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Thermostability and Thermoactivity of Enzymes from Hyperthermophilic Archaea

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Abstract—Enzymes from hyperthermophilic microorganisms are characteristically thermostable and thermoactive at extremely high temperatures. Information about the basis for the structure and function of these novel proteins is beginning to emerge. However, there are very few generalizations that can be drawn at this point that can be derived from the limited number of studies that have focused on biocatalysis and thermostability at extremely high tempratures.

Introduction

One of the most significant findings in the field of biochemistry in the past decade is the realization that protein structure and function need not cease as temperatures approach 100 °C and higher.[1–3] Whereas there have been isolated cases in which enzymatic activity at such temperatures was observed, it is now apparent that proteins produced by hyperthermophilic microorganisms (i.e. those growing above 90 °C with an optimum above 80 °C) are intrinsically stable and active at very high temperatures. The bases for this extreme thermostability are not completely clear although some clues are emerging from recent studies. What is also unclear is how biocatalysis at extremely high temperatures can be utilized to fulfil existing needs for hydrolytic or synthetic reactions or, more importantly, to create novel opportunities. Perhaps, the greatest contribution that will emerge from the study of proteins from hyperthermophilic microorganisms is discerning the mechanisms for protein stabilization and function at any temperature. This prospect provides a rationale for closer examination of their biophysical and biochemical characteristics.

Sources of Hyperthermophilic Microorganisms

Hyperthermophilic microorganisms have been isolated from numerous sites of geothermal activity.[4] These range from terrestrial hot springs in locations such as Yellowstone National Park, Mt St Helens, Iceland and New Zealand, to shallow marine hot springs in Vulcano, Italy to deep sea hydrothermal vents in the Atlantic and Pacific Oceans. Thus far, there have been some differences between the groups of organisms found in terrestrial as opposed to marine sites, possibly associated with the differences in salt levels in these locations. Most marine hyperthermophiles can be found at both shallow and deep sea geothermal habitats. Those isolated from deep sea sites can be cultivated under gas pressures close to one atmosphere. However, it has been shown in certain cases that pressures of 200–500 atmospheres can have a marked effect on cellular metabolic function as well as on enzyme stability and activity.[5,6] However, the pressures used to elicit these effects are often considerably higher than the prevailing hydrostatic pressures at the sites from which the organisms/enzymes have been isolated. In the case of *Methanococcus janaschii*,[7] a hyperthermophilic methanogen so far found only in deep sea sites, pressure has been shown to extend its growth temperature range as well as stabilizing and activating a hydrogenase produced by the organism.[5] More work is needed to understand the role that pressure plays in the physiology of hyperthermophilic organisms and the effect that it has on their constituent biomolecules.

Phylogenetic Classification of Hyperthermophilic Microorganisms

Because it has been proposed that life evolved from thermal environments,[8] the phylogenetic placement of hyperthermophilic microorganisms has received a great deal of attention in recent years. Woese *et al.*[9] proposed a phylogenentic system that places most hyperthermophiles among the domain Archaea which is distinguished from the Bacteria and Eucarya on the basis of 16S ribosomal RNA sequence as well as biochemical and structural features. Only members of the Thermotogales[10] and the genus *Aquifex*[11] are hyperthermophilic bacteria. The Archaea, which also contain halophilic and methanogenic microorganisms, are characterized by the extreme environments in which they thrive. Recent advances in phylogeny have many implications,[12] including providing some direction in identifying potential sources of unique proteins. Coupled with physiological information, phylogenetic placement of a given hyperthermophile provides some clues as to the types of proteins that a given organism produces. For example, neither xylanase nor xylose isomerase activities have been identified as yet in the hyperthermophilic Archaea,[13] although several species of the Thermotogales produce both enzymes.[14,15] Also, an interesting modified Entner–Doudoroff pathway, also referred to as pyroglycolysis,[16,17] has been found only among the thermophilic Archaea. This is also the case for

the eukaryotic multicatalytic proteinase complex which has been identified in an extremely thermophilic archaeon, *Thermoplasma acidophilum*,[18-22] but not found in bacteria. As more is learned about the biochemical and phylogenetic relationships of the three kingdoms of microorganisms, insights into evolutionary aspects of protein structure and function will follow.

