

## Physical bases of thermal stability of proteins: A comparative study on homologous pairs from mesophilic and thermophilic organisms

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We used classical molecular dynamics simulation method to investigate physical factors responsible for the increased thermal stability of proteins from thermophilic and hyperthermophilic organisms. Subject of investigation were two pairs of homologous proteins from the functional classes of: 1) cold shock proteins from *Escherichia coli* (mesophilic) and *Bacillus caldolyticus* (thermophilic) and 2) acylphosphatases from *Bos taurus* (mesophilic) and *Pyrococcus horicoshii* (hyperthermophilic). The simulations were performed for three different temperatures: 298 K, 373 K and 500 K. The results confirmed the common opinion that salt bridges and internal hydrogen bond networks stabilize thermostable proteins at high temperature. In addition, we found that at high temperatures the packing defects, in terms of cavity formation, increase with a preference to the mesophilic protein. Since cavities are a destabilizing factor, we conclude that due to specific packing organisation of proteins of extremophilic organisms, these proteins are more resistant to temperature induced cavity formation, which contributes to their enhanced tolerance towards increase in temperature.

**Key words:** Thermostability, extremophiles, molecular dynamics, packing defects.

### INTRODUCTION

Proteins from thermostable organisms are characterized by higher thermal stability in comparison with their mesophilic counterparts. The most discussed factors contributing to thermal stability of proteins are the optimization of electrostatic interactions [1], the optimization of protein-solvent interactions [2], salt bridges and hydrogen bond networks [3, 4]. Molecular packing and reduction of packing defects (cavity formation) can also be considered as a factor involved in the mechanism of thermal stabilization of proteins [5].

There are a few overall structural characteristics that discriminate proteins from mesophilic and (hyper)thermophilic organisms such as amino acid content, secondary and quaternary structure. These differences preferably occur at the protein-solvent interface rather than in the protein interior. Polar non-charged residues in thermophilic proteins have been found to change into glutamate and lysine and non-polar amino acids to substitute iso-

leucine [6]. In hyperthermophiles, isoleucine and to a lesser extent valine residues have been proved to form most of the hydrophobic contacts of the structurally conserved regions [7]. The secondary structure accounts for a larger fraction of residues with  $\alpha$ -helices and  $\beta$ -strands conformations in the thermophilic proteins. Consequently, it has been discovered that the content of lower structured irregular regions is smaller in thermophiles [8]. The comparison between Fe-Superoxide dismutases (Fe-SOD's) has revealed that the thermophilic counterparts have fewer and longer loops, more  $\alpha$ -helices and turns, and decreased length of  $\beta$ -strands [9].  $\alpha$ -Helices have showed to be involved in the greater apolar contact area in the hyperthermophilic proteins. Despite all the differences in amino acid contents and secondary structures, there are practically no or very small differences in the three-dimensional organization of the homologous mesophilic/thermostable proteins.

The above observations, however, do not reveal in detail the driving forces responsible for the shift of the melting point to higher temperature of proteins from (hyper)thermophilic species. Since the chemical content of these classes of proteins is practically the same, the answer to the question of thermal stability should be sought in the delicate balance of

