Stability Domains, Substrate-induced Conformational Changes, and Hinge-bending Motions in a Psychrophilic Phosphoglycerate Kinase

A MICROCALORIMETRIC STUDY

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The cold-active phosphoglycerate kinase from the Antarctic bacterium Pseudomonas sp. TACII18 exhibits two distinct stability domains in the free, open conformation. It is shown that these stability domains do not match the structural N- and C-domains as the heat-stable domain corresponds to about 80 residues of the C-domain, including the nucleotide binding site, whereas the remaining of the protein contributes to the main heat-labile domain. This was demonstrated by spectroscopic and microcalorimetric analyses of the native enzyme, of its mutants, and of the isolated recombinant structural domains. It is proposed that the heat-stable domain provides a compact structure improving the binding affinity of the nucleotide, therefore increasing the catalytic efficiency at low temperatures. Upon substrate binding, the enzyme adopts a uniformly more stable closed conformation. Substrate-induced stability changes suggest that the free energy of ligand binding is converted into an increased conformational stability used to drive the hinge-bending motions and domain closure.

Phosphoglycerate kinase (PGK2; EC 2.7.2.3) catalyzes the reversible high energy phosphoryl transfer from 1,3-diphospho-D-glycerate to Mg2++-ADP to yield Mg2++-ATP and 3-phospho-D-glycerate (3-PGA) in the glycolytic pathway. Besides its key metabolic function in living organisms, PGK has been widely used as a model enzyme to study domains in proteins and their relative motions (1, 2). Indeed, the structure of PGK consists of two globular domains of nearly equal size, referred to as the N- and C-terminal domains, linked by a hinge helix. In this structure, the C terminus of the protein crosses back the hinge region and folds into the N-domain, bringing the N- and C-extremities in close proximity where they generally interact via ion pairs (3, 4). The triose substrate binds to a basic patch in the N-domain (5), whereas the nucleotide substrate is bound to the C-domain (6). Crystalllographic studies have shown that the free or single substrate-liganded forms adopt an open conformation in which the distance between the substrates is too long to permit catalysis, probably to avoid the futile hydrolysis of 1,3-PGA or ATP (7). By contrast, the binding of both substrates induces a closed conformation resulting from hinge-bending motions of 32° at the level of the hinge helices and leading to domain closure into a catalytically competent conformation with well aligned substrates for phosphoryl transfer (7, 8).

The stability and folding of yeast PGK has been extensively investigated and deconvolution of its single DSC heat absorption peak suggests the occurrence of two overlapping transitions that have been tentatively assigned to the N- and C-domains (9). Furthermore, folding studies using guanidinium chloride as denaturation agent have suggested that the C-domain is the most stable domain, although contradicting results have been reported (9–13). In this context, the PGK from the Antarctic bacterium Pseudomonas sp. TACII18 (PsPGK) represents a unique model to study stability domains in PGK. The high level of amino acid sequence similarity of PsPGK with phosphoglycerate kinases from other species has allowed the building of a homology model based on the available crystal structures (14). Furthermore, crystals of PsPGK have been obtained (15), and its structure solved at 2.1 Å resolution indeed reveals a general fold identical to that of other monomeric PGKs. However, the preliminary characterization of PsPGK by DSC (14) has shown that it possesses two well defined transitions in the unliganded conformation (see also Fig. 1). It follows that the enzyme contains two types of structures or regions differing by their intrinsic stability and responsible for the occurrence of a heat-labile domain (melting point (Tm) ~50 °C) and a heat-stable domain (Tm ~60 °C) when thermal unfolding is recorded by DSC. Such stability domains within the protein may or may not correspond to the structural N- and C-domains. One cannot exclude for instance that each structural domain contains both heat-labile and heat-stable structures that unfold sequentially. We report here the unusual stability pattern of this enzyme and the complex effects of substrate binding inducing hinge-bending motions. The results have several implications in folding studies of PGK and of proteins in general as well as in the field of enzyme adaptations to extreme temperatures.

