

Chapter 15

Heterologous Production of Thermostable Proteins and Enzymes

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Abstract In the last decade, the genes encoding hyperthermophilic proteins and enzymes have been extensively expressed in heterologous organisms such as *Escherichia coli*, and their good productions have been achieved. However, some difficulties are often encountered when attempting to produce these proteins in the mesophilic hosts. This chapter focuses on the recent efforts made to overcome problems in heterologous production of hyperthermophilic enzymes: (1) successful production of hetero-oligomeric dye-linked L-proline dehydrogenases by use of effective promoters, (2) a typical procedure for the in vitro refolding of inclusion bodies composed of several hyperthermophilic enzymes (malate dehydrogenase, lysine dehydrogenase, and agmatinase), and (3) heat-induced structural conversion of hyperthermophilic glutamate dehydrogenase. This information could be useful in successful production of hyperthermophilic proteins and enzymes.

Keywords Hyperthermophilic proteins and enzymes • Heterologous expression • Inclusion bodies • Mesophilic hosts

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15.1 Introduction

Thermophilic enzymes have been attracting much interest from industry because they offer several major biotechnological advantages over their mesophilic counterparts: (1) once produced in mesophilic hosts, they are easy to purify through heat treatment, which could provide a comparatively inexpensive supply of proteins and enzymes; (2) they have a higher resistance to a variety of denaturants, including organic solvents and detergents; and (3) they show greater stability under various storage conditions. Moreover, their higher stability permits more detailed examination of their structural features and functionality. The knowledge gained from such studies could lead to the development of a wide range of biotechnological applications. One of the early successes in the area of industrial enzymes was the application of thermostable amino acid dehydrogenases to the enantiomeric-specific synthesis and analysis of amino acids (Ohshima and Soda 1989, 2000).

Hyperthermophiles are a group of microorganisms that exhibit optimum growth at temperatures above 80°C (Stetter et al. 1990; Adams 1993). Almost all of these organisms are classified as archaea, the third domain of life (Woese et al. 1990), and show a strong potential to serve as a new source of enzymes that are much more stable than their counterparts from mesophiles or moderate thermophiles (Vieille and Zeikus 2001). In addition, structural comparison of hyperthermophilic and mesophilic enzymes is expected to provide us with a better understanding of the mechanisms by which highly thermostable proteins are stabilized. As genomic sequence data for hyperthermophiles have accumulated over the last decade, their genes have been extensively expressed in heterologous organisms such as *Escherichia coli*. Construction of a heterologous expression system is particularly important for expressing genes from hard-to-grow hyperthermophiles; however, difficulties are often encountered when attempting to produce these proteins in *E. coli*. These difficulties include weak expression of the gene of interest due to ineffective promoters, formation of inclusion bodies, and improper folding of the protein at low temperatures. Considerable effort has been made to overcome these problems, and interesting findings dealing with heat-induced protein folding in vitro have been reported. In this chapter, we address these recent efforts with an emphasis on the methods for successful production of hyperthermophilic enzymes.

15.2 Production of Hetero-Oligomeric Dye-Linked L-Proline Dehydrogenase

Dye-linked dehydrogenases (dye-DHs) catalyze the oxidation of various amino acids, sugars, organic acids, amines, and alcohols in the presence of artificial electron acceptors such as 2,6-dichloroindophenol (DCIP) and ferricyanide (Fig. 15.1). Dye-DHs generally function as mediators of electron transfer from a reduced substrate to an electron transfer system used to produce energy for the cell. Within cells, these

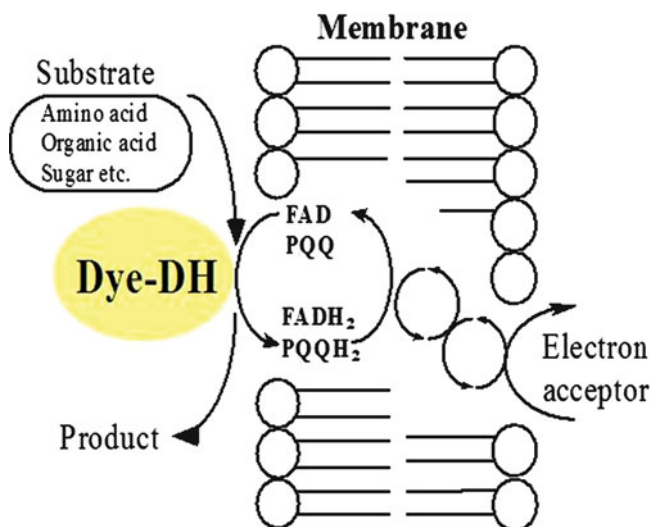


Fig. 15.1 Schematic diagram of dye-DHs

enzymes usually present in a membrane-associated or membrane-bound form, as a component of a complex containing several other proteins. Many types of dye-DHs have been identified in mesophilic microorganisms, and it has been suggested that they have the potential for use as specific elements in biosensors (Frew and Hill 1987). So far, however, their instability has limited the practical application of mesophilic dye-DHs. If dye-DHs are present in hyperthermophiles, their high stability would increase our ability to obtain useful information about their structures and functions and make them more amenable to practical application.

For several years, we have been extensively screening hyperthermophiles for dye-DHs. During that period, we have identified several dye-DHs in hyperthermophilic archaea, including a dye-linked L-proline dehydrogenase (LPDH) (Sakuraba et al. 2001; Kawakami et al. 2004, 2005; Satomura et al. 2010, 2011), a dye-linked D-proline dehydrogenase (Satomura et al. 2002), a dye-linked D-lactate dehydrogenase (Satomura et al. 2008), and a quinoprotein aldose sugar dehydrogenase (Sakuraba et al. 2010). We found that these enzymes remain stable for prolonged periods under a variety of conditions and some are applicable to a novel type of electrochemical detector for L-proline and D-proline (Tani et al. 2008, 2009; Zheng et al. 2010). Among these dye-DHs, LPDHs identified in the order Thermococcales have unique hetero-oligomeric structures.

