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Adaptation Strategy of Thermophiles toward Hyperthermophily and Their Molecular Bases

Tadayuki Imanaka

Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510

Received May 10, 2007; E-mail: imanaka@sbchem.kyoto-u.ac.jp

I reflect on some of our studies on the hyperthermophilic archaeon, *Thermococcus kodakaraensis* KOD1 and its enzymes. The strain can grow at temperatures up to 100 °C, and also represents one of the simplest forms of life. As expected, all enzymes, DNA, and cytoplasmic membrane displayed remarkable thermostability, and we have determined some of the basic principles that govern this feature. To our delight, many of the enzymes exhibited unique biochemical properties and novel structures not found in mesophilic proteins. Here, I will focus on some enzymes whose three-dimensional structures are characteristic of thermostable enzymes. I will also add some examples on the stabilization of DNA and cytoplasmic membrane.

1. Background of Research

Thermophiles are now classified by their growth temperature as follows: thermophiles above 55 °C, moderate thermophiles above 65 °C, extreme thermophiles above 75 °C, and hyperthermophiles above 90 °C. In the 1970's, I was focused on moderate thermophiles, namely *Bacillus stearothermophilus*, and one of the highlights of this period was that our group succeeded in the development of a transformation system in this strain.[1] This recombinant technology led to many exciting findings in the following years. In the 1980's, I was one of many who were taken by surprise with the discovery of hyperthermophiles, first isolated by Stetter and his colleagues.[2] I was fascinated that these hyperthermophiles could grow, or even preferred to grow, in boiling water. From then on, I have dedicated a major portion of my scientific activities to these "hot bugs," and this will most likely be the case in the years to come.

2. Molecular Basis of Protein Thermostability

When enhancement of protein (thermo)stability is desired, there are a number of strategies available, taking into account four major interactions within a protein; covalent bonds via disulfide bridges, ionic interactions, hydrogen bonds, and hydrophobic interaction (Fig. 1).[3] Addition of any of these four types of interactions may be considered in order to enhance the thermostability of a protein. Another alternative may be to introduce proline residue at β-turn structures (the proline rule). Here, I will show some examples of protein thermostability.

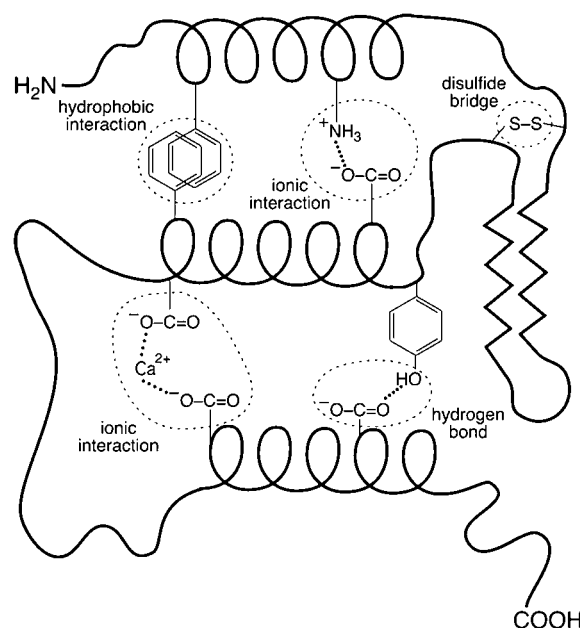### 2.1 Significance of Hydrophobic Interaction to the

Fig. 1. Major interactions that contribute to the stability of a folded protein.

Thermostability. An increase in thermostability of a neutral protease from *Bacillus stearothermophilus* (NprT) was achieved from a rational approach by comparing its sequence with the thermostable thermolysin from *B. thermoproteolyticus*. The enzymes were 85% identical, while the thermostability of NprT at 75 °C was significantly lower than that of thermolysin. Taking into account the three-dimensional structure of
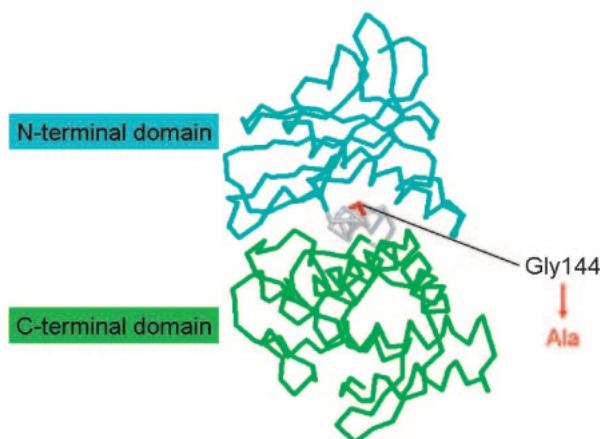
Fig. 2. Structure of thermolysin from *Bacillus thermo-proteolyticus* used for site-directed mutagenesis of neutral protease from *B. stearothermophilus*.
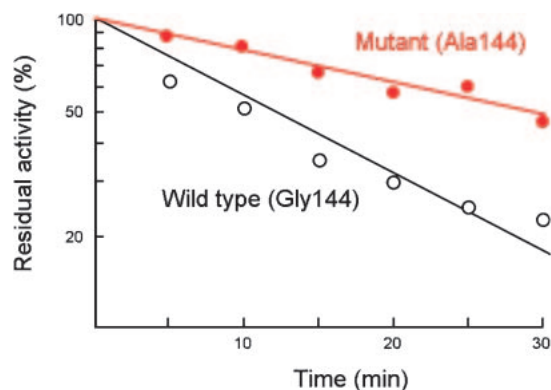

Fig. 3. G144A mutant displays higher thermotolerance than the wild-type enzyme.
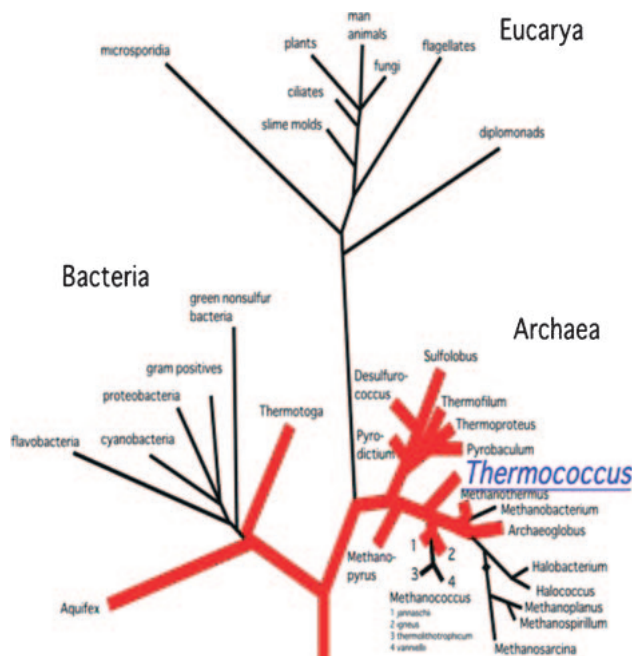

