Enzyme Vs. Extremozyme

What Makes Extremozymes Function Under Harsh Conditions ?

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Extremozymes that function in the extreme environmental conditions of temperature, pressure, pH, and salinity are of great importance for industrial processes and scientific research. Apart from understanding the principle behind the stability of such enzymes, there has been considerable effort to isolate enzymes from extremophiles (organisms that live in extreme environmental conditions) as well as modify enzymes from mesophiles (organisms that live in normal environmental conditions) to convert them into extremozymes.

Enzymes are biocatalytic protein molecules that enhance the rates of biological reactions by 10^6 to 10^{23} fold over the uncatalyzed reactions. A number of enzymes have been isolated from organisms for a variety of industrial processes and scientific research. Glucose isomerase, amylase, lipase and proteases are used in a variety of food, beverages and soap and detergent industries, while Taq polymerase T_4 lysozyme, ribonuclease and malate dehydrogenase are enzymes used in research laboratories. A major limitation of most enzymes used in the industries/ research laboratories is that they are very unstable even under ambient temperature let alone extreme conditions of temperature, pressure and pH. But life exists in extreme conditions of the environment like glaciers, hot springs, deep seas and the Dead Sea. Thus the metabolism and the nature of enzymes from these organisms, collectively called extremophiles, are likely to help us understand and manipulate protein stability. Thus there is not only a need to search for enzymes from *extremophiles* but also to understand the basis of the tolerance of naturally occurring extremozymes to high temperature or pH so that enzymes from mesophiles can be engineered to behave like extremozymes.

Structure and Stability of Enzymes

A general structure of an enzyme is outlined in *Figure 1*. Enzymes are made up of a linear sequence of amino acids stringed together by a peptide bond; this is referred to as the primary structure of the enzyme. Side chains of amino acids (charged, polar, apolar and hydrophobic) interact among themselves due to physical forces (hydrogen bonds, hydrophobic [1], electrostatic and Van der Waals interactions) acting between them and adopt a local conformation to form secondary structures. These secondary structures further interact and fold into a three dimensional structure with spatial disposition of its side chain to form tertiary structure. Tertiary structure is acquired by decreasing free energy (10-15 Kcal/mol) due to disulfide bond and other physical interactions. Sometimes two different polypeptides (subunits) having a tertiary structure interact in space and form a stable structure called quaternary structure. It is in the tertiary and quaternary structural forms that enzymes are functional to catalyze reactions under physiological conditions.

Enzymes Vs. Extremozymes: Structural Features

Natural extremozymes have been isolated from thermophiles,

[1] Hydrophobic interaction in protein arises due to aggregation of hydrophobic amino residues by removing water from the interior of the protein leading to increase in entropy.

Figure 1. Hierarchy of protein structures. (A) Primary structure, amino acid sequence in the protein chain, (B) Secondary structure, α-helix, (C) Secondary structure, β-sheets between two linear amino acid strands, (D) Tertiary structure, β-chain of haemoglobin and (E) Quaternary structure of the four separate chains of haemoglobin assembled into an oligomeric protein. Note that secondary structures, α-helix and β-sheet, are primarily stabilized by hydrogen bonds, tertiary structure of haemoglobin is stabilized by disulfide bond, and other physical forces and quarternary structure of haemoglobin is stabilized by physical interactions.
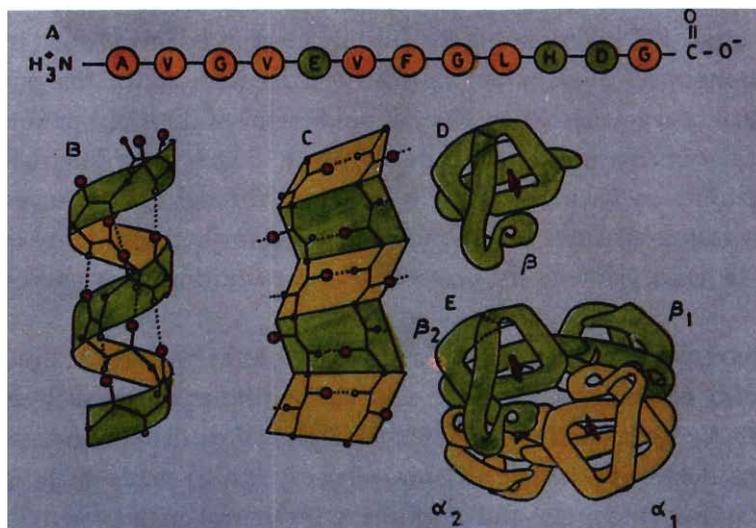
halophiles, psychrophiles, acidophiles and alkaliphiles. But structural feature of the enzymes from psychrophile, acidophile and alkaliphile is not known. Here, a structural comparison is made between enzymes from mesophiles and natural extremozymes from thermophiles and halophiles.

