

Metabolic Enzymes from Psychrophilic Bacteria: Challenge of Adaptation to Low Temperatures in Ornithine Carbamoyltransferase from *Moritella abyssi*

Ying Xu,^{1*} Georges Feller,² Charles Gerday,² and Nicolas Glansdorff¹

J. M. Wiame Research Institute, Microbiology, Free University of Brussels, B-1070 Brussels,¹ and Laboratory of Biochemistry, Institute of Chemistry B6, University of Liège, B-4000 Liège,² Belgium

Received 21 August 2002/Accepted 30 December 2002

The enzyme ornithine carbamoyltransferase (OTCase) of *Moritella abyssi* (OTCase_{Mab}), a new, strictly psychrophilic and piezophilic bacterial species, was purified. OTCase_{Mab} displays maximal activity at rather low temperatures (23 to 25°C) compared to other cold-active enzymes and is much less thermoresistant than its homologues from *Escherichia coli* or thermophilic prokaryotes. In vitro the enzyme is in equilibrium between a trimeric state and a dodecameric, more stable state. The melting point and denaturation enthalpy changes for the two forms are considerably lower than the corresponding values for the dodecameric *Pyrococcus furiosus* OTCase and for a thermolabile trimeric mutant thereof. OTCase_{Mab} displays higher K_m values for ornithine and carbamoyl phosphate than mesophilic and thermophilic OTCases and is only weakly inhibited by the bisubstrate analogue δ -*N*-phosphonoacetyl-L-ornithine (PALO). OTCase_{Mab} differs from other, nonpsychrophilic OTCases by substitutions in the most conserved motifs, which probably contribute to the comparatively high K_m values and the lower sensitivity to PALO. The K_m for ornithine, however, is substantially lower at low temperatures. A survey of the catalytic efficiencies (k_{cat}/K_m) of OTCases adapted to different temperatures showed that OTCase_{Mab} activity remains suboptimal at low temperature despite the 4.5-fold decrease in the K_m value for ornithine observed when the temperature is brought from 20 to 5°C. OTCase_{Mab} adaptation to cold indicates a trade-off between affinity and catalytic velocity, suggesting that optimization of key metabolic enzymes at low temperatures may be constrained by natural limits.

Intracellular biosynthetic enzymes are usually exposed to low substrate concentrations, in contrast to extracellular enzymes. Optimizing their catalytic efficiency (k_{cat}/K_m) in psychrophilic (cold-adapted) organisms may thus be challenging, since improving k_{cat} at low temperatures by decreasing the activation enthalpy may have a cost in terms of affinity for the substrate(s) of the reaction (18, 35). The study of cold-active enzymes is thus an important topic in terms of physiology and metabolic evolution (for recent reviews, see references 6, 21, 25, 44, and 60).

No cold-active ornithine carbamoyltransferase (OTCase; EC 2.1.3.3) had been characterized until now. However, the presence of this enzyme in microorganisms adapted to the full range of environments compatible with life makes it an excellent candidate for investigations of protein evolution and of molecular adaptations to extreme conditions. OTCase catalyzes the conversion of ornithine and carbamoyl phosphate (CP) into citrulline and inorganic phosphate in the de novo pathway for arginine biosynthesis and in the detoxifying urea cycle. Biosynthetic and urea cycle OTCases are usually homotrimers of 33- to 40-kDa subunits (7, 9), except in the hyperthermophilic archaeon *Pyrococcus furiosus*, where OTCase is a dodecamer (51). In *Pseudomonas aeruginosa*, a dodecameric catabolic OTCase catalyzes the reverse reaction (conversion of citrulline into ornithine and CP in the presence

of inorganic phosphate) in the deiminase pathway for arginine degradation; the allosteric properties of this enzyme, which are intimately linked to the dodecameric state, account for this ability despite a very unfavorable equilibrium constant (50). Crystal structures have been established for the trimeric OTCases of *Escherichia coli* and humans (26, 29, 47) and for the two known dodecameric transferases (50, 51).

This report presents the biochemical characterization of a biosynthetic OTCase from a new species of γ -*Proteobacteria*, *Moritella abyssi* (OTCase_{Mab}), isolated from Atlantic Ocean sediments at a depth of 2,815 m and a temperature of 2°C (57). With a maximal growth rate at 4°C and a maximal growth temperature of 13°C, the organism is one of the strictest psychrophilic bacteria isolated until now (25) and was chosen for this reason. OTCase_{Mab} was found to be distinctly psychrophilic and to differ from its homologues by a number of properties, illustrating the natural constraints which may limit the functional adaptation of metabolic enzymes at low temperatures.

MATERIALS AND METHODS

Strains and culture conditions. *E. coli* strains were grown at 30 or 15°C in medium 853 (24) as a liquid medium and with 1.5% agar (Difco) as a solid medium. For bacteria containing recombinant plasmids, the media contained kanamycin and chloramphenicol at 50 $\mu\text{g ml}^{-1}$.

Construction of an overexpressing strain. Restriction enzymes and T4 ligase were purchased from Boehringer Mannheim and used according to the manufacturer's instructions. The *argF* gene of *M. abyssi* strain 2693 (57, 59) was amplified by PCR with oligonucleotides 5'-GGAATTCATATGGAAAATTTATTATCAGTTAAAGATTTA-3' (start codon underlined) and 5'-CGGGATCCCTACTTTCTTAACTGTTTGTGTGC-3' to produce *Nde*I and *Bam*HI restriction sites at the ends of the fragment. The *Nde*I restriction site was designed to

* Corresponding author. Mailing address: J. M. Wiame Research Institute, Microbiology, Free University of Brussels (VUB), 1, Ave. E. Gryson, B-1070 Brussels, Belgium. Phone: 32 2 526 72 81. Fax: 32 2 526 72 73. E-mail: xuying@vub.ac.be.

overlap the ATG start codon and the *Bam*HI site after the TAA stop codon. The amplified fragment was inserted into the PCR-2 vector (Invitrogen) to verify the *argF* sequence (46) and subsequently was cloned into the pET24a vector (Novagen), which was then used to transform *E. coli* BL21-codonPlus(DE3)-RIL (Stratagene) competent cells; this rare-codon-usage-improved strain was found to be necessary to obtain satisfactory expression.