Fig. 4. Universal phylogenetic tree of living organisms based on 16S or 18S ribosomal RNA sequences. The three domains, Archaea, Bacteria, and Eucarya, are shown.
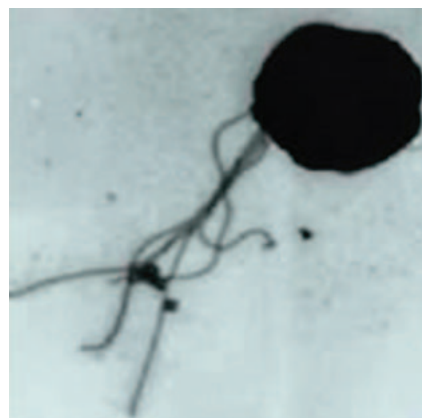

Fig. 5. Electron microscopic observation of *Thermococcus kodakaraensis* KOD1.

thermolysin, a single mutation G144A was chosen as a candidate to increase the thermostability of NprT. The glycine residue was supposed to be located in an α-helix that connected the N- and C-terminal domains of the enzyme (Fig. 2).[4] The mutation was expected to stabilize the α-helix, and increase internal hydrophobicity of the enzyme. Furthermore, the G144A mutation introduces only a small methyl group, minimizing any structural or functional interruption that may be caused by introduction of a new side chain. Indeed, this single mutation led to a significant increase in the thermostability of NprT (Fig. 3).[5] This is a good example of the fact that an increase in internal hydrophobicity of an enzyme and stabilization of a secondary structure α-helix leads to an increase in the thermostability of a protein.

2.2 Isolation of Hyperthermophilic Archaeon. Hyperthermophiles are referred to as microorganisms that optimally grow at temperatures above 80 °C,[6] or as microorganisms that can grow at temperatures above 90 °C.[7] When a phylogenetic tree of all living organisms is constructed based on 16S or 18S ribosomal RNA sequences, three domains are exhibited; Eucarya, Bacteria, and Archaea (Fig. 4).[8,9] Hyperthermophiles occupy branches nearest to the root of the tree, suggesting that hyperthermophiles may be the nearest organisms to the last common ancestor of life.

In 1993, we isolated a hyperthermophilic archaeon from a solfatara at Kodakara Island, Kagoshima, Japan.[10] The strain exhibited rapid growth on amino acids with elemental sulfur as a terminal electron acceptor, and was later designated *Thermococcus kodakaraensis* KOD1 (Fig. 5).[11]

T. kodakaraensis KOD1 exhibits cell growth from 60 to 100 °C. The optimum pH and NaCl concentrations for growth are 7.0 and 3%, respectively. While the strain does grow by fermentation of yeast extract and tryptone, elemental sulfur significantly enhances the growth rate. Excess reducing equivalents are used to reduce the sulfur to hydrogen sulfide, and released in the gas phase. KOD1 cells are irregular cocci with a diameter of approximately 1 μm. The cells are highly motile and harbor several polar flagella. The genome of strain KOD1 turned out to have a length of 2088737 bp.[12] The small size of the chromosome, and the high growth rate at high temperatures
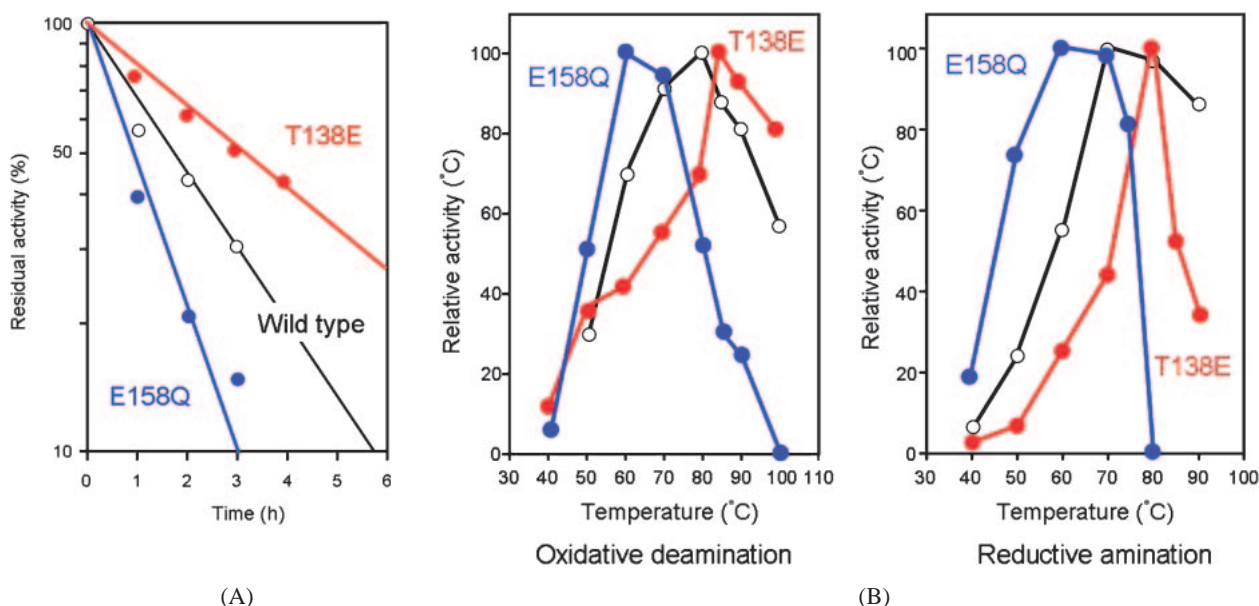
Fig. 6. (A) Thermotolerance of wild-type and mutant GDH from *T. kodakaraensis*. (B) Optimum temperature of wild-type and mutant GDH from *T. kodakaraensis*.

brought us to examine the strain and its proteins in detail. Fortunately, the knowledge that we have obtained from this strain has been well above our initial expectations. In this report, I would like to share some of what we have learned in the past years from strain KOD1.

2.3 Significance of Ion Pairs to the Thermostability. As the importance of ion pairs toward protein thermostability has been stressed in many cases, addition or removal of an ion pair should have significant effects. A clear example is provided by mutagenesis studies of glutamate dehydrogenase from *T. kodakaraensis* KOD1 (*Tk*-GDH). The GDH from *Pyrococcus furiosus* (*Pf*-GDH) and *Tk*-GDH are 83% identical in terms of primary structure. However, while *Pf*-GDH displays a half-life of 12 h at 100 °C, that of *Tk*-GDH is 4 h. The three-dimensional structure of *Pf*-GDH has been determined, and exists in a stable hexameric form. A structural model of *Tk*-GDH was constructed based on the structure of *Pf*-GDH. A difference was observed between two structures at the monomer–monomer interface. In *Pf*-GDH, there is a large ion pair network comprised of six residues, Arg35, Asp132, Glu138, Arg164, Arg165, and Lys166. Glu138 is located at the center of the network, interacting with Arg165 and Lys166. In the case of *Tk*-GDH, Glu138 was replaced by threonine residue. When a T138E mutation was introduced into *Tk*-GDH, an increase in both thermostability and optimal temperature was observed (Figs. 6A and 6B).[13] At one of the two-fold axes of the proteins, Glu158 is at the center of another ion pair network, interacting with Arg124 and Arg128. An E158Q mutation would interrupt this network, and is presumed to destabilize the protein. As expected, the E158Q mutant protein of *Tk*-GDH displayed a lower optimal temperature for activity and decreased thermostability (Figs. 6A and 6B).[13]

2.4 A Structural Basis for Protein Thermostability: *O*[6]-Methylguanine-DNA Methyltransferase. We have pursued attempts to elucidate the three-dimensional structures of various proteins from the hyperthermophilic archaeon *T. kodakaraensis* KOD1. These include DNA polymerase,[14,15] homing endonuclease II, *O*[6]-methylguanine-DNA methyltransferase (MGMT),[16–19] aspartyl-tRNA synthetase,[20–22] and ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco)[23,24] (Fig. 7).

