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Structural, Kinetic, and Calorimetric Characterization of the Cold-active Phosphoglycerate Kinase from the Antarctic *Pseudomonas* sp. TACII18*

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The gene encoding the phosphoglycerate kinase (PGK) from the Antarctic *Pseudomonas* sp. TACII18 has been cloned and found to be inserted between the genes encoding for glyceraldhyde-3-phosphate dehydrogenase and fructose aldolase. The His-tagged and the native recombinant PGK from the psychrophilic *Pseudomonas* were expressed in *Escherichia coli*. The wild-type and the native recombinant enzymes displayed identical properties, such as a decreased thermostability and a 2-fold higher catalytic efficiency at 25 °C when compared with the mesophilic PGK from yeast. These properties, which reflect typical features of cold-adapted enzymes, were strongly altered in the His-tagged recombinant PGK. The structural model of the psychrophilic PGK indicated that a key determinant of its low stability is the reduced number of salt bridges, surface charges, and aromatic interactions when compared with mesophilic and thermophilic PGK. Differential scanning calorimetry of the psychrophilic PGK revealed unusual variations in its conformational stability for the free and substrate-bound forms. In the free form, a heat-labile and a thermostable domain unfold independently. It is proposed that the heat-labile domain acts as a destabilizing domain, providing the required flexibility around the active site for catalysis at low temperatures.

Temperature is by far the most important environmental factor governing the distribution of life on Earth. Habitats of permanently cold temperature have been successfully colonized by a wide variety of organisms, termed psychrophiles, which have the ability to grow efficiently at temperatures close to the freezing point of cellular water (1). Psychrophilic organisms succeed in maintaining adequate metabolic fluxes by the production of cold-active enzymes able to efficiently catalyze biochemical reactions at low temperatures (2). When compared with their mesophilic counterparts these enzymes display a higher specific activity and a strongly reduced thermostability. This is probably due to an increased conformational flexibility which is now regarded as the main adaptive feature to low temperatures (3–5). It is thought that the higher flexibility of psychrophilic enzymes allows them to better accommodate their substrates and to undergo the fast conformational changes required for catalysis at low energy cost (4).

Phosphoglycerate kinase (PGK)[1] (EC 2.7.2.3), is a key enzyme of glycolysis which catalyzes the reversible phosphoryl transfer from 1,3-biphosphoglycerate to Mg-ADP, resulting in the formation of 3-PGA and Mg-ATP. The enzyme is monomeric, except in the extremophilic archaea *Pyrococcus woesei* and *Sulfolobus solfataricus,* in which PGK have been identified as a dimer and tetramer, respectively (6, 7). PGK is composed of two domains of approximately equal size connected by a well conserved hinge region. To date, more than 40 primary structures have been elucidated from various sources. Throughout evolution the enzyme has shown a high degree of conservation at the level of the primary and tertiary structures (8). Most of the conserved residues lie in the active site cleft, the substrate-binding sites and the hinge region. As shown by crystallographic studies, the triose substrate binds to a basic patch in the N-terminal domain (9) whereas the nucleotide substrate binds to the C-terminal domain (10). Six three-dimensional structures have been solved by x-ray crystallography: the PGK from horse muscle (11–13), yeast (14, 15), pig muscle (9, 16), *Bacillus stearothermophilus* (10), *Trypanosoma brucei* (17), and *Thermotoga maritima* (18). Until recently, the reported structures were in a conformational state incompatible with substrate catalysis. The *T. brucei* ternary complex has been crystallized in the catalytically competent conformation, however, and has shown that the substrates are close to each other and well aligned for phosphoryl transfer (17). Interestingly, the PGK complete hinge closure occurs only when both substrates are bound and depends on their synergistically induced conformational changes. The hinge bending motion of PGK is accomplished by a 32° rotation with respect to the unliganted horse PGK structure (12).

PGK has been used as a model to investigate protein folding and thermostability (8). For this purpose extensive kinetics (19), thermodynamics (20), and structural investigations comparing mesophilic and thermophilic PGK have been undertaken during the last years, attempting to point out the properties leading to thermostability. In the present work we have isolated a cold-active and heat-labile PGK from the Antarctic psychrophile *Pseudomonas sp.* TACII18. The corresponding gene has been cloned and overexpressed in *Escherichia coli*. The psychrophilic character of the enzyme has been analyzed

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ251129.

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[1] The abbreviations used are: PGK, phosphoglycerate kinase; 3-PGA, 3-D-phosphoglyceric acid; PCR, polymerase chain reaction.

by kinetic and thermodynamic characterization and the structural parameters possibly involved in its cold adaptation have been determined by protein modeling.

EXPERIMENTAL PROCEDURES

Sources—The Antarctic bacteria *Pseudomonas* sp. TACII18 was isolated from frozen organic debris near the French Antarctic base J.S. Dumont d'Urville (60° 40 min S; 40° 01 min E). *E. coli* XL1-blue (Stratagene) was used for DNA cloning and *E. coli* BL21 (DE3)(*hsdS cIts 857 ind1 sam7 nin5 lacUV5, gene1*) was used for protein production.