Physiology of Hyperthermophilic Microorganisms

Sulfur plays a central role in the bioenergetics of hyperthermophilic microorganisms. In most cases, elemental sulfur is included in growth media during the isolation of hyperthermophiles. While a sulfur requirement is not always obligate, its reduction or oxidation is noted during the growth of most aerobic and anaerobic hyperthermophiles studied to date. During aerobic growth, sulfur oxidation to sulfate typically occurs in highly acidic media of approximately pH 2.0. Sulfur oxidation (or ferrous iron oxidation) is energetically coupled to the pumping of protons out of the cell to maintain internal pH close to neutral as well as to the conservation of energy for use in cellular processes. As temperature approaches and exceeds 100 °C, anaerobic organisms are most abundant. In this case, soluble polysulfides, the product of nucleophilic attack of insoluble elemental sulfur, are converted to hydrogen sulfide and its hydrolysis products.[23] While for chemolithotrophic hyperthermophiles, such as *Pyrodictium brockii*, hydrogen–sulfur autotrophy is the bioenergetic basis for growth and sulfur reduction likely involves a membrane-associated system.[24,25] For heterotrophic hyperthermophiles, the significance of sulfur reduction, in addition to fermentative processes, such as pyro-glycolysis,[16] is not completely clear. For *Thermotoga maritima*, sulfur reduction is purportedly associated with de-bottlenecking metabolic pathways through the removal of inhibitory molecular hydrogen.[26] However, for *Pyrococcus furiosus*, sulfur reduction has been linked to an energy-conserving process as evidenced by the doubling of growth yields in the presence of sulfur in maltose-limited chemostats.[27] In fact, the hydrogenase produced by *P. furiosus* [28] has been shown to function as a sulfur reductase.[29] In any event, more must be done to determine the metabolic impact of sulfur oxidation and reduction at extremely high temperatures. Also, the effect of sulfur species on methanogenic hyperthermophiles and their roles in mixed high temperature communities should be investigated.

Enzymes from Hyperthermophilic Microorganisms

It is not surprising that many enzymatic activities previously identified in mesophilic microorganisms can be found in hyperthermophiles. Of course, for organisms thriving at such high temperatures, nature has designed a thermostable matrix within which such biocatalytic reactions can occur. The variations in biocatalytic mechanisms and protein structures that occur with increasing temperature have only recently been studied and very limited information is yet available. However, there are certain aspects of hyperthermophilic enzymes that are worth closer examination even at this early point.

Hyperthermophilic enzymes are not necessarily more specifically active than their mesophilic counterparts

Chemical reactions are typically accelerated with increasing temperature. In biological systems, this increase is ultimately limited by the lability of substrates, cofactors or, in most cases, the biocatalyst itself. However, hyperthermophilic enzymes operate at significantly higher temperatures than their mesophilic and moderately thermophilic counterparts, even though in many cases the same metabolic reaction is being catalyzed. As to whether hyperthermophilic enzymes, in general, catalyze reactions at higher rates since they operate at elevated temperatures, there are insufficient data yet to be conclusive. However, one should not expect the enzyme from a hyperthermophilic source to necessarily be more active than a less thermostable counterpart under their respective optimal conditions. The fact that an organism, at any temperature, must orchestrate a complex cascade of enzymatic reactions for growth and survival implies that the kinetics of these reactions have some temporal relationship. While there are hyperthermophiles that do have rapid growth rates (some with reported doubling times under 30 min), others have relatively slow growth rates (doubling times of 2–8 h). Just as is the case at lower temperatures, biocatalysis at higher temperatures must take place in the context of a cell's metabolic processes.

