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Stabilization of *Taq* DNA Polymerase at High Temperature by Protein Folding Pathways From a Hyperthermophilic Archaeon, *Pyrococcus furiosus*

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Abstract: *Pyrococcus furiosus*, a hyperthermophilic archaeon growing optimally at 100°C, encodes three protein chaperones, a small heat shock protein (sHsp), a prefoldin (Pfd), and a chaperonin (Cpn). In this study, we report that the passive chaperones sHsp and Pfd from *P. furiosus* can boost the protein refolding activity of the ATP-dependent Cpn from the same hyperthermophile. The thermo-stability of *Taq* polymerase was significantly improved by combinations of *P. furiosus* chaperones, showing ongoing protein folding activity at elevated temperatures and during thermal cycling. Based on these results, we propose that the protein folding apparatus in the hyperthermophilic archaeon, *P. furiosus* can be utilized to enhance the durability and cost effectiveness of high temperature biocatalysts.

Keywords: *Pyrococcus furiosus*; molecular chaperones; heat shock proteins; chaperonin; prefoldin and DNA polymerase; biocatalysis

INTRODUCTION

Hyperthermophiles are defined as microorganisms that grow optimally at or above 80°C. Their high temperature resistance raises questions regarding the protein chaperones that can fold proteins at very high temperatures. In common with other hyperthermophiles, *Pyrococcus furiosus*, an archaeon that grows optimally at 100°C, encodes a reduced set of protein chaperones compared with eukaryotes or Archaea with lower growth temperatures (Laksanalamai et al., 2004). In the *P. furiosus* genome (Robb et al., 2001), only two chaperones, the small heat shock protein (sHsp) and the Hsp60 (chaperonin), have been annotated, expressed, and characterized so far. In addition, several putative chaperones, such as prefoldin (Pfd), HtpX, and Nascent peptide Associated Complex (NAC) have been identified (Laksanalamai et al., 2004). The most extensively studied chaperone in *P. furiosus* is the sHsp, which is an α-crystallin homolog with conserved sequence motifs in common with sHsps and crystallins from all domains of life (Chang et al., 1996; Haley et al., 2000; Kim et al., 1998; Laksanalamai et al., 2001, 2003; van Montfort et al., 2001). Several lines of evidence indicate that sHsps can prevent denatured proteins from aggregating but are unable to refold non-native proteins in a catalytic fashion (Chang et al., 1996; Laksanalamai et al., 2001). Hsp60s on the other hand catalyze ATP-dependent protein folding (Hartl, 1996; Hartl and Hayer-Hartl, 2002). In this study, we tested the known chaperones from *P. furiosus* singly and in combinations to enhance the stability of DNA polymerases during functional enzyme-driven processes such as PCR. In addition to the sHsp and Hsp60, we also expressed and purified the *P. furiosus* Pfd α and β subunits, chaperones that had previously been shown to function together with Hsp60 complexes in studies with a related archaeon, *Pyrococcus horikoshii* (Okochi et al., 2002, 2005). In this study, we showed clearly that combinations of molecular chaperones from *P. furiosus* can improve thermostability of Taq polymerase.

Abbreviations used: sHsp, small heat shock protein; Pfd, prefoldin; Cpn, chaperonin; NAC, Nascent peptide Associated Complex.

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MATERIALS AND METHODS

Preparation of Recombinant Chaperones

Pyrococcus furiosus sHsp and Hsp60 were cloned, expressed, and purified as previously described by Laksanalamai et al. (2001) and Emmerhoff et al. (1998), respectively. The genes encoding *P. furiosus* Pfd α and β subunits were identified based on the homology between the *P. furiosus* and *P. horikoshii* sequences (Okochi et al., 2002). The genes were amplified by PCR using the following primers: Pfd α: 5′