Cloning the pgk Gene—A polymerase chain reaction (PCR) was performed on *Pseudomonas* sp. TACII18 genomic DNA using *Taq* DNA polymerase (Life Technologies, Inc.) and degenerated primers. The sense primer 5′-ACIGTIAARATGACNGA-3′ was derived from the N-terminal amino acid sequence of the purified PGK and the antisense primer 5′-RAAIACICCIRCIGGNCCRTT-3′ was chosen on the basis of a highly conserved sequence involved in ATP binding, corresponding to residues 334 to 340 in yeast PGK. A 935-base pair fragment was amplified and cloned into *E. coli* XL1-blue using the pCR-SCRIPT cloning kit (Stratagene). This fragment was completely sequenced. The entire *pgk* gene sequence and flanking regions were obtained from an enriched genomic DNA bank by PCR screening. Briefly, the purified genomic DNA was partially digested with *Sph*I and the resulting fragments were separated according to their size on a 10–40% sucrose gradient. The fraction containing fragments from 6 to 8 kilobases was ligated to the *Sph*I-digested pUC18 vector and was used to transform *E. coli* XL1-blue competent cells. The resulting genomic DNA bank was then PCR-screened using specific primers chosen from the 935-base pair fragment sequence. This led to the detection of a 7.5-kilobase fragment containing the entire *pgk* gene which was subsequently sequenced on both strands.

Construction of the pgk Expression Vectors—The *pgk* gene was isolated from the 7.5-kilobase fragment by PCR using *Pfu* DNA polymerase (Stratagene) with the following primers: 5′-GGAACTGCGACATATGACCGTG-3′, containing a *Nde*I restriction site (underlined) and 5′-CATTTTCAGGCTTTGGCGC-3′ (stop codon is underlined), corresponding to the C terminus enzyme sequence. The PCR product was cloned into *E. coli* XL1-blue using the pCR-SCRIPT cloning kit and sequenced. This construction was then digested with *Nde*I and *Hin*dIII to isolate the entire *pgk* gene which was subsequently ligated into the *Nde*I and *Hin*dIII sites of the expression vectors pET22b and pET28a. The resulting recombinant plasmids were named pT22-pgk and pT28-pgk, respectively. Following expression, these two constructs produced, respectively, the native and the His-tagged PGK recombinants.

Production and Purification of the Wild-type and Recombinants Enzymes—*Pseudomonas* sp. TACII18 was grown in Pfm broth (20 g/liter tryptone, 1.5 g/liter K_2HPO_4, 1.5 g/liter $MgSO_4$, 10 g/liter glycerin) for 5 days at 4 °C. Cells were collected by centrifugation at 20,000 × *g* for 20 min, resuspended in 200 ml of buffer A (10 mM Tris-HCl, 0.1 mM EDTA, 1 mM dithiothreitol, 20 μM phenylmethylsulfonyl fluoride, 20 μM benzamidine, pH 7.5), disrupted by pressure at 28 kpsi in a cell disrupter (Constant Systems Ltd., United Kingdom) and centrifuged for 30 min at 20,000 × *g*. The resulting supernatant was subjected to a protamine sulfate treatment (0.1% w/v) to remove nucleic acids and then to ammonium sulfate fractionation. The active fraction, collected between 45 and 75% ammonium sulfate saturation, was loaded onto a Phenyl-Sepharose column (2 × 31 cm) equilibrated with buffer A containing 30% ammonium sulfate. Elution was carried out using a decreasing ammonium sulfate gradient from 30 to 0%. Pooled fractions with PGK activity were dialyzed against 3 × 2 liters of buffer A, applied to a Q-Sepharose column (1.5 × 18 cm) equilibrated with the same buffer, and eluted by a 0.2 M NaCl gradient. The enzyme was further purified, using the same conditions as the latter step, on a weak anion exchanger (DEAE-agarose). Finally, the remaining contaminants were removed by high performance liquid chromatography (Beckman, Gold System) on a polyethyleneimine column (Rainin, 0.1 × 10 cm) equilibrated in buffer A and eluted by a 0.1 M NaCl gradient.

