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Thermodynamic analysis of substrate induced domain closure of 3-phosphoglycerate kinase

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ABSTRACT

The energetic changes accompanying domain closure of 3-phosphoglycerate kinase, a typical hinge-bending enzyme, were assessed. Calorimetric titrations of the enzyme with each substrate, both in the absence and presence of the other one, provide information not only about the energetics of substrate binding, but of the associated conformational changes, including domain closure. Our results suggest that conformational rearrangements in the hinge generated by binding of both substrates provide the main driving force for domain closure overcoming the slightly unfavourable contact interactions between the domains.

1. Introduction

Biological recognition and the associated allosteric conformational changes are essential for the function and regulation of living systems (cf. recent review [1]). Interdomain communications operating in multidomain proteins/enzymes usually represent examples of sophisticated mechanisms, which are rarely described in detail.

Previously, we have described a detailed molecular mechanism of domain movement of a typical two-domain enzyme, 3-phosphoglycerate kinase (PGK) [2]. PGK catalyzes the phospho-transfer from 1,3-bisphosphoglycerate (1,3-BPG) to MgADP producing 3-phosphoglycerate (3-PG) and MgATP. One of the two substrates, 3-PG [3] or 1,3-BPG binds to the N-domain, while the nucleotide substrate, MgATP [4] or MgADP [5] binds to the C-domain. By comparative graphical analysis of various crystal structures [2,6] and by mutagenesis studies [7] we could identify the main hinge of PGK molecule at β-strand L in the interdomain region that essentially determines the relative domain positions. An extension of an H-bond network upon simultaneous binding of both substrates was found that possibly regulates the shape of the main hinge of β-strand L and thereby directs closure of the two domains during the catalytic cycle of PGK.

It is generally accepted that the relative domain movements are essential for functioning of multidomain proteins [8]. Domain closure can often create an optimal environment for catalysis, orients properly the substrate reactive groups and/or impedes side reactions. Typical examples of such mechanism are the kinases catalyzing transfer of a phospho-group from one substrate to another [9].

In order to better understand the event of domain closure, the thermodynamic (energetic) aspects of the motion are equally important. Isothermal titration calorimetry (ITC) is one of the most adequate methods for quantitative measurement of the free energy changes that occur upon ligand/substrate binding and during the accompanied conformational transition [10]. There are only a few examples in the literature where energetics of domain reorientation is estimated [11–13].

In this work we present ITC ligand binding studies with human PGK (hPGK). Separate binding of either of the two substrates to PGK, although causes well-defined changes in the hinge-region [3,5,14], does not cause substantial domain movement, in agreement with both crystallographic [3–5] and small angle X-ray

Abbreviations: AMP-PNP, β,γ-imido-adenosine-5′ triphosphate; 1,3-BPG, 1,3-bisphosphoglycerate; 3-PG, 3-phosphoglycerate; PGK, 3-phospho-D-glycerate kinase or ATP, 3-phospho-D-glycerate 1-phosphotransferase (EC 2.7.2.3); hPGK, human PGK; ITC, isothermal titration calorimetry; SAXS, small angle X-ray scattering

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scattering (SAXS) [2] data. Complete domain closure occurs only upon binding of both substrates. Thus, from the measured heat effects that accompany substrate binding we have attempted to estimate the energetic terms related to the conformational transition leading to the active closed form of the enzyme molecule.

2. Materials and methods

2.1. Chemicals

Na-salts of 3-PG and ADP were from Boehringer, while that of β,γ-imido-adenosine-5′ triphosphate (AMP-PNP) was from Sigma. The complexes of MgADP and MgAMP-PNP were formed by addition of $MgCl_2$ (Sigma) to ADP and AMP-PNP, respectively. The dissociation constants of MgADP and MgAMP-PNP were taken to be 0.6 [15] and 0.08 mM [16], respectively. All other chemicals were reagent grade commercial preparations.

2.2. Enzymes

The hPGK gene was overexpressed in the *Escherichia coli* BL21-CodonPlus (DE3)-RIL (Stratagene) strain, extracted by sonication and purified as described [7,17]. For the experiments, the protein was dialyzed against 50 mM Tris–HCl, 1 mM EDTA buffer (pH 7.5) containing 1 mM mercaptoethanol and stored in a frozen state at −80 °C.

