Pyrococcus furiosus α-Amylase Is Stabilized by Calcium and Zinc†

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ABSTRACT: The hyperthermophilic archeon *Pyrococcus furiosus* produces an extracellular α-amylase that belongs to glycosyl hydrolases' family 13. This enzyme is more thermostable than its bacterial and archaeal homologues (e.g., *Bacillus licheniformis* TAKA-term and *Pyrococcus kodakaraensis* KOD1 α-amylases, respectively) even without adding Ca^{2+} ions. Unlike the TAKA-therm amylase that contains no cysteine, the *P. furiosus* enzyme contains five cysteines (C152, C153, C165, C387, and C430), only four of which (C152, C153, C387, and C430) are conserved in the *P. kodakaraensis* α-amylase. To test the potential function of cysteines in *P. furiosus* α-amylase stability, these five residues were substituted with Ser or Ala—either one-by-one or in sequence—to produce eight mutant enzymes. Mutation C165S dramatically destabilized *P. furiosus* α-amylase. At the same time, the quadruple mutant enzyme C152S/C153S/C387S/C430A (mutant SSCSA) was as thermostable as the wild-type enzyme. Mutant SSCSA and wild-type α-amylases were strongly destabilized by dithiothreitol and ethylenediaminetetraacetic acid, suggesting that metal binding can be involved in this enzyme's thermostability. Inductively coupled plasma-atomic emission spectrometry showed the presence of Ca^{2+} and Zn^{2+} metal ions in *P. furiosus* α-amylase. Although Ca^{2+} is known to contribute to α-amylase's stability, the absence of two out of the three conserved Ca^{2+} ligands in the *P. furiosus* enzyme suggests that a different set of amino acids is involved in this enzyme's Ca^{2+} binding. We also provide evidence suggesting that Cys165 is involved in Zn^{2+} binding and that Cys165 is essential for the stability of *P. furiosus* α-amylase at very high temperatures.

Hyperthermophilic microorganisms (those with optimal growth above 80 °C) produce enzymes that are optimally active at temperatures that would rapidly denature most mesophilic enzymes (*1*). Comparing proteins that have similar folding and function but different stability permits us to identify stabilizing structural elements. These studies are especially valuable for enzymes such as α-amylases, for which thermostability is a key property for their industrial use. α-Amylases are used in industrial starch liquefaction, a starch processing step that typically takes place between 95 and 105 °C. Because of its high thermostability, *Bacillus licheniformis* α-amylase is the major industrially used α-amylase (*2*). Although substantial increases in *B. licheniformis* α-amylase stability have been reported (*3*, *4*), this enzyme is still far from ideal because it requires added Ca^{2+} for stability. A promising alternative may involve α-amylases from hyperthermophiles that operate near the upper limit of the temperature range for biological catalysis.

We recently cloned and characterized the extracellular α-amylase from the hyperthermophilic archaeon *Pyrococcus furiosus* (*Pfu*)[1] (*5*). This enzyme belongs to glycosyl hydrolases' family 13 (also known as the α-amylase family), and it contains family 13's four typical conserved sequences (*6*). Family 13 enzymes share common structural features: (i) a $(\beta/\alpha)_8$ barrel catalytic domain (domain A), (ii) a long $\beta_3 \rightarrow \alpha_3$ loop in the $(\beta/\alpha)_8$ barrel that constitutes a separate domain (domain B), and (iii) a C-terminal domain (domain C). *Pfu* α-amylase is believed to fold in the same way. The archaeal enzyme is 35.7% identical to *B. licheniformis* α-amylase, and 87% identical to *Pyrococcus kodakaraensis* KOD1 α-amylase, another highly thermostable archaeal α-amylase. *Pfu* α-amylase, however, is significantly more thermostable (half-life of 13 h at 98 °C in the absence of added Ca^{2+}) than these two enzymes. A feature that sets *Pfu* α-amylase apart from its homologues is the presence in this enzyme of five cysteines (i.e., Cys152, Cys153, Cys165, Cys187, and Cys430). *P. kodakaraensis* α-amylase contains only four cysteines. Among bacterial thermostable amylases, the *B. stearothermophilus* α-amylase contains one cysteine, and the *B. licheniformis* enzyme contains none. Tomazic and Klibanov (*7*) showed that irreversible inactivation of *B. stearothermophilus* α-amylase at temperatures above 90 °C is partially due to O_2-oxidation of its unique cysteine. Deami-

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[1] Abbreviations: BSA, bovine serum albumin; DM, *n*-dodecyl-*β*-D-maltoside; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; ICP-AES, inductively coupled plasma-atomic emission spectroscopy; LB, Luria−Bertani; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; Ni−NTA, nickel−nitrilotriacetic acid; PAR, 4-(2-pyridylazo)resorcinol; *Pfu*, *Pyrococcus furiosus*; PMPS, *p*-hydroxymercuriphenyl sulfonate; SB, Super broth; SDM, site-directed mutagenesis; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; T_{opt}, optimum temperature for activity.

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dation of amide residues, thiol-catalyzed disulfide interchange, β-elimination of disulfides, formation of scrambled structures, and hydrolysis of peptide bonds at aspartate residues cause irreversible protein inactivation at temperatures close to 100 °C (*7*−*12*). Low asparagine, glutamine, and cysteine contents—common for hyperthermostable proteins, compared to their less stable counterparts—is believed to contribute to protein stability at temperatures above 90 °C (*13*). Indeed, *Pfu* α-amylase contains significantly fewer asparagines plus glutamines than the *B. licheniformis* enzyme (31 vs 47, respectively). *Pfu* α-amylase's high cysteine content, however, is unique and surprising. The work reported here focuses on understanding the possible role of cysteine residues in *Pfu* α-amylase thermal stability and activity.

Previous reports suggested that *Pfu* α-amylase does not require Ca^{2+} ions for activity and thermostability (*5, 14*). This conclusion was based on thermostability studies of the enzyme treated with EDTA at room temperature. It was also indirectly supported by the absence in *Pfu* α-amylase of two out of the three Ca^{2+} ligands highly conserved in α-amylases. Here we show that *Pfu* α-amylase contains one or more tightly bound Ca^{2+} ion(s) and one Zn^{2+} ion. One or more Ca^{2+} ions are essential for *Pfu* α-amylase activity and stability. The Zn^{2+} ion only has a stabilizing function.

MATERIALS AND METHODS

Strains and Cultivation. Escherichia coli DH5α was used for subcloning experiments, XL2-blue was used for SDM, and BL21(DE3) (Stratagene, La Jolla, CA) was used for protein expression. *E. coli* was routinely grown in LB medium (*15*) containing ampicillin (100 μg/mL) when required. For protein overexpression, 1 L flasks containing 250 mL of SB medium (*5*) were inoculated with 1/500 volume of fresh saturated culture and incubated for 12−13 h at 37 °C, with shaking at 250 rpm. Bacteria were harvested by centrifugation.