The PGK recombinants were overproduced using the T7 system (21). *E. coli* BL21 (DE3) cells, carrying the expression vectors pT22-pgk and pT28-pgk, were grown at 18 °C in L-broth containing 100 mg/liter ampicillin (pT22-pgk) or 80 mg/liter kanamycin (pT28-pgk), until $A_{550\ nm}$ reached 0.6. The cells were induced for a further 20 h with 0.5 mM isopropyl-1-thio-β-galactopyranoside. The purification of the native recombinant enzyme was carried out by the procedure used for the wild-type enzyme, but as a result of the high expression level and the different contaminants from *E. coli*, the two first chromatographic steps were sufficient to purify the enzyme to homogeneity. On the other hand, the His-tagged recombinant PGK was purified in one chromatographic step using affinity chromatography on a Ni-PDC column (Affiland, Belgium). The cell lysate of *E. coli* carrying the recombinant plasmid pT28-pgk was applied to a Ni-PDC column equilibrated with 50 mM Na_2HPO_4, 0.5 M NaCl, pH 6.5. The column was washed extensively using the same buffer, then by the buffer without NaCl. The elution was performed by 0.1 M imidazole, pH 7. The level of contamination from the endogenous host *E. coli* PGK was estimated to be less than 0.5% by comparison with the PGK activity in the cell lysate of *E. coli* carrying an empty pET22b or pET28a vector cultivated under the same conditions.

Enzyme Assay—The enzyme activity was measured in a coupled assay with glyceraldhyde-3-phosphate dehydrogenase (Roche Molecular Biochemicals) by monitoring the oxidation of NADH at 340 nm using a Kontron Uvikon 931 spectrophotometer (22). Assays were performed in 30 mM triethanolamine-HCl, pH 7.5, containing 50 mM KCl, 0.1 mM EDTA, 0.15 mM NADH, 5 mM $MgCl_2$, 40 mM $(NH_4)_2SO_4$, and 1 μg/ml glyceraldhyde-3-phosphate dehydrogenase at 25 °C. For determination of kinetic parameters, the concentration of one substrate was kept constant at 10 mM ATP or 5 mM 3-PGA while the concentration of the other was varied.

Molecular Modeling of Cold-active PGK—The *Pseudomonas* PGK structural model was based on the three-dimensional structures of PGK from yeast (15), *B. stearothermophilus* (10), *T. maritima* (18), and *T. brucei* (17). Such a strategy offers the advantage of minimizing the number of loops that are generated. Starting from sequence alignment using the BESTFIT program (23), the percentage of identity between the *Pseudomonas* PGK sequence and the templates was determined. The structurally conserved regions were also determined from the multiple sequence alignment generated with the PILEUP program (24). Following model building, energy minimization was achieved using the AMBER Molecular Mechanics 4.1 framework so as to avoid bad molecular contacts (25). Finally the geometric features were analyzed by the INSIGHT II program (Molecular Simulations, San Diego, CA) on a Silicon Graphics workstation. The ionic and aromatic interactions were determined using a cut-off distance of 3.5 Å and 4.5 Å between interacting groups, respectively.

Thermal Unfolding—Heat-induced unfolding of *Pseudomonas* and yeast PGK (Sigma) was analyzed by fluorescence spectroscopy and differential scanning calorimetry. All measurements were performed in 10 mM Tris-HCl, pH 7.5, containing 1 mM dithiothreitol. The change in fluorescence emission at 310 nm was recorded after excitation at 280 nm using a Perkin-Elmer LS50 spectrophotometer at a scan rate of 90 °C/h. The absorbance of the samples at 280 nm was below 0.1 and the pre- and post-transition baseline slopes were used to normalize the raw data (26). Differential scanning calorimetry was performed in a MicroCal MCS-DSC instrument at a scan rate of 90 °C/h and under 1 atm nitrogen pressure. The protein samples were dialyzed extensively against the buffer. The protein concentration was around 2 mg/ml in all experiments. The stability of the enzyme in closed conformation was measured in the presence of 5 mM 3-PGA and 5 mM Mg-ADP. The effect of sulfate ions was studied by addition of 10 mM Na_2SO_4. Thermograms were analyzed according to a non-two-state model using the MicroCal Origin software 2.1.

RESULTS

Cloning and Sequencing the pgk Gene—A 7.5-kilobase genomic DNA fragment from the Antarctic bacteria *Pseudomonas* sp. TACII18 was found to contain the entire *pgk* gene (accession number AJ251129). The latter consists of one open reading frame encoding a protein of 386 residues with a calculated mass of 40110 Da without the formylmethionine (Fig. 1). Further analysis of the isolated genomic fragment using the BLAST similarity search program (27) led to the identification of two partial open reading frames corresponding to the C-terminal sequence of glyceraldehyde-3-phosphate dehydrogenase (61 residues) in 5′ of the *pgk* gene and the N-terminal sequence of fructose aldolase (35 residues) in 3′ of the *pgk* gene.

