformational state of β -strand L. The H-bond between the OH-group of Ser 392/Bs370/Tm373/Tb395 and the peptide N-atom of Gly 394/Bs372/Tm375/Tb397, characteristic of 3-PG binding, may be also present here, but in a weaker form. The exceptional behaviour of certain nucleotide complexes (such as of MgATP or MgAMP-PCP) may be related to the different binding modes of these nucleotides to PGK in some crystals, showing no interactions with helix 13 [17,27,42].

Fig. 3C illustrates the atomic interactions of the same region as determined in the completely closed crystal structure of Tb PGK ternary complex (coloured as red). Most of the contacts are formed separately in 3-PG and MgADP binary complexes (indicated by cyan and green, respectively), only few additional contacts (Table 2) are formed. Among them there is a new contact between the NE atom of Arg 38/Bs36/Tm36/Tb39 and the peptide O-atom of Thr 393/Bs371/Tm374/Tb396, which further strengthens the interaction between the two domains. This contact, however, is not really characteristics of the closed conformation, since this bond also exists in the open conformations of all three pig muscle PGK ternary complexes. There are, however, two other, more interesting peptide H-bonds (denoted by black arrows in Fig. 3C) from the same atom of Gly 372/Bs350/Tm353/Tb375(N) to Ser 392/Bs370/Tm373/Tb395(O) and to Gly 394/Bs372/Tm375/Tb397(O). Neither of these bonds exists in any open PGK conformations. The first one creates a new contact of β L with the closest other strand, β K, i.e. between the β -strands before and after helix 13, in sequence. The second one connects the N-terminus of helix 13 to the end of β L, i.e. to the N-terminus of the following helix 14. In this way the helices before and after β L, in sequence, are connected. These new H-bonds, characteristic of the ternary complex, constitute an extension of the H-bond network (part of which already exists in the respective binary complexes), as a consequence of the simultaneously formed H-bonds on both sides of the hinge, under the

concerted action of 3-PG and MgADP. In spite of the small number of the new contacts, the shape of β -strand L is substantially changed (cf. Fig. 1). Thus, the bonds operating in the binary complexes now act together to bring β L into a conformation strikingly different from either binary complex. Taken together, these changes actually can be considered as operation of a double-sided molecular switch, controlled by simultaneous action of the two substrates, which can fix β -strand L in a conformation optimal for the closed state.

In the case of the other known, not fully closed structure of the ternary complex of Tm PGK with 3-PG and the MgATP analogue, MgAMP-PNP [19] one can find a slightly different solution of the same problem. Although the structure of the binary complex with MgAMP-PNP is lacking, from analysis of the contacts operating in the main molecular hinge of the closed Tm PGK structure (coloured as orange, Fig. 3D) a variation of the mechanism may be deduced. In addition to the contact between Arg38/Bs36/Tm36/Tb39(NH) and the peptide O-atom of Thr 393/Bs371/Tm374/Tb396, observed already in the open structure of 3-PG binary complex, here again a new contact is formed between the NE atom of Arg 38/Bs36/Tm36/Tb39 and the OH-group of Thr 393/Bs371/Tm374/Tb396, i.e. in the way different from the case of the closed structure of Tb PGK. This observation raises the possibility that besides the peptide O-atom of this Thr, its conserved side chain may also have a direct role in domain closure. The new H-bonding interaction, found above for Tb PGK between the atoms of Gly 372/Bs350/Tm353/Tb375(N) and Ser 392/Bs370/Tm373/Tb395(O), also operates in Tm PGK (marked as black arrow) and completes the H-bonding interactions between β -strands L and K, similar to the closed structure of Tb PGK. There is, however, a striking difference between the two closed structures: the H-bond between Gly 372/Bs350/Tm353/Tb375(N) and Gly 394/Bs372/Tm375/Tb397(O), characteristic of Tb PGK, is replaced by the bond between Gly 373/Bs351/Tm354/Tb376(N) and Thr 393/Bs371/Tm374/Tb396(O) atoms in Tm PGK (marked as black arrow). Thus, these two bonds are formed alternatively in the two closed structures. At the same time the contact between the OH-groups of the two serines (392/Bs370/Tm373/Tb395 and 398/Bs376/Tm379/Tb401), characteristic of the MgADP-bound structures of both binary and the ternary complexes, is suspended in case of MgAMP-PNP binding to Tm PGK.