DNA Manipulations and SDM. DNA manipulations were performed using routine protocols (*15*). DNA was recovered from agarose gels with the Geneclean II kit (BIO 101, La Jolla, CA). Sequences were analyzed using the ThermoSequenase radiolabeled terminator cycle sequencing kit (U. S. Biochemical Corp., Cleveland, OH). To express *Pfu* α-amylase as a fusion with an N-terminal His-tag, the *Pfu amyA* gene was amplified by PCR without its signal peptide, and it was cloned into the *Nhe*I and *Hin*dIII sites of pET28b, yielding plasmid pET281. Oligonucleotide 5′-AGCTAGCTTGGAGCTTGAAGAGGGAG-3′ was the forward primer. Sequence AAATCA encoding the two N-terminal residues Lys-Tyr was substituted with GCTAGC, encoding Ala-Ser, to create the *Nhe*I site. Oligonucleotide 5′-TTTAAGCTTATTTGCACTTCTCCCATTG-3′ was the reverse primer. Sequence AAGCTT creates a *Hin*dIII site downstream of the gene's transcription termination signal.

SDM was performed using the QuikChange Site-Directed Mutagenesis procedure (Stratagene) with mutagenic primers synthesized by the Michigan State University Macromolecular Structure Facility. Eight α-amylase mutants were constructed. (For convenience, these mutants are designated by a five-letter code, in which residues 152, 153, 165, 387, and 430 are indicated by "C" for Cys, "S" for Ser, or "A" for Ala. In this system, for example, SSCCC would stand for an α-amylase in which Cys152 and Cys153 are substituted with Ser.) Mutations were confirmed by complete sequencing of the gene.

Enzyme Purification. All procedures were performed at room temperature, if not otherwise specified. Cell pellets were resuspended in one-third of the initial culture volume, with 50 mM Tris-HCl (pH 8.5). The cell homogenate was prepared by passing the cell suspension through a French pressure cell at 15 000 lb/in.^{2} 2 times. For the wild-type, SSCSC, and SSCSA enzymes, cell homogenates were incubated for 30 min at 90 °C. Incubations for the CCSCC and SSSSA mutant enzymes were carried out at 60 °C. Soluble fractions were recovered after centrifugation at 45 000 rpm for 2 h. DM (Anatrace Inc., Maumee, OH) was added at 0.1% (w/v) final concentration, and the soluble fraction was incubated for 2 h with gentle stirring. Ni−NTA agarose (Qiagen, Valencia, CA), preequilibrated with 50 mM Tris-HCl (pH 8.5) containing 0.1% DM (buffer A), was added to the crude extract at approximately 1 mL of resin per 1 mg of protein. The mixture was incubated overnight at 4 °C with gentle shaking, and then packed into a chromatography column. The packed resin was washed with 10 volumes of buffer A, 2 volumes of buffer A containing 1 M KCl, and then again with 2 volumes of buffer A. The His-tag-containing protein was eluted with buffer A containing 100 mM imidazole. The eluted fraction was concentrated to approximately 1 mg of protein/mL using a centrifugal filter device (Millipore, Bedford, MA) equipped with a 30 000 Da cutoff membrane, and then dialyzed 3 times against buffer A. The purity of the eluted protein was judged by SDS−PAGE. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA), with BSA as the standard.

α-Amylase Assays. α-Amylase activity was determined by measuring the amount of released reducing sugar during enzymatic hydrolysis of 1% soluble starch in 50 mM sodium acetate (pH 5.6) at different temperatures for 10 min. A control without enzyme was used. The amount of reducing sugar released was measured by a modified dinitrosalicylic acid method (*16*). One unit of amylase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar as glucose per minute under the assay conditions. The effect of temperature on the enzymes' activities was determined by performing standard enzyme assays at different temperatures.

Thermostability Studies. For stability studies at high temperatures, enzymes were diluted to 10 μg/mL final concentration in buffer A. Fifty microliter samples covered with 20 μL of mineral oil were incubated at 115 or 90 °C in 0.5 mL, 30 × 8 mm screw-cap microtubes (Sarstedt, Newton, NC). After various incubation periods, samples were withdrawn and tested for residual α-amylase activity at 60 °C under standard assay conditions.

Metal Content Determination. To minimize contamination of the protein samples with metals, buffers were prepared with ultrapure MilliQ water. Buffers and salts were of high purity (SigmaUltra, Sigma). Plasticware and glassware were cleaned exhaustively with 97% ethanol, and then rinsed with MilliQ water. The enzymes, concentrated between 1 and 3.5 mg/mL, were incubated with 2 mM EDTA at 20 or 90 °C for 50 min. After incubation, the samples were dialyzed at

4 °C, first against buffer A containing 1 mM EDTA, then 3 times against buffer A. One milliliter of each sample was used for ICP-AES analysis (Animal Health Diagnostics Laboratory, MSU, East Lansing). Dialysis buffers used for the experiment were also sent for ICP-AES as controls.

Spectroscopic Methods. Spectrophotometric titration of Zn^{2+} in *Pfu* α-amylase with PMPS (*17*−*19*) was performed in a Beckman DU650 spectrophotometer, using quartz microcuvettes (1 cm path length). The enzyme was extensively dialyzed against 40 mM HEPES/NaOH (pH 7.2) (buffer B) before titration. Buffer B, PMPS, and PAR solutions were prepared using MilliQ water. The release of Zn^{2+} from *Pfu* α-amylase was followed after adding successive 1.2−10 μL samples of 1−10 mM PMPS to 300 μL of *Pfu* α-amylase in buffer B containing 0.1 mM PAR. Absorbances at 250 and 500 nm were recorded 5 min after each PMPS addition. A cuvette containing 300 μL of buffer B plus 0.1 mM PAR was used first as the blank, then as a negative control during the experiment. Absorbances from this cuvette were systematically subtracted from the *Pfu* α-amylase data. Initial OD_{250} and OD_{500} of the sample cuvettes were measured before adding the first PMPS sample. These initial ODs were also subtracted from the experimental readings to directly measure ΔOD values and to eliminate variations due to cuvette differences.

Other Analytical Methods. Dynamic light scattering was performed on a DynaPro-801 dynamic light scattering instrument (Protein Solutions, Inc., Charlottesville, VA). The protein sample (0.1 mg/mL) was in buffer A, and it was filtered prior to injection. Molecular weight determinations by mass spectrometry were performed on a Voyager Elite MALDI-TOF mass spectrometer (PerSeptive Biosystems, Foster City, CA) with 10 μL of a 7.5 μM protein solution in MilliQ water.