LPDH catalyzes the oxidation of L-proline to Δ^1 -pyrroline-5-carboxylate (P5C) in the presence of an artificial electron acceptor. Two different types of LPDH, PDH1 and PDH2, have been identified in the anaerobic hyperthermophile *Pyrococcus horikoshii* OT-3 (Kawakami et al. 2005). PDH1 is a heterooctameric complex ($\alpha_4\beta_4$; molecular mass, 440 kDa) containing FAD, FMN, Fe, and ATP (Kawakami et al. 2005), while PDH2 is a tetrameric complex ($\alpha\beta\gamma\delta$; molecular mass, 120 kDa).

Structural analysis of the PDH1 complex showed the enzyme to be a unique diflavin dehydrogenase containing a novel electron transfer system totally different from that of the PDH2 complex, which contains L-proline dehydrogenase, NADH dehydrogenase, a ferredoxin-like protein, and a protein of unknown function (Kawakami et al. 2004; Tsuge et al. 2005). Proteins homologous to PDH1 and PDH2 are widely distributed among the hyperthermophilic archaea that belong to the phylum Euryarchaeota, including *Thermococcus profundus* (Sakuraba et al. 2001; Kawakami et al. 2004, 2005). For detailed analysis of the structures and functions of these LPDHs, heterologous expression of their genes in *E. coli* is required.

15.2.1 Identification and Cloning of the Gene Encoding PDH1

To identify organisms that produce LPDHs, we screened for enzymes using native PAGE coupled with activity staining. When we ran the crude extract of *P. horikoshii* OT-3 on a polyacrylamide gel, we detected two distinguishable activity bands for LPDH. This means that at least two types of LPDH (PDH1 and PDH2) are produced by this organism. After purification, PDH1 was found to be composed of two different subunits ($\alpha 1$ and $\beta 1$) with molecular masses of 56 and 43 kDa, respectively. The native molecular mass of PDH1 is 440 kDa, indicating PDH1 has an $\alpha 4\beta 4$ structure.

The N-terminal amino acid sequence of the $\alpha 1$ subunit was determined to be MRPLDLTEKR, which corresponds to the underlined amino acid sequence in MLMRPLDLTEKR from the putative protein encoded by the open reading frame (ORF; PH1363) predicted by the genome analysis. This means that the ATG situated 7 bp downstream from the 5'-terminus of the predicted ORF is the proper initial codon for the $\alpha 1$ gene. The N-terminal amino acid sequence of the $\beta 1$ subunit was determined to be MLPEKSEIVV, which corresponds to that of the predicted PH1364 gene product. The $\alpha 1$ and $\beta 1$ genes are arranged in tandem ($\alpha 1$ - $\beta 1$) and were estimated to encode proteins with molecular masses of 55,316 and 42,685 Da, respectively. Similar gene clusters have been observed in the genomes of *P. furiosus* (PF1245–PF1246) and *P. abyssi* (PAB1842–PAB1843), suggesting that homologues of this enzyme are widely distributed within the order Thermococcales in the archaeal domain.

To avoid nucleotide incorporation errors, we did not use the PCR method to clone the PDH1 gene. Instead, an oligonucleotide probe was synthesized based on the DNA sequence in the *P. horikoshii* genome database, after which the probe was labeled with ^{32}P and used as a specific probe for southern and colony hybridizations. To prepare the PDH1 gene, genomic DNA was digested with SphI and KpnI, and the resultant fragments were separated on agarose gels. Approximately 8.0 kbp of fragments were extracted from the gel and inserted between the SphI and KpnI sites of plasmid pUC19. The recombinant plasmid was then used to transform *E. coli* JM109 cells growing on a Luria-Bertani (LB) plate containing ampicillin. Thereafter, the transformants were subjected to colony hybridization,

which enabled the plasmid pPDH1, containing the PDH1 gene (insert length, 8.1 kbp), to be obtained.

15.2.2 Expression of the PDH1 Gene and Purification of Its Product

We initially attempted to express the PDH1 gene using the plasmid pPDH1, but no functional product was obtained. We therefore introduced the gene into pET11a (Novagen) and were then able to successfully express the gene. As mentioned, PDH1 forms a complex comprised of $\alpha 1$ and $\beta 1$ subunits. As a first step, separate systems for expressing the $\alpha 1$ and $\beta 1$ genes were developed, and then a double expression system for the genes was constructed. Two sets of PCR primers were prepared to construct the expression plasmids for $\alpha 1$ and $\beta 1$. In each set, the forward primer introduced a unique NdeI restriction site that overlapped the 5'-initiation codon, and the reverse primer introduced a unique SmaI or BamHI restriction site proximal to the 3'-end of the termination codon. PCR was carried out using pPDH1 as the template, after which the amplified fragments were digested with NdeI and SmaI for $\alpha 1$ or with NdeI and BamHI for $\beta 1$. For ligation to $\alpha 1$, plasmid pET11a was digested with BamHI, blunted, and then further digested with NdeI. For ligation to $\beta 1$, plasmid pET11a was digested with NdeI and BamHI. The $\alpha 1$ and $\beta 1$ gene fragments were introduced into pET11a after linearizing the plasmid with NdeI and blunted BamHI to generate pET11a/ $\alpha 1$, and with NdeI and BamHI to generate pET11a/ $\beta 1$. To construct the double expression system, pET11a/ $\alpha 1$ was digested with ClaI, blunted, and further digested with SphI. The resultant fragment containing $\alpha 1$ and the T7 promoter were introduced into pET11a/ $\beta 1$ digested with SphI and BglII (the BglII site had already been blunted) to generate the expression plasmid pEPDH1 (Fig. 15.2), which was then used to transform *E. coli* strain BL21 CodonPlus RIL (DE3) cells (Stratagene).

