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Review

Biomolecular stability and life at high temperatures

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Abstract. It is not clear what the upper temperature limit for life is, or what specific factors will set this limit, but it is generally assumed that the limit will be dictated by molecular instability. In this review, we examine the thermal stability of two key groups of biological molecules: the intracellular small molecules/metabolites and the major classes of macromolecules. Certain small molecules/metabolites are unstable in vitro at the growth temperatures of the hyperthermophiles in which they are found. This instability appears to be dealt with in vivo by a range of mechanisms including rapid turnover, metabolic channelling and local stabilisation. Evidence to date suggests that proteins have the potential to be stable at substantially higher temperatures than those known to support life, but evidence concerning degradative reactions above 100 °C is slight. DNA duplex stability is apparently achieved at high temperature by elevated salt concentrations, polyamines, cationic proteins, and supercoiling rather than manipulation of C-G ratios. RNA stability seems dependent upon covalent modification, although secondary structure is probably also critical. The diether-linked lipids, which make up the monolayer membrane of most organisms growing above 85 °C are chemically very stable and seem potentially capable of maintaining membrane integrity at much higher temperatures. However, the in vivo implications of the in vitro instability of biomolecules are difficult to assess, and in vivo data are rare.

Key words. Archaea; biomolecules; DNA; enzymes; lipids; membranes; proteins; RNA; stability; thermophiles; thermostability.

Introduction

Studies of life at high temperatures (> 75 °C) are comparatively recent, but have been a fertile field, providing new impetus to discussions on the origin of life and molecular (in)stability, and broadening the environmental 'envelope' within which we expect to find life. The latter potentially provides a key set of parameters to guide the current and future design of strategies for detection of life on planets other than Earth.

In this review we will deal with the molecular factors which are characteristic of organisms living at temperatures above 75 °C and which might act as an upper temperature limit. However, much of the focus will be on the higher-temperature ranges, since the molecular differences between low- and high-temperature life manifest themselves most obviously above 90 °C. Life above 75 °C is confined to the bacteria and the archaea, and only members of the latter are capable of growth above 95 °C. This makes it difficult in many cases to separate those features that are related to a high-temperature existence from those which are present for

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phylogenetic reasons. (We will not speculate on why no eucaryotes exhibit thermophily, except to point out that other major biochemical and physiological pathways and properties are also absent from this group, including methanogenesis and extreme halophilicity.) Taxonomic trees constructed from ribosomal RNA (rRNA) sequences show the archaeal kingdom, with its high proportion of thermophiles and hyperthermophiles, nearest the root. Among the bacteria, *Aquifex* ($t_{max} = 95$ °C) is more deeply rooted than *Thermotoga* ($t_{max} = 90$ °C), which is more deeply rooted than *Thermus* ($t_{max} = 80$ °C) [1, 2] (fig. 1), and other thermophilic bacteria such as *Dictyoglomus* and *Thermodesulfobacterium* are also deeply rooted. The simplest explanation of these trees is a high-temperature origin for the earliest living organisms, although the trees are a subject of considerable debate [3], and it is argued by some that the deep branching of the hyperthermophilic bacterial 16S rRNA sequences is an artefact arising from low rates of evolution. The evidence for a thermophilic origin of life is therefore indicative rather than overwhelming. Protagonists of the 'RNA world' suggest that a high-temperature origin for life is unlikely because the nonliving systems preceding such organisms are likely to have been based on RNA, which is unstable at high temperatures. However, only minor modifications are needed to stabilise RNA in vitro and in vivo [4], and organisms capable of growth at 110 °C do contain functional RNA.

Arguments over the nature of the universal ancestor notwithstanding, the thermophiles and hyperthermophiles have provided new insights into a wide range

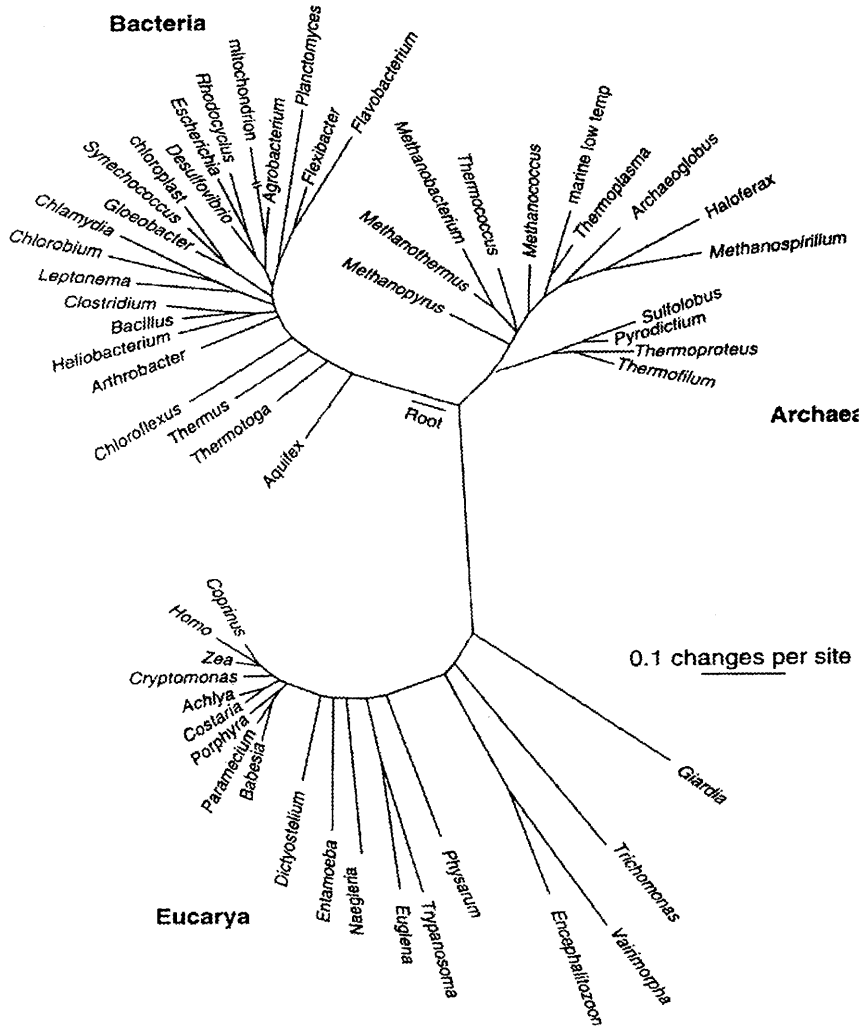

Figure 1. Universal phylogenetic tree based on rRNA sequences (after [2]).