Homology Modeling and Protein Structure Visualization. Homology modeling was performed using SWISS-MODEL (GlaxoWellcome Experimental Research, Geneva, Switzerland) (*20*−*22*). The *Pfu* α-amylase amino acid sequence was sent to SWISS-MODEL for modeling in the first approach mode. The model (containing the coordinates for *Pfu* α-amylase residues 8 to 230) was visualized using Insight II on a Silicon Graphics workstation at the MSU Biochemistry Department Computer Facility.

RESULTS

Effects of N- and C-Terminal His-Tags on Pfu α-Amylase Activity and Stability. Before starting any SDM experiments on *Pfu* α-amylase cysteine residues, we investigated what effect the presence of an N- or C-terminal His-tag had on the enzyme's activity and stability properties. *Pfu* α-amylase was expressed as a fusion with a C-terminal and an N-terminal His-tag, from plasmids pET213 (*5*) and pET281, respectively. The N- and C-terminal His-tags do not significantly affect *Pfu* α-amylase's specific activity (data not shown), and they do not affect the enzyme's T_{opt} (Figure 1A). The C-terminal His-tag shifted the enzyme's activity curve toward lower temperatures by almost 10 °C, though (Figure 1A). While the N-terminal His-tag increases the enzyme kinetic inactivation rate approximately 2-fold at 115 °C, the C-terminal His-tag does not destabilize the enzyme at 115 °C. The C-terminal His-tag even marginally stabilizes

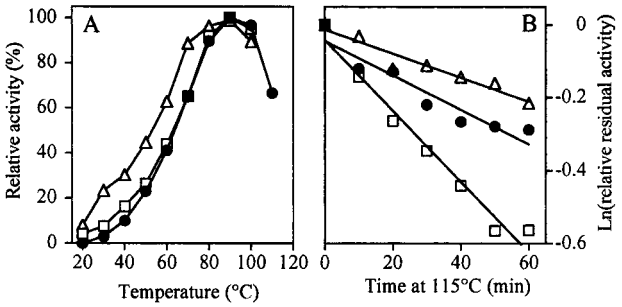

FIGURE 1: Effects of N- and C-terminal His-tags on *Pfu* α-amylase activity (A) and stability (B). (●) Wild-type recombinant *Pfu* α-amylase without His-tag; (□) *Pfu* α-amylase fused with an N-terminal His-tag; (△) *Pfu* α-amylase fused with a C-terminal His-tag. Six independent assays were performed with the purified enzymes.

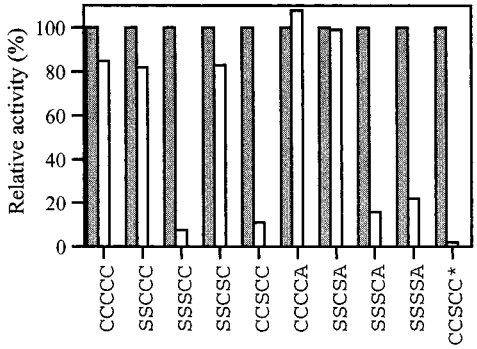

FIGURE 2: Initial screening of *Pfu* α-amylase cysteine mutants for thermostability. Gray bars, α-amylase activity in the soluble crude extract before heat treatment, fixed as 100%; white bars, relative remaining α-amylase activity in the soluble crude extract after a 15 min heat-treatment at 100 °C. CCSCC*: CCSCC enzyme without His-tag.

Pfu α-amylase at 115 °C (Figure 1B). For this reason, plasmid pET213 (expressing the *Pfu* α-amylase with the C-terminal His-tag) was chosen as the template for SDM, and all the mutant enzymes characterized in the rest of this study contain a C-terminal His-tag.

Construction and First Screening of Pfu α-Amylase Cysteine Mutants. Plasmid pET213 was subjected to single or successive rounds of SDM, creating a series of eight single and multiple mutant genes, in which Cys codons were substituted with Ser or Ala codons. To test the mutant enzymes for altered thermostability, crude extracts containing the overexpressed enzymes were incubated for 15 min at 100 °C. Denatured proteins were removed by centrifugation, and the remaining α-amylase activity was measured on the supernatant (Figure 2). Crude extracts of the wild-type enzyme (CCCCC) and of mutants SSCCC, SSCSC, SSCSA, and CCCCA (i.e., the thermostable mutants) retained full activity after this heat treatment. Crude extracts of mutants CCSCC, SSSCC, SSSCA, and SSSSA (i.e., the thermosensitive mutants) showed a dramatic decrease in activity. These latter four mutant enzymes have mutation Cys165Ser in common, whereas the stable mutants carry an intact Cys165. To confirm that these results were not biased by the presence of a C-terminal His-tag, the CCSCC mutant enzyme was expressed in the absence of His-tag. The CCSCC enzyme with no His-tag (i.e., CCSCC* in Figure 2) is as unstable at 100 °C as the Cys165 mutants carrying a His-tag. The wild-type enzyme (CCCCC), two thermostable mutants (SSCSC

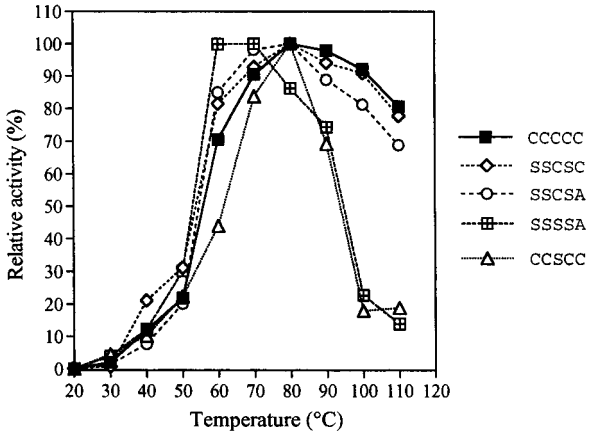

FIGURE 3: Effect of temperature on the activity of *Pfu* α-amylase and its cysteine mutants. Assays were performed with the purified enzymes.

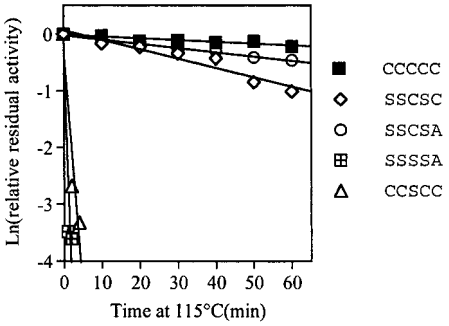

FIGURE 4: Stability of *Pfu* α-amylase and its cysteine mutants at 115 °C. Assays were performed with the purified enzymes.

and SSCSA), and two thermosensitive mutants (CCSCC and SSSSA) were purified, and their activities and thermostabilities were further characterized.