In the limited data available, there are cases where hyperthermophilic enzymes lead to higher reaction rates and cases where they do not. For example, Table 1 shows kinetic information for α-glucosidases from three thermophilic microorganisms evaluated at the temperature optimum for growth. Clearly, the enzyme from the hyperthermophile *P. furiosus* has a V_{max} (evaluated at the organisms' optimal growth temperatures) comparable to that of enzymes from less thermophilic sources. However, the catalytic efficiency (e.g. k_{cat}/K_m) of the *P. furiosus* enzyme[1,32] is considerably higher than that for the *Bacillus thermoglucosidus* enzyme.[31] It is interesting to note that k_{cat}/K_m for the *P. furiosus* α-glucosidase appears to be maximal at the optimal growth temperature of the organism. This enzyme, which catalyzes the hydrolysis of maltose to glucose, is at the beginning of the central glycolytic pathway of *P. furiosus* when grown on maltose.[27] Even though V_{max} for this enzyme increases to 310 U/mg from 140 U/mg as the temperature increases from 98 to 110 °C, the improvement in rate appears to happen at the expense of catalytic efficiency. Whether this will be true for other enzymes is not known; data for V_{max} and K_m as a function of temperature are typically not measured because of the narrow thermostability range of most enzymes.

In other cases, there is variability among enzymes from various sources with common catalytic functions. For example, Table 2 shows a comparison among thermostable xylose isomerases isolated from a variety of bacteria and

includes one isolated from the hyperthermophile, *Thermotoga maritima*;[14] these activities are reported at temperatures close to the optimum growth temperature of the particular organism. The hyperthermophile exhibits the highest V_{max} for xylose conversion to xylulose among those listed. However, the xylose isomerase from the next most thermophilic species, *Thermus aquaticus*, has a relatively low V_{max}[33] among those listed. Catalytic efficiencies (k_{cat}/K_m) estimated from information provided in the literature suggest that the *T. maritima* enzyme is not a more effective catalyst by this measure than enzymes functioning best at lower temperatures; note that the temperature optimum for this enzyme is approximately 105 °C[14] so that the V_{max} could be somewhat higher. The turnover number (as measured by k_{cat}), however, does seem to be higher at higher temperatures. This trend was noted above when considering the change of these parameters with temperature for the *P. furiosus* α-glucosidase.

Both of the cases cited here do no more than illustrate that enzyme function at elevated temperatures must be evaluated for each instance and that no general trend is apparent.

Hyperthermophiles may have developed alternatives to labile cofactor requiring biocatalytic reactions

Central pathways in a microorganism typically include biotransformations catalyzed by enzymes requiring cofactors. Many universal organic metabolites and cofactors, in particular NADH and NADPH, are labile at temperatures near 100 °C which presents problems if these are necessary for key metabolic reactions. It has been

Table 1. Kinetic constants for thermophilic α-glucosidases

Organism	T_{opt} (°C)	K_m (mM)	V_{max}* (U/mg)	k_{cat} (1/min)	k_{cat}/K_m (1/mM-min)	Reference
Bacillus caldovelox	60**	N/A	140	4,200	N/A	30
Bacillus thermoglucosidus	60**	0.023	183	1,260	5,570	31
Pyrococcus furiosus	60	0.018	9	1,270	70,300	1,32
	70	0.019	19	2,680	141,030	
	80	0.026	41	5,710	219,420	
	90	0.039	84	11,820	303,120	
	98**	0.047	140	19,760	416,550	
	110	0.110	310	43,400	319,550	

*Kinetics determined for *p*-nitrophenyl-α-D-glucopyranoside.