EXPERIMENTAL PROCEDURES

Mutagenesis of PsPGK—The pgk gene from the Antarctic bacterium Pseudomonas sp. TACII18 has been cloned in the expression vector pET22b as previously described (14). Genetic engineering was performed on the gene or on gene fragments inserted in the plasmid pSP73. Mutations were introduced by polymerase chain reaction as described (16). The mutation Trp305 → Phe was introduced by changing the codon TGG into TTC on the gene fragment generated by SalI-PstI digestion. The mutation Phe135 → Trp was introduced by changing the codon TTT into TGG. Deletion of domain coding regions was carried out by inverse PCR using silent primers, 24 nucleotides in length, starting in 5’ at codon for Thr1 (reverse primer) and for Ala172 (forward primer) for the

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2 The abbreviations used are: PsPGK, phosphoglycerate kinase from Pseudomonas sp. TACII18; DSC, differential scanning calorimetry; 3-PGA, 3-phospho-D-glycerate; AMP-pNP, 5′-adenylylimidodiphosphate; Mops, 3-(N-morpholino)propanesulfonic acid.

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deletion of the N-domain and starting in 5’ at codon for Leu<sup>170</sup> (reverse primer) and at the stop codon (forward primer) for the deletion of the C-domain. The amplification products were purified (QIAquick PCR purification kit, Qiagen), phosphorylated by T4 polynucleotide kinase and circularized by ligation before transformation. The sequence of these constructions was checked by double-strand sequencing on an ALF DNA sequencer Express II (Amersham Biosciences) and transferred to the expression vector using appropriate restriction sites.

**Protein Expression and Purification**—The recombinant proteins were expressed in *Escherichia coli* BL21(DE3) grown at 18 °C as described previously (14), except that Terrific broth was used for cultivation and induction with 0.5 mM 3-(1-pyridinio)-1-propanesulfonate to delay expression and induction with 0.5 mM isopropyl 1-thio-

**Analytical Procedures**—N-terminal amino acid sequences were determined using a pulsed liquid-phase protein sequencer (Procise 492, Applied Biosystems, PerkinElmer Division) fitted with an on-line phenylthiohydantoin analyzer. Electrospray mass spectrometry of the proteins (5–10 μg) was performed on a Q-TOF Ultima mass spectrometer (Micromass). Absorbance spectra were recorded on an Uvikon XS spectrophotometer (Bio-Tek Instruments). Fluorescence spectra were recorded on an SLM-Aminco model 8100 spectrofluorimeter (Spectronic Instruments). Circular dichroism spectra were acquired under constant nitrogen flush using a J-810 spectropolarimeter (Jasco Corp.) in a 0.2-cm path length cell for the far-UV region. All spectrophotometric analyses were performed at 20 °C.

**Differential Scanning Calorimetry**—Measurements were performed using a MicroCal MCS-DSC instrument as detailed (17). Samples (~3 mg/ml) were dialyzed overnight against 30 mM Mops, pH 7.5. Thermograms of the isolated domains and for reversibility tests were recorded in the presence of 0.5 mM 3-(1-pyridinio)-1-propanesulfonate to delay aggregation. Thermograms were analyzed according to a non-two-state model in which the \( T_m \) the calorimetric enthalpy (\( \Delta H_{cal} \)), and the van’t Hoff enthalpy (\( \Delta H_{vH} \)) of individual transitions are fitted independently using the MicroCal Origin software (version 2.9). The magnitude and source of the errors in the \( T_m \) and enthalpy values have been discussed elsewhere (18). Fitting standard errors on a series of three DSC measurements made under the same conditions in the present study were found to be ±0.1 K on \( T_m \) and ±1% on both enthalpies. Fitting standard errors on four batches of purified PsPGK were found to be ±1 K on \( T_m \) and ±10% on both enthalpies.

