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Hydrophobic environment is a key factor for the stability of thermophilic proteins

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ABSTRACT

The stability of thermophilic proteins has been viewed from different perspectives and there is yet no unified principle to understand this stability. It would be valuable to reveal the most important interactions for designing thermostable proteins for such applications as industrial protein engineering. In this work, we have systematically analyzed the importance of various interactions by computing different parameters such as surrounding hydrophobicity, inter-residue interactions, ion-pairs and hydrogen bonds. The importance of each interaction has been determined by its predicted relative contribution in thermophiles versus the same contribution in mesophilic homologues based on a dataset of 373 protein families. We predict that hydrophobic environment is the major factor for the stability of thermophilic proteins and found that 80% of thermophilic proteins analyzed showed higher hydrophobicity than their mesophilic counterparts. Ion pairs, hydrogen bonds, and interaction energy are also important and favored in 68%, 50%, and 62% of thermophilic proteins, respectively. Interestingly, thermophilic proteins with decreased hydrophobic environments display a greater number of hydrogen bonds and/or ion pairs. The systematic elimination of mesophilic proteins based on surrounding hydrophobicity, interaction energy, and ion pairs/hydrogen bonds, led to correctly identifying 95% of the thermophilic proteins in our analyses. Our analysis was also applied to another, more refined set of 102 thermophilic–mesophilic pairs, which again identified hydrophobicity as a dominant property in 71% of the thermophilic proteins. Further, the notion of surrounding hydrophobicity, which characterizes the hydrophobic behavior of residues in a protein environment, has been applied to the three-dimensional structures of elongation factor-Tu proteins and we found that the thermophilic proteins are enriched with a hydrophobic environment. The results obtained in this work highlight the importance of hydrophobicity as the dominating characteristic in the stability of thermophilic proteins, and we anticipate this will be useful in our attempts to engineering thermostable proteins.

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Key words: protein stability; thermophilic proteins; surrounding hydrophobicity; EF-Tu proteins.

INTRODUCTION

Elucidating the molecular mechanisms responsible for the stability of thermophilic proteins is an important task for engineering stable proteins. Enhancing protein stability is required for biotechnological processes that require catalysis at elevated temperatures, for increased product/substrate solubility, viscosity and catalysis, and to prohibit biological contamination. To achieve this goal, two different approaches have been used: experimental attempts have been made to increase the stability of enzymes and proteins for industrial applications[1–5] while, conversely, computational methods have been developed in attempt to understand the major factors for the stability of thermophilic proteins. Computational methods are themselves broadly based on two approaches: (i) comparison of a thermophilic protein structure with its mesophilic homologue[6,7] and (ii) comparisons of amino acid features from mesophilic and thermophilic families.[8,9]

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Both experimental and computational analyses have revealed the importance of specific interactions for the stability of thermophilic proteins based on amino acid replacements as determined from thermodynamic data for proteins and their corresponding mutants.[10] Major contributions come from hydrophobic interactions, hydrogen bonds, and electrostatic interactions.[11–13] For instance, Kumar *et al.*[14] reported that the stability of thermophilic proteins is achieved by an increase in the number of ion pairs and salt bridges. The importance of electrostatic interactions on protein stability has also been stressed by other researchers.[15–17] Vogt *et al.*[18] examined a set of 16 families of proteins and reported that hydrogen bonding is the major predictor for the stability of thermophilic proteins. Further, thermophiles have a greater number of main chain hydrogen bonds than mesophiles.[19] The influence of hydrophobic interactions has been demonstrated through the site-specific replacement of amino acids and include such properties as the number of aromatic clusters, the packing geometry of aromatic residues, the main chain hydrophobic free energy, and the compactness and packing of water accessible residues.[20–26] Although various interactions are reported to be important for stability coming from different perspectives, systematic analysis with a unified approach has not yet been completely explored. Such an exploration is necessary in order to understand the relative importance and contributions of different interactions.