Table 1. Metabolite and coenzyme stabilities.

Metabolite/coenzyme	% remaining after	
	1 h/95 °C	3 h/105 °C
NAD	<5	n.d.
FAD	100	85
FMN	75	65
Pyridoxal phosphate	40	0
Glucose	100	100
Glucose-6-phosphate	100	70
Glucose-1,6-diphosphate	90	50
Gluconate	100	100
6-Phosphogluconate	100	90
Glycerate	100	100
3-Phosphoglycerate	100	100
Acetate	100	100
Acetyl phosphate	<10	n.d.
CoASH	100	45
Acetyl CoA	100	75
ATP	40	0
ADP	50	0
AMP	95	60

Solutions (10 mM) in distilled water containing 1 mM KI were heated in sealed glass tubes, and degradation assessed by changes to the electrospray mass spectrum [5].

of biochemical and physiological processes. Indeed, studies on this relatively small and select group of organisms may have a greater influence on our understanding of fundamental biomolecular processes than virtually any other biotype.

While at this stage of our knowledge it may be most rational to ask how life has managed to adapt to the difficulties imposed by sub-80 °C temperatures, the question most commonly asked of the hyperthermophiles (living optimally at between 85 and 113 °C) is, What molecular adaptations are responsible for their ability to survive and grow at such temperatures? Two subsidiary and closely related questions are, What molecular instability (or other process) dictates the upper limit of life? and Where key metabolic components are known to be unstable at temperatures well below this limit, how do hyperthermophiles retain metabolic activity up to this limit? This review attempts to present and discuss some of the current evidence bearing on these questions.

Throughout, we have used the term 'thermophile' in the general sense of growing optimally above 75 °C, and to include hyperthermophiles growing optimally at temperatures well above this.

Metabolism, metabolites and cofactors

Many low molecular weight metabolites and coenzymes have quite short half-lives even at 95 °C (see, for example, table 1). The known presence of these compounds in hyperthermophiles suggests the existence of a number of mechanisms by which metabolite/coenzyme thermal instability may be overcome at high growth temperatures.

Microenvironment protection

The thermal stability of many metabolites is highly dependent on conditions. For example, adenosine 5′-triphosphate (ATP) stability is greatly affected by pH and metal ions [6, 7], so microenvironment protection is possible. This may also be the case for NADH, which in dilute buffer at around pH 7 has a half-life of only a few minutes at 95 °C, but which at higher pH is much more stable [8–10]. In any event, since ATP and NAD(P) are both used as coenzymes in archaea growing at 105 °C, such organisms obviously have a way of circumventing this instability, and the exploitation of microsites which have less destabilising conditions is one possibility.

Metabolic channelling

It has become clear over the last decade that cytoplasm is not a homogeneous medium in which enzymes are dissolved. But while the idea of cytoplasmic microstructure is now well accepted [11], its physiological implications are less clear. Nevertheless, a strong case has been made that channelling of intermediates between physically associated enzymes which are sequential members of a metabolic pathway can have major effects. These include reduction in intermediate metabolite concentration and increase in pathway flux [12–14]. Van de Casteele et al. [15, 16] have suggested that the thermal instability of carbamoyl phosphate can be circumvented at high temperatures by the physical juxtaposition of the enzyme producing the metabolite (carbamoyl phosphate synthetase) and the next enzyme in the metabolic pathway (ornithine carbamoyl transferase), which consumes the metabolite. Evidence for such a juxtaposition has been obtained for *Pyrococcus furiosus* [17].

Given the extent of cytoplasm microstructure, and the relatively undeveloped state of this field, enzyme-enzyme association may turn out to be a potent and widespread mechanism by which metabolite instability can be circumvented. Currently there is no evidence to indicate that the phenomenon is more widespread in thermophiles, but this is not necessarily an argument against it.

Catalytic efficiency

Sterner et al. [18] have suggested that the thermal instability of phosphoribosyl anthranilate (an intermediate in the tryptophan biosynthesis pathway; $t_{1/2} = 39$ s at

80 °C) is overcome in *Thermotoga maritima* by the very high efficiency of the phosphoribosyl anthranilate isomerase enzyme. The high efficiency is largely due to a very low K_m, although the k_{cat} is relatively high.

As a group, enzymes from thermophiles tend to have similar k_{cat} values to their mesophilic homologues, and there is likewise no evidence for systematically lower K_m values for thermophilic enzymes. However, it must be borne in mind that few systematic comparisons between the kinetic properties of enzymes from thermophiles and mesophiles have been carried out, and given the wide variation among mesophilic enzymes, it would not necessarily be easy to draw conclusions from such a comparison. Nevertheless, on the basis of current evidence an increase in catalytic efficiency in thermophilic enzymes does not seem likely to be a widespread strategy.

Substitution or deletion

In this case, the thermal instability of a metabolite is circumvented by use of an alternate pathway or by using a more stable alternative compound.

NAD(P) and non-haem iron protein. There is a strong association between the use of non-haem iron proteins instead of NAD(P) and thermophily [19]. NAD and NAD(P) are unstable at 95 °C ($t_{1/2} \sim 2$ min) [10], whereas non-haem iron proteins have the potential to be quite stable and functional at 100 °C [19, 20]. Makund and Adams [21] have shown that *P. furiosus* oxidises glucose to pyruvate via a nonphosphorylated Entner-Doudoroff pathway in which the redox reactions are all catalysed by ferredoxin-linked oxidoreductases. In the less primitive archaea, *Sulfolobus* and *Thermoplasma* [22], glucose is also catabolised via this nonphosphorylated pathway. Glucose dehydrogenase and glyceraldehyde dehydrogenase are NAD/P-linked [23, 24], but pyruvate decarboxylation is ferredoxin-linked [25].