Expression and purification of recombinant OTCase_{Mab}. Exponentially growing cells of *E. coli* BL21-codonPlus(DE3)-RIL harboring plasmid pXY144 were induced with 1 mM isopropyl-1-thio- β -D-galactopyranoside (IPTG), harvested after 20 h of induction at 15°C, and washed in 0.9% NaCl. Cells were disintegrated with a high-pressure cell disrupter (Basic Z model; Constant Systems Ltd.) at 28 MPa in 40 mM phosphate buffer (pH 7.0). The extracts were freed of cell debris by centrifugation two times, first for 30 min at 12,000 \times g and then for 1 h at 100,000 \times g. The supernatant was applied to a 15Q XK16/20 column (Pharmacia) pre-equilibrated with 10 mM phosphate buffer (pH 6.5) and washed with the same buffer. A salt step gradient (0 to 220 and 220 to 450 mM KCl) was then applied to the column, and the activities of eluted fractions were tested. The enzyme eluted as the major peak at about 300 to 400 mM KCl. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and native PAGE of eluted fractions revealed that the *M. abyssi* OTCase peak contained more than 95% pure protein. Typically, 100 mg of pure protein was obtained from 1 liter of culture.

Purified *E. coli* ArgF and *P. aeruginosa* OTCase, which were used to calibrate gel filtration chromatography, were obtained from Catherine Tricot (J. M. Wiame Institute); δ -N-phosphonoacetyl-L-ornithine (PALO), synthesized by D. Gigot (Brussels University), was a gift from C. Bompain.

N-terminal amino acid analysis. The N-terminal sequence (six amino acids) of the recombinant enzyme was determined by automated Edman degradation with a Procise 494 protein sequencer (Applied Biosystems).

SDS-PAGE. SDS-PAGE was performed by using a Pharmacia PhastSystem with a discontinuous buffer system and homogeneous 12.5% gels. Gels were stained with Coomassie brilliant blue. The Benchmark protein ladder (Life Technology) was used as a standard to estimate subunit molecular masses.

Native molecular mass determination. The molecular mass of native OTCase_{Mab} was determined by gel filtration with a Pharmacia AKTA fast protein liquid chromatography system fitted with a Hiloal 16/60 Superdex 200 prep-grade column and the following buffers: 10 mM phosphate buffer (pH 7.0) and this buffer plus 0.1, 0.15, 0.2, 0.3, or 0.5 M NaCl. About 0.174 mg of pure OTCase (in 0.1 ml) was applied to a column that had been equilibrated and calibrated with each buffer by using Pharmacia standards (about 100 μ g of each protein), OTCase from *E. coli* (trimeric, 110 kDa), and *P. aeruginosa* (dodecameric, 420 kDa).

DSC. Differential scanning calorimetry (DSC) was performed by using a MicroCal MCS-DSC instrument at a scan rate of 1°C/min and under a nitrogen pressure of 2 atm. Samples were desalted by gel filtration with a PD10 column (Pharmacia) equilibrated with 10 mM phosphate buffer (pH 7.0) supplemented with 0.0 M salt, 0.3 M KCl, 0.3 M NaCl, 0.5 M NaCl, or 0.1 M Na₂SO₄. These buffers were used in the reference cell and for buffer baseline determination. Thermograms of OTCase were analyzed according to a single non-two-state transition model in which the melting point, calorimetric enthalpy, and effective or van't Hoff enthalpy of unfolding were fitted independently by using MicroCal Origin software (version 2.9).

Enzyme assays. Activity was measured by citrulline colorimetry as described previously (3). The reaction mixture contained, in a final volume of 2.0 ml, 30 mM Tris HCl buffer (pH 9.0), 100 mM L-ornithine, and 20 mM CP for the standard assay unless otherwise specified. The reaction was started by OTCase addition. Incubation was carried out for 10 min at 20°C unless otherwise specified. Under the conditions applied, OTCase activity increased linearly with the protein concentration, and the reaction rate remained linear. Specific activity was expressed as micromoles of citrulline produced minute⁻¹ milligram of protein⁻¹. The protein concentration was determined by the method of Lowry et al. (35a).

In order to measure residual OTCase activity after thermal denaturation, the enzyme (final concentration, 0.0035 mg/ml) was incubated in the absence of substrates for 15 min at different temperatures in 10 mM Tris HCl (pH 7.5) or potassium phosphate (pH 7.0); the pH was adjusted at each temperature. Samples were withdrawn and kept at 0°C, and their activity in the same buffer was measured under standard conditions.

Half-lives of activity ($t_{1/2}$) were determined by incubating the enzyme in 10 mM Tris HCl (pH 7.5), potassium phosphate (pH 7.0), or piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 7.0) at different temperatures for different times; the pH was adjusted at each temperature. Samples were withdrawn and kept at 0°C, and their activity in the same buffer was measured under standard conditions.

TABLE 1. Quaternary structure determination by gel filtration on Superdex 200

NaCl (mM)	%	
	Trimers	Dodecamers
0	100	0
150	15	85
300	10	90
500	2	98
1,000	0	100

RESULTS

Sequence analysis of *M. abyssi argF*. In *M. abyssi*, the OTCase-encoding gene *argF* was found to be part of a large divergent operon of which the genes identified so far are clustered in the order *ECBFGH(A)*, with *argE* constituting the left wing of the operon (59). Alignment of OTCase sequences by the Clustal program revealed that *M. abyssi argF* is clearly homologous to other OTCase genes. Remarkably, the amino acid sequence of OTCase_{Mab} seems to be more closely related to those of the trimeric OTCase of *Thermus thermophilus* (42.6% identity) and the dodecameric OTCase of the hyperthermophilic archaeon *P. furiosus* (43% identity) than to many others. This paradox is due to the complex phylogeny of OTCases, which appears to reflect the parallel development of subfamilies resulting from the differential loss of paralogous copies already present in the last common ancestor of the three domains (30). That psychrophilic and thermophilic enzymes would appear in the same subgroup is not surprising in itself, since relatively few structural features may account for large differences in temperature profiles (28).

Another striking feature is that the CP and ornithine binding motifs are only partly conserved; T56 (*E. coli* residue numbering) in the CP site (ST56RTR) is replaced by a leucine in OTCase_{Mab}, and L275 in the ornithine site (HCL275P) is replaced by a glutamine. The other known instances of deviations from these canonical sequences are the substitutions T56G in the phaseolotoxin-resistant OTCase from *Pseudomonas syringae* (27), T56L in the OTCase from *T. thermophilus* (40), and T56M in the OTCase from *Pisum sativum* (53). In no known OTCases other than that of *Moritella* does the ornithine binding site appear to be modified, except in cold-adapted mutants of *P. furiosus* OTCase (43). The OTCase from the closely related psychrophilic organism *Moritella profunda* (57) displays the same characteristics (Y. Xu, unpublished results). As for other genes of *M. abyssi* (59; Xu, unpublished), the codons AGA, AUA, and CUA, which are rare in *E. coli*, were found to be used several times in the sequence. This finding explains why an *E. coli* host improved for rare codon usage was required for *M. abyssi argF* expression.