MGMT repairs DNA lesions in a single-step reaction by transferring methyl groups from *O*[6]-methylguanine of DNA to the cysteine residue of its own molecule. The enzyme from strain KOD1, *Tk*-MGMT, is a monomeric protein composed of 174 amino acid residues.[25] Recombinant *Tk*-MGMT exhibits methyltransferase activity, and is stable at 90 °C for 30 min. We crystallized the protein, and its three-dimensional structure was elucidated at 1.8 Å resolution. This structure was compared with its counterpart from *Escherichia coli* (AdaC, C-terminal fragment of Ada protein).[26] We observed seven intra-helix ion pairs in *Tk*-MGMT, while none were detected in AdaC. It is presumed that these intra-helix ion pairs contribute to reinforcement of the stability of the alpha-helices. Furthermore, the number of inter-helix ion pairs was also higher in *Tk*-MGMT (four pairs) compared to AdaC (two pairs), stabilizing the internal packing of the tertiary structure (Fig. 8).[17,18]

Differences in the content of hydrophobic, polar, and charged residues were less than expected, as shown in Table 1. The only alteration observed was a 6% increase in charged residues in *Tk*-MGMT compared to AdaC. However, discrepancies became more evident when we focused on the solvent-accesible surface area (SASA) of the two proteins. Although the SASA of *Tk*-MGMT (8160 Å^2) and AdaC (8339 Å^2) were comparable, the percentage of charged residues in the SASA of *Tk*-MGMT (54%) was considerably higher than that of AdaC (35%). This was also accompanied by a decrease in hydrophobic and polar residues in the SASA from 32% to 24%. These comparisons clearly indicate that in the thermostable *Tk*-MGMT, there is a shift of hydrophobic residues to the interior of the molecule, and charged residues to the surface of the molecule.[18] It should also be noted that *Tk*-MGMT
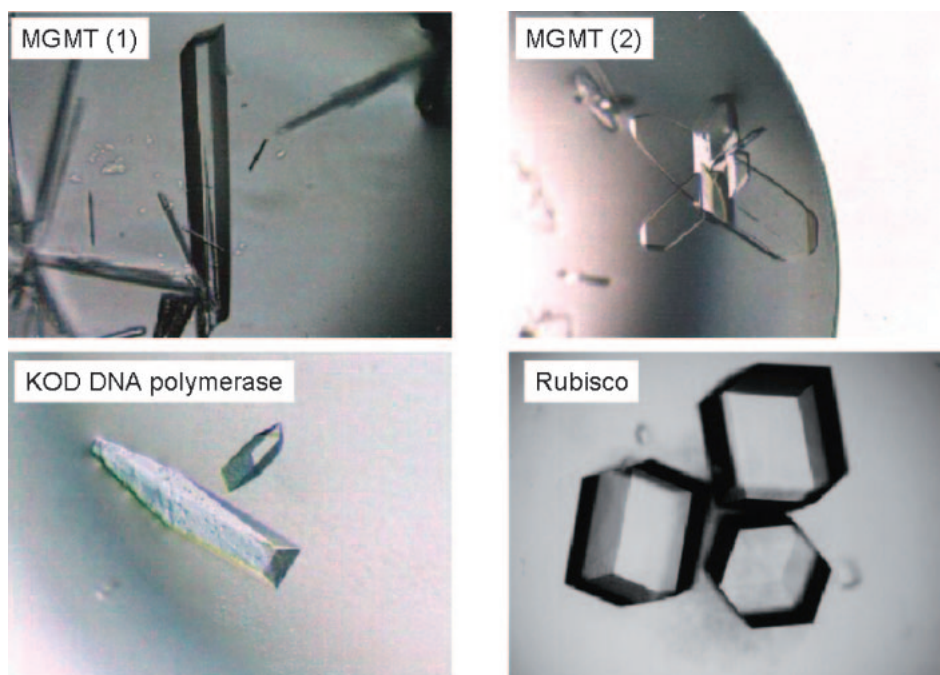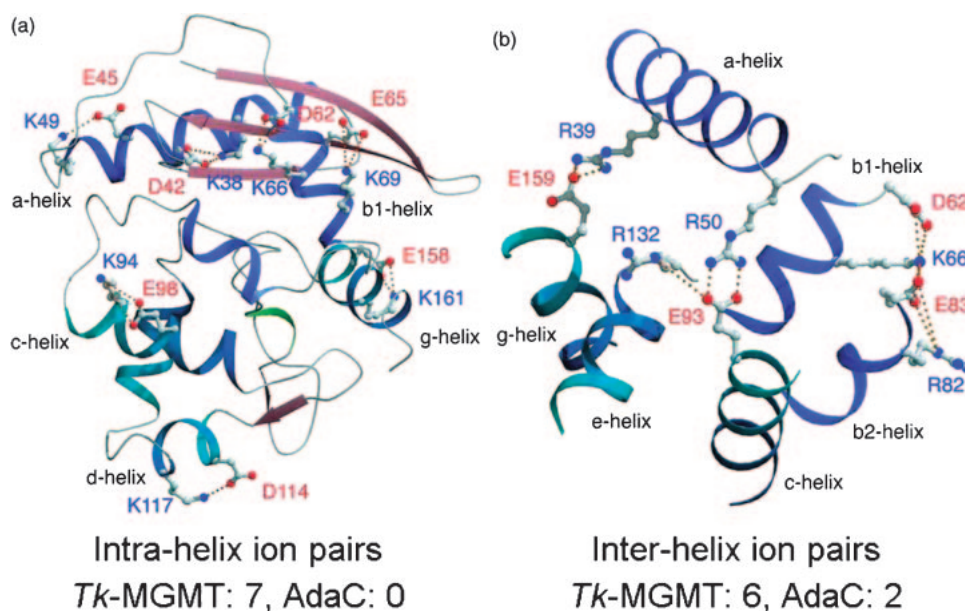
Fig. 7.   Crystals of various proteins from *T. kodakaraensis* KOD1.

Fig. 8.   Comparison of intra-helix and inter-helix ion pairs.

(17 residues) contains more aromatic residues than AdaC (8 residues), generating aromatic clusters within the protein to stabilize the internal packing of the protein (Fig. 9).

These observations suggest that the presence of both ion pairs and hydrophobic interaction can stabilize internal packing of its tertiary structure, leading to thermostability of the protein.

