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Mechanisms and Applications of Enzymatic Thermostability

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Imagine a hot summer day. What happens when the temperature climbs to a point above 100° F (38° C)? Depending on the place and the typical weather found in that location, reactions vary. Ice cream sales will probably go up. People may curse global warming as they seek shade. Others will head for the beach or community pool. Most forms of life, including human beings, aren't accustomed to that level of heat.

One special group of microorganisms, however, would find 100° F heat downright chilly. They are far more comfortable in temperatures nearly three times as warm as the hottest summer day. These thermophilic creatures live mostly in aqueous habitats near 70-80° C, yet a few specimens thrive in water at temperatures above the boiling point. How any living thing could continue to function under these conditions is a mystery, as most enzymes and other proteins necessary for life are denatured and/or destroyed at such high temperatures. Obviously, some stabilizing structural feature unique to thermophilic proteins must be the key to survival for the thermophilic organisms.

The race is on in the world of microbiologists to determine what mechanism(s) offer the proteins of thermophilic microorganisms such unsurpassed heat stability. Other scientists are researching the helpful roles that these creatures could have in scientific applications. Few contestants compete in this race, however, due to the technical difficulties encountered in studying these organisms while continuing to give them the proper environment. Nevertheless, in spite of sparse research and few publications devoted to thermophilic microorganisms, these creatures are slowly finding a niche in research and industrial applications, both as catalysts and as models for mechanisms of protein stability.

Background

Overview of Characteristics

Like most scientific conventions, the taxonomic system of naming living things is under constant revision. Previously, bacteria and archaea were grouped together in prokaryotic Kingdom Monera; some publications still use this classification. Carl Woese at the University of Illinois suggested a new classification scheme, which places the domain above the kingdom in the taxonomic hierarchy. Microorganisms today are segregated into three domains: bacteria (eubacteria), archaea (archaebacteria) and eukarya (eukaryotes). The new system reflects the fact that archaea are more closely related to eukaryotes than to the other domain of prokaryotes, the eubacteria (Campbell, 1993). Despite differences in evolutionary origin, however, both the archaea and bacteria domains contain members with the amazing ability to survive at incredibly high temperatures. The title "thermophile" applies to all organisms with optimal biological temperatures above 50° C (Kristjansson, 1992). Microorganisms that prefer heat above and beyond 80-85° C are referred to as hyperthermophiles.

Members of the domain archaea are more commonly known for their odd methods of metabolism and bizarre choices of environment than for their thermostability. For example, many sulfur and methane-metabolizing microorganisms belong to the archaea. Most of the archaea are obligate anaerobes, deriving their energy from organic compounds, sulfur or hydrogen, instead of oxygen gas (Brock, 1994). Halophiles are archaea that live in areas of extreme salinity. The Dead Sea, which holds water ten times more salty than seawater, contains few organisms other than halophiles (Brock, 1994). Whatever their other requirements, however, most archaea share the common desire for a hot environment. With the exception of the halophiles, all archaea are hyperthermophilic and seek out environments with temperatures

well in excess of 90° C (Kristjansson, 1992). Some grow at low pH and are called hyperacidothermophiles. With their strange mixture of unique characteristics, the archaea live in the harshest environments on earth. Special proteins in the archaeal cell wall and cytosol suggest that archaea could possibly survive in temperatures up to 150° C (Brock, 1994).

Overall, there are fewer hyperthermophilic eubacteria than hypothermophilic archaea. Species of *Thermotoga* are the only true hyperthermophiles, as they may be found in temperatures up to 90° C (Kristjansson, 1992). Most eubacterial species in hot environments are only thermophilic, with temperature optima around 50-60° C. Some bacteria, however, cheat death from high heat by forming tough, heat-resistant outer coatings called endospores (Brock, 1994). No archaea species have this ability. Even for the eubacteria, however, endospores are only a temporary emergency maneuver for the eubacterium suddenly exposed to high heat. Eubacteria cannot use their endospore-forming skills to live permanently at hypothermophilic temperatures. More eubacteria than archaea are aerobic, probably because oxygen more readily dissolves in water at the moderately warm temperatures of thermophilic bacteria than the blazingly hot environments of most archaea. As in the archaea, metabolism in the eubacteria varies. The eubacterial domain contains photosynthetic members as well as chemoautotrophs that digest compounds of sulfur, hydrogen, or iron (Kristjansson, 1992).

Habitat

Thermophilic bacteria colonize any environment with the proper combination of extreme heat, pH and metabolic precursors. Numerous species of thermophilic eubacteria and archaea thrive in hot springs, such as those found in Yellowstone National Park. Many more species live in the depths of the ocean, near hydrothermal vents. Species from the bottom of the ocean are typically hyperthermophiles, as the combination of geothermal heat and intense pressures in

these areas allow water temperatures to exceed 100° C. A few thermophiles with less stringent heat requirements live a little closer to the ocean's surface (Zimmer, 1995). Other habitats for thermophilic microorganisms include volcanic sediments, the Dead Sea, and the Great Salt Lake (Adams & Kelly, 1995). Slightly unorthodox thermophiles settle in outflows from geothermal power plants or home hot water heaters (Borman, 1991).

History

Since the early part of this century, scientific curiosity regarding thermophilic microorganisms has made them the subjects of much research. *Clostridium thermocellum*, thermophiles which belong to the same species as the microorganisms that cause botulism and tetanus, have been subjects of study since their discovery in 1926 (Brock, 1994). Due to its useful applications, *Thermus aquaticus* is probably the most heavily scrutinized thermophilic microorganism to date. In 1969, Thomas Brock and Jim Brierly discovered *Thermus aquaticus* in the hot springs of Yellowstone National park (Borman, 1991). Today's molecular biologists know *Thermus aquaticus* well, as they use its DNA polymerase I in a nucleic acid amplification technique called the Polymerase Chain Reaction (PCR). Advances in undersea exploration within the last two decades have brought exponential growth in the discovery of new thermophilic species. State-of-the-art submersibles, such as the Alvin (U.S.) and the Cyana (France), routinely collect samples of thermophilic microorganisms 2 or 3 miles below the ocean's surface (Borman, 1991). Thanks to these slightly dangerous submarine missions, fifty percent of known thermophilic bacteria and eighty percent of known archaea were discovered in the last twenty-five years (Kristjansson, 1992).

Even as more and more species of thermophilic microorganisms were caught and categorized in the last few decades, new archaeal species were usually dismissed as unique

eubacterial freaks-of-nature. Biologists lumped archaea together with all other microorganisms in Kingdom Monera. Many current publications still fail to cleanly separate the archaea from the thermophilic eubacteria. Some authors, however, quite correctly remind readers that archaea and thermophilic eubacteria have little in common other than high optimal growth temperatures. The Yellowstone Hot Springs show "head-spinning" levels of archaeal diversity, indicating that archaea may be the most common taxonomic class on the planet, certainly more common than the eubacteria (Zimmer, 1995). Studies which say that archaea may be the "dominant" type of microbe in deep ocean water support this conclusion, since the ocean covers more than half of the earth's surface (Zimmer, 1995).