Other examples of the tendency for non-haem iron protein to replace NAD/P in more thermophilic organisms include the finding [26, 27] that in *Sulfolobus* the 2-oxoglutarate oxido-reductase is ferredoxin-linked, rather than NAD/P linked, the non-haem iron protein linkage of the aldehyde oxidoreductase from ES-4 [28] (already known in *P. furiosus*), the 2-keto isovalerate ferredoxin oxidoreductase from several hyperthermophilic archaea [29] and the indole pyruvate oxidoreductase in *P. furiosus* [30]. The examples above are from Archaea, but an increased dependence upon non-haem iron protein rather than NAD has also now been shown in one of the most deeply rooted and thermophilic of the bacteria, *Thermotoga*. This occurs at the pyruvate-ferredoxin oxidoreductase step, although the enzyme mechanism is different to that found in Archaea [31]. Thus, although the use of non-haem iron proteins in place of NAD(P) is associated with archaea, occurring in methanogens and halophiles as well as in thermophiles, it also occurs in thermophilic bacteria. This suggests that although it seems likely to be a primitive characteristic [19], there is a correlation with thermophily.

Acetyl phosphate. Acetyl phosphate is particularly unstable relative to acetate and acetyl coenzyme A (CoA) (table 1), and its use as a metabolic intermediate would seem to pose particular difficulties in hyperthermophiles. Shafer et al. [32] have found that in hyperthermophiles the interconversion of acetyl CoA to acetate is direct rather than proceeding via acetyl phosphate. So far it seems that the absence of acetyl phosphate from this pathway is an archaeal characteristic rather than a thermophilic one, so it is not clear whether this absence is imposed by thermophily, or if the use of acetyl phosphate arose after the separation of the other two kingdoms. Of course, if the universal ancestor was a thermophile, and most closely related to the archaeal kingdom, this question does not arise.

Other phosphorylated compounds. It is possible that the presence of an Enter-Douderoff pathway based on non-phosphorylated carbohydrates in thermophilic archaea [33] arises because of the greater stability of these intermediates compared with the phosphorylated forms. However, the in vitro differences in stability are small (table 1). Nevertheless, although the relative importance of the phosphorylated and nonphosphorylated pathways is not clear within the thermophilic archaea, the nonphosphorylated pathway appears to be confined to this group.

The most important phosphorylated metabolites are of course ATP and adenosine 5′-diphosphate (ADP). The stability of this class of compounds is in the order pyrophosphate (PP)/AMP > ADP > ATP, and ATP is relatively unstable at 95 °C (table 1). There is some evidence for the use of the more stable compounds (PP and ADP) instead of ATP in thermophilic archaea. For example, Kengan et al. [34] have found that in *Pyrococcus* both hexokinase and PFK are, uniquely, ADP-linked. In the case of hexokinase, glucose is normally phosphorylated by ATP, which is less stable than ADP. In the case of PFK, F-6-P is also normally phosphorylated by ATP; Siebers and Hensel [35] have shown that in *Thermoproteus tenax* pyrophosphate is used, but this is also the case in some primitive eukaryotic parasites.

The four mechanisms described here are by no means mutually exclusive, and other mechanisms, such as rapid synthesis, are also possible. In summary, some key coenzymes and metabolites, including NAD(P), acetyl phosphate and ATP are quite unstable above 100 °C. While the presence of some of these compounds in archaea growing above 100 °C indicates the existence of mechanisms for circumventing this instabil-

ity in 'modern' organisms, there is also evidence that in many thermophiles more stable compounds (e.g. non-haem iron protein) replace some or all of the functions of these [e.g. NAD(P)].

Enzymes and proteins

Denaturation

It has been known for some time that some enzymes from thermophiles and hyperthermophiles (e.g. [36]) have significant half-lives at >100 °C (table 2). A variety of detailed structural studies have compared such proteins to much less stable variants from mesophiles (e.g. [44–46]). Considering these results as a whole, the most significant finding is that although there are structural differences between very stable and 'normal' enzymes, there is no pattern of systematic structural differences. Within a closely related group of enzymes there may be a pattern of changes differentiating the more and less stable variants, but for a different group of enzymes the changes will be different: overall, in structural terms the differences between very stable and less stable enzymes are no greater than the differences within the groups [47, 48]. This finding is entirely in keeping with early theoretical studies indicating that the tertiary structures of proteins are only marginally stable (e.g. [49]), and with results showing that only a few amino acid substitutions are required to bring about significant changes in thermal stability [50, 51].

The conformational stability of a protein (defined as the difference in free energy between the folded and unfolded states, ΔG) is the sum of a large number of weak, noncovalent interactions, including hydrogen bonds, salt bridges, van der Waals interactions and the hydrophobic effect, and the destabilising forces arising largely from conformational entropy. The sum of these stabilising interactions is about 1 MJ mol^{-1}. Destabilising forces are of a similar magnitude, and ΔG, the difference between the two, is only of the order of 40 kJ mol^{-1}. Point mutational studies have shown that replacement of a single amino acid (and thus apparent removal of a single stabilising interaction) can have a significant effect on stability without any detectable effect on the three-dimensional structure [52]. Such 'individual' interactions can contribute up to 25 kJ mol^{-1}, so it is evident that only a few additional interactions will be needed to account for the additional stability of enzymes from hyperthermophiles. However, the thermodynamic consequences of such a single amino acid mutation are rarely limited to deletion (or addition) of a single noncovalent interaction, since the global effect on ΔG must include changes in the unfolded as well as the folded state.

There is no evidence that in any protein all the amino acid residues are participating in stabilising interactions. Because of this, and because each additional interaction can have such a marked stabilising effect, it is difficult to determine what might be the upper limit for conformational stability. The temperature dependence of hydrophobic interactions suggests that these may be weak at 140 °C [53], so that this might seem to set an upper limit. But the hydrophobic interaction and the magnitude of its contribution to protein stability is incompletely understood [53, 54]. Since stability also depends on other interactions, as well as on any factors that may destabilise the unfolded state, then even if hydrophobic interactions are much weaker above 140 °C, this may not represent an upper temperature limit for protein stability.

