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ORIGINAL PAPER

Comparison of the structural basis for thermal stability between archaeal and bacterial proteins

Yanrui Ding · Yujie Cai · Yonggang Han · Bingqiang Zhao

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Abstract In this study, the structural basis for thermal stability in archaeal and bacterial proteins was investigated. There were many common factors that confer resistance to high temperature in both archaeal and bacterial proteins. These factors include increases in the Lys content, the bends and blanks of secondary structure, the Glu content of salt bridge; decreases in the number of main–side chain hydrogen bond and exposed surface area, and changes in the bends and blanks of amino acids. Certainly, the utilization of charged amino acids to form salt bridges is a primary factor. In both heat-resistant archaeal and bacterial proteins, most Glu and Asp participate in the formation of salt bridges. Other factors may influence either archaeal or bacterial protein thermostability, which includes the more frequent occurrence of shorter 3_{10}-helices and increased hydrophobicity in heat-resistant archaeal proteins. However, there were increases in average helix length, the Glu content in salt bridges, temperature factors and decreases in the number of main–side chain hydrogen bonds, uncharged–uncharged hydrogen bonds, hydrophobicity, and buried and exposed polar surface area in heat-resistant bacterial proteins. Evidently, there are few similarities and many disparities between the heat-resistant mechanisms of archaeal and bacterial proteins.

Keywords Bacterial protein · Archaeal protein · Thermostability · Structural parameters

Abbreviations

HTH_AR	Hyperthermophilic archaeal proteins
TH_AR	Thermophilic archaeal proteins
ME_AR	Mesophilic archaeal proteins
HTH_BC	Hyperthermophilic bacterial proteins
TH_BC	Thermophilic bacterial proteins
ME_BC	Mesophilic bacterial proteins
t(HTH–TH_AR)	*t* test value between hyperthermophilic and thermophilic archaeal proteins
t(HTH–TH_BC)	*t* test value between hyperthermophilic and thermophilic bacterial proteins
t(HTH–ME_AR)	*t* test value between hyperthermophilic and mesophilic archaeal proteins
t(HTH–ME_BC)	*t* test value between hyperthermophilic and mesophilic bacterial proteins
t(TH–ME_AR)	*t* test value between thermophilic and mesophilic archaeal proteins
t(TH–ME_BC)	*t* test value between thermophilic and mesophilic bacterial proteins
HTH_AR SD	Standard deviation of hyperthermophilic archaeal proteins
HTH_BC SD	Standard deviation of hyperthermophilic bacterial proteins
TH_AR SD	Standard deviation of thermophilic archaeal proteins
TH_BC SD	Standard deviation of thermophilic bacterial proteins

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Y. Ding (✉) · Y. Han · B. Zhao
Key Laboratory of Advanced Process Control for Light Industry, Department of Computer Science and Technology, JiangNan University, Wuxi 214122, People's Republic of China
e-mail: yr_ding@yahoo.com.cn

Y. Cai
Key Laboratory of Industrial Biotechnology, School of Biotechnology, JiangNan University, Wuxi 214122, People's Republic of China

ME_AR SD	Standard deviation of mesophilic archaeal proteins
ME_BC SD	Standard deviation of mesophilic bacterial proteins
p(HTH–TH_AR)	*p* value between hyperthermophilic and thermophilic archaeal proteins
p(HTH–TH_BC)	*p* value between hyperthermophilic and thermophilic bacterial proteins
p(HTH–ME_AR)	*p* value between hyperthermophilic and mesophilic archaeal proteins
p(HTH–ME_BC)	*p* value between hyperthermophilic and mesophilic bacterial proteins
p(TH–ME_AR)	*p* value between thermophilic and mesophilic archaeal proteins
p(TH–ME_BC)	*p* value between thermophilic and mesophilic bacterial proteins

Introduction

Currently, nearly 100 microorganisms, with growth temperatures greater than 80°C, have been discovered and isolated (http://pgtdb.csie.ncu.edu.tw/). Most of these families belong to the kingdom of archaea. The underlying reasons for the ability of archaea to thrive at high temperatures, while most bacteria can only live at normal temperatures are intriguing. Within the 16S rRNA-based universal phylogenetic tree (Pace 1997), heat-resistant microorganisms occupy all of the short, deep branches closest to the root, which provides strong evidence of a hyperthermophilic last common ancestor (Stetter 2006). Archaea possess unique characteristics that are distinct from bacteria (Stetter 1999) which implies that archaeal and bacterial proteins are different in the molecular basis of stability. Therefore, elucidation of the differences in the structural basis for thermostability in archaeal and bacterial proteins can provide insight into alternative mechanisms by which thermophilic proteins could withstand higher temperatures, and can also improve our understanding of evolutionary processes.

