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ORIGINAL PAPER

Structural determinants increasing flexibility confer cold adaptation in psychrophilic phosphoglycerate kinase

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Abstract

Crystal structures of phosphoglycerate kinase (PGK) from the psychrophile *Pseudomonas sp*. TACII 18 have been determined at high resolution by X-ray crystallography methods and compared with mesophilic, thermophilic and hyperthermophilic counterparts. PGK is a two-domain enzyme undergoing large domain movements to catalyze the production of ATP from 1,3-biphosphoglycerate and ADP. Whereas the conformational dynamics sustaining the catalytic mechanism of this hinge-bending enzyme now seems rather clear, the determinants which underlie high catalytic efficiency at low temperatures of this psychrophilic PGK were unknown. The comparison of the three-dimensional structures shows that multiple (global and local) specific adaptations have been brought about by this enzyme. Together, these reside in an overall increased flexibility of the cold-adapted PGK thereby allowing a better accessibility to the active site, but also a potentially more disordered transition state of the psychrophilic enzyme, due to the destabilization of some catalytic residues.

Keywords Phosphoglycerate kinase · Psychrophile · Enzyme · Hinge bending · *Pseudomonas sp.* · TACII 18 · Cold adaptation

Abbreviations

PGK	Phosphoglycerate kinase
3-PG	3-phosphoglycerate
1,3-PG	1,3-bisphosphoglycerate
AMP-PNP	Adenylyl imidophosphate

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Introduction

Extremophilic micro-organisms grow in a wide variety of extreme conditions as acidic and alkaline environments, high pressure, high salinity and temperatures ranging from − 25 °C (Mykytczuk et al. 2013) to 122 °C (Takai et al. 2008). Habitats of cold temperature have been colonized by a number of different organisms known as psychrophiles, which are able to grow at temperatures near the freezing point of cellular water (Low et al. 1973). Psychrophilic bacteria have been able to survive within these conditions by developing several molecular adaptations. At low temperatures, enzymes from these organisms display a higher specific activity and a lower thermostability than their mesophilic counterparts (Feller and Gerday 2003), and from a molecular point of view, this enzyme adaptation seems to be favored by a lower number of salt bridges, hydrogen bonds, hydrophobic clusters and proline residues in loop regions. A lower arginine/(arginine + lysine) ratio as well as an increased hydrophobicity at the surface of the protein have also been observed (Feller and Gerday 2003; Jaenicke 1990; Smalås et al. 2000; Siddiqui and Cavicchioli 2006; Feller 2013). Altogether, these factors should lead to an increased flexibility or dynamics of the enzyme, particularly around

the catalytic site, which may explain the high specific activity at low temperature and the lower thermostability (Siddiqui and Cavicchioli 2006). A number of three-dimensional structures of cold-adapted enzymes determined by X-ray crystallography indicate how the flexibility of these enzymes is maintained in a cold environment and how their stability at higher temperature could be improved. Together, these characteristics have resulted in a growing interest of the biotechnology industries for psychrophilic enzymes (Gerday et al. 2000; Struvay and Feller 2012).

Phosphoglycerate kinases (EC 2.7.2.3, PGK) catalyze the phosphate transfer from 1,3-bisphosphoglycerate (1,3-BPG) to Mg-ADP resulting in the formation of 3-phosphoglycerate (3-PG) and Mg-ATP during glycolysis. The protein is active in its monomeric state except those from some hyperthermophilic organisms as *Pyrococcus woesei* (Hess et al. 1995) and *Sulfolobus solfataricus* (Jones et al. 1995). The first determined three-dimensional structure of PGK from horse muscle (Banks et al. 1979) showed that the enzyme has two domains: an N-terminal domain and a C-terminal domain of equal size and separated by a helical linker. Each domain plays a distinct role in substrate recognition; the N-terminal domain binds 1,3-BPG using a basic patch (Harlos et al. 1992) and Mg-ATP is bound to the C-terminal domain (Davies et al. 1993). These enzymes operate via a hinge-bending motion of the two domains which brings the substrates at spatial proximity to facilitate phosphate transfer (Bernstein and Hol 1997; Marston et al. 2010; Inoue et al. 2010; Cliff et al. 2010).

Here, we report the crystal structures in both native and product bound forms of PGK from a psychrophilic bacteria *Pseudomonas sp.* TACII 18 (henceforth PsyPGK) isolated from Antarctic ice water. It has been shown that this enzyme displays a high catalytic rate constant k_{cat} to compensate for the reduction of chemical reaction rates at low temperature, but also a high affinity (low K_m) for the nucleotide, thereby improving the k_{cat}/K_m ratio which is the relevant efficiency parameter for intracellular enzymes (Bentahir et al. 2000). Microcalorimetric studies have revealed that the psychrophilic PGK is heat labile with, however, a small heat-stable domain including the nucleotide-binding site. It has been proposed that the main heat-labile domain acts as a destabilizing domain, providing the required flexibility around the active site for catalysis at low temperature, whereas the heat-stable domain provides a compact structure improving the nucleotide-binding affinity (Zecchinon et al. 2005).

In the light of the well-characterized catalytic mechanism of PGK, comparative studies with PGK three-dimensional structures from the mesophilic *T. brucei* (Bernstein et al. 1998), the thermostable *B. stearothermophilus* (Davies et al. 1993) and the hyperthermophilic *T. maritima* (Auerbach et al. 1997) may contribute to our understanding of molecular mechanisms governing adaption to extreme temperatures.

Materials and methods

Crystallization

The recombinant protein expressed and purified as described previously (Bentahir et al. 2000) was crystallized using the hanging drop vapor diffusion method at room temperature under two different conditions as described earlier (Mandelman et al. 2001). One in which only protein concentrated to 10 mg ml^{-1} was used, a second one in which protein at a concentration of 10 mg ml^{-1} was mixed with 2.1 mM AMP-PNP, 4.2 mM 3-PGA, 1 mM DTT and 10 mM Tris–HCl at pH 7 prior to crystallization. For both conditions, crystals grew in a mixture of 30% polyethylene glycol (PEG) 4000, 0.2 M $MgCl_2$ and 0.1 M Tris–HCl at pH 8.4. Droplets of 2 µl of protein solution mixed with 2 µl of precipitant solution were equilibrated over a reservoir containing 500 µl of the precipitant solution. These drops were incubated at 290 K. Crystals were cryo-protected by stepwise soaking in a solution containing 5, 10 and 15% ethylene glycol during 2 min at each concentration.