In general terms, there is strong evidence for an inverse correlation of conformational stability with specific activity, via molecular flexibility (e.g. [47, 55]). Whereas enzyme activity is dependent upon flexibility, a less flexible enzyme will be more stable. The broadest general evidence for this is the finding that, at any given temperature, as a group, enzymes from thermophiles are more stable, less flexible and less active than those from mesophiles [47, 48, 55–59]. Together with the general structural and functional identity of stable and less stable enzymes, this leads to the view that the instability of enzymes from mesophiles is a functional requirement rather than because of any restraint on achieving higher stability [47, 55]. It is required so that enzymes have sufficient flexibility to perform their catalytic functions: i.e. enzymes tend to be denatured at temperatures not very far above their evolved or 'design' temperature because too much stability would mean not enough flexibility for effective catalysis, whereas too little stability would mean too short a useful lifetime. An additional requirement for instability can be inferred from the finding that, irrespective of whether or not they are denatured, stable proteins are more resistant to proteolysis [60]. A balance between stabilizing and destabilizing interactions is required to

Table 2. Stability of some enzymes at 100 °C.

Enzyme (Source)	$T_{1/2}$ at 100 °C
Cellobiohydrolase (*Thermotoga*) [37]	>200 min
β-Glucosidase (*Thermotoga*) [38]	90 min
Xylanase (*Thermotoga*) [39]	20 min
Xylosidase (*Thermotoga*) [38]	150 min
Esterase (*Sulfolobus*) [40]	60 min
Hydrogenase (*Pyrococcus*) [41]	120 min
Amylase (*Pyrococcus*) [42]	360 min
DNA-dependent RNA polymerase (*Thermoproteus*) [43]	>120 min

$T_{1/2}$ values are half-lives of activity under specified, but not identical, conditions.

meet the conflicting demands of stability on the one hand, and catalytic function and cellular turnover on the other. As a consequence, if we consider only conformational stability, we may postulate that the reason we have not found proteins that are stable much above 130 °C is because we have not found organisms growing much above 110 °C (rather than vice versa).

However, the influence of molecular flexibility on stability and activity is poorly defined. Conformation stability is a global property of an enzyme [49, 51], but it is not clear whether the influence of flexibility on stability is regional or global. It seems likely that flexibility at particular points in the structure is much more critical for stability than elsewhere. With respect to activity, although certain local motions at or near the active site must occur over time scales similar to those of substrate turnover, it is not clear whether these are coupled to faster motions, locally or globally. It has been found that enzyme activity at low temperatures is unaffected by the cessation of fast anharmonic global dynamics (< 100 ps time scales) [61, 62]. It is difficult to understand how dynamics mediate the apparent inverse correlation between stability and activity if the dynamics required for activity is local, while that required for stability is global; but if this is the case, it will certainly hold out good prospects for the engineering of enzymes which are both more stable and more active. For a more detailed discussion see [47, 61, 62].

Degradation

Thermophilic proteins are conformationally stable at high temperatures [20, 36], but the effect of irreversible degradative processes [63, 64] on these proteins at high temperatures is unclear. In contrast to denaturation (and leaving aside the special case of disulphide bonds), the irreversible processes of protein inactivation arise from changes in covalent bonding. The most common at high temperatures are deamidation of the amide side chain of Asn and Gln residues, succinimide formation at Glu and Asp, and oxidation of His, Met, Cys, Trp and Tyr. These reactions have high activation energies and are thus greatly accelerated by high temperatures, and so have the potential to play a particularly important role in the inactivation of enzymes at high temperatures. At least some of the chemical mechanisms for irreversible degradation in proteins require local molecular flexibility. A survey of environments around Asp and Asn resides in known three-dimensional protein structures suggests that the rigidity of the folded protein greatly decreases the intramolecular imide formation necessary for degradation. In the numerous X-ray crystal structures studied, the peptide-bond nitrogen could not approach the side-chain carbonyl carbon closely enough to form the succinimide ring [65]. At 37 °C the rate of deamidation has been shown to be higher for small peptides with high flexibility than for proteins when comparing the same amino acid sequence [66], and higher in denatured than in native proteins [67]. In other words, the resistance to degradation of a protein is linked to its conformational integrity. This contention is supported by studies using thermally stable proteins in which the conformation is known to be retained for significant periods at the high temperatures at which degradative reactions occur. Hensel et al. have shown that the rate of deamidation for a thermostable glyceraldehyde phosphate dehydrogenase from *Pyrococcus woesei* at 100 °C is increased after denaturation of the enzyme [68] (and after dialysing away the denaturing guanidinum hydrochloride). Furthermore, for the same enzyme from *Methanothermus fervidus*, deamidation occurred more readily at 85 °C in a less stable chimaeric form of the enzyme derived from a construct containing both thermophilic and mesophilic genes. Similarly, the addition of phosphate, known to stabilise the conformation of the dehydrogenase, decreased the rate of peptide bond hydrolysis at temperatures ranging from 85 to 100 °C [68]. Support for the view that the loss of conformation precedes irreversible degradative reactions comes from studies of deamidation (ammonia release) and loss of activity of the very stable xylanase from *Thermotoga* strain FjSS 3B1 in the range 95–100 °C [47]. Both the onset and progress of deamidation occurred later than those of activity loss, consistent with a dependence of deamidation upon loss of conformation.

In pig muscle myokinase, which is a very stable enzyme despite its mesophilic origin, studies on peptide bond hydrolysis give similar results. In the native and denatured enzyme at 95 °C [47] the rate of peptide bond hydrolysis is always lower than the rate of activity loss, although agents which affect the rate of activity loss such as SDS plus mercaptoethanol (faster) and substrate (slower) have a similar effect on peptide bond hydrolysis. These correlations are consistent with a dependence of degradation on loss of conformation. There is thus growing evidence that the degradative reactions to which proteins are subject are slower or do not occur in conformationally intact proteins, at least up to 100 °C. In other words, the upper temperature limit for protein stability may after all be determined by the conformational integrity of the protein, although we must bear in mind that few studies on conformational or degradative stability above 100 °C have been made.