2.3. Site-directed mutagenesis

Residue N336 of hPGK was mutated into alanine, and the residue T393 was deleted as described previously [7,18].

2.4. Isothermal calorimetric titration (ITC) binding studies

ITC was performed using a MicroCal VP-ITC type micro-calorimeter (MicroCal Incorporate) at 20 °C. Temperature equilibration prior to experiments was allowed for 1–2 h. All solutions were thoroughly degassed before use by stirring under vacuum. The titrating samples of the first ligand were prepared in the dialysis buffer of the protein. The titrating sample of the second ligand was prepared in a solution containing the saturating concentration of the first ligand, since in the latter case the protein sample was initially saturated with the first ligand. A typical titration experiment consisted of consecutive injections of 5 μl of the titrating ligand (approximately in 60 steps), at 3 min intervals, into the protein solution in the cell with a volume of 1.42 ml. The titration data were corrected for the small heat changes observed in control titrations of ligands into the buffer. The data were analyzed by assuming a 1:1 binding stoichiometry using the software MicroCal Origin 5.0.

3. Results and discussion

3.1. Antagonistic binding of the substrates to hPGK

Fig. 1 shows a typical titration of hPGK with 3-PG both in the absence (A) and presence (B) of the MgATP-analogue nucleotide MgAMP-PNP [16]. In all cases the best fit to the experimental data was obtained by assuming a 1:1 stoichiometry of binding, similar to previous results with MgADP and MgATP or with the analogue MgAMP-PNP [4]. Table 1 summarizes the K_d values together with the derived thermodynamic parameters of binding of 3-PG and the nucleotides MgADP or MgAMP-PNP (both separately and in the presence of each other) to the wild-type hPGK. These nucleotides were chosen to substitute the substrate MgATP in the ternary complexes, since they cannot react with 3-PG, thus one can study substrate binding without the heat effect of the enzyme reaction. It was shown that these non-functioning ternary complexes could satisfactorily mimic the functioning ternary complex of PGK*3PG*MgATP [2,19–23].

3-PG binding itself is clearly an enthalpy-driven process (Table 1), possibly due to the dominating ionic interactions in its binding [3]. However, in the presence of either MgAMP-PNP or MgADP binding of 3-PG is weakened and the contribution of the entropy-factor to its binding is increased relative to the enthalpy factor. Considering all K_d values, it is clear that in the ternary complexes binding of both 3-PG and either nucleotide is weakened compared to their separate binary complexes. This phenomenon, called sub-

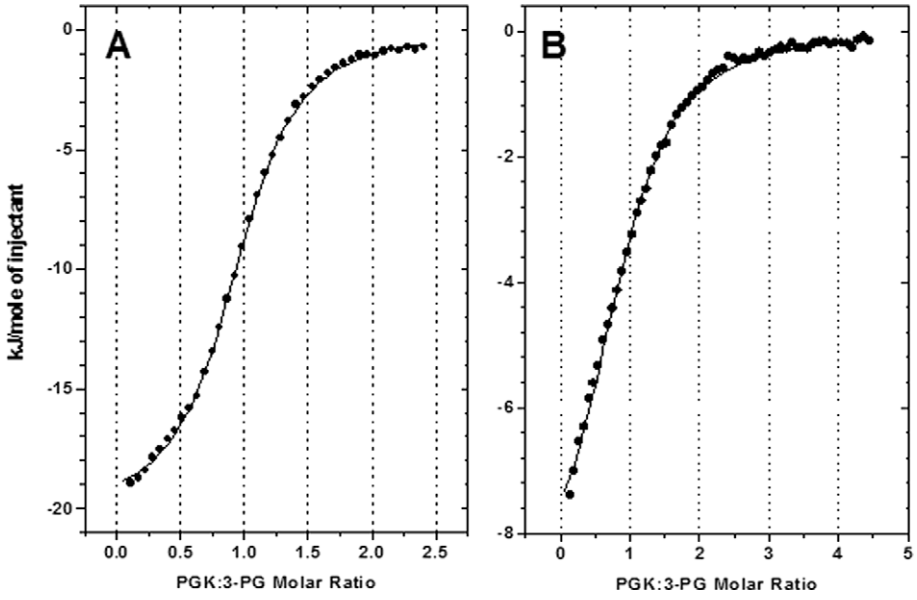


Fig. 1. Titration of wild-type hPGK with 3-PG in the absence (A) and presence (B) of MgAMP-PNP. Solutions of hPGK [in concentrations of 0.50 and 0.63 mM for (A) and (B), respectively] either in the absence (A) or in presence (B) of 10 mM AMP-PNP and 25 mM $MgCl_2$ were placed into the cell and titrated with 8 mM (A) and 12 mM (B) 3-PG. Solid lines represents the best fits of the data assuming a single binding. The dissociation constants and the derived thermodynamic data are in Table 1.