In this work, we have analyzed the importance of different interactions by computing structure-based parameters such as surrounding hydrophobicity, ion pairs, hydrogen bonds, inter-residue interaction energy, long-range order, and multiple contact index in a set of 373 thermophilic proteins and their mesophilic counterparts. We found that the hydrophobic environment is the major factor for the stability of thermophilic proteins and 80% of the considered pairs obeyed this finding. The importance of ion pairs, hydrogen bonds, and van der Waals interactions is supported by their presence in 68%, 50%, and 62% of the thermophilic proteins. The combination of hydrophobicity, interaction energy, and ion pairs/hydrogen bonds increased the discrimination accuracy of up to 95%. Thus, in this work, we have compared the relative contributions of different interactions contained within two large datasets and revealed that the hydrophobic properties of amino acid residues along with interaction energies and ionic bonds account for the majority of stability conferred upon thermostable proteins.

MATERIALS AND METHODS

Dataset

We used a dataset of 373 pairs of thermophilic and mesophilic proteins compiled by Glyakina *et al.*[25] for our analyses (Supplementary Table S1). The dataset has the following features: (i) multi-domain proteins were divided into separate, single domains, (ii) a domain has no more than 400 residues, (iii) if one partner of the pair had a longer sequence at the amino or carboxyl terminus, the extended segment of residues was truncated, (iv) the difference in the length between the proteins in a pair was not more than 10%, (v) the number of residues that lack 3D coordinates were not more than 10%, and (vi) the structural alignment score computed with Maxsub was more than 70%.

In addition, we used a dataset of 102 aligned mesophilic and thermophilic protein pairs presented in Greaves and Warwicker,[27] which is different from the original set of 373 pairs. Lastly, we tested our approach on both ancestral and modern elongation factor-Tu proteins.

Computation of surrounding hydrophobicity

Amino acid residues in a protein molecule are represented by their α-carbon atoms and each residue is assigned with the hydrophobicity index obtained from thermodynamic transfer experiments.[28,29] The surrounding hydrophobicity (H_p) of a given residue is defined as the sum of hydrophobic indices of various residues, which appear within an 8 Å radius limit.[30]

$$H_p(i) = \sum_{j=1}^{20} n_{ij} h_j \qquad (1)$$

where n_{ij} is the total number of surrounding residues of type j around ith residue of the protein and h_j is the experimental hydrophobic index of residue type j in kcal/mol.[28,29] The average surrounding hydrophobicity of a protein is then the sum of the H_p values of all the residues normalized by the total number of residues.

The limit of 8 Å is sufficient to characterize the hydrophobic behavior of amino acid residues[31] and to accommodate both local and non-local interactions.[32,33] Further, an 8 Å limit has been used in several studies, such as to understand the folding rate of two-state proteins,[34,35] protein stability after mutations,[36] thermal stability of proteins,[37] and to determine the transition state structures of proteins.[38]

Computation of inter-residue interaction energy

We calculated the interaction energy between atoms in protein structures using the AMBER potential,[39] which is widely used in protein folding and stability analysis. It is given by:

$$E_{\text{inter}} = \sum [A_{ij}/r_{ij}^{12} - B_{ij}/r_{ij}^{6} + q_i q_j/\epsilon r_{ij}] \qquad (2)$$

where $A_{ij} = \varepsilon_{ij}^{*}(R_{ij}^{*})^{12}$ and $B_{ij} = 2\ \varepsilon_{ij}^{*}(R_{ij}^{*})^{6}$; $R_{ij}^{*} = (R_i^{*} + R_j^{*})$ and $\varepsilon_{ij}^{*} = (\varepsilon_i^{*}\ \varepsilon_j^{*})^{1/2}$; $R^{\star}$ and $\varepsilon^{\star}$ are, respectively, the

