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Review

Protein thermostability in Archaea and Eubacteria

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ABSTRACT. In order to survive at high temperatures, thermophilic prokaryotes (Archaea and Eubacteria) adopt different strategies. Among several important contributing factors for stability of proteins are CG-rich codons, the ratio of charged amino acids compared to uncharged amino acids, ionic interactions, amino acid preferences and their distribution, post-translational modifications, and solute accumulation. However, these factors may differ from taxon to taxon, both within and between species depending upon the composition of proteins found in these organisms. This is exemplified in the case of differences in strategies adopted by soluble proteins and membrane proteins. Therefore, it appears that no single factor or combination of factors together can be universally attributed to the provision of thermal stability in proteins.

Key words: Archaea, Hyperthermophiles, Mesophiles, Thermophiles, Protein, Stability

INTRODUCTION

Most organisms living at moderate optimum growth temperature (OGT) of 24° to 40°C are mesophiles. Organisms living at the higher OGT of 50° to 70°C are thermophiles and at an OGT of greater than 80ºC are hyperthermophiles, including members of domains Archaea and Eubacteria. Archaea are probably the earliest living organisms; they occupy diverse habitats and are found among many ecological groups including the thermophiles, halophiles and psychrophiles (Woese et al., 1990; Doolittle, 1995; Zlatanova, 1997; Makarova and Koonin, 2003; Farias and Bonato, 2003). Although there are many thermophile and hyperthermophile Archaea, few Eubacteria are found at these elevated temperatures (Kreil and Ouzounis, 2001; Bao et al., 2002). Since these prokaryotes live under extreme conditions, certain characteristic features have enabled them to survive in these environments. Some of these features are modifications in the metabolic pathways: for example, the synthesis of co-factors such as heme, acetyl CoA, acyl CoA, and folic acid may be either greatly reduced or totally absent in thermophiles because of constraints of high temperature. This trend is seen in Archaea growing at OGT of around 60°C and increases with the increase in OGT (Kawashima et al., 2000). The Archaeal membrane and wall may also contribute to the thermal stability of the cellular structure. This may be due to unique chemical compositions, lipid side chain branching and ether linkages and the structural modifications that these may undergo when the temperature and salinity change (van de Vossenberg et al., 1995; Mathai et al., 2001; Futterer et al., 2004).

Here, we do not attempt to provide a comprehensive review of the vast literature on the strategies adopted to provide molecular and functional thermostability. Instead, we restrict our emphasis to Archaeal proteins and, where possible, comparisons with proteins from thermophilic Eubacteria. For convenience, we will employ the term “thermophile” to include both thermophiles and hyperthermophiles unless specified otherwise.

PROTEIN STABILITY

Thermophiles are under constant destabilizing effects of high temperatures. To counteract ill effects of temperature on proteins, thermophiles adopt strategies that are characteristically different from mesophiles. Some of the strategies are replacement of Arg with Tyr, presence of sulfate ions (Kallwass et al., 1992), the type of metal ions as co-factors, thermamines, chaperones, substrate, flexibility and or rigidity of proteins (Vieille et al., 2001), protein mobility (Panasik et al., 2000) and pressure due to deep sea habitat (Konisky et al., 1995). Besides these, slow unfolding of proteins in Archaea (Zeeb et al., 2004) but efficient refolding in thermophilic Eubacteria (Tokunaga et al., 2004), smaller size of protein and higher compactness ratio may also influence stability of proteins (Spassov et al., 1995; Chakravarty and Varadarajan, 2000; Hickey and Singer, 2004). However, some reports suggest that compactness ratio of proteins in thermophiles and mesophiles is not significantly different (Kumar et al., 2000b). It appears that these strategies are important but not universally adopted by thermophilic prokaryotes. In this review, we focus on protein-stabilizing factors such as CG-rich codons, the ratio of charged to uncharged amino acids, ionic interactions, amino acid preferences and their distribution, neutral nature of amino acids, and solute accumulation.