primer, CCATATGGAAAACAATAAGGAATTGGAAA-AGGTTGCT and 3′ primer, CCTCGAGTCACTTCTTAAG-CTTGAAGCTCATTGCTTG; Pfd β: 5′ primer, CCATAT-GAATCCCAGGGTGTGCCAAGTGGATTTAGG and 3′ primer, CCTCGAGTCATCCAGCGGTTGGAGGTCTTA-GGGCTGCCTGAATC. The underlined letters indicate the restriction sites used for insertion. The PCR products were subsequently cloned into the expression vector, pET19b (Novagen, San Diego, CA) and transformed into BL21 competent cells (Novagen). The recombinant Pfds were affinity purified on a nickel column (Ni-NTA, Qiagen, Valencia, CA) based on their N-terminal his-tags according to the product protocol.

Effect of the Small Heat Shock Protein on PCR Reactions

PCR reactions (45 μL final volume) were prepared as follows: 1× *Taq* polymerase buffer (100 mM Tris-HCl, pH 9.0, 500 mM KCl, and 15 mM $MgCl_2$), 1 mM dNTP, 1 μM each of forward and reverse primers, 20 ng of DNA template. A stock solution of *Taq* polymerase (5U/μL, Cat: FB600030 Lot: 005975) was diluted five- and tenfold to provide final concentrations of 0.025, 0.005, and 0.0025 U/μL in the PCR reactions. From a stock solution of a purified recombinant sHsp in Buffer A (25 mM potassium phosphate buffer, pH 7.0, 2 mM dithiothreitol, and 1 mM EDTA) (Laksanalamai et al., 2001), sHsp was added to the PCR reaction providing a final concentration of 0.2 μg/μL. Control reactions without sHsp were performed with the addition of buffer A and water to adjust the total volume to 50 μL. The effect of sHsp on the concentration of *Taq* polymerase in the reaction mixtures was examined by PCR amplification with *P. furiosus* genomic DNA and a pair of primers that generate a 700 kb fragment.

Effect of Chaperones on Thermostability of *Taq* DNA Polymerase at 100°C

Commercial Taq polymerase enzyme was diluted in 10 mM Tris-HCl pH 8.0 and 0.5M NaCl to the final concentration of 0.5 U/μL in the presence of various combinations of the recombinant chaperones including individual subunits of Pfd, Pfd complex, Hsp60, and sHsp. Heat inactivation of the polymerase was carried out at 100°C and the samples were removed at the indicated times. The residual activity of the polymerase was subsequently measured by quantitative primer extension assays. Mixtures (25 μL) were incubated in PTC-150 Minicycler (MJ Research, Waltham, MA) at 100°C. Samples (3 μL) were removed at defined times of incubation and assayed for primer extension activity. The primer-extension assay was performed with a fluorescent duplex substrate containing a primer–template junction (PTJ). The duplex was prepared by annealing a 5′-end labeled with fluorescein 20-nt long primer with a 30-nt long template:

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FL-GTAATACGACTCACTATAGGG  ->extension
    ||||||||||||||||||||
   CATTATGCTGAGTGATATCCCAAAAACCCCC  .
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DNA polymerase reaction mixtures (15 μL) contained dATP, dTTP, dCTP, and dGTP (1 mM each), 4.5 mM $MgCl_2$, detergents Tween 20 and Nonidet P-40 (0.2% each), 0.2 μM PTJ, and appropriately diluted Taq polymerase. The primer extension was initiated by addition of the polymerase and carried out for 3 min at 70°C in PTC-150 Minicycler (MJ Research). The extension products were applied on an ABI Prism 377 DNA sequencer (Applied BioSystems, Foster City, CA) and the florescent signals were analyzed as described in (Pavlov et al., 2002).

RESULTS AND DISCUSSIONS

Molecular chaperones function to restrain denatured proteins from aggregation and/or to assist protein folding (Barral et al., 2004; Hartl and Hayer-Hartl, 2002; Yon, 2001; Young et al., 2004). In this study, we used *Taq* DNA polymerase, an important enzyme in biotechnology (Dalton, 1999, 2001; Pavlov et al., 2004), as a model substrate for examining the effects of chaperones on stability and durability of *Taq* polymerase, by incorporating chaperones into PCR reaction mixtures.