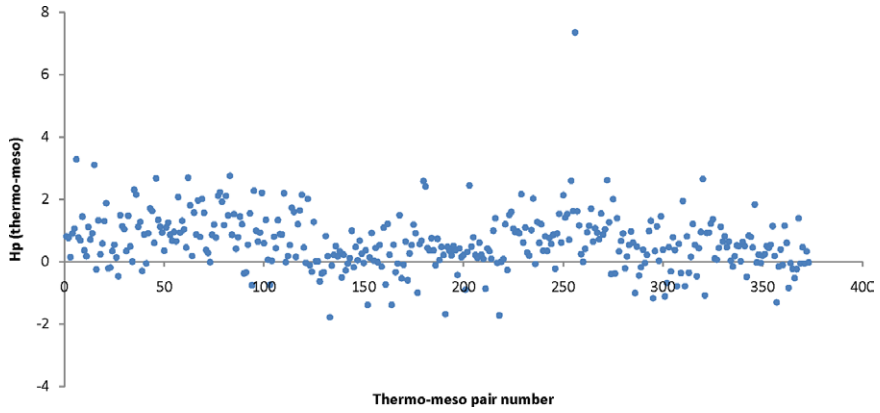

Figure 1

Difference of surrounding hydrophobicity between thermophilic and mesophilic proteins in a dataset of 373 protein pairs. The positive value shows that H_p is higher in thermophilic proteins. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

van der Waals radius and the well depth, and these parameters are obtained from Cornell et al.[39]; q_i and q_j are, respectively, the charges for the atoms i and j, and r_{ij} is the distance between them. We used the distant dependent dielectric constant ($\varepsilon = r_{ij}$) to take into account of the dielectric damping effect of the Coulomb interactions, as used in other studies.[40]

Computation of ion pairs and hydrogen bonds

We identified ion-pairs in a protein based on the distance between the positively (N^+) and negatively charged (O^-) atoms. A cutoff of 3.5 Å is used to define the cutoff distance for the formation of an ion-pair.[41] The number of hydrogen bonds in a protein is computed using the program HBPLUS.[42]

Estimation of long-range order

The long-range order (LRO) for a protein was computed using knowledge of long-range contacts (contacts between two residues that are close in 3D space but distant in primary sequence) in protein structure.[35] LRO for a specific residue is calculated using the number of long-range contacts for that residue. It is given by:

$$\mathrm{LRO}_i = \sum_{j=1}^{N} n_{ij}/N;\ n_{ij} = 1 \text{ if } |i-j| > 12;\ n_{ij} = 0 \text{ otherwise}, \quad (3)$$

where i and j are two residues in which the C_α distance between them is $\leq$ 8 Å and N is the total number of residues in a protein. The LRO for a whole protein can be obtained by summing up the LRO values obtained for all the residues in the protein.

Multiple contact index

Multiple contact index of a protein is defined using three parameters: (i) distance between amino acid residues in space, (ii) primary sequence separation between the residues, and (iii) number of residues that have multiple contacts. It is given by[43]:

$n_{ci} = \Sigma n_{ij}$; $n_{ij} = 1$ if $r_{ij} < 7.5$ Å; $|i\text{-}j| > 12$ residues; 0 otherwise;

$$\mathrm{MCI} = \sum n_{mi}/N;\ n_{mi} = 1 \text{ if } n_{ci} \geq 4;\ 0 \text{ otherwise}, \quad (4)$$

where n_c is the number of contacts for each residue and r_{ij} is the distance between the C_α atoms of residues i and j. N is the total number of residues.

RESULTS AND DISCUSSION

Role of hydrophobicity in thermophilic proteins

We computed the surrounding hydrophobicity of all amino acid residues in a protein using Eq. (1) and estimated the average value. The calculation was conducted for all the mesophilic and thermophilic proteins in the dataset of 373 proteins. The difference between the hydrophobic behavior of thermophilic and mesophilic proteins is shown in Figure 1. We noticed that about 80% of the thermophilic proteins have higher surrounding hydrophobicity compared to their mesophilic homologues. We also examined the tendency in another dataset of 102 protein pairs and 71% of these pairs showed the same pattern. These results highlight the importance of hydrophobicity for the stability of thermophilic proteins as reported in the literature.[44]