X-ray data collection

For the complex, X-ray diffraction was performed in house on a Nonius FU 581 generator and CuKα X-rays were focused with Osmic mirrors, and for the crystal of native PGK, the data were collected at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France, on the beamline BM-30A/FIP. The crystals were flash-frozen in supercooled N_2 gas produced by an Oxford Cryosystems Cryostream (600 series) and maintained at 100 K during data collection. Diffraction data were collected up to 2.1 (complex)- and 2.0 (native)-Å resolution, respectively, on a MAR345 image plate detector (MAR Research) using a crystal being oscillated 1° per frame around the φ-axis. Indexing and integration of the diffraction images, as well as scaling and merging of the reflections, were performed with DENZO and SCALEPACK (Otwinoswki and Minor 1997). For both conditions, crystals belong to the trigonal space group $P3_2$, with data collection statistics as described in Supplementary Table 1. One molecule is present in the asymmetric unit and the Matthews coefficient (V_M) of 2.1 $Å^3Da^{-1}$ indicates a solvent content of 39% (Matthews 1968).

Determination and refinement of the structures

The crystal structure of PsyPGK determined from a crystallization solution containing the substrate was solved by the molecular replacement method using the program AMoRe

(Navaza 2001) and the structure of *Thermotoga maritima* phosphoglycerate kinase (Auerbach et al. 1997) as a search model. No solution was found using the entire molecule, but a search using domains N (residues 2–168) and C (residues 185–399) separately omitting the helical linker between the two domains gave a solution as described earlier (Mandelman et al. 2001). Manual fitting and replacement of amino acid residues were carried out using COOT (Emsley et al. 2010) and alternated with maximum-likelihood refinement using the program "phenix.refine" (Afonine et al. 2012) to final R- and Rfree factors of 17.4 and 22.2%. Electron density was partly lacking for some of the side chains in the three-dimensional structure including Lys 27, Lys 102, Lys 116, Lys 183, Lys 231, Lys 248, Lys 327, Asp 348, and Tyr 358. In all refinements, data in the range 46.0–2.1 were used. Data refinement statistics are given in Supplementary Table 1. The free R-factor (Brünger 1992) was calculated on the basis of 5% of the data being randomly selected. Analyses with the program PROCHECK (Laskowski et al. 1993) indicate that 92.3% of the non-glycine and non-proline residues were in the most favorable region of the Ramachandran plot (Ramakrishnan and Ramachandran 1965) and the remaining in additional allowed regions. This crystal structure was used for phasing the native data, for which data collection and refinement statistics as described in Supplementary Table 1. Alternating manual fitting (COOT, (Emsley et al. 2010)) and maximum-likelihood refinement ("phenix.refine", (Afonine et al. 2012)) were performed to final R- and Rfree factors of 17.9 and 22.9%. Electron density for the native structure was partly lacking for the side chains of residues Gln12, Lys102, Lys116 Lys248, Lys 183 and Ser266.

Structure analysis

Protein sequences were aligned using CLUSTALW (Thompson et al. 1994) and rendered with the program ESPript (Robert and Gouet 2014). The secondary structure as indicated at the top of the alignment was calculated using the program DSSP (Kabsch and Sander 1983).

Structural superposition of Cα backbones was performed using the program "SUPERPOSE" (Krissinel and Henrick 2004; Winn et al. 2011) and the rmsd was calculated with NPS@ (Combet et al. 2000). Volumes and surfaces are calculated with GRASP (Nicholls 1993) and the number of hydrogen bonds was calculated using HBPLUS (McDonald and Thornton 1994). The hydrophobic surface area was calculated with the program BICEP (van Dijk et al., 2016), and the number of salt bridges was calculated using PIC (Tina et al. 2007). Three-dimensional structure representations were generated using PyMOL (DeLano 2002).

Atomic coordinates of PsyPGK have been deposited in the Protein Data Bank under accession numbers 6I06 (native) and 6HXE (complex).

Results

Overall structure description of psychrophilic PGK

The 387 amino acid residues phosphoglycerate kinase was crystallized in two open forms (one free and one with the product bound) under two distinct conditions and refined to 2.1-Å and 2.0-Å resolution, respectively. Data collection and refinement statistics are summarized in Supplementary Table 1.

The RMSD between the two structures was calculated to be 0.5 Å The overall fold of PsyPGK, shown in Fig. 1, is very similar to the other known phosphoglycerate kinase structures. Two domains are separated by a helical linker (residues 165–179): the N-terminal domain (residues 2–164) which is made up of 4 α-helices and 8 β-strands forming a Rossmann fold and the C-terminal domain (residues 180–387) containing 8 α-helices and 8 β-strands.

The largest differences between the three-dimensional structures compared herein are localized in the N-terminal domain. A two-turn helix between strand β5 and strand β6 in the thermophilic and hyperthermophilic enzymes is replaced by a one-turn helix in the mesophilic enzyme, and is completely lost in the psychrophilic enzyme in which this stretch is unstructured (Fig. 2). This loss arises from a six-residue deletion, as indicated by the sequence alignment (Fig. 3). In the structure obtained by co-crystallization, a molecule of 3-PG (product of the catalyzed reaction) interacts with the main chain carbonyls of Ala264, Glu 265 and Ala267, as well as with the side chains of Asp123 and Lys156 from a symmetry-related molecule, in addition to three water molecules (Fig. 4). 3-PG may contribute to the stabilization of loop 262–270 and loop 308–318 which interact via a hydrophobic interaction between Phe263 and Phe315. This interaction does not seem mandatory for the proper structuring of the loops since this is already seen in the Apo enzyme. However, it is interesting to notice that the two aromatic residues are also part of a hydrophobic cluster involving Phe318.

Comparative studies with PGK counterparts

The psychrophilic enzyme was compared with counterparts with which it displays high sequence identity (41.6% with the mesophile (Ms) *T. brucei* PGK, 48.1% with the thermophile (Th) *B. stearothermophilus* PGK and 46.1 with the hyperthermophile (Ht) *T. maritima* PGK). Three-dimensional structures of MsPGK, ThPGK and HtPGK superimposed on PsyPGK had rmsd values of 2.36/3.25 Å for MsPGK, 1.06/2.95 Å for ThPGK and 2.97/3.86 Å for HtPGK based on 113/387 (N-ter domain/entire protein) Cα atoms, respectively.