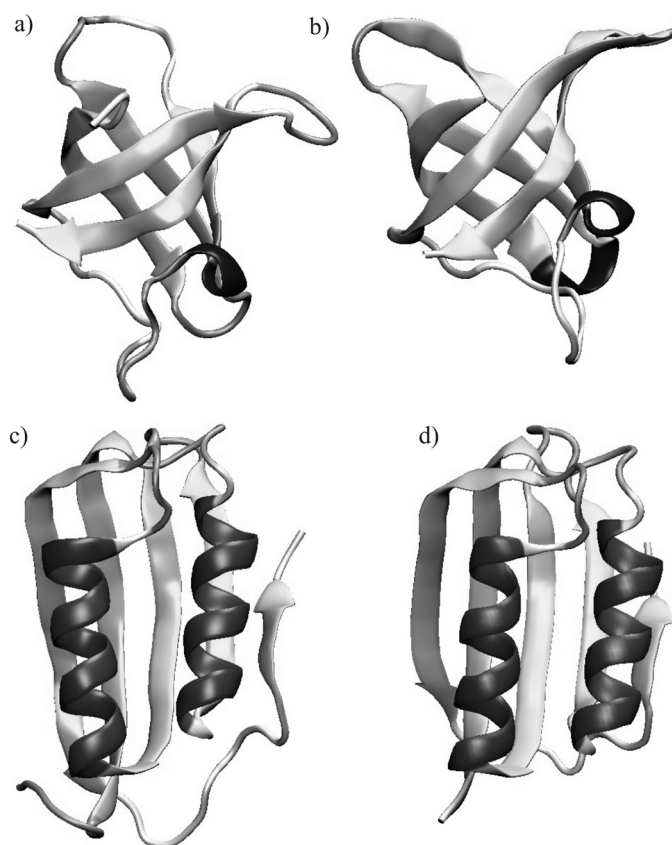
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non-covalent interactions. Vogt et al. [10] have investigated 16 families of proteins in which 80% have manifested correlation between the thermal stability and the increase in the number of internal hydrogen bonds, salt bridges and existence of polar surface. These authors have found that proteins connect to 22 extra solvent molecules per 10 °C rise in melting temperature by increasing their polar surface area. It has also been found that the increase in the internal hydrogen bonding in thermophilic proteins is mainly due to links between buried donor/acceptor pairs belonging to the main chains. The abundance of stable intermolecular and intramolecular hydrogen bonds has been found to attribute to the mechanical rigidity of the proteins, a factor enhancing their thermal stability [11]. In thermostable proteins a large number of side chain alternative H-bonds are formed with rise in temperature[12].

The electrostatic interactions have been found to have an important role for the thermal stability of proteins, especially in increasing the number of the salt bridges [13]. Danciulescu et al. [14] have investigated the electrostatic interactions calculating the free energy contributions for the nucleotide-binding domain of homologous mesophilic and hyperthermophilic Glutamate dehydrogenases from *E. Coli* and *T. maritima*. The mobility and the dynamics of the salt bridges proved to be crucial. According to

their calculations the specific heat capacity of the hyperthermophilic protein is higher than the heat capacity of the mesophilic one.

In our earlier investigations we have noticed that protein from (hyper)thermophilic organisms are characterized by somewhat lower packing density [5]. We have also confirmed that the packing defects in term of cavity formation are reduced in (hyper)thermophilic proteins. Hence, the destabilizing effect of the cavities is diminished in these proteins. These results, as well as the vast majority of the conclusions discussed above, are based on three-dimensional X-ray protein structures. It is well documented that proteins in solution are dynamic and occupy more than a single conformation. It is interesting to see to what extent the above observations can be confirmed when dynamic properties of proteins are taken into account. For this purpose we employed MD simulation for two pairs of homologous proteins from mesophilic and (hyper) thermophilic organisms. The first homologous pair consists of the cold shock proteins from *Escherichia coli* (mesophilic, in the following abbreviated as M-csp) and *Bacillus caldolyticus* (thermophilic, T-csp) and 2) acylphosphatases from *Bos taurus* (mesophilic, M-acp) and *Pyrococcus horicoshii* (hyperthermophilic, H-acp). The structures of the two pairs are illustrated in Figure 1. As seen, the



**Fig. 1.** X-ray crystal structures of the investigated proteins: a) M-csp; b) T-csp; c) M-acp and d) H-acp

three dimensional similarity within the pairs is remarkable, nevertheless the unfolding temperatures differ dramatically (see also Table 1).

Their X-ray structures were downloaded from Protein Data Bank (PDB) [15]. Our results are in accord with the common opinion that electrostatic interactions and hydrogen bonding are important factor for the thermal stability of proteins from extremophiles. The average percentage of occupancy per amino acid pair, participating in a salt bridge and per hydrogen bond in hyperthermophilic proteins has showed distinct separation compared to the mesophilic and thermophilic proteins. The hyperthermophiles formed longer-lasting, stable hydrogen bonds. In addition, we have shown that thermophilic and especially hyperthermophilic proteins from the investigated pairs are more resistant towards temperature induced cavity formation. Since cavities are destabilizing component of protein structure, we conclude that proteins from thermophiles gain stability via diminishing temperature induced cavity formation.