It is reasonable to assume that the formation of the new H-bond between Gly 373/Bs351/Tm354/Tb376(N) and Thr 393/Bs371/Tm374/Tb396(O) atoms is greatly helped by simultaneous interactions of both the β - and γ -phosphate O-atoms of MgAMP-PNP with

the same Gly Tm354(N) atom. In this way, from one side, the nucleotide effect is transmitted towards the peptide O-atom of Thr 393/Bs371/Tm374/Tb396 in β L, i.e. exactly where the effect of 3-PG is transmitted from the other side through the above shown interactions with Arg 38/Bs36/Tm36/Tb39. These two sided interactions of Thr 393/Bs371/Tm374/Tb396 in Tm PGK, under the simultaneous control of both 3-PG and the nucleotide, may be considered here as a variant of the above suggested double switch mechanism. The characteristic differences, that can be observed both in the molecular contacts (Figs. 3C and 3D) and in the conformations of β L (cf. Fig. 1) in the two differently closed PGK structures direct our attention to the fact that this hinge region is a very mobile part of the molecule, which possibly adopts different conformations between the completely open and the closed states.

Molecular dynamics calculations by Chandra *et al.* [45] could also generate domain closure and describe the process at the level of relative movements of the key helices (numbered 7, 13 and 14) of the interdomain region. Their model does not deal with either any atomic details or the conformational change of β L, and only qualitatively support (the hinge-bending is overestimated) the existence of the other hinges at the C- and N-termini of the interdomain helix 7, as deduced earlier from the X-ray data obtained for Tb PGK [18]. Our previous graphical analysis [29] goes far beyond these works by identifying the main hinge point of PGK at β L. Moreover, here we give a detailed description of the operation of this main molecular hinge at atomic level. It seems also plausible that the main hinge itself directs the operation of the other hinges at the termini of helix 7 through the permanent molecular contacts existing in the well-structured interdomain region. A specific way of propagation of the conformational effect through the atomic contacts of completely conserved side-chains has been described elsewhere [29,30] and makes the above picture of the molecular events more complete. In accordance with the primary role of β L, no characteristic changes of molecular contacts of the conserved side-chains occur at the termini of helix 7, there are notable changes only in the backbone H-bond pattern, especially at its C-hinge [21]. Site-directed mutagenesis with yeast PGK at the termini of helix 7 either did not support [46] or did not provide clearly conclusive results [47] in favour of the importance of the hinges at helix 7, in agreement with their possible secondary role. Other mutations at the interdomain region of yeast PGK (according to the mammalian PGK numbering Glu 192 in helix 7 [48] and His 390 in β L [49-51]) did not produce dramatic effects on the kinetic properties. It was concluded therefore, that cumulative effect of multiple interactions of the side chains in the interdomain region (rather than individual residues) is likely to be responsible for the efficient

mechanism of ligand induced domain movement [50]. No mutagenesis studies have been carried out with the conserved Ser and Thr side of β L in order to test their importance in operation of the main hinge.

Based on our present experimental data and molecular graphical analysis, the essential molecular event of domain closure may be summarised as follows: two bound substrates simultaneously direct the operation of a double molecular switch at the β -strand L, leading to substantial conformational changes of this main molecular hinge. Thus, under the synergistic action of both bound substrates their separate minor effects are enlarged resulting in a major change in the shape of β -strand L, possibly at the expense of their binding energy. This primary conformational change at β L is transmitted through the interaction of the conserved residues towards the other important hinges (e.g. termini of helix 7) and drives their operation as described earlier [29,30].

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LEGENDS to the FIGURES

Fig. 1 Comparison of the open and closed conformations PGK molecule and illustration of the hinge at β -strand L

The $C\alpha$ traces of the molecules of open conformations of the 3-PG binary complex of pig muscle (blue, [11]) and of MgADP binary complex of Bs (green, [12]) PGK-s, as well as closed conformations of MgADP*3-PG ternary complex of Tb (red, [43]) and MgAMP-PNP*3-PG ternary complex of Tm (orange, [19]) PGK-s are superposed according to the backbone atoms of the core β -sheets of the C-terminal domain. The β -strand L in all structures is highlighted by ribbons and the bound substrates (analogues) are shown by ball and stick models.

