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MINIREVIEW

Hyperthermophilic enzymes – stability, activity and implementation strategies for high temperature applications

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Current theories agree that there appears to be no unique feature responsible for the remarkable heat stability properties of hyperthermostable proteins. A concerted action of structural, dynamic and other physicochemical attributes are utilized to ensure the delicate balance between stability and functionality of proteins at high temperatures. We have thoroughly screened the literature for hyperthermostable enzymes with optimal temperatures exceeding 100 °C that can potentially be employed in multiple biotechnological and industrial applications and to substitute traditionally used, high-cost engineered mesophilic/thermophilic enzymes that operate at lower temperatures. Furthermore, we discuss general methods of enzyme immobilization and suggest specific strategies to improve thermal stability, activity and durability of hyperthermophilic enzymes.

Introduction

In general, it is agreed that living organisms can be grouped into four main categories as defined by the temperature range that they grow in: psychrophiles, mesophiles, thermophiles and hyperthermophiles [1]. The origin of extremophilic organisms has long been debated. Based on the analysis of 16S and 18S rRNA gene sequence data, it was shown that, in the evolutionary history of the three domains of living organisms, bacterial and archaeal hyperthermophiles are closest to the root of the phylogenetic tree of life [2]. Therefore, it has been postulated that hyperthermophiles actually precede mesophilic microorganisms [3]. Intuitively, this is in agreement with current theories about the environmental conditions on the surface of Earth when life emerged. According to this theory, all biomolecules evolved to be functional and stable at high temperatures, and adapted to low temperature environments. However, another theory suggests that hyperthermophiles arose from mesophiles via adaptation to high temperature environments. This hypothesis is based on the supposition that ancestral RNA could not be stable at elevated temperatures [4,5].

The first hyperthermophilic organisms from the *Sulfolobus* species was discovered in 1972 in hot acidic springs in Yellowstone Park [6]. Subsequently, over 50 hyperthermophiles have been discovered in

Abbreviations
ADH, alchohol dehydrogenase; G-C, guanine-cytosine.

environments of extreme temperatures: near or above 100 °C. Examples of environments that, until recently, were considered as being inhospitable to life include volcanic areas rich in sulfur and 'toxic' metals and hydrothermal vents in the deep sea (approximately 4 km below sea level) of extremely high pressure [7]. Recently discovered hyperthermophiles have been observed to grow at temperatures as high as 121 °C [8]. Interestingly, hyperthermophilic microorganisms do not grow below temperatures of 50 °C and, in some cases, do not grow below 80–90 °C [7]. Yet, they can survive at ambient temperatures, in the same way that we can preserve mesophilic organisms in the fridge for prolonged times. Hyperthermozymes, in particular, are essentially inactive at moderate temperatures and gain activity as temperatures increase [9].

Hyperthermozyme function at elevated temperatures is a unique attribute that may enable their use in a plethora of biotechnological and biocatalytic applications, where the opportunities are relevant to (a) how we might employ hyperthermostable enzymes for applications where extreme temperatures are required and (b) how we can engineer enzymes in general to maintain their functionality over a broad range of temperatures. In this minireview, we aim to highlight some of the unique characteristics of hyperthermophilic proteins, at the genome, transcriptome and proteome level, which allow for functionality at high temperatures. Moreover, strategies will be discussed with respect to optimizing the thermostability and activity of free as well as immobilized enzymes. The end goal is to provide a system that is able to operate under temperatures higher than those currently employed in systems based on mesophilic and thermophilic biocatalysts.

Hyperthermostability: genomic and proteomic considerations

The survival of hyperthermophiles necessitates a cellular machinery that operates at extreme temperatures. Thus, all aspects of the complex biomolecular systems have to be functional at high temperatures (i.e. individual proteins, genetic coding material, transcription/translation systems, etc.). By comparing differences between mesophilic, thermophilic and hyperthermophilic biomolecules, it is anticipated that a clearer understanding of the major factors that allow for enzymatic activity at higher temperatures will be provided.

Genome-transcriptome level considerations

Although thermal denaturation of dsDNA is known to be affected by its nucleotide composition [10,11] and that an increase in guanine-cytosine (G-C) content could result in an increase in DNA thermostability, it has been shown that no correlation exists between G-C content and the optimal growth temperature (T_{opt}) of bacterial organisms [10]. Others suggest that, when specific families of prokaryotes (i.e. bacteria and archaea) are analyzed, there may be significant increases in G-C content that coincide with an increase in T_{opt} [12]. However, it has also been observed that for some cases, a decrease in the frequency of SSS and SSG codons occurs with an increase in T_{opt}, which obscures the uniform increase in G-C content [13].