Quaternary structure of recombinant OTCase. As shown by N-terminal sequencing and a nucleotide sequence comparison, the recombinant monomer contains 301 amino acid residues (32,873 Da) starting at Met1. The molecular mass of the recombinant OTCase was estimated by native PAGE and gel filtration. In contrast with all other OTCases examined so far, we found that the pure enzyme was present as a mixture of trimers and dodecamers, with the relative proportions of the

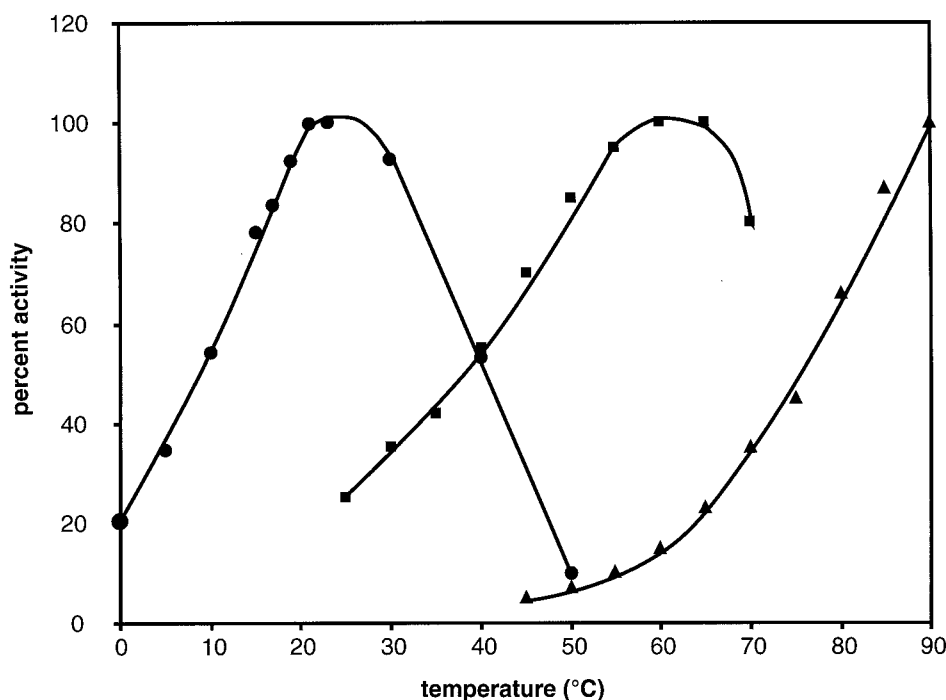


FIG. 1. Temperature dependence of OTCase_{Mab} activity (circles) compared to that of *E. coli* (squares) (56) and *P. furiosus* (triangles) (32) homologues. The *P. furiosus* curve represents the reverse reaction. Activity assays were performed under the conditions described in Materials and Methods. The data presented are the means of triplicate measurements; the standard errors of the means were less than 10%.

two forms depending on the salt concentration (Table 1). In addition, the trimeric fraction could reassociate into dodecamers when the salt concentration was increased. The salt requirement of *M. abyssi*, which is a marine bacterium, can be met with half-strength seawater (57), but the intracellular salt concentration is not known. Assuming intracellular concentrations of free Na⁺ and K⁺ of between 0.15 and 0.3 M in moderate halophiles (23, 49), it appears that in vivo, the main fraction of OTCase_{Mab} is in the dodecameric form.

Temperature dependence of activity and kinetic parameters. The apparent optimal temperatures for the activity of OTCase_{Mab} were found to be about 23 to 25°C, in sharp contrast to those of its mesophilic and thermophilic homologues (Fig. 1). However, about 37% of this maximal activity could still be recorded at 5°C. Almost identical results were obtained with the native enzyme in crude extracts of *Moritella* and with the recombinant enzyme in *E. coli* cell lysates (data not shown).

The optimal pHs for the reaction at 20°C were found to be between 9.0 and 10.0 (data not shown), whereas *E. coli* and *T. thermophilus* OTCases display broad optimal activity at about pH 8.0 (31, 45). As expected for an anabolic OTCase (9), the reaction catalyzed by OTCase_{Mab} displayed no allosteric kinetics. The enzyme was inhibited by excess ornithine, a common feature of OTCases (9). In *E. coli*, this effect has been attributed to noncompetitive inhibition of CP (31). Interestingly, a similar substrate inhibition of OTCase_{Mab} by excess ornithine (above 25 mM) was noticeable only at the lowest temperature investigated, 5°C (Fig. 2A), whereas at 20°C, a very high ornithine concentration (above 150 mM) was necessary to induce inhibition. The kinetic parameters for ornithine and CP (Fig.

2) are reported in Table 2, and the results are compared to the cognate values obtained for other OTCases. The K_m values for both substrates were, in general, much higher for OTCase_{Mab}, especially for ornithine. The latter parameter displayed a strong temperature dependence, whereas the K_m value for CP remained practically unaffected by changes in temperature. When compared at environmental temperatures, the high K_m and low k_{cat} values of OTCase_{Mab} at 5°C were responsible for the weak k_{cat}/K_m ratios. The bisubstrate analogue PALO, a competitive inhibitor of both CP and ornithine (42), inhibits OTCase_{Mab} only weakly at 20°C, with an apparent K_i of $11.5 \pm 0.4 \mu\text{M}$ (mean and standard error of the mean); the K_i for *E. coli* OTCase is 0.8 μM . As shown in Table 1, increasing the NaCl concentration favored oligomerization of the trimeric form of the enzyme into a dodecamer. Increasing the NaCl concentration up to 0.5 M in Tris buffer at 20°C decreased the k_{cat} parameter of the reaction by 31%; the corresponding value for phosphate buffer was 24%. This high NaCl concentration had only a limited effect on K_m values; a 1.5-fold increase for ornithine and a 2-fold increase for CP were recorded.