PROTEIN STABILITY AND CG CONTENT

Higher CG content in coding sequences in thermophiles (Bao et al., 2002; Saunders et al., 2003) as compared to mesophiles (McDonald et al., 1999; Zhu et al., 1999; Kreil and Ouzounis, 2001) affects the amino acid content and hence protein stability with some exceptions (Farias and Bonato, 2003; Paz et al., 2004; Hickey and Singer, 2004). The abundance of CG-rich codons is reported for amino acids such as Ala, Pro, Trp, Met, Gly, Glu, Arg, and Val (although Val is exceptional as it is GTX-rich coded and Met ATX-coded amino acids) as compared to Ile, Phe, Tyr, Asn, Lys, Gln, Thr, His, Ser, and Asn, in thermophiles but not in mesophiles (Kreil and Ouzounis, 2001). The authors attribute the observed unexpected higher values of Met and Val to the fact that Met is the start codon for almost all proteins, and that Met and Val belong to a conserved group of the interchangeable residues Ile, Leu, Met, and Val. However, the present study does not indicate differences between the two domains because the Eubacteria taken for this study are not hyperthermophiles and are of the ancient lineage close to Archaea. Lobry and Chessel (2003) agree with the contentions of Kreil and Ouzounis (2001) regarding the high CG content of the genome clearly affecting amino acid composition in proteins, although they further elaborate that there may be preferences for AGG, ATA, AGA, AAG, and to avoid CAA, CGT. However, Farias and Bonato (2003) and Paz et al. (2004) contradict these findings. Further, detailed analysis by the latter authors shows a preference for purine-rich tracts and predominantly for adenine in mRNA. They conclude that preference for purine-rich codons, particularly for charged amino acids, may have happened due to their contribution in providing thermal stability to protein. They suggest that purine-purinic codons could be a result of mutations that helped in the survival of thermophiles. Therefore, it appears that although earlier studies did find a positive correlation of high CG content of coding sequences with increasing OGT, recent studies after complete genome analysis of more thermophiles suggest that this correlation is not valid in the case of mRNA. However, these studies do find a positive correlation of high CG content in tRNA and rRNA sequences with high OGT (Gorgan, 1998; Bao et al., 2002; Hickey and Singer, 2004). Hickey and Singer (2004) suggest the need to look into other kinds of genomic biases besides the CG content of coding sequences that affect amino acid composition. It is therefore apparent that CG-rich codons may be important in the case of some organisms; it is not the sole universal factor in providing thermal stability to proteins. The following sections discuss other equally important factors that influence thermal stability in proteins.

RATIO FOR CHARGED AND POLAR AMINO ACIDS

Higher ratios for charged amino acids, especially at the protein surface, increase ion interactions which provide thermal stability to proteins (Szilágyi and Závodszky, 2000; Fukuchi et al., 2003; Nakashima et al., 2003; Saunders et al., 2003; Suhre and Claverie, 2003; Tanaka et al., 2004). Comparisons between mesophiles and thermophiles show that amino acids such as Glu, Arg, Tyr, Asp, and Lys are abundant in thermophiles as compared to Ala, Asn, Gln, Thr, Ser, and Val (Szilágyi and Závodszky, 2000; Paz et al., 2004). The ratios of these amino acids are important for flexibility or lack of it in proteins (Parthasarathy and Murthy, 2000); they help in tetramerization (L-isoaspartyl-O-methyltransferase from *Sulfolobus tokodaii*) and are critical for thermal stability (Tanaka et al., 2004). A comparison between thermophiles and hyperther-