Table 2. Kinetic constants for thermophilic xylose isomerases for xylose conversion to xylulose

Organism	T (°C)	K_m (mM)	V_{max}* (U/mg)	k_{cat} (1/min)	k_{cat}/K_m (1/mM-min)	Reference
Streptomyces olivochromogenes	60	33	21.8	2564	77.7	34
Bacillus stearothermophilus	60	100	44.5	4900	49.0	34
Thermoanaerobium thermosulfurogenes	65	20	15.7	3140	157.0	35-37
Thermoanaerobacter B6A	65	16	17.6	3520	220.0	35,37
Thermus aquaticus	70	15	15.9	516	34.4	33
Thermotoga maritima	90	74	68.4	3420	46.2	14

*μmol/min per mg.

established, nonetheless, that hyperthermophiles do contain several different types of dehydrogenases and these are thermally stable and participate in energetic and biosynthetic pathways. For example, glutamate dehydrogenase has been purified and characterized from several hyperthermophiles growing near 100 °C.[38–42] In their molecular and catalytic properties, these enzymes are very similar to the analogous enzyme from mesophilic bacteria and show a high degree of sequence identity. Their one distinct property, of course, is that they are much more thermally stable, with half-lives at 100 °C of many hours. Thus, these hyperthermophilic dehydrogenases do not appear to have any specific features, such as a high affinity for NAD(P)H, which suggest they function to stabilize these nucleotides at extreme temperatures. On the other hand, they are found at extremely high cellular concentrations (up to 20 % of the cytoplasmic protein), much greater than their physiological role would suggest. A stabilizing role for these enzymes, therefore, cannot be ruled out. Other dehydrogenase-type enzymes isolated from the hyperthermophilic Archaea include glyceraldehyde-3-phosphate dehydrogenase[43,44] alcohol dehydrogenase[45] and polysulfide dehydrogenase.[46] None of these enzymes are present at extraordinary cellular concentrations or have extremely high affinities for nicotinamide nucleotides. Yet to be determined is if conditions *in vivo* (e.g. salt concentrations) act to stabilize thermolabile cofactors.

A second option for hyperthermophiles is not to utilize labile intermediates such as nicotinamide cofactors in key pathways. In *P. furiosus,* for example, the classical requirement of NAD/NADH-requiring reactions in the oxidative degradation of sugars appears to have been circumvented through the use of a novel 'pyrosaccharolytic' pathway for the conversion of glucose to acetate (for a review, see ref. 47). This is based on a pathway found in aerobic Archaea[48] which differs from the conventional one in that it lacks many of the phosphorylated intermediates, which might possibly be more thermolabile. The hyperthermophilic version also differs in that the three oxidation steps between glucose and acetate are catalyzed not by dehydrogenase-type NAD-reducing enzymes but by ferredoxin-reducing oxidoreductases. Ferredoxin is a small (M_r 7500), iron–sulfur containing redox protein that has been purified from several hyperthermophiles.[49,50] It is distinguished by its extreme thermostability; for example, the pure protein from *P. furiosus is* unaffected after 12 h at 95 °C. Clearly, its replacement of NAD in such a central metabolic pathway is consistent with nucleotide instability at hyperthermophilic temperatures. However, some of the organic intermediates in this same pathway, for example, glyceraldehyde, are rapidly hydrolyzed *in vitro* at temperatures above 80 °C, suggesting that other stabilizing mechanisms must be in effect.

The third (and perhaps only other) option for these organisms is to maintain certain solutes at cytoplasmic concentrations such that they bestow a general 'thermoprotectant' effect, which might extend to cofactors, intermediates, enzymes and also DNA. For example, *P. woesei* contains K^+ ions and the novel sugar, di-inositol-1,1'-phosphate, at concentrations of approximately 0.8 M.[51] However, the intracellular concentrations of K^+ and Na^+ ions in the hyperthermophile *Thermoproteus tenax* are less than 100 mM.[43] Hence, if cytoplasmic solutes do serve a role in stabilizing biological molecules in the hyperthermophiles, there appears not to be a universal mechanism. Clearly, much remains to be understood about how hyperthermophiles stabilize a range of biological molecules.

