



Thermodynamic stability of proteins adapted to extreme temperatures

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ABSTRACT

Small cold shock proteins from bacteria are model proteins for many biophysical studies as a result of their small size and compact folding. Here we report a stability study of these proteins from the Antarctic psychrophile *Pseudoalteromonas haloplanktis*, the mesophile *Escherichia coli* and the hyperthermophile *Thermotoga maritima*. When analyzed by differential scanning calorimetry, the three proteins unfolded fully reversibly according to a perfect two-state model, which are strict prerequisites to apply equilibrium thermodynamic equations. The parameters obtained by differential scanning calorimetry allowed to calculate the thermodynamic stability of the three proteins and to draw their bell-shaped stability curves, i.e. the free energy of unfolding as a function of temperature. These curves provided fundamentals of stability for proteins adapted to extreme temperatures. For instance, the maximal stability of the three proteins was reached around room temperature and is not shifted toward high temperature for the hyperthermophilic protein, nor to low temperature for the psychrophilic one. The latter is heat-labile but is also cold-labile. Therefore, cold unfolding appears as a new but unsuspected factor limiting life at low temperatures. The distinct temperature-induced dynamics of these proteins are also discussed.

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1. Introduction

The range of environmental temperatures compatible with life has been substantially enlarged these last decades. At high temperature, the archaeon *Pyrolobus fumarii* growing at 113 °C was long regarded as the most resistant microorganism [1]. But latter, *Methanopyrus kandleri* thriving at 122 °C and enduring exposure up to 130 °C has established the current upper temperature limit for life under *in situ* high pressure [2,3]. At the other extremity of the temperature scale, the brine veins between winter polar sea ice crystals host metabolically active psychrophilic bacteria at –20 °C [4,5]. Furthermore, *Planococcus halocryophilus* isolated from Arctic permafrost was found to divide at –15 °C and to be metabolically active at –25 °C [6], which possibly represents the lowest temperature for sustained life before dormancy.

Obviously, a vast array of adaptations at all cellular levels, including macromolecules is required to sustain microbial life under these extreme environmental temperatures. In this respect, proteins and enzymes ensure a critical role by driving the cell cycle and metabolism. Thermophilic and hyperthermophilic proteins have to maintain a functional native state at elevated temperatures that are not compatible for mesophilic proteins which would otherwise unfold. Their robust and heat-stable structure is the result of improvement in strength and number of all types of weak interactions and structural factors sta-

bilizing the protein conformation [7–9]. At low temperature, enzymes have to remain active and proteins have to maintain adequate functional dynamics at temperatures that slow down molecular motions. This is reached by adopting a mobile and flexible structure through reduction of all factors required to maintain protein conformation. But the price to pay for such an improved structural dynamics is the pronounced heat-lability of most psychrophilic proteins [10–12]. It should be emphasized that the same types of weak interactions and structural factors are altered in psychrophilic proteins, leading to their low stability, but are improved in thermophilic proteins to reach their stable conformation. Accordingly, a precise description of protein stability as a function of temperature is needed to analyze the adaptation to extreme temperatures.

In this respect, small cold shock proteins (Csp) from bacteria are models for many biophysical studies owing to their small size (~7 kDa) and compact folding into a β -barrel structure. These proteins are overexpressed during a sudden drop of the medium temperature and act as nucleic acid chaperones, inhibiting the formation of secondary mRNA structures at low temperatures, thereby facilitating the initiation of translation [13,14]. Their unfolding/refolding pathways have been recorded by various techniques including transverse urea gradient gel electrophoresis [15], differential scanning microcalorimetry [16], stopped-flow kinetic [17] or force-dependent folding using magnetic tweezers [18]. Several NMR structures are available, including from a psychrophile [19], from mesophiles such as *E. coli* [20] and from the hyperthermophile *T. maritima* [21], both latter being investigated here. We report a microcalorimetric study of Csp from the Antarctic psychrophile *Pseudoalteromonas haloplanktis* thriving around 0 °C [22,23], from the mesophile *E. coli* and from the hyperthermophilic bacterium *Thermotoga maritima* isolated from geothermi-

Abbreviations: Csp, small cold shock protein; CspP, Csp from *P. haloplanktis*; CspM, Csp from *E. coli*; CspT, Csp from *T. maritima*; DSC, differential scanning calorimetry.