The transformed cells were grown for 8 h at 37°C in SB medium (1.2% tryptone peptone, 2.4% yeast extract, 1.25% K₂HPO₄, 0.38% KH₂PO₄, and 0.5% glycerol) containing ampicillin, after which isopropyl β -D-thiogalactopyranoside (IPTG) was added to 1 mM, and cultivation was continued for an additional 4 h. The transformants exhibited a high level of LPDH activity, which was not lost upon incubation at 90°C for 20 min. The enzyme was purified to homogeneity from the cell extract using heat treatment followed by successive Butyl Toyopearl and Superdex 200 gel filtration column chromatography steps. About 50 mg of the purified enzyme was obtained from 2 L of *E. coli* culture. The purified PDH1 showed the same mobility as the native enzyme on native PAGE, and the N-terminal amino acid sequences of the recombinant $\alpha 1$ and $\beta 1$ subunits were confirmed to be identical to those of the native enzyme. These results suggest that $\alpha 1$ and $\beta 1$ were properly expressed in *E. coli* only when they were cloned under the control of a strong promoter, but that the heterooctameric complex of the enzyme can be successfully produced in *E. coli*.

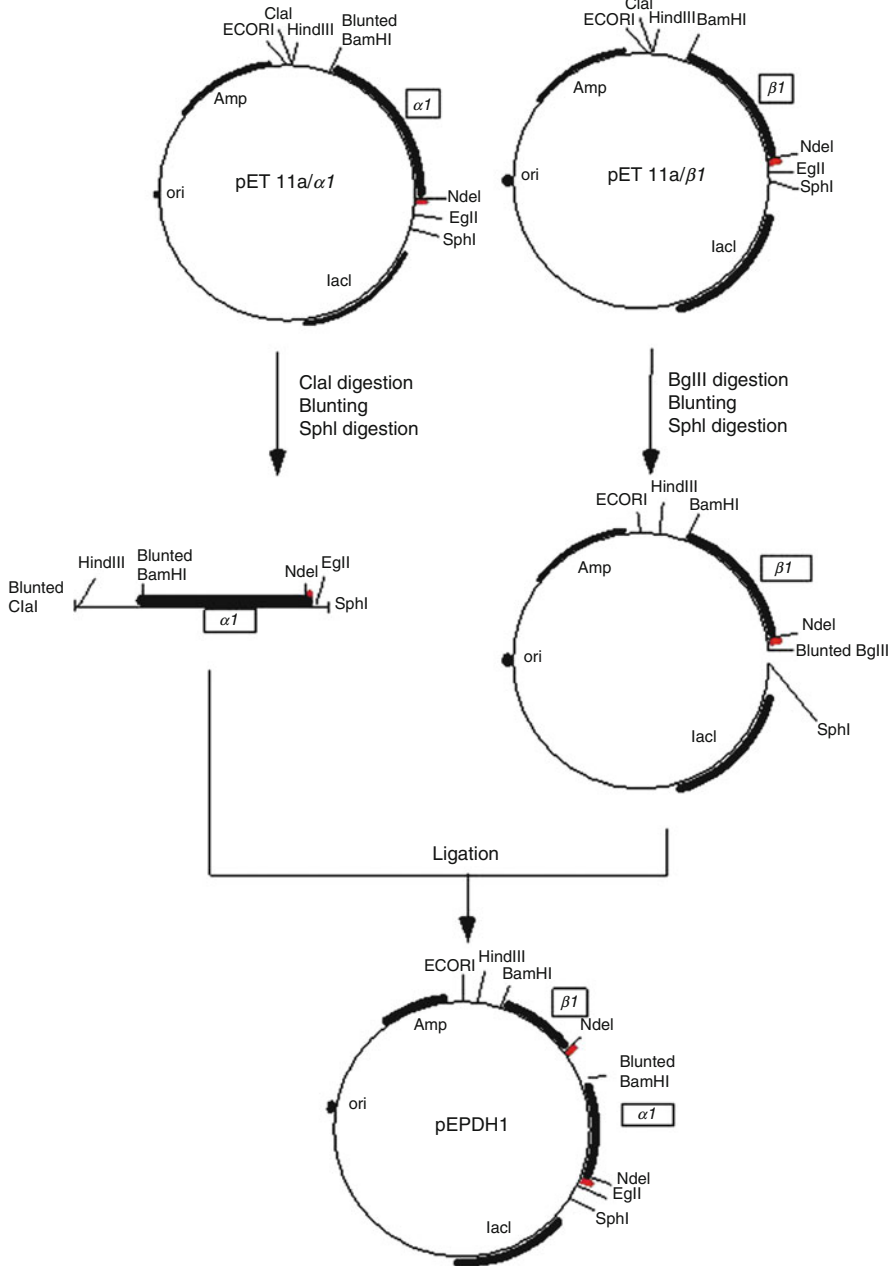


Fig. 15.2 Construction of a double expression system for the $\alpha 1$ and $\beta 1$ genes encoding the PDH1 subunits