Purification and Characterization of the Wild-type PGK—Following cultivation of the psychrophile *Pseudomonas* TACII18 at 4 °C, four successive chromatographic steps were required to achieve purification of its intracellular PGK. Only 7% of the initial PGK activity was recovered, corresponding to 0.5 mg of enzyme/liter of culture. SDS-polyacrylamide electrophoresis gel analysis shows that the enzyme is pure at homo-

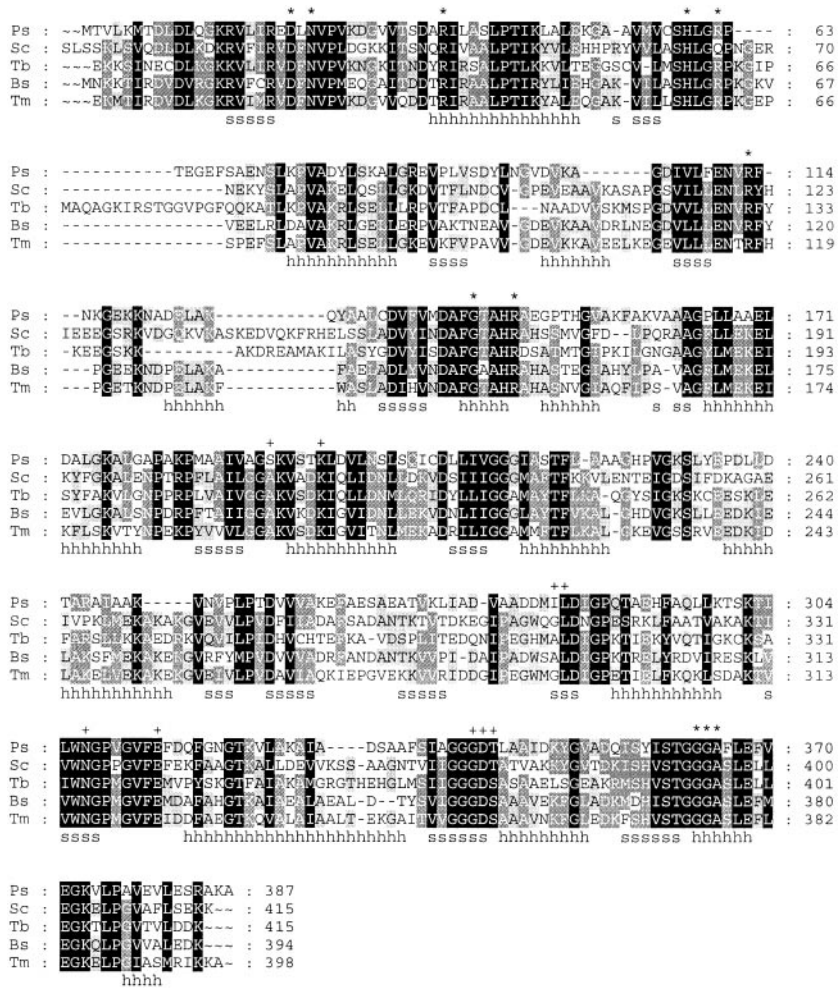

FIG. 1. **Multiple sequence alignment of PGK.** Amino acid sequence from the psychrophile *Pseudomonas* sp. TACII18 (*Ps*), the mesophiles *Saccharomyces cerevisae* (*Sc*), and *T. brucei* (*Tb*), the thermophile *B. stearothermophilus* (*Bs*), and the hyperthermophile *T. maritima* (*Tm*). Secondary structure elements are indicated (according to Ref. 18), where *s* is strand and *h* is helice. * residues involved in 3-PGA binding and + residues involved in ATP binding according to Ref. 17.

geneity, displaying an apparent molecular mass of about 40 kDa. Unlike the mesophilic PGK from yeast which shows a neutral pI (28) and an optimal pH for activity at pH 8.0, the pI of the psychrophilic enzyme is acidic, pI 4.9, and it is active over a broad pH range (6.5–10), with an optimal activity at pH 8.0 (data not shown). The cold-adapted enzyme is activated by sulfate ions at concentrations below 20 mM and is inhibited at concentrations above 50 mM, as already reported for mesophilic PGK (29, 30). The first 15 amino acids of the N terminus were determined by Edman degradation and were found to be similar to that deduced from the gene sequence, displaying a removed formylmethionine and sharing substantial homology with other PGK sequences from different sources.

Heterologous Expression in E. coli—Two different constructs were prepared in which the coding sequence of the *pgk* gene was cloned at the *Nde*I site of pET22b and pET28a plasmid, giving rise to the native and the His-tagged (at the N terminus) recombinant PGK, respectively. Because of the high expression levels, which ranges between 30 and 50% of the soluble proteins obtained both for the native and the His-tagged recombinant PGK, the purification yielded 180 and 100 mg of each enzyme, respectively. The mass of the native recombinant PGK, as determined by electrospray mass spectroscopy, was found to be 40,128 Da, in good agreement with the value calculated from the sequence. Moreover, the specific activity of the native recombinant enzyme was identical to that of the wild-type PGK while that of the His-tagged recombinant protein was seriously altered, as shown in Table I.

Temperature Dependence and Kinetic Parameters—Thermal inactivation of the wild-type, the native and His-tagged recombinant PGK as compared with that of the mesophilic counterpart from yeast was analyzed by measuring the residual activity after various incubation times at 50 °C (Fig. 2*A*). As expected, the half-life of activity of the wild-type and the native recombinant PGK are similar. Furthermore, both enzymes exhibit a very reduced thermostability, giving evidence of their psychrophilic character (31). Surprisingly, the thermostability of the His-tagged recombinant PGK was significantly increased, although remaining lower than that of the mesophilic enzyme.