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cally heated seafloor sediment around 80–90 °C [24] and noted CspP, CspM and CspT, respectively. As these Csp unfolded reversibly according to a perfect two-state model in microcalorimetry, it was possible to determine unambiguously their thermodynamic stability as a function of temperature. To the best of our knowledge, this is the first report of thermodynamic stability for proteins originating from such a large range of environmental temperatures.

2. Materials and methods

2.1. Expression and purification

The three Csp are referenced in UniProtKB under entries Q3ICV2 for CspP, P0A9X9 for CspM and O54310 for CspT. The corresponding genes were synthesized (with, for CspT, codon optimization for expression in *E. coli*) and inserted into the expression vector pET22b at the NdeI site. Colonies of the *E. coli* BL21 (DE3) cells transformed with this vector were used to inoculate a 25 ml preculture of Terrific broth TB (12 g/l Bactotryptone (Difco), 24 g/l yeast extract (Difco), 4 ml/l glycerol, 12.54 g/l K_2HPO_4 , 2.31 g/l KH_2PO_4 , pH 7.2) containing 100 mg/l ampicillin which was run at 18 °C for 1 h. Two-ml aliquots were used to inoculate 250 ml of TB in 2-L Erlenmeyer flasks and the cultures were incubated overnight at 18 °C and 250 rpm. Protein expression was induced by 1 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG) and following further 24-h incubation at 18 °C, the cells were harvested by centrifugation at 20000g for 30 min at 4 °C. As Csp were also found in the culture supernatant, the latter was treated by Benzonase, RNase and $MgSO_4$ for 1h then brought to 90% saturation $(NH_4)_2SO_4$ and the precipitated proteins were collected by centrifugation. The cell pellet was resuspended in 10 mM Tris-HCl, pH 7.2 containing Benzonase and a protease inhibitor cocktail (Complete, Roche), then disrupted in a pre-chilled high-pressure cell disrupter (Constant Systems) at 28 kpsi, and centrifuged at 20000 g for 30 min. For CspP and CspM, the lysis supernatant was then brought to 90% saturation in $(NH_4)_2SO_4$. The precipitated proteins collected by centrifugation were suspended in 10 mM Tris-HCl, pH 7.2. This fraction and the pellet of culture supernatant were pooled and diluted to 30% saturation $(NH_4)_2SO_4$ and the soluble fraction was recovered after centrifugation at 45000g. This fraction was loaded on a Phenyl-Sepharose CL-4B column (2.5 × 40 cm) equilibrated in 10 mM Hepes, 1 mM PMSF, 30% saturation in $(NH_4)_2SO_4$, pH 7.2. Proteins were eluted with a decreasing gradient from 30% to 0% saturation in $(NH_4)_2SO_4$. Fractions containing the Csp were then loaded on a Q-Sepharose FF column (2.5 × 40 cm) equilibrated in 10 mM Hepes, 1 mM PMSF, pH 7.2 and eluted with a linear gradient from 0 to 0.4 M NaCl. Fractions containing the Csp were concentrated by ultrafiltration on membrane with 1 kDa cut-off and loaded on a Superdex 75 column (2.5 × 100 cm) and eluted by 10 mM Tris-HCl, pH 7.2. CspT was purified essentially as described previously [16]. Purified proteins samples (~5 mg ml⁻¹) were stored at -70 °C.