Mesophilic and Thermophilic enzymes: The primary, secondary, tertiary and where appropriate, quarternary structural parameters were in general found to be strikingly similar between thermophilic enzymes and their mesophilic counterparts. Enhanced thermostability is a consequence of a small number of amino acid replacements to promote additional non-covalent interactions without perturbations in the overall protein conformation.

In 1995, Prof. Waksman and his coworkers from School of Medicine, Washington University, St. Louis, USA compared the structural features of DNA polymerase from *Thermus aquaticus*, a thermophile and *E. coli*, a mesophile. They compared the C –terminal domain of *E. coli* polymerase designated as 'Klenow pol 1' with a similar fragment from *T. aquaticus* DNA polymerase designated as 'Klentaq 1'. Striking differences leading to enhanced stability of Klentaq 1 are presented in *Figure 2*. Apart from the differences shown in *Figures 2a* and *b*, a total of 17 hydrophobic residues are more in Klentaq1 than its counterpart Klenow pol 1, which buries 21% of the enzyme surface against 14.7% in Klenow pol 1 (some of the hydrophobic replacements in Klentaq1 are shown in *Figure 2c*). Thermostability in Klentaq1 is partly achieved through an enhanced hydrophobic core. A significant increase in thermostability of Klentaq 1 is due to the presence of three additional ion pairs.

Recently, a statistical analysis was made on the preferred amino acids between thermophilic and mesophilic enzymes such as DNA polymerase 1, glyceraldehyde-3-phosphate dehydrogenase, ferridoxin and malate dehydrogenase. Amino acids such as alanine, threonine and arginine were found more often in
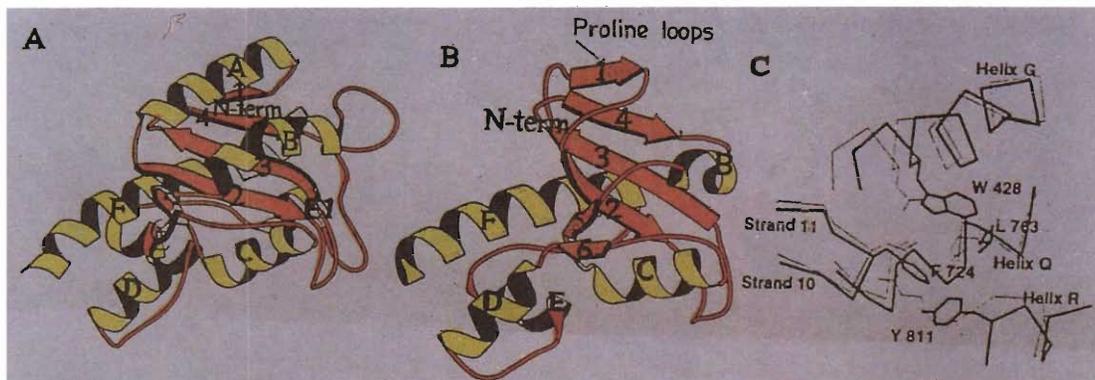
Free energy change (ΔG) between native and denatured protein is defined as $\Delta G = \Delta H - T\Delta S$. Decrease in enthalpy arises mainly due to disulfide bonds, hydrogen bonds, electrostatic and Van der Waals interactions while increase in entropy is largely due to hydrophobic interactions.