mophiles shows that the number of charged amino acids (which may include Lys) is very high in hyperthermophiles, whereas thermophiles have a preference of Arg over Lys. However, Asp and Met are unstable at high temperatures and therefore their percentage decreases in hyperthermophiles (Szilágyi and Závodszky, 2000). This finding contradicts Tanaka et al. (2004) who report that Tyr and Asp interactions are critical for thermal stability particularly in hyperthermophiles. Although Asp residues particularly in Asp-Pro combination may be susceptible to hydrolysis of peptide bonds, they get protection by either substitution or by higher conformational rigidity (Vieille et al., 2001). The authors also discuss the reasons for preference for different amino acids in their review. In addition, it is suggested that temperature-induced deaminations have acted against selection of polar or non-charged amino acids (Chakravarty and Varadarajan, 2000; Vieille et al., 2001) contrary to the report by Lobry and Chessel (2003). However, Kreil and Ouzounis (2001) find an abundance of amino acids (mentioned in previous section) in thermophiles due to the pressure of CG-rich codons and not the charges that these amino acids have. In other reports, variation in the ratio of charged amino acids versus uncharged amino acids has been observed, and it is therefore suggested that the ratio of charged compared to uncharged amino acids alone cannot be responsible for thermal stability (Chakravarty and Varadarajan, 2000; Yamagishi, 2000; Vieille et al., 2001; McDonald, 2001). However, the differences in amino acids of psychrophilic Archaea and hyperthermophiles (Saunders et al., 2003) and a study of proteins related with transcription and replication in thermophiles (Paz et al., 2004) support the concept of higher proportions of charged amino acids in thermophiles as compared to mesophiles. On the other hand, it is not necessary that the adaptations in thermophiles are the opposite of psychrophiles (Russell, 2000).

Therefore, it appears that in some thermophiles, irrespective of the CG-rich codons, the ratio of charged to uncharged amino acids may be an important factor in the stability of protein. We feel that investigation of post-translational modifications of amino acids could also possibly settle this issue, since Febbraio et al. (2004) recently found stabilizing effects of lysyl methylation as a post-translational modification. Further, we speculate that prior to translation there may be certain mechanisms prevailing in these organisms that would provide charges to uncharged amino acids or vice versa, but possibly such changes remain undetected in the *in vitro* analysis.

AMINO ACID PREFERENCE

Several lines of investigations suggest that amino acid preference may vary from protein to protein within an organism or may be taxon specific (Kawashima et al., 2000). This is because in some thermophiles Ala is preferred over Tyr, whereas in others Val or Gly is preferred over Ile. However, Ile is preferred in *Methanococcus* (McDonald et al., 1999) and *Picrophilus torridus* (Futterer et al., 2004). A comparative study between hyperthermophilic Archaea *Methanococcus jannaschii* and thermophilic Eubacteria *Bacillus stearothermophilus* (OGT 85° and 60°C, respectively) shows that Lys is preferred in Archaea but not in Eubacteria. In *B. stearothermophilus*, preference is Gly over Ile and Ala over Tyr, but in *Methanococcus*, Tyr is preferred over Arg (McDonald et al., 1999). Since comparison was between only two genomes with different OGT, this cannot be a universal example of the differences between the two domains. However, almost universal preference for Glu and Lys over Gln and His in hyperthermophiles is reported (De Farias and Bonato, 2002; Farias and Bonato, 2003). Nevertheless, since there are variations in preference for other amino acids between mesophiles,

thermophiles and hyperthermophiles, it is apparent that these variations are not only organism specific but are also protein specific within the organism.

NEUTRAL NATURE OF PROTEINS AND SOLUTE ACCUMULATION

In addition to preference for amino acids, thermophiles may adopt alternative strategies for stabilizing protein. In this regard, although not clearly established, studies report a correlation between increase in OGT above 60°C and the neutral nature of proteins. This may be achieved by increasing intracellular salt concentrations even though the preference may be for charged amino acids (Kawashima et al., 2000). Increase in intracellular salt concentrations may be due to membrane permeability to water, which reduces with increase in temperature in Archaea. It is reported that aquaporins are preferentially permeable to protons than to sodium. This preferential permeability increases with increase in temperature and is considered essential for the growth of thermophiles (van de Vossenberg et al., 1995; Mathai et al., 2001; Futterer et al., 2004). Solutes such as di-myo-inositol-phosphate, di-mannosyl-di-myo-inositol-phosphate, diglycerol-phosphate, mannosylglycerate, and mannosylglyceramide may provide osmoprotection and thermoprotection, which are not found in prokaryotes that do not live in extreme environments (Santos and da Costa, 2002). However, in *Picrophilus torridus* intracellular pH is 4.6 unlike other thermoacidophiles, which maintain neutral pH. Therefore, in this case both extracellular and intracellular proteins counteract low pH stress in addition to temperature stress (Futterer et al., 2004).