2.2. Protein characterization

The purification process was followed by electrophoresis on Tricine-SDS-PAGE [25]. The protein concentration was determined by the BCA Protein Assay (Pierce). N-terminal amino acid sequence

of the recombinant proteins was determined by automated Edman degradation using a pulsed-liquid-phase protein sequencer Procise 494 (Applied Biosystems). Mass determination was performed on an ESI-Q-TOF instrument (Waters, Micromass) in positive ion mode. Samples (10 mM) were analyzed in 30% acetonitrile, 0.5% formic acid, 25 mM ammonium acetate. Spectra deconvolution technique of calculation was the maximum entropy (Max ent1).

2.3. Differential scanning calorimetry

Measurements under standard conditions were performed using a MicroCal VP-DSC instrument at a scan rate of 60 K h⁻¹ and under ~25 psi positive cell pressure. Samples (~2–3 mg ml⁻¹) were dialyzed overnight against 30 mM Mops, 100 mM NaCl, pH 7.2. Reference baselines were recorded using the dialysis buffer. After cell loading, protein concentration was determined on the remaining sample by the bicinchoninic acid method (Pierce). Thermograms were analyzed according to a non-two-state model in which the melting point T_m , the calorimetric enthalpy ΔH_{cal} and the van't Hoff enthalpy ΔH_{eff} of individual transitions are fitted independently using the MicroCal Origin software (version 7). The magnitude and source of the errors in the T_m and enthalpy values have been discussed elsewhere [26]. Fitting standard errors on a series of five DSC measurements made under the same conditions in the present study were found to be ± 0.1 K on T_m and $\pm 4\%$ on both enthalpies.

3. Results and discussion

3.1. Protein characterization

Three genes have been selected in order to investigate small cold shock proteins (Csps) from bacteria thriving at extreme temperatures, namely Csp from the Antarctic psychrophile *P. haloplanktis* (CspP), the mesophile *E. coli* (CspM) and the hyperthermophile *T. maritima* (CspT). Cysteine-free homologs having the highest identity with the CspA protein from *E. coli* (CspM) were selected. The native protein sequences were used, without any additional tag. The multiple sequence alignment between the three Csp is displayed in Fig. 1. These proteins possess a similar size (66–70 aa), with about 56–59% identity (80–83% similarity) between each pair and an overall identity of 44% (72% similarity). Interestingly, the bias in amino acid composition reported for proteins adapted to extreme temperatures [27,28] is not clearly observed in the small Csp (see Supporting Information). The superimposition of the three Csps structures is displayed in Fig. 2 which highlight the excellent conservation of the β -barrel conformation.

The N-terminal amino acid sequence of the recombinant proteins has been determined by automated Edman degradation and the M_r has been calculated by mass spectrometry on an ESI-Q-TOF instrument. As shown in Table 1, the N-terminal formyl methionine (*fMet*) from CspP is totally removed, but 65% are cleaved in CspM whereas *fMet* remain uncleaved in CspT. This is in line with previous observations showing that N- and C-terminal extremities, as preferential site for unzipping, are loose or relaxed in psychrophilic proteins but are buried or constrained by various interactions in thermophilic proteins [27,29]. Accordingly, during synthesis,

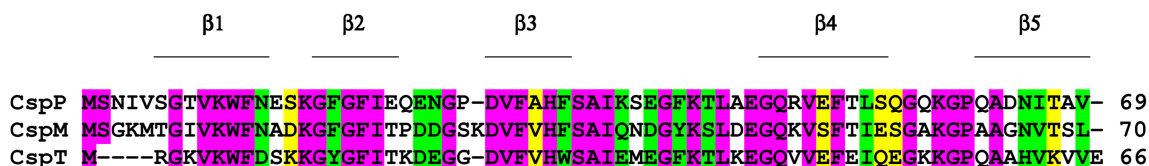


Fig. 1. Multiple sequence alignment of the three Csp. Amino acids are colored according to similar side chain properties: purple, identity; green, similarity; yellow, partial similarity. The five β -strands are also delineated according to 21 (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