Effect of Temperature on the Enzymes' Activities. The five enzymes were purified from crude extracts by Ni−NTA affinity chromatography. As *Pfu* α-amylase noticeably aggregated during purification and concentration (data not shown), the detergent DM was added to the purification buffers and to the crude extracts prior to purification. Purified proteins were tested for activity at temperatures ranging from 20 to 100 °C (Figure 3). Mutants SSCSC, SSCSA, and CCSCC had the same T_{opt} as CCCCC (80 °C), while mutant SSSSA was optimally active between 60 and 70 °C. Mutants SSSSA and CCSCC rapidly lost activity above 80 °C, in contrast to CCCCC, SSCSC, and SSCSA, which retained 70−80% activity at 110 °C. None of the mutations affected the enzyme's specific activity in any significant way (data not shown).

Effect of Temperature on the Enzymes' Stabilities. The thermostability of the wild-type and mutant enzymes was tested by incubating the enzymes at 115 °C for different periods of time, and by measuring their residual activity at 60 °C (Figure 4). While enzymes CCCCC, SSCSC, and SSCSA had half-lives of 44, 63, and 44 min at 115 °C, respectively, mutants CCSCC and SSSSA were completely inactivated after 20 and 15 min at 115 °C, respectively. Since the mutations were introduced on the same plasmid construct and since the enzymes were expressed and purified using the same method, any differences in specific activity or thermostability are caused by the cysteine mutations. The data presented in Figure 4 show that mutation Cys165Ser

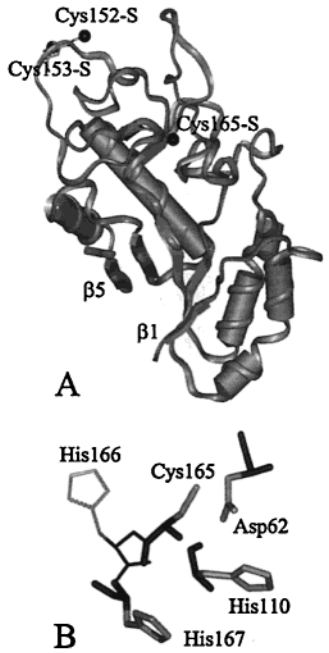

FIGURE 5: 3D model of *Pfu* α-amylase. (A) Ribbon representation. Cylinders represent α-helices, and arrows represent β-strands. Strands 1 and 5 of the $(\alpha/\beta)_8$ barrel are labeled. The sulfur atoms of Cys152, Cys153, and Cys165 are represented by black spheres. (B) Residues potentially forming the Zn^{2+}-binding site. Backbone and side-chain atoms are in dark and light gray, respectively.

dramatically decreases *Pfu* α-amylase thermostability. No other cysteine substitution causes the same dramatic effect on thermostability, since mutant SSCSA (i.e., containing a single cysteine, Cys165) is as thermostable at 115 °C as the wild-type enzyme.

Homology Modeling of Pfu α-Amylase. Although *Pfu* α-amylase shows low similarity to the α-amylase family enzymes of known 3D structure [including *B. licheniformis* and barley α-amylases (35.7% and 27% identical, respectively)], the extensive number of available structures allowed a partial homology modeling of this enzyme. Using the Automated Comparative Protein Modeling Server at SWISS-MODEL, a partial model of *Pfu* α-amylase (residues Glu8−Leu230) was obtained. Obtaining the complete model using this automated method was impossible due to multiple deletions present in the *Pfu* α-amylase and due to low similarity between target and template sequences. The structures of four family 13 enzymes were used as templates for this model: *B. licheniformis* α-amylase (1.7 and 2.2 Å resolution), barley α-amylase (2.8 Å resolution), *Pseudomonas stutzeri* α-glycosidase (2.2 Å resolution), and maltotetraose-forming exo-amylase (2.2 Å resolution). The *Pfu* α-amylase partial model included most of the $(\beta/\alpha)_8$ barrel and the complete B domain, but not the C domain (Figure 5A). This model, however, allowed us to tentatively locate Cys152, Cys153, and Cys165 in the enzyme structure. The large distance between Cys152−Cys153 and Cys165 excludes the possibility of disulfide bonds Cys152−Cys165 or Cys153−Cys165 being formed. On the other hand, Cys165, located on the surface of domain B, could participate in a hypothetical intermolecular Cys165−Cys165 disulfide bridge.

Effect of Reducing Agents on Pfu α-Amylase's Stability and Denaturation Behavior. The CCCCC and SSCSA

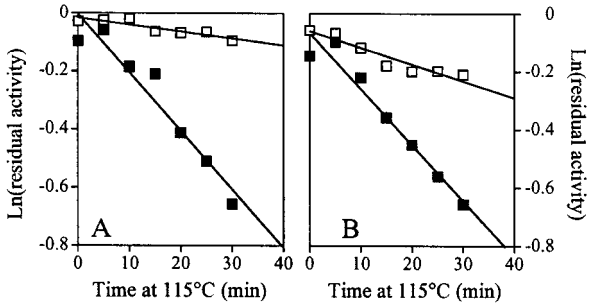

FIGURE 6: Effect of DTT on the thermostabilities of *Pfu* α-amylase CCCCC (A) and of its mutant SSCSA (B). Open symbols, inactivation in the absence of DTT; closed symbols, inactivation in the presence of 10 mM DTT.

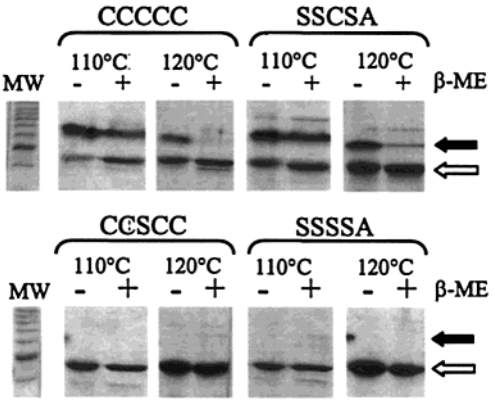

FIGURE 7: Denaturation behavior of *Pfu* α-amylase and its cysteine mutant derivatives at 110 and 120 °C in the absence and presence of β-mercaptoethanol [β-ME, 5% (v/v) final concentration]. Samples were incubated at 110 or 120 °C for 10 min in 2-times-concentrated SDS−PAGE sample buffer prior to being loaded on an SDS−polyacrylamide gel. The SDS final concentration in the samples was 2%. MW, molecular weight markers; open arrow, monomer; closed arrow, dimer.

enzymes were incubated at 115 °C in the presence and absence of DTT. DTT decreased the half-life of CCCCC by a factor of 10 (from 347 to 33 min) and the half-life of SSCSA by a factor of 3 (from 115 to 33 min) (Figure 6). In the SSCSA mutant, the only disulfide bound that could be reduced by DTT would have to be an intermolecular Cys165−Cys165 bound. The effect of DTT on the CCSCC enzyme's stability was tested. Ten millimolar DTT decreased the half-life of CCSCC by a factor of 2.6 (from 231 to 87 min) at 90 °C, but the reducing agent had no significant destabilizing effect on CCSCC at 95 °C. CCSCC's half-life at 95 °C decreased from 13 to 10 min in the presence of 10 mM DTT.