Nucleic acids

The question of how hyperthermophiles maintain the structure and integrity of their DNA and RNA in vivo

has been the focus of considerable research over the past 20 years. Based on early studies of the effects of high temperature on nucleic acids [69], it has been reasonably assumed that hyperthermophiles, as compared with mesophiles, would be required to cope with a considerably greater burden of both chemical degradation and duplex destabilisation.

At high temperatures DNA undergoes denaturation by strand separation. However, it has long been known that duplex stability in vitro could be manipulated over a wide temperature range by addition of salts. The discovery that some hyperthermophiles contained molar concentrations of potassium di-inositol-1,1′-phosphate [70] or tripotassium cyclic-2,3-diphosphoglycerate [71], suggested a possible in vivo mechanism for stabilisation of nucleic acid secondary structure. (It must be noted that these compounds also stabilise protein conformation at high temperatures in vitro.) However, not all hyperthermophiles contain high intracellular ion concentrations.

Polycationic polyamines, which increase the melting temperature of DNA and protect *S. solfataricus* ribosomes from thermal inactivation in vitro [72], have also been observed in hyperthermophiles [73]. Concentrations of putrescine (H_2N-$(CH_2)_4$-NH_2), spermidine (H_2N-$(CH_2)_3$-NH-$(CH_2)_4$-NH_2), norspermidine (H_2N-$(CH_2)_3$-NH-$(CH_2)_3$-NH_2, thermospermine (H_2N-$(CH_2)_3$-NH-$(CH_2)_3$-NH-$(CH_2)_4$-NH_2) and spermine (H_2N-$(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3$-NH_2) of up to 0.4 g% (d.w. cell biomass) were detected in various *Sulfolobus* strains. A comprehensive analysis of the polyamines in 75 bacterial and archaeal isolates from mesophilic to hyperthermophilic sources has been carried out [74]. The results showed that some polyamines (norspermine and norspermidine) occurred only in the hyperthermophilic archaea, but that there was no significant correlation between total intracellular polyamine concentration and the growth temperature of the source organism. However, the hyperthermophilic archaea were found typically to contain a greater diversity of polyamines than other organisms.

Stabilisation of nucleic acid duplex structure may also be achieved by increasing the G–C ratio, a strategy which the hyperthermophiles appear to eschew, there being no obvious correlation between percentage G + C content and optimum growth temperature in even the most hyperthermophilic of the archaea (table 3). Grogan [82] notes that the molar percentage G + C in the 16S RNAs of this same group of organisms is in all cases significantly higher (typically 63–69%).

Alternative mechanisms for the stabilisation of DNA secondary structure include supercoiling and association with cationic proteins. The discovery of a novel ATP-dependent topoisomerase I activity ('reverse gyrase', generating positive supercoils [83]) in all the hyperthermophilic archaea tested [84] (and in some hyperthermophilic bacteria [85]) led to the proposal that this was a specific mechanism for DNA stabilisation, and possibly a true 'hyperthermophilic characteristic' [86]. Subsequent studies (e.g. [87]) have shown that the presence of positive supercoils per se was not specifically required for thermostabilisation of DNA. It therefore seems likely that the torsional constraints of supercoiling, whether positive or negative, provide substantial but similar increases in T_m. The true in vivo role of topoisomerases in hyperthermophiles is currently unclear. Evidence that reverse gyrase and topoisomerase II (relaxing) activities in *Desulfurococcus amylolyticus* were regulated both by temperature and growth phase [88] suggests that these enzymes play a complex but important role in the superhelicity of the genome.

Table 3. Molar percentage G+C values for hyperthermophilic archaea.

Organism	T_{opt} (°C)	Mol % G+C	Reference
Methanopyrus kandleri	98	60	[75]
Pyrobaculum islandicum	100	46	[76]
Pyrococcus abyssi	96	44–45	[77]
Pyrococcus furiosus	100	38	[78]
Pyrodictium abyssi	97	59	[79]
Pyrodictium occultum	105	62	[80]
Pyrolobus fumarii	106	53	[81]

DNA topology is also affected by interaction with cationic proteins, numerous examples of which have been identified in hyperthermophiles (table 4). These small basic proteins bind DNA in vitro with substantial increases in T_m, and have been variously shown to bend DNA [89] or form nucleosome structures [96]. For example, the HMf family of archaeal histones (originally identified in *M. fervidus*) are homologues of eucaryal nucleosome core histones and have been shown to bind to and compact archaeal DNA both in vitro and in vivo [97–99]. Histone-like proteins from *Sulfolobus* [100] have no eucaryal homologues but, like the HMf proteins, also compact DNA and increase the T_m of DNA in vitro. The driving force for the evolution of these proteins may therefore have more to do with DNA packaging and nucleosome formation [101] than DNA stabilisation. For more detail on DNA topology and nucleosome structure, the reader is directed to a recent review [97].

The structural stabilisation of ribonucleic acids in hyperthermophiles is particularly important in transfer RNAs (tRNAs), where there is a requirement for the maintenance of a complex three-dimensional structure in the absence of other macromolecular associations. The strategy employed by the hyperthermophilic

Table 4. Hyperthermophilic histone-like proteins.

Protein	Source organism	Homologues	References
HMf family	*Methanothermus fervidus*	Eucaryal nucleosome core histones H2A, H2B, H3 and H4	[89]
	Methanobacterium thermoautotrophicum		[90]
	Thermococcus zilligii		[91]
	Methanopyrus kandleri		[92]
	Pyrococcus spp. (*HPy*)		[93]
Sac family	*Sulfolobus acidocaldarius*	Eukaryal SH3 domains	[94]
DNABPII	*Thermotoga maritima*	*E. coli* HU histone-like proteins	[95]

archaea would seem to be largely one of posttranscriptional modification. Studies of modified sugars and bases in thermophilic archaea and bacteria have revealed a number of novel modifications, some of which are archaea-specific [102]. The importance of these modification with respect to thermostability is not clear, although it was noted [102] that the greatest number of different ribose methylated nucleosides were observed in the most hyperthermophilic organism (*Pyrodictium occultum*, $T_{opt} = 105$ °C) whereas the least thermophilic organisms (*Thermoplasma acidophilum* (55 °C) and *Methanobacterium thermoautotrophicum* (65 °C) contained the fewest. Clearer evidence for the role of posttranscriptional modification of ribonucleotides is found in the study of Edmonds et al. [103], which reported the liquid chromatography/mass spectrometry analysis of the ribonucleosides from *Pyrococcus furiosus* grown at 70, 85 and 100 °C. Three modified nucleosides (fig. 2) were shown to increase in relative abundance as a function of cell culture temperature. Each of these nucleosides was localised to regions where it contributed to conformational rigidity [4].