Thermal inactivation and structural stability. The inactivation curves, especially in Tris buffer, did not follow first-order kinetics but were distinctly biphasic, suggesting the presence of two populations of enzyme molecules with different stabilities. Very similar results were obtained with the native enzyme in crude extracts of *Moritella* and with the recombinant enzyme in *E. coli* cell lysates. These findings can be tentatively related to the equilibrium between the trimeric and the dodecameric forms, which have different stabilities (see below). Table 3 compares the half-lives of activity of OTCase_{Mab} obtained from the first part of the inactivation curves with data obtained

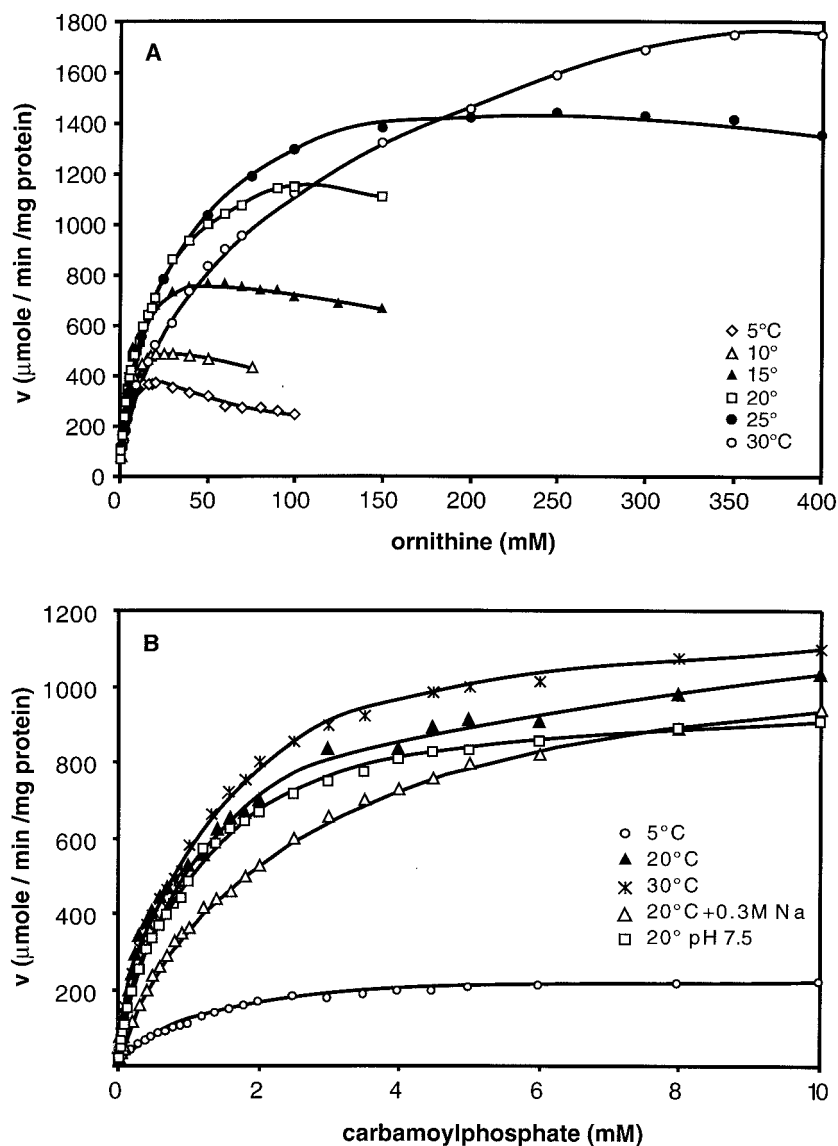


FIG. 2. Effects of the concentrations of L-ornithine (A) and CP (B) on the velocity (v) of the reaction catalyzed by $OTCase_{Mab}$ under standard conditions (unless otherwise indicated) in the presence of 20 mM CP and 100 mM ornithine, respectively. Na, NaCl.

from mesophilic and thermophilic homologues. Like other $OTCases$, $OTCase_{Mab}$ is more stable in potassium phosphate buffer than in Tris HCl buffer; stability in PIPES buffer was 1.5 times higher than that in Tris HCl buffer. Indeed, phosphate is a product of the reaction known to stabilize $OTCases$ against thermal inactivation (31, 33). These half-lives also illustrate the heat-labile character of $OTCase_{Mab}$.

Heat-induced unfolding of $OTCase_{Mab}$ recorded by DSC was found to be irreversible, with a large deviation from the two-state model. Kinetically driven protein unfolding is scan rate and protein concentration dependent (52). Accordingly, $OTCase$ thermograms were recorded at a constant scan rate (1 K/min) and a constant enzyme concentration (Table 4). Heat-induced unfolding was recorded at salt concentrations at which either the trimeric form (no salt added) or the dodecameric form (0.5 M NaCl) of $OTCase$ prevails. As shown in Fig. 3, the latter condition induced strong enzyme stabilization, with

marked increases in both the melting point (the midpoint of the transition) and the heat absorbed during unfolding (area under the peak). At a physiological salt concentration (0.3 M), both NaCl and KCl displayed the same stabilization efficiencies. However, the anion concentration played a crucial role in this effect, because at the same ionic strength (0.1 M Na_2SO_4), the sulfate ion had a weaker stabilization capacity. Table 4 also illustrates the very weak structural stability of $OTCase_{Mab}$ compared to that of its thermophilic homologue as far as the melting point and enthalpy of unfolding are concerned.

DISCUSSION

$OTCase_{Mab}$ is the first enzyme of its family to have been studied in an obligate psychrophilic organism. Its anabolic function may be inferred from the repressibility of its synthesis and the inclusion of the cognate gene in a biosynthetic operon

TABLE 2. Kinetic parameters for different OTCases

Organism	Max growth rate temp (°C)	Assay conditions (pH)	Assay temp (°C)	K_m Apparent (mM) for:		k_{cat} (s^{-1})	k_{cat}/K_m for:		Source or reference	
				Ornithine	CP		Ornithine	CP		
<i>M. abyssi</i>	4	Tris (9.0) ^a	30	45.0	0.9	690	15	766	This work	
			25	22.49		750	33		This work	
			20	8.0 ^b	1.0 ^b	694	87	694		This work
			15	5.67		546	97			This work
			10	3.34		382	114			This work
			5	1.78 ^b	1.1 ^b	235	132	214		This work
<i>Saccharomyces cerevisiae</i>	30	Tris (8.5)	30	0.9	0.2	354	393	1,770	13	
<i>E. coli</i> W	37	Tris (8.0)	37	2.4 ^b	0.2 ^b	1,732	722	8,660	31	
<i>T. thermophilus</i>	75	Tris (7.0)	55	0.1	0.1	74	740	740	45	
			70			146 ^c				
<i>P. furiosus</i>	102	Tris (7.3) or PIPES (7.0)	55	0.13	0.13	35	269	269	33	
			95			175 ^c				

^a There were no significant differences in K_m values at pH 7.5 and 20°C.

^b Real K_m .

^c Extrapolated due to CP thermolability.