Table 1
Dissociation constants and the thermodynamic data of substrate binding to wild-type hPGK. In the various ES complexes S_1 and S_2 denote 3-PG and nucleotide (MgADP or MgAMP-PNP) ligands, respectively. The data were determined from ITC binding experiments as described in Section 2. The K_d values are given in mM, while the thermodynamic quantities are in kJ/mol. The error of the determinations of the first and the second titrations are ±4 and 6%, respectively.

Type of complex	Binary complexes			Ternary complexes			
Ligands	3-PG	MgADP	MgAMP-PNP	3-PG and MgADP		3-PG and MgAMP-PNP	
Type of titration	$E + S_1$	$E + S_2$	$E + S_2$	$ES_2 + S_1$	$ES_1 + S_2$	$ES_2 + S_1$	$ES_1 + S_2$
K_d	0.033	0.054	0.26	0.18	0.29	0.12	0.57
ΔG	−25.1	−23.9	−20.1	−21.0	−19.9	−22.0	−18.3
ΔH	−20.2	−13.3	−5.2	−7.5	−10.7	−7.5	−9.8
$T\Delta S$	4.9	10.6	14.9	13.5	9.2	14.4	8.5

strate antagonism, is a characteristic property of PGK [20–22,24]. Since each of the interacting substrates binds to separate domains, substrate antagonism can be considered as a manifestation of domain–domain interaction and its possible mechanism has been explained [20,22]. In fact, the absence of antagonism was demonstrated in the open crystalline state of PGK [20]. Thus, domain closure and substrate antagonism are related phenomena.

3.2. Estimation of the free energy requirement of the conformational transitions upon substrate binding to hPGK

Scheme 1 depicts the free energy changes accompanying binding of the first (ΔG_1 or ΔG_2) and the second (ΔG_{12} or ΔG_{21}) substrate, respectively, as derived from the experiments.

Thus, the functionally competent closed ternary complex, ES_1S_2, can be formed in two alternative ways, as expected on the basis of the random binding of the two substrates to PGK (cf. [24] and references therein). Since there are structural [3,5,14] and enzymological [4,14] evidences for occurrence of well-defined conformational changes in the hinge-region (ordering of helix 13) already in the open conformation upon binding of any of the first substrate, the respective free energy changes that accompany the first substrate binding include the pure binding energies (ΔG_1^B or ΔG_2^B) plus free energy changes of the subsequent conformational rearrangement in the hinge (ΔG_1^H and ΔG_2^H):

$$\Delta G_1 = \Delta G_1^B + \Delta G_1^H \quad (1)$$

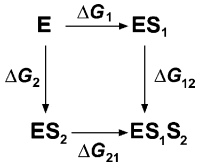
and

$$\Delta G_2 = \Delta G_2^B + \Delta G_2^H \quad (2)$$

ΔG_1^H and ΔG_2^H are supposed to be negative to provide a driving force for the structural transformation of the hinge. Substrates bind to separate domains of PGK. Therefore, we can assume that binding of the first substrate does not directly influence binding of the second one, and in fact, the induced conformational changes in the hinge are additive [3,5,14]. Thus, the respective free energy changes that accompany binding of the second substrate include ΔG_2 or ΔG_1 (as defined by Eqs. (1) and (2)) plus the free energy changes that originate from the contact formation between the two domains upon closure ΔG_2^C and ΔG_1^C:

$$\Delta G_{12} = \Delta G_2 + \Delta G_2^C \quad (3)$$

and



Scheme 1.