## COMPUTATIONAL METHODS

Classical Molecular Dynamics (MD) simulations were carried out for two pairs of homologous proteins. The biological units of the mesophilic proteins are monomers, whereas those of the thermophilic and hyperthermophilic proteins are dimers. For the consistency of our calculations only the monomers were used.

The possible contribution of intermolecular interactions within the tertiary structures to thermal stability is out of the scope of this study.

Based on the sequence analysis performed using BLASTP algorithm [16], we found that the thermostable cold shock protein and the acylphosphatase have a structural identity with their mesophilic counterparts of 92% and 84%, respectively.

All the MD simulations were performed, using GROMACS v.4.5.3 [17, 18] with CHARMM27 force field [19] and TIP3P explicit water model

[20]. The systems were set up in dodecahedron boxes with 1nm distance between the sides of the box and the protein surfaces. Water molecules were added, along with sodium ( $\text{Na}^+$ ) and chloride ( $\text{Cl}^-$ ) counter ions in order to achieve ionic strength of 0.1 M. The systems were minimized using Steepest Descent method. The restraints of the atoms were released at three stages: 1) the minimizations were performed with all non-hydrogen atoms, restrained with 500 kJ/mol/nm<sup>2</sup>; 2) only non-hydrogen atoms of the main chain restrained with 200 kJ/mol/nm<sup>2</sup> and 3) the minimizations were performed without position restraints. Next, MD simulations were conducted in periodic boundary conditions, in NVT ensemble. The systems were heated by 10 K every 2 ps to the desired temperatures: 298, 373 and 500 K. For the purpose of keeping the temperature constant, Berendsen thermostat was used. The constant temperature MD simulations were performed using leap-frog integrator with 2 fs time step. The hydrogen atoms were constrained with LINKS algorithm, implemented in GROMACS. The length of simulations was 6 ns at 298 K and 10 ns at 373 K and 500 K.

The analysis of the trajectories with rmsd, the salt bridges and hydrogen bonding evaluation was processed with VMD [21]. The secondary X-ray structure of all the molecules was investigated with STRIDE [22].

For the purposes of this study we define a void as room within the protein moiety which is inaccessible to the solvent and small enough so that no solvent molecules can be situated there. Cavity is an internal space in the protein where at least one solvent molecule can be introduced. The identification of voids and cavities as well as their volumes were calculated using the method and the parameters described earlier [5].

Salt bridge/hydrogen bond and void/cavity formation were calculated taking 50 snapshots over the last 2 ns of the MD simulations at all temperatures. The length of simulation at 298 K was 6 ns, during which time the protein structures reached equilibrium. The high temperature simulations were stopped

**Table 1.** Mesophilic/thermophilic and mesophilic/hyperthermophilic pairs

Organism	Functional class	PDB entry	Name	Number of residues	Biological unit	Melting temperature, $T_m$ [°C]
<i>Escherichia coli</i>	Cold shock protein	1MJC	M-csp	69	Monomer	57.60 [31]
<i>Bacillus caldolyticus</i>	Cold shock protein	1C9O	T-csp	66	Dimer	76.90 [32]
<i>Bos taurus</i>	Acyl-phosphatase	2ACY	M-acp	98	Monomer	53.80 [33]
<i>Pyrococcus horicoshii</i>	Acyl-phosphatase	1W2I	H-acp	91	Dimer	111.50 [25]

at 10 ns when they began to show increased deviations from the X-ray structure in the last 2 ns. For the analysis of the results, we took the averages of the rmsd values when the temperature perturbation starts to affect the protein structure. Then the first step of denaturation occurs and the structures leave their equilibrium conformation(s).

The assessment of salt bridge and hydrogen bonds formation was made on the basis of a cut-off distance of 3.6 Å and 3.2 Å respectively, whilst the cut-offs of 40° and 20° of the acceptor-donor-hydrogen angle (according VMD nomenclature) were used. Data were collected over the last 2 ns of the simulation.