The thermodependence of the catalytic constant of the wild-type, the recombinants, and the yeast PGK is illustrated in Fig. 2*B*. It is noteworthy to underline the psychrophilic behavior of both the wild-type and recombinants PGK which exhibit superior catalytic properties at low and moderate temperatures and a lower optimum of temperature with respect to the mesophilic PGK (32). The k_{cat} thermodependence of the wild-type and the

TABLE I

Purification of the wild-type and the recombinants PGK from Pseudomonas TACII18 (for 1-liter of culture)

Native and His-tagged are the recombinants PGK expressed in *E. coli*.

	Crude extract		Purified enzyme		Recovery
	Total proteins	Specific activity	Total proteins	Specific activity	
	mg	*units/mg*	*mg*	*units/mg*	%
Wild-type	3500	1.5	0.5	750	7
Native	594	300	180	750	75
His-tagged	565	122	100	550	80

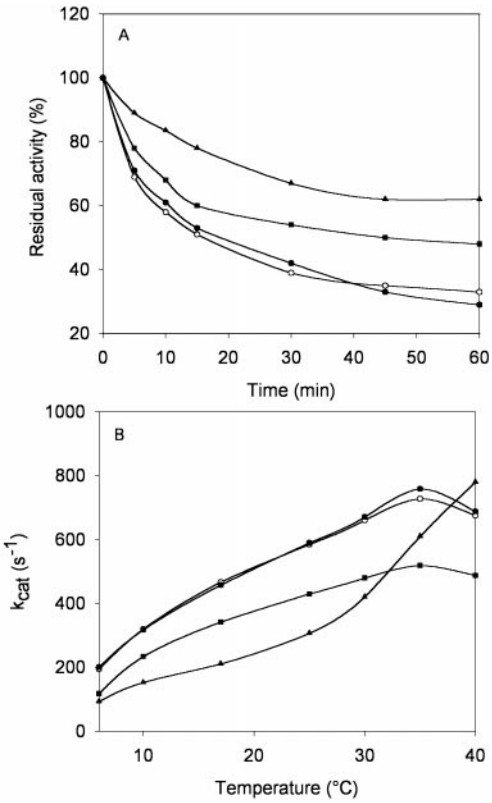

FIG. 2. **Stability and thermodependence of *Pseudomonas* and yeast PGK.** *A,* thermal inactivation of the wild-type (●), the native recombinant (○), the His-tagged PGK (■) from *Pseudomonas* compared with that of the mesophilic enzyme from yeast (▲). *B,* thermodependence of k_{cat} for the wild-type, the native recombinant and the His-tagged PGK in comparison with the mesophilic PGK. Symbols as in *A*.

native recombinant PGK is identical, whereas the k_{cat} values recorded for the His-tagged enzyme are much lower, although still higher than those of the yeast PGK (Fig. 2*B*). This clearly indicates that the His-tag peptide affects both the thermostability and the catalytic properties of the His-tagged PGK.

The kinetic parameters determined at 25 °C for the wild-type, the recombinants and the yeast enzyme are summarized in Table II. The wild-type and the native recombinant enzymes exhibit identical kinetic parameters and display a 2-fold higher catalytic efficiency when compared with that of the mesophilic PGK. This 2-fold catalytic efficiency is, however, totally impaired in the case of the His-tagged enzyme which displays a k_{cat}/K_m ratio similar to that of the yeast PGK.

Protein Modeling—In order to analyze the structural parameters responsible for the cold adaptation of the psychrophilic PGK, a three-dimensional model was built using the known three-dimensional structures of PGK from *B. stearothermophilus, S. cerevisae, T. brucei,* and *T. maritima* as templates. These enzymes share an amino acid sequence identity of between 43 and 48% with the psychrophilic PGK. As shown in Fig. 1, these amino acid identities are distributed uniformly through the sequences and mainly correspond to secondary structure elements, functional residues, and strongly conserved amino acids in all PGK. As a result, the structural model displays a very similar fold consisting of two domains of almost equal size connected by a hinge region (Fig. 3). Analysis of the model was focused on structural factors which possibly reduce thermostability and increase flexibility, parameters which are believed to be responsible for the higher catalytic efficiency (31, 32). As listed in Table III, the parameters derived from the primary structure (amino acid content) of psychrophilic, mesophilic, thermophilic, and hyperthermophilic PGK fail to reveal significant differences which could be attributed to temperature adaptation. The number of Gly and Pro residues, which affect the local mobility of the chain, is not altered in the cold-active PGK. Arginine residues, which have the potential to form multiple ion pairs and H-bonds, are, however, slightly less abundant. There are no specific modifications in the N- and C-terminal caps of α-helices altering charge-dipole interaction in these secondary structures. By contrast, the lower number of salt bridges and the reduced number of aromatic interactions determined on the model of the cold-adapted enzyme are consistent with its psychrophilic character. Furthermore, the number of these weak interactions increases significantly from the psychrophile to the hyperthermophile. Nearly all ion pairs of the psychrophilic PGK are found at the enzyme surface. This feature is illustrated in Fig. 3, showing an increase in surface charges from psychrophilic to thermophilic PGK.