During the past four decades, the molecular basis of protein thermal stability has expanded as a research area. Investigations of protein thermostability using experimental and theoretical approaches include site-directed mutagenesis (Sommaruga et al. 2008; Kotzia and Labrou 2009; Jaouadi et al. 2010), structural comparison (Smith et al. 1999; Robinson-Rechavi et al. 2006), and molecular dynamics simulation (Lazaridis et al. 1997; Liu and Wang 2003; Liu et al. 2008). Protein thermostability is influenced by many factors, such as amino acid preference, secondary structure propensity, the number of salt bridges, the number and type of hydrogen bonds, hydrophobic packing, the amount and type of solvent accessible surface area, loop size and cavity number, and the B-factor (Robinson-Rechavi et al. 2006; Fukuchi and Nishikawa 2001; Farias and Bonato 2003; Szilagyi and Zavodszky 2000; Ge et al. 2008; Missimer et al. 2007; Makhatadze et al. 2003; Vogt et al. 1997; Paiardini et al. 2008; Chan et al. 1995; Eriksson et al. 1992; Ishikawa et al. 1993; Parthasarathy and Murthy 2000).

Site-directed mutagenesis and molecular dynamics simulation are effective methods to investigate single protein thermostability. Another advantageous method is systematic analysis of specific protein families from the same domain by structural comparison. These types of analytical approaches provide significant insight into specific mechanisms, but it is difficult to determine the common rules of protein thermostability.

Additionally, the dataset used in structural comparison is composed of proteins with different thermostabilities, or even different superkingdoms of life. As mentioned, most thermophilic proteins are from archaea, and most mesophilic proteins are from bacteria. Structural comparisons are usually between thermophilic archaeal and mesophilic bacterial proteins. Therefore, the structural differences between thermophilic and mesophilic proteins may be related to either protein thermostability or features of specific superkingdoms.

Studies in recent years have indicated that there are disparities in the heat-resistant mechanism between archaeal proteins and bacterial proteins (Trivedi et al. 2006). Hyperthermophilic archaea use "structure-based" physical mechanisms, while some bacteria use "sequence-based" physical mechanisms to cope with the ambient temperature (Berezovsky and Shakhnovich 2005). Archaeal organisms resist high temperatures by substituting non-charged polar amino acids with Glu, Lys and non-polar amino acids with Ile on protein surfaces (Mizuguchi et al. 2007).

In this paper, the protein structures of all prokaryotes with sequenced genomes were selected and classified into bacterial and archaeal proteins to study structural differences that confer thermal stability. Some common features allow both archaeal and bacterial proteins to resist high temperatures. However, there are many factors that only influence either archaeal or bacterial protein thermostability. We determined that there are few similarities and many disparities between the heat-resistant mechanisms of archaeal and bacterial proteins. Therefore, the archaeal and bacterial proteins should be distinguished when considering protein thermostability.

Materials and methods

Dataset

Almost all heat-tolerant microorganisms are prokaryotes. In this paper, the objective is to study the protein structures of all prokaryotes with sequenced genomes. The prokaryotes were taken from the NCBI database. These protein structures of selected prokaryotes were retrieved from the PDB database (Berman et al. 2000) using PISCES to eliminate sequences redundancies (Wang and Dunbrack 2003). These proteins are grouped into three classes according to protein thermostability based on the PGTdb database (Huang et al. 2004). In the analysis, hyperthermophiles are defined as microorganisms with optimal growth temperatures greater than 85°C, while those with optimal growth temperatures between 40 and 85°C were classified as thermophiles. The final dataset is composed of 18 hyperthermophilic archaea, 16 thermophilic archaea, 2 mesophilic archaea, 2 hyperthermophilic bacteria, 39 thermophilic bacteria, and 185 mesophilic bacteria. The details are listed in Table S1. From the dataset, we can see that most hyperthermophiles are archaea and most mesophiles are bacteria.

Compute the structural parameters

The properties of secondary structure

The DSSP program was used to compute the proteins secondary structure. The DSSP program was developed by Kabsch and Sander (1983) to compute protein secondary structure. There are seven secondary structures that can be computed by the DSSP, H = alpha helix; B = isolated β-bridge; E = β-strand; G = 3_{10}-helix; T = turn; S = bend; blank = rest.

The content of secondary structure refers to the number of secondary structure elements per 100 amino acids in a protein structure. The number of secondary structures, average length of each secondary structure, and the distribution of 20 types of amino acids in secondary structure were calculated in Perl. Then we carried out *t* test to determine which factor is important.

Hydrogen bonds

The hydrogen bonds of each protein were computed by HBPlus3.0 (Linux) (McDonald and Thornton 1994). The parameters were set as in Vogt's paper (Vogt et al. 1997). The hydrogen bonds were classified into six types: main–main chain, side–side chain, main–side chain, charged–charged, uncharged–uncharged, and charged–uncharged. Here, the number of hydrogen bond indicates number per 100 amino acids in each protein.