Substrate lability rather than enzyme stability can be the bottleneck in biocatalysis at high temperatures

While there are many potential biocatalytic steps that might take advantage of hyperthermophilic enzymes, it is often the thermolability of a substrate or product which is limiting. This is a very different situation than is encountered in mesophilic systems. Thus, attempts to utilize the intrinsic thermostability of hyperthermophilic enzymes may ultimately be limited in some cases.

Problems with the thermal lability of certain substrates are reflected in the metabolic strategies seen in hyperthermophiles. For example, as growth temperature increases for the hyperthermophilic heterotrophs, the direct utilization of glucose as a carbon/energy source is less apparent. This is presumably a result of the sensitivity of simple sugars, such as glucose, to unwanted side reactions in the presence of certain compounds at elevated temperatures. For example, both *P. furiosus* (T_{opt} = 100 °C)[52] and *Thermococcus litoralis* (T_{opt} = 88 °C)[53] will grow on maltose but not glucose, presumably because of the lability of glucose at these temperatures in the presence of certain chemical species in their growth environments. However, maltose is converted to glucose, after it is transported, through the action of an α-glucosidase present in both organisms.[32] This route for glucose utilization presumably minimizes its exposure to destabilizing temperatures or, perhaps, isolates glucose from unwanted side reactions which may be less likely in its intracellular environment. The same argument might also apply to amino acid processing because complex peptide mixtures are typically used in culture media in place of single amino acids. Uptake of intact peptides might be a route to minimize deamination of labile amino acids (e.g. glutamine and asparagine) at elevated temperatures prior to their utilization in fermentation pathways.

Thermostable DNA polymerases from hyperthermophilic sources have become a mainstay in molecular biology with their use in the polymerization chain reaction (PCR). Here, the reported temperature optimum for enzymes from hyperthermophiles is typically around 75 °C, although the polymerases from *P. furiosus*[54] and *T. litoralis*[55] certainly are much more thermostable. Here, the template DNA is not stable at temperatures approaching 100 °C so that there is no convenient way to determine enzymatic activity at these temperatures. The DNA from hyperthermophilic organisms, which has been shown to be stabilized through a host of binding proteins[56], is apparently manipulated *in vivo* to facilitate reproduction at such high temperatures.[57]

Gene sequences available thus far for hyperthermophilic proteins shed little light on detailed strategies for thermostability

Over the past several decades, the protein chemistry literature has been laden with studies addressing the intrinsic bases for 'moderate' thermostability. Armed with deduced amino acid sequences from genes encoding particular proteins and with occasional three-dimensional structures of one or more proteins in a homologous series covering a certain temperature range, certain rules have been proposed for thermostabilization. More recently, site-directed mutagenesis has been used for the systematic testing of existing thermostability heuristics as well as for the development of new generalizations. Suffice to say that while general trends responsible for protein thermostability can be identified in some cases, the issue is no less complex than it has ever been. Moreover, the recent availability of several enzymes and proteins from hyperthermophilic organisms growing near 100 °C[2,47] opens up a completely new aspect of protein stability, especially since proteins *in vitro* can be destroyed at 80–100 °C by the hydrolysis of certain peptide bonds, deamination of asparaginyl residues, and cleavage of disulfide bonds.[58] It might be expected that studies of these hyperthermophilic proteins will confirm proposed mechanisms of thermostability in some cases while refuting prevailing wisdom in others. Of greater importance perhaps is the identification of strategies that the hyperthermophiles may have employed, strategies that might be utilized through recombinant techniques to stabilize commercially-relevant enzymes.