Interestingly, at the level of RNA, there is a growing body of work suggesting that a correlation does exist between G-C content and T_{opt} [14]. A survey of the small subunit rRNA sequences from archaeal, bacterial and eukaryotic lineages (mesophiles, thermophiles and hyperthermophiles) revealed that there is a significant correlation of the G-C content of the paired stem regions (Watson–Crick base pairing) of the 16S rRNA genes, with the actual length of the stem, and with their T_{opt} [15].

In spite of attempts to correlate the G-C content of hyperthermophilic genomes with their T_{opt}, it should be noted that experiments performed *in vitro* and statistical genomic analyses may not accurately represent the situation *in vivo*. It is generally accepted that the DNA and RNA of hyperthermophilic microorganisms are also stabilized through a combination of mechanisms, including increased intracellular electrolyte concentrations, binding of positively charged proteins and histones and spatially confined atomic fluctuations due to macromolecular crowding [16,17]. In addition, supercoiling plays an important role in stability of chromosomal DNA; all hyperthermophilic bacteria and archaea have the enzyme reverse gyrase, which affects DNA topology and appears to be essential for growth at extreme temperatures [18].

Proteome level considerations

It is generally acknowledged that, although hyperthermophilic proteins may have similar functions as their mesophilic counterparts, there may be intrinsic differences that allow them to maintain structural stability and activity at elevated temperatures. In general, protein stability at extreme temperatures above 90 °C is a complex issue that has been attributed to many factors: (a) amino acid composition (including a decrease in thermolabile residues such as Asn and Cys); (b) hydrophobic interactions; (c) aromatic interactions, ion pairs and increased salt bridge networks;

(d) oligomerization and intersubunit interactions; (e) packing and reduction of solvent-exposed surface area; (f) metal binding; (g) substrate stabilization; (h) a decrease in number and size of surface loops; and (i) modifications in the α-helix and β-sheet content [19–26].

Apart from the above mentioned intrinsic factors, extrinsic factors also have been demonstrated to contribute to protein stability in the context of a biological cell. This mainly concerns the so-called compatible solutes, a wide range of small stabilizing molecules (including sugar-derivatives such as trehalose, mannosyl-glycerate and di-myo-inositol-phosphate) [27]. Another factor usually forgotten when discussing hyperthermophlic proteins is their stability at intracellular conditions. Protein stability studies are generally conducted in dilute protein solutions *in vitro*. Such studies are likely to provide meaningful results when secreted, extracellular proteins are considered. However, these conditions may not represent the real situation found inside the cell: macromolecular crowding and naturally occurring small molecules such as metabolites and sugars are expected to play a significant role in protein stability [28,29].

Recent work has shown that the denaturation temperature (T_d) of the globular protein, CutA1, from the hyperthermophile *Pyrococcus horikoshii* OT3 approaches 150 °C [30]. Upon comparing the crystal structures of CutA1 from *Escherichia coli*, *Thermus thermophilus* and *P. horikoshii* OT3 (T_{opt} of 37, 75 and 95 °C, respectively), it was observed that there was a drastic increase in the number of intrasubunit ion pairs (1, 12 and 30, respectively) as T_{opt} increased. Moreover, this increase in intrasubunit ion pairs was directly related to the relative decrease in neutral amino acids and a significant increase in polar amino acids (i.e. Asp, Glu, Lys, Arg and Tyr). It is thought that the increased presence of ion pairs confers thermal stability due to the significantly reduced desolvation penalty for ion pair formation at increased temperatures [31].

Work by Szilagyi and Zavodszky [32] categorized thermophilic proteins based on the T_{opt} of the microorganism. They compared the crystal structures of proteins from moderate thermophilic microorganisms (T_{opt} = 45–80 °C) and extreme thermophilic microorganisms ($T_{opt} \approx$ 100 °C). It was observed that the number of ion pairs increased with increasing growth temperature, whereas other parameters, such as hydrogen bonds and the polarity of buried surfaces, do not directly correlate with T_{opt}. Furthermore, the authors concluded that proteins from moderate and extreme thermophilic organisms are stabilized via different mechanisms. However, although these trends are consistent with previous studies, it should be noted that not all proteins from hyperthermophiles are hyperthermostable. There are proteins from hyperthermophilic organisms that denature at temperatures between 70 and 80 °C and, conversely, proteins from thermophilic organisms that exhibit melting temperatures of approximately 100 °C.