(59). The apparent optimal temperature of OTCase_{Mab}, 23 to 25°C, is one of the lowest reported for enzymes isolated from psychrophilic organisms (6, 11, 15, 37, 39). At 5°C, a temperature close to that allowing maximal growth of the organism (4°C), the activity of the enzyme is still about 37% maximum. Cold-active enzymes are frequently thermolabile, a fact which is understandable, since they have to be flexible enough to be active at a low energy cost (14). OTCase_{Mab} is indeed considerably less resistant to thermal inactivation (Table 3) and has a weaker conformational stability (Table 4) than its *E. coli* or thermophilic counterparts (32, 33, 45). Psychrophilic enzymes counteract the inhibitory effect of low temperatures on activity by reducing the temperature dependence of the reaction rate (low activation enthalpy). Because this low activation enthalpy reflects the smaller number of enthalpy-driven interactions that have to be broken to reach the activated transition state, it has been proposed that the activity of a psychrophilic enzyme is heat labile, as these interactions also contribute to the active-site architecture (18, 35). However, the salt-dependent oligomerization state (trimeric or dodecameric) of OTCase_{Mab} also seems to be involved in its stability.

TABLE 3. Half-lives of activities of psychrophilic, mesophilic, and thermophilic OTCases

Organism	Temp (°C)	Half-life ^a in the following buffer (pH):		
		Potassium phosphate (7.0)	PIPES-NaOH (7.0)	Tris HCl (7.5)
<i>M. abyssi</i>	50	60	14	9
	55	40	4.5	3
	60	2		
<i>E. coli</i>	68	43 ^b		5
<i>P. furiosus</i>	100	65	60	40

^a Reported in minutes.

^b Value was determined with 10 mM potassium phosphate in 300 mM Tris HCl (pH 8.0).

This involvement of quaternary structure in the stability of OTCase activity is particularly clear for *P. furiosus* OTCase, which is a very stable dodecamer whose architecture and stability are dependent mainly on a network of hydrophobic interactions between trimers (7, 51). A mutant of *P. furiosus* OTCase (E25Q/M29A/W33A) in which the intertrimeric interactions are lost becomes much less thermostable and exclusively trimeric while retaining the same global kinetic properties of the wild-type enzyme (7). The half-life of the residual activity at 85°C dropped from 150 min (300 min in 0.2 M KCl) for the wild-type dodecameric *P. furiosus* OTCase to 2.5 min (13 min in 200 mM KCl) for the mutant trimeric enzyme, illustrating the involvement of quaternary structure in the stability of activity. Accordingly, the unusual equilibrium between trimers and dodecamers in OTCase_{Mab}, which arises from weak interactions between trimers, should also contribute to the heat lability of its activity. As shown by DSC (Table 4 and Fig. 3), the salt-dependent oligomerization state of OTCase_{Mab} strongly affects the stability of the molecule, favoring a more stable dodecamer. The difference in stability between the trimeric and the dodecameric forms of OTCase_{Mab} has two possible origins: the preferential exclusion of the cosolvent arising from the high salt concentration (34) and the oligomeric state

TABLE 4. DSC parameters for thermal unfolding of OTCase_{Mab} and *P. furiosus* OTCase

Organism	Buffer supplement	OTCase (μM)	T_m (°C)	Calorimetric enthalpy (kcal mol ⁻¹)
<i>M. abyssi</i>	None	48.7	56.6	66
	0.1 M Na ₂ SO ₄	46.6	66.4	123
	0.3 M NaCl	47.3	70.5	145
	0.3 M KCl	48.7	70.5	144
	0.5 M NaCl	49.0	71.7	155
<i>P. furiosus</i> ^a		43.2	104.0	842

^a Data are from B. Clantin (personal communication).

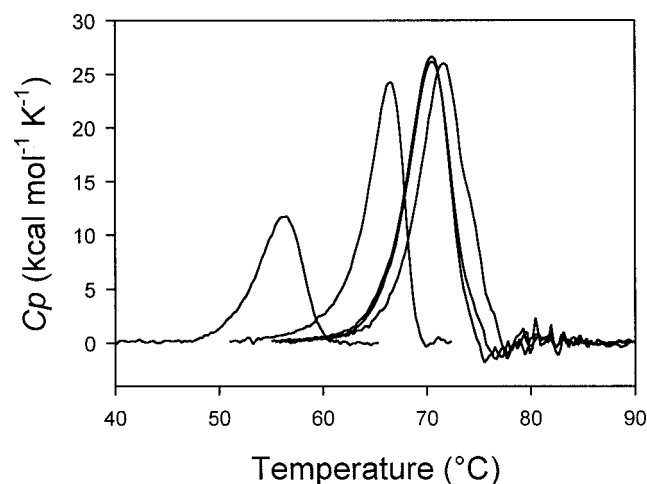


FIG. 3. Heat-induced unfolding of $OTCase_{Mab}$ recorded by DSC. Curves show the following, from left to right: without added salt, with 0.1 M Na_2SO_4 , with 0.3 M NaCl or KCl (superimposed curves), and with 0.5 M NaCl. The melting point corresponds to the top of the denaturation peak, and the calorimetric enthalpy corresponds to the area under the peak. Thermograms have been baseline subtracted and normalized for protein concentration. C_p , heat capacity change for unfolding; K, degrees Kelvin.

of the enzyme. As both parameters are interdependent, their relative contributions cannot be firmly established. It should be noted, however, that the increase in stability recorded for $OTCase$ is in the upper range of values reported for the effects of salts on proteins at a neutral pH (2). Accordingly, it can be assumed that the oligomeric state also contributes to the greater stability of the dodecameric $OTCase$.

What could be the significance of a dodecameric state for $OTCase_{Mab}$, an enzyme which is neither allosteric, as in *P. aeruginosa*, nor thermophilic, as in *P. furiosus*? The fact that it has to function in its natural environment under relatively high pressures (28 MPa at the site of capture) must be considered. The positive effect of salt on the oligomeric state of $OTCase_{Mab}$ suggests that a substantial fraction of the enzyme may be dodecameric in vivo, at least under atmospheric pressures. However, it is not yet known whether increasing pressures will tend to dissociate this enzyme into trimers or, on the contrary, stabilize the dodecameric state. Very little is known about genuinely psychropiezophilic enzymes (25).

Comparing $OTCases$ from organisms whose G+C contents are not divergent enough to obscure biologically significant differences (*Moritella*, 42%; *E. coli*, 48 to 52%, *Thermotoga maritima*, 46%) makes it obvious that as a percentage of the total, the sum of the charged residues (KRED) available to provide stabilizing interactions is slightly lower in *Moritella* (21.3%) than in *E. coli* (23.1%) and much lower in *Moritella* than in the hyperthermophilic bacterium *T. maritima* (28.8%; M. Van de Castele, unpublished data, NCBI accession number Y10661), particularly with regard to arginine (3.0, 3.3, and 6.1%, respectively), which can play an important role in protein stabilization through its high level of hydrophilicity and hydrogen-bonding capacity (40). Moreover, the thermolabile residues asparagine and glutamine are more represented in *Moritella* and *E. coli* (8.0 and 8.1%, respectively) than in *T.*

maritima (5.7%). These observations are probably related to the psychrophilic nature of $OTCase_{Mab}$, but direct three-dimensional studies are required to examine their significance.