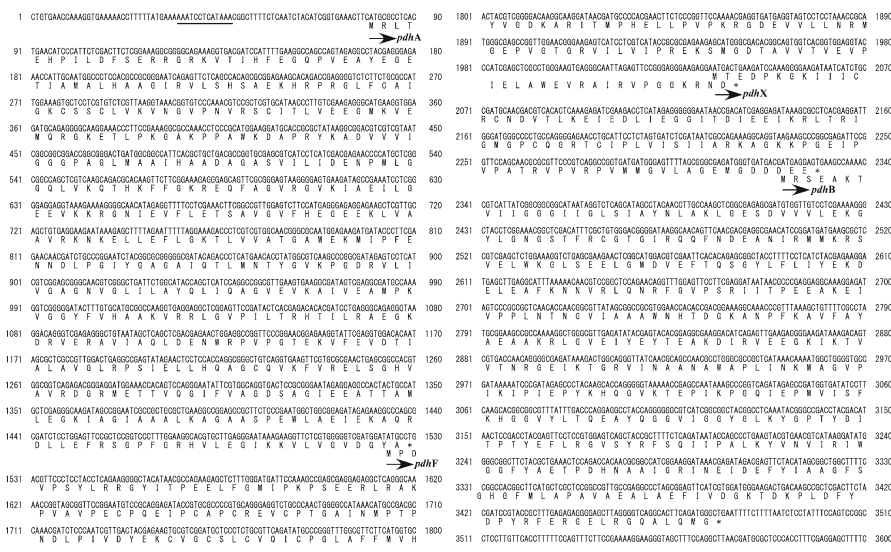


Fig. 15.3 Nucleotide and deduced amino acid sequences of a 3.6-kbp DNA fragment containing *pdhA*, *pdhF*, *pdhX*, and *pdhB* from *T. profundus*. The 3'-ends of *pdhA*, *pdhF*, and *pdhX* partially overlap the 5'-ends of *pdhF*, *pdhX*, and *pdhB*, respectively. A deduced promoter sequence is underlined. The figure cited in reference Kawakami et al. (2004) was modified in part

15.2.3 Expression of the Gene Encoding Heterotetrameric LPDH

The gene encoding the heterotetrameric LPDH from *T. profundus* ($\alpha\beta\gamma\delta$; a PDH2 homolog) forms an operon comprised of *pdhA*, *pdhF*, *pdhX*, and *pdhB*, in that order (Fig. 15.3). Within the operon, the 3' parts of *pdhA*, *pdhF*, and *pdhX* partially overlap the 5' parts of *pdhF*, *pdhX*, and *pdhB*, respectively. To clone this gene cluster, degenerate oligonucleotides were designed to be DNA probes for the genes, based on an amino acid sequence determined to be part of the N-terminal sequence of the α subunit of the natural LPDH, and were labeled with ^{32}P . The genomic DNA was then digested with PstI, and the resultant fragments were separated on agarose gels. An approximately 6-kb PstI fragment, which gave a positive signal on southern hybridizations labeled with the probe, was inserted into the PstI site of plasmid pUC18, which was then used to transform *E. coli* JM109 cells. The transformants were selected by colony hybridization on an LB plate containing ampicillin, which enabled us to obtain a plasmid, pUPDH19, containing the heterotetrameric LPDH gene (insert length; 5.8 kbp).

The pUPDH19 was digested with NruI and ligated to produce the expression vector pUPDH, which was used to transform *E. coli* JM109 cells. The cells were then grown for 12 h at 37°C in SB medium containing ampicillin and IPTG. The transformants exhibited highly thermostable LPDH activity, and the recombinant enzyme was purified to homogeneity from the cell extract using heat treatment

followed by successive Butyl Toyopearl and Red Sepharose CL-4B column chromatography steps. The N-terminal amino acid sequences of the α , β , γ , and δ subunits of the purified enzyme coincided with those deduced from the respective gene sequences, and the α : β : γ : δ molar ratio was calculated to be about 1:1:1:1 based on SDS-PAGE analysis. These results indicate that, in contrast to the *P. horikoshii* PDH1, the gene cluster for the *T. profundus* LPDH was directly expressed using its own promoter and the heterotetrameric enzyme complex was successfully produced in *E. coli*.

Because archaeal transcription systems are generally closer to eukaryal systems than to bacterial ones, it is not surprising that most archaeal genes are expressed in *E. coli* only when they are cloned under the control of strong promoter such as the T7 promoter. In the order Thermococcales, however, *E. coli* consensus promoter-like sequences are often found within intergenic regions, and some of the genes can be expressed in *E. coli* without introduction of strong promoters. Upstream of the start codon in *pdhA*, which encodes the α subunit of the *T. profundus* LPDH, a promoter-like sequence, AAATCCTCATAAA, was identified (Fig. 15.3). The presence of this region might explain why *T. profundus* LPDH gene is directly expressed in *E. coli*.

15.3 In Vitro Refolding of Hyperthermophilic Enzymes

In *E. coli*-based expression systems, the recombinant protein is often produced as an insoluble aggregate called an inclusion body. The formation of such inclusion bodies results from an anomaly in the protein folding. To produce the protein in a soluble form, a co-expression or fusion system that includes the target protein and a molecular chaperone has to be developed. For hyperthermophilic enzymes, an expression system entailing the fusion of the target protein with an archaeal FK506 binding protein (FKBP) has recently been reported (Ideno et al. 2004). The archaeal FKBP from a hyperthermophilic archaeon, *Thermococcus* sp. KS-1 (TcFKBP18), possesses both peptidyl-prolyl *cis-trans* isomerase activity and chaperone-like activity that increases the refolding yield from unfolded protein by suppressing irreversible protein aggregation (Iida et al. 1998; Ideno et al. 2001). For example, a putative rhodanese from the aerobic hyperthermophilic archaeon *Aeropyrum pernix* forms insoluble aggregates when expressed alone in *E. coli*. However, by modifying its gene (APE_2595.1) so that the rhodanese was expressed as a fusion protein, connecting to the C-terminal end of TcFKBP18 significantly increased its production in the soluble fraction. Using this approach, the percentage of the soluble form of the expressed protein reached 28% of the host soluble proteins (Ideno et al. 2004). That said, it is still unclear whether this FKBP fusion system is sufficient to reduce inclusion body formation with other hyperthermophilic enzymes. In addition, it has been reported that co-expression of APE_2595.1 with TcFKBP18 gene does not improve protein

production in the soluble fraction of *E. coli* (Ideno et al. 2004). This may indicate that intermolecular interaction between the refolding protein and TcFKBP18 enhances neither the proper folding nor the soluble production of the target protein, although intramolecular interactions do.

On the other hand, inclusion bodies composed of several hyperthermophilic enzymes have been properly refolded, and soluble enzyme preparations have been successfully obtained through the use of conventional protein-folding procedures, which entail solubilization of the aggregate using a denaturant, refolding through removal of the denaturant, and purification of the refolded protein. Although these conventional protocols are insufficient for use in all the cases, careful optimization of the folding conditions can often enable efficient refolding of the target proteins. In this section, we introduce a typical procedure for the *in vitro* folding of a hyperthermophilic enzyme.