Enhancement of *Taq* Polymerase Performance in PCR Reactions by sHsp

We reported previously that the sHsp from *Pyrococcus furiosus* (Pfu-sHsp) can prevent bovine glutamate dehydrogenase from aggregating at 50°C (Laksanalamai et al., 2001). During PCR reactions, the temperature fluctuates over a wide range, in this case, 50–95°C. Since the source of *Taq* polymerase is the thermophilic bacterium *Thermus aquaticus*, with a maximal growth temperature of 70°C (Nold and Ward, 1995), exposure to 95°C can lead to the denaturation of *Taq* DNA polymerase during extended cycling. This experiment was performed based on the hypothesis that sHsp provides thermal stability by preventing precipitation of denatured *Taq* polymerase, thus, allowing the polymerase to undergo spontaneous refolding. To test this, decreased starting concentrations of *Taq* polymerase were used to establish conditions in which DNA amplification was limited by enzyme availability. The final concentrations of *Taq* polymerase are 0.025, 0.005, and 0.0025 U/μL in the reactions. Controls were identical reactions without the addition of sHsp. We found that at a higher concentration of Taq polymerase (0.025 U/μL), PCR products are generated in both cases suggesting that sHsp does not interfere with the polymerase activity. Lower amounts of *Taq* polymerase molecules reduced the final amounts of PCR products visualized by gel electrophoresis (Fig. 1, lanes 3 and 5) and that no detectable PCR products occurred in the PCR reaction with 0.0025 U/μL (Fig. 1, lane 7). With the addition of Pfu-sHsp, the level of PCR product was unaltered in the reactions with undiluted *Taq* polymerase and PCR products were still retained at 0.005 and 0.0025 U/μL of *Taq* polymerase (Fig. 1, lanes 4 and 6, respectively). The visible PCR products in lanes 4 and 6 are convincing proof that Taq polymerase is

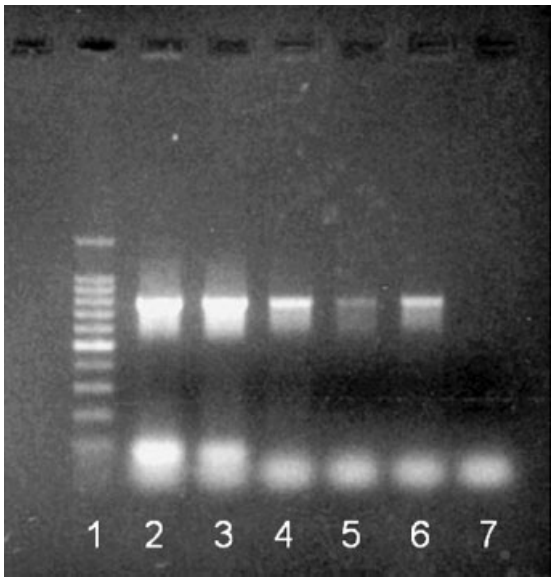

Figure 1. Effect of sHsp from *P. furiosus* on Taq polymerase enzyme stability in PCR reactions. **Lane 1** is a 100-bp DNA ladder. **Lanes 2** and **3** are controls with non-diluted enzyme at 0.025 U/μL with and without sHsp, respectively. **Lanes 4** and **5** are PCR products from reactions with fivefold dilutions of Taq polymerase at 0.005 U/μL with and without sHsp, respectively. **Lanes 6** and **7** are PCR products from reactions with tenfold dilutions of Taq polymerase at 0.0025 U/μL with and without sHsp, respectively.

extended by chaperone action. US patent 6,579,703 was issued on the basis of a similar result, showing that the sHsp, which does not require an ATP hydrolysis, is able to function as an efficient passive chaperone without interfering with the polymerase activity of its target protein. This function resembles the stabilizing action of α-crystallins, which perform similar functions in eye lenses. Unlike the α-crystallin, sHsps can function at very high temperature.