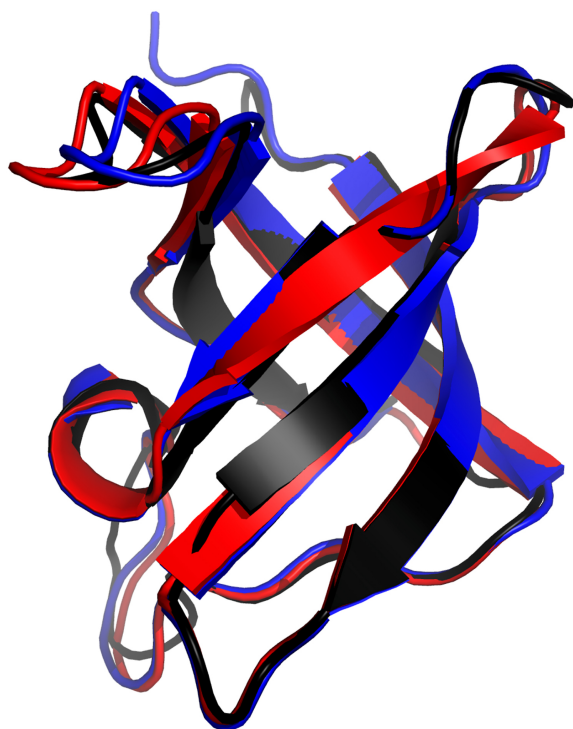


Fig. 2. Superimposition of the Csp structures from *P. haloplanktis* (blue, AlphaFold DB model), *E. coli* (black, PDB code 1MJC) and *T. maritima* (red, AlphaFold DB model) showing the conservation of the β -barrel conformation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1

Theoretical and experimental molecular weight of Csp derived from sequence and mass spectrometry analyses.

	N-terminal residue	theoretical mass	experimental mass
CspP	Ser (100%)	7378.1	7379
CspM	Ser (65%) Met (35%)	7272.0 7403.3	7271 7403
CspT	Met (100%)	7472.6	7473

the methionine-aminopeptidase from *E. coli* should have an easy access to *f*Met in recombinant CspP, a moderate access in CspM and no access to *f*Met in CspT. Furthermore, the excellent agreement between theoretical and experimental masses (Table 1) also indicates that no detectable post-translational modifications have occurred during synthesis and purification of the three Csp.

3.2. Structural stability recorded by microcalorimetry

Heat-induced unfolding of the three Csp has been recorded by differential scanning calorimetry (DSC). In these experiments, the protein solution is heated at a constant rate in the microcalorimeter cell and, as unfolding is an endothermic process, the calorimeter records the heat absorption peak (the thermogram) between the native state (before the transition) and the unfolded state (after the transition) as shown in Fig. 3. Reversibility of unfolding was demonstrated by two consecutive DSC scans as illustrated in Fig. 3. The excellent superimposition of both thermograms for the three Csp indicates reversibility, while the area below the transition allows to quantify reversibility. Table 2 shows an unusual high degree of reversibility of heat induced unfolding, even for CspT which was heated up to 100 °C, a temperature which promotes aggregation of the unfolded state for most proteins.

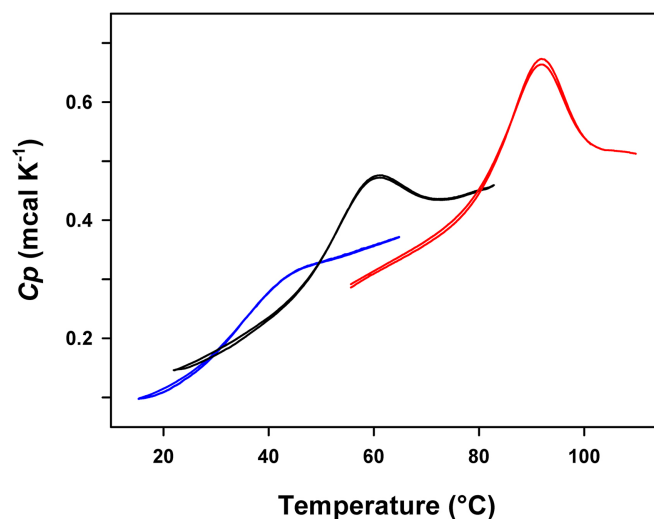


Fig. 3. Raw DSC thermograms, from left to right, of CspP (blue), CspM (black) and CspT (red). Two consecutive scans were performed: after the first scan of heat-induced unfolding, the protein solution was cooled in the microcalorimeter cell (no record) and a second scan was performed. Superimposition of both thermograms demonstrates reversibility of unfolding. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2

Microcalorimetric parameters for Csp unfolding.