## RESULTS AND DISCUSSION

*Rmsd and rmsf (root mean square deviation and fluctuation) analysis.* The average rmsd values for the non-hydrogen atoms of the proteins simulated at different temperatures are summarized in Table 2. At almost all simulated temperatures the T-csp and H-acp proteins were characterized by smaller changes in the average rmsd values than their mesophilic counterparts, which relates to their greater structural stability. The fluctuations of residues in the structures were investigated by calculating the rmsf for the non-hydrogen atoms from an average structure for the last

2 ns at all temperatures. Structural elements fluctuating more than the threshold of 0.15 nm are presented in Table 3. During the last 2 ns of the simulations all the proteins fluctuated at distance-separated unique residues with preferably highly mobile, solvent-exposed secondary structures such as turns and coils. Fluctuations in  $\alpha$ -helices and  $\beta$ -strands were either in their beginnings or ends where they are followed or preceded by the mobile residues.

At 373 K the difference in rmsd values of the cold shock proteins was minimal (M-csp-2.105 Å and T-csp-2.002 Å). The rmsf per residue involved fluctuation in coordinates of coils, turns and a  $\beta$ -strand (Glu56) in M-csp and well-structured elements as parts of  $\beta$ -strands (Lys5, Gly23-Ser24; Gln53- Gly54 and Lys65) in T-csp. The rmsf difference between the M-acp and H-acp acylphosphatases at 373 K became 0.244 Å. The alternations in M-acp consist of fluctuations in turns,  $\alpha$ -helices (Lys31; His60; Lys68) and  $\beta$ -strand (Arg77). In H-acp the fluctuating regions were mostly parts of  $\beta$ -strands and  $\alpha$ -helices (Arg13, Arg28, Leu53, Arg70, Arg77).

At 500 K M-csp experienced greater structural fluctuations in larger number of residues than its thermophilic homolog. The change in coordinates preferably included big blocks of  $\beta$ -strands and a visual loss of its 3–10 helix structure (Phe34-Ala36), which was transformed into a turn and

**Table 1.** Average rmsd and standard deviation (sd) values of the non-hydrogen atoms from the X-ray structure of the M-csp/T-csp and M-acp/H-acp homologous pairs at 298 K, 373 K and 500 K

Protein	Average rmsd [Å] at 298 K	Average rmsd [Å] at 373 K	Average rmsd [Å] at 500 K	Average $\Delta$ rmsd [Å] (298K-373K)	Average $\Delta$ rmsd [Å] (298K-500K)
M-csp	1.915 ± 0.260	2.105 ± 0.219	2.635 ± 0.468	0.190	0.720
T-csp	1.515 ± 0.206	2.002 ± 0.272	2.418 ± 0.287	0.487	0.903
M-acp	1.231 ± 0.089	1.684 ± 0.197	2.815 ± 0.675	0.453	1.584
H-acp	1.356 ± 0.199	1.440 ± 0.133	1.849 ± 0.308	0.084	0.493

**Table 3.** Total number of amino acid pairs, participating in the formation of salt bridges and their population over 50 frames of the last 2 ns of the simulations

Protein Id	Total number of amino acid pairs participating in salt bridges			Average occupancy of salt bridges per amino acid pair [%]		
	298 K	373 K	500 K	298 K	373 K	500 K
M-csp	1	3	5	40.38	35.9	18.46
T-csp	6	6	11	42.66	49.36	27.97
M-acp	6	9	17	52.20	38.46	25.11
H-acp	17	16	20	54.44	59.62	39.13



coil. The flexibility of a turn (Asn39- Tyr42) and a bend (Ser57- Pro62) caused larger deviation from the X-ray structure and distortion of the structure. During the simulations similar changes were observed for T-csp, which also fully lost one of its 3-10 helix (Asn11- Lys13) and partially another 3-10 helix (Phe30- Ala32) structures to irregular regions (turn and coil).

The second pair of proteins, which has difference in the melting temperatures bigger than the first homologous pair, showed significant separation in the rmsd values ( $\sim 1 \text{ \AA}$ ). The mesophilic protein reached higher rmsd values especially in the last 2 ns of the simulation- consistent with changes in the secondary structure and initial stage of denaturation. The main change in the secondary structure in M-acp was in one  $\beta$ -strand (Ile75- Val85). In some of the snapshots the  $\beta$ -strand was disrupted in residues His81, Asn82 and Glu83. Residues from another  $\beta$ -strand and residues close to the N- and C- termini (Asn41, Tyr11, Val85) manifested high fluctuations. These structural deviations are displayed as major peaks in Figure 2c. The major difference between the X-ray of H-acp and the simulated structure at 500 K was in  $\beta$ -strand and  $\alpha$ -helix (Tyr11- Arg13; Trp21; Arg25; Arg28; Gln64, Arg70- Arg73).