Enzymes CCCCC, CCSCC, SSCSA, and SSSSA were incubated in denaturing conditions (110 or 120 °C, in SDS−PAGE sample buffer) in the presence and absence of β-mercaptoethanol, before being analyzed by SDS−PAGE. When denatured at 110 °C in the absence of reducing reagent, proteins containing an intact Cys165 (i.e., CCCCC and SSCSA) showed an additional band that migrated slower than the band corresponding to the α-amylase monomer (Figure 7). This additional band could correspond to a hypothetical denatured α-amylase dimer, whose monomers are maintained together by a covalent Cys165−Cys165 bound. This additional band was observed even in the samples containing β-mercaptoethanol. At 120 °C, the additional band observed with enzymes CCCCC and SSCSA was still present when denaturation was performed in nonreducing conditions, but it almost completely disappeared in the presence of β-mercaptoethanol. This additional band was never observed with the CCSCC and SSSSA mutants.

Pfu α-Amylase's Molecular Mass. The stability properties of the *Pfu* α-amylase cysteine mutants, DTT's detrimental effect on the stability of enzymes CCCCC and SSCSA, and the apparent denaturation behavior of these two enzymes (as seen after SDS−PAGE, Figure 7) suggest that *Pfu* α-amylase is potentially a dimeric enzyme. Since all other known α-amylases belonging to glycosyl hydrolases' family 13 are monomeric enzymes, we determined the molecular mass of the *Pfu* native α-amylase using two techniques. The enzyme's hydrodynamic radius was determined by light scattering to be 3.2 nm. The polydispersity parameter of 0.404 nm (i.e., 12.7% of the hydrodynamic radius) indicates that the protein sample fits a standard monomodal Gaussian distribution—in other words, the protein was present in a homogeneous, single form. The 3.2 nm hydrodynamic radius corresponds to a 48 kDa protein, close to the calculated 50 kDa molecular mass of the monomer.

If the enzyme formed a dimer that was stabilized by an intersubunit disulfide bridge (i.e., a covalent bond), we would obtain two peaks of 50 and 100 kDa by MALDI-TOF-MS. Several independent molecular weight determinations were performed on the wild-type and various mutant enzymes. No difference in size distribution was detected between CCCCC and the Cys165Ser mutants. In all cases, a strong signal was obtained around 50 kDa, whereas only a trace signal was found around 100 kDa (data not shown).

Determination of Pfu α-Amylase's Metal Content. The *Pfu* α-amylase molecular weight determination by light scattering indicates without a doubt that this enzyme is monomeric. Since Cys165 can no longer form a stabilizing intersubunit disulfide bridge in a monomeric enzyme, we explored Cys165's potential stabilizing role as a metal ligand. We determined the metal contents of the recombinant *Pfu* α-amylase and of its cysteine mutant derivatives. The enzymes were extensively dialyzed against 50 mM Tris (pH 8.5). Dialysis buffers and protein solutions were analyzed by ICP-AES. Significant amounts of Ca^{2+} and Zn^{2+} were detected in the four enzymes tested (Table 1). No other metals were present in the enzymes at any significant level (data not shown). Because of a large variability in the AES results, in particular for Ca^{2+}, the results listed in Table 1 reflect individual experiments. Despite this variability, though, the Ca^{2+} contents of the four tested enzymes suggest that *Pfu* α-amylase contains at least two Ca^{2+} cations. To our surprise, the CCCCC and SSCSA enzymes contained a significant amount of Zn^{2+}, approximately 1 mol/mol of enzyme. These two enzymes contain at least twice as much Zn^{2+} as mutants CCSCC and SSSSA do, suggesting that Cys165 is a Zn^{2+} ligand.

Because ICP-AES gave us only qualitative results, and because Zn^{2+} is a common contaminant in glassware, plasticware, and aqueous solutions, we confirmed the presence of Zn^{2+} in *Pfu* α-amylase by titration with the strongly dissociating sulfhydryl reagent PMPS (Figure 8). PMPS forms covalent complexes with cysteine residues in proteins. Formation of these complexes can be followed by absorbance at 250 nm. In mesophilic Zn^{2+}-containing enzymes, cysteines typically react with PMPS in a stoichiometric fashion that

Table 1: Metal Content of *Pfu* α-Amylase (CCCCC) and of Its Cysteine Mutant Derivatives, As Determined by ICP-AES

enzyme	Ca^{2+}/molecule[a] (mol/mol)	Zn^{2+}/molecule[a] (mol/mol)
no EDTA treatment		
CCCCC	3.24	0.86
SSCSA	7.90	1.80
CCSCC	2.87	0.52
SSSSA	4.60	0.41
after EDTA treatment at 20 °C		
CCCCC	2.98	0.92
SSCSA	7.86	0.96
CCSCC	5.14	0.09
SSSSA	2.55	0.05
after EDTA treatment at		
90 °C	0.59	0.04
CCCCC	0.67	0.46
SSCSA	0.06	0.08
CCSCC	0.38	0.02
SSSSA	0.72	bl[b]

[a] Metal concentrations in the dialysis buffers were subtracted from the concentrations in the corresponding protein samples prior to determining the metal content per enzyme molecule. [b] bl: background level.