Upon comparing citrate synthases from the hyperthermophilic *Pyrococcus furiosus* (T_{opt} = 100 °C), the thermophilic *Thermoplasma acidophilum* (T_{opt} = 55 °C), the mesophilic mammal (pig; T_{opt} = 37 °C), and the psychrophilic bacterium (Antarctic strain DS2-3R; T_{opt} = 4 °C), it was observed that subunit contacts are crucial for enhancing the thermostability of these homodimeric enzymes [33]. Specifically, it was shown, using three site-directed mutants of *P. furiosus* and *T. acidophilum* citrate synthases, that ionic interactions are essential to their thermal stability. Indeed, ionic interactions, including ionic networks, are thought to be crucial among enzymes with activities around 100 °C [33]. Finally, it was also shown that thermostability does not guarantee thermoactivity. This final point is of particular interest because it highlights the delicate balance between thermostability and thermoactivity that must be considered when employing hyperthermozymes for biotechnological and biocatalytic applications.

Protein molecules are not fixed structures, as depicted in crystallographic representations. Rather, they exhibit a dynamic nature as described by their conformational flexibility, which in turn depends on the fluctuations of the protein atoms. Earlier work [9], which was later confirmed for other homologues proteins [34], suggested that the flexibility of a hyperthermostable protein is lower than that of thermophilic and mesophilic proteins at room temperature and increases with temperature, so as to allow for enzymatic activity near 100 °C. It is only upon achieving these high temperatures that sufficient molecular flexibility (via atomic motions) exists to facilitate the necessary conformational changes required for enzymatic activity (e.g. binding, releasing the substrate, etc.) [9].

Opportunities for biotechnological applications

Perhaps the quintessential example of a successful biotechnological application of thermozymes is the use of Taq polymerase, isolated from *Thermus aquaticus* [35], for PCR [36]. The groundbreaking discovery that proteins from hyperthermophilic microorganisms could be

expressed in mesophiles (e.g. *E. coli*) without losing their conformation, heat stability or activity not only lead to further characterization, but also initiated research on applying them to biocatalysis and biotechnology fields. Obviously, the ability of hyperthermostable proteins to be functional at elevated temperatures presents a number of potential opportunities: (a) the enzymatic processing of many natural polymers is significantly limited by their solubility, this barrier could be overcome by increasing the operating temperatures; (b) the viscosity of the medium increases as temperature is raised; (c) diffusion limitations of the reactants and of the products are minimized; (d) favorable thermodynamics (i.e. for endothermic reactions) would result in increased yields when the reaction is performed at high temperatures; (e) the reactions kinetics are faster at high temperatures; (f) enzymatic processing at temperatures near or above 100 °C minimizes the risk of bacterial contamination in food and drug biosynthesis applications; (g) enzyme immobilization may increase heat stability and therefore, improve biocatalyst performance; and (h) protein engineering by rational design and/or random mutagenesis of hyperthermostable enzymes may result in even more thermostable enzymes.

Several enzymes have already replaced many traditional synthetic chemistry processes. To date, the majority of industrially used enzymes are from bacteria and fungi; the result of 'natural evolution'. In some cases, their properties have been improved through: (a) rational design using combinatorial approaches (i.e. 'computational evolution') [37] and (b) random approaches using high-throughput systems (i.e. 'laboratory evolution') [38–40].

The profit motivation for substituting traditional enzymes with hyperthermostable counterparts is enormous, given that the global enzyme market currently exceeds €4 billion per year. The challenge is obvious: rather than investing more effort in generating mutant mesophilic proteins that operate at high temperatures, a more straightforward approach may be to search the existing protein database for the appropriate hyperthermophilic enzyme that normally functions at higher temperatures. Utilizing this approach would obviously avoid the expensive and laborious enzyme engineering process, and revolutionize industrial and biotechnological processes. Obviously, this approach relies on the availability of hyperthermophile orthologs: enzymes with improved stability, and with similar substrate specificity, enantioselectivity and catalytic activity.