$$\Delta G_{21} = \Delta G_1 + \Delta G_1^C \quad (4)$$

By replacing Eqs. (1) and (2) into Eqs. (3) and (4), we obtain:

$$\Delta G_{12} = \Delta G_2^B + \Delta G_2^H + \Delta G_2^C \quad (5)$$

and

$$\Delta G_{21} = \Delta G_1^B + \Delta G_1^H + \Delta G_1^C \quad (6)$$

According to the thermodynamic laws the energy balance of either of the two ways from the open substrate free E form to the closed ternary complex ES_1S_2 should be the same:

$$\Delta G_1 + \Delta G_{12} = \Delta G_2 + \Delta G_{21} \quad (7)$$

or in detail:

$$\Delta G_1^B + \Delta G_1^H + \Delta G_2^B + \Delta G_2^H + \Delta G_2^C = \Delta G_2^B + \Delta G_2^H + \Delta G_1^B + \Delta G_1^H + \Delta G_1^C \quad (8)$$

Thus, it follows:

$$\Delta G_1^C = \Delta G_2^C = \Delta G^C \quad (9)$$

where ΔG^C is the free energy change associated with domain–domain interaction formed upon domain closure.

Although the complete energy requirement of the open-closed transition is the sum of $\Delta G_1^H + \Delta G_2^H + \Delta G^C$, from the data only ΔG^C, characteristic of the closure itself, can be calculated:

$$\Delta G^C = \Delta G_{12} - \Delta G_2 \quad \text{and}$$
$$\Delta G^C = \Delta G_{21} - \Delta G_1 \quad (\text{cf. Eqs. (3) and (4)}).$$

Table 2 summarizes the results with wild-type hPGK for both kinds of ternary complexes. In all cases, ΔG^C is a relatively low, but positive value (around 4 kJ/mole), comparable to the energy of a single H-bond, which is estimated to be 5.4 kJ/mole [25]. Accordingly, the contact energy of domain interactions in the

Table 2
Changes in the free energy (in kJ/mole) that accompany formation of the ternary complexes in cases of wild-type PGK and its mutants, N336A and T393del. In the ES complexes S_1 and S_2 denote 3-PG and nucleotide (MgADP or MgAMP-PNP) ligands, respectively. The error of ΔG^C is about ±70%.

Nature of ΔG		Wild-type hPGK		S_2: MgADP	
		S_2: MgADP	S_2: MgAMP-PNP	N336A	T393del
Formation of the ternary $E*S_1*S_2$ complexes	$\Delta G_1 + \Delta G_{12}$[a]	45.0	43.4	41.4	46.9
	$\Delta G_2 + \Delta G_{21}$[a]	44.9	42.1	41.2	45.8
ΔG^C	$\Delta G_{21} - \Delta G_1$[b]	4.0	3.1	3.2	1.7
	$\Delta G_{12} - \Delta G_2$[b]	4.1	1.8	3.0	0.6

[a] See Eq. (7).
[b] See Eqs. (3) and (4).

closed form is slightly unfavourable. The small energy input of ΔG^C required by formation of domain–domain interactions is covered by the energy release (ΔG_1^H or ΔG_2^H) during completion of the conformational rearrangement of the hinge. Otherwise, this low positive value of ΔG^C is consistent with the antagonistic substrate binding: the unfavourable domain interactions always weaken the binding of the second substrate. The small value of ΔG^C assures relatively easy domain movements during catalysis. This picture is entirely consistent with the conclusion of transient kinetic measurements that domain closure is possibly not the rate limiting step on the kinetic pathway of PGK [26].

3.3. Test of the restriction or absence of domain closure with mutant hPGKs

In order to test further the energetics of domain movements, we have carried out similar experiments with two single site mutant hPGKs exhibiting reduced (N336A) [18] and completely abolished (T393del) [7] domain closure abilities. Typical titration experiments with T393del are illustrated in Fig. 2. The thermodynamic parameters are summarized in Table 3. The substrate binding abilities of these mutants (as expressed in K_d values) are hardly changed, but the substrate antagonism, characteristics of the wild-type enzyme, is restricted (N336A) or completely abolished (T393del). These findings further support the view that domain closure and substrate antagonism are closely related phenomena for PGK.

If we calculate a value of ΔG^C from the binding data of these mutants, we obtain low values, close to zero (cf. Table 2), reflecting diminishing domain interactions. These data are consistent with reduction of domain motion of N336A mutant [18] and with its complete prevention upon perturbing the shape of β-stand L by deleting T393 [7].