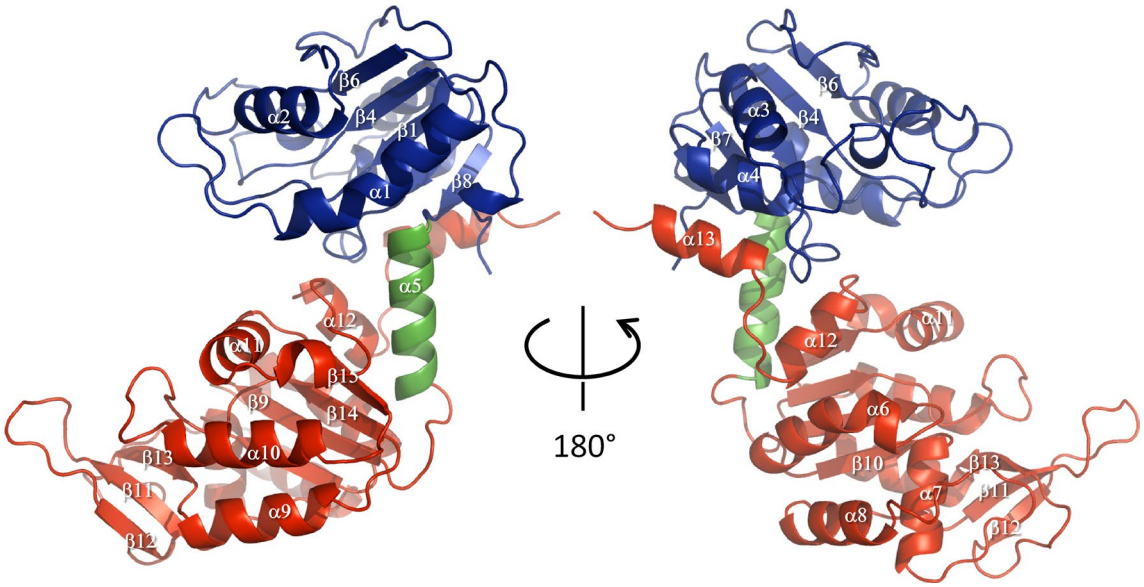

Fig. 1 Overall three-dimensional structure of the psychrophilic phosphoglycerate kinase (PDB-ID 6I06). N- and C-terminal domains are colored in blue and red, respectively. The helical linker is depicted in green. This figure was generated with PyMOL (DeLano 2002)

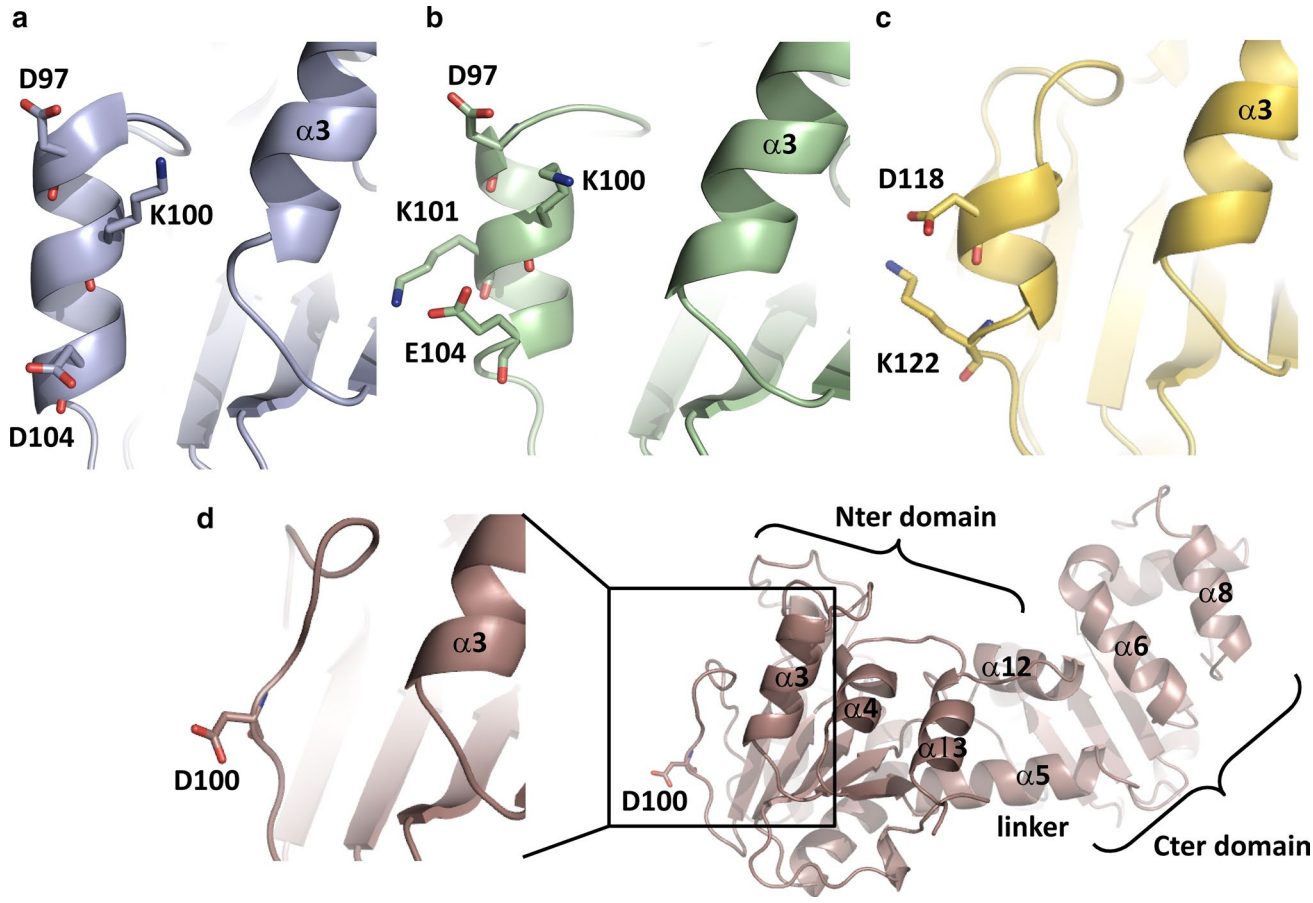

Fig. 2 Close-up on a region in the amino terminal part of the enzymes in which a surface-exposed α-helix holding a number of charged residues and found in meso-, thermo- and hyperthermophilic counterparts is unstructured in the psychrophilic enzyme. **a** ThPGK, **b** HtPGK, **c** MsPGK, **d** PsyPGK