To date, however, only limited amino acid sequence information is available for proteins and enzymes from the hyperthermophilic Archaea, and the three-dimensional structure of only one hyperthermophilic protein is known. In fact, the complete amino acid sequences of only seven enzymes from the hyperthermophilic Archaea are available: for glyceraldehyde-3-phosphate dehydrogenase[59] and glutamine synthetase[60] from *P. woesei*, for the DNA polymerases from *P. furiosus*[61] and *Thermococcus litoralis*,[62] for the α-amylase[63] from *P. furiosus*, and for the glutamate dehydrogenases from *P. furiosus*[64] and from the novel deep sea isolate ES-4.[41] Comparisons of these sequences with those of the corresponding mesophilic and moderately thermophilic enzymes have led to some conclusions on amino acid preference. For example, Zwickl *et al.*[59] found for *P. woesei* GAPDH that serine and glycine are discriminated against, alanine is preferred, and that there is a striking increase in the phenylalanine content and decrease in the number of aspartate, cysteine and methionine residues in the hyperthermophilic enzyme. Such generalizations, however, afford little help in trying to stabilize thermolabile proteins, and it has now become clear that insights into mechanisms of 'hyper-thermostability' will require detailed structural comparisons. Until there is a dramatic improvement in our abilities to predict the pathways of protein folding and ultimate structural conformations from sequence information, the amino acid sequences of hyper-thermophilic proteins will remain virtually useless as tools for assessing stabilizing mechanisms.

The inadequacy of amino acid comparisons is readily illustrated with recent studies on electron transfer proteins such as rubredoxins and ferredoxins from hyperthermophiles. These redox proteins are ideal candidates for studying the issue of hyperthermostability, as they are small (50–70 residues) proteins and there is a large database of amino acid sequences together with several three-dimensional structures available for the mesophilic versions of these redox proteins. Hence, a detailed comparison of the amino acid sequence of the rubredoxin from *P. furiosus* with those of 14 mesophilic rubredoxins gave no clue as to why the former should be stable at 95 °C for 24 h, while the most stable mesophilic analog rapidly denatured at 80 °C.[65] Moreover, the same conclusion was reached when the ferredoxins of *P. furiosus*, *T. litoralis* and ES-4 were compared both with each other and with the ferredoxins from over 30 mesophilic species.[50]

On the other hand, structural studies of the hyperthermophilic redox proteins have given the first clues into mechanisms of stabilizing proteins at extreme temperatures. The three-dimensional structure of *P. furiosus* rubredoxin was recently determined by three independent techniques—crystallography,[66] NMR[67] and molecular modeling.[68] All gave virtually identical structures. Moreover, they were remarkably similar to the known crystal structures of the mesophilic proteins.[69,70] For example, the hydrophobic core of hyperthermophilic protein exhibited high sequence and structural homology to the mesophilic proteins. Hence, hyperthermostability appeared to be conferred by relatively minor changes involving mainly surface residues. The most striking difference was at the N-terminal region. In the *P. furiosus* protein, the N-terminal residue is specifically incorporated into the H-bonding network of a triple stranded β-sheet, and this is thought to prevent the β-strands from 'unzipping' at high temperature. In contrast, the high conserved N-terminal of all of the mesophilic proteins, which is one residue longer than the hyperthermophilic protein, is highly disordered, and is thought to be the first part of the protein to denature at high temperatures.

That hyperthermostability is conferred by relatively minor changes in protein structure, at least in small redox proteins, was recently confirmed by the determination of the secondary structure of *P. furiosus* ferredoxin by 2D NMR.[71] In essence, this protein contains an extension of the secondary structural elements found in mesophilic ferredoxins, while the hydrophobic core is highly conserved. Hence a loop-helix-double-stranded β-sheet structure in the mesophilic protein was 'replaced' by a slightly longer helix, a much shorter loop, and a triple-stranded β-sheet in the hyperthermophilic protein. Moreover, the β-sheet is expanded in the *P. furiosus* protein to include the C- and N-terminal residues, and these specifically interact via a salt bridge. Thus, in the two hyperthermophilic proteins for which structural