Salt bridges

The positively charged amino acids are Lys, Arg and His, and the negatively charged amino acids are Asp and Glu. When the nitrogen atoms in Lys, Arg and His are within 4 Å of the side chain carbonyl oxygen atoms in Asp and Glu, salt bridges are formed. For a given pair of salt bridge forming residues, if more than one nitrogen–oxygen pair of atoms can be within 4 Å, the salt bridge was counted only once (Kumar et al. 2000). First, we computed all atom–atom distances in each protein, and then, we selected the atoms from charged amino acid with a distance of less than 4 Å between them. Using the above definition for a salt bridge, the salt bridges were computed using a Perl program. In this paper, we statistically computed all salt bridges in the selected protein chain, the ratio of salt bridge network to the total number of salt bridges, the composition of each charged amino acid in the total number of salt bridges, the ratio of total charged amino acid formed salt bridges and the ratio of each type of charged amino acid in the salt bridges. The number of salt bridges indicates the number of salt bridges per 100 amino acids in each protein.

Solvent accessible surface area

The solvent accessible surface area (ASA) is the surface area of a protein that is accessible to a solvent. The ASA is calculated with a solvent of a particular radius to 'probe' the surface of the molecule. In this paper, the ASA is calculated following Lee and Richards' (1971) definition. The 'probe radius' is 1.4 Å, which approximates the radius of a water molecule. The ASC (The Analytic Surface Calculation Package Linux) (Eisenhaber and Argos 1993; Eisenhaber et al. 1995) can calculate the proteins' ASA. Here, formal charge is used to divide atoms into polar and non-polar atoms (Tsai and Nussinov 1997). When the formal charge of an atom is less than 0.25, the atom is regarded as a non-polar atom. The ASA_{Non} refers to the non-polar atoms' ASA, while the ASA_{Pol} refers to the polar atoms' ASA. If the ASA of residue X is greater than 50% of the ASA calculated for tripeptide GLY–X–GLY in an extended conformation, then this residue is regarded as an exposed residue. If the ASA is less than 20% of the ASA calculated for the tripeptide GLY–X–GLY, it is regarded as an internal residue (Fraczkiewicz and Braun 1998). The ASA_{Buried} refers to the area buried by atoms belonging to other residues, and the ASA_{Surf} refers to the exposed surface area (Tsai and Nussinov 1997). Here, the ASA means the ASA per 100 amino acids.

Compactness

The compactness (Z) was calculated according to the definition proposed by Zehfus and Rose (1986). The Z is defined as the ASA of a structure divided by its minimum possible surface area (Tsai and Nussinov 1997), so the Z is calculated as

$$Z = \mathrm{ASA_{Surf}}/(36\pi \mathrm{VOL}^2)^{\frac{1}{3}}.$$

The $\mathrm{ASA_{Surf}}$ is the exposed ASA of a protein. The VOL is the volume, which is calculated as (Zehfus 1994)

$$\mathrm{VOL} = \sum_i \frac{1}{3}\mathrm{ASA}_i(N_i V_i).$$

The VOL can be calculated using the program VIODOO (Kleywegt and Jones 1994).

Hydrophobicity

According to the types of atoms, the ASA include non-polar ASA ($\mathrm{ASA_{Non}}$) and polar ASA ($\mathrm{ASA_{Pol}}$). Hydrophobicity (H) means the ratio of the buried non-polar in total non-polar ASA, the H is calculated using following formula

$$H = \mathrm{ASA}^{\mathrm{Non}}_{\mathrm{Buried}}/\left(\mathrm{ASA}^{\mathrm{Non}}_{\mathrm{Buried}} + \mathrm{ASA}^{\mathrm{Non}}_{\mathrm{Surf}}\right).$$

The number and volume of cavity

The packing density of a protein is related to the number and volume of cavities in the protein structure. A cavity is defined as the spatial structure that can accommodate at least one water molecule in the protein structure. The program VIODOO (Kleywegt and Jones 1994) is used to compute the number and volume of cavity in the protein structure.

The temperature factor

The temperature factor is a parameter of protein structure determined by X-ray. The PDB file of the protein records the temperature of each atom. The program BAVERAGE in ccp4-5.0.2 (Collaborative Computational Project 4 1994) can compute the average temperature of side chain and main chain.

Results and discussion

Secondary structural properties

Secondary structure contents

Secondary structure contents, especially helices and sheets, play an important role in stabilizing proteins. The data for archaeal proteins in Table S2 demonstrate that the contents of B and G increase with greater protein thermostability. In other words, HTH_AR have more isolated β-bridges and 3_{10}-helices in structures than TH_AR and especially ME_AR. For bacterial protein, the contents of B, E, T, S, and blank decrease with greater protein thermostability. The p values of B, T, and blank between HTH_BC and ME_BC and between TH_BC and ME_BC are smaller than 0.05. Therefore, B, T and blank are major factors that influence bacterial protein thermostability. There is no common trend for secondary structure content in archaeal and bacterial proteins. However, there is a negative relationship between B, T and blank for bacterial protein thermostability, as opposed to the positive relationship between B, T and blank for archaeal protein thermostability.