Csp	T_m °C	ΔH_{cal} kcal mol ⁻¹	ΔH_{eff} kcal mol ⁻¹	$\Delta H_{cal}/\Delta H_{eff}$	reversibility %
CspP	41.0	35.0	35.3	0.99	≥ 99
CspM	58.8	59.3	59.7	0.99	≥ 98
CspT	91.6	88.8	87.8	1.01	≥ 96

Errors are ± 0.1 °C on T_m and $\pm 4\%$ on ΔH_{cal} and ΔH_{eff} .

Fig. 4 displays the normalized thermograms of the three Csp after baseline subtraction of the raw data and normalization for protein concentration while Table 2 reports the related calorimetric parameters. The melting point T_m recorded at the top of the transition dras-

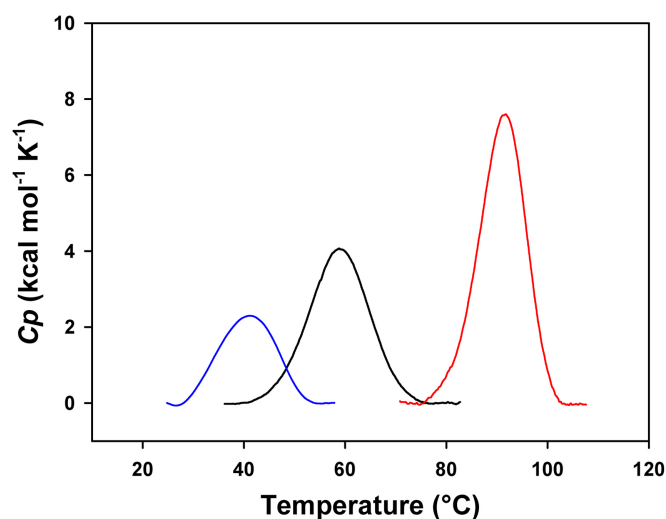


Fig. 4. Normalized thermograms, from left to right, of CspP (blue), CspM (black) and CspT (red). Raw DSC data (as shown in Fig. 3) were baseline subtracted and normalized for protein concentration. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

tically increases from CspP to CspT, illustrating the large differences in stability between the three Csp. The calorimetric enthalpy ΔH_{cal} calculated from the area under the heat absorption peak [30] also significantly increases from the psychrophilic Csp to the thermophilic protein. The ΔH_{cal} parameter corresponds to the total amount of heat absorbed during unfolding which is required to disrupt all enthalpy-driven interactions involved in maintaining the compact structure: it evolves in the order CspP < CspM < CspT. The same order is observed for the ΔH_{eff} parameter measured by the relative amount of the heat absorbed to a given temperature and is equivalent to the van't Hoff enthalpy. More importantly, the ratio $\Delta H_{cal}/\Delta H_{eff}$ close to unity indicates that the three Csp unfold according to a perfect two-state model [30].