The comparative analysis of kinetic parameters among various $OTCases$ proved highly significant in terms of functional adaptation to cold. Ideally, adapting an enzyme to cold would mean optimizing both K_m and k_{cat} . Such a trend was noted for enzymes from Antarctic or Arctic fishes and some bacterial enzymes (4, 14, 21). However, a survey of cold-adapted enzymes showed that optimization of the k_{cat}/K_m ratio is far from universal (25, 60); for instance, markedly high K_m values have been reported for psychrophilic glutamate dehydrogenase (12), citrate synthase (22), aspartate carbamoyltransferase (48, 58), aspartate aminotransferase (5), triosephosphate isomerase (1), DNA ligase (20), elongation factor Tu (36), subtilisin (41), xylanase (8), and alpha-amylase (10). $OCTase_{Mab}$ obviously belongs to this group, as shown by the high K_m values for both ornithine and CP as well as by the high K_i for the bisubstrate analogue PALO, indicating a low substrate binding affinity. Therefore, it appears that numerous psychrophilic enzymes improve k_{cat} values at low temperatures at the expense of K_m values. Several aspects seem to be involved in this adaptive strategy. (i) From the kinetic and thermodynamic theories, it is well known that weak substrate binding is catalytically advantageous (17); indeed, the ground-state enzyme-substrate complex falls in a less deep energy pit, therefore reducing the energy barrier (and increasing k_{cat}) for the reaction (14). (ii) The large activation entropy variation in psychrophilic enzymes suggests large conformational movements between a loose active site in the free state and the tightly bound transition state (18, 35); such loosely structured active sites should bind a substrate weakly. (iii) According to the folding-funnel hypothesis and to achieve the flexibility required for an enzyme to function efficiently at a lower temperature, a larger number of conformational states must be available to the enzyme; therefore, the enzyme may exhibit a higher K_m and, possibly, a lower affinity for the substrate(s) of the reaction (18, 35). (iv) Finally, the specific amino acid substitutions noted for the ornithine and CP binding sites of $OCTase_{Mab}$ are possible structural determinants of such weak substrate binding. It is worth mentioning that mutants of *P. furiosus* $OTCase$ selected in vivo to complement yeast mutants lacking $OTCase$ at 30 and even 15°C displayed a much higher K_m value for ornithine and a much higher K_i value for the bisubstrate analogue PALO than the wild type, a lower apparent optimal temperature, a higher k_{cat} value (up to fivefold), and a markedly increased thermolability (43); this example is one of the rare cases (see also references 38 and 54) where it could be observed directly that thermolability was not a property acquired during genetic drift at a low temperature independent of the changes responsible for kinetic adaptation.

In conclusion, $OTCase_{Mab}$ appears to be suboptimal in its physiological temperature range as far as the k_{cat}/K_m ratio is concerned. The observed trade-off between affinity and catalytic velocity indicates the limits that can be encountered in optimizing the kinetic parameters of metabolic enzymes at low temperatures. From the point of view of evolution, this means that in many cases, a psychrophilic organism may respond to part of the pressure to adapt to low temperatures by making more of an imperfect enzyme; regulatory effects would there-

fore be expected to be important elements in evolutionary strategy. Furthermore, by comparing the thermodynamic and kinetic features of different series of homologous enzymes, it may be possible to determine whether such natural shortcomings of optimization result mainly from the barrier set by the enthalpic-entropy balance or are significantly influenced by structural constraints of evolutionary origin, for example, by the fact that the ancestor of a psychrophilic enzyme was adapted to higher temperatures. In this respect, it is interesting that in the polyphyletic pattern of OTCases, OTCase_{Mab} falls in a broad subgroup containing several thermophilic enzymes (30). Although it has become doubtful that the last common ancestor of the three domains of life was a hyperthermophile (19; reviewed in reference 55), it could still have been a moderate thermophile.

