BIOCATALYSIS AT EXTREME TEMPERATURES

ENZYME SYSTEMS NEAR AND ABOVE 100 °C

EDITED BY
Michael W. W. Adams
and Robert M. Kelly

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Biocatalysis at Extreme Temperatures

Enzyme Systems Near and Above 100 °C

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Foreword

THE ACS SYMPOSIUM SERIES was founded in 1974 to provide a medium for publishing symposia quickly in book form. The format of the Series parallels that of the continuing ADVANCES IN CHEMISTRY SERIES except that, in order to save time, the papers are not typeset, but are reproduced as they are submitted by the authors in camera-ready form. Papers are reviewed under the supervision of the editors with the assistance of the Advisory Board and are selected to maintain the integrity of the symposia. Both reviews and reports of research are acceptable, because symposia may embrace both types of presentation. However, verbatim reproductions of previously published papers are not accepted.

Preface

WHEN APPROACHED BY PROFESSOR PRASAD DHURJHATI of the University of Delaware to organize a symposium on Biocatalysis Near and Above 100 °C, we were faced with a dilemma. While this area had aroused its share of scientific and technological curiosity, it was not clear that enough had been accomplished to serve as a basis for such a symposium. Most efforts had focused on the ecology of geothermal environments and the growth physiology of hyperthermophilic microorganisms. The pioneering work of individuals such as Holger Jannasch of Woods Hole Oceanographic Institute, Karl Stetter of the University of Regensberg, and John Baross of the University of Washington had led to the discovery of a growing list of novel microorganisms, many of which have optimal growth temperatures at or above 100 °C. However, the purification and characterization of the enzymes from these organisms had only begun, largely due to the difficulties encountered in working with biological systems at such high temperatures. As such, biocatalysis at high temperatures remains on the fringes of biology and biotechnology.

Nonetheless, a number of interesting studies concerned enzymes derived from hyperthermophiles, many of which are still in progress. The preliminary results from many of these studies are intriguing and potentially have great impact beyond the ecology and microbial physiology of geothermal environments. Already molecular biology has been expanded through the use of thermostable DNA polymerases in the polymerase chain reaction (PCR), and thermostable enzymes have been employed in the bioprocessing of starch. However, the enzymes employed in these applications, while stable relative to most enzymes, have limited durability at temperatures approaching and exceeding 100 °C. Some enzymes from hyperthermophiles (bacteria with optimal growth temperatures near or above 100 °C) are known to have half-lives that are orders of magnitude longer than those of the enzymes currently used. However, the extent to which these hyperthermophilic enzymes can be utilized remains to be seen.

This book contains contributions from a diverse set of research efforts that are focused in some way on biocatalysis at elevated temperatures. In organizing this volume, we attempted to provide coverage ranging from microbiology to the molecular biology and biotechnology of high-temperature enzymes. Much to our delight, contributors were willing to present and discuss recent results, helping to make the perspective

presented here forward-looking and optimistic. Whether these enzymes are used directly in biotechnology or information gained from their study can be extended to improve our understanding of biocatalysis, this research area will undoubtedly make its mark in many scientific areas. We hope that this first symposium that seeks to address these issues will lead to others with similar intentions.

We thank the contributors to this volume for their willingness and cooperation. Their frank discussion of ongoing research projects is appreciated. We also are grateful to Barbara Tansill of ACS for her encouragement and patience. Finally, our thanks to the U.S. Department of Energy, the National Science Foundation, the National Institutes of Health, and the Office of Naval Research, whose support has fostered this emerging research area.

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Chapter 1

Biocatalysis Near and Above 100 °C

An Overview

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Enzymes are typically labile molecules and thus are adversely affected when exposed to any type of extreme conditions. As such, biocatalysis, in either a physiological or biotechnological sense, has usually constrained to a rather narrow range of temperature, pH, pressure, ionic strength and to an aqueous environment. In fact, given its physiological role, and the need at times to regulate enzymatic activity, this is appropriate. Unfortunately, the use of biological catalysts for technological purpose necessitates that enzymes be stable and functional in non-physiological environments. The challenge then is to either isolate enzymes more suitable for a particular application or be able to modify existing enzymes systematically to improve their stability and/or function. While a number of thermostable enzymes have been studied previously, the focus here is on thermostable enzymes produced by high temperature microorganisms.