2.5 Significance of Quaternary Structure to Thermostability: Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase. Rubisco is found predominantly in higher plants, algae, cyanobacteria, and photosynthetic bacteria.[27,28] It is the most abundant enzyme on our planet, and plays one of the most impor-

tant roles in our ecosystem. Rubisco catalyzes the covalent addition of carbon dioxide to ribulose 1,5-bisphosphate, producing two molecules of 3-phosphoglycerate.[29] The function and the abundance of the enzyme provide a major link between inorganic and organic carbon in our biosphere. Through the Calvin cycle, the fixed carbon is then converted into sugars and other cell material, which will ultimately be utilized as the carbon and energy source of virtually all heterotrophic organisms. The significance of Rubisco has attracted scientists for decades, consequently leading to an extraordinary accumulation of knowledge on the enzyme. In almost all organisms, Rubisco is a hexadecamer consisting of eight large (L) and

Table 1. Comparison of Solvent-Accessible Surface Areas (SASAs)

	Tk-MGMT	AdaC
No. of residues in crystal structure	169	165
No. of hydrophobic residues	75	78
	(44%)	(47%)
No. of polar residues	46	50
	(27%)	(30%)
No. of charged residues	48	37
	(28%)	(22%)
Total SASA	8160 Å^2	8339 Å^2
SASA of hydrophobic residues	1935 Å^2	2638 Å^2
	(24%)	(32%)
SASA polar residues	1797 Å^2	2752 Å^2
	(22%)	(33%)
SASA of charged residues	**4428 Å^2**	**2949 Å^2**
	(54%)	**(35%)**

eight small (S) subunits, known as the Type I enzyme. Another type of Rubisco, found in some β-purple bacteria, is a homodimer of L subunits only and referred to as the Type II enzyme. While the primary structures of Rubisco L subunits of the same type are highly similar (>70%), those of Type I and Type II enzymes display less than 50% similarity.

Through genome analysis of strain KOD1, we came upon an open reading frame that displayed similarity to previously known Rubiscos. At first we were skeptical as to whether the ORF encoded a genuine Rubisco. The deduced amino acid sequence showed only ≈50% similarity to the previously known types of Rubisco, and moreover, why would a heterotrophic hyperthermophilic archaeon need a Rubisco?

Phylogenetic analysis of Rubiscos indicated that *Tk*-Rubisco clustered with the other archaeal sequences, and was distinct from Type I and Type II enzymes in terms of primary struc-

ture. We expressed the gene in *Escherichia coli*, and purified the recombinant enzyme. Activity measurements revealed that *Tk*-Rubisco could catalyze the carboxylation and oxygenation of ribulose 1,5-bisphosphate, indicating that the protein was a *bona fide* Rubisco.[30,31] In fact, as *Tk*-Rubisco displayed activity at temperatures as high as 100 °C, its specific activity was higher than any previously characterized Rubisco. We also found that *Tk*-Rubisco exhibited an unprecedentedly high carboxylase specificity. The native *Tk*-Rubisco consisted solely of large subunits,[23] and allowed us to perform further structural analysis with the recombinant protein.

Observation of the purified enzyme by electron microscopy indicated a pentagonal structure, indicating that *Tk*-Rubisco was a decamer composed only of large subunits, with a pentagonal ring-like structure (Fig. 10A).[23] We further crystallized the protein using ammonium sulfate as a precipitant, and determined the structure of *Tk*-Rubisco at 2.8 Å resolution.

As indicated by the above results, *Tk*-Rubisco showed a novel (L$_2$)$_5$ decameric structure (Fig. 10B).[24] Compared to previously known Type I enzymes, each L$_2$ dimer was inclined approximately 16° to form a toroid-shaped decamer with unique L$_2$–L$_2$ interfaces. Differential scanning calorimetry (DSC), circular dichroism (CD), and gel permeation chromatography (GPC) showed that *Tk*-Rubisco maintained its secondary structure and decameric assembly even at high temperatures. Despite the low sequence homology, monomer (L) and dimer (L$_2$) structures of *Tk*-Rubisco were well conserved compared with those of Type I and Type II enzymes. In its unique L$_2$–L$_2$ interface which includes rims of the L–L dimerization surface, an intensive ionic network was observed. This ionic network was considered to stabilize not only the pentagonal (L$_2$)$_5$ assembly, but also the L–L association in each dimer itself. It might contribute especially to the structural thermostability of the active sites, which are also located at the L–L dimerization surface. When the ionic network was
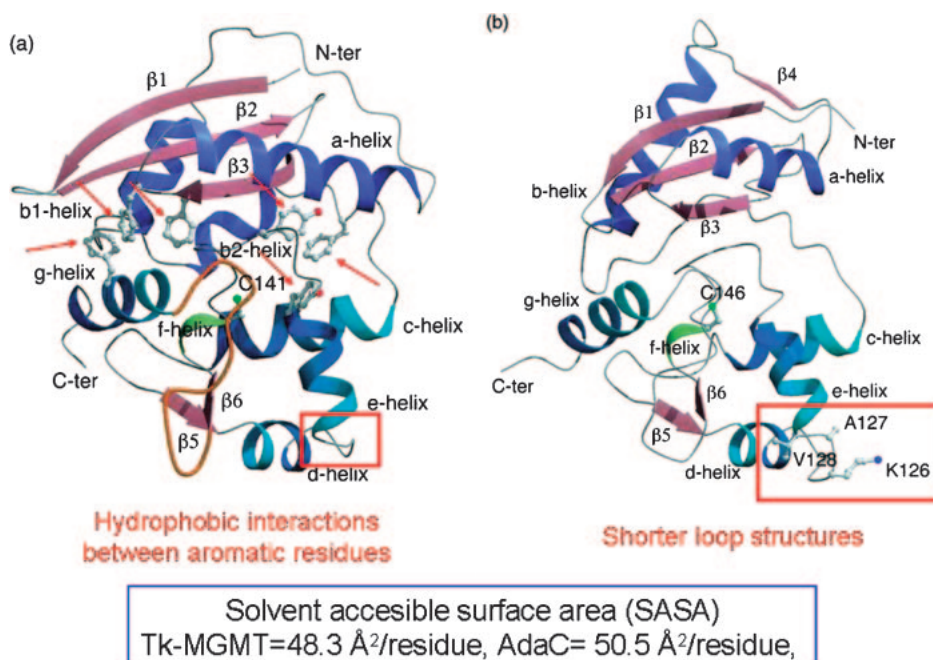
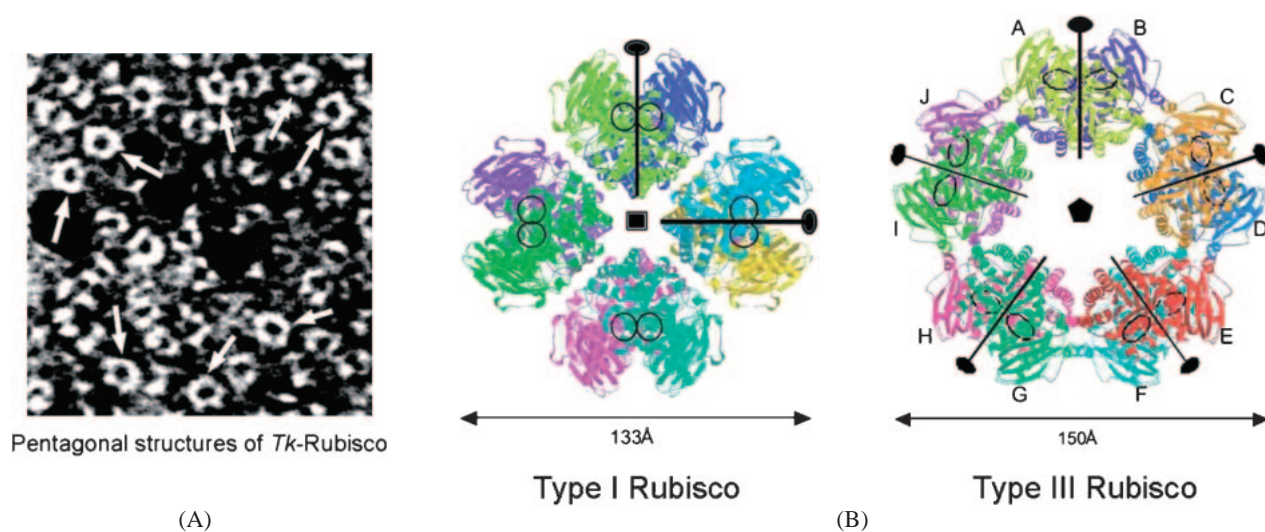Fig. 9. Structural comparison between *Tk*-MGMT (a) and AdaC (b).