The members of the archaeal domain were probably on the evolutionary scene long before the eubacteria began to appear. Ribosomal RNA comparisons of archaea and ancient species indicate that archaea first appeared on Earth 3.5 billion years ago and have changed very little since their arrival (Zimmer, 1995). In fact, some scientists believe that archaea resemble the "primordial creatures" representing the first life on earth. If the ribosomal RNA evidence is valid, one could argue that archaea and thermophilic eubacteria are not closely related at all. In fact, the archaebacteria and eukaryotes probably split from a common ancestor long after the eubacteria diverged from the first ancestor of all life and evolved in new directions (Apenzeller, 1997). Proof for the close family ties between eukarya and archaea also emanates from studies showing that archaeal RNA polymerase mirrors certain forms of eukaryotic RNA polymerase. Also, archaeal genes contain introns, which are found in eukaryotes but not eubacteria. Douglas Clark of the University of California at Berkley sums up this whole argument by saying, "thermophilic [archaea] haven't adapted to a hot environment, [but] all the other organisms have adapted to a low temperature environment" (Borman, 1991).

Protein Stability in Thermophilic Organisms

It is a wonder that thermophilic microorganisms can even survive, much less thrive, at temperatures at or near the boiling point. The majority of biomolecules cannot exist in these conditions. Most enzymes, for example, are highly thermolabile and become irreversibly inactivated by any one of several mechanisms at temperatures at and above 40-50° C. These mechanisms include deamidation of asparagine residues, hydrolysis of peptide bonds near aspartic acid residues, destruction of cystine residues, and the formation of incorrect structures (Ahern & Klibanov, 1985). Irreversible inactivation should not be confused with reversible inactivation. In the reversible type, slight alterations in folding occur in the protein structure, and the enzyme returns to its normal appearance after the temperature is lowered. With regard to irreversible inactivation, the changes are permanent. These alterations include covalent changes, in which the enzyme is chemically altered, and/or noncovalent changes that lead to aggregated or incorrectly folded enzyme (Figure 1).

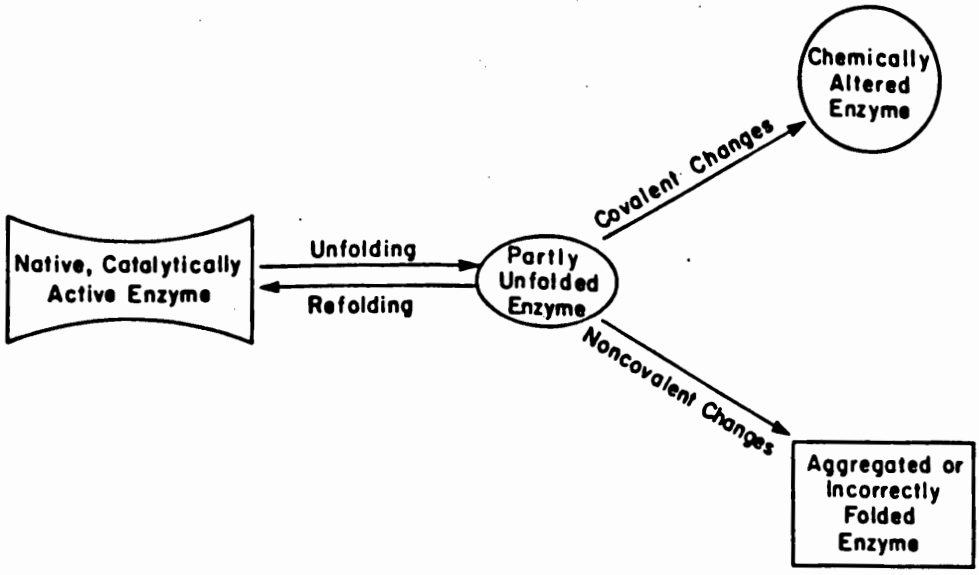


Fig.1. Diagram of the events in thermal enzymatic inactivation. (Klibanov, 1983.)

Irreversibly inactivated enzymes exist in abnormal conformations and cannot carry out their normal duties of catalysis, since substrate recognition depends upon the shape of the enzyme's active site. Since so many biological processes depend on enzymatic catalysis, how these thermophilic microorganisms protect their proteins from denaturation at such high temperatures is still largely a mystery. The mystery is intensified by the fact that thermophilic and mesophilic enzymes are typically quite similar in every respect except for a few minor structural details. The formation of these structural variances, such as hydrogen bonds and electrostatic interactions, releases energy. This benefits the protein's stability because objects that have lower energy and entropy are more stable; the energy release brings the protein from a high energy, high entropy state to a lower, more stable state. In the formation of these structures, however, the protein does not use more than twenty kJ/mol of energy. Mesophilic and thermophilic proteins, therefore, do not differ by more than thirty to sixty kJ/mol of energy (Cowan, 1995). For a protein molecule with a total energy on the order of several thousand kJ/mol, this reduction in energy is only a drop in the bucket. The true mystery, then is how the energies of mesophilic and thermophilic enzymes differ by only a few dozen kJ/mol, yet thermophilic enzymes have the amazing stability that would be expected from a molecule with a much lower amount of total energy. Several proposed solutions to the mystery of protein stability in thermophilic microorganisms have already been tested or currently await further comparison with experimental data.

Mechanism #1: Polypeptide Chain Alterations

Although the mesophilic and thermophilic versions of an enzyme catalyze the same reaction and typically possess similar structures, they unquestionably are not identical molecules. Thermophilic and mesophilic counterparts often have essentially the same three-dimensional

structure, yet only thirty to forty percent of the amino acid sequences match (Adams & Kelly, 1995). Some researchers believe that these differences in amino acid composition play the largest role in providing enzymatic thermostability. As proof for this belief, researchers cite examples of common amino acid substitutions that occur in several thermophilic microorganisms. Certain amino acids that destabilize the protein at high temperatures must be replaced with non-temperature-sensitive amino acids in order to give proteins thermostability. However, there is a catch to this hypothesis. At this point in time, few scientists completely agree on which amino acid substitutions contribute to the stability of the thermostable enzyme.