It is now a fact that life can be found at temperatures at or above the normal boiling point of water. Bacteria have been isolated from sites as diverse as the ocean floor and terrestrial hot springs proliferating at these temperatures. As the number of microorganisms isolated from geothermal environments grows, there is increasing interest in the intrinsic characteristics of their constituent biomolecules. The enzymes from these bacteria are not only significant for their potential as biocatalysts but as model systems to which nature has endowed incredible levels of thermostability. It will be up to scientists and technologists interested in the properties of these proteins to devise ways to probe the most fundamental aspects as well as envision ways in which biocatalysis at elevated temperatures can be put to good use.

The prospect of biocatalysis at high temperatures and in normally denaturing environments is not a new one. Studies focusing

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on ribonuclease A and on several α-amylases that are stable at temperatures in the vicinity of 100°C have shown that enzymes from mesophilic or moderately thermophilic sources may possess high levels of thermostability. In fact, there have been numerous attempts to systematically improve the thermostability of a given protein through site-directed mutagenesis or to infer the bases for thermostability by comparing amino acid sequences of homologous systems. However, while the idea of biocatalysis near and above 100°C may not be new, the levels of thermostability at these temperatures found in enzymes from high temperature bacteria exceed any that have previously been examined. Previous work on enzymology at high temperatures focused on enzymes that, from a thermostability standpoint, were uncommon. Now, the expanding sources of high temperature bacteria provide a diverse source of extremely thermostable enzymes for both basic scientific studies as well as for biotechnological opportunities.

There is no doubt that the study and use of enzymes from extremophiles is in its earliest stages. Although prospects from both basic and applied perspectives are appealing, only a relatively small number of researchers have significant efforts in this emerging field. This is not surprising since one must develop a range of capabilities including biomass generation in order to make significant progress in reasonable periods of time. Fortunately, there are an increasing number of efforts along these lines which makes progress on many fronts increasingly likely.

Despite the fact that the study of enzymes from high temperature bacteria is not well-developed, there has, nonetheless, been considerable progress on many associated issues. This volume contains a diverse set of contributions that, taken together, provide some perspective of where this field is now and where it is heading. As is clear from examining the diverse expertise collected here, many different approaches and motivations are apparent. These range from investigations into the fundamental and molecular characteristics of high temperature enzymes to their physiological significance to ways in which they can be utilized for technological purpose. A brief discussion of these contributions is in order.

Chapter 2 provides a perspective on the genesis of efforts on biocatalysis at elevated temperatures. There are still only a relatively small number of microorganisms with extremely high optimal growth temperatures populating only a handful of genera. The initial efforts focusing on the biochemistry of the enzymes from these bacteria were motivated by an interest in comparative physiology and biochemistry relative to mesophilic counterparts. Chapter 3 provides some perspective on the challenges facing one interested in this emerging area of science and technology. Two case studies featuring enzymes from *Pyrococcus furiosus* illustrate the many differences one encounters in studying biocatalysis at elevated temperatures. Chapter 4 further demonstrates the process through which high temperature enzymes are identified and isolated with a discussion of thermostable ureases from organisms in hot springs at Yellowstone National Park.

Much of the work done thus far in this area has emerged from a interest in comparative physiology between mesophiles and

thermophiles. The enzymes discussed in Chapters 5 and 6 illustrate this point. Chapter 5 focuses on the respiratory enzymes implicated in the physiological function of *Pyrodictium brockii.* Not only does this chapter provide an interesting perspective on sulfur respiration but demonstrates an efficient use of very small amounts of biological material - a critical requirement in many cases in this field. Chapter 6 focuses on glutamate dehydrogenase in *P. furiosus* which apparently is under regulatory control as part of the bacterium's nitrogen metabolism.

Chapters 7 and 8 discuss the effect of additional stresses on enzymes from high temperature bacteria. Chapter 7 reviews the status of enzymology in organic solvents and includes coverage of prospects for catalyzing reactions in non-aqueous media and at elevated temperatures. The prospect of organic synthesis in this environment is presented. Many high temperature bacteria come from locations of extremely high pressure; their enzymes must therefore deal with this additional stress. Chapter 8 provides some background on pressure effects on biocatalysis, including the additional affect of temperature.