The surrounding hydrophobicity profiles of a typical pair of mesophilic–thermophilic proteins (1jnr: adenylyl-

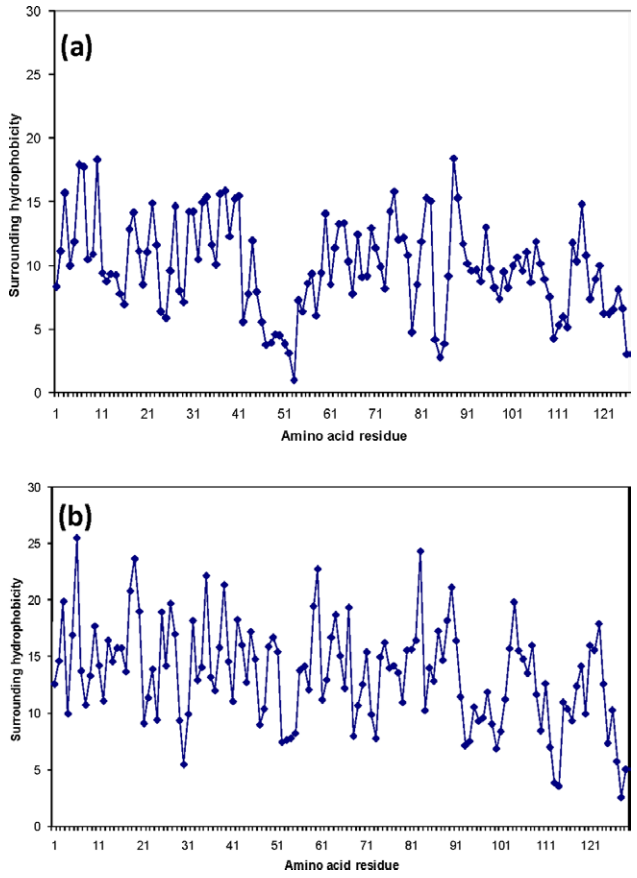

Figure 2

Surrounding hydrophobicity profiles for (a) 1nek and (b). 1jnr [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

sulfate reductase from *Archaeoglobus fulgidus* and 1nek: succinate dehydrogenase from *Escherichia coli*) are shown in Figure 2(a,b). These proteins have a structural alignment score (Maxsub value) of 79.8 across 114 aligned residues. The proteins have similar structures and the mean root mean square deviation between all atoms is less than 3 Å. We observed that several residues in 1jnr have a surrounding hydrophobicity of more than 20 kcal/mol whereas none of the residues in 1nek have a value higher than this cutoff. Further analysis showed that the difference between the average surrounding hydrophobicities in these proteins is 3.28 kcal/mol. The superimposition of amino acid residues in these proteins is shown in Figure 3, which reveals the hydrophobic packing of 1jnr, possibly responsible for the increased stability as achieved via the patches of residues such as I13-N22, E40-V59, F71-Y79, and W92-S98.

The variation of difference in surrounding hydrophobicity in the set of 373 protein pairs is presented in Figure 4. We found that few pairs have a H_p difference of more than 3 kcal/mol but that 32% of the considered pairs (121 pairs) have H_p values $>$ 1 kcal/mol in thermophiles than their respective mesophiles. These results demonstrate the importance of hydrophobic interactions for the stability of thermophilic proteins.