Several lines of evidence suggest that chaperones from hyperthermophiles can function cooperatively (Konieczny and Liberek, 2002; Okochi et al., 2002, 2005; Veinger et al., 1998). Since a single chaperone improved PCR reactions by reducing the amount of *Taq* polymerase required, we then examined the cooperative effects of several *P. furiosus* molecular chaperones on the apparent thermostability of *Taq* polymerase quantitatively by means of primer extension assays followed by product quantitation as previously described by Pavlov et al. (2002). Combinations of the chaperones were used in the experiments including sHsp, Hsp60, and Pfds. Hsp60-sHsp and Hsp60-Pfd combinations were tested for their ability to stabilize *Taq* polymerase.

Stabilization of *Taq* Polymerase by a Combination of sHsp and Hsp60

Since sHsps and Hsp60s are known to be passive and active protein chaperones, respectively, we hypothesize that sHsp retained denatured *Taq* polymerase in a soluble form and the

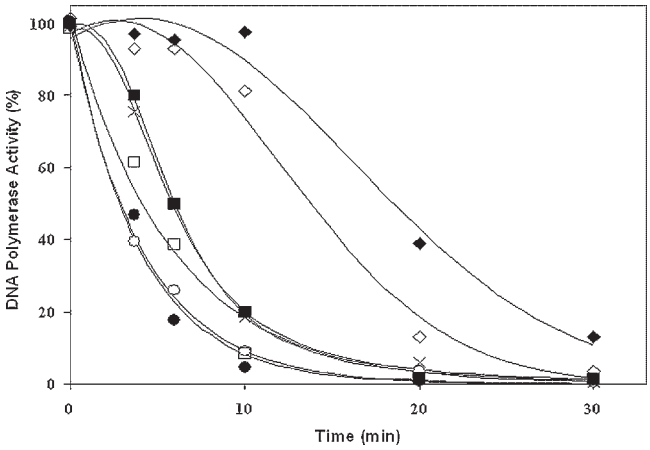

Figure 2. Effect of chaperones on thermostability of Taq DNA polymerase in the presence of *P. furiosus* molecular chaperones (sHsp and Hsp60). Inactivation of Taq polymerase in the presence of individual subunits of sHsp (**X**), Hsp60 (□), Hsp60-Mg^{2+}-ATP (■), sHsp and HSP60 (◇), and sHsp and Hsp60-Mg^{2+}-ATP (◆). The controls are reactions without the addition of chaperones (○) and with the addition of Mg^{2+} and ATP (●).

soluble denatured proteins were subsequently refolded by Hsp60s. Figure 2, open and closed circles, revealed that *Taq* polymerase was rapidly denatured at 100°C and was reduced to less than 10% of the starting activity after 10 min of exposure to 100°C with and without Mg^{2+} and ATP. We then used the combination of sHsp and Hsp60 to study this hypothesis. The level of protection by the Hsp60 alone (Fig. 2, closed and open squares) was comparable to that of the sHsp (Fig. 2, crosses) and also consistent with the previous experiment. When both sHsp and Hsp60 were present without Mg^{2+} and ATP, 80% of the initial polymerase activity was observed (Fig. 2, open diamonds) whereas 100% polymerase activity remained in the reaction at 10 min with the addition of Mg^{2+} and ATP (Fig. 2, closed diamonds)

Stabilization of *Taq* Polymerase by a Combination of Prefoldin and Hsp60

A handover mechanism of non-native protein substrates from Pfd to the Hsp60s has been reported in the hyperthermophile, *P. horikoshii*, suggesting cooperative functions of these two chaperones (Klunker et al., 2003; Okochi et al., 2002, 2004). Our data support the model suggested by these authors for cooperative protein folding. Since *P. furiosus* has two non-identical Pfd subunits, we examined the individual subunits of Pfd (α and β) and the Pfd complex. We found that the levels of protection of *Taq* polymerase by Pfd β and Pfd complex alone (Fig. 3, closed triangles and crosses) were comparable to those of the controls with the addition of Mg^{2+} and ATP or without the addition of chaperones (Fig. 3, closed and open circles, respectively). Surprisingly, the addition of the Pfd α destabilized the *Taq* polymerase (Fig. 3, open triangles) compared to the denaturation of *Taq* polymerase without chaperones presented (Fig. 3, open and closed circles). The Hsp60 alone caused a slight improvement of approximately twofold compared to controls (Fig. 2, closed and open