It is difficult with such limited information to begin to generalize as to the mechanisms of extreme protein thermostability. However, it is possible to extend present knowledge of protein stability to attempt to provide some basis for further study. Chapters 9 through 11 come at this in different ways. Chapter 9 uses insights developed from theoretical studies of protein thermostability to provide a thermodynamic basis for higher levels of thermostability. While meaningful experimental data are not available to test the current hypothesis, the arguments presented are persuasive and merit additional attention. Chapter 10 presents a correlative approach to predict enzyme properties from very limited information. This framework could be very useful is assessing the relative merits of particular enzymes in a given situation. Chapter 11 then describes the ambitious task of deriving insights into the intrinsic features of thermostability using molecular dynamics.

Possibly the most exciting development in biotechnology in recent years has been the use of the polymerase chain reaction to amplify quantities of DNA to useful levels. A thermostable DNA polymerase is central to this technology and several have now been isolated from high temperature bacteria. A more fundamental question arises as to how these organisms stabilize/destabilize DNA at such high temperatures. Chapter 12 provides some intriguing insights into how the replication process proceeds at elevated temperatures and the role of DNA-binding proteins in this regard. From a technological perspective, Chapter 13 discusses a new DNA polymerase isolated from *P. furiosus* and its properties relative to other enzymes now in use for the same purpose.

In one sense this volume provides no definitive answers to the question of what underlies extreme thermostability or how biocatalysis proceeds at high temperatures. However, the contributions here do provide a perspective on the field and suggest the directions in which it is headed. It is clear that progress has been made and, because of the potential benefits to be derived from studies of enzymes from high temperatures bacteria, more attention to this field is merited.

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Chapter 2

Metabolic Enzymes from Sulfur-Dependent, Extremely Thermophilic Organisms

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Microorganisms growing near and above 100°C were discovered only in the last decade. Most of them depend upon elemental sulfur for growth. Both the organisms and their enzymes have enormous potential in both basic and applied research. To date only a few metabolic enzymes have been characterized. The majority are from two sulfur-dependent organisms, from the archaeon, *Pyrococcus furiosus,* and from *Thermotoga maritima,* the most thermophilic bacterium currently known. In this chapter we review the nature of the sulfur-dependent organisms, their evolutionary significance, and the properties of the enzymes that have been purified so far.

The existence of life forms that not only survive but actually thrive at temperatures near and even above the normal boiling point of water is a very recent discovery in biology. The field began in 1982 when Karl Stetter of the University of Regensburg isolated microorganisms from shallow marine volcanic vents off the coast of Italy that grew reproducibly above 100°C (*1*). This immediately led, with considerable success, to the search for organisms with similar properties. However, an understanding of how biochemical processes are able to take place at such temperatures has been less forthcoming. Indeed, it was only in 1989 that the first metabolic enzymes were purified and characterized from an organism capable of growing at 100°C (*2,3*). Similarly, the first insights into the factors that lead to protein "hyperthermostability" are only just emerging (Chapter 11), as are potential mechanisms for stabilizing DNA (Chapter 12). The main objectives of this chapter are to describe the "extremely thermophilic" organisms known at present, and to summarize the properties of the enzymes and proteins that have been purified to date from the sulfur-dependent species.

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The Classification of Extreme Thermophilic Organisms

As shown in Figure 1, well over a dozen different genera are now known that are able to grow optimally at temperatures of 80°C or above (*4-7*). These are referred to as extremely thermophilic or hyperthermophilic organisms, although the latter term is frequently restricted to those organisms that grow optimally at or above 100°C. As indicated, all but one of the extreme thermophiles have been classified within what is now termed the *Archaea* domain of life. These contrast with the numerous thermophilic organisms belonging to the *Bacteria* domain that have been isolated over the years, most of which have temperature optima for growth (T_{opt}) below 70°C (*8*).