Sheet hydrogen bonding patterns that are too short will be designated as isolated β-bridge. More isolated β-bridge can allow HTH_AR and TH_AR to resist high temperatures, but not bacterial proteins. 3_{10}-helices often exist in the α-helix terminal or as a connector between two β-strands. Pal and Basu (1999) reported that a large fraction of independent two-turn and longer 3_{10}-helices formed novel super-secondary structural motifs with α-helices and β-strands. These motifs may be related to protein folding or local conformational relaxation. Szilagyi and Zavodszky (2000) also determined that the number of 3_{10}-helices decreased dramatically compared with mesophilic GAPDH, and these authors indicated that the 3_{10}-helices were transformed mainly into α-helices and β-strands. According to the statistical method results in Table S2, the thermostability of hyperthermophilic archaeal proteins is related to the motif formed by 3_{10}-helices, along with α-helices and β-strands. Certainly, the inter-transition between 3_{10}-helices and α-helices seems to play a key role.

The length of secondary structure

The average length of secondary structures, especially loop length, affects protein thermostability (Thompson and Eisenberg 1999; Russell et al. 1994). The average lengths of all protein secondary structures are listed in Table S3. For archaeal proteins, the average length of E and S increases with greater thermostability. Thus, the heat-resistant archaeal proteins have longer β-sheets and loops than mesophilic proteins. The p value of G between TH_AR and ME_AR is 0.008, which indicates a remarkable reduction in the average length of G. Therefore, heat-resistant archaeal proteins have many shorter 3_{10}-helices in their structures. For bacterial proteins, the average length of B decreased and the average length of H and T increased with greater thermostability. Remarkably, the p(HTH–TH_BC), p(HTH–ME_BC) and p(TH–ME_BC) of H are all less than 0.05, which indicates that the

average length of H is a major factor determining bacterial protein thermostability.

Thompson and Eisenberg (1999) found that thermophilic sequences had fewer exposed loop regions than mesophilic sequences. There are also deleted, exposed loop regions in thermophilic sequences that reduce the entropy of unfolding. According to our result presented in Table S3, the short loop does not contribute to the thermostability of archaeal proteins. Karpen et al. (1992) determined that short 3_{10}-helices are usually present on protein surface and may form binding surfaces or active sites. The occurrence of many shorter 3_{10}-helices may be one reason that heat-resistant archaea can grow in a high-temperature environment. The helix content does not affect bacterial protein thermostability, but the average helix length does confer stability.

The distribution of amino acids in secondary structures

Thermophilic and mesophilic proteins differ in the distribution of amino acids in secondary structures, especially in α-helices (Haney et al. 1997; Russell et al. 1997). As shown in Table 1, several types of amino acids exhibit no significant change in β-bridges with greater thermostability of archaeal proteins, which indicates that β-bridges do not affect thermostability. Lys changes dramatically in most of the remaining secondary structures, which indicates that Lys significantly affects thermostability. For bacterial proteins, the content of Ala and Gln decreases dramatically and the content of Glu and Lys increases sharply in most secondary structures when thermostability increases.

Obviously, the decreased Gln content may minimize deamidation. Ala is the best helix-forming residue (Kumar and Bansal 1998), but the underlying reason that the Ala content is lower in hyperthermophilic bacterial proteins is still unknown. As shown in Table 1, the helix content is higher in mesophilic bacterial proteins than in heat-resistant proteins indicating the reason that the Ala content is lower in hyperthermophilic bacterial proteins. Furthermore, these results show that the helix content does not contribute much to the thermostability of bacterial proteins. Glu is a thermolabile amino acid, but some thermophilic proteins are known to have a high Glu content (Chakravarty and Varadarajan 2000; Haney et al. 1999). Here we show that there is a higher content of Glu and Lys in most secondary structures. Lys and Glu are charged amino acids and can form salt bridges to stabilize protein structure. However, Asp and Arg are also charged amino acids; we discuss later why there is not a higher content of these two amino acids in bacterial proteins.

In summary, Lys contents are higher in most secondary structures of thermo-tolerant archaeal and bacterial proteins. Therefore, Lys plays an important role in maintaining thermostability for proteins of both kingdoms. In addition, the bends and blanks of amino acids change significantly for these two protein types, and bend and blank are highly related to protein thermostability. The number and length of these two secondary structures and, the distribution of amino acids can affect protein thermostability.

Hydrogen bonds

Hydrogen bonds are classified into six types: main–main chain, side–side chain, main–side chain, charged–charged, uncharged–uncharged, and charged–uncharged. As shown in Table 2, the results of our studies indicate that total hydrogen bonds, side–side chain hydrogen bonds, and main–side hydrogen bonds negatively relate to archaeal protein thermostability. Other types of hydrogen bonds have no obvious effects on archaeal protein thermostability. Furthermore, there are more main–main chain hydrogen bonds, main–side chain hydrogen bonds, uncharged–uncharged hydrogen bonds, and charged–uncharged hydrogen bonds for ME_BC compared to HTH_BC and TH_BC. Therefore, we do not assert whether hydrogen bonds can improve protein thermostability because different types of hydrogen bonds have different effects on thermostability. Notably, main–side hydrogen bonds decrease with greater thermostability for both archaeal and bacterial proteins. Furthermore, the main–side hydrogen bonds and the uncharged–uncharged hydrogen bonds are major factors based on p(HTH–TH_BC), p(HTH–ME_BC) and p(TH–ME_BC).