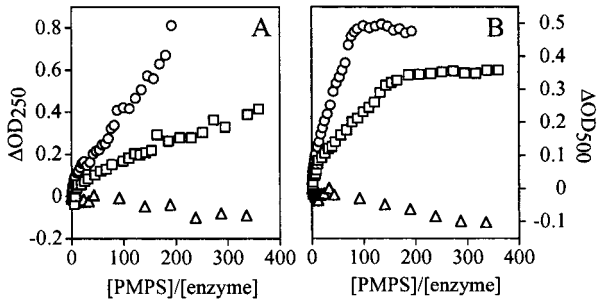

FIGURE 8: Titration of Zn^{2+} in *Pfu* α-amylase (CCCCC and CCSCC) with the sulfhydryl reagent PMPS in the presence of the dye PAR. The titration was followed at 250 nm (A) and at 500 nm (B). (□) Data for CCCCC at an enzyme concentration of 4.6 μM; (○) data for CCCCC at an enzyme concentration of 8.6 μM; (△) data for CCSCC at an enzyme concentration of 6.8 μM.

is illustrated by the linear increase of OD_{250} with PMPS concentration (*18*, *19*, *23*). Absorbance at 250 nm reaches a plateau when all the cysteines have reacted with PMPS. The [PMPS]/[enzyme] ratio at which OD_{250} becomes constant indicates the number of cysteines that have reacted with PMPS. In this instance, following the formation of the PMPS−sulfhydryl chromophore at 250 nm does not indicate whether PMPS is really incorporated into the CCCCC enzyme: OD_{250} increases very slowly, and no plateau is reached, even at [PMPS]/[enzyme] ratios above 200 (Figure 8A). This constant OD_{250} increase cannot be attributed to the absorbance of PMPS, itself, at 250 nm, since we systematically subtracted a negative control (i.e., buffer plus PAR plus increasing amounts of PMPS) from our data.

All the Zn^{2+} released from a protein upon PMPS reaction with cysteines forms complexes with the PAR dye almost instantly, if PAR is present in excess in the solution. Formation of the $(PAR)_2Zn^{2+}$ complex can be followed at 500 nm: OD_{500} increases linearly as Zn^{2+} is progressively released from the CCCCC enzyme. OD_{500} reaches a plateau when all the Zn^{2+} has been released. This maximum OD_{500} value is a direct indication of how much Zn^{2+} was initially present in the protein. With an extinction coefficient of 6.6 $\times 10^4$ M^{-1} min^{-1} at 500 nm for $(PAR)_2Zn^{2+}$ at 20 °C (*18*), a complete release of one Zn^{2+} ion from *Pfu* α-amylase (at 4.6 and 8.6 μM concentrations) gives theoretical ΔOD_{500}'s of 0.3 and 0.57, respectively. In Figure 8B, formation of the $(PAR)_2Zn^{2+}$ complex is slow: plateaus are reached only at [PMPS]/[CCCCC] ratios above 100. At both enzymes' concentrations, though, the ΔOD_{500} reaches maxima of 0.36 and 0.5. These maxima correspond to the equivalents of 1.2 and 0.87 mol of Zn^{2+}/mol of CCCCC enzyme, respectively. These results confirm the presence of 1 mol of Zn^{2+}/mol of *Pfu* α-amylase, as suggested by the ICP-AES results (Table 1). Since PMPS specifically reacts with sulfhydryl groups, these results also confirm that at least one cysteine is a Zn^{2+} ligand in *Pfu* α-amylase.

When the same titration was performed with CCSCC, neither OD_{250} nor OD_{500} increased with the addition of PMPS to the enzyme solution (Figure 8). These results indicate that Cys152, Cys153, Cys387, and Cys430 are not accessible to react with PMPS at 20 °C. These results also indirectly suggest that all the Zn^{2+} released from CCCCC was released upon PMPS reaction with Cys165 only.

Because all the metal analyses were performed on His-tag-containing enzymes, and because histidines are well-known Zn^{2+} ligands, we verified that the CCCCC enzyme expressed and purified in the absence of His-tag also contained 1 mol of Zn^{2+}/mol of enzyme. We obtained an average of 0.87 ± 0.17 mol of Zn^{2+}/mol of enzyme in four independent titrations.

EDTA Treatment and Pfu α-Amylase's Metal Content. To determine how tightly Ca^{2+} and Zn^{2+} are bound to *Pfu* α-amylase, the CCCCC enzyme and the cysteine mutants were treated with EDTA at different temperatures, dialyzed extensively against metal-free buffer, and then submitted to ICP-AES (Table 1). EDTA treatments at temperatures below 75 °C had no effect on CCCCC and SSCSA's metal contents (see EDTA treatment at 20 °C). A 50 min EDTA treatment at 90 °C was necessary to remove a significant amount of the Ca^{2+} and most of the Zn^{2+} from CCCCC and SSCSA. EDTA treatment of mutants CCSCC and SSSSA at 20 °C did not deplete these enzymes of their Ca^{2+}, suggesting that Cys165 is not involved in Ca^{2+} binding. On the other hand, the same EDTA treatment at 20 °C was enough to remove almost all the Zn^{2+} initially present in these two enzymes (Table 1). This result and the fact that CCSCC and SSSSA contain less Zn^{2+} than CCCCC and SSCSA confirm that Cys165 is a Zn^{2+} ligand. As expected from the results with CCCCC and SSCSA, an EDTA treatment at 90 °C is able to remove a significant fraction of the Ca^{2+} present in mutants CCSCC and SSSSA.

Roles of Ca^{2+} and Zn^{2+} in Pfu α-Amylase's Activity. Room temperature EDTA treatments did not affect *Pfu* α-amylase activity or thermostability. These results initially suggested that this enzyme did not require Ca^{2+} (*5*). EDTA treatments at temperatures higher than 75 °C, though, led to significant enzyme inactivation. Figure 9 shows the time course of *Pfu* α-amylase inactivation by EDTA at 90 °C. Even after a 30 min EDTA treatment at 90 °C, almost full activity could be restored by immediately adding an excess of Ca^{2+} and by heating the enzyme for an additional 5 min at 90 °C. Adding Zn^{2+} instead of Ca^{2+} did not restore activity (data not shown). When *Pfu* α-amylase was EDTA-treated for 30 min at 90 °C, then cooled to room temperature and dialyzed 3 times against buffer A, heating the enzyme at 90 °C for 30 min in

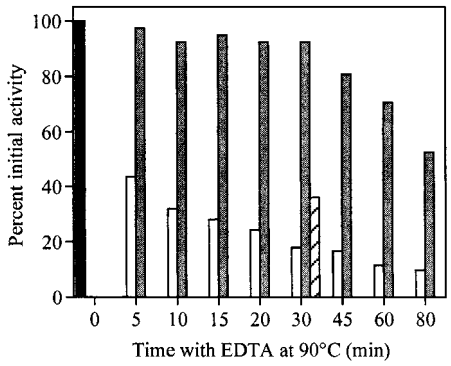

FIGURE 9: Effects of EDTA treatment and Ca^{2+} on CCCCC activity. Black bar: Activity of the holoenzyme in buffer A. White bars: The enzyme (5 μg/mL) was incubated at 90 °C in the presence of 2 mM EDTA for increasing periods of time and then cooled in a room temperature water bath. Gray bars: After EDTA treatment at 90 °C, 5 mM $CaCl_2$ was immediately added to the solution, and the enzyme was incubated for an additional 5 min at 90 °C before being cooled in a room temperature water bath. Hatched bar: After a 30 min EDTA treatment at 90 °C, the enzyme was extensively dialyzed against buffer A. The enzyme was then incubated at 90 °C for 30 min in the presence of 2 mM $CaCl_2$. Residual and restored activities were measured at 60 °C.