IONIC INTERACTIONS

Among other strategies, investigations confirm that charge-to-charge interactions (Chakravarty and Varadarajan, 2000; Del Vecchio et al., 2002) and cation-Pi interactions render stability (Lo et al., 1999; Kumar et al., 2000a,b; Das and Gerstein, 2000; Bruins et al., 2001). These interactions particularly among long chains of Tyr with Lys in thermophiles, but His and Arg in mesophiles are important (Gromiha et al., 2002; De Farias and Bonato, 2002; Chakravarty and Varadarajan, 2002; Farias and Bonato, 2003). Amino acid content in thermophiles and mesophiles may not be significantly different. However, hydrogen bonds formed between hydrophobic and hydrophilic groups are due to Tyr and Cys (Gromiha, 2001), and hydrogen bonds networking both in the core and side chains stabilize proteins (Kumar et al., 2000a,b). On the other hand, Szilágyi and Závodszky (2000) report a high number of ionic bonds in hyperthermophiles as compared to thermophiles and low in mesophiles, but no differences in the number of hydrogen bonds. Frankenberg et al. (1999) consider ionic bonds important for protein stability in Eubacteria *Thermotoga maritima* also. However, a recent finding that an increased number of hydrogen bond is important for the stability of proteins supports reports on the importance of hydrogen bonds in the stability of proteins (Irimia et al., 2004).

In addition to amino acid-amino acid interactions that provide core and side chain stability, ionic bonds between subunits may help in stabilizing proteins (Nordberg et al., 2003) which may lead to a higher melting temperature (Kumar and Nussinov, 2001a). Disulfide bridges may also provide thermal stability to proteins even above 100°C (Kumar et al., 2000b; Sedlak et al., 2001; Vieille et al., 2001; Mallick et al., 2002; Roovers et al., 2004). Regarding this strategy there are differences between the two domains. Thermophilic and hyperthermophilic Archaea

show a significantly higher number of disulfide bridges as compared to thermophilic Eubacteria. However, thermophiles in general have a higher number of disulfide bridges as compared to mesophiles (Mallick et al., 2002). Contrary to this, other findings suggest that disulfide bridges may not be as important for stability in some proteins (Lo et al., 1999; Tanaka et al., 2004). It is possible that buried salt bridges are more stabilizing than exposed ones (Kumar and Nussinov, 2001a,b). At the same time, the situation is compounded by their report that ion interactions keep on changing due to the breaking of old and building of new salt bridges and changes in charges. However, it is increasingly being reported that hydrogen bonds, co-operative electrostatic interactions, side chains, and salt bridges (Tanaka et al., 2004), particularly in solvent exposed surfaces of proteins, helical structures and negative charges at the N-terminus are important for thermal stability (Szilágyi and Závodszky, 2000; Kumar et al., 2000b). The same is true in the case of thermophilic Eubacteria (Tomschy et al., 1994).

DISTRIBUTION OF AMINO ACIDS AND STRUCTURE OF PROTEINS

In addition to a preference for certain amino acids, there may be differences in their distribution, which may provide different structural adaptations that affect thermostability (Jaenicke, 1996; Gianese et al., 2002). Vieille et al. (2001) believe that the conserved core in mesophiles and thermophiles does not contribute to stability at high temperature, but that the less conserved regions are important. This is supported by the report that amino acid compositions, particularly on the outer surface of folded protein, are important for the thermal stability of proteins (Lindsay and Creaser, 1977; Argos et al., 1979; Fukuchi and Nishikawa, 2001; Fukuchi et al., 2003). Specific associations or avoidance of amino acids in loops or core (Kumar et al., 2000b), variations in amino acid content at the C-terminus or N-terminus and shortening of the surface exposed loop add to stability in prokaryotes as reported by Spassov et al. (1995), Tanner et al. (1996), Macedo-Ribeiro et al. (1997) and Tanaka et al. (2004) but contrary to that reported by Chakravarty and Varadarajan (2000). In small heat shock proteins, it has been found that the carboxy terminal and not the amino terminal helps in assembling the subunits of this chaperon which enables thermoprotection in Archaea specially when the temperature goes beyond 103°C (Laksanalamai et al., 2003). In L-isoaspartyl-O-methyltransferase from *Sulfolobus tokodaii*, long C-terminal loop and alpha helix, which contain Tyr and Asp, help in tetramerization and are critical for thermal stability (Tanaka et al., 2004). Studies reveal that Arg is favored over Pro, His and Cys in the alpha helix to provide stability to protein in thermophilic Eubacteria and Archaea (Kumar et al., 2000b). Contrary to the latter, Cys (Tanaka et al., 2004) and Pro are not considered important for protein stability (Lo et al., 1999; Chakravarty and Varadarajan, 2000). However, their metabolic costs or the type of protein may govern preferential distribution of amino acids (for example, membrane proteins and ribosomal proteins). Although, this study is based on comparisons of proteins from mesophilic and thermophilic Eubacteria (McDonald, 2001), the same may be true in the case of Archaea.