Fig. 2 Comparison of the experimental the calculated SAXS scattering curves for PGK

The experiments were carried out in the absence of substrates (1), as well as in the presence of 20 mM 3-PG (2), 10 mM ATP plus 12.5 mM $MgCl_2$ (3), 2.5 mM ADP plus 12.5 mM $MgCl_2$ (4), 20 mM 3-PG, 10 mM ATP plus 12.5 mM $MgCl_2$ (5) and 20 mM 3-PG, 2.5 mM ADP plus 12.5 mM $MgCl_2$ (6). The logarithm of the scattering intensity (I) is plotted as a function of the momentum transfer s . In (A) the successive curves are displaced down by one logarithmic unit for better visualization. Dots (with error bars): experimental data, solid lines: best fits from the crystallographic models (cf. Table 1) computed by CRY SOL [41]. In (B) comparison of the higher angle scattering from an open form (substrate-free PGK, (1)) and from a closed form (3-PG*MgATP ternary complex, (5)) is displayed. The curves (1) and (5) are representative for open and closed forms, respectively, i.e. the curves (2), (3) and (4) are similar to (1); the curves (6) is similar to (5).

Fig. 3 Mechanism of operation of the hinge at β -strand L under the synergistic action of both substrates

Molecular contacts including β -strand L and its surroundings are shown in the open conformations of the 3-PG binary complex of pig muscle (blue, [11]) (A) and of MgADP binary complex of Bs (green, [12]) (B) PGK-s, as well as in the closed conformations of MgADP*3-PG ternary complex of Tb (red, [43]) (C) and MgAMP-PNP*3-PG ternary complex of Tm (orange, [19]) (D) PGK-s. The β -strand L and the sequentially following helix 14 are highlighted by ribbons and the $C\alpha$ traces of the β -strands J and K are also shown. The bound substrates (or analogues) are shown by ball and stick models. The permanent H-bonds are shown as dashed black lines. The contacts formed upon 3-PG and

nucleotide binding are indicated by cyan and light green arrows, respectively (A, B, C, D). The H-bonds formed upon domain closure are indicated by black double-sided arrows (C, D).

Table 1

Table 1: Comparison of SAXS experimental parameters with those of the crystallographic models.

SAXS experiments			Discrepancy χ^2 between the scattering from crystallographic models and experimental data					
Investigated PGK complexes	R _g (experimental), Å ^a		Open crystal structures				Closed crystal structures	
	GNOM	Guinier	Pig PGK (no ligand)	Bs PGK MgADP binary	Pig PGK MgATP binary	Pig PGK 3-PG binary	Tm PGK ^c ternary1	Tb PGK ^c ternary2
<i>No ligand</i>	23.9±0.5	24.2±0.1	2.746	4.332	3.524	3.158	9.135	9.560
<i>Binary</i>								
3-PG	23.3±0.7	23.8±0.2	2.678	5.329	3.297	1.958	6.052	6.125
MgATP	23.2±0.6	23.4±0.2	3.855	2.848	2.409	3.389	3.179	3.910
MgADP	23.5±0.7	23.7±0.2	1.486	3.235	1.627	1.140	5.151	6.193
<i>Ternary</i>								
MgATP*3-PG	22.5±0.5	23.0±0.2	6.140	6.044	4.656	5.307	2.247	1.611
MgADP*3-PG	23.4±0.5	23.9±0.2	2.303	3.522	2.795	2.049	2.018	2.922
R _g (theoretical), Å ^b			24.25	24.34	24.02	23.97	23.26	22.64
Molecular mass, kDa ^b			43.7	43.2	43.6	43.8	43.7	45.3

^aThe experimental R_g values are computed by two alternative methods, using the program GNOM and Guinier approximation, respectively.

^b Radius of gyration and molecular mass of the high resolution models as retrieved from the PDB.

^cternary1 and ternary2 denote MgAMP-PNP*3-PG and MgADP*3-PG ternary complexes, respectively.

The minimum values of discrepancy (in bold) indicate the best correlation between SAXS data and crystallographic model. Only the ternary complexes exhibit good agreement with the closed crystal structures, while the unliganded PGK and the binary complexes better correlate with the open crystal structures. The trends of correlation are highlighted by frames.

The small variations in the calculated molecular mass are due to different numbers of residues resolved in different crystal structures. The influence of these variations was checked by deleting appropriate numbers of amino acids from more complete models. These deletions yielded only marginal impact on the discrepancy values compared to the opening and closure of the structure confirming that the small variations in mass did not influence any of the above conclusions.