Hydrogen bonds play an important role in maintaining protein structure. However, the influence of hydrogen bonds on protein thermostability has always been controversial (Dill 1990; Pace et al. 1996). Some studies have

Table 1 The significantly changed amino acid in secondary structure with thermostability increasing

	α-helix	β-bridge	β-strand	Bend	3_{10}-helix	Turn	Blank
Archaeal							
Decrease	DQST	–	–	S	–	–	CDW
Increase	–	–	K	KP	E	K	K
Bacterial							
Decrease	ADGHQST	QG	MQS	AQ	AQ	AQT	ADLQS
Increase	EK	K	EFIKPRV	K	KG	KR	EHIK

Table 2 The change of hydrogen bond with protein thermostability

Hydrogen bond	HTH_AR average	TH_AR average	ME_AR average	HTH_AR SD	TH_AR SD	ME_AR SD	*t*(HTH–TH_AR)	*p*(HTH–TH_AR)	*t*(HTH–ME_AR)	*p*(HTH–ME_AR)	*t*(TH–ME_AR)	*p*(TH–ME_AR)
Total hydrogen bond↓	**90.371**	**92.474**	**107.352**	21.236	30.649	11.265	−0.944	0.346	−1.782	0.076	−1.082	0.280
Side–side chain↓	**6.653**	**9.654**	**10.475**	5.972	14.708	10.498	−2.902	***0.004***	−0.812	0.462	−0.124	0.901
Main–main chain	35.044	42.686	35.003	23.336	32.483	25.489	−3.023	***0.003***	0.004	0.997	0.525	0.600
Main–side chain↓	**9.781**	**9.954**	**15.386**	6.842	7.894	13.001	−0.272	0.786	−0.962	0.390	−0.931	0.404
Charged–charged	8.110	6.166	7.838	5.933	5.893	7.143	3.772	***0.000***	0.101	0.919	−0.625	0.533
Uncharged–uncharged	24.610	38.234	29.196	14.765	28.909	21.386	−6.504	***0.000***	−0.684	0.494	0.694	0.488
Charged–uncharged	19.498	18.267	24.062	15.936	12.989	20.579	0.956	0.340	−0.633	0.527	−0.627	0.564
	HTH_BC average	TH_BC average	ME_BC average	HTH_BC SD	TH_BC SD	ME_BC SD	*t*(HTH–TH_BC)	*p*(HTH–TH_BC)	*t*(HTH–ME_BC)	*p*(HTH–ME_BC)	*t*(TH–ME_BC)	*p*(TH–ME_BC)
Total hydrogen bond	87.178	90.702	89.120	15.945	15.808	19.895	−1.714	0.087	−0.769	0.442	2.248	***0.025***
Side–side chain	5.803	8.322	8.199	3.749	7.441	12.273	−4.761	***0.000***	−1.548	0.122	0.295	0.768
Main–main chain↓	**34.142**	**35.462**	**37.583**	18.934	21.725	19.462	−0.531	0.597	−1.389	0.165	−2.695	***0.007***
Main–side chain↓	**8.876**	**11.221**	**13.357**	6.383	8.054	8.462	−2.776	***0.007***	−5.467	***0.000***	−7.043	***0.000***
Charged–charged↑	**7.991**	**7.509**	**7.229**	5.035	5.468	5.177	0.681	0.496	1.157	0.247	1.438	0.151
Uncharged–uncharged↓	**22.261**	**27.660**	**30.678**	11.915	16.851	20.754	−3.384	***0.001***	−5.428	***0.000***	−4.094	***0.000***
Charged–uncharged↓	**18.922**	**20.298**	**21.769**	10.778	13.135	12.240	−0.968	0.336	−2.067	***0.043***	−3.172	***0.002***

↑ Indicates the factor increases with the growth of protein thermostability

↓ Indicates the factor decreases with the growth of protein thermostability

The bold and italic number indicates the *p* value is smaller than 0.05

The bold and italic factor indicates it is a major influence factor

The bold number indicates the average value of factor in some kind of protein

shown that the numbers and types of hydrogen bonds could increase protein thermostability (Vogt et al. 1997; Zhang et al. 2007; Bougault et al. 2003; Shortle 1992; Brouns et al. 2005), while others have presented different conclusions (Szilagyi and Zavodszky 2000; Panasik et al. 2000; Lo Leggio et al. 1999). Kumar et al. (2000) divided hydrogen bonds into three classes (i.e., main–main chain, main–side chain, and side–side chain hydrogen bonds) and determined that only side–side chain hydrogen bonds increased in the monomers of most thermophilic proteins. Therefore, not all hydrogen bond types can affect thermostability.