Therefore, it appears that protein stability may be due to structural adaptations governed by amino acid content and distribution. It has been found that helical structures formed due to AXXXA and GXXA stabilize protein motifs (Kleiger et al., 2002). The numbers of alpha helices are high in mesophiles and thermophiles but the beta strands are more in hyperthermophiles (Szilágyi and Závodszky, 2000). Hydrophobic core and aliphatic side chains could also be responsible for protein stability (Argos et al., 1979; Lindsay, 1995; Haney et al., 1997; Vieille et al.,

2001; Futterer et al., 2004; Irimia et al., 2004), but this is contrary to the observations of Kumar et al. (2000b). It has been investigated that position of side chain on amino acids, for example β- and γ-side chains (Kumar and Nussinov, 2001a), reduction of solvent-exposed surface, increase in links of ion pairs, and loops with secondary structure stabilize proteins (Spassov et al., 1995; Tanner et al., 1996; Macedo-Ribeiro et al., 1997; Irimia et al., 2004). Recently, crystallographic studies of L-isoaspartyl-O-methyltransferase from *Sulfolobus tokodaii* revealed the importance of oligomerizations of proteins for thermal stability (Tanaka et al., 2004). Therefore, it appears that thermophilic prokaryotes adopt different structural folds to protect proteins against high temperature.

MEMBRANE PROTEINS

Many features that render stability to soluble proteins do not hold true in case of membrane proteins. Investigations report that integral membrane proteins avoid Glu, Lys and Asp unlike soluble proteins (Lobry and Chessel, 2003). However, higher amounts of Ala, Gly, Ser, Asp, and Glu and low amounts of Cys are present in transmembrane proteins of thermophiles. Although disulfide bonds formed due to Cys render thermostability to soluble proteins, it does not seem to work in case of membrane proteins. These amino acids including Cys may provide stronger interhelical hydrogen bonds, tighter packing, or both resulting in thermostability. Also unlike in soluble proteins, Pro is preferentially present in helices of transmembrane proteins of thermophiles because it prevents misfolding especially in hydrophobic regions (Schneider et al., 2002). It is reported that charged amino acids are avoided in membrane proteins of thermophiles (Lobry and Chessel, 2003), but the ratio for charged and polar amino acids is not significantly different between the two domains (Schneider et al., 2002; Suhre and Claverie, 2003). However, like the soluble proteins, it appears that membrane proteins depend on ion-pair interactions for stability at high temperature. This is further supported by the study on membrane-associated chitinase (Chi70) of thermophiles where ion interactions with Arg on the surface of the protein play an important role in providing thermal stability compared to disulfide bridges (Andronopoulou and Vorgias, 2003). S-layer glycoproteins in extremophilic Archaea are stabilized by the absence of Cys, presence of long stretch of hydrophobic amino acids and magnesium ions (Eichler, 2001; Claus et al., 2002). However, aquaporins of Archaea *Methanothermobacter marburgensis* (OGT of 65°C) show no significant differences in amino acid sequence when compared to mesophiles or hyperthermophiles. Thus, the factors that provide thermostability to aquaporins remain elusive (Kozono et al., 2003). Probably like cytosolic proteins, the membrane proteins also depend on multiple factors for stability. This is evident from the study of membrane-associated enzymes such as glycosyl transferases in which differences in charge of amino acids, amphiphilicity and hydrophobicity for the N- and C-domains affect thermal stability (Edman et al., 2003).