Adams and Kelly suggest that the replacement of asparagine and glutamine favors thermostability, as these residues have side chains prone to losing amino groups at higher temperatures. This loss detrimentally changes the structure of the protein due to sudden increases in negative charge (Adams & Kelly, 1995). D. A. Cowan agrees that amino group loss poses a problem for protein structure. In Cowan's model, however, only asparagine and not glutamine residues are replaced at non-catalytic positions with increasing temperature. Cowan also asserts that asparagine residues are not replaced because they contribute to negative charge build-up, as previously suggested, but because this negative charge can attack and cleave adjacent peptide linkages via a cyclic imide intermediate (Cowan, 1995). Additional research indicates that an increase in glutamic acid residues can actually stabilize proteins at higher temperatures by promoting the formation of stable helical structures (Kristjansson, 1992). Since glutamine becomes glutamic acid after losing its amino group, these findings also refute Adams and Kelly's claim that both asparagine and glutamine decrease thermostability.

At high temperatures, proline amino acid residues often replace unstable residues. In fact, Cowan lists an increase in proline residues as a general stabilizing mechanism employed by

most thermostable proteins (Cowan, 1995). A paper documenting the structural mechanisms of thrermostability found in *Thermus aquaticus* DNA polymerase I provides evidence for this idea (Korolev et al., 1995). Experiments in this paper examined the x-ray crystal structures of *Thermus aquaticus* DNA polymerase I and its mesophilic counterpart, *E. coli* DNA polymerase I. According to the authors, alanine to proline substitutions results in increased stability. In fact, a proline-rich loop in the *Thermus aquaticus* enzyme replaces an entire helix found in the Klenow fragment of *E. coli* DNA polymerase I. These added prolines aid in giving the *Thermus aquaticus* enzyme its superior thermostability.

Thermostability depends on more than simple amino acid substitution; the location of the substitution is also critically important. Many substitutions occur at the N-terminus areas of the protein, because the N-terminus is one of the least stable parts of any mesophilic protein exposed to high temperatures. Some attribute this instability to the fact that the N-termini of mesophilic proteins are not incorporated into organized structures and have a tendency to "unzip" under heat stress (Adams, 1993). The rubredoxin found in thermophilic *Pyrococcus furiosus* lacks the standard N-terminal methionine residue and has a few other substitutions around amino acid position 15. These alterations allow the N-terminus to join the hydrogen-bonded network of a nearby beta-sheet. At the high optimal growth temperatures of *Pyrococcus furiosus*, the hydrogen bonds stabilize the N-terminus and keep it from disintegrating. The authors of the *Thermus aquaticus* paper agree that reorganization of the N-terminus adds to the stability of thermophilic enzymes. In their studies of DNA polymerase I from thermophilic *Thermus aquaticus* and mesophilic *Escherichia coli*, they discovered that the N-terminus of the thermostable enzyme has fewer unfavorable electrostatic interactions and an enhanced interface with the large domain when compared to its mesophilic counterpart in *Escherichia coli* (Korolev

et al., 1995). Again, the added stability of the N-terminus contributes to the overall stability of the protein.

Mechanism #2: Bonding

Within the topic of enzyme stabilization mechanisms, amino acid replacement is only the tip of the iceberg. Other stabilization strategies involve several other types of amino acids in complex bonding patterns. All major types of bonding, including covalent disulfide interactions, ionic bonds, hydrogen bonds, and van der Waals bonding play a role in creating the structure of a thermostable enzyme. According to Cowan (1995), the free energy of folding is typically –30 to –60 kJ/mole (or –7 to –15 kcal/mole). The ability of minor structural changes to impart relatively large changes in protein stability can be understood when it is considered that a typical intramolecular interaction contributes between –2 and –20 kJ/mole (-.5 to –5 kcal/mole) of energy.

In 1989, a trio of researchers at the University of Oregon published a paper describing the thermostability attributable to multiple disulfide bonds in a protein's structure (Matsumura et al., 1989). The majority of native proteins possess disulfide bonds that connect different chains. These bonds aid in giving the protein its three-dimensional shape. In the right combination, the position and number of these bonds result in exceptional thermostability. In experiments for the paper, the University of Oregon researchers mutated a disulfide-free enzyme, phage T4 lysozyme. In these manipulations, they added up to three disulfide bridges at specific locations in the lysozyme molecule. By observing all possible combinations of mutations, they found that no single disulfide linkage seemed to have a great effect, but the combination of all three linkages had an additive effect on decreasing entropy. The activity of the enzyme was unaffected by these changes, and the altered protein denatured at temperatures approximately

twenty-three degrees higher than the inactivation temperatures of normal T4 lysozyme. Theoretically, the disulfide bridges increase the thermostability of the protein by reducing the number of conformations possible for the folded enzyme. In effect, they lowered the entropy of the molecule, which corresponds to a decrease in energy and an increase in stability.

Hydrogen and ionic bonding contribute to protein thermostability for many of the same reasons discussed in the disulfide bond paper. Cowan briefly mentions the amount of stability given by these types of bonding (Cowan, 1995). Roy M. Daniel describes the contribution of these bonds in depth. Daniel argues that a few additional salt bridges or hydrogen bonds takes the thermostable enzymes down to a lower, more stable energy state (Daniel, 1985). The authors of the *Thermus aquaticus* DNA polymerase I paper report that three additional ion pairs found in the thermostable enzyme, but not in the mesophilic *Escherichia coli* counterpart, play a vital role in stabilizing that enzyme (Korolev et al., 1995). They are not the only reasons for stability, but they certainly add to stability by reducing the energy of the thermostable form.

Lastly, many thermophilic microorganisms stabilize enzymes by increasing the internal hydrophobic interactions of these proteins. Since an increase in interior hydrophobic interaction draws the core chains closer together, the surface area of the protein decreases as a result. A few different theories attempt to explain why this particular characteristic increases the thermostability of the protein. The simplest explanation states that, as with other types of bonding, additional hydrophobic interactions lower the total energy and entropy of the protein, which increases the stability (Daniel, 1985). Adams and Kelly suggest that smaller surface areas minimize interactions with denaturing solvent particles (Adams & Kelly, 1995). Whatever the explanation, strong support abounds for the idea that changes in hydrophobic interactions and surface area aid in thermostability. Both Peter Privalov (Adams & Kelly, 1995) and Cowan

emphasize the importance of this "molecular packing" to protein stability (Cowan, 1995). Douglas C. Rees at the California Institute of Technology examined the surface area of aldehyde ferredoxin oxidoreductase (AOR) from the thermophile *Pyrococcus furiosus* and found that it is much smaller than he expected for a protein of its size (Adams & Kelly, 1995). Countless other thermophilic proteins follow the same pattern.