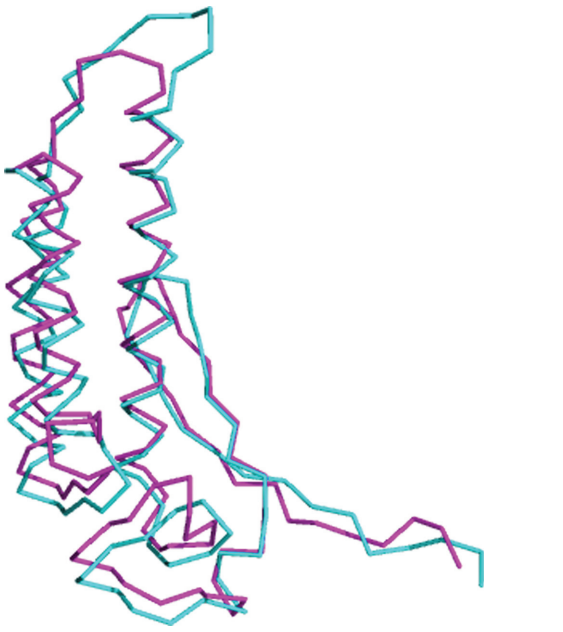

Figure 3

Ribbon model for the superimposition of the proteins 1nek (blue) and 1jnr (magenta). These proteins have similar structures and the rmsd between all atoms is 2.9 Å. The packing of residues in 1jnr can be clearly seen from the figure.

Hydrophobic behavior of mesophilic and thermophilic proteins computed with C_β atoms

We have computed the surrounding hydrophobicity of amino acid residues using C_β atoms and a cutoff distance of 6.5 Å (C_α atoms for Gly) in addition to our analysis

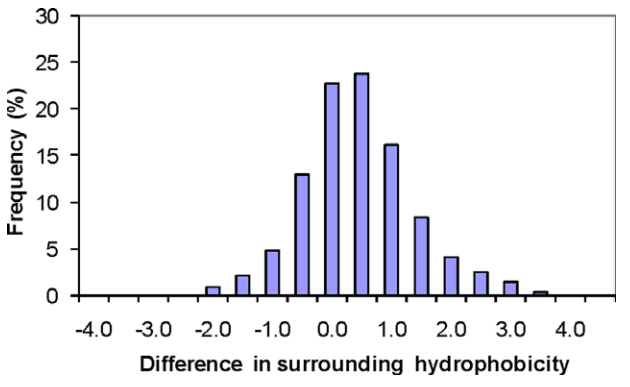

Figure 4

Frequency of thermophilic–mesophilic pairs based on differences in surrounding hydrophobicity. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

of C_α atoms. We noticed that a minimum distance of 6.5 Å is required to accommodate these residues, which are further away in protein space but also account for hydrophobic interactions. The C_β atoms make more contacts in protein structures compared with C_α atoms at a specific cutoff distance and we noticed that the range of surrounding hydrophobicity of residues obtained with 8 Å limit of C_α atoms is similar to those obtained with 6.5 Å radius of C_β atoms. Our analysis showed that the average surrounding hydrophobicity of 292 thermophilic proteins (78.3%) are higher than that of mesophilic proteins. This observation is similar to that obtained with the computation of surrounding hydrophobicity using C_α atoms with a distance cutoff of 8 Å.

Surrounding hydrophobicity of multidomain proteins

We have evaluated the contribution of hydrophobicity by considering all the domains together in a protein, which accounts for both intra- and inter-domain interactions. We found that 80.2% of the analyzed proteins have higher hydrophobicity in thermophilic proteins than mesophilic ones. We then repeated the analysis using proteins only with multidomains and we observed that 82.9% of the thermophiles are more hydrophobic than the mesophiles. This analysis emphasizes the role of hydrophobicity for the stability of thermophilic proteins regardless of domain structure.

We have also computed other factors, such as ion pairs, hydrogen bonds and interaction energy with all domains together. We noticed that the trends are similar to those obtained with individual domains as discussed in the Methods section.