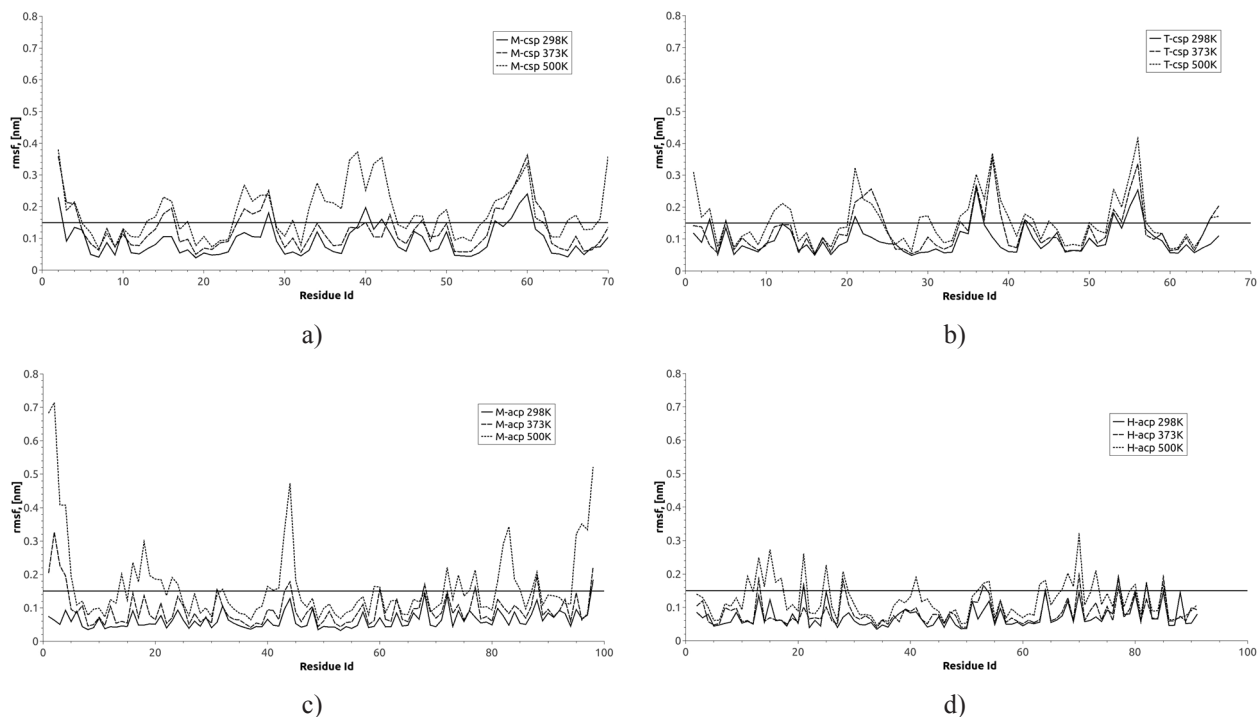
The  $\Delta$ rmsd values in Table 2 were calculated as difference between rms deviations of the simulated

structures at 373 K and 500 K and the average rms deviations of the equilibrated molecule at 298 K. The average  $\Delta$ rmsd of H-acp does not show large deviation in coordinates from the equilibrated structure at 298 K unlike the other simulated proteins.