**RESULTS**

**General Properties**—The thermogram of PsPGK (Fig. 1a) shows that its heat-induced unfolding is characterized by two distinct heat absorption peaks separated by a temperature interval of about 10 °C (TABLE ONE) and henceforward referred to as the heat-labile (\( T_m = 49.8 °C \)) and the heat-stable (\( T_m = 60.6 °C \)) transitions (with \( T_m \), the melting point at the top of the transition). In our experimental conditions, no aggregation occurred in the heated sample and a stable post-transition baseline was recorded allowing the calculation of a progress baseline accounting for both transitions (Fig. 1a). On this basis, an estimation of the heat capacity difference between the unfolded and the native states can be made for the whole molecule (\( \Delta C_p = 7.47 \text{ kcal mol}^{-1} \text{ K}^{-1} \)) and the heat-labile (\( \Delta C_p = 5.93 \text{ kcal mol}^{-1} \text{ K}^{-1} \)) and the heat-stable (\( \Delta C_p = 1.54 \text{ kcal mol}^{-1} \text{ K}^{-1} \)) transitions. A \( \Delta C_p \) value of 7.5 kcal mol<sup>-1</sup> K<sup>-1</sup> has been reported for yeast PGK (11), and the specific value of 19.3 cal/mol residue for PsPGK (386 amino acid residues) is in perfect agreement with data reported for large proteins (17). As far as heat absorption during unfolding is concerned, the normalized surface \( \Delta H_{cal, corr} \) corre-

**TABLE ONE**

| Protein and ligand | \( T_m^1 \) | \( \Delta H_{cal1} \) | \( \Delta H_{vH1} \) | \( T_m^2 \) | \( \Delta H_{cal2} \) | \( \Delta H_{vH2} \) | \( \Delta H_{cal} \) |
|------------------|----------------|------------------|------------------|----------------|----------------|------------------|------------------|
| Free PsPGK       | 49.8           | 160              | 98               | 60.6           | 57             | 147              | 217              |
| + 5 mM ADP       | 54.4           | 165              | 102              | 60.9           | 63             | 139              | 226              |
| + 5 mM 3-PGA     | 55.1           | 161              | 112              | 60.8           | 53             | 158              | 212              |
| + 5 mM Mg<sup>2+</sup>-ADP, 3-PGA (closed) | 60.5 | 220              | 250              | 219 |
| + 40 mM Na<sub>2</sub>SO<sub>4</sub>      | 56.3           | 228              | 154              | 222 |
| Free N-domain    | 51.6           | 11               | 6                | 11             |                |                  |                  |
| Free C-domain    | 58.3           | 48               | 141              | 48             |                |                  |                  |
| + 5 mM Mg<sup>2+</sup>-ADP     | 60.4           | 96               | 152              | 99             |
sponding to all enthalpy-driven interactions disrupted during unfolding) below the heat-labile transition is nearly twice that of the heat-stable transition (Fig. 1b; TABLE ONE), indicating that a large fraction of the native PsPGK conformation has a low stability. As shown in Fig. 2, thermal unfolding of the heat-labile transition is fully reversible. In addition, the $\Delta H_{cal}/\Delta H_{vH}$ ratio for this transition (where $\Delta H_{vH}$ is the van’t Hoff enthalpy calculated from the slope of the transition) is $\approx 1.5$, indicating that the heat-labile transition may be composed by more than one cooperative unit, as confirmed below. By contrast, thermal unfolding of the heat-stable transition is not reversible and, as a result, the $\Delta H_{cal}/\Delta H_{vH}$ ratio decreases to $\approx 0.35$. It should be noted that unfolding of the latter transition drastically reduces the reversibility of the heat-labile transition (Fig. 2). This suggests that the protein regions involved in both transitions are structurally connected. Addition of 5 mM dithiothreitol does not improve the reversibility, indicating that oxidation of the three free cysteines (Cys$^{56}$, Cys$^{137}$, and Cys$^{207}$) is not a major determinant of the irreversible unfolding.