Figure 2. Comparison of the structures of Klentaq1 and Klenow pol 1. (A) Small domain of Klenow pol 1, (B) Small domain of Klentaq 1 and (C) Superimposed cluster of aromatic residues in Klentaq1 (thick lines) replacing a cluster of charged residues in Klenow pol 1 (thin lines). Only residues in Klentaq1 are labelled. Amino acids at positions equivalent to trp-428, Phe-724, Leu-763 and Tyr-811 of Klentaq1 are Asn-524, Asp-819, Arg-858 and Arg-909, respectively in Klenow pol 1. Differences between small domains of Klentaq1 and Klenow pol 1 from A & B; a) Helix A of Klenow pol 1 is replaced by proline-rich loop, b) Extensive loop seen between helices E and F has been deleted in Klentaq1, c) Helix F is much shorter in Klentaq1 than Klenow pol 1, and d) The loop between strand 4 and helix B is 10 amino acids shorter and Helix B itself is 6 amino acids shorter than Klenow pol 1. Consequently, N-terminal domain in Klentaq1 is much more compact (40X40X29 A° in Klentaq1 against 50X50X37 A° in Klenow pol 1).

enzymes from thermophiles than in those from mesophiles. Details are presented in *Figure 3*.
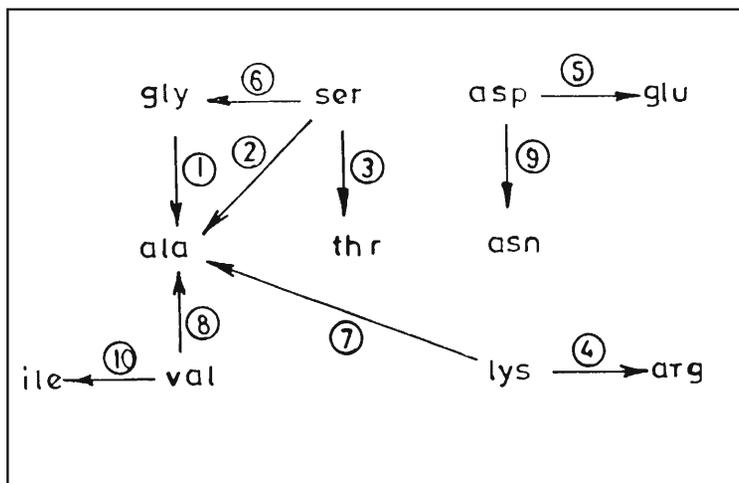

Figure 3. Preferred amino acid changes observed in a comparison between thermophilic and mesophilic proteins. Data is derived from a comparison of DNA polymerase 1, ferridoxin, glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase. Arrows point to the change from the mesophilic to the thermophilic proteins. Number indicates the preference for the given amino acid exchange.
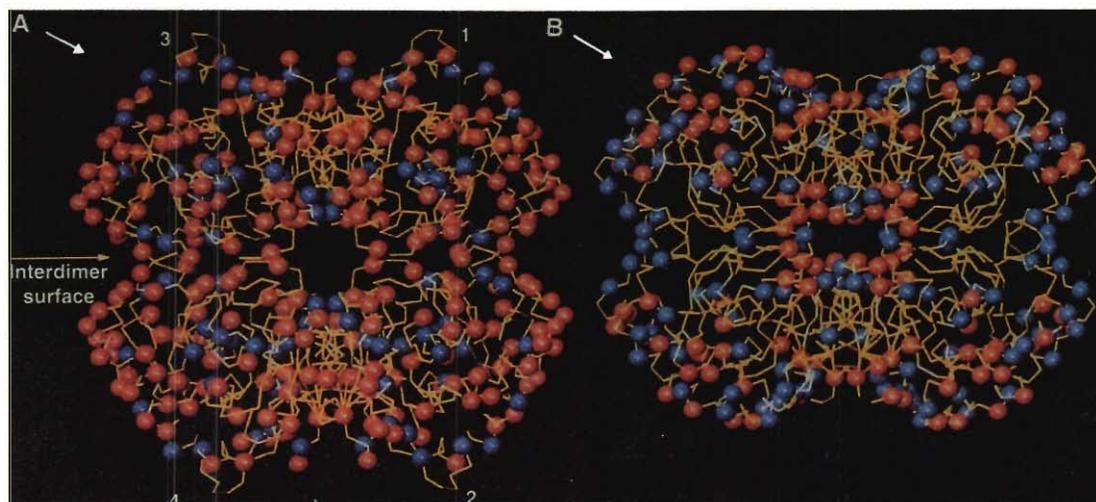
Mesophilic and Halophilic Enzymes: High salt concentration disrupts the structure of water and hence destabilizes enzymes. But it does not happen in the case of enzymes from halophiles. Recently, Sussman and coworkers from Weizmann Institute of Science, Israel, isolated halophilic malate dehydrogenase (hMDH) from Archaebacterium (*Haloarcula marismortui*) and compared its structural features with a mesophilic counterpart, dogfish malate dehydrogenase (dfMDH). It was observed that an excess of acidic residues was distributed on the enzyme surface (*Figure 4*) and more salt bridges[2] were present in hMDH; 15 intramolecular and 8 intermolecular as compared to its mesophilic counterpart, where 1-3 intramolecular and no intermolecular salt bridges were observed. Other structural features that contribute to the stabilization of hMDH are the incorporation of alanine into helices and the introduction of negatively charged amino acids near the amino termini, both of which stabilize the α-helix as a result of interaction with the positive part of the α-helix dipole