The requirement for specific stabilisation of rRNA at hyperthermophilic temperatures remains uncertain, possibly because the complex topology and RNA-protein interactions of these molecules reduces the potential impact of deleterious conformational changes. However, posttranscriptional modifications in the 16S and 23S rRNAs of *Sulfolobus solfataricus* have been investigated [103]. Ribose O-2′ methylations occurred most frequently, and for some nucleotides, successive increases in the degree of methylation ($> 10\%$) were detected in cells grown at 63, 75 and 83 °C. The authors tentatively concluded that these modifications may play a role in 'the secondary and tertiary stabilisation of rRNA'.

Chemical degradation of bases at high temperature is potentially a serious threat to the genetic stability of hyperthermophiles. The most common covalent modifications are the loss of bases from one strand to generate apurinic or apyrimidinic sites, and base deaminations (particularly deamination of cytosine and 5-methyl cytosine). It has been suggested that both processes should be greatly enhanced at high temperatures, chemical damage to the genome of a hyperthermophile growing at 100 °C being possibly as much as 3000-fold more rapid than in an *Escherichia coli* genome at 37 °C [104]. The authors are not aware of any experimental verifica-

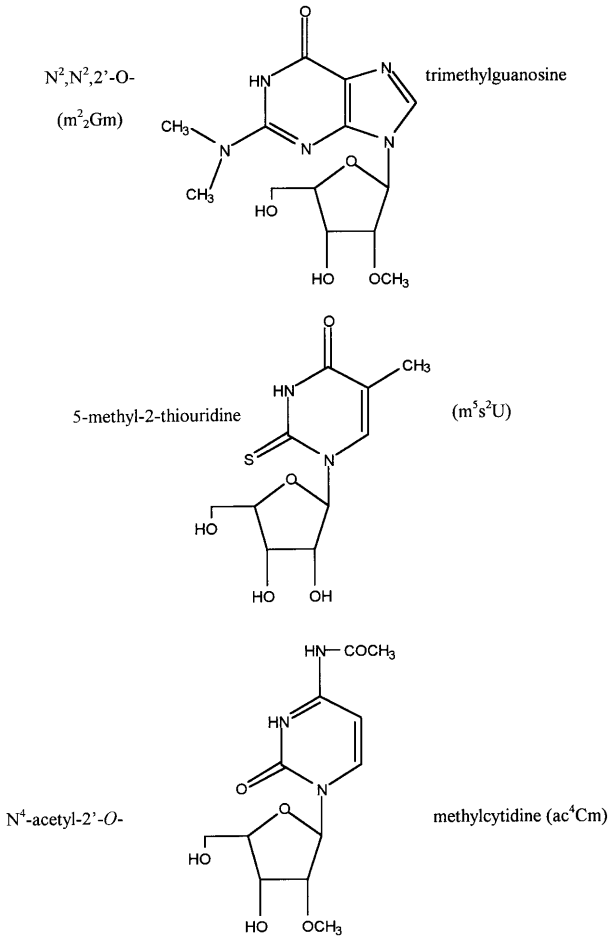

Figure 2. Modified nucleosides implicated in stabilisation of hyperthermophile tRNA.

tion of these calculations. Not all the known enzymes required for the repair of these modifications have been investigated in hyperthermophiles, but archaeal homologues of well-characterised bacterial and eucaryal DNA repair systems continue to be discovered. For example, homologues of homologous recombination components *recA/RAD51* [105] and a putative SOS repair system component (dinF homologue [106]) have been identified in *Pyrococcus*, whereas nucleotide excision repair has been demonstrated in *Methanobacterium thermoautotrophicum* extracts [107]. Uracil DNA glycosylase activity (the repair enzyme for cytosine deamination) has also been detected in a number of hyperthermophilic bacteria and archaea [108]. The importance of DNA repair mechanisms in hyperthermophiles is reviewed in more detail by Grogan [82].

Lipids and membranes

The basic components of the thermophilic microbial cell membrane, the membrane lipids, play a key role in thermophily. The maintenance of membrane fluidity, transport functions, intracellular solute concentrations, chemiosmotic gradients and membrane protein stability are but a few examples of the functions of these molecules. Furthermore, the membrane composition must adapt to fluctuations in environmental temperature in order to retain these functions while still possessing the chemical stability necessary to avoid degradation at high temperature (e.g. in *Pyrodictium abyssi*, $T_{max} = 110$ °C) and by combinations of high temperature and very low pH (e.g. *Sulfolobus solfataricus*, $T_{max} = 85$ °C, $pH_{min} = 1.5$).

The molecular strategies employed by the two phylogenetically distinct groups of hyperthermophiles, the crenarchaeota (archaea) and the thermotogales and aquificales (bacteria) for maintaining stable and adaptable membranes are substantially different. In the thermophilic archaea, more stable ether bonds replace the ester linkages of bacterial and eukaryotic cells, and the 'bilayer' is replaced by a monolayer generated by C_{40} transmembrane phytanyl chains. As ether linkages are not restricted to the archaea, but are also found in some thermophilic bacteria, the presence of these stable chemical structures may be a true 'thermophilic' adaptation. For a detailed analysis of the composition of archaeal membrane lipids, the reader is directed to several recent reviews [109–111].

The hyperthermophilic bacteria show evidence of lipid composition mimicking that in the archaea, possible support for the contention that hyperthermophily evolved independently in the different lineages [112]. The hyperthermophilic bacteria variously possess diether links with fatty alcohols, tetraesters and long-chain aliphatic diols. All are heavily decorated with a wide variety of phospho-, glyco-, sulfoglyco-, sulfophospho- and phosphoglyco-derivatives.