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REFERENCES

- Alvarez, M., J. P. Zeelen, V. Mainfroid, F. Rentier-Delrue, J. A. Martial, L. Wyns, R. K. Wierenga, and D. Maes. 1998. Triose-phosphate isomerase (TIM) of the psychrophilic bacterium *Vibrio marinus*. Kinetic and structural properties. *J. Biol. Chem.* **273**:2199–2206.
- Arakawa, T., R. Bhat, and S. N. Timasheff. 1990. Why preferential hydration does not always stabilize the native structure of globular proteins? *Biochemistry* **29**:1924–1931.
- Archibald, R. M. 1944. Determination of citrulline and allantoin and demonstration of citrulline in blood plasma. *J. Biol. Chem.* **156**:121–142.
- Bentahir, M., G. Feller, M. Aittaleb, J. Lamotte-Brasseur, T. Himri, J. P. Chessa, and C. Gerday. 2000. Structural, kinetic and calorimetric characterization of the cold-active phosphoglycerate kinase from the antarctic *Pseudomonas sp.* TACII18. *J. Biol. Chem.* **275**:11147–11153.
- Birolo, L., M. L. Turino, B. Fontanella, C. Gerday, K. Mainolfi, S. Pascarella, G. Sannia, F. G. Vinci, and G. Marino. 2000. Aspartate aminotransferase from the Antarctic bacterium *Pseudoalteromonas haloplanctis* TAC 125. Cloning, expression, properties and molecular modelling. *Eur. J. Biochem.* **267**:2790–2802.
- Cavicchioli, R., K. S. Siddiqui, D. Andrews, and R. K. Sowers. 2002. Low-temperature extremophiles and their applications. *Curr. Opin. Biotechnol.* **13**:253–261.
- Clantin, B., C. Tricot, T. Lonhienne, V. Stalon, and V. Villeret. 2001. Probing the role of oligomerization in the high thermal stability of *Pyrococcus furiosus* ornithine carbamoyltransferase by site-specific mutants. *Eur. J. Biochem.* **268**:3937–3942.
- Collins, T., M. A. Meeuwis, I. Stals, M. Claeysens, G. Feller, and C. Gerday. 2002. A novel family 8 xylanase: functional and physicochemical characterization. *J. Biol. Chem.* **277**:35815–35818.
- Cunin, R., N. Glansdorff, A. Piérard, and V. Stalon. 1986. Biosynthesis and metabolism of arginine in bacteria. *Microbiol. Rev.* **50**:314–352.
- D'Amico, S., C. Gerday, and G. Feller. 2001. Structural determinants of cold adaptation and stability in a large protein. *J. Biol. Chem.* **276**:25791–25799.
- Davail, S., G. Feller, E. Narinx, and C. Gerday. 1994. Cold adaptation of proteins. Purification, characterization and sequence of the heat-labile subtilisin from the antarctic psychrophile *Bacillus* TA41. *J. Biol. Chem.* **269**:17448–17453.
- Di Fraia, R., V. Wilquet, M. A. Ciardello, V. Carratore, A. Antignani, L. Camardella, N. Glansdorff, and G. di Prisco. 2000. NADP⁺-dependent glutamate dehydrogenase in the Antarctic psychrotolerant bacterium *Psychrobacter sp.* TAD1. Characterization, protein and DNA sequence, and relationship to other glutamate dehydrogenases. *Eur. J. Biochem.* **267**:121–131.
- Eisenstein, E., J. C. Osborne, Jr., I. M. Chaiken, and P. Hensley. 1984. Purification and characterization of ornithine transcarbamylase from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **259**:5139–5145.
- Feller, G., and C. Gerday. 1997. Psychrophilic enzymes: molecular basis of cold adaptation. *Cell. Mol. Life Sci.* **53**:830–841.
- Feller, G., F. Payan, F. Theys, M. Qian, R. Haser, and C. Gerday. 1994. Stability and structural analysis of alpha-amylose from the antarctic psychrophile *Alteromonas haloplanctis* A23. *Eur. J. Biochem.* **222**:441–447.
- Feller, G., D. d'Amico, and C. Gerday. 1999. Thermodynamic stability of an cold-active amylose from the Antarctic bacterium *Alteromonas haloplanctis*. *Biochemistry* **38**:4613–4619.
- Fersht, A. 1985. Enzyme structure and mechanism. W. H. Freeman & Company, New York, N.Y.
- Fields, P. A., and G. N. Somero. 1998. Hot spots in cold adaptation: localized increases in conformational flexibility in lactate dehydrogenase A (4) orthologs of Antarctic notothenioid fishes. *Proc. Natl. Acad. Sci. USA* **95**:11476–11481.
- Galtier, N., N. Tourasse, and M. Gouy. 1999. A non-hyperthermophilic common ancestor to extant life forms. *Science* **283**:220–221.
- Georlette, D., Z. O. Jonsson, F. Van Petegem, J. Chessa, J. Van Beeumen, U. Hubscher, and C. Gerday. 2000. A DNA ligase from the psychrophile *Pseudoalteromonas haloplanctis* gives insights into the adaptation of proteins to low temperatures. *Eur. J. Biochem.* **267**:3502–3512.
- Gerday, C., M. Aittaleb, J. L. Arpigny, E. Baise, J. P. Chessa, J. M. François, I. Petrescu, and G. Feller. 1999. Cold enzymes: a hot topic, p. 257–275. *In* R. Margesin and F. Schinner (ed.), *Cold-adapted organisms. Ecology, physiology, enzymology, and molecular biology*. Springer-Verlag, Heidelberg, Germany.
- Gerike, U., M. J. Danson, N. J. Russell, and D. Hough. 1997. Sequencing and expression of the gene encoding a cold-active citrate synthase from an Antarctic bacterium, strain DS2–3R. *Eur. J. Biochem.* **248**:49–57.
- Gilboa, H., M. Kogut, S. Chalamish, R. Regev, Y. Avi-Dor, and N. J. Russell. 1991. Use of ²³Na nuclear magnetic resonance spectroscopy to determine the true intracellular concentration of free sodium in a halophilic eubacterium. *J. Bacteriol.* **173**:7021–7023.
- Glansdorff, N. 1965. Topography of cotransducible arginine mutations in *Escherichiacoli* K12. *Genetics* **51**:167–179.
- Glansdorff, N., and Y. Xu. 2002. Microbial life at low temperatures: mechanisms of adaptation and extreme biotopes. Implications for exobiology and the origin of life. *Recent Res. Dev. Microbiol.* **6**:1–21.
- Ha, Y., M. T. McCann, M. Tuchman, and N. M. Allewell. 1997. Substrate-induced conformational change in a trimeric ornithine transcarbamoylase. *Proc. Natl. Acad. Sci. USA* **94**:9550–9555.
- Hatziloukas, E., and N. J. Panopoulos. 1992. Origin, structure, and regulation of *argK*, encoding the phaseolotoxin-resistant ornithine carbamoyltransferase in *Pseudomonas syringae* pv. *phaseolicola*, and functional expression of *argK* in transgenic tobacco. *J. Bacteriol.* **174**:5895–5909.
- Jaenicke, R. 1991. Protein stability and molecular adaptation to extreme conditions. *Eur. J. Biochem.* **202**:715–728.
- Jin, L., B. A. Seaton, and J. F. Head. 1997. Crystal structure at 2.8 Å resolution of anabolic ornithine transcarbamylase from *Escherichia coli*. *Nat. Struct. Biol.* **4**:622–625.
- Labeledan, B., A. Boyen, M. Baetens, D. Charlier, P. Chen, R. Cunin, V. Durbecq, N. Glansdorff, G. Hervé, C. Legrain, Z. Liang, C. Purcareau, M. Roovers, R. Sanchez, T. L. Thia-Toong, M. Van de Castelee, F. Van Vliet, Y. Xu, and Y. Zhang. 1999. The evolutionary history of carbamoyltransferases: a complex set of paralogous genes was already present in the last universal common ancestor. *J. Mol. Evol.* **49**:461–473.
- Legrain, C., and V. Stalon. 1976. Ornithine carbamoyltransferase from *Escherichia coli* W. Purification, structure and steady-state kinetic analysis. *Eur. J. Biochem.* **63**:289–301.
- Legrain, C., V. Villeret, M. Roovers, D. Gigot, O. Dideberg, A. Piérard, and N. Glansdorff. 1997. Biochemical characterisation of ornithine carbamoyltransferase from *Pyrococcus furiosus*. *Eur. J. Biochem.* **247**:1046–1055.
- Legrain, C., V. Villeret, M. Roovers, C. Tricot, B. Clantin, J. Van Beeumen, V. Stalon, and N. Glansdorff. 2001. The ornithine carbamoyltransferase of *Pyrococcus furiosus*. *Methods Enzymol.* **331**:227–235.
- Lin, T. Y., and S. N. Timasheff. 1996. On the role of surface tension in the stabilization of globular proteins. *Protein Sci.* **5**:372–381.
- Lonhienne, T., C. Gerday, and G. Feller. 2000. Psychrophilic enzymes: revisiting the thermodynamic parameters of activation may explain local flexibility. *Biochim. Biophys. Acta* **1543**:1–10.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
- Masullo, M. P. Arcari, B. de Paola, A. Parmeggiani, and V. Bocchini. 2000. Psychrophilic elongation factor Tu from the Antarctic *Moraxella sp.* Tac II 25: biochemical characterization and cloning of the encoding gene. *Biochemistry* **39**:15531–15539.
- McDonald, I. J., C. Quadling, and A. K. Chambers. 1963. Proteolytic activity of some cold-tolerant bacteria from Arctic sediments. *Can. J. Microbiol.* **9**:303–315.
- Merz, A., M. C. Yee, H. Szadowski, G. Pappenberger, A. Cramer, W. P. Stemmer, C. Yanofsky, and K. Kirschner. 2000. Improving the catalytic activity of a thermophilic enzyme at low temperatures. *Biochemistry* **39**:880–889.
- Morita, R. Y. 1975. Psychrophilic bacteria. *Bacteriol. Rev.* **39**:144–167.
- Mrabet, N. T., A. Van den Broeck, I. Van den brande, P. Stanssens, Y.