[2] Salt bridge is formed due to the presence of an array of positive and negative amino residues at the surface and to some extent in the interior of the protein.

Based on the understanding of physical forces and selectivity of the amino acid residues in extremozymes, it is now possible for researchers to engineer industrially/academically important enzymes. Simultaneously there are efforts to isolate extremozymes from natural sources.

Sturdy Enzymes – Nature's Success

Many micro-organisms live under extreme conditions of heat (100–120° C, deep sea vent) cold (0–4°, sea ice), salinity (>3 M NaCl, soda lake), acidity (below pH 1, sulphur springs) and alkalinity (above pH 10, soda lake) from which extremozymes have been isolated. F C Lowyer of the California Institute of Technology, USA, isolated a thermophilic enzyme called Taq polymerase from heat loving bacteria, *Thermus aquaticus.* This is the most successful example of an extremozyme. It catalyzes the polymerization of DNA at temperatures upto 90° C *in vitro* and is used in a well known recombinant DNA technique called 'polymerase chain reaction' (PCR). This technique is used to amplify specific regions of DNA from a study sample using appropriate primers, short stretches of nucleotides complementary to the gene of interest which are needed for the extension of the DNA sequence by the polymerase. Yet another thermostable enzyme, vent polymerase, functions at greater than 100° C and has been isolated from a micro-organism of the deep sea vent. Other extremozymes have also been isolated from thermophilic (*Methanopyrus kandleri*), psychrophilic (*Polaromonas vacuolata*), alkalinophilic (*Nitronobacteria gregoryi*), acidophilic (*Sulpholobus acidocaldarius*) and halophilic (*Haloferax volcanii*) organisms.

Designing Extremozymes by Enzyme Engineering

Several biochemical and/or genetic approaches have been successfully utilized to enhance the stability of the enzymes towards heat.

Site Directed Mutagenesis[3] is one of the best approaches to engineer enzymes based on the previous knowledge of similar enzymes from an extremophile. Crystal structure of the enzyme provides clues to change certain amino acids which may confer stability in terms of additional disulfide bond, hydrophobicity and salt bridges. Appropriate changes are introduced at the desired nucleotides on a cloned copy of the gene. Recently

[3] Site directed mutagenesis is a technique to substitute a required amino acid for pre-existing amino acid at the gene level. There are several methods to bring about such a change.

Figure 5. Ribbon drawing of Lactobacillus casei thymidylate synthase viewed perpendicular to the 2-fold axis. The modelled disulfide bonds between 155 & 188' and 188 & 155' are indicated at the top (courtesy P Balaram).

P Balaram and coworkers from Indian Institute of Science, Bangalore, India have engineered two disulfide bridges across the dimer interface of thymidylate synthase that brings two subunits in close proximity and stabilizes them (*Figure 5*).

The engineered enzyme is able to function at 55°C with its original rate of catalysis and is stable even at 70°C. Site directed mutagenesis has been extensively used to engineer disulphide bonds in the case of T_4 lysozyme. Similar approach has also been used to change single/multiple amino acids in order to stabilize proteins like T_4 lysozyme, λ-repressor, proteinases like barnase I and barnase II.

Random mutagenesis is a strategy used to make random mutations by UV radiation or chemical mutagens and select for mutants with altered properties. It does not require the previous knowledge of the enzymes. Thermostable versions of enzymes like T_4 lysozyme, staphylococcal nuclease and λ-repressor have been isolated by using this technique.