It is nevertheless suggested that there is no universal combination of the factors that may be responsible for thermal stability of soluble proteins or membrane proteins. These combinations are specific, which vary from protein to protein (Vieille et al., 1996; Jaenicke and Bohm, 1998; Chen et al., 2000; Jaenicke, 2000; Kumar et al., 2000b; Szilágyi and Závodszky, 2000). Although, there are differences in amino acid compositions of thermophiles and mesophiles, the differences between cytoplasmic and membrane proteins within thermophile species are more significant (Lobry and Chessel, 2003).

From the preceding information, it is apparent that in thermophilic prokaryotes protein stability is due to various adaptations that provide flexibility or rigidity in extreme environments. Lobry and Chessel (2003) suggest that the selective pressures in thermophiles have not acted on proteins but on DNA, which resulted in preferences for codons and amino acids. They also suggest that temperature cannot be the sole determining factor at least for the synonymous codon usage. We agree with the suggestions that these adaptations could be due to stress such as salt concentrations and pH in addition to temperature that differ in the organisms. So far, in most of the studies, emphasis is on temperature as being the only common factor that destabilizes proteins. Studies emphasizing other common factors in addition to temperature would probably reveal similarities in thermal adaptations. In addition to this, the majority of reports are on thermophilic and hyperthermophilic Archaea with few examples about Eubacteria that reveal just a couple of differences between the two domains regarding the strategies used for thermal protection to proteins. Further, comparisons have been made between thermophiles (including hyperthermophiles) and mesophiles but not the two domains with the aim to seek phylogenetic differences. Two more factors compound the situation. First, the thermophilic Eubacteria used for studies are considered close to Archaea in lineage and appear to share many genes with them possibly due to horizontal gene transfer. Second, structure and functions of many gene products that are unique to Archaea still remain predicted and uninvestigated (Makarova and Koonin, 2003). We suggest that genome expression strategies such as frameshifting, either due to overlapping genes or inteins, in some Archaea, for example, *Aeropyrum pernix* (proteins and their sequence positions are available at ftp://ftp.ncbi.nih.gov/genomes/Bacteria/Aeropyrum_pernix/NC_000854.ptt) should be investigated. Violation of universal code rules (based on the report of Srinivasan et al., 2002 on amber codon being used for pyrrolysine amino acid in *Methanosarcina barkeri*) should also be considered to determine whether they have any influence on protein stability. In the future, an understanding of the structures and functions of these unique Archaeal gene products and discoveries in more thermophilic bacteria (if more exist) will probably help in comparing the strategies of the two domains. In studies that report no difference between the two domains in the strategies employed for thermal protection, it is suggested that there are no phylogenetic differences regarding thermal protection. However, Kumar et al. (2000b) do suggest that the differences and inconsistencies between thermophiles and mesophiles could be due to phylogenetic differences and not solely due to factors rendering thermostability.

So far, comparisons made between proteins of thermophiles and mesophiles have revealed the differences between strategies to survive at high temperatures. However, there is no comparison of proteins involved with complete specific metabolic pathways. Possibly such comparisons may resolve the contradictory findings. For example, heat shock proteins in all the three domains have a higher proportion of charged amino acids (Paz et al., 2004). Therefore, a high ratio for charged amino acids appears to be the common strategy to stabilize proteins at high temperature in the three domains and that temperature (at least in this case) is the determining factor for choice of amino acids. Hence, comparisons of proteins of complete metabolic pathways may help in understanding the specific strategies that are common and necessary for surviving at high temperature.

From the foregoing information, it is apparent that thermophilic prokaryotes have adapted to high temperature, or possibly because of their unique features they have been able to survive in these conditions. It appears that all aforementioned factors either alone or in combination help

in protein stability even though amino acid content, ratio, number of hydrogen bonds, hydrophobicity, etc., may or may not be significantly different in proteins from mesophiles and thermophiles. Nonetheless, it is evident that the adaptations have occurred in DNA, RNA and protein. Whether evolutionary forces induced changes in DNA that are reflected in expression or whether it was the necessity of undergoing modification at the expression level that the DNA had to undergo changes still remains debatable.

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