3.4. General considerations

From the very scarce data available in the literature about the energy requirement of domain closure it is difficult to reach generalization. In case of creatine kinase a value of $\Delta G = -38.2$ kJ/mole, was attributed to domain closure as derived from ITC studies, however, in contrast to PGK, binding of ATP and Mg^{2+} is noted to be synergistic [11]. For maltose-binding protein using combination of sophisticated experimental approaches, NMR, fluorescence spectroscopy and mutational studies a similar value, but with opposite sign, $\Delta G = +30$ kJ/mole, was obtained [12]. Furthermore, from a computer simulation study with the glutamate receptor ligand binding domain an about $\Delta G = +18$ kJ/mole was estimated for the open-closed transition [27]. The slightly positive ΔG^C obtained in this study also indicate that domain interactions alone do not favour the closed form. Instead, the additive conformational changes in the hinge upon binding of both substrates appear to provide the driving force for domain closure. The variation of the available data suggests substantially different mechanism of

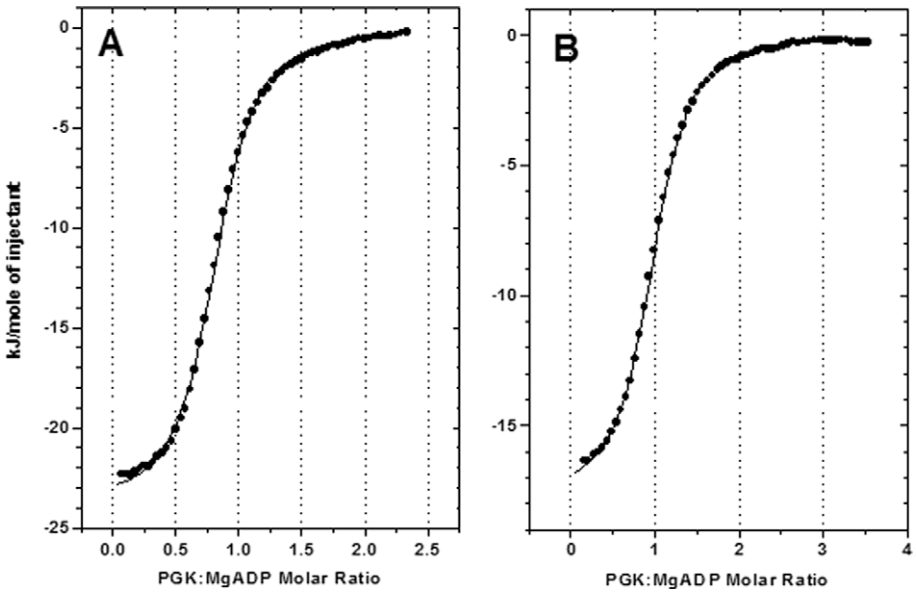


Fig. 2. Titration of T393del mutant with MgADP in the absence (A) and presence (B) of 3-PG. Titrations of T393del hPGK (in concentration of 0.8 mM) were carried out with 8 mM MgADP (formed from 8 mM ADP and 15 mM $MgCl_2$) in the absence (A) and in presence of 10 mM 3-PG (B). In both cases 15 mM $MgCl_2$ was added in advance to the protein solution placed into the calorimeter cell. The best fits of the data to the model of single binding site are shown by solid lines. The dissociation constants and the obtained thermodynamic data are in Table 3.

Table 3
Dissociation constants and the thermodynamic data of substrate binding to N336A and T393del mutant hPGKs. In the ES complexes S_1 and S_2 denote 3-PG and MgADP, respectively. The data were determined from ITC binding experiments as described in Section 2. The K_d values are given in mM and the thermodynamic quantities are in kJ/mol. The error of the determinations of the first and the second titrations are ±4 and 6%, respectively.

Mutant forms	N336A				T393del			
Type of titration	$E + S_1$	$ES_2 + S_1$	$E + S_2$	$ES_1 + S_2$	$E + S_1$	$ES_2 + S_1$	$E + S_2$	$ES_1 + S_2$
K_d	0.065	0.248	0.18	0.66	0.14	0.28	0.025	0.032
ΔG	−23.5	−20.3	−20.9	−17.9	−21.7	−20.0	−25.8	−25.2
ΔH	−10.0	−6.6	−4.1	−12.9	−37.7	−23.0	−23.5	−17.5
$T\Delta S$	13.5	13.6	16.9	5.0	−16.0	−3.1	2.3	7.8

domain motions for the investigated proteins. In order to draw more general conclusions about energetics of domain closure further experimental data with other multidomain proteins are required.

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