Some hyperthermostable proteins, with optimal operation temperatures at or above 100 °C, are summarized in Table 1. Novel hyperthermostable enzymes, of known or unknown functions, are constantly being discovered, presenting a huge potential for being employed in a number of applications, including starch processing, cellulose degradation and ethanol production, pulp bleaching, leather and textile processing, chemical synthesis, food processing, and the production of detergents, cosmetics, pharmaceuticals, etc. [41–50].

Thermal stability and enzymatic activity upon immobilization

Successful implementation of hyperthermozymes to many applications depends on their ability to retain activity upon exposure to the harsh conditions required for most enzymatic reactions: non-natural solvents, high temperature and pressure. In addition to these constraints, many processes require the enzyme to be removable from the reaction medium, reusable or at least recyclable, while not contaminating the product stream by its presence. Enzyme immobilization on the surface of a carrier may address many of the issues listed above. Methods commonly employed for this purpose are covalent bonding [51,52], entrapment [53–55] and physical adsorption [56–58]. Adsorption is considered as the dominant mechanism of interaction of a protein with a surface and, in principle, is the initial event that precedes immobilization through covalent bonding or encapsulation. In general, the immobilized enzyme acquires an increased stability at high temperatures [59–61]. However, the key to successfully utilizing enzymes for biotechnological applications is to ensure that upon immobilization the enzyme remains functional.

Protein adsorption mechanisms and events

The interaction of proteins with surfaces often leads to their adsorption (i.e. excess accumulation of protein at the interface compared to the bulk). Physical adsorption is a mild method of immobilization. Protein adsorption events are largely directed by interfacial phenomena in the vicinal region between the surface and the adsorbing species within the bulk contacting medium [57,62,63]. These interfacial phenomena are mainly driven by electrostatic and hydrophobic interactions. Electrostatic interactions can be repulsive or attractive, depending on the net charges of the surface and of the protein. Hydrophobic interactions are thermodynamically favorable because they increase the system entropy by reducing the extent of unfavorable interactions between polar solvent molecules and hydrophobic moieties (i.e. the hydrophobic patches of

Table 1. Hyperthermostable enzymes with commercial interest and optimal activity over 100 °C in aqueous media.