The relationship between the *Archaea* and the *Bacteria* is shown in Figure 2. During the 1960's, two forms of life were recognized based on features at the cellular level. One was the *Eucaryotae,* which represented all "higher" life forms, and these were distinguished by the presence of a membrane-defined nucleus. The other was the *Procaryotae,* which included all bacteria, and these lacked a nucleus. During the 1970's techniques were developed to classify organisms at the molecular level using sequences of proteins and nucleic acids. A fundamental dichotomy in the procaryotes was then shown to exist by Woese and coworkers on the basis of 16S rRNA sequences (*9, 10*). This confirmed in quantitative terms the close relatedness of the the vast majority of bacteria (procaryotes) and the fundamental difference between them and the eucaryotes. However, two groups of organisms, methanogenic bacteria and extremely halophilic bacteria, were shown to be specifically related to each other, but widely separated from all other bacteria and also from eucaryotes. The organisms in the methanogen/halophile branch were termed archaebacteria to distinguish them from all other bacteria, or eubacteria. This separation was also reflected in the biochemistry of the two groups: the archaebacteria have different cell walls and cell membranes, and contain a range of enzymes and cofactors not present in other bacteria. Indeed, the genetic machinery of the archaebacteria in many ways more resembles that of eucaryotes rather than eubacteria.

As more molecular sequences became available, it was possible to discern the relationship between these three domains of life, and a universal phylogenetic tree was proposed by Woese and colleagues in 1990 (*11*). In fact, they recommended that the three domains of life be referred to as *Bacteria, Archaea,* and *Eucarya,* since, at the molecular level, the archaebacteria resembled other (eu)bacteria no more than they did eucarya(otes), and so they should no longer be termed bacteria. These definitions will be used herein.

A rather surprizing conclusion from the universal tree shown in Figure 2 is that the *Eucarya* and *Archaea* have a common ancestor that is not shared by the *Bacteria.* Moreover, the first organisms to have diverged from the *Eucarya/Archaea* lineage were the extremely thermophilic archaea. Thus, investigations into the molecular properties of the extreme thermophiles may give some insights into the development of the eucaryotic cell. Furthermore, as shown in Figure 1, there is one extremely thermophilic genus that is not classified within

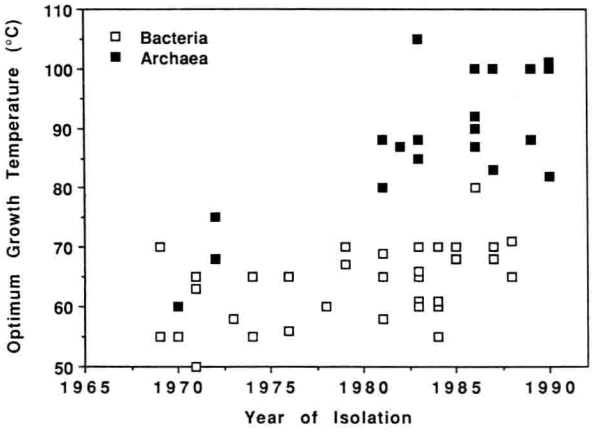

Figure 1. Thermophilic organisms isolated over the last twenty years. Data taken from Table I and Refs. 7, 8.

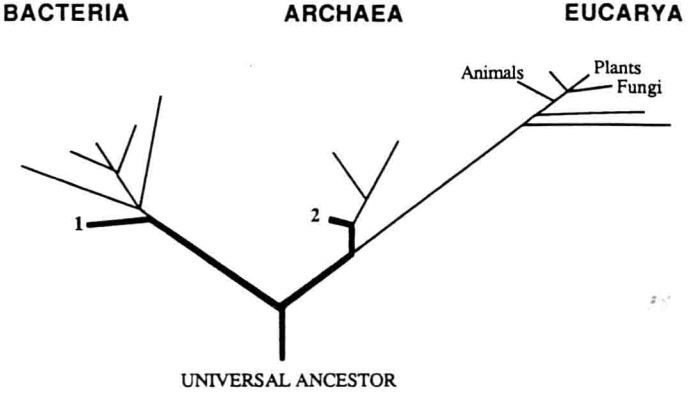

Figure 2. The universal phylogenetic tree showing the three domains of life. Known extremely thermophilic organisms include the novel bacterium, *Thermotoga maritima* (designated 1) and the S°-dependent archaea (designated 2). It is proposed that the extent of extreme thermophily, shown by the heavy line, includes the Universal Ancestor. Evolutionary time is proportional to the distance along any one line. Present time is represented by the ends of the lines. (Adapted from reference 11.)

the *Archaea* domain, and this is *Thermotoga* (*13*). As shown in Figure 2, in addition to being the most thermophilic, *Thermotoga* is also the most ancient bacterial genus currently known, the first to have diverged from the "Universal Ancestor". Extremely thermophilic organisms therefore appear to be the remnant of some ancestral form of all extant life, having evolved when the earth was much hotter than it is at present (*12*). Of course, this is contrary to traditional wisdom, which has regarded thermophily as an adaptation. That is, thermophiles were thought to represent a few "conventional" organisms that originally grew at "normal" temperatures but had somehow adapted so that they could flourish at higher temperatures. However, the remarkable conclusion from the evolutionary analysis is that extreme thermophiles are the most ancient organisms currently known. The rest of the life forms on this planet may therefore themselves be the result of temperature adaptations - adaptations to lower (less than 100°C) temperatures.