Mechanism #3: External Factors

Oddly enough, the third class of mechanisms for enzymatic thermostability does not involve any changes in amino acid composition or protein structure. These mechanisms are completely dependent upon interactions between the protein and outside atoms or molecules. One recent paper documents the thermostability imparted by the disaccharide trehalose to proteins that are normally denatured at high temperatures (Carninci et al., 1998). The authors offer several explanations for the incredible stabilizing abilities of trehalose. The "glass state theory" postulates that the trehalose molecules enter a quasi-glass-like state in the presence of heat. All molecular motion is slowed in this fluid, including the rate of protein degradation by solute molecules. The breakdown of thermolabile enzymes still occurs, but at a rate far below normal. A second theory states that trehalose replaces water molecules in protein surface hydrogen bonds. The trehalose bonds are more stable than the water bonds, and share this stability with the protein. The problem with these theories, however, is that saccharides that should behave like trehalose offer little thermostability to proteins when subjected to the same heat extremes. Trehalose must have additional stabilizing features that are currently unknown.

Tungsten atoms also star in thermostabilization schemes. As previously mentioned, *Pyrococcus furiosus* AOR acquires stability through internal hydrophobic interactions; tungsten cofactors, however, also add to the thermostability of AOR (Chan et al., 1995). Thermostable

AOR contains two subunits, each holding a molybdopterin molecule that coordinates with a tungsten cofactor. As in other stabilizing mechanisms, the tungsten interaction minimizes the surface area of the protein, reduces its entropy, and leads it to a lower, more stable energy state. This interaction is of utmost importance for *Pyrococcus furiosus* specimens, which absolutely require tungsten in the environment for survival. The researchers that discovered this mechanism are not convinced, however, that the tungsten method will prove to be a common means of thermostability in thermophilic organisms as a whole.

My Research Project

For my undergraduate research project at Western Washington University, I also entertained questions of protein stability. The work was based upon the research of another student, Matt Kaeberlein, who measured the rate of irreversible inactivation of mesophilic and thermophilic glyceraldehyde-3-phosphate dehydrogenase (GAPD) dissolved in solvents of varying ionic strength and composition. Mesophilic GAPDs were destabilized in the presence of NaCl, KCl, and NH_4Cl, whereas the thermophilic enzymes were not significantly destabilized by any of the salts tested. These results show that the chemical structure of the salt and not just the ionic strength are important for thermostability. In the concluding remarks of his thesis, he suggested that a future student could determine the mechanism(s) involved in this irreversible inactivation (Kaeberlein, 1997).

Since I already had interest in the biochemistry of thermophilic microorganisms when I began working with Dr. Sal Russo, my research adviser, I chose to research the problem proposed by Matt Kaeberlein. Before I started work on the project, Dr. Russo and I examined the four main mechanisms of irreversible inactivation, as printed in a paper by Tim Ahern and Alexander Klibanov (Ahern & Klibanov, 1985). Since the deamidation of asparagine residues

appeared to be the most common mechanism, I intended to prove whether this same mechanism had a role in destabilizing mesophilic and thermophilic GAPD. My research, however, did not single out asparagine as the only source of deamidation. In certain systems, such as the lysozyme system investigated by Ahern and Kilbanov, only asparagine residues lose their amino groups at high temperatures (Ahern & Klibanov, 1985). Whether or not glutamine joins asparagine in releasing its amino group depends up on the reaction conditions and the unique properties of the molecule in question (Robinson & Rudd, 1974). Since GAPD had not been examined for this inactivation mechanism before, I did not know if glutamine would be involved or not. Therefore, I proceeded under the guise that both asparagine and glutamine were involved in irreversible inactivation.

I began my research in January of 1998 and compiled the data for a Chemistry Department seminar in May of 1998. My main goal was to obtain evidence for deamidation during the thermal inactivation of GAPD. To denature the GAPD, I placed 1 ml of a 2 mg/ml chicken GAPD solution in several sealed glass tubes. The buffer for this solution was 0.015 M sodium pyrophospate and 0.03 M sodium arsenate at pH 8.5. These tubes were heated at 100° C for 0, 20, 40, 60, 80, or 100 minutes. When each tube had cooled, I poured the solution out of the tube and removed the denatured proteins by filtering with a Millipore Centricon-30 protein concentrator. After filtration, ten microliters of the solution were diluted to 1 ml with phosphate/arsenate buffer and put aside for a kinetics assay. The remainder of the milliliter was used in an assay for ammonium ions in solution.

The spectrophotometric kinetics assay for GAPD activity simply tests whether or not the molecules of GAPD in a given solution can perform their normal catalytic duties. The conditions inside the spectrophotometric cuvet mimic the environment inside the cell; a water

cycling system maintains a constant temperature of 25°C and the phosphate/arsenate buffer keeps the system at a constant pH. The prepared cuvet contains 2.6 ml of additional phosphate/arsenate buffer, 0.1 ml of 7.5 mM NAD^+ (a cofactor), 0.1 ml of 0.1 M dithithreitol, and the 1 ml of enzyme solution previously set aside. At the start of the kinetics run, glyceraldehyde-3-phosphate (the substrate) is added to the cuvet and the spectrophotometer begins to take A_{340} measurements every two seconds for a duration of five minutes. Any active enzyme present catalyzes the following reaction:

glyceraldehyde-3-phosphate + NAD^+ + P_i → 1,3-diphosphglycerate + NADH + H^+

The A_{340} measurements change during the kinetics run because NADH absorbs at that wavelength, whereas NAD^+ does not. The absorbance measurements rise as the reaction proceeds. When my samples were tested, I found that the sample heated for 0 minutes was the only one with activity (Figure 2). Since they lost the ability to catalyze the reaction, all of the other samples were irreversibly inactivated by heating and have a kinetic rate of zero on the graph.

The assay for detection of ammonium ions depends upon the spectrophotometric absorbance of hypobromite at 330 nm (Howell & Boltz, 1964). In preparing solutions for the assay, ammonium is reacted with hypobromite to produce bromide ions. The equation for this reaction is as follows:

$$2NH_3 + 3BrO^- \rightarrow N_2 + 3Br^- + 3H_2O$$

Hypobromite ions absorb light at 330 nm, while bromide ions cannot absorb at this wavelength.