Fig. 10.   (A) Electron micrographs of recombinant *Tk*-Rubisco stained with uranyl acetate. The electron micrograph was recorded at a magnification of (×50000). (B) Decamer structures of Type I Rubisco and *Tk*-Rubisco (Type III). Ribbon diagram is shown. Each monomer is shown in different colors and labeled from A to J.
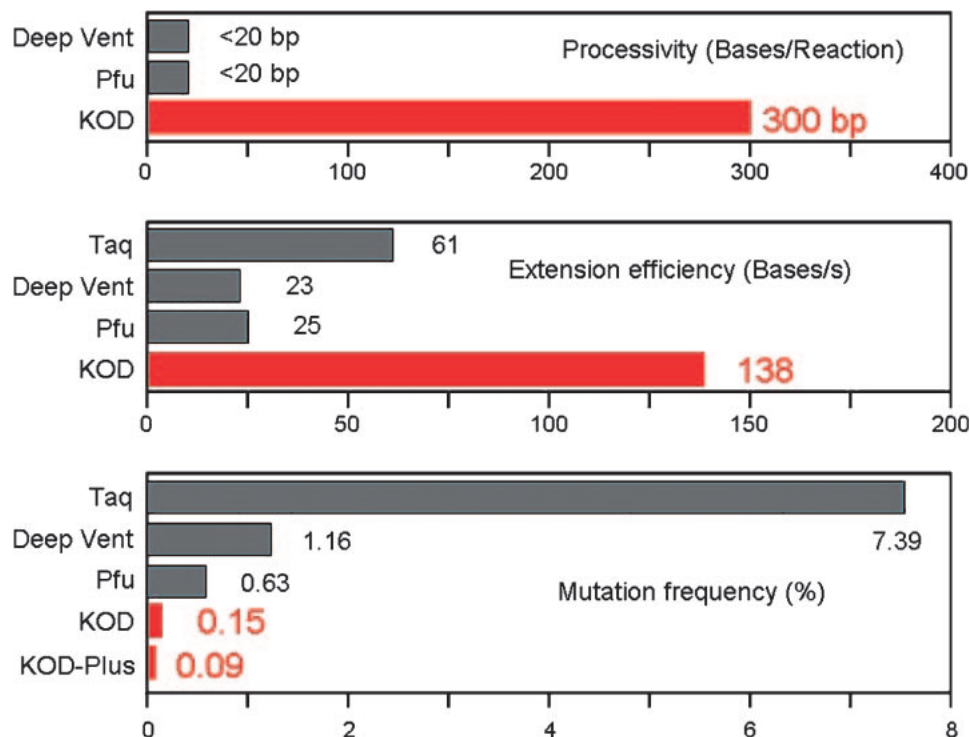

Fig. 11.   A comparison of various thermostable DNA polymerases in terms of processivity (upper panel), extension efficiency (middle panel), and mutation frequency (lower panel).

broken, the themostability decreased, though the L_2 structure was maintained. These results clearly indicate that the quaternary structure is important to increase thermostability of the enzyme.[32]

In spite of the accumulation of knowledge on the biochemical and structural features of *Tk*-Rubisco, little progress has been made in elucidating the physiological role of the enzyme. Recently, we found that *Tk*-Rubisco is involved in a new metabolic pathway, AMP degradation.[33]

2.6 Significance of S–S Bond: DNA Polymerase.   Recom-

binant KOD DNA polymerase was purified, and its characteristics were studied and compared with those of DNA polymerases from various (hyper)thermophiles (Fig. 11).[34] The frequency of deoxyribonucleotide misincorporation was very low (0.35%), indicating that KOD DNA polymerase was an enzyme with high fidelity. Processivity, or the persistence of sequential nucleotide polymerization, of the enzyme was 10- to 15-fold greater than those for *Pfu* DNA polymerase and Deep Vent DNA polymerase. The extension rate of KOD DNA polymerase was 106 to 138 bases s^{-1}, which is much

higher than those of *Pfu* (25 bases s^{-1}), Deep Vent (23 bases s^{-1}), and *Taq* (61 bases s^{-1}) DNA polymerases. It turned out that KOD DNA polymerase was a superb enzyme in terms of fidelity (low mutation frequency), processivity, and elongation speed for PCR (polymerase chain reaction).

The excellent performance of KOD DNA polymerase tempted us to further determine its three-dimensional structure.[14,15] The enzyme had a disk-like shape with dimensions of 60 Å × 80 Å × 100 Å (Fig. 12). The enzyme was comprised of four distinct domains with subdomains; N-terminal domain, Exonuclease domain, Polymerase domain (including Palm and Fingers subdomains), and the Thumb domain (including Thumb-1 and Thumb-2 subdomains). Two disulfide bonds were found in the polymerase domain, and these bonds may contribute to the thermostability of the enzyme. Based on our recent results of tertiary structural studies, we are now clarifying the mechanisms that lead to the high fidelity and elongation rate of KOD DNA polymerase. Further elucidation of the structure–function relationship will provide better understanding of DNA polymerases and will suggest strategies to engineer better PCR enzymes.

2.7 Significance of Chaperonin. It is well known that chaperonin is important for protein folding (Fig. 13). Strain KOD1 has two chaperonins CpkA and CpkB, and they are mainly expressed and functioned at lower and higher temperatures, respectively.[35–37] When chaperonin is added into a protein solution in vitro, it prevents the inactivation and aggregation of protein at high temperature. Some other proteins such as prefoldin are also induced by heat shock and exhibit the same function as chaperonin. These proteins can support the growth of hyperthermophiles at high temperatures.