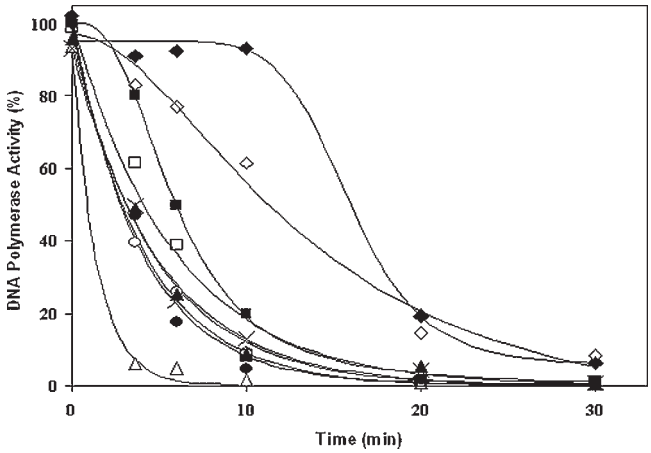

Figure 3. Effect of chaperones on thermostability of *Taq* DNA polymerase in the presence of *P. furiosus* molecular chaperones (prefoldin and Hsp60). Inactivation of *Taq* polymerase in the presence of individual subunits of prefoldin, prefoldin α (△) and β (▲), prefoldin complex (**X**), Hsp60 (□), Hsp60-Mg^{2+}-ATP (■), prefoldin and HSP60 (◇), and prefoldin and Hsp60-Mg^{2+}-ATP (◆). The controls are reactions without the addition of chaperones (○) and with the addition of Mg^{2+} and ATP (●).

squares, respectively). This effect occurred both with and without the addition of Mg^{2+} and ATP. In the presence of the Hsp60 and Pfd complex, 60% of the DNA polymerase activity was retained after 10 min at 100°C without ATP (Fig. 3, open diamonds) whereas 95% of the polymerase activity was retained in the complete reaction with Mg^{2+} and ATP (Fig. 3, closed diamonds).

Our results established that the *P. furiosus* chaperones in vitro can function together. It appears that co-chaperones such as Pfd or sHsp are essential for optimal Hsp60 turnover as they facilitate its performance by fivefold (Figs. 2 and 3). We have used *Taq* polymerase, a crucial enzyme in biotechnology applications such as PCR and cycle sequencing, as a model enzyme. This evidence indicates that chaperones from hyperthermophiles have potential applications in biotechnology. Using these chaperones from hyperthermophilic organisms could potentially improve a wide range of biotechnology applications demanding prolonged enzyme function at elevated temperatures.

In addition to these biotechnology applications, our data could also define the protein folding pathways in *P. furiosus*. Our results indicated that Pfds and sHsps have analogous roles as they both improve the efficiency of Hsp60 catalysis. Hsp60 and Pfd subunits in *P. furiosus* are constitutively expressed (data not shown) whereas the sHsp is highly induced by heat shock treatment at 105°C (Laksanalamai et al., 2001). In addition, after *P. furiosus* cells are removed from heat shock conditions and restored to growth conditions (95°C), the levels of mRNA and protein appear to decrease rapidly as detected by Northern and Western blot, respectively (Laksanalamai, Lowe and Robb, unpublished results). This suggested that the co-chaperone functions of the Pfds and Hsp60 may be sufficient for cells to contain protein folding problems at their normal growth temperature whereas elevated levels of sHsp may be required in addition to the Pfds under heat shock conditions.

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