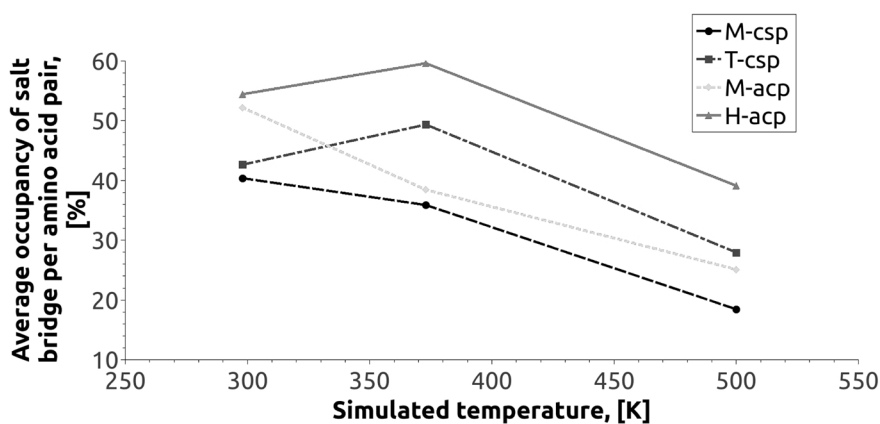
In the acylphosphatase pair the fluctuations around the average structure over the trajectory of the hyperthermophilic protein, were smaller in average than its mesophilic counterpart.

The 1MJC/1C9O pair are proteins with small molecular weight per chain (7280 Da and 7547 Da) [23, 24], without or with small hydrophobic cores, which would lead to high, close rates of fluctuations for both structures. Fluctuation difference should be sought in the M-acp/H-acp pair (weights of 11.326 kDa and 10.369 kDa per chain) [25, 26]. The mesophilic proteins are less fluctuating at 373 K with change in position mainly in irregular regions compared to their thermostable counterparts, where the fluctuations occur mostly in  $\alpha$ -helices and  $\beta$ -strands. Therefore, the thermostable proteins are more mobile at moderately high temperatures (373 K) without experiencing overall significant distortion in structure at 500 K.

*Salt bridges and internal hydrogen bonds.* The number of salt bridges for the homologous pairs of proteins was calculated over the last 2 ns of the MD trajectories for 298 K, 373 K and 500 K (Table 3).



**Fig. 2.** Average rmsf plots per residue for the last 2 ns of the MD simulation at 298 K, 373 K and 500 K: a) M-csp; b) T-csp; c) M-acp and d) H-acp



**Fig. 3.** Average population of salt bridges per amino acid pair at 298 K, 373 K and 500 K in mesophilic M-csp (dashed line) and thermophilic T-csp (dash-dotted line) cold shock proteins and mesophilic M-acp (dotted line) and hyperthermophilic H-acp (solid line) acylphosphatases

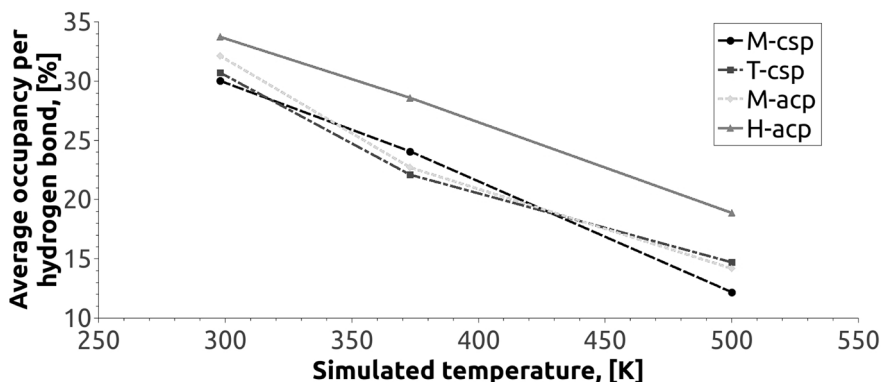
As expected [2] the proteins from thermostable organisms are characterized by a larger number of salt bridges than their mesophilic counterparts (Table 3). Since salt bridges are dynamic formation, i.e. they may break and form during simulations [27, 28], the quantity relevant for the structural stability of proteins is their lifetime. As seen in Figure 3 and Table 3 there is a weak tendency the proteins from the extremophilic organisms to have a longer lifetime at room temperature. Due to increasing of the kinetic energy when temperature increases the overall salt bridge lifetime (occupancy) reduces. However, this reduction is lower for the thermostable and especially for the hyperthermostable protein. Hence, the role of salt bridges as stabilizing factor is less reduced in proteins from (hyper)thermophilic organisms. The physical foundation of this effect has been discussed [29]. Based on X-ray structure and continuum dielectric model it has been shown that due to reduction of permittivity of solvent water, the charge-charge interaction energy increases, whereas the desolvation penalty reduces. These two factors increase the stabilization effect of electrostatic in-

teractions. In this work we illustrate the same effect using more detailed representation of the protein/solvent system, including structural flexibility of the protein molecule.