3. The Importance of High Temperature for Maturation of Thermostable Proteins: Glutamate Dehydrogenase

An intriguing characteristic of thermostable proteins was revealed during studies on glutamate dehydrogenase of *T. kodakaraensis* KOD1 (*Tk*-GDH). The native enzyme purified from KOD1 cells was obtained solely in a hexameric form and displayed high specific activity.[38] In contrast, when we expressed the gene in *Escherichia coli* and purified the recombinant protein, we obtained a mixture of hexamers and monomers. Only the recombinant hexamers exhibited GDH activity, but the specific activity was significantly lower than the native hexameric enzyme. Comparison of CD spectra between the native and recombinant hexamers revealed that their structures were markedly different (Fig. 14). The results suggested that the temperature of protein production might be important for proper assembly of thermostable proteins.[39] We pursued this possibility by incubating the recombinant protein at high temperature (80 °C, 15 min), and found that the CD spectra of the heat-treated protein displayed a similar profile to that of the native *Tk*-GDH. The specific activity of the protein, although still lower than the native enzyme, also increased 2.6-fold. Likewise, heat treatment of the inactive monomeric form of the recombinant enzyme converted a portion of the monomers to a hexameric form. From these studies, we realized that high temperature plays a pivotal role (heat maturation) in the proper folding and oligomerization of thermostable enzymes (Fig. 15).
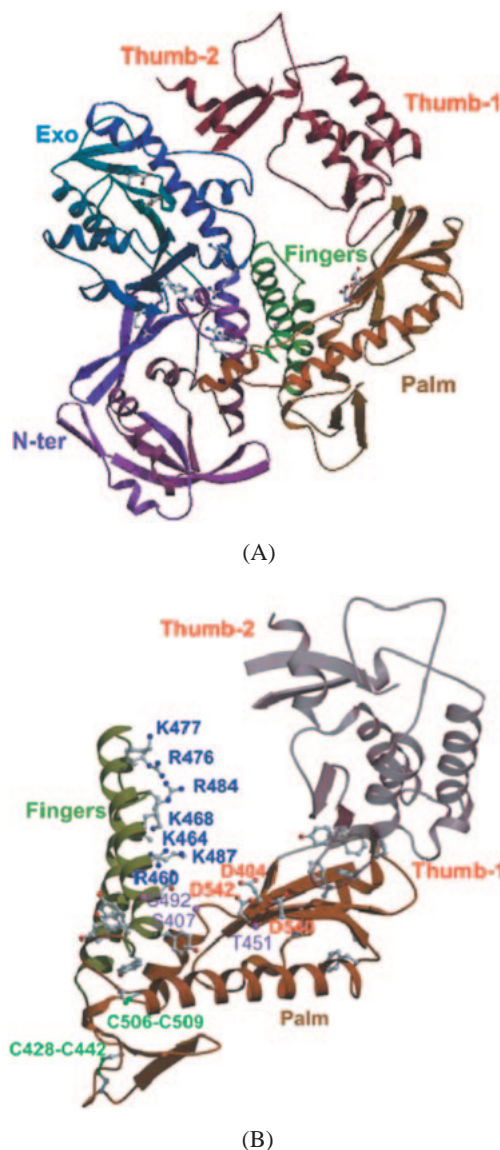

(A)

(B)

Fig. 12. Three-dimensional structure of KOD DNA polymerase. A, Overall structure of KOD DNA polymerase. The structure is composed of domains and subdomains, which are indicated as follows. N-Terminal domain (N-ter, purple), Exonuclease domain (Exo, blue), Polymerase domain including Palm (brown) and Fingers (green) subdomains, and the Thumb domain (red) including Thumb-1 and Thumb-2 subdomains. B, The polymerase domain of KOD DNA polymerase. Conserved acidic residues (D404, D540, and D542) are represented by ball-and-stick models and indicated in red. Basic residues in the Fingers domain are also represented by ball-and-stick models and indicated in blue.

4. Stabilization Mechanism of DNA at High Temperature

4.1 Significance of Histone and Polyamine. When a solution of linear DNA is heated, double-stranded DNA is melted and two single-stranded DNA molecules appear. How is the chromosomal DNA of hyperthermophile stabilized at high temperatures such as boiling point? *T. kodakaraensis* KOD1 possesses two histones (basic proteins, HpkA & HpkB) that
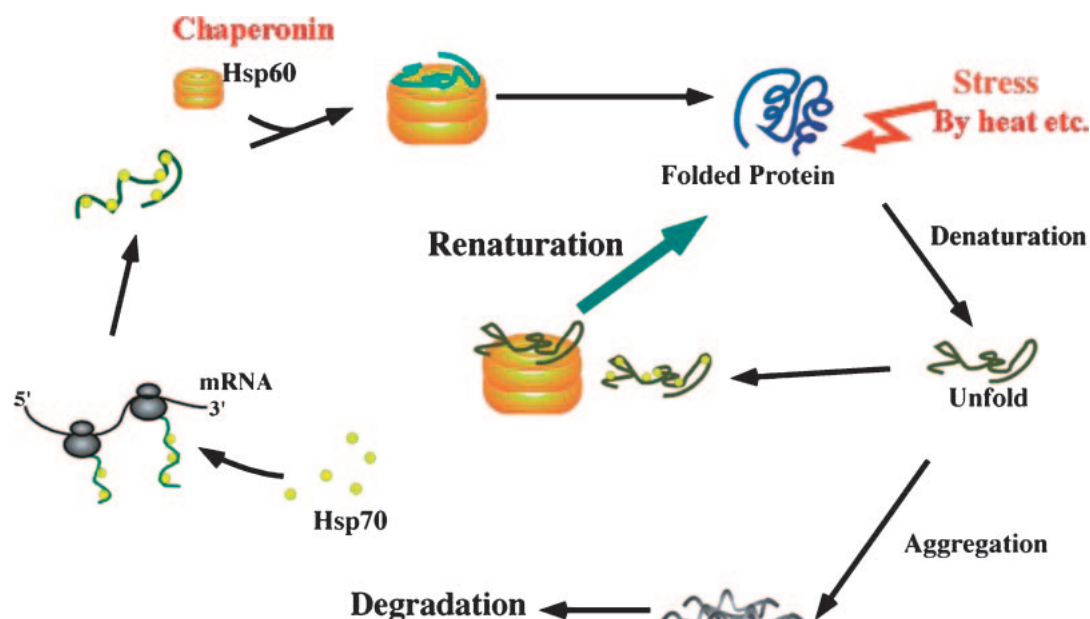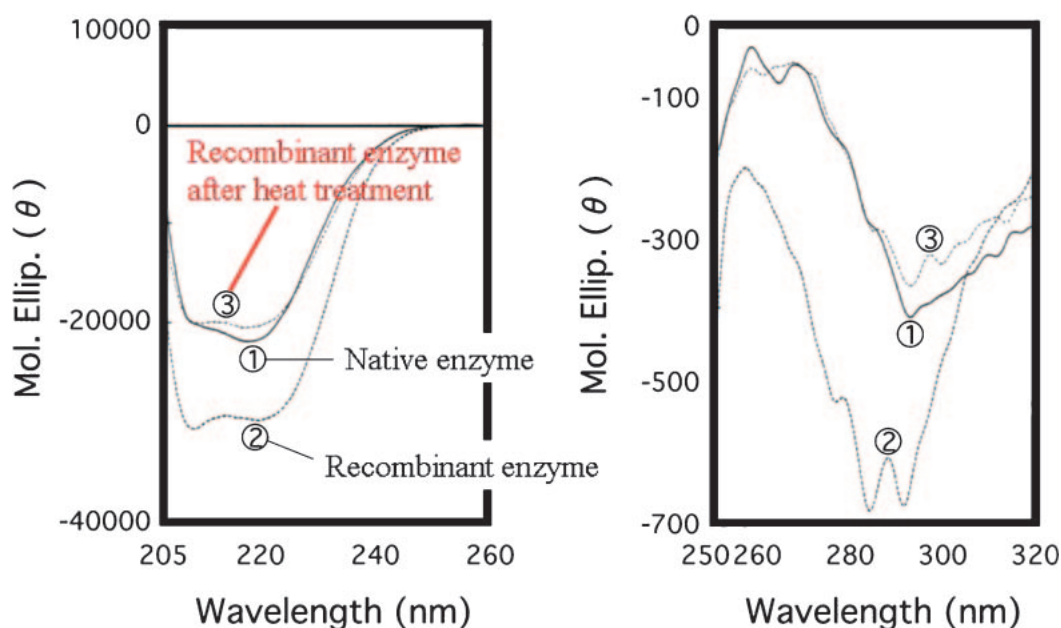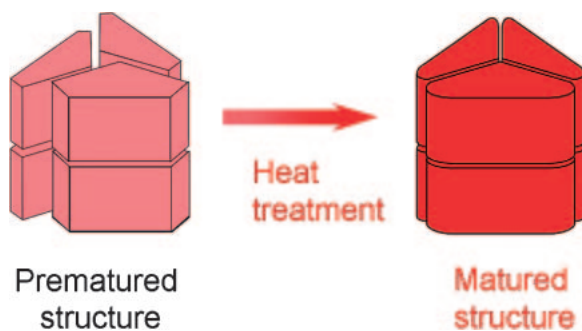
Fig. 13.   Process of protein folding.