Table 3. Enzymes purified from sulfur-reducing hyperthermophilic Archaea

Enzyme	M_r[a]	T_{opt}[b]	$t_{50\%}$[c]	Function	Source[d]	Ref.
Hydrolases						
Protease	52 (α)	100°	1.5 h/95°	Peptide hydrolysis	Dm	77
Protease	66 (α)	115°	33 h/98°	Peptide hydrolysis	Pf	76
Amylase	132 (α_2)	100°	2 h/120°	Starch hydrolysis	Pf	63,75
Amylase	70 (α)	100°	6 h/100°	Starch hydrolysis	Pw	79
Amylopullulanase	140 (α)	118°	20 h/98°	Starch degradation	ES	80,61
α-Glucosidase	125 (α)	110°	48 h/98°	Maltose hydrolysis	Pf	32
β-glucosidase	230 (α_4)	105°	13 h/110°	Cellobiose hydrolysis	Pf	82
Oxidoreductases						
Aldehyde oxidoreductase	85 (α)	> 95°	6 h/80°	Glycolytic enzyme	Pf	16
Pyruvate oxidoreductase	120 ($\alpha\beta\gamma\delta$)	> 95°	0.3 h/90°	Pyruvate oxidation	Pf	83
Formaldehyde oxidoreductase	280 (α_4)	> 95°	2 h/80°	Unknown	Tl	84
Indolepyruvate oxidoreductase	180 ($\alpha_2\beta_2$)	> 95°	6 h/80°	Peptide fermentation	Pf	85
Dehydrogenases						
Glutamate dehydrogenase	270 (α_6)	95°	10 h/100°	Glutamate oxidation	Pf	39
Glutamate dehydrogenase	270 (α_6)	95°	10 h/100°	Glutamate oxidation	ES	41
Glutamate dehydrogenase	270 (α_6)	95°	2 h/100°	Glutamate oxidation	Tl	42
GAPDH[e]	150 (α_4)	nd[f]	0.7 h/100°	Glycolysis ?	Pw	59
GAPDH (NAD)	196 (α_4)	nd	0.3 h/100°	Unknown	Tt	52
GAPDH (NADP)	156 (α_4)	nd	0.5 h/100°	Unknown	Tt	52
Polysulfide dehydrogenase	92 ($\alpha\beta$)	> 95°	12 h/95°	Sulfur reduction	Pf	46
Alcohol dehydrogenase	200 (α_4)	80°	2.0 h/85°	Unknown	Tl	45
Hydrogenases/Redox Proteins						
Hydrogenase	118 ($\alpha\beta$)	> 90°	1 h/98°	H_2 oxidation	Pb	25
Hydrogenase	160 ($\alpha\beta\gamma\delta$)	> 95°	2 h/100°	H_2 production	Pf	28
Ferredoxin	7 (α)	> 95°	> 24 h/95°	Electon transfer	Pf	49
Ferredoxin	7 (α)	> 95°	> 24 h/95°	Electon transfer	Tl	50
Rubredoxin	5 (α)	> 95°	> 24 h/95°	Unknown	Pf	65
Isomerases/Invertases						
Sucrose α-glucohydrolase	114 (α)	105°	48 h/95°	Unknown	Pf	85
Nucleic acid modifying enzymes						
DNA polymerase	93 (α)	> 75°	20 h/95°	Replication	Pf	54
DNA polymerase	93 (α)	> 75°	7 h/95°	Replication	Tl	55

[a]Molecular weight in kDa (the number of subunits is given in parenthesis).
[b]Optimum temperature for catalytic activity *in vitro*.
[c]Time required to lose 50% of catalytic activity after incubation at the indicated temperature.
[d]The sources of the enzymes are: Pf, *Pyrococcus furiosus*; Pw, *P. woesei*; Tl, *Thermococcus litoralis*; ES, ES-4; Tt, *Thermoprotus tenax*; Pb, *Pyrodictium brockii*; Dm, *Desulfurococcus mucosus*.
[e]GAPDH: glyceraldehyde-3-phosphate dehydrogenase.
[f]nd, Not determined due to the instability of the substrates at high temperature.

information is available, both incorporate their N-terminal residues into secondary structural elements. Although it remains to be established if this is a general feature of this type of protein, the strategy is, in hindsight, perhaps an obvious one, in that highly flexible and disordered N- or C-terminal regions could be the sites where denaturation of mesophilic proteins is initiated.