- Laroche, A. M. Lambeir, G. Matthijssens, J. Jenkins, M. Chiadmi, H. van Tilbeurgh, J. Janin, W. J. Quax, I. Lasters, M. De Maeyer, and S. J. Wodak. 1992. Arginine residues as stabilizing elements in proteins. *Biochemistry* **31**:2239–2253.
41. Narinx, E., E. Baise, and C. Gerday. 1997. Subtilisin from psychrophilic antarctic bacteria: characterization and site-directed mutagenesis of residues possibly involved in the adaptation to cold. *Protein Eng.* **10**:1271–1279.
 42. Penninckx, M., and D. Gigot. 1978. Synthesis and interaction with *Escherichia coli* L-ornithine carbamoyltransferase of two potential transition-state analogues. *FEBS Lett.* **88**:94–96.
 43. Roovers, M., R. Sanchez, C. Legrain, and N. Glansdorff. 2001. Experimental evolution of enzyme temperature activity profile: selection in vivo and characterization of low-temperature-adapted mutants of *Pyrococcus furiosus* ornithine carbamoyltransferase. *J. Bacteriol.* **183**:1101–1105.
 44. Russell, N. J. 2000. Toward a molecular understanding of cold-activity of enzymes from psychrophiles. *Extremophiles* **4**:83–90.
 45. Sanchez, R., M. Baetens, M. Van de Castele, M. Roovers, C. Legrain, and N. Glansdorff. 1997. Ornithine carbamoyltransferase from the extreme thermophile *Thermus thermophilus*. Analysis of the gene and characterization of the protein. *Eur. J. Biochem.* **248**:466–474.
 46. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
 47. Shi, D., H. Morizono, Y. Ha, M. Aoyagi, M. Tuchman, and N. M. Allewell. 1998. A resolution crystal structure of human ornithine transcarbamoylase complexed with N-phosphonoacetyl-L-ornithine. Catalytic mechanism and correlation with inherited deficiency. *J. Biol. Chem.* **273**:34247–34254.
 48. Sun, K., L. Camardella, G. di Prisco, and G. Hervé. 1998. Properties of aspartate transcarbamylase from TAD1, a psychrophilic bacterium from Antarctica. *FEMS Microbiol. Lett.* **164**:375–382.
 49. Ventosa, A., J. J. Nieto, and A. Oren. 1998. Biology of moderately halophilic aerobic bacteria. *Microbiol. Mol. Biol. Rev.* **62**:504–544.
 50. Villeret, V., C. Tricot, V. Stalon, and O. Dideberg. 1995. Crystal structure of *Pseudomonas aeruginosa* catabolic ornithine transcarbamoylase at 3.0-Å resolution: a different oligomeric organization in the transcarbamoylase family. *Proc. Natl. Acad. Sci. USA* **92**:10762–10766.
 51. Villeret, V., B. Clantin, C. Tricot, C. Legrain, M. Roovers, V. Stalon, N. Glansdorff, and J. Van Beeumen. 1998. The crystal structure of *Pyrococcus furiosus* ornithine carbamoyltransferase reveals a key role for oligomerization in enzyme stability at extremely high temperatures. *Proc. Natl. Acad. Sci. USA* **95**:2801–2806.
 52. Vogl, V., C. Jatzke, H. J. Hinz, J. Benz, and R. Huber. 1997. Thermodynamic stability of annexin V E17G: equilibrium parameters from an irreversible unfolding reaction. *Biochemistry* **36**:1657–1668.
 53. Williamson, C. L., M. R. Lake, and R. D. Slocum. 1996. Isolation and characterization of a cDNA encoding a pea ornithine transcarbamoylase (*argF*) and comparison with other transcarbamoylases. *Plant Mol. Biol.* **31**:1087–1092.
 54. Wintrode, P. L., K. Miyzaki, and F. H. Arnold. 2000. Cold adaptation of a mesophilic subtilisin-like protease by laboratory evolution. *J. Biol. Chem.* **275**:31635–31640.
 55. Xu, Y., and N. Glansdorff. 2002. Was our ancestor a hyperthermophilic prokaryote? *Comp. Biochem. Physiol. A* **133**:677–688.
 56. Xu, Y., Z. Liang, C. Legrain, V. Villeret, J. Van Beeumen, and N. Glansdorff. 1999. Genes and enzymes involved in arginine and pyrimidine biosynthesis in psychrophilic *Vibrio* strains from the deep sea, p. 319–333. In R. Margesin and F. Schinner (ed.), *Cold-adapted organisms. Ecology, physiology, enzymology, and molecular biology*. Springer-Verlag, Heidelberg, Germany.
 57. Xu, Y., Y. Nogi, Y. C. Kato, Z. Liang, H. J. Rüger, D. De Kegel, and N. Glansdorff. *Moritella profunda* sp. nov. and *Moritella abyssi* sp. nov., two psychropiezophilic organisms isolated from deep Atlantic sediments. *Int. J. Syst. Evol. Microbiol.*, in press.
 58. Xu, Y., Y. F. Zhang, Z. Liang, M. Van de Castele, C. Legrain, and N. Glansdorff. 1998. Aspartate carbamoyltransferase from a psychrophilic deep-sea bacterium, *Vibrio* strain 2693. Properties of the enzyme, genetic organisation and expression in *Escherichia coli*. *Microbiology* **144**:1435–1441.
 59. Xu, Y., Z. Liang, C. Legrain, H. J. Rüger, and N. Glansdorff. 2000. Evolution of arginine biosynthesis in the bacterial domain: novel gene-enzyme relationships from psychrophilic *Moritella* strains (*Vibrionaceae*) and the evolutionary significance of N- α -acetyl ornithinase. *J. Bacteriol.* **182**:1609–1615.
 60. Zecchinon, L., P. Claverie, T. Collins, S. d'Amico, D. Delille, G. Feller, D. Georlette, E. Gratia, A. Hoyoux, M. A. Meuwis, G. Sonan, and C. Gerday. 2001. Did psychrophilic enzymes really meet the challenge? *Extremophiles* **5**:313–321.