Fig. 14.   CD spectra of native *Tk*-GDH (1), and hexameric forms of recombinant *Tk*-GDH prior to (2) and following (3) heat treatment (80 °C, 15 min). Far UV spectra are shown on the left panel, and near UV-spectra on the right panel.

Fig. 15.   A model describing the structural changes of *Tk*-GDH induced by heat treatment.

are essential for DNA compaction and nucleosome formation (Fig. 16), leading to higher melting temperature by 20 °C or more (Fig. 17).[40,41] In addition, high concentration of potassium ion and polyamines such as spermine and spermidine can stabilize the DNA as shown in Fig. 18.

4.2 Significance of Reverse Gyrase.   Reverse gyrase is worthy of remark from the standpoint of both DNA stabilization and evolution of life. The enzyme is specific for hyperthermophiles, because the enzyme gene (including homologues) has been found in every hyperthermophile ever analyzed without exception and none is found in thermophiles or mesophiles. Reverse gyrase is a kind of topoisomerase and gives a positive superhelix structure (Fig. 19), giving high

DNA binding model of archaeal histone   Atomic force microscopy
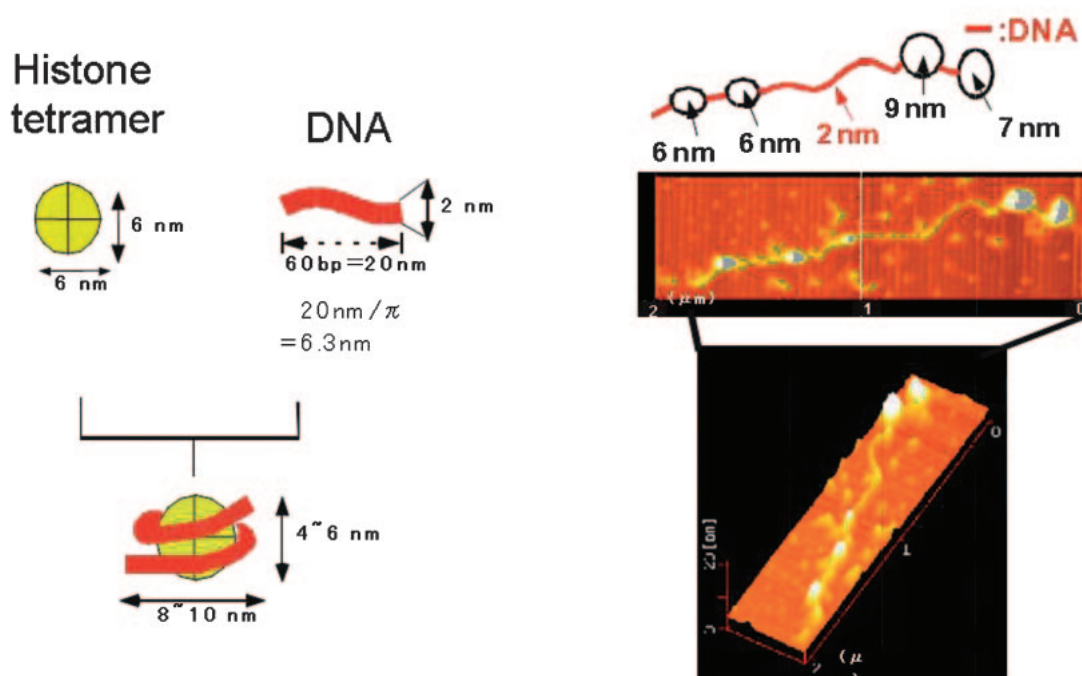

Fig. 16. DNA binding model of archaeal histone.

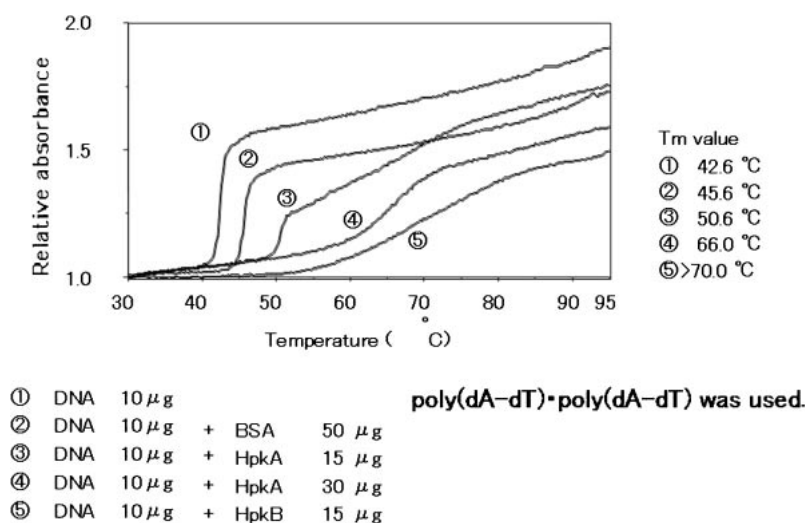|				
①	DNA	10 μg		
②	DNA	10 μg	+ BSA	50 μg
③	DNA	10 μg	+ HpkA	15 μg
④	DNA	10 μg	+ HpkA	30 μg
⑤	DNA	10 μg	+ HpkB	15 μg

poly(dA–dT)·poly(dA–dT) was used.
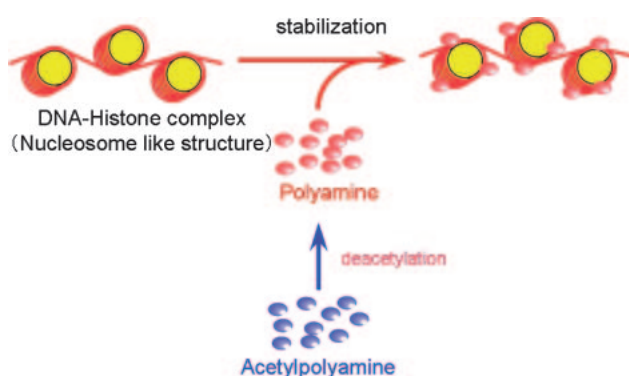
Fig. 17. Melting curve of DNA.