Salt bridge

The ratio of salt bridge networks to total salt bridges is higher in HTH_AR than TH_AR, which is also higher than ME_AR, as shown in Table S4. From the amino acid composition of salt bridge, our results indicate that as protein thermostability increases, the content of Lys and Glu in the salt bridge also increases. From analysis of the ratio of salt bridge formed by charged amino acids, most Arg, Lys and Glu residues were determined to participate in salt bridge formation. This result indicates that hyperthermophilic and thermophilic proteins may select for some types of charged amino acids to form salt bridges. In heat-tolerant archaeal proteins, Glu can be used to form salt bridges more efficiently than Asp. Arg can be used to form salt bridges more efficiently than Lys because Arg is smaller than Lys.

For bacterial proteins, the total number of salt bridges increases dramatically with greater thermostability because p(HTH–ME_BC) is 0.021 and p(TH–ME_BC) is 0. Accordingly, salt bridges significantly contribute to the ability of the organism to withstand high temperatures. From the charged amino acid content in salt bridges, only Glu was determined to significantly increase. Thus, heat-tolerant bacterial proteins maximize the use of the negatively charged amino acid Glu to form salt bridges, while mesophilic bacterial proteins utilize the negatively charged amino acid Asp. The ratio of charged amino acids forming salt bridges also increased remarkably in heat-resistant proteins, especially for Glu and Asp. While the number of charged amino acids in mesophilic proteins is equivalent the number of heat-resistant proteins, the thermostability is quite distinct due to the greater utilization of negatively charged amino acids to form salt bridges.

These results demonstrate that salt bridges are the most important factor determining heat resistance in archaeal or bacterial proteins. Previous studies have shown that salt bridges and salt bridge networks are major factors that affect protein thermostability (Alsop et al. 2003; Hakamada et al. 2001; Karshikoff and Ladenstein 2001; Das and Gerstein 2000). However, archaeal and bacterial proteins are very different in the ability to form salt bridges under high-temperature resistance.

Surface area, compactness and hydrophobicity

As shown in Table 3, the number of buried residues, buried polar surface area, and hydrophobicity increases in archaeal proteins with greater protein thermostability, while there is a decrease in the exposed polar surface area. The total polar surface area, total non-polar surface area, buried polar surface area, buried non-polar surface area, exposed polar surface area, exposed non-polar surface area, hydrophobicity and compactness are related to thermostability for bacterial proteins, which is significantly different than archaeal proteins.

Chan et al. (1995) determined that the growth of buried atoms contributes to the extreme thermostability of aldehyde ferredoxin oxidoreductase (AOR) from *Pyrococcus furiosus*, and the results of this present study corroborate that conclusion. Increasing polar surface area is related to protein thermostability (Haney et al. 1997; Vogt and Argos 1997). From the average solvent-contact surface area (ASA), Knapp et al. (1997) determined that the ASA is not related to protein thermostability. If the surface area is divided into non-polar surface area, polar surface area, and charged-residue surface area, the surface of thermophilic glutamate dehydrogenase has fewer hydrophobic residues and more charged residues. However, Kumar et al. (2000) determined that heat-resistant and mesophilic proteins had similar surface areas and packing densities, and these authors concluded that heat-resistant and mesophilic proteins had similar hydrophobicity cores. Our results indicate that the polar surface area in archaeal proteins can affect protein thermostability.

Haney et al. (1997) ascertained that heat-resistant proteins have an increased hydrophobicity. The results in Table 3 show that hydrophobicity is greater in heat-tolerant archaeal proteins compared to mesophilic proteins, which is consistent with Haney's conclusion. Kumar et al. (2000) found that thermophilic protein compactness was similar to mesophilic proteins. Karshikoff and Ladenstein (1998) computed the cavity volume and compactness of many thermophilic and mesophilic proteins, and compactness did not affect protein thermostability. From our results, compactness has no relationship to protein thermostability in archaeal proteins.

Cavity number and volume

Both cavity number and volume are related to protein packing density. Cavity volume is very small compared to whole protein volume. However, protein packing density is very important when considering the energy that stabilizes

Table 3 The change of surface area, compactness and hydrophobicity with protein thermostability