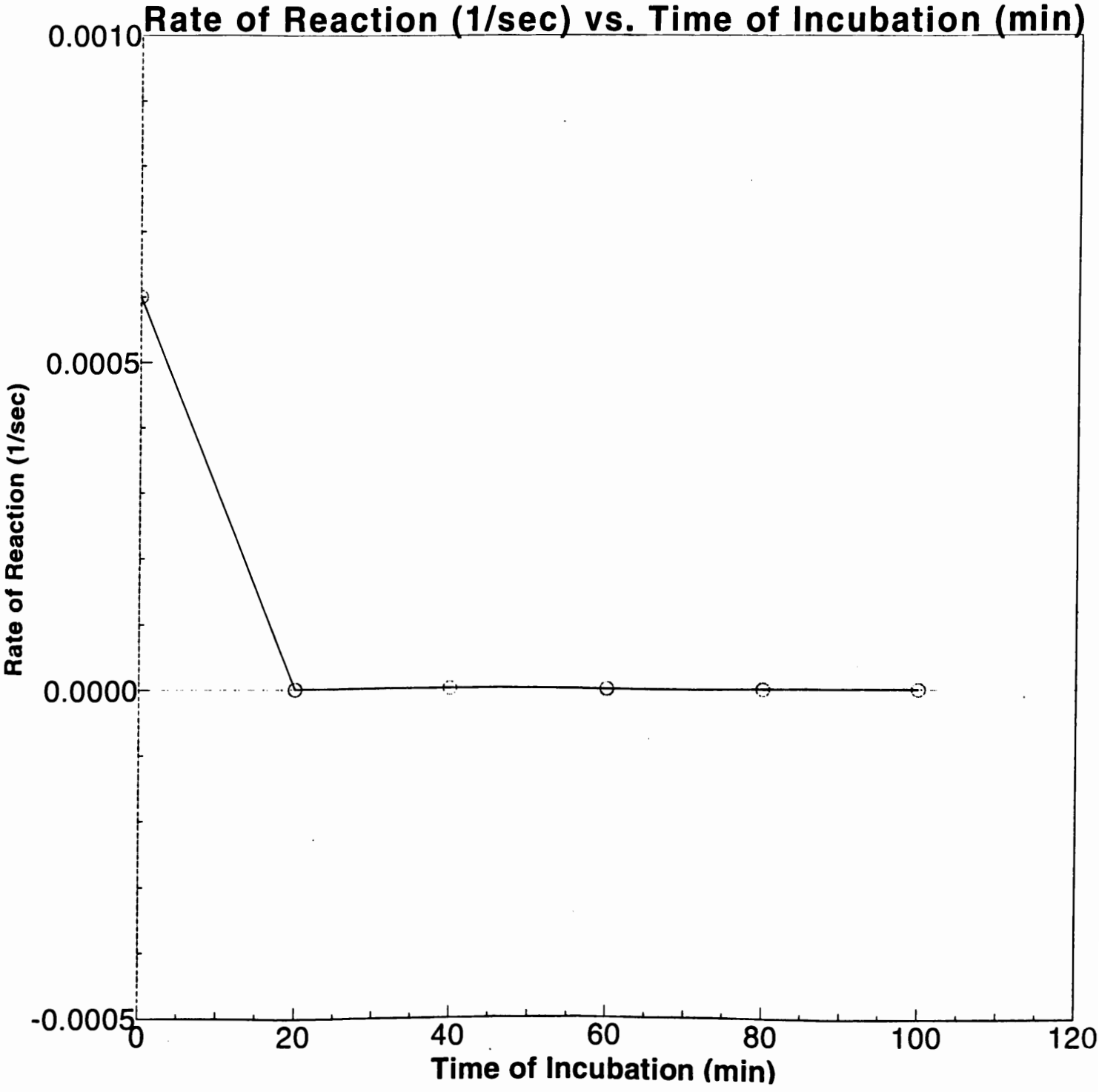


Figure 2. Comparison of kinetics measurements and time of incubation at 100°C. All enzyme samples that were heated for more than 20 minutes showed no activity.

The blank solution in this assay contains unreacted hypobromite and ammonia-free water. The same quantity of hypobromite is added to all samples containing ammonium ions. Since the hypobromite disappears and bromide is produced, high levels of ammonium correspond to low absorbance levels at 330 nm. Before any proteins were analyzed, I produced a standard curve by performing the reaction with known quantities of NH_4Cl documenting the decreasing absorbance values (Figure 3). Under heat stress, deamidation appears to be a mechanism of irreversible inactivation in GAPD solutions. As the time of inclubation increases, the levels of excess ammonium ions in solution increase and the A_{340} measurements fall (Figure 4).

Unfortunately, the spectrophotometric method does not ideally suit this application, as I often see peaks of interference around 260-280 nm on the spectrophotometric reports. I am not entirely sure where this interference is coming from. The wavelength indicates that a contaminating polypeptide may be causing the erroneous peaks, as proteins absorb in this area. The filtering step should remove all intact molecules of GAPD; therefore, this contamination may come from small pieces of polypeptide that broke off from GAPD in the heating step. If future students choose to continue this work, they may decide to view this breakage as a possible inactivation mechanism. They may also choose another assay for ammonium ions to circumvent the interference problems encountered in my method. Dr. Russo has suggested using an amino acid analyzer, which can detect free ammonium, in this capacity.

Applications

Unanswered questions in the research on thermophilic bacteria are too numerous to count. In spite of the enormous research opportunities in this field, however, few scientists are accepting the task of answering these questions. This lack of participation is mainly due to the