Thermal Unfolding—Heat-induced unfolding of the Antarctic *Pseudomonas* and yeast PGK was monitored by fluorescence spectroscopy and differential scanning calorimetry. The temperature dependence of the fluorescence signal reveals a specific denaturation pattern for the psychrophilic enzyme. Indeed, the fluorescence signal of the yeast enzyme increases with temperature, up to the so-called hyperfluorescent intermediate previously characterized (33), according to a sharp and apparently cooperative transition (Fig. 4*B*). By contrast, the fluorescence signal of the psychrophilic PGK decreases with temperature and denaturation occurs over a larger temperature range (Fig. 4*A*). This indicates distinct modifications in the solvent accessibility and in the environment of the aromatic residues during the unfolding process. In addition, the experimental data slightly deviates from the monoexponential curve corresponding to a two-state unfolding model, suggesting two denaturation steps.

The occurrence of two well separated transitions is clearly demonstrated by DSC experiments (Fig. 4*C*). Such a biphasic profile of the heat capacity function is accounted for by either two thermodynamic domains within the protein (34) or by the coexistence of two enzyme populations, corresponding to the free form and to an enzyme-ligand complex (35). Electrospray mass spectroscopy confirms that the mass of the psychrophilic enzyme sample is homogenous. One can therefore conclude that the cold-active PGK is composed of two different calorimetric units. Irreversibility of the unfolding reaction for both PGK and deviation from the two-state model (indicated by large values of ΔH_{eff}, calculated from the slope of the transition, in comparison with ΔH_{cal} calculated from the area under the heat absorption peak) only allow the comparison of the melting point, T_m, and of the experimental enthalpy of unfolding, ΔH_{cal}. As shown in Table IV, the psychrophilic PGK is characterized by a heat-labile domain and a heat-stable domain in respect to the T_m of the yeast enzyme. However, the total amount of heat absorbed during unfolding is similar for both PGK in the free state.

According to crystallographic studies (17, 18), both PGK were trapped in the closed conformation by incubation with 3-PGA and Mg-ADP. In this conformation, the thermogram of the liganted psychrophilic PGK displays, unexpectedly, no heat-stable domain and merging of both calorimetric units (Fig. 4*e*). The melting point of the resulting transition is slightly higher than that of the heat-labile domain but interestingly, unfolding of this conformation approximates a two-state model as far as the $\Delta H_{cal}/\Delta H_{eff}$ (130/135 kcal/mol) ratio is concerned. On the other hand, both the melting point and the unfolding enthalpy of the yeast PGK are increased in the presence of 3-PGA and ADP (Fig. 4*f*). As a result, the largest difference in

TABLE II
Kinetic parameters for the wild-type and recombinants PGK from Pseudomonas TACIII8 and for yeast PGK at 25 °C
Values are the mean of at least three determination sets. S.E. for k_{cat} and K_m was <5%.

	ATP			3-PGA		
	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m
	s^{-1}	mM	s^{-1} mM^{-1}	s^{-1}	mM	s^{-1} mM^{-1}
Wild-type	500	0.21	2380	537	0.53	1002
Native	519	0.24	2162	540	0.48	1126
His-tagged	411	0.38	1071	362	0.89	406
Yeast	345	0.37	930	335	0.59	560

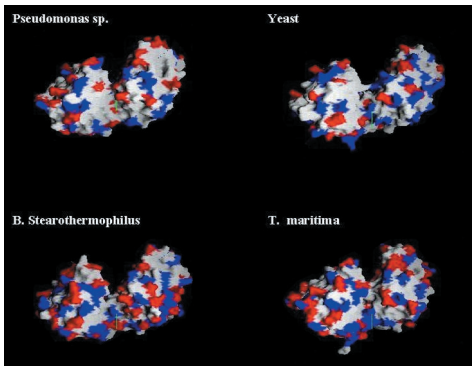

FIG. 3. **Comparison of the electrostatic potential at the surface of PGK.** Enzyme from the psychrophilic *Pseudomonas sp.* strain, the mesophilic yeast, the moderate thermophile *B. stearothermophilus,* and the hyperthermophile *T. maritima*. The psychrophilic enzyme displays a reduced number of charges at the surface. Positive charges are in *blue* and negatives charges in *red*. The figure was generated using the program GRASP (45). The N domain (*left*) is connected to the C domain (*right*) by the hinge region and delineate the active site cleft (*open* to the *top*).