Fig. 18. Stabilization mechanism of DNA.

thermostability to DNA. When the reverse gyrase gene was deleted from the chromosome,[42] the Δ rgy mutant strain grew slowly at 80 °C or higher and could not grow at 93 °C, while the wild-type strain could grow at 100 °C.[43] These results clearly show that reverse gyrase is essential to stabilize chromosomal DNA at high temperatures.

5. Stabilization of Cytoplasmic Membrane at High Temperature

Generally speaking, cytoplasmic membrane is composed of bi-layers of phospholipid molecules, which are labile at high temperatures. The cytoplasmic membrane of hyperthermophile has the following features in comparison with those of meso-
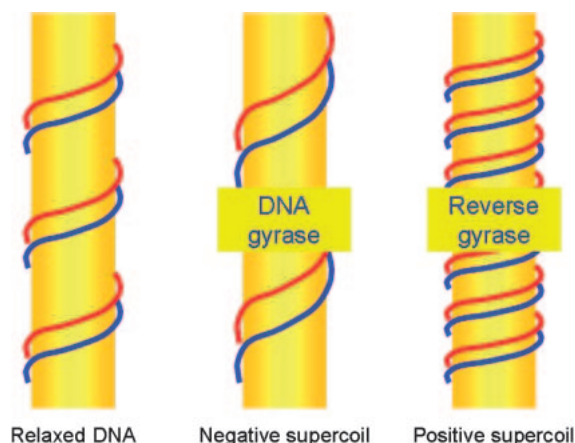
Fig. 19.   Function of reverse gyrase.

philes. (1) An ether bond between hydrocarbon and glycerol (ester bond in mesophiles). (2) Hydrocarbon chains are composed of isoprenoid (linear alkane or alkene in mesophiles). (3) Caldarchaeol in which two archaeol molecules are connected is sometimes used for membrane. Therefore, one caldarchaeol molecule is facing to inside and outside of the membrane. These complex characteristics are important to stabilize membrane (Fig. 20).[44–46]

6. Future Perspectives

The examples mentioned above describe some of the interesting aspects of enzymes, DNA, and cytoplasmic membranes from hyperthermophilic archaea. Besides their exceptional thermostability, many display distinct substrate and cofactor specificities, and novel primary and/or quaternary structures. Until now, our studies have been mainly biased towards biochemical and structural studies on the enzymes of strain KOD1. In future studies, we are planning to focus a little more on the cell itself, with its diverse metabolic and regulatory pathways. Elucidating the functions of hypothetical genes will be one of the main tasks to address. We have determined the complete genome sequence of strain KOD1,[12] and 2306 genes at most are present on the genome, and in some way, contribute to the activities in the cell. The relatively small number of genes implies that the mechanisms supporting life in strain KOD1 should also be relatively simple. We have also exploited an excellent gene disruption (replacement and deletion) method in this hyperthermophile;[47] the physiological function of the target gene (and gene product) can be directly analyzed. We hope this will lead us to better understand some of the basic principles of life. There is still a long ride ahead, but we are certain that it will be a "hot" one.
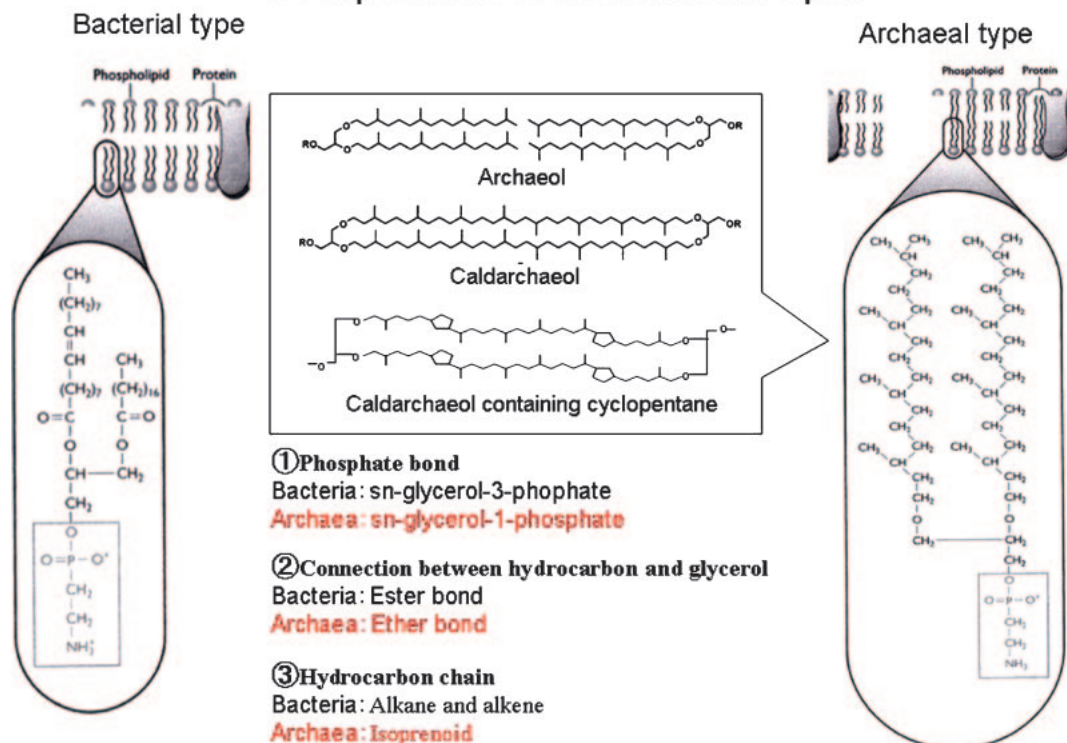
Fig. 20.   Comparison of membrane lipid.

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Tadayuki Imanaka, born in Dalian, Manchuria in 1945, graduated from Osaka University in 1967, completed his master's degree at the same University in 1969, and became a research associate (instructor) there in 1970. He received his Ph.D. degree in 1973. He was appointed Associate Professor at Osaka University in 1981 and promoted to Professor in 1989. He became Professor at Kyoto University in 1996. He spent a couple of years (1973–1974) as a post doctoral research associate at MIT. He attended the 46th Japanese Antarctic Research Expedition from 2004 to 2005. He is now a member of Science Council of Japan since 2005. His current research interests include extremophiles such as hyperthermophiles and their application. He was awarded Saito Prize of the Society of Fermentation Technology, Japan (1987), Biotechnology award of Society for Bioscience and Bioengineering, Japan (2001), Arima prize of Japanese Biotechnology Association (2001), Fellow of the American Academy of Microbiology (2003), The Chemical Society of Japan Award (2004), among others. He has been appointed as an Editorial Board Member of many international journals.