15.3.1 Cloning and Expression of the Gene Encoding Malate Dehydrogenase

Tartrate oxidation activity was found in the crude extract of *A. pernix*, and the enzyme was identified as (S)-malate dehydrogenase (ApeMDH). Because there were no earlier reports of an MDH exhibiting strong catalytic activity toward tartrate, we endeavored to express the ApeMDH gene (APE_0672.1) in *E. coli* and characterize the purified enzyme (Kawakami et al. 2009).

The gene was amplified by PCR using a set of oligonucleotide primers in which a unique NdeI restriction site that overlapped the 5'-initiation codon was introduced in the forward primer and a unique BamHI restriction site proximal to the 3'-end of the termination codon was introduced in the reverse primer. Chromosomal *A. pernix* DNA was used as the template. The amplified 0.9-kb fragment was digested with NdeI and BamHI and ligated with the expression vector pET11a linearized with NdeI and BamHI to generate pET/ApeMDH. *E. coli* BL-21 CodonPlus (DE3)-RIL cells were then transformed with pET/ApeMDH, after which the transformants were cultivated for 8 h at 37°C in LB medium supplemented with ampicillin. Thereafter, expression was induced by adding 1 mM IPTG to the medium, and cultivation was continued for an additional 3 h at 37°C.

In the *E. coli* transformants, ApeMDH was present in both the soluble and insoluble fractions but was found mainly as an inclusion body. The MDH activity in the soluble fraction was unaffected by incubation at 90°C for 10 min, confirming that APE_0672.1 does indeed encode a hyperthermostable MDH. However, the protein in the soluble fraction migrated as an aggregate on native PAGE. Because changing the cultivation temperature or the conditions under which the cells were disrupted did not prevent this aggregation, we decided to carry out refolding of the inclusion body.

Solubilization of inclusion body

Centrifuge tube

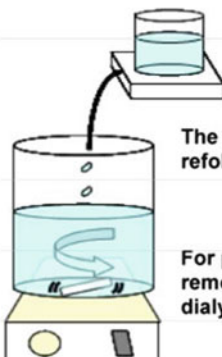


Denaturant solution was slowly added to the centrifuge tube without disturbing the pellet.

The tube was then left to stand overnight, which allowed the inclusion body to dissolve spontaneously.

The tube was centrifuged to remove any insoluble materials.

Refolding



The solubilized protein was gently dropped into refolding buffer containing 0.4 M L-arginine.

For proteins that tend to form aggregates, removal of the L-arginine in a stepwise fashion by dialysis is strongly recommended.

Fig. 15.4 Scheme of the procedure for refolding ApeMDH. Essentially the same method has been used for the refolding of agmatinase and LysDH from *P. horikoshii*

15.3.2 Refolding and Purification of the Recombinant MDH

The scheme for a typical refolding procedure is illustrated in Fig. 15.4. To purify ApeMDH from the inclusion bodies, *E. coli* transformants were collected (20 g wet weight from 2 L of culture), washed with 0.85% NaCl, suspended in 100 ml of 10 mM Tris-HCl buffer (pH 7.5), and disrupted by sonication. The homogenate was centrifuged at 20,000 $\times g$ for 10 min at 4°C, and the pellet was resuspended by sonication in 100 ml of 10 mM Tris/HCl (pH 7.5) buffer containing 1 mM EDTA and 4% Triton X-100. The suspension was then incubated for 30 min at room temperature and centrifuged again. This procedure was repeated twice. The resultant pellet was washed twice with 200 ml of Milli Q water, and then the denaturant solution

(10 ml; 50 mM Tris-HCl buffer (pH 7.5) containing 6 M guanidine HCl, 0.2 M NaCl, and 1 mM EDTA) was slowly added to the centrifuge tube without disturbing the pellet. The tube was then left to stand overnight at 4°C, which allowed the inclusion body to dissolve spontaneously, after which the tube was centrifuged at 20,000 ×g for 10 min at 4°C to remove any insoluble material. The protein concentration of the resultant supernatant was calculated based on the absorbance at 280 nm (Gill and von Hippel 1989) and adjusted to 1 mg/ml by dilution with the denaturant solution.

The solubilized ApeMDH (200 mg of enzyme in 200-ml solution) was gently dropped into refolding buffer (1.5 L of 0.1 M Tris/HCl (pH 7.5) containing 2 mM EDTA and 0.4 M L-arginine) and then incubated for 36 h at 4°C. The resultant enzyme solution (1.7 L) containing the refolded ApeMDH was then concentrated to a volume of 50 ml using Vivaflow 50 ultrafiltration modules (30000 MWCO module × 6) (Sartorius Stedim Biotech), dialyzed against 50 mM Tris/HCl (pH 7.5) buffer containing 0.2 M NaCl, and centrifuged at 20,000 ×g for 10 min at 4°C to remove the insoluble material. The supernatant was then further concentrated to a volume of 15 ml using an Amicon Ultra Centrifuge Filter (30000 MWCO) (Millipore), and an aliquot (5 ml) of the resultant solution was applied to a Superdex 200 gel filtration column (2.6 × 60 cm, GE Healthcare) equilibrated with 50 mM Tris/HCl (pH 7.5) buffer containing 0.2 M NaCl. The eluate was fractionated, and the active fractions were pooled and used for biochemical and structural experiments. All solutions used for the refolding procedures were filtered through 0.45-μm membrane filters (Advantec) to remove dust and any other impurities.

The protein in the washed inclusion body was over 95% pure, as judged from the SDS-PAGE, and the amount of protein solubilized by the guanidine HCl was estimated to be about 200 mg based on the absorbance at 280 nm. The refolded enzyme catalyzed malate dehydrogenation, confirming that active ApeMDH was successfully recovered from the inclusion body. After further purification using gel chromatography, the mobility of the refolded ApeMDH in native PAGE was the same as that of the natural ApeMDH. With this procedure, about 70 mg of purified ApeMDH were obtained from 2 L of culture.