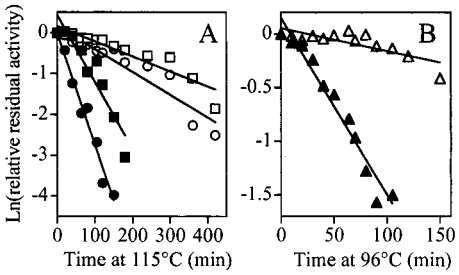

FIGURE 10: Effect of Ca^{2+} on *Pfu* α-amylase stability. (A) Stability of the CCCCC (□, ■) and SSCSA (●, ○) enzymes at 115 °C in buffer A (■, ●) and in the presence of 2 mM $CaCl_2$ (□, ○). (B) Stability of the CCSCC enzyme at 96 °C in buffer A (▲) and in the presence of 2 mM $CaCl_2$ (△). Residual activity was measured at 60 °C.

the presence of 2 mM Ca^{2+} also partially restored activity (Figure 9). Again, adding Zn^{2+} instead of Ca^{2+} to the dialyzed enzyme had no reactivating effect (data not shown). These data indicate that, in contrast to what we published earlier (*5*), Ca^{2+} is essential for *Pfu* α-amylase activity. These results also indicate that Ca^{2+} is the only cation required for activity.

Role of Ca^{2+} in Pfu α-Amylase's Stability. Added to enzymes that had not been EDTA-treated, Ca^{2+} (2 mM) increased the half-lives of CCCCC 4 times (from 43 to 173 min) and of SSCSA 4.5 times (from 26 to 116 min) at 115 °C. Ca^{2+} increased the half-life of CCSCC 8 times (from 43 to 345 min) at 96 °C (Figure 10). Mutant CCSCC's thermostability in the presence of Ca^{2+}, though, was still significantly lower than that of the wild-type holoenzyme, even with no added metals. These results indicate that Ca^{2+} is required for complete *Pfu* α-amylase thermostability, but they also indicate that Ca^{2+} is not the only stabilizing factor in this enzyme.

DISCUSSION

Effects of N- and C-Terminal His-Tags on Pfu α-Amylase's Activity and Stability. Fusing a His-tag to a protein is a widely used approach to simplify protein purification (*24*−*26*). We applied this technique to *Pfu* α-amylase to obtain identical purification conditions for the wild-type enzyme and for mutants with altered thermostability. In most cases, C- or N-terminal His-tags do not affect protein properties such as stability or temperature range for activity. Here an N-terminal His-tag significantly decreases the kinetic stability of the enzyme at 115 °C, and the C-terminal His-tag shifts *Pfu* α-amylase's activity curve toward lower temperatures by approximately 10 °C. The C-terminal His-tag does not change the temperature for optimal activity, though. It is interesting to note that the C-terminal His-tag does not change the enzyme's stability. The C-terminal His-tag may affect the conformation of *Pfu* α-amylase's C-terminal domain (domain C) as well as the conformation and dynamics of the active site in a way that makes the enzyme more active at lower temperatures. (Although domain C's function in catalysis is not determined, deleting this domain inactivates α-amylases [(*6*), Savchenko et al., unpublished data].) Since all the enzymes we compared contain the same C-terminal His-tag, the temperature shift between the wild-type and the His-tag-containing enzymes does not interfere with our results on Cys165's function in α-amylase stabilization.

Cys165 Is a Key Residue for Pfu α-Amylase's Stability. Pfu extracellular α-amylase is one of the most thermostable enzymes in the α-amylase family. Because the presence of five cysteines in such a thermostable enzyme was surprising, the five cysteines were mutated either individually or in sequence. The Cys165Ser substitution dramatically destabilized *Pfu* α-amylase. Substitutions of any of the four other cysteines were not destabilizing. Even the quadruple SSCSA derivative retained the thermostability properties of the wild-type enzyme. These results identified Cys165 as a key residue for *Pfu* α-amylase thermostability. α-Amylases highly similar to the *Pfu* enzyme have been characterized from other *Thermococcal* species. We compared the cysteine content and the thermostability of these enzymes with those of the *Pfu* α-amylase to determine if the stabilization mechanism involving Cys165 was widespread in archaeal hyperthermophilic α-amylases (Table 2). Neither the *P. kodakaraensis* (*27*) nor the *T. profundus* (*28*, *29*) α-amylase contains Cys165. Both enzymes contain the four other cysteines present in the *Pfu* enzyme. Both enzymes are less thermostable than their *Pfu* homologue (Table 2). *P. kodakaraensis* α-amylase is more thermostable than the *Pfu* α-amylase CCSCC mutant, though, suggesting the existence of different stabilization mechanisms in the *P. kodakaraensis* enzyme. It would be interesting to determine if introducing the equivalent of Cys165 in the *P. kodakaraensis* enzyme makes it a more thermostable enzyme than the *Pfu* α-amylase.

Pfu α-Amylase Is a Monomeric Enzyme. Three lines of evidence suggest that *Pfu* α-amylase is a dimeric enzyme: (i) it behaves as a 100 kDa protein by gel filtration (*5*); (ii) it behaves as a dimer in SDS−PAGE; and (iii) the low stability of the Cys165Ser mutants as well as DTT's destabilizing effect on CCCCC and SSCSA suggest that Cys165 participates in a Cys165−Cys165 disulfide bridge between two α-amylase monomers. Each of these results can be interpreted in a way that does not imply the existence of a dimer: First, the gel filtration experiment was performed in the absence of DM (*5*). Since *Pfu* α-amylase is highly