Enzyme	Microorganism	Microorganism $T_{opt.}$ (°C)	Protein $T_{opt.}$ (°C)	Optimal pH	Molecular mass (kDa)	Reference
α-Amylase (α-glucosidic bonds)	*Pyrococcus furiosus*	100	106	6.5–7.5	129 (α_2)	[83]
	Pyrococcus furiosus	100	100	4.5	54	[84]
	Pyrococcus woesei	100	100	5.5	68	[85]
	Staphylothermus marinus	90	100	5.0	–	[86]
	Methanococcus jannaschii	85	120	5.0–8.0	–	[87]
Pullulanase type II (α-1,6 glycosidic bonds)	*Pyrococcus woesei*	100	100	6.0	90	[88]
	Pyrococcus furiosus	100	102	6.0	89	[83]
	Pyrodictium abyssi	98	105	9.0	–	[89]
Pullulan hydrolase III (α-1,6 and α-1,4 glycosidic bonds)	*Thermococcus aggregans*	85	100	6.5	83	[90]
Phospho-glucose/mannose isomerase	*Pyrobaculum aerophilum*	100	102	7.4	65 (α_2)	[91]
Glucose isomerase	*Thermotoga maritima*	80	105	6.5–7.5	180 (α_4)	[92]
β-Mannosidase	*Pyrococcus furiosus*	100	105	7.4	220 (α_4)	[93]
α-Glucosidase	*Thermococcus* strain AN1	80	130	–	63	[45]
	Thermococcus hydrothermalis	80	120	5.5	57	[94]
	Pyrococcus woesei	100	100	5.0–5.5	90	[95]
	Sulfolobus solfataricus	88	> 120	4.5	80	[96]
	Pyrococcus furiosus	100	115	5.0–6.0	135	[97]
β-Glucosidase	*Pyrococcus furiosus*	100	105	–	232 (α_4)	[98]
	Pyrococcus horikoshii	95	> 100	6.0	35	[99]
α-Galactosidase	*Thermotoga neapolitana*	80	103	7.0–7.5	61	[100]
Threonine (alcohol) dehydrogenase	*Pyrococcus furiosus*	100	100	10.0	155	[101]
Alcohol dehydrogenase	*Pyrococcus furiosus*	100	100	6.1–8.8	32	[102]
Carboxypeptidase	*Pyrococcus furiosus*	100	100	6.2–6.6	59	[103]
Aminopeptidase	*Pyrococcus horikoshii*	95	100	7.0–7.5	330 (α_8)	[104]
	Thermococcus strain NA1	80	> 100	6.0–7.0	40	[105]
	Pyrococcus furiosus	100	> 100	8.0	38	[106]
Glukokinase	*Pyrococcus furiosus*	100	105	–	93	[107]
Sucrose α-glucohydrolase	*Pyrococcus furiosus*	100	110	–	114	[108]
Serine protease	*Desulfurococcus mucosus*	88	105	–	52	[109]
Thiol protease	*Thermoc. kodakaraensis* KOD1	95	> 100	7.0	45	[110]
Metalloprotease	*Pyrococcus furiosus*	100	100	6.3	124 (α_6)	[111]
β-1,4-endoglucanase	*Pyrococcus furiosus*	100	104	6.0–7.0	30	[112]
Pyruvate kinase	*Pyrobaculum aerophilum*	100	> 100	6.0	205 (α_4)	[113]
	Aeropyrum pernix	93	> 100	6.1	207 (α_4)	[113]
	Thermotoga maritima	80	> 100	5.9	190 (α_4)	[113]
Methylthioadenosine phosphorylase	*Pyrococcus furiosus*	100	125	7.4	180 (α_4)	[114]
	Sulfolobus solfataricus	87	120	7.4	160 (α_6)	[115]
Fructose 1,6-biphosphate aldolase	*Thermoc. kodakaraensis* KOD1	95	> 100	5.0	312 (α_{10})	[116]
2-keto-3-deoxygluconate aldolase	*Sulfolobus-solfataricus*	87	100	–	133 (α_4)	[117]
Glucokinase	*Aeropyrum pernix*	93	> 100	6.2	36	[118]
ADP-dependent glucokinase	*Pyrococcus furiosus*	100	> 100	7.5	98 (α_2)	[119]
	Thermococcus litoralis	85	> 100	7.5	52	[119]
Glucanotransferase	*Thermococcus* strain B1001	85	110	5.0–5.5	83	[120]
4-α-glucanotransferase	*Pyrococcus furiosus* KOD1	100	100	6.0–8.0	77	[121]
Esterase	*Pyrococcus furiosus*	100	100	7.6	–	[122]
Metalloproteinase	*Aeropyrum pernix* K1	90	100	5.0–9.0	52	[123]
Aminoacylase	*Pyrococcus furiosus*	100	100	6.5	170 (α_4)	[124]

the protein and the hydrophobic surface of the sorbent).

The difficulty faced when discussing protein adsorption mechanisms arises from the fact that proteins are highly spatially organized, with various substructures that have differing stabilities, hydrophilicities and charges at given environmental conditions, such as temperature, concentration, ionic strength and pH. Thus, the diverse chemical and physical properties of proteins and surfaces provide multiple interaction

pathways that facilitate adsorption. It is this innate nature of proteins and surfaces that makes it difficult to predict the mechanism of protein adsorption, thus making it difficult to control the process and consistently generate a surface filled with stable and functional enzymes [57].

A common problem associated with the adsorption of enzymes is the conformational changes observed upon adsorption. Such a structural modification may ultimately lower or even diminish the catalytic efficacy of adsorbed enzymes; as discussed below, activation of enzyme activity may occur in rare cases. This excludes any discussion on enzymes that only become active upon adsorption. In general, however, protein immobilization strategies aim to minimize surface-induced conformational changes of adsorbed proteins.

The effect of adsorption on protein structure, thermostability and enzymatic activity was recently highlighted in a series of studies involving hyperthermostable glucanase from *P. furiosus* [60,61,64]. The conformation of the enzyme in the adsorbed state was determined using spectroscopically 'invisible' particles. It was found that thermal stability and enzymatic activity were dependent on the resulting structure of the adsorbed protein and that this structure was affected by the sorbent hydrophilicity. The denaturation temperatures of the free enzyme in solution and adsorbed to hydrophilic or hydrophobic surfaces were 109, 116 and 133 °C, respectively [61]. Compared to solution free enzyme, adsorption to hydrophobic sorbents led to slightly distorted secondary and tertiary structures [65]. In all cases, the specific enzymatic activity of the enzyme did not change upon adsorption.