Role of packing for the stability of thermophilic proteins

It is generally thought that increased thermostability of thermophilic proteins involves tight packing of the interior of a protein. We computed surrounding hydrophobicity of residues in the interior of both mesophilic and thermophilic proteins to address this notion. Residues with a solvent accessibility of less than 5% are considered to be interior residues. We observed that the average hydrophobicity is marginally higher in thermophilic proteins (18.5 kcal/mol) compared with their mesophilic (17.7 kcal/mol) homologues. This result confirms the influence of core packing on the stability of thermophilic proteins. On the other hand, we also computed the average surrounding hydrophobicity of surface residues (solvent accessibility is more than 75%) and values of 7.8 kcal/mol and 7.3 kcal/mol are obtained for thermophilic and mesophilic proteins, respectively. These results indicate that core residues are tightly packed while surface residues are more loosely packed in thermophilic proteins as reported in the literature.[25]

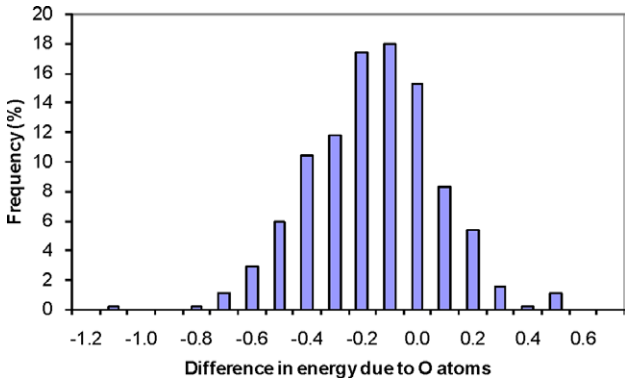

Figure 5

Frequency of thermophilic–mesophilic pairs based on differences in interaction energy due to oxygen atoms; negative energies represent favorable interactions. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Relationship between thermal stability and hydrophobicity in EF-Tu proteins

We computed the average hydrophobicity of five ancestral and three modern EF-Tu proteins from different organisms and compared the H_p values with their melting temperatures.[45] We obtained a correlation of 0.86 and 0.85 for the ancestral and modern proteins, respectively. The combination of all the EF-Tu proteins showed a correlation of 0.78 between hydrophobicity and thermal stability.

Interaction energy between amino acid residues

We computed the interaction energy between all atoms in mesophilic and thermophilic proteins and compared differences between them. We divided atoms into different groups, such as all atoms, main chain-main chain atoms, main chain-side chain atoms, side chain-side chain atoms, nitrogen (N), carbon (C), and oxygen (O) atoms. We observed that the contribution due to oxygen atoms was stronger than that due to other individual atoms as well as the combinations of atoms. Figure 5 shows the frequency of thermophilic-mesophilic pairs based on interaction energies due to oxygen atoms. We noticed that the interaction energy is favorable (negative values) for 68% of the thermophilic proteins in the considered set of 373 pairs.

Contribution due to ion pairs and hydrogen bonds

We computed the number of ion pairs and hydrogen bonds in the dataset of 373 mesophilic and thermophilic proteins and determined the differences between them. The results obtained for ion pairs and hydrogen bonds

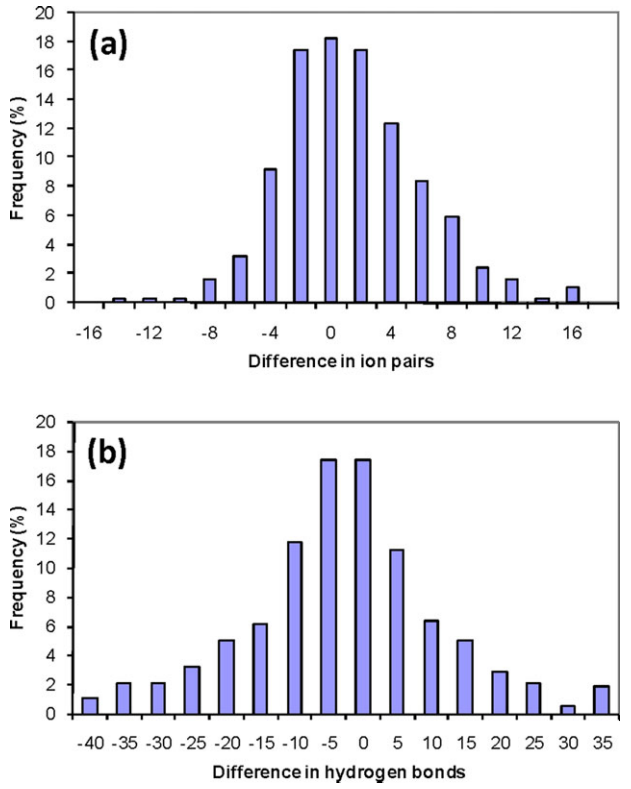

Figure 6

Frequency of thermophilic–mesophilic pairs based on difference in (a) ion pairs and (b) hydrogen bonds. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