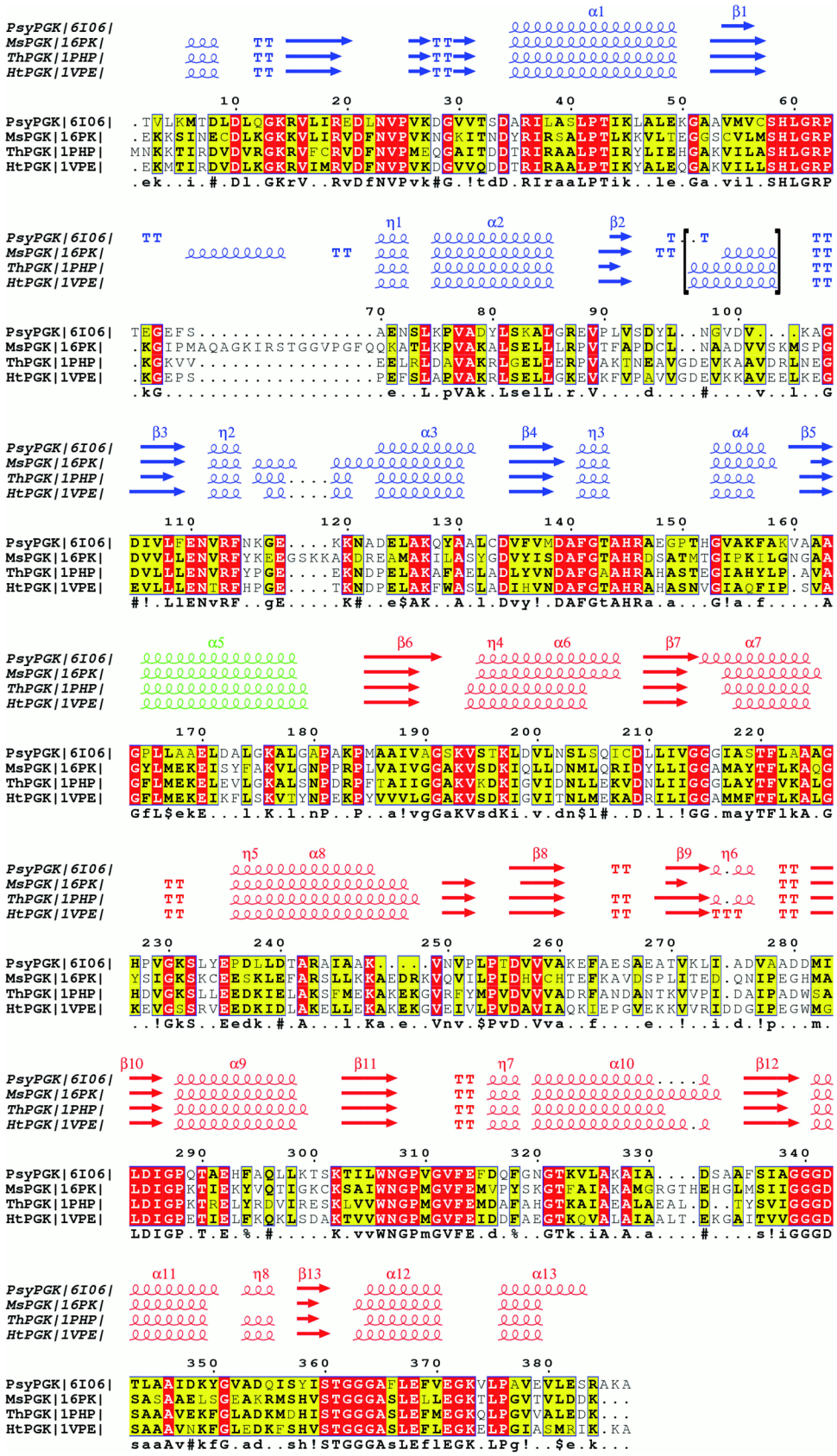

Fig. 3 Sequence alignments of PsyPGK (6I06), MsPGK (16PK), ThPGK (1PHP) and HtPGK (1VPE) performed using CLUSTALW (Thompson et al. 1994). Secondary structures of all the counterparts, calculated with the DSSP algorithm (Kabsch and Sander 1983), are presented above the alignments. The N-terminal domain is colored in blue, the helical linker (α5) in green and the C-terminal domain in red. α-Helices are represented by α, β-strands by β and 3_{10} helices by η. Identities are shown on red background and similar residues on yellow background. For the psychrophilic enzyme, the missing helix between strand β2 and strand β3 is indicated between brackets. Figure rendering was done using the program ESPript (Robert and Gouet 2014)