Several examples of adsorption-induced activation of enzymes exist and the thermostable lipases are of particular interest because they have the potential for being employed in a myriad of biotech applications [66]. In aqueous media, lipases are usually found in a conformation where a 'flap' blocks the active center [67] and only upon adsorption to colloidal drops of oil is this conformation perturbed enough to allow for enzymatic activity [68]. Work with the lipase QL from *Alcaligenes* sp. showed that physical adsorption on a hydrophobic surface led to: (a) a 135% increase in enzymatic activity, relative to the free enzyme; (b) a 20 °C increase of the optimal temperature for enzymatic activity; and (c) surface regeneration [69], unlike immobilization through chemical grafting.

Therefore, when designing an efficient means of introducing hyperthermozymes to the reaction mixture, it is evident that both the enzyme's and the sorbent's physical and chemical properties must be considered. A general observation is that the majority of proteins tend to adsorb relatively well on hydrophobic surfaces. However, when interacting with hydrophobic surfaces, enzymes generally appear more susceptible to conformational perturbations as compared to adsorption on hydrophilic surfaces [56,57]. Moreover, conditions such as pH and ionic strength can affect the adsorbed amount of the enzyme. For example, it has been observed that changes in pH may lead not only to increased protein adsorption, but also to higher specific activity than the free enzyme [70]. Furthermore, adsorption-induced conformational changes are less when adsorption occurs at pH values near the protein's pI and that this is responsible for an increase in activity [71].

In physical adsorption, proteins become immobilized on the surface of the sorbent through multiple contact points resulting from the interaction between the sorbent and charged and/or hydrophobic amino acid side chains. Depending on the adsorbing conditions, as well as the protein and surface properties, these interactions, which individually are marginally stable, may result in irreversible immobilization of the protein at the interface when considered in total. Also, depending on the solution conditions (e.g. pH, ionic strength, the presence of a detergent), physically adsorbed enzymes may be displaced from the surface of the carrier [72].

Covalent bonding

It is generally accepted that some of the main benefits associated with covalent immobilization include: (a) increased thermal stability; (b) an ability to scale up to reactor applications; (c) ease of interaction with solution compared to encapsulated enzymes; and (d) decreased probability of the enzyme being displaced from the surface and contaminating the reaction solution. Strategies for the covalent immobilization of enzymes have been reviewed elsewhere [51,73]; this minireview rather focuses on correlating protein stability and activity upon bonding, particularly highlighting mild, multipoint attachment techniques [52,74,75].

Optimizing the multipoint covalent immobilization of thermophilic esterases from *Bacillus stearothermophilus* to agarose gels, yielded: (a) 30 000 and 600-fold increases in half-life compared to free and single-point attached enzymes, respectively; (b) retention of 65% of residual activity (cf. soluble) upon bonding; and (c) retention of 70% activity (cf. immobilized) after 1 week of exposure to organic solvents [75]. The case for optimizing the surface–enzyme interaction to retain activity is further highlighted by work conducted on

modified epoxy supports, where it was shown that some surfaces preserved 75–100% of the activity (cf. free enzyme), whereas other combinations lead to full inactivation of the enzyme [74]. Moreover, epoxy modification of the gel surface leads to the precise control of the covalent bonds formed with the enzyme [52].

Despite the various successful cases realized in covalently attaching enzymes to surfaces, the means of attachment can lead to enzyme inactivation. It has been shown that unreacted functional groups can further react with bonded enzymes that are active, resulting in their inactivation after long periods of incubation at high operating temperatures [76]. Thus, a major immobilization criterion involves neutralizing these reactive groups to prevent the surface from adversely affecting the half life of the enzyme.

Encapsulation

Enzyme encapsulation has the potential to provide a microenvironment that increases thermal stability and facilitates enzymatic activity at high temperatures. Although treated separately, encapsulation includes both adsorption and covalent bonding strategies with the difference that, in this case, the enzyme is confined at least on two dimensions by the encapsulating material. This section focuses on correlating protein stability and activity using traditional and novel encapsulation schemes that employ a variety of materials: silica based materials (e.g. sol-gel matrices, mesoporous silica) [28,53,77], aluminosilicates [55], polymers [54,78] and organoclays [79,80].