**Mutagenesis Study of the Structural Domains—**PsPGK contains one single Trp residue at position 305 in the C-domain. As shown by heat-induced unfolding monitored by fluorescence (excitation wavelength: 295 nm), this Trp is quenched in its microenvironment. Indeed, unfolding results in fluorescence increase (Fig. 1c) and the appearance of a hyperfluorescent intermediate. The temperature of maximal dequenching closely corresponds to the melting point of the heat-labile transition observed by DSC. The mutation Trp$^{305}$ → Phe totally abolishes the dequenching transition (data not shown) therefore ascertaining the assignment of the fluorescence signal to Trp$^{305}$. Interestingly, the DSC thermogram of this mutant displays an altered heat-labile transition (Fig. 3), as indicated by the decrease of $T_m$ (−5 °C) and of $\Delta H_{cal}$ (−30%), whereas the heat-stable transition remains almost unaffected. One can conclude that the C-domain mainly contributes to the heat-labile transition. On the other hand, the mutation Phe$^{135}$ → Trp has been introduced in the N-domain and the DSC trace of this mutant (Fig. 3) also exhibits a drastic destabilization of the heat-labile transition ($T_m$ = 8 °C, $\Delta H_{cal}$ = −50%). These results indicate that both N and C structural domains of PsPGK contribute to the heat-labile DSC transition. Accordingly, each DSC transition of PsPGK cannot be assigned to the unfolding of a specific structural domain. One can also note a slight increase of the $T_m$ for the heat-stable transition in both mutants. This can be tentatively related to the relaxation of weak interactions between interacting domains in the protein when one of these domains is destabilized (9, 11).

When both mutations are combined in the double mutant Phe$^{135}$ → Trp/Trp$^{305}$ → Phe, it is expressed and purified as an inactive and partly folded protein, as demonstrated by the weak DSC heat
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absorption peak (Fig. 3, inset). It was found that after addition of 20 mM sulfate ions, the double mutant recovered 20% of the wild-type PsPGK activity. In the presence of sulfate, the DSC thermogram of the mutant exhibits two slight transitions (Fig. 3, inset). This indicates that the sulfate ion behaves like a folding effector of PsPGK.

The Isolated N and C Structural Domains—To further characterize the contribution of the PsPGK structural domains in both DSC transitions, the isolated N- and C-domains were expressed in E. coli from the pgk gene truncated in the middle of the first hinge helix. The plasmid construct coding for the N-domain contained the nucleotide sequence from Met-Thr1 to Leu170 (mature protein numbering; 18,107.9 Da for 170 aa), whereas the sequence for Met-Thr1–Ala172 to Ala386 was used for the C-domain (21,996.3 Da for 216 aa). It should be recalled that because the C-terminal end of PGK folds into the N-domain, truncation for the C-domain (21,999 Da) for 216 aa) results in the expression of an N-domain devoid of 15 residues that remain attached to the recombinant C-domain. N-terminal amino acid sequencing and mass spectrometry analyses (N-domain: 18,107 Da; C-domain: 21,999 Da) indicated that the initiating formylmethionine has been removed and that no other post-translational modification occurred during the expression and purification processes.

The UV absorbance spectra well reflect the aromatic amino acid content of the analyzed proteins. The N-domain (six Phe, three Tyr) lacking Trp has a low absorption coefficient (ε_{280 nm} = 3,960 M^{-1} cm^{-1}) and its spectrum is dominated by Tyr residues, with three slight shoulders from Phe between 260 and 270 nm (Fig. 4a). Having an additional Trp residue, the absorption coefficient of the C-domain (nine Phe, three Tyr, one Trp) is much higher (ε_{280 nm} = 9,530 M^{-1} cm^{-1}). An equimolar mix of both N- and C-domains restores the absorbance value of the native PsPGK but the imperfect superimposition of the spectra indicates slight differences in the aromatic microenvironment within the recombinant domains. The fluorescence emission spectrum of PsPGK (Fig. 4b) is poorly defined as a result of the above-mentioned quenching of Trp305. Similarly, the N-domain fluorescence spectrum is dominated by Tyr residues. However, the pronounced red shift of the C-domain fluorescence spectrum shows that Trp 305 is dequenched and therefore, this residue is not in its native microenvironment in the recombinant domain. It follows that the equimolar mix of both domains restores the maximal fluorescence intensity of the native PsPGK but at a higher emission wavelength. The far-UV circular dichroism spectrum of the native PsPGK (Fig. 4c) displays two strong ellipticity minima at 208 and 222 nm indicating a high content of secondary structures. The spectra of both isolated N- and C-domains also present these minima but at a weaker ellipticity, especially for the N-domain. As a result, the equimolar mix of both domains reveals a lower secondary structure content as compared with the native enzyme. Finally, the near-UV CD spectra indicate an asymmetric environment of aromatic residues (and possibly of cysteines) typical of a folded conformation in the three proteins and the equimolar mix (Fig. 4d). From these spectrophotometric analyses, it can be concluded that the isolated N- and C-domains possess well organized secondary structures and a defined tertiary structure which however differ from the native state of PsPGK.