Lipids of the thermophilic archaea

The 'core' lipids of the archaea are largely based on saturated isoprenoid chains linked to a glycerol backbone by ether bonds. Common structures include the monomeric diphytanylglycerol ethers (fig. 3a) and the dimeric dibiphytanyldiglycerol tetraethers and dibiphytanyl glycerol nonitol tetraethers (fig. 3b). The former, termed archaeols, are found in all archaea, whereas the latter, termed caldarchaeols and nonitolcaldarchaeols, are found only in the thermophilic archaea. The caldarchaeols and nonitolcaldarchaeols exhibit further modification by containing up to four cyclopentane rings in each of the C_{40} biphytanyl chains (figs 3c–f). The addition of cyclic structures in the transmembrane portion of the lipid appears to be a thermoadaptive response, resulting in enhanced membrane packing and reduced membrane fluidity [113]. However, not all hyperthermophilic archaea contain cyclopentane-modified dibiphytanyldiglycerol tetraethers. It is suggested, though not proven, that in such organisms the polar head groups contribute to membrane rigidity [114, 115].

With the publication of lipid analyses of an increasingly diverse range of hyperthermophilic archaea, the diversity of lipids is rapidly increasing with, for example, the identification of fatty acids in the marine archaeon *P. furiosus*, unsaturated diether lipids in *Methanopyrus kandleri* [116], 36-membered macrocyclic diether lipids in *Methanococcus jannaschii* (fig. 3g) [117] and tetraether lipids containing a covalent cross-link at the centre of the isoprenoid chains in *Methanothermus fervidus* (fig. 3h) [118]. There is currently no suggestion that any of these more exotic structures have a specific role in thermophily.

The ether lipids of the thermophilic archaea are typically glycosylated at C_3 and C_6 of the glycerol and nonitol backbones, respectively. It has been suggested [119] that interglycosyl headgroup hydrogen-bonding interactions further stabilise the membrane structure, possibly by reducing lateral lipid mobility. The C_1 atom of the 'opposite' glycerol backbone is phosphorylated, typically with P-inositol, or P-ethanolamine. The orientation of the phosphate groups to the inner face of the archaeal membrane results in a high negative charge density on the inner membrane surface. Without shielding or charge compensation, this high anionic charge density might be expected to destabilise the membrane. Although nothing is known of the mechanisms preventing such putative destabilisation, the high intracellular K^+ concentration of some hyperthermophiles may well be implicated in preventing this destabilising effect.

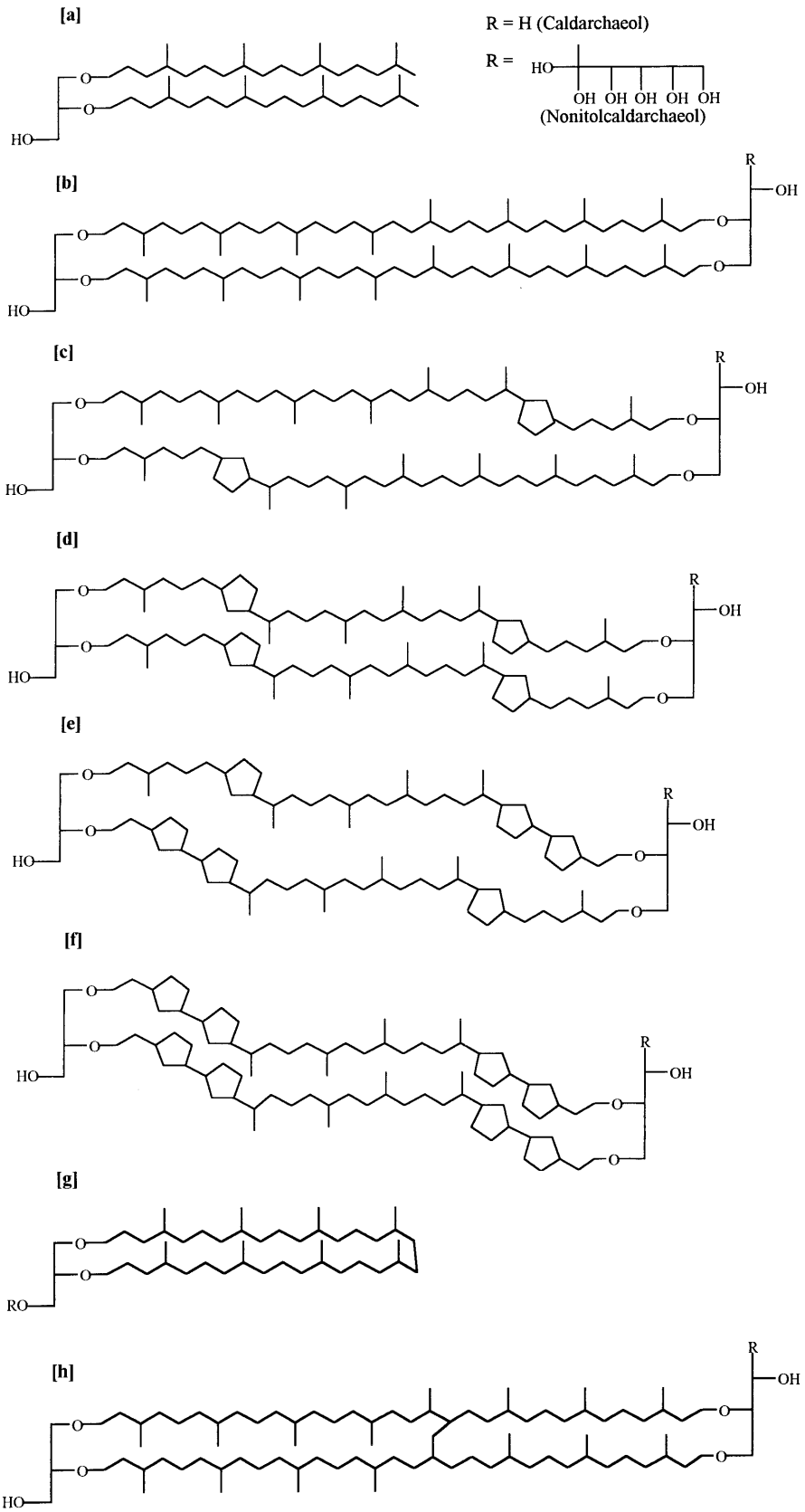

Figure 3. Archaeal lipid architecture. (a) Diphytanyl glycerol diethers, (b) dibiphytanyl diglycerol tetraethers, (c–f) internal cyclisation in dibiphytanyl diglycerol tetraethers, (g) macrocyclic diphytanyl glycerol diether, (h) internal covalent cross-linking in dibiphytanyl diglycerol tetraether.