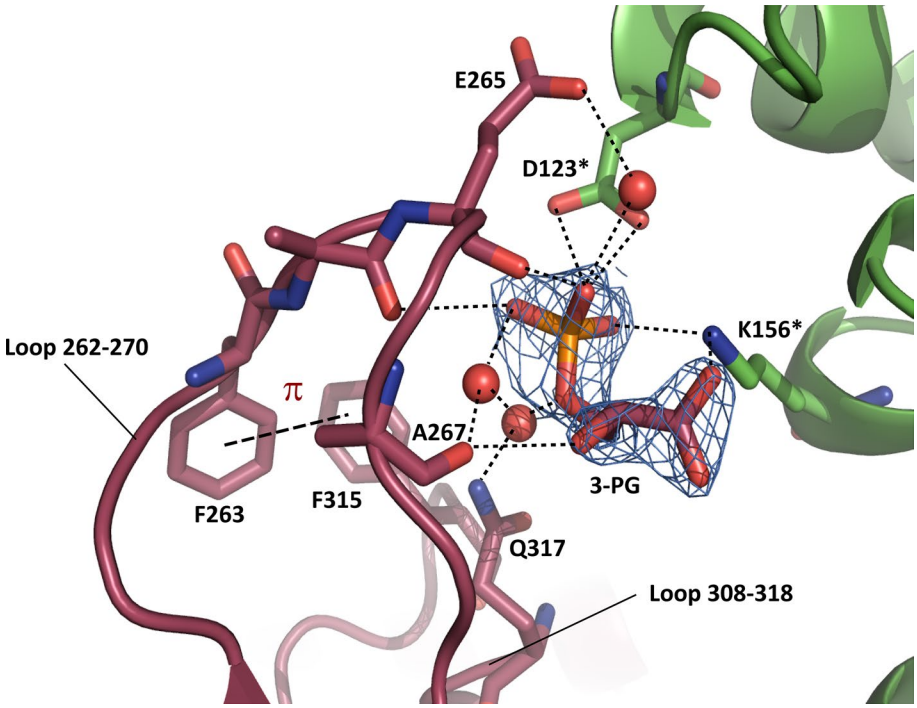

Fig. 4 Reaction product (glycerate 3-phosphate) binding contributes to stabilize loop 262–270 and loop 308–318. Stars indicate residues in a symmetry-related molecule. The 2Fo–Fc omit map is contoured at 1.3 σ around glycerate 3-phosphate (3PG)

Amino acid composition

The overall amino acid composition of the four enzymes does not show significant differences apart from an increase in the number of alanine residues in PsyPGK (Table 1). This residue may play a crucial role in thermostability, due to its flexible nature, and is often found in loop regions of proteins. Furthermore, PsyPGK displays a lower Lys and Arg content, therefore, decreasing the potential to form stabilizing ion pairs and H-bonds. By contrast, the proline content is unchanged, whereas this imino acid tends to rigidify the protein structure and is often found in β-turns and α-helices (Watanabe et al. 1997).

Hydrophobicity

Hydrophobic interactions play a major role in thermostability as they contribute to maintain the core of the protein. The percentages of hydrophobic residues (alanine, valine, isoleucine, leucine, phenylalanine, tryptophan, proline and methionine) in each enzyme are 51% in PsyPGK, 43% in MsPGK, 48% in ThPGK and 48% in HtPGK. The difference in hydrophobicity of the psychrophilic enzyme is mainly due to a higher percentage of alanine as noted above, but also a slight increase in the percentage of leucine and, at a lower degree, of valine. These residues tend to replace some polar residues at the surface of the protein, where they are thermodynamically unfavorable, being exposed to the solvent, thereby increasing the overall flexibility of the outer shell. Similar observations have been made for other structures of

Table 1 Environmental temperatures and percentages of each amino acid residue in the four phosphoglycerate kinases studied with charged residues highlighted in bold

Tenv	PsyPGK ~0 °C	MsPGK 37 °C	ThPGK 50–60 °C	HtPGK 85–90 °C
Ala	15.5	9.1	12.4	9.5
Arg	**2.3**	**3.6**	**5.1**	**3.0**
Asn	2.6	3.0	3.3	2.3
Asp	**7.0**	**4.5**	**7.9**	**6.0**
Cys	0.8	1.4	0.3	0.0
Gln	1.8	2.0	0.5	1.5
Glu	**5.4**	**5.7**	**8.4**	**9.3**
Gly	8.8	10.2	8.9	9.5
His	**1.3**	**1.8**	**2.0**	**1.5**
Ile	4.7	6.1	5.3	6.5
Leu	11.1	9.5	9.6	9.0
Lys	**7.5**	**9.5**	**8.1**	**10.8**
Met	1.6	3.4	2.3	2.3
Phe	3.9	2.5	3.8	4.3
Pro	4.1	4.5	3.8	4.5
Ser	5.2	7.7	2.8	4.3
Thr	4.7	5.0	3.3	3.8
Trp	0.3	0.5	0.5	0.8
Tyr	1.6	2.7	2.0	0.8
Val	10.1	7.0	9.6	10.5

psychrophilic enzymes as citrate synthase from an Antarctic bacterial strain DS2-3R (Russell et al. 1998), α-amylase from *Pseudoalteromonas haloplanctis* (Aghajari et al. 1998), and metalloprotease from *Pseudomonas sp.* (Aghajari et al. 2003). Calculations of the hydrophobic surface area for the respective structure are 4300 Å^2 for PsyPGK, 3059 Å^2 for MsPGK, 3183 Å^2 for ThPGK and 2859 Å^2 for HtPGK which are in agreement with this tendency.

It has been reported that in the absence of substrates, the psychrophilic protein has two domains with different thermostabilities: one similar to the mesophilic PGK and one with lower stability (Bentahir et al. 2000). Parker et al. have shown, in studies on yeast PGK that the N-terminal domain unfolded 70 times faster than the C-terminal domain (Parker et al. 1996). When analyzing the structure of PsyPGK, we observed a cluster of polar amino acids in the core of the N-terminal domain including Arg19, Glu20, Cys57, Ser58, Tyr129, Ser150 and Thr151. This cluster is not found in mesophilic and thermophilic counterparts where Glu20 is replaced by a valine and Cys57 and Tyr129 by hydrophobic residues. The hydrophobic effect has been shown to play an important role in the stability of proteins (Dill 1990); hence, the substitutions observed may be responsible for the loss of hydrophobic interactions in the core of the protein and could explain the low stability of the N-terminal domain of PsyPGK.

The presence of these polar amino acids in the core of the protein and the increased number of valine, alanine and leucine at the surface of the protein suggest that the N-terminal domain may be the more thermolabile domain and could be responsible for the lower stability of the psychrophilic enzyme at higher temperatures.