Covalent modification is a process in which a chemically reactive component is attached covalently to the reactive side chain of an amino acid in the enzyme. Considerable stabilization has been achieved using covalent modifiers like carbohydrates, lipids, alkyl and aromatic residues. This technique has been used to stabilize amylases, lysozymes, ribonucleases and lipases. For instance, glutaraldehyde has been used to stabilize amylase.

Multiple covalent immobilization or cross linking: In this technique, the enzyme is immobilized on the polymer surface either covalently or through physical interaction(s). This technique has resulted in an impressive increase in stability. For example, immobilized trypsin on polyethylglycol (PEG) is 1000 times more stable than the soluble form, and immobilized lipase on PEG is 140 times more stable than the soluble form.

Protein-protein non-covalent associations: Subtilisin thermostability has been increased by 20°C by making a complex with its inhibitor (a protein molecule). The enhanced thermostability is due to increased hydrophobic interactions.

Antibody binding: In lysozyme-antibody interaction, three salt-links, 10 hydrogen bonds and 74 Van der Waals interactions are established to thermostabilize lysozyme. The thermostability of α-amylase, glucoamylase and subtilisin have also been increased substantially by this approach.

Organic Solvents[4]: Usually enzymes do not function in organic solvents. Dordick, from the University of Iowa, has isolated enzymes from halophilic and mesophilic bacteria and converted them into extremozymes. These extremozymes function in a variety of organic solvents and are thermostable. Enzymes isolated from halophiles and mesophiles were freeze-dried in the presence of high salt concentration and then the powdered form of freeze-dried enzymes were added into organic solvents which showed considerable activity. Salt dehydrates the enzyme and sticks to it, shielding it from water. A sufficient amount of water is present to retain suitable charge distribution at the active site and the conformation of the enzyme. In organic solvents the thermostability was also increased due to the rigid nature of enzymes. Some industrially important enzymes like lipases, proteases, lysozymes and ribonuclease have also been made to function in a variety of organic solvents.

Extremozymes have been envisaged to have tremendous applications in industries and academia in the years to come.

[4] Enzymatic reactions in organic solvents reduce the rate of microbial growth, enhance thermostability, control the substrate specificities and shift the reaction equilibrium.
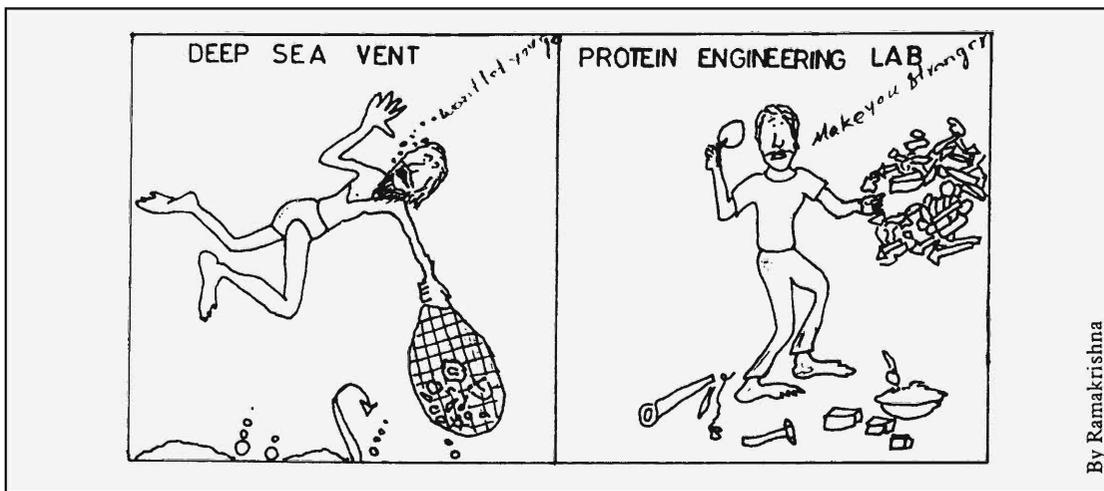
Thus the lessons learnt from natural extremozymes can be effectively utilized to end the long agonising hours that enzymologists spend in the cold room both at the laboratories and the industries.

Suggested Reading

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