15.3.3 Refolding of Other Hyperthermophilic Enzymes

Agmatinase and L-lysine 6-dehydrogenase (LysDH) from *P. horikoshii* were refolded from inclusion bodies using a method that was essentially the same as the one described above (Goda et al. 2005b; Yoneda et al. 2010). The agmatinase inclusion bodies were solubilized in a denaturant solution composed of 50 mM Tris/HCl (pH 8.0), 6 M guanidine HCl, 200 mM NaCl, and 1 mM EDTA. The solubilized enzyme was then added to refolding buffer (0.1 M Tris/HCl (pH 8.0), 0.4 M L-arginine, 2 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)) at 4°C and further incubated for about 40 h. The resultant enzyme solution was concentrated and dialyzed to remove L-arginine, and the active enzyme

was successively recovered in the supernatant after centrifugation. The efficiency of the refolding was calculated to be 67% based on the absorbance at 280 nm. After the refolded agmatinase was further purified using a gel filtration column, about 45 mg of soluble homogeneous enzyme was obtained from 200 ml of *E. coli* culture.

To refold the LysDH from *P. horikoshii*, the inclusion bodies were solubilized in 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM β -mercaptoethanol, 6 M guanidine HCl, 0.2 M NaCl, and 1 mM EDTA. The solubilized LysDH was added to a refolding buffer containing 0.1 M Tris-HCl (pH 7.5), 5 mM β -mercaptoethanol, 0.4 M L-arginine, 2 mM EDTA, and 0.1 mM PMSF and incubated for 36 h at 4°C, after which the resultant enzyme solution was concentrated and dialyzed to remove L-arginine. The refolded LysDH solution was then heated at 80°C for 10 min and clarified by centrifugation. The protein in the supernatant was then further purified by running it on a gel filtration column. In the *E. coli* transformants, LysDH was present in both the soluble and insoluble fractions but was found mainly as an inclusion body. When soluble enzyme was purified from the crude extract of the transformant cells using heat treatment and Ni²⁺-chelating affinity chromatography, only 1.7 mg of the purified enzyme was obtained from 7.3 g of cells (wet weight). However, the refolding procedure increased the yield to 7.8 mg of the purified enzyme from the same starting material.

In all cases, 0.4 M L-arginine was added to the refolding buffer to prevent irreversible protein aggregation. For proteins that tend to form aggregates, removal of the L-arginine in a stepwise fashion by dialysis is strongly recommended. If the native protein contains metal ions, EDTA should be omitted from the denaturant solution and refolding buffer. Alternatively, an excess of the respective metal ion should be added to the refolding buffer. Similarly, addition of other ligands that bind to the native protein is required.

15.4 Heat-Induced Structural Conversion of Hyperthermophilic Enzymes

When the properties of native and recombinant hyperthermophilic enzymes are compared, a majority of the hyperthermophilic enzymes expressed in *E. coli* retain all of the native enzyme's biochemical properties, including its thermostability and its optimal pH and optimal activity at high temperatures. Thus, most proteins from hyperthermophiles are thought to be able to fold properly even at temperatures much lower (e.g., room temperature) than their physiological temperatures (80–100°C). On the other hand, over the last decade, there have been several reports showing that recombinant enzymes derived from hyperthermophiles and expressed in *E. coli* are not present in the same form as the corresponding native enzymes.

Glutamate dehydrogenases (GDHs; EC 1.4.1.2-4) are a widely distributed group of oligomeric oxidoreductases whose structural characteristics and structure-function relationships have been well studied. Some GDHs derived from hyperthermophiles

are expressed as inactive forms in *E. coli*, but in vitro application of heat is sufficient to convert the inactive recombinant form to one more similar to the active native form. This suggests that high temperature has a crucial effect on the folding, oligomerization, and subunit arrangement of hyperthermophilic GDHs. DiRuggiero and Robb (Diruggiero and Robb 1995) originally described the heat-induced activation of inactive recombinant *P. furiosus* GDH. In that case, the enzyme was expressed in *E. coli* as a mixture of both monomeric and hexameric forms, but the inactive monomers in the solution assembled into the active hexameric form upon application of heat. Similar heat-induced activation of hexameric GDH was reported for the recombinant GDH from *T. kodakaraensis* (formerly *Pyrococcus* sp. KOD1)(Abd Rahman et al. 1997). Heating the enzyme at 90°C in vitro induced the conversion from a low-activity form to a high-activity form, but the specific activity of the heat-activated enzyme was still not as high as that of the native enzyme (Abd Rahman et al. 1997), suggesting an additional factor is required for the full activation of *T. kodakaraensis* GDH. Although similar heat-induced structural conversions of several other thermostable proteins have been reported (Schultes and Jaenicke 1991; Rehaber and Jaenicke 1992; Siddiqui et al. 1998; Schroder et al. 2004), the mechanisms of these conversions are not yet fully understood. To obtain more detailed information, we cloned and expressed the gene encoding GDH from the hyperthermophilic archaeon *Pyrobaculum islandicum* (pis-GDH) in *E. coli* (Kujo et al. 1999). Unlike the two aforementioned enzymes, the recombinant pis-GDH always assumed a hexameric form but exhibited extremely low specific activity, nevertheless. Heating the inactive enzyme increased its activity to a level comparable to that seen with the native enzyme. This suggests that the mechanism underlying the heat-induced activation of recombinant pis-GDH differs from those underlying the activation of *P. furiosus* and *T. kodakaraensis* GDHs. We therefore used small-angle X-ray scattering (SAXS) and 1-anilinonaphthalene-8-sulfonic acid (ANS) fluorescence analyses to examine the structural changes that accompany activation of recombinant pis-GDH (Goda et al. 2005a).