Charge-mediated interactions

The number of charged residues increases from the psychrophilic PGK to the mesophilic one, and from the mesophilic to thermophilic and hyperthermophilic PGKs (24% in PsyPGK, 25% in MsPGK, 31% in ThPGK and 31% in HtPGK, Table 1). Increasing charges are related with thermostability because these residues are able to make hydrogen bonds and salt bridges as confirmed within these studies (Supplementary Table 2). Indeed, salt-bridge networks at the surface of thermophilic and hyperthermophilic proteins are frequently observed (Yip et al. 1995). For the PGKs studied here, this tendency is confirmed (Fig. 5, Supplementary Fig. 1) with 0.02 salt bridges per residue in PsyPGK, 0.04 in MsPGK, 0.06 in ThPGK and 0.06 in HtPGK. It is also worth noting that the loss of one helix between strands β5 and β6 may contribute to the overall softness of the surface of the enzyme by decreasing the number of charged residues turned towards the solvent (Fig. 2). Finally, the number of salt bridges between the two domains increased with increased thermostability, with 0 inter-domain salt bridges in PsyPGK, 1 in MsPGK, 3 in ThPGK and 3 in HtPGK.

Hydrogen bonds

Despite the low energy of these interactions, they play a very important role in maintaining the three-dimensional structure of the protein by their large number (Creighton 1991), and indeed the number of hydrogen bonds decreases drastically in the psychrophilic enzyme compared to the other PGKs investigated herein (Supplementary Table 3).

Aromatic interactions

Aromatic interactions play an important role in protein stability (Burley and Petsko 1985). They are mediated by the Π electron cloud of aromatic rings being close in space (between 4.5 and 7 Å). Whereas the number of aromatic interactions is not strikingly different (9 in PsyPGK, 6 in MsPGK, 11 in ThPGK and 6 in HtPGK), these interactions are differently organized in the respective structures (Fig. 6). Both domains in the psychrophilic enzyme are stabilized only locally by two clusters of three

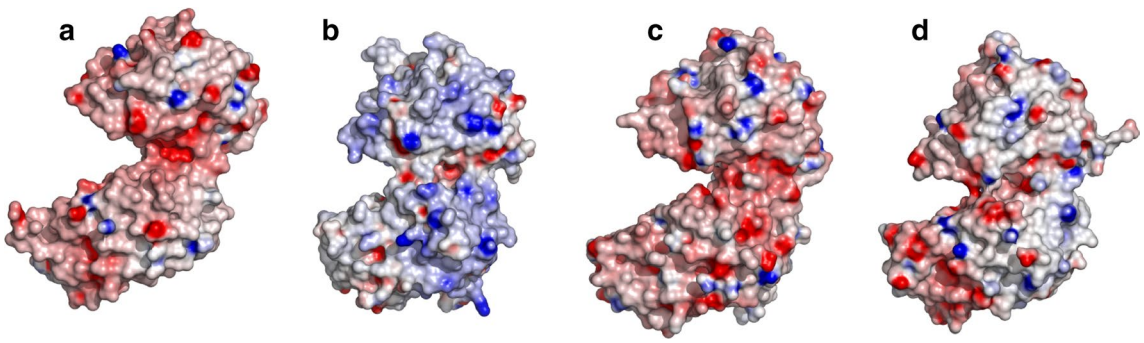

Fig. 5 Surface representations of PsyPGK (**a**), MsPGK (**b**), ThPGK (**c**) and HtPGK (**d**) displayed at the same potential level. Color codes are red for negative charges and blue for positive charges. The figure was generated with GRASP (Nicholls 1993)