Membrane stability

While few if any studies have been carried out on the chemical stability of purified ether lipids, the stability of artificial membranes (liposomes) has attracted considerable attention. Whether membrane fluidity is driven by 'homeoviscous adaptation' (maintenance of constant membrane fluidity) [120] or 'homeophasic adaptation' (maintenance of a liquid-crystal state) [121], the membranes of hyperthermophiles have clearly evolved mechanisms for maintaining a liquid crystal state at very high temperatures. Thermoadaptative mechanisms in bacteria include alterations in acyl chain length, saturation, branching and/or cyclisation [122]. Only in archaea are membrane-spanning lipids employed.

Membranes composed of C_{40} membrane spanning (bolaform amphiphilic) tetraether lipids maintain a constant thickness of 2.5–3.0 nm [113], somewhat thinner than typical C_{18} phosphodiester bilayer membranes. Nevertheless, these archaeal membranes are much more physically stable than those formed from phosphodiesters. For example, large (600 nm) vesicles generated from *T. acidophilum* ether lipids were found to be more resistant to physical disruption by high temperature and surface active agents such as phenol, alcohols and detergents than dipalmitoyl phosphatidylcholine vesicles [123]. In a more extensive study of liposome stability, Sprott et al. [124] compared liposomes prepared from the ether lipid extracts of a number of archaea with egg phosphatidylcholine and dipalmitoyl phosphatidylcholine liposomes. In virtually all instances, the archaeal ester lipid liposomes showed higher levels of stability to temperature, pH, serum proteins and long-term oxidative effects than the ester lipid vesicles. Perhaps not surprisingly, the former were also highly resistant to the addition of phospholipases. The use of fluorescent probe techniques to measure the thermal stability of *S. acidocaldarius* lipid liposomes over a temperature range of 25–85 °C showed little variation in proton permeability across the temperature range [125]. The low permeability of these membranes was attributed to steric hindrance by the methyl side groups of the hydrophobic chains. X-ray analysis of Langmuir-Blodgett films of *S. solfataricus* lipids showed decreasing order with increasing temperature, but no loss of periodic organisation at temperatures below 100 °C [126].

The remarkable physical and chemical stability of archaeal ether lipid liposomes, attributed to the presence of the ether linkage, to the intimate packing of the phytanyl chains and to the reduced degree of molecular translational freedom in a boliform structure, has provided a significant incentive to the practical utilisation of such liposomes. A number of applications, including drug delivery systems [124] and bioelectronics components [127], have been proposed.

Conclusions

Since the discovery in the late 1960s of microorganisms living at temperatures above 70 °C, numerous laboratories around the world have focussed specifically on the biochemistry and physiology of life at high temperatures. So where do we now stand in terms of our understanding of the molecular and physiological basis of high-temperature life processes and the factors which dictate the upper limits? We must conclude that we still lack a clear understanding of the mechanisms by which thermophiles and hyperthermophiles address the issue of biomolecule stability at high temperatures. Despite research efforts spanning nearly 30 years and the huge expansion of our understanding of high-temperature microbial ecology, physiology, biochemistry and genetics, there are many fundamental issues still to be resolved in this field.

One major difficulty is the shortage of data on biomolecule stability above 100 °C. A second is that most of the available data is, unsurprisingly, for in vitro rather than in vivo conditions. Given the complexity of intracellular conditions in terms of potentially stabilising environments and interactions, in vitro data cannot be more than a very rough guide to the true stabilities of molecules in vivo. We already know that several vital biomolecules which are quite unstable in vitro at 100 °C are present and apparently participating in the metabolism of organisms growing at 100–113 °C. This observation underlines the necessity for caution in applying in vitro data to judgements on which factors might limit life at high temperatures. Nevertheless, from the data available it is difficult to see how protein or lipid/membrane stability is likely to be a limiting factor below, say, 140 °C. Indeed, the evidence to date suggests that associations with such macromolecular structures may be a strategy for stabilising more thermolabile molecules. The issue is much less clear-cut in respect of nucleic acids and their polymers, but mechanisms for stabilising these, including covalent modification and association with proteins, do seem to be available. For low molecular weight metabolites there is potentially an even wider range of mechanisms for stabilisation or circumventing instability. However, the difficulty for the cell may lie in the range of functions and properties of such molecules. A relatively modest range of generic stabilising strategies (i.e. additional weak interactions, covalent modification, association with other macromolecules) seems sufficient for each of the major classes of macromolecules of the cell. It is not obvious that the variety of stabilisation strategies needed to deal with the wide range of chemistries and functions of small molecules is sufficient to cope with the effects of temperatures above 115 °C. However, this reservation may simply indicate how difficult

it is to avoid our inherent 'mesocentric' bias. Almost all the biochemistry we know has been learnt from organisms very well adapted to life at 20–40 °C.

There is still a strong tendency to see high temperature as an obstacle to be overcome by thermophiles, rather than as presenting advantages, such as faster (nonenzymic) reaction rates, lowered viscosity, faster diffusion and raised solubilities. While it is obvious that some intracellular components, essential in mesophiles, seem to be unstable at 100 °C, it is not inconceivable that the instability of these or other molecules is an advantage in organisms evolved to exploit this. We should also remember that, from an organismal point of view, hyperthermophiles living at 100–110 °C are as well adapted to their immediate environments as are mesophiles living at 37 °C.

Finally, we stress that the question, ''What molecules or molecular factors will be responsible for establishing the upper temperature limit of life?,'' is still a very open one. Not only are the in vitro data available to us very uneven in coverage, but more seriously, the in vivo data are entirely inadequate. To remedy the latter will require considerable experimental ingenuity and a better knowledge of intracellular conditions than we currently possess.

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