15.4.1 Preparation of Recombinant pis-GDH

Native pis-GDH was purified from *Pb. islandicum* cells using previously described procedures (Kujo and Ohshima 1998). In addition, the enzyme was expressed in *E. coli* as follows. The expression vector pKGDH1 was constructed as described previously (Kujo et al. 1999), after which the *pis-gdh* gene in pKGDH1 was subcloned into a pET11a vector to obtain a better producer. The DNA fragment containing *pis-gdh* was then amplified by PCR using two primers containing NdeI and BamHI restriction sites, after which the PCR product was inserted into the NdeI and BamHI sites of pET11a, yielding pEGDH2. *E. coli* BL21 (DE3)-codon plus-RIL cells carrying the recombinant plasmid were grown at 37°C in LB medium (2.1 L) containing ampicillin. After cultivation for 9 h, expression of the enzyme was induced by addition of 1 mM IPTG, and the cultivation was continued for an additional 3 h. The cells were then collected (11.6 g wet weight); suspended in 10 mM potassium

phosphate buffer (pH 7.2) containing 10% glycerol, 1 mM EDTA, and 0.1 mM DTT (buffer A); and disrupted by sonication. After centrifugation ($16,000\times g$ for 15 min), the soluble fraction was used as the crude extract. Further purification of the recombinant enzyme was carried out at 4°C to prevent heat-induced activation of the enzyme. The crude extract was applied to a Red Sepharose CL-4B column (Ohshima and Sakuraba 1986) previously equilibrated with buffer A. After washing the column with buffer A, the enzyme was eluted with the same buffer containing 0.5 M NaCl, and the active fractions were pooled. The enzyme solution was then dialyzed against buffer A and applied to a DEAE-Toyopearl column (Tosoh) equilibrated with buffer A. When the column was washed with the same buffer, the enzyme was found in the eluate, as it was not adsorbed onto the column. The enzyme solution was therefore applied to a hydroxyapatite column (GIGAPITE K-100S) (Seikagaku Kogyo) equilibrated with buffer A. After being washed with the same buffer, the enzyme was eluted with a linear gradient of 10–300 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 7.2) containing 10% glycerol, 1 mM EDTA, and 0.1 mM DTT. The active fractions were pooled, dialyzed against buffer A, and used as the purified enzyme preparation. At each step, an aliquot of the enzyme solution was dialyzed against buffer A and heated at 90°C for 15 min to assess the heat-treated enzyme activity.

15.4.2 *Properties of Recombinant pis-GDH*

The extract from *E. coli* expressing recombinant pis-GDH exhibited a low level of enzyme activity that was markedly increased by heating at 90°C for 30 min. Likewise, the specific activity of the purified recombinant enzyme was much lower than that of the native enzyme, but heating (from 37 to 110°C) enhanced its activity. The effect of heat was both temperature and time dependent, with heating to 90°C for 15 min resulting in an increase in the enzyme's activity to a level comparable to that seen with native pis-GDH (Table 15.1). Gel filtration of the recombinant pis-GDH showed an activity peak corresponding to a molecular mass of 280 kDa. In addition, the recombinant enzyme migrated as a single band on SDS-PAGE, from which the molecular mass was calculated to be 47 kDa. Thus, like native pis-GDH, the most active recombinant enzyme was a hexamer (Kujo and Ohshima 1998). The kinetic parameters for oxidative deamination by the native and recombinant GDHs are summarized in Table 15.1. Note that the K_m values of the heat-activated enzyme for NAD and L-glutamate are similar to those of the native enzyme.

15.4.3 *Structural Differences Between Inactive and Heat-Activated Recombinant pis-GDHs*

Measurements of SAXS were carried out with inactive and heat-activated recombinant pis-GDHs, and the values obtained before and after activation are summarized in Table 15.2. The radius of gyration, $R_{g,z}$ (Kratky et al. 1951; Guinier and Fournet 1955)

Table 15.1 Kinetic properties of native and heat-activated recombinant pis-GDHs

pis-GDH	Specific activity	Km (mM)	
	($\mu\text{mol}/\text{min}/\text{mg}$)	L-glutaminate	NAD
Native	3.51	0.16	0.019
Heat-activated	3.65	0.16	0.021

Table 15.2 Structural parameters of inactive and heat-activated recombinant pis-GDHs determined by SAXS

State	$R_{g,z}$ (\AA)	$M_{w,w}$ (kDa)	D_{max} (\AA)
Inactive	54.6 ± 0.1	280 ± 26	145 ± 3
Heat-activated	46.5 ± 0.1	299 ± 14	124 ± 3

reflects the molecular shape and size, while D_{max} gives the maximum particle dimension. In addition, the weight average molecular mass, $M_{w,w}$, was estimated as described previously (Glatter and Kratky 1982; Kajiwara and Hiragi 1996). The $R_{g,z}$ values for the inactive and heat-activated enzymes were determined to be 54.6 \AA and 46.5 \AA , respectively. That the radius of gyration of the inactive enzyme is 8 \AA larger than that of the activated form means that heat can cause the nascent recombinant pis-GDH to undergo a change in its quaternary structure from a loose assembly of subunits to a much more compact one. Correspondingly, D_{max} decreased from 145 \AA for the inactive enzyme to 124 \AA for the heat-activated enzyme, confirming that heat causes the enzyme molecule to become substantially smaller. On the other hand, the values of $M_{w,w}$ were the same, within the experimental error, clearly indicating that the molecular masses of the two proteins were unaffected by heat. These values are nearly the same as the molecular mass of hexameric pis-GDH (280 kDa).