Physiology of Extreme Thermophiles

The genera of extremely thermophilic organisms currently known are depicted in Table I. Species of the genera *Pyrococcus, Pyrobaculum, Pyrodictium, Hyperthermus* and *Methanopyrus,* together with ES-4, grow optimally at or above 100°C, and these will be termed "hyperthermophiles". Most of the genera shown in Table I are represented by only one or two species. The majority have been found in geothermally-heated marine environments, in both shallow (several meters below sea level) and deep water (several kilometers below sea level). Two of the organisms shown in Table I, ES-1 and ES-4, have yet to be formally classified. Both were recently isolated by Baross and colleagues near deep sea hydrothermal vents. Stetter and coworkers have also recently extended the known habitats of extremely thermophilic bacteria. They isolated several different bacteria from within the crater and from the open sea plume of an erupting submarine volcano, located 40 m below sea level (*44*). These organisms included relatives (by DNA hybridization) of species of *Pyrodictium, Pyrococcus, Archaeglobus* and *Thermococcus,* bacteria that had been previously found only in volcanic vents off the coast of Italy. They also isolated a novel bacterium that showed no DNA homology with any of the extreme thermophiles tested. Some of the extreme thermophiles therefore appear to be spread in the open oceans, and are able to remain viable even under such cold and aerobic conditions.

Except for three methanogenic genera and one novel sulfate-reducing genus (Table I), all of the extremely thermophilic archaea are termed sulfur-dependent organisms, since, to a greater or lesser extent, they obtain energy for growth by the reduction or oxidation of elemental sulfur (S°). Only species of *Acidianus* and *Desulfurolobus* are able to grow aerobically, and they do so by oxidizing S° with O_2 to produce sulfuric acid. Accordingly, they grow optimally at low pH (near 2). They are also autotrophs and use CO_2 as a carbon source. Remarkably, these organisms are facultative aerobes, and are able to switch to an anaerobic growth mode using H_2 as an electron donor and

Table I. Extremely Thermophilic Genera

Genus	T_{max} (T_{opt})	%GC	*Habitat*[a]	*First Isolated*	*Ref.*
		BACTERIA			
Thermotoga	90° (80°)	46	m	1986	*13-15*
		ARCHAEA			
S°-DEPENDENT					
Thermoproteus	92° (88°)	56	c	1981	*16,17*
Staphylothermus	98° (92°)	35	d/m	1986	*4,18*
Desulfurococcus	90° (87°)	51	d/m/c	1982	*19-21*
Thermofilum	100° (88°)	57	c	1983	*22*
Pyrobaculum	102° (100°)	46	c	1987	*23*
Acidianus	96° (90°)	31	m/c	1986	*24*
Desulfurolobus	87° (81°)	33	c	1986	*25*
Pyrodictium	110° (105°)	62	d/m	1983	*7,26,27*
Thermodiscus	98° (90°)	49	m	1986	*4*
Pyrococcus	105° (100°)	38	d/m	1986	*28,29*
Thermococcus	97° (88°)	57	d/m	1983	*30-32*
Hyperthermus	110° (100°)	56	m	1990	*33*
"ES-1"	91° (82°)	59	d/m	1990	*34*
"ES-4"	108° (100°)	55	d/m	1990	*35*
SULFATE-REDUCING					
Archaeoglobus	95° (83°)	46	m	1987	*36-38*
METHANOGENIC					
Methanococcus	86° (85°)	31	d/m	1983	*39*
Methanothermus	97° (80°)	33	c	1981	*40,41*
Methanopyrus	110°(100°)	60	d/m	1990	*42,43*

[a] Species have been isolated from shallow marine vents (m), deep sea hydrothermal vents (d), and/or from continental hot springs (c). The sulfur-dependent genera are grouped in separate orders, except for *Hyperthermus*, ES-1 and ES-4. Modified from refs. 2 and 4.