In contrast to salt bridges, the number of intramolecular hydrogen bonds at room temperature practically does not differ within the counterparts of the two types of investigated proteins (Table 4). This reflects the similarity of the three-dimensional structures of the counterparts. As mentioned above, the measure of the contribution of hydrogen bonds to structural stability is their lifetime (the occupancy) rather than their number. Similarly to salt bridge occupancy, that of intramolecular hydrogen bonds reduces with temperature. However, in this case the reduction is practically the same for all four proteins under consideration (see also Figure 4). The thermostable protein shows a bit longer average lifetime at 500 K MD simulation run, however the difference between the counterparts is too small to make general conclusion. In the case of Acp pair, the average hydrogen bond lifetime for the hyperthermophilic protein at 500 K simulation is about

**Table 4.** Values of the hydrogen bonds for the proteins at all the simulated temperatures for 50 frames of the last 2 ns of each of the simulations

Protein Id	Total number of hydrogen bonds			Average occupancy per hydrogen bond [%]		
	298 K	373 K	500 K	298 K	373 K	500 K
M-csp	62	66	103	30.02	24.07	12.19
T-csp	60	83	97	30.71	22.10	14.71
M-acp	102	130	167	32.13	22.71	14.19
H-acp	100	107	127	33.73	28.59	18.88



**Fig. 4.** Average population per hydrogen bond at 298 K, 373 K and 500 K in mesophilic M-csp (dashed line) and thermophilic T-csp (dash-dotted line) cold shock proteins and mesophilic M-acp (dotted line) and hyperthermophilic H-acp (solid line) acylphosphatases

25% longer than that for the mesophilic counterpart. Thus, on the basis of the two pairs of proteins we conclude that in the case of meso/thermophilic pair the contribution of intramolecular hydrogen bonds to protein thermal stability is not so pronounced as that of salt bridges. We hypothesise that the hydrogen bonding is “used” as stabilising instrument for hyperthermophilic proteins.

*Void volumes and cavities.* Voids and cavities are integral characteristics, which result from all non-covalent interactions, as well as from the amino acid content of the protein molecules. It is well documented that amino acid substitution leads to cavity formation, which reduces the stability of the

protein molecule [30]. In general, cavities are considered as packing defects destabilizing the native structure. Voids also may lead to decreasing of the thermal stability, due to reduction of Van der Waals interactions. On the other hand, voids make folded protein structure to tolerate the increase of vibrational entropy with elevation of temperature. It is a general question to investigate to what extent thermo- and hyperthermophilic proteins can be discriminated from their mesophilic counterparts in terms of voids and cavities.

The calculated void and cavities volumes are presented in Table 5. The void volumes remain almost unchanged in the simulation at 298 K and 373 K. In

**Table 5.** Average number of cavities and void volumes per atom over 50 frames of the last 2 ns of the MD simulations at 298 K, 373 K and 500 K. In brackets are given the calculated void volumes per atom/average number of cavities/cavity volumes over the last 2 ns of additional 5 ns simulations for H-acp

Temperature [K]	M-csp mesophile			T-csp thermophile		
	Average number of cavities	Average cavity volume [ $\text{\AA}^3$ ]	$V_{\text{void}}/\text{atom}$ [ $\text{\AA}^3$ ]	Average number of cavities	Average cavity volume [ $\text{\AA}^3$ ]	$V_{\text{void}}/\text{atom}$ [ $\text{\AA}^3$ ]
298	0.57	14.35	3.76	0.96	14.14	3.92
373	1.80	21.23	3.90	0.73	15.19	3.86
500	1.92	18.13	3.77	1.75	17.11	3.91
Temperature [K]	M-acp mesophile			H-acp hyperthermophile		
	Average number of cavities	Average cavity volume [ $\text{\AA}^3$ ]	$V_{\text{void}}/\text{atom}$ [ $\text{\AA}^3$ ]	Average number of cavities	Average cavity volume [ $\text{\AA}^3$ ]	$V_{\text{void}}/\text{atom}$ [ $\text{\AA}^3$ ]
298	1.78	12.92	4.19	1.35	14.24	4.15
373	2.39	17.41	4.18	1.96	14.78	4.17
500	4.10	13.90	3.06	(1.66)	(17.45)	(4.15)
				2.29	21.47	4.00