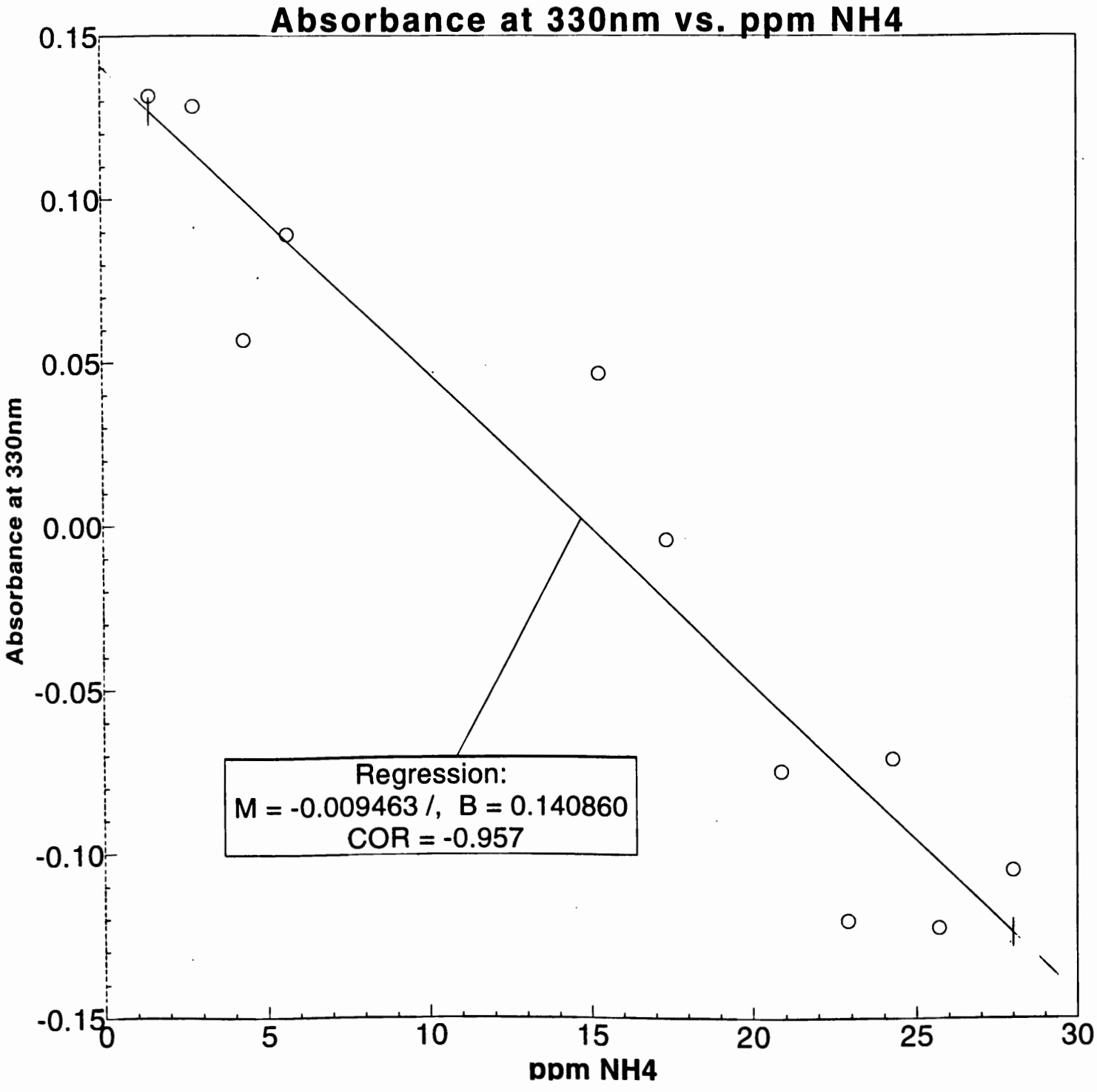


Figure 3. Standard curve for spectrophotometric ammonium assay.

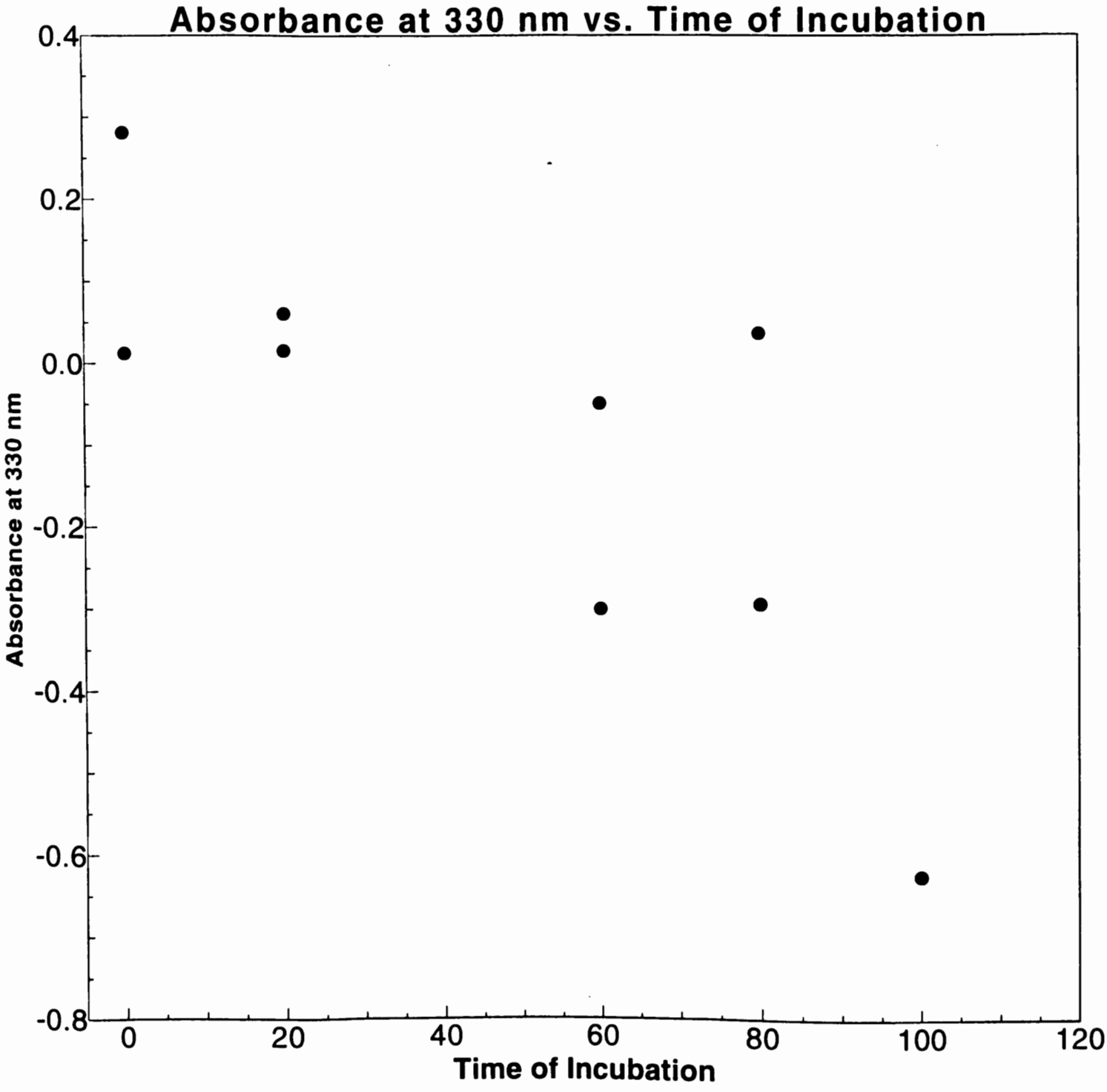


Figure 4. Graph comparing the A_{330} of ammonium solutions derived from denatured GAPD solutions with the time of incubation at 100°C. The trend in the data points reveals that ammonium release increases with increasing incubation.

fact that thermophilic microorganisms are notoriously difficult to work with in large-scale cultures (Borman, 1991). Researchers often must build a special apparatus to contain the organism if it is of a species that must grow under conditions of low pH, high pressure, or high salinity. For example, some thermophilic microorganisms produce metabolic byproducts that corrode standard stainless steel fermentors; special fermentors made of noncorrosive glass must be used to contain them (Adams & Kelly 1995). With regard to the species that grow easily in standard laboratory conditions, researchers can still hit roadblocks in attempting to document enzymatic features. Activity assays are often very difficult, as standard laboratory reagents are often destroyed at the high temperatures favored by thermophilic enzymes, or equipment is not built to handle these temperatures. Some researchers have attempted to circumvent these problems by genetically altering mesophilic *Escherichia coli* so that it will express the proteins normally made by a thermophilic species (Borman, 1991). With *Thermotoga* and *Pyrococcus*, two thermophilic species that grow at temperatures below 100° C, scientists have had a limited amount of success in this task. For example, Reinhard Hensel of the Max Planck Institute successfully expressed the gene for glyceraldehyde-3-phosphate dehydrogenase from a *Pyrococcus* species in a strain of *Escherichia coli* (Adams & Kelly, 1995). Others, however, have had problems expressing genes from hyperthermophiles that live above 100° C. Occasionally, the thermostable protein needs to be incubated at high temperatures before it is activated. In other cases, the recombinant protein has a much higher molecular weight than the native thermostable protein, suggesting that the recombinant protein is actually an aggregate (Adams & Kelly, 1995).