Table 2

Table 2: **Lists of the atomic contacts in the main hinge region at β -stand L.** Atomic distances are given in Å-s for the most characteristic open and closed crystal structures, selected from the total investigated 10 ones. The H-bonding and ionic interactions that are formed upon substrate binding or upon domain closure are indicated in bold.

Structural elements involved	Pig muscle PGK (open)				<i>B. stearotherm.</i> PGK (open)			<i>T. maritima</i> PGK (closed)			<i>T. brucei</i> PGK (closed)		
	Atom1	Atom2	Substrate free	3-PG binary	Atom1	Atom2	MgADP binary	Atom1	Atom2	ternary	Atom1	Atom2	ternary
H1- β L	R38:NE	T393:O	5.81	4.04	R36:NE	T371:O	5.57	R36:NE	T374:O	4.40	R39:NE	T396:O	2.88
	R38:NH1	T393:O	6.12	3.53	R36:NH1	T371:O	6.73	R36:NH1	T374:O	5.19	R39:NH1	T396:O	4.64
	R38:NH2	T393:O	5.16	2.68	R36:NH2	T371:O	4.76	R36:NH2	T374:O	2.90	R39:NH2	T396:O	2.93
	R38:NE	T393:OG1	8.21	5.70	R36:NE	T371:OG1	7.52	R36:NE	T374:OG1	3.30	R39:NE	T396:OG2	5.33
3PG-H1	3-PG:O1	R38:NH1		3.52				3-PG:O1	R36:NH1	2.71	3-PG:O1	R39:NH1	2.74
	3-PG:O2	R38:NH1		4.06				3-PG:O1	R36:NH2	3.07	3-PG:O1	R39:NH2	3.21
Nucleotide – H13 and β J		T375:N			ADP: β O1	S353:N	2.89	^a A-NP: β O1	S356:N	2.92	ADP: β O1	S378:N	2.95
		T375:OG1			ADP: β O1	S353:OG	2.60	^a A-NP: β O1	S356:OG	2.42	ADP: β O1	S378:OG	2.72
		N336:OD1			ADP: β O3	N316:ND2	2.79	^a A-NP:N	N317:OD1	3.02	ADP: β O3	N338:ND2	3.04
		G373:N			ADP: β O2	G351:N	3.31	^a A-NP: β O2	G354:N	3.25	ADP: β O2	G376:N	3.42
		G373:N						^a A-NP: γ O2	G354:N	2.96			
β K-H14	G371:O	S398:OG	5.45	2.76	G349:O	S376:OG	7.01	G352:O	S379:OG	5.45	G374:O	S401:OG	6.41
β K- β J	G371:O	N336:ND2	6.55	4.21	G349:O	N316:ND2	3.04	G352:O	N317:OD1	3.39	G374:O	N338:ND2	2.72
β K- β L	I370:O	S392:N	2.97	2.90	I348:O	S370:N	2.90	V351:O	S373:N	2.65	I373:O	S395:N	2.70
	G372:N	S392:O	4.62	4.16	G350:N	S370:O	5.36	G353:N	S373:O	2.73	G375:N	S395:O	3.46
	G372:N	G394:O	4.47	4.90	G350:N	G372:O	7.20	G353:N	G375:O	4.84	G375:N	G397:O	3.24
within β L	T393:OG1	G394:N	3.30	3.37	T371:OG1	G372:N	3.31	T374:OG1	G375:N	3.32	T396:OG1	G397:N	3.42
	S392:OG	T393:N	3.51	3.10	S370:OG	T371:N	3.09	S373:OG	S374:N	3.07	S395:OG	T396:N	3.01
	S392:OG	G394:N	4.03	2.97	S370:OG	G372:N	3.42	S373:OG	G375:N	3.23	S395:OG	G397:N	2.84
H13- β L	G373:N	T393:O	6.40	6.54	G351:N	T371:O	7.75	G354:N	T374:O	3.31	G376:N	T396:O	5.10
β L-H14	S392:OG	S398:OG	4.38	4.58	S370:OG	S376:OG	2.72	S373:OG	S379:OG	5.04	S395:OG	S401:OG	3.00
	G394:O	S398:OG	3.04	2.63	G372:N	S376:OG	3.49	G375:O	S379:OG	2.99	G397:O	S401:OG	2.59

^aA-NP stands for AMP-PNP (see Abbreviations)

Figure 1
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Figure 2
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Figure 2

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