the case of the 500 K MD simulations, the mesophilic M-acp showed dramatic reduction of the void volume. The same tendency but with smaller magnitude is observed for the mesophilic M-csp. It can be attributed to initiation of unfolding at which the protein interior becomes accessible to the solvent. The overall similarity of void volumes within the two pairs suggests that this parameter does not reveal difference between mesophilic and (hyper)thermophilic proteins.

The number of cavities gradually increases with temperature in all investigated proteins. This dependency on temperature is clearly pronounced for the mesophilic proteins. Another interesting feature uncovered by the computations is that, for these proteins the increase in the cavities' number and volume is observed already at 373 K, compared to the thermostable proteins, where this change occurs at 500 K. The larger structure of H-acp needed to be simulated longer for this phenomenon to be observed. Therefore, we performed MD simulations with H-acp at 373 K for 5 more nanoseconds, and collected statistics over the last 2 ns. The void volumes and average number of cavities changed their values from 4.17 Å<sup>3</sup> and 1.96 to 4.15 Å<sup>3</sup> and 1.66, respectively. These results suggest that thermophilic and hyperthermophilic proteins are characterized by structural organization resistant to temperature induced packing defects. This resistance correlates with the melting temperatures of the investigated proteins.

## CONCLUSIONS

Our comparative computational study on two homologous pairs of mesophilic- thermophilic/hyperthermophilic proteins showed that the molecules from the extremophilic species have a larger flexibility than that of the mesophilic counterparts. It has been observed that the number of charged groups participating in salt bridges increases, as obeying the correlation mesophilic < thermophilic < hyperthermophilic proteins. In parallel the reduction of salt bridge lifetimes at high temperature is less for the proteins from extremophiles, hence salt bridges as stabilising factor are more resistant towards increasing of temperature. It was hypothesized that the hydrogen bond network becomes relevant stabilizing factor in hyperthermophilic proteins, whereas for the thermophilic species its stabilizing role is not pronounced. Finally, the results have showed that thermophilic and hyperthermophilic proteins are characterized by structural organization resistant to temperature induced packing defects.

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## ФИЗИЧНИ ОСНОВИ НА ТЕРМИЧНАТА УСТОЙЧИВОСТ НА ПРОТЕИНИ: СРАВНИТЕЛНО ИЗСЛЕДВАНЕ НА ХОМОЛОЖНИ ДВОЙКИ ОТ МЕЗОФИЛНИ И ТЕРМОФИЛНИ ОРГАНИЗМИ

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(Резюме)

За изследване на физичните фактори, отговорни за повишена термична стабилност на белтъци от термофилни и хипертермофилни организми, използвахме симулационен метод, базиран на класическата молекулярна динамика. Предмет на изследването са две двойки хомоложни белтъци от функционалните класове на: 1) „студ-стрес“ (cold shock) белтъци от *Escherichia coli* (мезофилен) и *Bacillus caldolyticus* (термофилен) и 2) ацил фосфатази от *Bos Taurus* (мезофилен) и *Pyrococcus horicoshii* (хипертермофилен). Проведени бяха симулации на белтъците при три различни температури: 298, 373 и 500 К. Резултатите потвърдиха общото мнение, че солеви мостове и мрежи от вътрешномолекулни водородни връзки стабилизират термостабилните белтъци при висока температура. В допълнение ние установихме, че при „високи“ температури дефектите на пакетиране се увеличават (чрез формиране на кухини), най-осезаемо при мезофилните белтъци. Тъй като кухините са дестабилизиращ фактор, ние стигнахме до заключението, че особеното опаковане на белтъци от екстремофилни организми, по-устойчиви към температурно-индуцираното формиране на кухини, допринася за подобряване на тяхната толерантност към висока температурата.