Until the beginning of this decade, the Polymerase Chain Reaction, which utilizes DNA polymerase from *Thermus aquaticus*, was considered to be the "only commercial use of enzymes

from thermophilic bacteria" (Borman, 1991). Although PCR may still be the main use for thermostable proteins, new applications for these molecules are being studied every day. If someone does not already have a patent for a new commercial application for thermostable enzymes, someone will in the very near future.

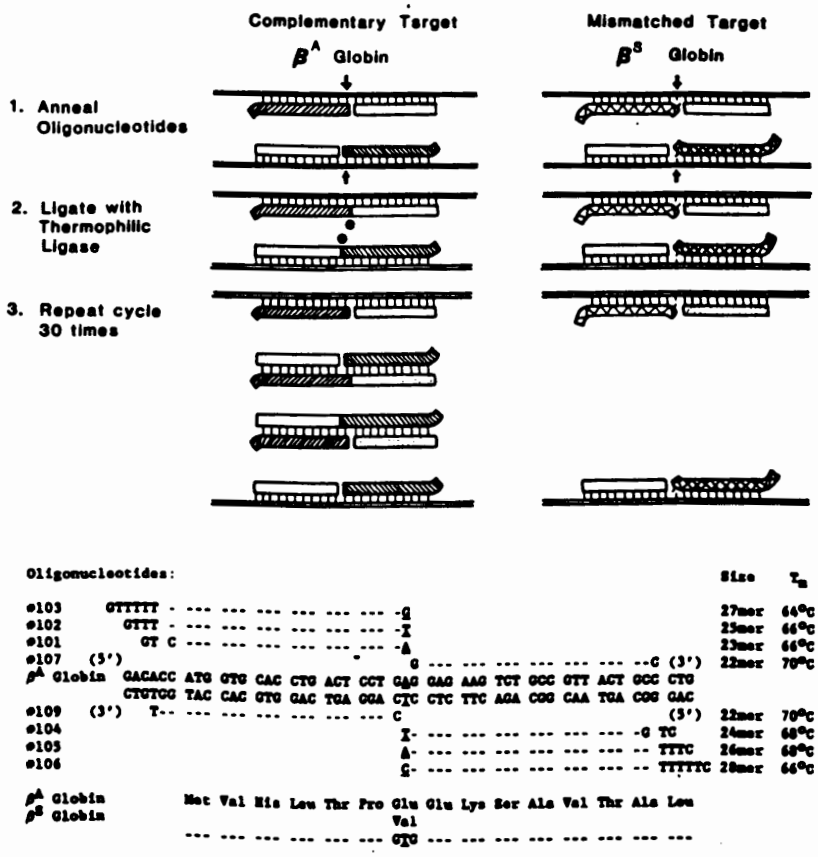
The PCR method revolutionized the world of genetics by providing a simple, quick method for amplifying any portion of DNA for genetic analysis. This process has three basic steps. First, the DNA sample is heated to a temperature around 90° C to separate the double helix into two single strands. A lower annealing temperature allows primers specific for the section of DNA to be amplified to bind to the DNA at the proper location. An elongation step at a slightly higher temperature then allows the DNA polymerase in solution to elongate the DNA double helix at the sites where primers have been added. The cycle then repeats, beginning at the DNA denaturing step, several dozen more times. Mesophilic DNA polymerase could be used for this application, but since it becomes irreversibly inactivated at the 90° C temperature, new enzyme would have to be added with each cycle. DNA polymerase from *Thermus aquaticus* eliminates this need to add fresh enzyme with each cycle because it can survive the high temperatures.

The flaw in the traditional PCR method stems from the fact that the enzyme from *Thermus aquaticus*, unlike other mesophilic DNA polymerases, has no proofreading function. Most DNA polymerases sense mismatches in the growing double strand and cannot continue to the next template base until they have corrected the mistake. *Thermus aquaticus* DNA polymerase lacks this ability and continues chain elongation whether or not the newest addition to the chain is correct. After the completion of the PCR process, the product DNA sequences must be inspected to exclude those that contain errors. Recent discoveries in protein

thermostability could eliminate this final step from the PCR process or replace PCR with a new, mistake-proof method of DNA amplification.

One new method for DNA amplification avoids the mistakes in traditional PCR by replacing *Thermus aquaticus* DNA polymerase with an entirely different thermostable enzyme. A recent publication from Francis Barany at Cornell University Medical College describes an amplification reaction that uses thermostable DNA ligase instead of DNA polymerase. Enzymes from *Thermus aquaticus* still command the spotlight in this reaction, as the DNA ligase in Barany's reaction originates from cells of this species. Instead of building the DNA clones from two pieces of template DNA, as in traditional PCR, Barany's method introduces into solution multiple copies of pre-synthesized, single-stranded oligonucleotides complementary to sections of the desired DNA sequence, as well as a few copies of the target DNA. The oligonucleotides anneal to the DNA target in solution, and the *Thermus aquaticus* DNA ligase seals the breaks between oligonucleotides to produce a double-stranded DNA molecule. The reaction mix is heat denatured at 94° C, which splits the double helix in two and makes the newly joined oligonucleotides target DNA for the next cycle. Theoretically, there are no mistakes in the product because DNA ligase cannot seal mismatched oligonucleotides (Figure 5). Any mismatches denature with the heating step, freeing the individual oligonucleotides to form correct matches in the next cycle (Barany, 1991). Barany's method certainly appears to be an excellent method for exponential reproduction of target DNA, but it remains to be seen if geneticists and molecular biologists accustomed to PCR will switch to this new method.

Another alternative to PCR circumvents the mistakes of the traditional method simply by using the mesophilic DNA polymerases that have proofreading abilities. Of course, using mesophilic enzymes resurrects the original problems at the heart of *in vitro* DNA amplification.