Nevertheless, the DSC thermograms of these isolated domains reveal their individual contribution to the biphasic profile of the native PsPGK (Fig. 5). The recombinant C-domain unfolds at a temperature closely corresponding to the heat-stable transition and accounts for about 80% of heat absorption (ΔH_{cal} = 48 kcal mol^{-1}). By contrast, the N-domain is strongly destabilized and only accounts for less than 10% (ΔH_{cal} = 11 kcal mol^{-1}) of the heat-labile transition. It seems therefore that only the C-domain contributes to the heat-stable DSC transition, whereas the structures of both domains responsible for the heat-labile DSC transition have not a compact conformation in the recombinant fragments. The spectroscopic and calorimetric data therefore indicate that the heat-labile structures in the recombinant domains tend to have molten globule-like properties, i.e. a defined but non-compact conformation with a low intrinsic conformational stability (Figs. 4 and 5). This contrasts with the well folded isolated domains obtained from more stable PGKs (10, 19, 20).

Interestingly, the isolated C-domain still binds the substrate Mg^{2+}-ADP (Fig. 5, inset, and TABLE ONE), as revealed by the large (ΔH_{cal} + 50
Substrate-induced Stability Changes—Crystallographic studies have shown that the PGK from *Trypanosoma brucei* can be trapped in the closed, active conformation (7) upon incubation with one substrate (Mg\(^{2+}\)-ADP) and one product (3-PGA). In these conditions, the DSC thermogram of *Ps* PGK (Fig. 6) reveals that substrate binding and the resulting hinge-bending motions drive the free (open) *Ps* PGK into a closed, compact and uniformly more stable conformation, as indicated by the single sharp transition at a higher \(T_m\) (+2 °C). The \(\Delta H_{cal}/\Delta H_{rot}\) ratio of ~0.9 also indicates that unfolding of the closed conformation is cooperative and approaches a two-state mechanism which however is poorly reversible (~6%; data not shown). The nonhydrolyzable nucleotide analog AMP-pnp has been used to study hinge bending but the crystal structures of the porcine 3-PGA-AMP-pnp (21) and the yeast 3-PGA-AMP-pnp (22) complexes do not adopt a closed conformation. As a matter of fact, the thermogram of *Ps* PGK incubated with Mg\(^{2+}\) AMP-pnp and 3-PGA (Fig. 6) is indicative of a less compact and stable conformation (estimated from the sharpness and \(T_m\) of the transition, respectively) than that recorded for the closed conformation. It should also be noted that in the absence of Mg\(^{2+}\), the conformational changes promoted by ADP and 3-PGA are less pronounced (Fig. 6), underlining that the true physiological substrate is the complex Mg\(^{2+}\)-ADP.

Crystallographic studies have shown that the individual substrates do not induce the closed conformation (5, 6). However, the DSC thermograms of *Ps* PGK incubated with one substrate (Fig. 6) display drastic alterations of both the heat-labile and heat-stable transitions, revealing significant modifications of the structure energetics. Finally, the DSC traces of *Ps* PGK in the presence of Mg\(^{2+}\)-ADP and of Mg\(^{2+}\)-ATP are markedly different suggesting that upon phosphoryl transfer to ATP, the enzyme undergoes a noticeable conformational change. It should be noted that in the absence of Mg\(^{2+}\), the conformational changes promoted by ADP and 3-PGA are less pronounced (Fig. 6), underlining that the true physiological substrate is the complex Mg\(^{2+}\)-ADP.