Thus, the limited data on hyperthermophilic proteins so far, and specifically those from small redox proteins, indicate that amino acid sequence comparisons give no insight into stabilizing mechanisms, and that the differences between analogous mesophilic and hyperthermophilic proteins arise from relatively minor changes in protein structure. Unfortunately, these results do not bode well for either understanding why complex hyperthermophilic enzymes are so stable, or for means to stabilize labile mesophilic enzymes, even if high resolution structures are available. This field is still very much in an early stage of development, as any predictions of why a particular hyperthermophilic protein is so stable must also be tested by specifically changing the proposed interactions, and this has yet to be carried out with a hyperthermophilic protein. Of course, compensatory interactions might occur, but elucidation of these would also be extremely informative and contribute to the information base. Such results would have obvious implications in the broader questions of protein folding and engineering, in particular, the relationship between sequence and structure.[72] It should also be noted that very recent studies on the thermodynamic behavior of proteins have convincingly shown that there is a convergence temperature for enthalpy and entropy changes with an upper limit near 113 °C, at which point the apolar contributions are zero.[73] In other words, at this temperature hydrophobic interactions cease to contribute to protein stability and the core of globular proteins will unfold to the denatured state. Although only limited calorimetric data on hyperthermophilic proteins are available so far, the glutamate dehydrogenases of *P. furiosus*[74] and ES-4[41] and the α-amylase from *P. furiosus*[75] all show transition temperatures (T_m) near 113 °C. In contrast, the iron–sulfur-containing ferredoxin from *P. furiosus* has a T_m value of 117 °C, and for the zinc-substituted form of *P. furiosus* rubredoxin the value is 124 °C,[76] both of which contain covalently coordinated metal atoms. It therefore remains to be established if proteins in the absence of coordinated metals are stable at temperatures above 113 °C.

Opportunities for High Temperature Biocatalysis

The enzymes that have been purified from the sulfur-reducing hyperthermophilic Archaea so far are listed in Table 3. They can be subdivided into hydrolases, ferredoxin-linked oxidoreductases, NAD(P)-dependent dehydrogenases, hydrogenases and small redox proteins, isomerases and invertases, and nucleic acid modifying enzymes. Clearly, several of the hydrolases represent candidates for replacing enzymes now used commercially in cases where additional thermostability is desirable. For example, in starch processing, temperatures near and above 100 °C are necessary to liquify starch prior to enzymatic hydrolysis so that thermostable enzymes are desirable. Numerous applications involving thermostable hemicellulases and cellulases can be envisioned in cases ranging from waste treatment to foods processing to pulp and paper technology.

Enzymes other than hydrolases will find use in situations where thermostability and thermoactivity are important. There are certain to be additional thermostable nucleic-acid modifying enzymes identified from hyperthermophiles; for example, there is great interest in isolating a thermostable reverse transcriptase so that thermal cycling approaches might be applied to RNA fragments. Also, DNA ligases will be utilized in aspects of molecular biology.

Less obvious, but perhaps of greater significance, are applications in which thermostable enzymes can be used for synthetic purposes. Peptide synthesis using thermostable proteases in aqueous and non-aqueous systems may alleviate some of the current problems in these processes with specificity and activity. Esterases and lipases from hyperthermophilic sources might be used in certain pharmaceutical processes. With additional emphasis placed on enzymatic routes in place of conventional organic synthesis, desirable specificities may be identified that advantageously utilize high temperatures. Several biotransformations involving saccharides and carbohydrates are possibilities.

In any event, the role of enzymes in a number of industries should be considered and re-considered, given the availability of more thermally robust systems. The tools of molecular biology, in conjunction with knowledge gained from structural studies of hyperthermophilic proteins, might be applied to less thermally stable enzymes to improve their stability characteristics. It would seem that by pushing up the temperature limits for biocatalysis new opportunities in both applied and fundamental arenas have been created.

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