Surface area (*H*, *Z*)	HTH_AR average	TH_AR average	ME_AR average	HTH_AR SD	TH_AR SD	ME_AR SD	*t*(HTH–TH_AR)	*p*(HTH–TH_AR)	*t*(HTH–ME_AR)	*p*(HTH–ME_AR)	*t*(TH–ME_AR)	*p*(TH–ME_AR)
Total polar surface area	390.285	485.289	365.119	307.704	377.994	270.260	−3.039	***0.003***	0.182	0.856	0.970	0.382
Total non–polar surface area	1022.099	1702.248	618.293	1192.015	2934.385	499.713	−3.065	***0.002***	0.755	0.451	0.824	0.411
Buried residue↑	**550.372**	**461.167**	**161.055**	782.404	687.589	133.160	1.338	0.181	1.111	0.268	0.973	0.331
Exposed residue	4702.112	4854.607	3275.693	2594.308	4044.028	720.244	−0.522	0.602	1.227	0.221	0.871	0.385
Buried polar surface area↑	**72.376**	**66.601**	**45.770**	72.212	60.523	62.119	0.953	0.341	0.818	0.414	0.759	0.448
Buried non–polar surface area	75.559	122.117	14.954	96.133	139.893	8.704	−4.044	***0.000***	1.407	***0.160***	9.949	***0.000***
Exposed polar surface area↓	**517.093**	**551.558**	**586.262**	244.796	305.057	255.639	−1.376	0.169	−0.600	0.580	−0.252	0.801
Exposed non–polar surface area	1376.673	1889.259	1007.921	1030.022	2782.981	210.681	–2.456	***0.015***	0.799	0.425	0.706	0.481
Hydrophobicity (*H*)	0.032	0.032	0.005	0.061	0.048	0.004	0.130	0.896	1.017	***0.310***	1.262	***0.208***
Compactness (*Z*)	0.377	0.539	0.190	0.432	0.461	0.179	–3.846	***0.000***	0.965	0.335	1.681	0.094
	HTH_BC average	TH_BC average	ME_BC average	HTH_BC SD	TH_BC SD	ME_BC SD	*t*(HTH–TH_BC)	*p*(HTH–TH_BC)	*t*(HTH–ME_BC)	*p*(HTH–ME_BC)	*t*(TH–ME_BC)	*p*(TH–ME_BC)
Total polar surface area↓	**401.319**	**570.814**	**709.477**	371.814	500.249	793.510	−3.352	***0.001***	−3.025	***0.003***	−6.090	***0.000***
Total non–polar surface area↓	**863.166**	**1032.103**	**1078.278**	579.490	698.485	1210.129	−1.844	0.065	−1.385	***0.166***	−1.066	***0.287***
Buried residue	419.687	672.701	436.417	780.290	1173.216	585.403	−1.687	0.092	−0.222	0.824	5.968	***0.000***
Exposed residue	4319.148	4320.388	4163.598	1181.067	1796.605	2513.737	−0.005	0.996	0.490	0.624	2.076	***0.038***
Buried polar surface area↓	**54.409**	**103.911**	**134.081**	57.508	99.608	167.912	−6.105	***0.000***	−9.927	***0.000***	−5.005	***0.000***
Buried non–polar surface area↓	**39.977**	**78.747**	**80.426**	48.550	110.371	125.346	−2.724	***0.007***	−2.516	***0.012***	−0.353	0.724
Exposed polar surface area↓	**534.971**	**656.733**	**826.230**	282.418	408.275	624.372	−3.143	***0.002***	−3.635	***0.000***	−7.483	***0.000***
Exposed non–polar surface area↓	**1320.686**	**1346.821**	**1390.448**	368.321	476.825	1030.516	−0.419	0.675	−0.528	***0.598***	−1.714	***0.087***
Hydrophobicity (H)↓	**0.013**	**0.026**	**0.030**	0.015	0.043	0.052	−5.340	***0.000***	−7.884	***0.000***	−2.176	***0.030***
Compactness (Z)↓	**0.300**	**0.347**	**0.406**	0.251	0.325	0.392	1.106	0.269	3.160	***0.002***	−4.198	***0.000***

↑ Indicates the factor increases with the growth of protein thermostability

↓ Indicates the factor decreases with the growth of protein thermostability

The bold and italic number indicates the *p* value is smaller than 0.05

The bold and italic factor indicates it is a major influence factor

The bold number indicates the average value of factor in some kind of protein

protein structure (Eriksson et al. 1992; Ishikawa et al. 1993). The number of cavities can affect packing density and structure regularity. The data in Table S5 shows that cavity number has no influence on archaeal and bacterial protein thermostability, but that cavity volume does affect thermostability. Furthermore, cavity volume is larger in heat-tolerant archaeal proteins than mesophilic proteins, but smaller in heat-tolerant bacterial proteins than mesophilic proteins.

Cavity volume decreases with greater thermostability (Russell et al. 1994), and our results are consistent with this conclusion for bacterial proteins. Vogt et al. (1997) determined that approximately half of the families studied had a higher packing density with greater thermostability. However, Yip et al. (1995) indicated that mesophilic and thermophilic glutamic dehydrogenase had similar packing densities. In this paper, we determined that cavity volume may affect archaeal and bacterial protein thermostability, while cavity number does not alter thermostability. Hubbard and Argos (1994) stated that the cavity volume and number increased with higher molecular weight. If reduction of the cavity number and volume can increase protein thermostability, the molecular weight of thermophilic proteins should be smaller than that of mesophilic proteins'. In our study, bacterial thermophilic proteins had small cavity volumes, which is consistent with the Hubbard's conclusion.