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![FIGURE 5. Normalized thermograms of *Ps* PGK (thick line) and of its isolated N- and C-domains. Inset, thermograms of the unliganded (thin line) and of the substrate-bound (thick line) isolated C-domain.](https://example.com/figure5)

![FIGURE 6. Substrate-induced stability changes of *Ps* PGK. Normalized thermograms of the free enzyme (thick lines) and of the substrate-bound enzyme (thin lines). The concentration of each substrate was 5 mM.](https://example.com/figure6)
noted that all these substrate-induced stability changes only affect the
$T_m$ values and the unfolding cooperativity of the transitions. Indeed, as
shown in TABLE ONE, there is no significant variation of the surface of
the transitions ($\Delta H_{cal}$) for the binary and ternary $P_s$PGK complexes,
whereas the sharpness ($\Delta H_{vH}$) and $T_m$ of the transitions are modified.

Substrate and Ion Binding—The available crystal structures of binary
and ternary PGK complexes and a multiple sequence alignment allowed
to identify the highly conserved residues of $P_s$PGK involved in substrate
and ion binding. 3-PGA binds to the N-terminal domain (residues
Asp$^{20}$, Asn$^{22}$, Arg$^{25}$, His$^{38}$, Arg$^{41}$, Gly$^{44}$, Arg$^{45}$, Gly$^{362}$, Gly$^{363}$,
and Ala$^{364}$) in the so-called basic patch, whereas ADP binds to the
C-terminal domain (residues Ser$^{191}$, Lys$^{196}$, Ile$^{282}$, Leu$^{283}$, Asn$^{306}$,
Glu$^{313}$, Gly$^{340}$, and Thr$^{342}$). Fig. 7 shows the effect of increasing concen-
trations of both substrates on the DSC thermograms of $P_s$PGK. It can be
seen that upon 3-PGA binding, the $T_m$ of the heat labile transition is
shifted to higher values whereas the heat-stable transition remains unaffected. This confirms that the structural N-domain bearing the 3-PGA binding site belongs to the heat-labile DSC transition. On the other hand, upon ADP binding the stability of the heat-labile transition is improved, but in this case, the surface ($\Delta H_{cal}$) of the heat-stable transition is also increased. This again confirms that the structural C-domain bearing the ADP binding site contributes to both the heat-
labile and heat-stable DSC transitions. Interestingly, a plot of the $T_m$
for the heat-labile transition as a function of the substrate concen-
tration can be fitted to a binding isotherm (Fig. 7, insets) providing
apparent $K_d$ values of 0.51 mM for 3-PGA and 0.38 mM for ADP. These
values are in agreement with the $K_m$ values previously determined by
standard kinetic methods (14).

The above-mentioned experiments have been performed in the
absence of Mg$^{2+}$. This ion was shown to interact with an acidic side
chain of the ADP binding site (7), corresponding to Asp$^{341}$ in the C-
domain of $P_s$PGK. Fig. 8 shows that increasing concentrations of MgCl$_2$
only affect the heat-stable DSC transition by decreasing its $T_m$. At
MgCl$_2$ concentrations higher than 2 mM, the heat-stable transition
tends to fuse with the heat-labile DSC transition that remains unaltered
but sample precipitation precluded further analysis of this phenome-
non. These results clearly demonstrate that the Mg$^{2+}$-binding site
belongs to the heat-stable DSC transition. Finally, the sulfate ion can be
viewed as a test case for our assignment of the DSC transitions because
it has been reported to bind both to the N- (Arg$^{61}$) and C- (Lys$^{195}$)
domains (23). And indeed, increasing Na₂SO₄ concentrations affect both the heat-labile and heat-stable DSC transitions (Fig. 8), which fuse in a conformation mimicking the substrate-induced closed conformation, however, at a lower Tₘ.