3.3. Thermodynamic stability of extremophilic Csp

A key feature of Csp unfolding is their reversible denaturation according to a single cooperative transition between the native (N) and the unfolded (U) state:



The validity of thermodynamic equations related to eq. (1) requires that N and U are in equilibrium and thus that both the reversibility and the two-state transition are unambiguously established, as demonstrated in the previous section. The thermodynamic stability of a protein that unfolds according to eq. (1) is described by the classical Gibbs-Helmholtz relation:

$$\Delta G_{N-U} = \Delta H_{N-U} - T \Delta S_{N-U} \quad (2)$$

The latter relation can be rewritten for any temperature (T) using the parameters determined experimentally by DSC:

$$\Delta G_{N-U}(T) = \Delta H_{cal} (1 - T/T_m) + \Delta C_p (T - T_m) - T \Delta C_p \ln (T/T_m) \quad (3)$$

where ΔC_p is the difference in heat capacity between the native and the unfolded state. This parameter reflects the hydration of nonpolar groups that are exposed to water upon unfolding. It can be estimated on raw data but this parameter has been calculated with high confidence for CspT at $1.1 \pm 0.1 \text{ kcal mol}^{-1} \text{ K}^{-1}$ [16]. Inspection of raw data and mainly model calculation of ΔC_p [31] failed to retrieve significant differences between the three Csp and accordingly the experimentally determined value of ΔC_p for CspT was used for the three proteins. Computing eq. (3) in a temperature range where the native state prevails in solution provides the protein stability curve [32], i.e. the free

energy of unfolding as a function of temperature, as displayed in Fig. 5. More commonly stated, this is the work required to disrupt the native state at any given temperature [30], which corresponds to the thermodynamic stability reported here. By definition, ΔG_{N-U} is zero at T_m , at equilibrium. At temperatures below T_m , the stability increases, as expected, and reaches a maximum then it decreases at lower temperatures (Fig. 5). As a result, this function predicts a temperature of cold-unfolding, which is generally not observed because it occurs below 0 °C [33]. Comparison of the stability curves for the three Csp allows to draw several general conclusions.

The high stability of CspT is reached by higher free energy values in the whole range of computed temperatures, whereas the low stability of CspP corresponds to lower free energy values resulting in the global lowering of its stability curve. This is the consequence of large differences in T_m and ΔH_{cal} values in eq. (3) and it follows that adjustments of the conformational stability are essentially provided by enthalpy-driven interactions. Interestingly, the temperature for maximal stability of the three Csp is reached near room temperature, without drastic shift towards high temperatures for CspT, nor towards low temperatures for CspP. Whatever the microbial source is, either from Antarctic frozen water or from Mediterranean hot springs, the maximum stability of their proteins is clustered around 20 °C. It should be noted that this has been previously reported for mesophilic proteins [34,35] and can now be extended to extremophilic proteins. This clustering has been explained by the fact that the hydrophobic effect, a major driving force in protein folding, is optimal around room temperature [35,36] but also because the various and sometimes opposite forces driving folding are optimally balanced in this temperature range [37].

The free energy function of the psychrophilic CspP shown in Fig. 5 indicates its weak stability at all temperatures, including low temperatures, when compared with CspM and CspT, whereas it would be expected that cold-adapted proteins should also be cold stable. The psychrophilic CspP is in fact both heat and cold labile. It is worth mentioning that cold unfolding of two psychrophilic proteins has been experimentally recorded at the temperature predicted by the stability curve [38,39]. This observation has two main consequences: first, it provides validation of the free energy function and of the approach used here and second, it suggests that cold denaturation of some key proteins in psychrophilic microorganisms could be an additional, though unsuspected, factor limiting life at low temperatures. As a matter of fact, cold denaturation of CspP in Fig. 5 is predicted at –18 °C (in the absence of protective effects from cellular components) and the lower temperature for metabolic activity in a psychrophile was recorded at –25 °C [6].

According to the bell-shaped stability curves of CspM and CspT in Fig. 5, the range of environmental temperature is located on the right side of the curve and accordingly, the thermal dissipative force is used to promote molecular motions in these proteins, by taking advantage of the conformational entropy rise with temperature. In sharp contrast, the range of environmental temperature for CspP is located on the left side of the stability curve. It follows that molecular motions in CspP at low temperatures are gained from the factors ultimately leading to cold unfolding, i.e. the hydration of polar and non-polar groups and the weakening of the hydrophobic effect [37]. The origin of dynamics in CspP is therefore drastically different from its mesophilic and hyperthermophilic homologs. The maximal stability of the Csp in Fig. 5 is reached when the entropic contribution cancels. At temperature below the maximal stability, both the enthalpy and the entropy change their sign: it follows that in this region of the stability curve, the enthalpic term is a destabilizing factor whereas the entropic contribution becomes the stabilizing factor of the native state [32]. It has been argued that this noticeable involvement of entropy reflects a relative disorder in psychrophilic macromolecules to maintain dynamics and function in the cold [40].