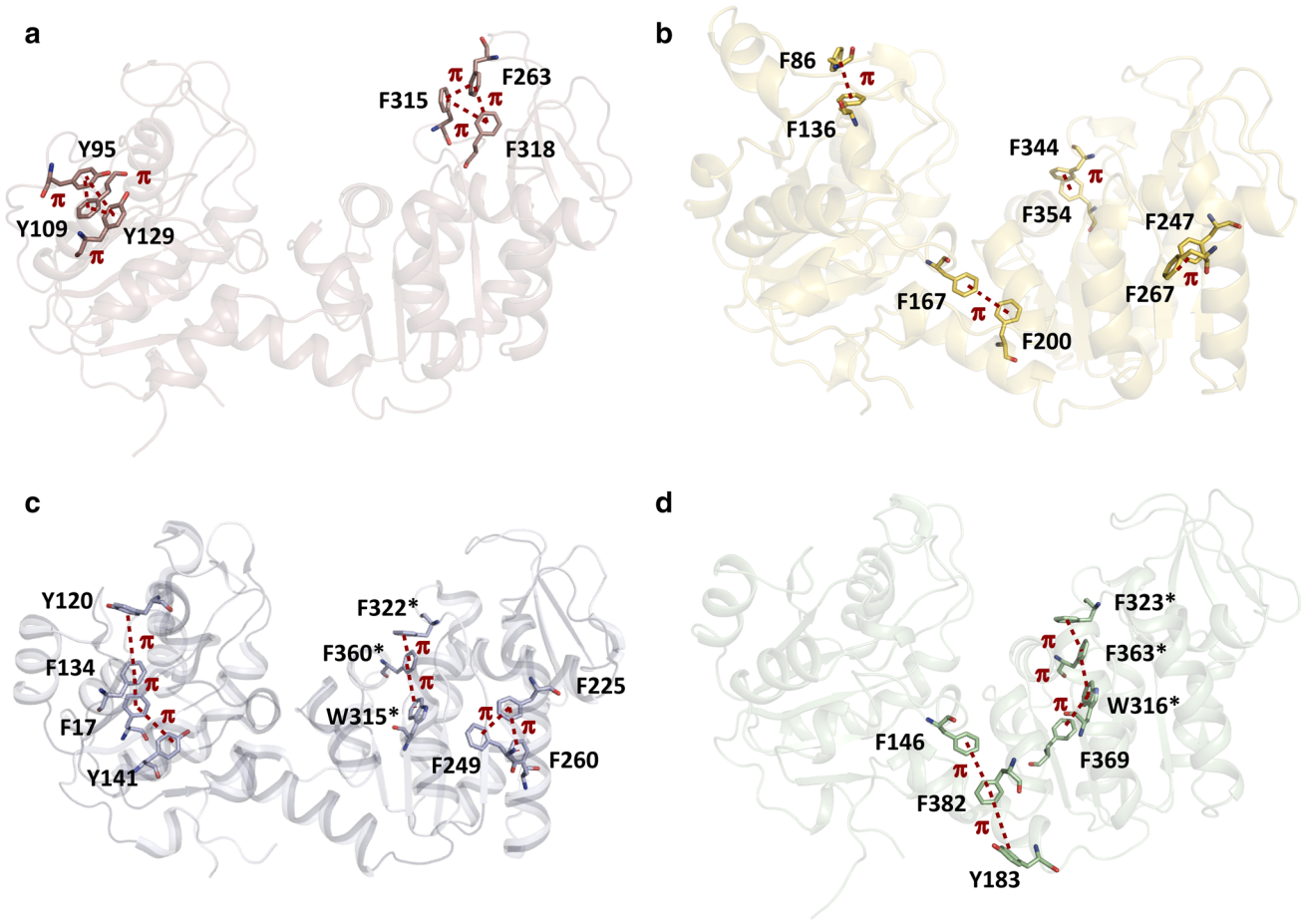

Fig. 6 Formation of compact (**a**), extended (**c, d**) or semi-compact (**b**) clusters of aromatic residues. The clusters are present in both N- and C-terminal domains for the psychrophilic and thermophilic enzymes, whereas the meso- and hyperthermophilic counterparts show additional stabilization at the hinge between the domains. (**a**) PsyPGK, (**b**) MsPGK, (**c**) ThPGK, (**d**) HtPGK. Conserved residues between ThPGK and HtPGK are indicated by a star

aromatic residues each. The thermophilic and hyperthermophilic enzymes are stabilized by a series of aligned aromatic residues, spanning a larger distance, which highly stabilize the two respective domains (ThPGK) or the hinge region and the C-terminal domain (HtPGK). Finally, the mesophilic enzyme displays clusters being something in between compact and extended clusters, and stabilizing also the hinge region and the C-terminal domain.

Specific adaptations

The residues forming the active site are very well conserved among the four different enzymes suggesting that a more subtle mechanism is responsible for the increased catalytic efficiency of the psychrophilic enzyme observed at low temperature.

Increased flexibility around the hinge region

It is now well known how the enzyme performs the hinge-bending motions to get the two domains close together (Banks et al. 1979; Cliff et al. 2010; Auerbach et al. 1997). We hypothesized that this movement could be favored in the psychrophilic enzyme. It is clear that the overall amino acid composition and the reduced number of weak interactions tend to destabilize the enzyme but most probably, more specific modifications may play a role in the bending motion. To address this issue, we first focused our attention on the three helices shown to form the inter-domain region and the surrounding regions (Marston et al. 2010; Inoue et al. 2010; Auerbach et al. 1997). These three helices (helices 5, 12 and 13 in PsyPGK) should be directly involved if an adaptive bending motion occurred in the psychrophilic enzyme. No differences could be found in the overall inter-domain organization. However, when observations were

enlarged to the surrounding regions, we observed that some interactions between residues of helix 5 (residue 165–178) and helix 1 (residue 35–49) in the N-terminal domain, present in PGKs from meso-, thermo- and hyperthermophilic organisms (Fig. 7), were totally lacking in the psychrophilic enzyme. More concretely, in thermophilic and hyperthermophilic PGKs, stabilization is mainly provided by an aromatic interaction between Phe169 and Tyr46 as well as a charge-mediated interaction between Glu172 and Arg7. In MsPGK, stabilization involves hydrogen bonds from Tyr191, Asn10 and Lys49, and a salt bridge between Lys49 and Glu53. Substitution of the aromatic Phe169 and charged Glu 172 (Ht- and ThPGKs)/Tyr191 and Glu194 (MsPGK) by a proline and an alanine, respectively, in PsyPGK (Pro165 and Ala168) which do not form similar interactions with the neighboring residues (Fig. 7), strongly suggests that the N-terminal domain should be under less constraint during the bending motion.

Destabilization of the 1,3-biPG-binding site

The reaction through which a phosphate monoester is transferred to ADP has been extensively studied. The 3D structure determination of transition state analog (TSA) complexes formed with trifluoromagnesate or tetrafluoroaluminate have largely contributed to the understanding of the mechanism (Cliff et al. 2010). In particular, TSA structures and mutational studies have shown that at least three basic residues are involved in the transfer process, namely Arg36, Lys193 and Lys197 in PsyPGK. Interestingly, Arg36 is positioned at the N-terminal of helix 1. In MsPGK, ThPGK and HtPGK, the N-terminal position of helix 1 is largely stabilized by a complex network of charge-mediated interactions, whereas in PsyPGK, only one interaction remains between Asp34 and Tyr80 (Supplementary Fig. 2). Furthermore, in the helix of the psychrophilic PGK, three polar or charged residues are substituted by the aliphatic residues Ala35, Leu38 and Leu46, Leu38 being in direct contact with the solvent. These reduced number of interactions and increased hydrophobicity support the idea that helix 1 is destabilized and more flexible, thus imparting a greater degree of movement to the catalytic residue, Arg36.

Another important feature related to the stabilization of the ligands bound to the enzyme is the presence of inter-domain bonds and in particular salt bridges observed in many organisms such as *T. brucei*, *T. maritima* or their human counterpart. Interestingly, one of the most conserved inter-domain charge-mediated interactions is lacking in the