**DISCUSSION**

**Stability and Structural Domains**—Several multidomain proteins display distinct stability domains resulting in multiphasic DSC thermograms (24). In some cases, a close correlation was found between structural and stability domains, i.e. each DSC transition can be assigned to the unfolding of a specific structural domain (25–29). However, PsPGK clearly demonstrates that such correlation is not always valid as, in this case, we have shown that the heat-stable DSC domain belongs to the structural C-domain, whereas both N and C structural domains contribute to the main heat-labile DSC domain. The size of the heat-stable domain in PsPGK can be estimated to 100 residues in a first approximation, assuming that all 386 residues contribute equally to ΔHₛ of the DSC transitions (TABLE ONE). A more precise estimation of 80 residues can be based on ΔCₚ of the transitions (5.93 and 1.54 kcal mol⁻¹ K⁻¹ for the first and second transitions, respectively) because only discrete modifications have been reported between the crystal structures of free and single substrate-bound PGKs (5, 6). The behavior of the isolated C-domain provides useful insights into the energetics of hinge-bending motions in PsPGK. Indeed, Fig. 5 (inset) and TABLE ONE show a drastic increase of ΔHₛ and of Tₘ for this isolated domain upon Mg²⁺-ADP binding. An estimation of the conformational stability in the free and substrate-bound states can be obtained using Equation 1.

\[
ΔG(T) = ΔHₛ(1 - T/Tₘ) + ΔCₚ(T - Tₘ) - TΔCₚ ln T/Tₘ \quad \text{(Eq. 1)}
\]

The validity of this approximation has been discussed elsewhere (30–32). The computed stability curves indicate an increase of the conformational stability (free energy of unfolding) of 5.5 kcal mol⁻¹ upon substrate binding. On the other hand, the free energy of substrate binding can be estimated to −5 kcal mol⁻¹ using the Kₐ value for the nucleotide (0.21 mM) and the following relation (Equation 2).

\[
ΔGₚ = -RT ln Kₐ \quad \text{(Eq. 2)}
\]

Comparison of both free energy estimates strongly suggests that the nucleotide binding free energy is transferred into conformational stability. However, such behavior of the heat-stable transition is not observed in the native PsPGK upon ADP (Fig. 7) and Mg²⁺ (Fig. 8) binding. One can therefore propose that in the native enzyme, the gain in conformational stability arising from substrate binding is now converted into the energy required for hinge-bending motions and domain closure. The lack of ΔΔHₛ difference between the free and closed forms of PsPGK (TABLE ONE) also supports this view. Finally, in a thoughtful study of PGK activation, the authors concluded that it cannot be decided whether anion activation is a consequence of domain closure or anion binding itself mediates the closure by connecting the two domains (23). From our results, it seems that the latter hypothesis is valid, as demonstrated by the apparent domain closure induced by the sulfate anion activator (Fig. 8).

**Activity at Low Temperatures**—The unusual stability pattern of PsPGK should also be considered in the context of the enzyme adaptations to catalysis at low temperatures. In order to catalyze the essential glycolytic step at temperatures close to 0°C found in the Antarctic environment, this enzyme possesses a high specific activity to compensate for the reduction of chemical reaction rates at such low temperatures. In addition, PsPGK displays a high affinity for the nucleotide, therefore improving the kₐ/Kₐ ratio which is the relevant parameter for intracellular enzymes (14). It has been argued that the high activity of cold-adapted enzymes is gained through an improved flexibility or mobility of the active site and of the structures bearing the active site residues in order to facilitate molecular motions in low thermal energy conditions (32–34). In the case of PsPGK, the active site is formed by the
interface of the N- and C-domains that have to move to bring the substrates in close proximity. Accordingly, the predominant weak stability of this enzyme can enhance the substrate-induced conformational changes (Figs. 6 and 7) and the resulting hinge-bending motions at low temperatures. It has also been suggested that during the course of evolution, some psychrophilic enzymes have reached a state close to the lowest stability compatible with the native state (17, 33, 35). This stability arising from the active site mobility (33, 36), whereas some psychrophilic enzymes have a reduced affinity arising from the active site mobility (33, 36), whereas PsPGK has a better affinity for the nucleotide. One can therefore propose that the heat-stable domain of PsPGK provides a more compact and structured interface of the N- and C-domains that have to move to bring the substrate-induced conformational changes (Figs. 6 and 7) and the resulting hinge-bending motions at low temperatures, not previously observed in other psychrophilic enzymes.

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