Temperature factor

The temperature factors can determine the mobility of residues. Higher temperature factors confer higher flexibility to protein structures. According to the results in Table 4, the temperature factors for main chain, side chain and whole side chain do not greatly increase thermostability for archaeal proteins. However, these three types of temperature factors change greatly for bacterial proteins. Thus, temperature factors contribute significantly to bacterial protein thermostability, but have no effect on archaeal protein thermostability. These data indicate that side chain temperature factors are greater in both archaeal and bacterial proteins than main chain temperature factors, which is consistent with the greater mobility of side chains compared to main chains. By calculating the average temperature factor, we find that main–main, side–side and whole chain temperature factors are all positively related to protein thermostability for both archaeal and bacterial proteins. Therefore, protein flexibility is an important factor in the degree of protein thermostability.

Protein stability is related to the balance of flexibility and rigidity (Wray et al. 1999). Tsou proposed the theory for the conformational flexibility of enzyme active sites,

Table 4 The change of temperature factor with protein thermostability

Temperature factor	HTH_AR average	TH_AR average	ME_AR average	HTH_AR SD	TH_AR SD	ME_AR SD	*t*(HTH–TH_AR)	*t*(HTH–TH_BC)	*p*(HTH–TH_AR)	*p*(HTH–TH_BC)	*t*(HTH–ME_AR)	*t*(HTH–ME_BC)	*p*(HTH–ME_AR)	*p*(HTH–ME_BC)	*t*(TH–ME_AR)	*t*(TH–ME_BC)	*p*(TH–ME_AR)	*p*(TH–ME_BC)
Main chain↑	**30.208**	**28.996**	**24.235**	16.709	14.050	13.435	0.814	3.065	0.416	***0.002***	−0.794	5.622	0.428	***0.000***	−0.748	6.471	0.455	***0.000***
Side chain↑	**36.109**	**35.554**	**28.765**	17.882	16.603	13.350	0.359	2.560	0.720	***0.011***	−0.913	4.782	0.362	***0.000***	−0.907	9.643	0.366	***0.000***
Whole chain↑	**34.012**	**33.582**	**26.589**	18.035	16.076	13.208	0.280	2.511	0.780	***0.012***	−0.916	4.629	0.361	***0.000***	−0.964	9.467	0.336	***0.000***
	HTH_BC average	TH_BC average	ME_BC average	HTH_BC SD	TH_BC SD	ME_BC SD												
Main chain↑	**33.923**	**27.594**	**23.834**	16.076	15.016	13.335												
Side chain↑	**39.533**	**33.687**	**27.758**	18.795	16.903	14.013												
Whole chain↑	**37.683**	**31.910**	**26.079**	19.141	17.006	13.926												

↑ Indicates the factor increases with the growth of protein thermostability

↓ Indicates the factor decreases with the growth of protein thermostability

The bold and italic number indicates the p value is smaller than 0.05

The bold and italic factor indicates it is a major influence factor

The bold number indicates the average value of factor in some kind of protein

which explains the requirement for enhanced flexibility in this region of the proteins (Tsou 1993; Zhang et al. 1999). Similarly, not only is the enzyme and substrate interaction required for conformational flexibility when there is heat stimulus, but proteins can accelerate conformational change to increase flexibility and cope with this stimulus. In addition, there was less Ser and Thr in mesophilic proteins (Parthasarathy and Murthy 2000), which indicates that Ser and Thr are less flexible. Glu and Lys increase and Ser and Thr decrease in high-temperature factor regions of thermophiles in comparison to mesophiles. Accordingly, we deduced that the change of these amino acid compositions may guarantee high protein structure flexibility. Meanwhile, more salt bridges are formed with increased Glu and Lys to improve structure rigidity. Therefore, heat-tolerant proteins must balance structural rigidity and flexibility to withstand the extreme heat.

Conclusions

We conclude that some factors may assist both archaeal and bacterial proteins in high-temperature resistance. There are many common factors that determine the resistance of archaeal and bacterial proteins to high temperature, and these include increases in Lys content, secondary structure bend and blank, and Glu content in salt bridges; decreases in main–side chain hydrogen bonds, exposed surface area, and changes in the bend and blank of amino acids. Certainly, the most exciting result is the use of charged amino acids to form salt bridges. Even when the amount of charged amino acids is the same, the number of formed salt bridges may vary between heat-resistant proteins and mesophilic proteins. In both heat-resistant archaeal and bacterial proteins, most Glu and Asp participate in the formation of salt bridges. Some factors only influence either archaeal or bacterial protein thermostability. There are many shorter 3_{10}-helices and increased hydrophobicity in heat-resistant archaeal proteins. However, there are increases in average helix length, Glu content in the salt bridge, and temperature factors and decreases in the main–side chain hydrogen bond, the uncharged–uncharged hydrogen bond, hydrophobicity, and the buried and exposed polar surface area in heat-resistant bacterial proteins. Evidently, there are few similarities and many disparities between the heat-resistant mechanisms of archaeal and bacterial proteins. Therefore, when studying protein thermostability, it should be taken into account that the factors/mechanisms underlying the thermostability of archaeal and bacterial proteins may differ.

Certainly, a further increase in the number of hyperthermophilic bacteria would further add to the generality of our conclusions; and, a further increase in the number of proteins from mesophilic archaea other than halophiles would further add to the accuracy of our calculations.

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