Although the crystal structure of heat-activated pis-GDH (PDB entry 1V9L) has been described previously (Bhuiya et al. 2005), the structure of the inactive recombinant enzyme remains unknown. We therefore used DAMMIN to carry out ab initio modeling of the low-resolution structure of the recombinant enzyme from the scattering curve (Svergun 1999). Because DAMMIN allows one to apply symmetry restrictions to a model's solution, we restored the low-resolution structure of the heat-activated pis-GDH assuming 32-point symmetry, like that of the crystal structure (Bhuiya et al. 2005), and the resultant model was well fitted to the crystal structure (Fig. 15.5a, b). We found that the heat-activated enzyme has one threefold axis passing through two identical trimers, which face one another, yielding a whole molecule having a cylindrical shape (Fig. 15.5a, b, lower row) with one trimer stacking on the other and forming a symmetrical interface (Fig. 15.5a, b, upper row). By contrast, the ab initio modeling of the inactive enzyme yielded better results with no internal symmetry than with 32-point symmetry. This suggests the inactive form may have no internal symmetry, despite the same total number of subunits; consequently, no subunit from one trimer superimposes on the corresponding subunit of the other (Fig. 15.5c, upper row). Another structural feature of the inactive pis-GDH is that it is not cylindrical. It is instead more spread out, especially

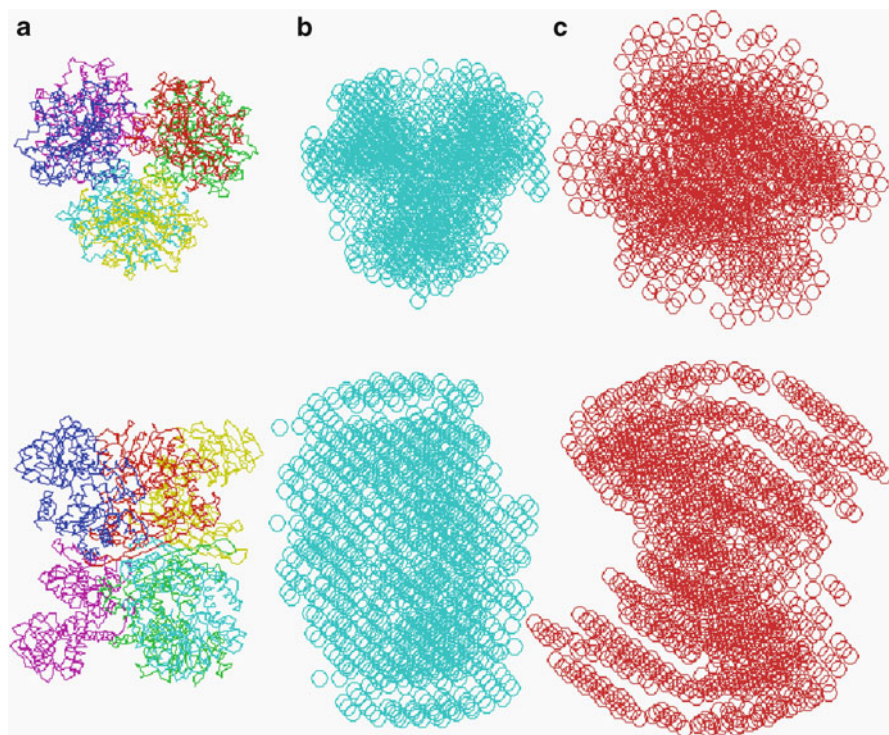


Fig. 15.5 Crystal and low-resolution structures of pis-GDH. **(a)** Crystal structure of heat-activated pis-GDH. **(b)** Low-resolution structure of the heat-activated enzyme with 32-point symmetry. **(c)** Low-resolution structure of the inactive enzyme with no symmetry. The *upper row* is the hexameric model viewed down the threefold axis. The *lower row* are views down the twofold axis. The figure cited in reference Goda et al. (2005a) was modified in part

at the surface of the molecule (Fig. 15.5c, lower row). This also suggests that the inactive enzyme is comprised of a loose and improper arrangement of subunits. The relative hydrophobicity of the recombinant proteins was evaluated as a function of ANS fluorescence intensity. When inactive pis-GDH was incubated with ANS, the resultant fluorescence spectrum (excitation wavelength, 350 nm) exhibited intense emission with a maximum at 446 nm. By contrast, the fluorescence spectra of the ANS-modified native and heat-activated enzymes were nearly identical and far weaker than that of the inactive enzyme. This means that there are a greater number of exposed hydrophobic residues on the surface of the inactive enzyme than on the native or heat-activated enzyme and suggests that activation causes those hydrophobic residues to be moved to the interior of the oligomer. The presence of buried hydrophobic residues at the intersubunit interface in the crystal structure (Bhuiya et al. 2005) of the active enzyme strongly suggests that the major source of stability against extremely high temperature is the much stronger hydrophobic interaction of these residues. The crystal structures thus substantiate the notion that, upon heat

activation, each subunit of the enzyme refolds, and the hydrophobic residues on the surface move to the intersubunit interface.

Overall, the process of heat-induced activation of pis-GDH expressed in *E. coli* can be summarized as follows: (1) the inactive enzyme is comprised of a loose and unstable arrangement of subunits that is perturbed by heat, leading the protein to settle into a tighter and more stable arrangement; (2) during this process, hydrophobic residues on the surface are brought to the interior interface between subunits where they promote the hydrophobic association of the subunits within the oligomer. These results strongly suggest that subunit rearrangement – i.e., a change in the quaternary structure of the hexameric recombinant pis-GDH – is essential for activation of the enzyme.

15.5 Conclusions

Hyperthermophilic enzymes have become model systems with which to study enzyme stability mechanisms and protein structure-function relationships. Moreover, these enzymes are now attracting a great deal of interest due to their potential to serve as both analytical tools and as biocatalysts for application. The recent advances in this field have been facilitated by the cloning and expression of hyperthermophilic genes in mesophilic hosts, although difficulties are still encountered with heterologous production of some hyperthermophilic enzymes. In this chapter, we presented an overview of several recently developed protocols for the proper production of these enzymes in *E. coli*, as well as protein-folding protocols. As additional related information leads to the production of still more recombinant proteins at high yields, the functional and structural analysis of hyperthermophilic enzymes and their industrial application will be greatly accelerated.

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