Sol-gels are commonly used for protein encapsulation. It has been shown that, upon silica entrapment, the mesophilic α-lactalbumin exhibited a 25–32 °C increase in thermal stability and did not fully denature at 95 °C, even after prolonged treatment [53]. However, this same system did not stabilize apomyoglobin [53]. Immobilization of horse heart cytochrome *c* by encapsulation into mesoporous silica led to improved stability and lifetimes of several months; heating to 100 °C for 24 h resulted in a residual activity of 61–74%, compared to the untreated free enzyme [55]. Polyacryalamide gels have also been used as an encapsulating material for various proteins, resulting in an increase in melting temperature [78]. Furthermore, it was observed that coencapsulation of yeast alchohol dehydrogenase (ADH) with a hyperthermophilic chaperone (group II) from *Thermococcus* strain KS-1 resulted in a significant increase in residual activity: ADH-only and ADH-chaperone yielded residual activities of 15% and 78%, respectively, after 5 days [81]. Intercalation of proteins between layered materials such as protein-organoclay lamellar composites may serve as an effective support providing increased protein stability [82]. The intercalation of glucose oxidase into functionalized phyllosilicate clay yielded systems where activity at denaturing pH values (i.e. between 6 and 9) was maintained at 90% of the free enzyme [80]; a trait ascribed mainly to increased electrostatic interactions between enzyme and surface.

Encapsulation provides a platform for protecting enzymes from thermal inactivation during prolonged exposure to elevated temperatures, provided that adequate interactions occur between the surface and the enzyme. The successful implementation of encapsulated hyperthermozymes obviously requires that the matrix materials are also able to withstand high temperatures.

Strategies for enhancing thermal stability and activity of hyperthermozymes

Crucial for the development and optimization of high-temperature biocatalysis systems is the need to gain further understanding of structural differences between hyperthermozymes and their mesophilic and thermophilic homologs, as well as the effect of immobilization on their structural rearrangement and resulting activity at high temperatures.

Through examining proteomic level differences between hypthermophilic proteins and their thermo/mesophilic counterparts, it is evident that Nature has employed multiple mechanisms to ensure high temperature activity. However, it appears that the resounding message for increasing the thermal stability of proteins revolves around three central tenents: (a) substitute polar for neutral amino acids so as to further increase the number of ion pair interactions; (b) delete surface loops to decrease molecular flexibility; and (c) minimize cavity volumes to increase packing density.

Because the adsorption configuration and conformational features at interfaces cannot yet be accurately predicted for enzymes, it is difficult to design a platform that works for any given enzymatic system and to find remedies to treat decreased activities of adsorbed enzymes. The delicate balance between thermostability and thermoactivity must be maintained when employing hyperthermozymes for biotechnological and biocatalytic applications. However, several studies on a range of enzymes indicate that successful immobilization strategies can lead to increased thermal stability, operation over a wide pH range, protection from non-natural solvents and higher specific

activities over prolonged operational lifetimes. It is important to consider that protein structural and chemical characteristics need to be correlated to the physical chemical properties of the carrier. As a general guideline: (a) hydrophilic surfaces may be preferred over hydrophobic surfaces; (b) electrostatic effects should be reduced by immobilizing at a solution pH near the pI; (c) surface concentration of enzymes should be maximized to inhibit denaturation events; (iv) there is the need to ensure carrier durability at the optimal, hyperthermozyme operating temperature; and (v) multipoint attachment strategies should be utilized, both to prevent protein leaching and to increase heat stability.

The integration of this information, combined with previous strategies used to enhance the thermostability of mesophilic and thermophilic proteins, should provide an efficient route for the development of catalytic systems based on hyperthermozymes. Research efforts should be focused on facilitating the transfer from meso/thermophilic to hyperthermophilic based catalytic systems.

Future focus

In the genomic era, new hyperthermophilic enzymes with novel properties will be discovered via thorough comparative genomic–proteomic analysis combined with high-throughput structural and functional characterization. The genomes of several hyperthermophilic microorganisms have been sequenced, whereas others are forthcoming (http://www.genomesonline.org/). Hyperthermophiles are hosts for a high number of genes, many of which encode proteins of unknown function. A wide range of thermostable and biologically novel enzymes for an array of potential applications is expected to become available simply by searching the ever expanding (meta-)genome sequence databases.

The characterization of these novel proteins has great potential for the chemical and pharmaceutical industries ('White Biotechnology'), as they are applied to the synthesis of chemical compounds that are currently difficult to synthesize using traditional synthetic methods. In addition, these natural enzymes will provide the basis for further protein engineering via the described computational and/or laboratory combinatorial approaches, undoubtedly ushering in a new stage of high temperature enzymatics.

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