(*Upper*) Diagram depicting DNA amplification/detection by using LCR. DNA is heat denatured, and four complementary oligonucleotides are hybridized to the target at a temperature near their melting temperature (65°C; t_m). Thermostable ligase will covalently attach only adjacent oligonucleotides that are perfectly complementary to the target (*Left*). Products from one round of ligations become targets for the next round, and thus products increase exponentially. Oligonucleotides containing a single-base mismatch at the junction do not ligate efficiently and, therefore, do not amplify product (*Right*). (*Lower*) Nucleotide sequence and corresponding translated sequence of the oligonucleotides used in detecting β^A- and β^S-globin genes. Oligonucleotides 101 and 104 detect the β^A target, whereas oligonucleotides 102 and 105 detect the β^S target when ligated to labeled oligonucleotides 107 and 109, respectively. Oligonucleotides 103 and 106 were designed to assay the efficiency of ligation of G–T or G–A and C–A or C–T mismatches when using β^A- or β^S-globin gene targets, respectively. Oligonucleotides have calculated t_m values of 66–70°C (15), just at or slightly above ligation temperature. The diagnostic oligonucleotides (101–106) contained slightly different length tails to facilitate discrimination of various products when separated on polyacrylamide denaturing gel.

Figure 5. Diagrams from Barany DNA ligase amplification paper. (Barany, 1991)

How can mesophilic enzymes function in cycle after cycle of high temperatures, without requiring someone to monitor the reaction and replace denatured enzyme after each cycle? The solution to this problem rests with the publication written by Carninci et al. (1998) on the thermostabilization of enzymes with trehalose. As previously mentioned, trehalose interacts with mesophilic proteins in a currently undetermined way to stabilize these proteins at levels far above their optimum temperatures of activity. Perhaps trehalose could be integrated into the traditional PCR method, where its mission would be to stabilize mesophilic DNA polymerase I at the high temperatures of DNA denaturation. Since mesophilic DNA polymerases have proofreading abilities, the use of these enzymes with trehalose should reduce the number of mistakes in PCR amplification products.

For new research on thermostable enzymes, there is more to life than just PCR. Researchers constantly invent new ways to use PCR methods in new and innovative ways, or dream up applications for thermostable enzymes that have absolutely nothing to do with DNA amplification. The trehalose PCR principles mentioned previously could be used to stabilize reverse transcriptases in the generation of cDNA libraries; these libraries would then be used in DNA sequencing reactions (Carninci et al., 1998). Francis Barany already has used his DNA ligase amplification reaction method to detect sickle cell genotypes in miniscule blood samples (Barany, 1991). Cowan also mentions that Barany's method shows promise for future work in screening for single nucleotide base lesions that cause serious genetic disorders, such as cystic fibrosis (Cowan, 1995). Since gene sequencing and the early detection of genetic diseases are at the heart of many research projects, most notably the Human Genome Project, these new methods can only increase the popularity of thermostable enzymes in genetic research.

A constant stream of new industrial applications for thermostable enzymes also exists in the research world. Most authors warn against thinking of thermostable enzymes as the next Holy Grail of industrial catalysis. Many thermostable enzymes have rates of catalysis comparable to their mesophilic counterparts. Therefore, using a thermophilic enzyme and increasing reaction temperatures does not always reduce the time required for completion of an industrial process (Cowan, 1995). Critics also argue that many industrial processes cannot benefit from high temperature catalysis because the desired product is unstable at high temperatures. Others say that the use of thermostable enzymes will never catch on because too many industries have invested all of their capital in equipment for low-temperature processes.

Nevertheless, skeptics of their usefulness cannot deny that more and more products with thermostable enzymes appear on the market each month; the use of enzymes from thermophilic sources has its advantages. Advertisements for thermostable enzymes praise these products for their ability to function at a wider range of temperatures. Any researcher that has inactivated an expensive tube of enzymes by leaving it out on a laboratory bench top will appreciate the flexibility of thermostable enzymes that remain active at both room and elevated temperatures. In spite of the difficulty of collecting a cultivating enzymes from thermophilic sources, Adams and Kelly maintain that, for "every enzyme on the market right now," there is a "more stable or higher temperature version [that] could be useful in certain situations" (Borman, 1991). Several industries are following this advice and searching for opportunities to use thermostable enzymes in their processes.

Fuel industries, for example, look to thermostable enzymes for many potential applications. Borman writes that hydrogen gas is an important intermediate at many chemical and petrochemical plants and could be produced on an industrial scale by thermostable

hydrogenases (Borman, 1991). Future coal providers may use thermophilic sulfur bacteria to remove sulfur deposits from coal (Borman, 1991). Desulfurized coal would burn more cleanly, releasing smaller amounts of the sulfur compounds that cause acid rain. Companies drilling for oil or natural gas may also benefit from thermostable enzymes. Oil well workers currently use sugar-hydrolyzing enzymes to thin the crude product and enhance flow from the well. Temperatures in the well often reach 80-100° C, which creates a denaturation problem for the mesophilic enzymes. Sugar-hydrolyzing enzymes from *Thermus neapolitana* can withstand the higher temperatures and offer an alternative to constant replacement of the mesophilic enzymes currently in use (Adams & Kelly, 1995).

Industrial food producers also see the thermostable enzyme as a possible boon to their businesses. In the future, sugar producers may use thermostable enzymes in starch hydrolysis. This reaction is currently performed at high temperatures in a somewhat inefficient manner by mesophilic enzymes (Borman, 1991). The use of thermostable enzymes, as they function best at elevated temperatures, could multiply the efficiency of this reaction. In general, a transition to high temperatures would benefit most food processing operations, as this would reduce the chances of bacterial contamination (Adams & Kelly, 1995). Theoretically, only the thermophilic biocatalyst, and not the contaminant, would be able to survive at extreme temperatures.

These are but a few of the possible laboratory and industrial applications currently on the drawing board for thermostable enzymes. Researchers have barely scratched the surface of applications from other areas of life. For example, the recent publicity over archaea's evolutionary origins demonstrates that comparisons of molecules from thermophilic and mesophilic species may someday be of chief importance to evolutionary explanations for the origin of life on this planet. The greatest application, however, may be to the newborn field of

protein engineering. Scientists are just beginning to consider how they can improve on the design of materials used in a variety of applications, from medicine to industry, by altering the proteins involved or introducing new proteins. Thermostable enzymes demonstrate that nature is the best tutor for protein engineers. Unraveling the mechanisms that thermostable enzymes use to survive in their harsh environments will provide invaluable clues for engineering stable enzymes in the laboratory.

Conclusion

Thermophilic microorganisms are unlike any other group of creatures on the Earth. The amazing and mysterious ease with which they live in hot and unforgiving environments baffles current researchers and will continue to mystify many more scientists for years to come. The many mechanisms of protein stability that have already been elucidated demonstrate that the popularity of these organisms as research subjects is steadily growing. As their usage in scientific work increases, the lessons learned in protein stability, metabolism, bioengineering and other subjects certainly will have useful connections to the field of microbiology. These principles, however, will also apply to other fields of science in ways that only the future can reveal.

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