are shown in Figure 6(a,b), respectively. The results show that the number of ion pairs could distinguish 68% of the mesophilic–thermophilic pairs of proteins. This agrees with the earlier observation that the number of ion pairs is important for the stability of thermophilic proteins.[14] On the other hand, the number of hydrogen bonds is higher for thermophilic proteins in 50% of the considered pairs while a reverse trend is observed for the remaining 50% of protein pairs. This analysis shows that hydrogen bonding is not a distinguishing factor for thermophilic proteins compared with their mesophilic counterparts.

Influence of long-range order and multiple contact index

Previous studies have shown that long-range order (LRO) and multiple contact index (MCI) play an important role in the folding of two-state proteins.[38,43,46] We have analyzed the influence of these parameters in correlation to the stability of thermophilic proteins. The results show that the LRO and MCI are higher only for 53% and 56% of the thermophilic proteins, respectively.

Combination of various interactions

We combined different interactions and the results obtained with various combinations are presented in Table I. We found that the combination of hydrophobicity, interaction energy due to oxygen atoms, and hydrogen bonds/ion pairs could distinguish 95% of the thermophilic proteins. Further analysis showed that the number of hydrogen bonds is higher only in those thermophilic proteins that have lower hydrophobicity. We examined the pattern with a different dataset of 102 protein pairs and the same combination of hydrophobicity, interaction energy due to oxygen atoms and hydrogen bonds/ion pairs could distinguish 91–93% of the thermophilic proteins.

We attempted to determine why our analyses were not able to identify all thermophilic proteins by using our unified approach. We determined that the failure to identify these proteins was due, in part, to the fact that they had fewer hydrogen bonds as well as fewer ion pairs. The influence of other factors such as additives, solutes, immobilization, chemical modifications in solution, etc. may attribute to their stabilities and would thus hinder our predictions/correlations.

CONCLUSIONS

We have determined the correlations of hydrophobic, electrostatic, hydrogen bonding, and van der Waals interactions among thermostable/mesostable protein-pairs using different terms. The contribution of hydrophobicity is higher in about 80% of the thermophilic proteins versus their mesophilic counterparts. A similar pattern is also observed in a set of ancestral and modern EF-Tu proteins. Further, the combination of hydrophobicity, interaction energy due to oxygen atoms and hydrogen bonds/ion pairs could distinguish 95% of the thermophilic proteins. Our study reveals the importance of hydrophobicity for the

Table I

Combination of Hydrophobicity, Ion Pairs, Hydrogen Bonds, and Interaction Energy

Term	Number of pairs	Percentage of pairs
H_p	296	79.4
Ion pairs	253	67.8
Hydrogen bonds	185	49.6
Interaction energy due to O atoms	254	68.1
H_p and ion pairs	342	91.7
H_p and hydrogen bonds	331	88.7
H_p and energy due to O atoms	345	92.5
H_p, energy, and HB	355	95.2
H_p, energy and ion	356	95.4
H_p, ion pairs, and hydrogen bonds	350	93.8
H_p and energy (all)	325	87.1
H_p, energy (all), and HB	342	91.7
H_p, energy (all), and ion pairs	349	93.6
H_p, energy (all), ion pairs, and hydrogen bonds	354	94.9
Energy (all)	232	62.2

H_p, surrounding hydrophobicity; HB, hydrogen bonds; Ion, ion pairs.

enhanced stability of thermophilic proteins with marginal contributions from other factors.

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