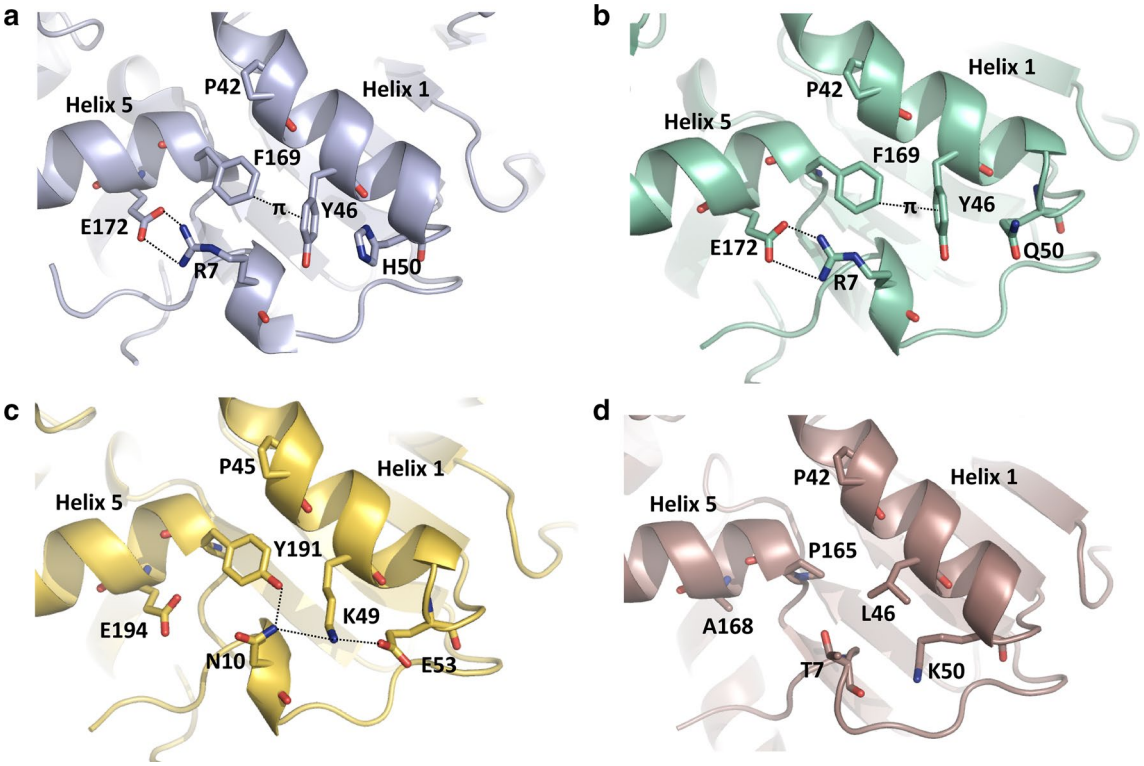

Fig. 7 Different stabilizing interactions in the hinge region: **a**, **b** an aromatic interaction between F169 and Y46 as well as a charge-mediated interaction between E172 and R7 are responsible for the (**a**) thermophilic and (**b**) hyperthermophilic enzyme stabilization. Hydrogen bonds between Y191, N10 and K49, and a salt bridge between K49 and E53 are responsible for the (**c**) mesophilic enzyme stabilization. No stabilizing interactions are seen for the psychrophilic enzyme (**d**). This figure was generated with PyMOL (DeLano 2002)

psychrophilic enzyme. In the HtPGK crystal structure in complex with ligands (AMP-PNP and 3-PG), this interaction involves Arg62 and Asp200. These residues are conserved in the thermophilic and mesophilic counterparts, whereas Asp200 is substituted by Thr196 in PsyPGK (Supplementary Fig. 3). Despite this substitution, an interaction between the charged arginine and the hydroxyl group of the threonine would still be possible, but would probably be weaker than that of the salt bridge. Noticeably, Thr196 is positioned between Lys193 and Lys197 which form salt bridges with the transferred phosphate indicating that in addition to Arg36, the three basic residues involved in the transfer are less constrained.

Altogether, the weakly stabilized hinge domain as well as specific modifications surrounding the catalytic residues should strongly increase the flexibility, likely to enhance the catalytic rate.

Discussion

The comprehensive comparison of homologous proteins that display different optimal temperatures appears as a valid approach to elucidate the molecular mechanism of cold adaptation.

We have studied phosphoglycerate kinases for which crystal structures of psychrophilic, mesophilic, thermophilic and hyperthermophilic counterparts are available. All display a considerable difference in thermal stability despite identical functions and very similar three-dimensional structures.

The high specific activity of psychrophilic enzymes has been attributed to an increased flexibility at low temperatures (Feller and Gerday 2003). This property seems to ensure a better positioning of substrates in the active site and a rapid conformational change during the catalysis at low temperature.

Herein, we have shown that different factors may play a role in flexibility, for instance an increased number of hydrophobic residues at the surface of the psychrophilic enzyme. These amino acids play a crucial role in stabilization of the three-dimensional structure when being in the core of the protein. At the surface, exposed to the solvent, they lead to destabilization of the structure and reduce thermostability (Privalov and Gill 1988).

Fewer salt bridges were found at the surface and between the two domains of PGK, and fewer aromatic interactions and a decreased number of hydrogen bonds were observed when its structure was compared to its meso- and thermophilic counterparts. Similar trends have been observed in other enzymes of differing thermostabilities. In addition, the psychrophilic phosphoglycerate kinase lacks an α-helix compared to its counterparts. Indeed, secondary structure elements contribute to protein stability (Baldwin et al. 1996) and play roles in local flexibility. Accordingly, the lack of such a helix should result in a more flexible protein (Jaenicke and Bohm 1998). Interestingly, it was reported that consecutive enzymes in a number of metabolic pathways can form complexes to transfer directly intermediary metabolites as it has been shown for GAPDH and PGK (Srivastava and Bernhard 1986; Srere 1987; Tomokuni et al. 2010). The formation of such weak and transient complexes is in agreement with functioning in cold environments (Sukenik et al. 2017), in which rapid responses are needed to cope with the low temperatures. An overall flexibility of PsyPGK, including a more flexible hinge domain coupled to destabilized conserved catalytic residues, is in favor of rapid modulation of the stability and the activity of these enzyme complexes.

Together, the overall increased flexibility of the cold-adapted PGK should allow a better accessibility to the active site compared to its counterparts and could contribute to explain the high specific activity at temperatures close to 0 °C. Differential scanning calorimetry studies have shown that in the presence of the substrate (Mg^{2+}-ADP) and the product (3-PG) PsyPGK is stabilized compared to the free enzyme (Zecchinon et al., 2005). This indicates that the binding site is sufficiently stable in the presence of ligands despite an overall flexibility. Also, one cannot exclude the possibility of a more disordered transition state of the psychrophilic enzyme, due to the destabilization of the three catalytic residues Arg36, Lys193 and Lys197, and which hence could improve the reaction rate as well.

In the light of our results presented herein and other earlier studies on citrate synthase from an Antartic bacterial strain DS2-3R (Russell et al. 1998), α-amylase from *Pseudoalteromonas haloplanctis* (Aghajari et al. 1998), metalloprotease from *Pseudomonas sp.* (Aghajari et al. 2003) and many others (Feller 2013), it appears evident that psychrophilic enzymes use various subtle strategies for cold adaptation.

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