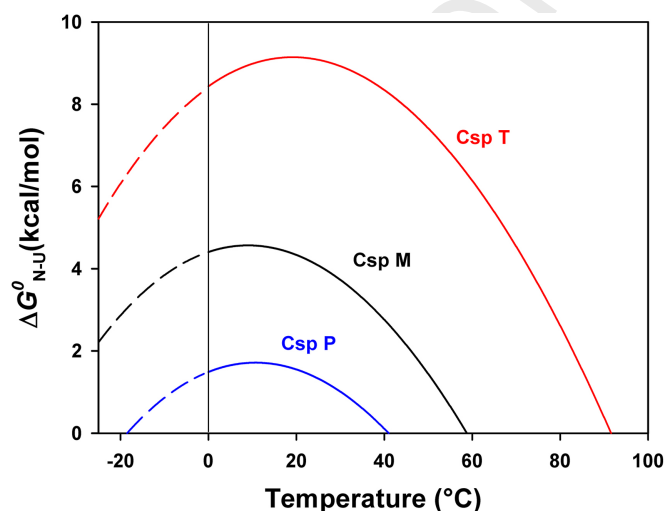


Fig. 5. Gibbs free energy of unfolding or thermodynamic stability of the three investigated Csp. The work required to disrupt the native state is plotted as a function of temperature. Functions are dashed below 0 °C. The melting points T_m (and $T_{m \text{ cold}}$ for Csp P) correspond to the X-axis when $\Delta G_{N-U} = 0$.

4. Conclusion

Some of the above-mentioned conclusions have been previously anticipated but by comparing non-reversible or non-two-state series [38,39,41]. Accordingly, the calculated thermodynamic stabilities were speculative and were taken with caution. These conclusions are now firmly validated by the reversible, two-state unfolding of the Csp reported here. Notably, the temperature for maximal stability around room temperature can be generalized to other proteins as the same behavior was reported for two psychrophilic proteins [39,41] and a hyperthermophilic one [16]. The available NMR structures provide insights into the molecular mechanisms responsible for the differences in Csp stability recorded here by microcalorimetry. In CspT, the side chain of Arg2, which is exclusively found in thermophilic Csp, participates in a peripheral ion cluster with neighboring Asp, Glu and Lys residues. It has been suggested that the thermostability of CspT is based on ionic interactions in this cluster around the Arg2 side chain [21]. Interestingly, this has been confirmed by side-directed mutagenesis of homologous mesophilic and thermophilic Csp [42]. The mesophilic Csp was converted to a highly thermostable form by introducing the equivalent of Arg2 and a Leu residue at the C-terminus which increased the hydrophobic stacking of side chains at the protein surface. As a result, both N- and C-terminus, which are preferential sites for unzipping, are constrained within the structure. By contrast, the NMR structure of a psychrophilic Csp, closely related to CspP, displays the opposite features [19]. It lacks salt bridges and has longer flexible loops with a loosely packed hydrophobic core, more solvent-exposed hydrophobic residues and a lower proportion of secondary structures as compared with mesophilic Csp. All the above-mentioned structural factors modulate the stability of the investigated Csp but mainly provide structural dynamics compatible with activity at extreme temperatures.

CCRediT authorship contribution statement

Lorenzo Grossi: Investigation. **Caroline Struvay:** Investigation. **Georges Feller:** Conceptualization, Investigation, Writing – review & editing.

Data availability statement

All data from this study are available in reported Figures and Tables.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biochi.2026.08.015>.

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