TABLE III
Main structural features related to temperature adaptation in psychrophilic, mesophilic, and thermophilic PGK

Parameters	Psychrophile, *Pseudomonas*	Mesophile		Thermophile	
		Yeast	*T. bruccistearothermophilus*	*B. stearothermophilus*	*T. maritima*
Gly content	34	37	43	35	38
Pro content	16	17	20	15	18
Arg content	9	12	16	20	13
Ion pairs (total)	10	17	20	29	24
Ion pairs (surface)	7	14	ND[a]	29	22
Aromatic interactions	1	5	3	6	5

[a] ND, not determined.

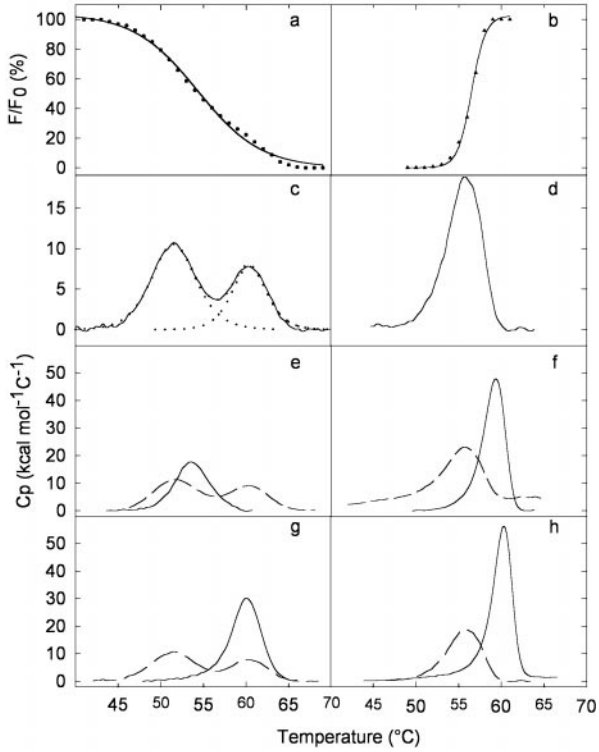

FIG. 4. **Thermal unfolding of *Pseudomonas* (*left*) and yeast (*right*) PGK.** *a* and *b,* relative change in intrinsic fluorescence (●) or (▲); *solid line*, regression curve for a two-model state. *c-h,* baseline-subtracted thermograms recorded by DSC. *c* and *d,* free enzymes (the deconvolution in two transitions is *dotted*). *e* and *f,* PGK in the presence of 5 mM 3-PGA and 5 mM Mg-ADP (*solid lines*). *g* and *h,* PGK in the presence of 10 mM Na_2SO_4 (*solid lines*). In *e-h*, the thermogram for the free enzymes is given as a *dashed line* for comparison.

stability between both investigated enzymes is found in the closed conformation (Table IV). In addition, we found that the sulfate ion has a remarkable effect on PGK stability. Binding of sulfate to the psychrophilic enzyme now induces the disappearance of the heat labile domain and the merging of the two calorimetric units at the level of the heat-stable domain (Fig. 4*g*), while the denaturation pattern of the sulfate-bound PGK from yeast is similar to that recorded in the closed conformation (Fig. 4*h*). Therefore, in the sulfate-stabilized form, the psychrophilic enzyme displays the same melting point and only a slightly lower unfolding enthalpy (16%) than the mesophilic PGK (Table IV).

DISCUSSION

This work has been undertaken in order to compare the psychrophilic PGK with the well characterized PGK from mesophilic and thermophilic strains and to analyze the strategy evolved by the cold-active PGK to reach psychrophily.

The *gapDH* and *fda* genes have been identified in 5′ and 3′ from the *pgk* gene, instead of the usual promoter region and terminator sequence, suggesting that these three glycolytic genes are clustered in an operon and are under the control of an upstream promoter. A similar operon organization for the same enzymes has been reported for *E. coli* (36), *Lactobacillus bulgaricus* (37), and *Borelia burgdorferi* (38). But in the case of the two latter microorganisms a *tim* gene is found instead of the *fda* gene, indicating a closer relationship of the Antarctic *Pseudomonas* with *E. coli*.

Life at low temperatures requires that psychrophilic organisms maintain sufficiently high metabolic fluxes despite thermal inhibition of enzyme reaction rates. In the case of intracellular enzymes, adaptation to cold can be achieved either by increasing $k_{cat,}$ or reducing K_m, or both (39). This assumption is confirmed in the case of the psychrophilic PGK which adjusts both k_{cat} and K_m parameters, leading to a 2-fold improved catalytic efficiency with respect to the mesophilic PGK at 25 °C. Among the limited number of cold-adapted intracellular enzymes studied so far, heat-lability is a common feature (40) but *Pseudomonas* PGK is the first intracellular enzyme found to display the two main characteristics of psychrophilic enzymes:

TABLE IV
Thermodynamic parameters of PGK unfolding recorded by microcalorimetry

	Pseudomonas TACIII8					Yeast	
	T_{m1}	T_{m2}	ΔH_1	ΔH_2	$\Sigma\Delta H$	T_m	ΔH
	°C			*kcal mol*$^{-1}$		°C	*kcal mol*$^{-1}$
Free enzyme	51.5	60.4	73	42	115	55.6	100
+5 mM Mg-ADP, 3PGA	53.8		130		130	59.1	160
+10 mM Na_2SO_4		60		140	140	60.1	167

a reduced thermostability and a higher catalytic efficiency. The higher specific activity of psychrophilic enzymes has been commonly related to their increased flexibility, also responsible for their low stability (41, 42). Unexpected support for this hypothesis is provided by the His-tagged psychrophilic PGK. Indeed, whereas the enzyme is stabilized by the His-tag peptide, its turnover number is drastically reduced. This is the first psychrophilic enzyme mutant showing the inverse relationship between stability and reaction rate. This peptide is linked to the solvent exposed N terminus which protrudes from the bottom of the N domain in Fig. 3, on the opposite side and far from the active site cleft. The poly-His peptide possibly interacts with oppositely charged residues on the protein surface, providing the extra stability. This mutant also demonstrates that the His-tag can markedly alter the enzyme properties, contrary to what is commonly assumed.

The progressive increase in the salt bridge content (Table III) and the surface charge (Fig. 3) from psychrophilic to thermophilic PGK strongly suggest their involvement in temperature adaptation by modulation of the external shell compactness. In addition, it is known that the extremities of a protein are preferential sites for denaturation (20, 39). In *T. maritima* and *B. stearothermophilus* PGK the N and C termini are linked by two salt bridges (D2-K397 and K3-E387, *T. maritima* PGK numbering) whereas only one connects the extremities of the mesophilic enzymes (K5-E413 in yeast). On the other hand, the psychrophilic PGK is devoid of linkage between its N and C extremities as a result of substitutions of the involved residues (Lys to Val and Glu to Ser). The presence of such salt bridges in mesophilic and thermophilic PGK can significantly increase the overall protein thermostability and their lack in the psychrophilic enzyme would therefore weaken its stability. Moreover, the lack of these interactions correlates well with the occurrence of two independent unfolding domains in the psychrophilic PGK as revealed by DSC. PGK achieves its closed conformation only when both substrates are bound. This catalytically competent conformation is stabilized by a newly formed ion pair, between residues Arg^{62} and Asp^{200}, upon closure of the catalytic interface. Whereas these two residues have been found to be totally conserved in mesophilic and thermophilic PGK, the aspartic residue is substituted in the psychrophilic PGK sequence by a threonine. This suggests therefore that the psychrophilic PGK adopts a less stable structure in the closed conformation: this is indeed demonstrated by DSC experiments performed in the presence of 3-PGA and ADP (Table IV).

Thermal unfolding recorded by differential scanning calorimetry reveals an unusual denaturation pattern for the psychrophilic enzyme as it is composed of a heat-labile and a heat-stable domain. However, one has to recall here that the overall stability and function of a macromolecule are governed by its least stable unit (34). Such an organization of the psychrophilic enzyme into thermodynamic units of different stability has profound implications for our current view of protein adaptation to cold. From the limited number of cold-adapted enzymes studied so far, it was concluded that they are characterized by a low conformational stability, that even multidomain proteins display a reduced stability of all calorimetric units and that they have evolved toward the lowest available stability of the native state (40, 43). The results obtained here for the Antarctic PGK demonstrate that this evolution can affect only one particular domain of the molecule. In this respect, two possible explanations are worth mentioning. On one hand, the concept of stabilizing domains has emerged from studies of proteins such as xylanases (44), in which the increased stability of one domain promotes the stability of the whole molecule. It is therefore tempting to consider the heat-labile unit of PGK as a destabilizing domain, providing the required flexibility around the active site in order to increase the catalytic rate at low temperature. On the other hand, a high flexibility of the entire enzyme molecule should be accompanied by a broader distribution of conformational states, leading to poor ligand binding and high K_m values (4). However, the psychrophilic PGK displays high k_{cat} associated with lower K_m resulting in an optimized k_{cat}/K_m ratio, which is the operational parameter for intracellular enzymes working at low substrate concentrations. Therefore, maintaining a stable and rigid domain could improve substrate binding while the labile and flexible unit could favor the reaction rate. By this way, the contradictory optimization of both kinetic parameters can possibly be realized. Finally, the finding that the sulfate activator strongly influences the structural stability of both psychrophilic and mesophilic PGK can be analyzed in the context of cold adaptation. Indeed, our results suggest that at a given step in the reaction pathway, simulated by sulfate binding, the psychrophilic PGK is able to reach a conformation close to that of its mesophilic homologues, as far as stability is concerned (Table IV). These perspectives should stimulate further kinetic and structural studies of enzymes adapted to extreme temperatures.

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