Table 2: Thermal Properties of Representative *Thermococcal* α-Amylases

origin	% identity to *Pfu* α-amylase	T_{opt} (°C)	Ca^{2+} requirement	cysteine content	thermostability
P. furiosus (*5*)	100	100	+	CCCCC	$t_{1/2}$ of 13 h at 98 °C (no Ca^{2+} added)
P. kodakara-ensis (*27*)	87	90	+	CCACC	90% active after 1 h at 90 °C (no Ca^{2+} added)
T. profundus (*28*, *29*)	71	80	+	CCACC	$t_{1/2}$ of 15 min at 90 °C (no Ca^{2+} added) $t_{1/2}$ of 4 h at 90 °C (5 mM Ca^{2+})

hydrophobic, its behavior as a 100 kDa protein by gel filtration could be explained by partial aggregation during that experiment. Second, we know that the denaturation step prior to SDS−PAGE does not completely unfold the *Pfu* α-amylase: the enzyme is still active after migration in a starch-containing SDS−polyacrylamide gel (*5*). We suspect that in these partially denaturing conditions Cys165 becomes available to react with Cys165 from another monomer and to form a disulfide bridge. This disulfide bridge is never observed in the Cys165 mutants CCSCC and SSSSA (Figure 7). Adding *β*-mercaptoethanol to the denaturation buffer reduces the quantity of disulfide bridges formed. It is interesting to note that, although in the 3D model of *Pfu* α-amylase Cys152 and Cys153 are close to the enzyme's surface, only Cys165 is in a conformation or in a location that allows it to form a disulfide bridge. The fact that only Cys165 reacts with the sulfhydryl reagent PMPS (Figure 8) reinforces this interpretation. Finally, DTT's destabilizing effect on CCCCC and SSCSA (Figure 5) can be interpreted by the fact that DTT can form stable coordination complexes with Zn^{2+}. [The Zn^{2+}−DTT complex has a stability constant of 10.3 (*30*).] In this instance, DTT would play a Zn^{2+}-chelator function similar to that of EDTA. In CCSCC, in which Zn^{2+} is not bound as tightly as in CCCCC or SSCSA, Zn^{2+} is probably bound tightly enough at 90 °C to still help stabilize the enzyme. At this temperature, DTT competes with the enzyme for Zn^{2+} and destabilizes the enzyme. At 95 °C, Zn^{2+} is probably not bound to the enzyme tightly enough to still have a stabilizing role in CCSCC. Adding DTT in these conditions has no impact on the enzyme stability.

Other lines of evidence suggest that *Pfu* α-amylase is a monomeric enzyme: (i) mass spectrometry indicates a molecular mass of 50 kDa (although we are aware that some disulfide bridges can be reduced in MALDI experiments); (ii) light scattering measurements show *Pfu* α-amylase as a 48 kDa protein; (iii) Cys165 is the only cysteine residue that reacts with PMPS (Figure 8), suggesting that Cys165 is not protected in a monomer−monomer interface; and (iv) all other family 13 α-amylases are monomeric enzymes. During light scattering, the protein solution is only exposed to a beam of light. The protein quaternary structure will not be altered during this process. For this reason, we trust our light scattering results over those of the other methods we used to determine the molecular mass and quaternary structure of *Pfu* α-amylase. Comparison of a large number of hyperthermophilic and mesophilic protein structures has shown that packing density is seldom higher (at the most, it can be marginally higher) in hyperthermostable enzymes. For *Pfu* α-amylase to be a dimer of 3.2 nm hydrodynamic radius, its packing density would have to be twice as high as that of an average mesophilic protein. For these reasons, we are convinced that *Pfu* α-amylase is a monomeric enzyme like the other family 13 α-amylases.

Pfu α-Amylase Is a Ca^{2+}- and Zn^{2+}-Containing Enzyme. Our ICP-AES results (Table 1) gave us only qualitative information about *Pfu* α-amylase's metal content. Repeating the experiment several times did not eliminate the large variability in the results. This variability may be due to the presence of DM in all our enzyme solutions. DM itself is not able to trap Ca^{2+} or Zn^{2+}, but it can solubilize part of the bacterial phospholipids that are present in the early stages of purification. Phospholipids are known to easily bind Ca^{2+}, but not Zn^{2+} (Shelagh Ferguson Miller, personal communication). During our purification procedure, the enzymes were commonly dialyzed or concentrated by ultrafiltration using 30 kDa molecular mass cutoff membranes, membranes that do not let phospholipids through. Thus, enzyme concentration steps also concentrated the phospholipids. Depending on the amount of Ca^{2+} bound to these phospholipids, their presence in the enzyme solution would affect our ICP-EAS results. Despite this variability, since the smallest Ca^{2+} content detected in Table 1 is 2.55 mol/mol of SSSSA after EDTA treatment at 20 °C, it is probably safe to conclude that *Pfu* α-amylase contains at least two Ca^{2+} ions. It is not uncommon for α-amylases to contain several Ca^{2+} cations. A good example of such an α-amylase is the *B. licheniformis* enzyme, which contains three Ca^{2+} (*4*). Because it is a widespread contaminant in glassware and plasticware, it could be argued that Zn^{2+} is present in *Pfu* α-amylase only by chance. The fact that only high-temperature EDTA treatments can deplete *Pfu* α-amylase of its Zn^{2+} proves otherwise. Not only does *Pfu* α-amylase bind Zn^{2+} specifically, but also it binds Zn^{2+} tightly.

Cys165 Is a Zn^{2+} Ligand. Although our ICP-AES data gave us only qualitative information about *Pfu* α-amylase's metal content, the facts that mutants CCSCC and SSSSA contained half the amount of Zn^{2+} contained in CCCCC and SSCSA and that only CCSCC and SSSSA lost their Zn^{2+} upon EDTA treatment at 20 °C were strong indications that Cys165 is a Zn^{2+} ligand. The release of approximately 1 mol of Zn^{2+} upon PMPS reaction with CCCCC confirmed that at least one of *Pfu* α-amylase's cysteines was a Zn^{2+} ligand. The fact that CCSCC did not release any Zn^{2+} upon reaction with PMPS allowed us to identify Cys165 as a Zn^{2+}-binding cysteine in *Pfu* α-amylase. The fact that [PMPS]/[enzyme] ratios of at least 120 were necessary to release all the Zn^{2+} from *Pfu* α-amylase suggests that, in contrast to mesophilic Zn^{2+}-containing enzymes, Cys165 is not readily accessible for equimolar reaction with PMPS. This observation also indirectly confirms that Zn^{2+} is specifically bound to Cys165. Our 3D model of *Pfu* α-amylase (Figure 5A) suggests that Cys152 and Cys153 are too distant from Cys165 to also be Zn^{2+} ligands. In our 3D model, we could identify only four residues (i.e., Asp62, His101, His166, and His168) that are typically found in Zn^{2+}-binding sites (*31*), and whose side chains are in proximity of Cys165 (Figure 5B). The side chain of His166 points away from Cys165,

though, suggesting that His166 is not part of the Zn^{2+}-binding site. If Asp62, His101, and His168 are the other Zn^{2+} ligands, mutants of these residues should bind less Zn^{2+} and be less thermostable than the wild-type enzyme.

Zn^{2+} Participates in Stabilizing Pfu α-Amylase. *Pfu* α-amylase requires Ca^{2+} for activity and stability. Before any stability experiment with Zn^{2+} can be performed, Ca^{2+} has to be added to the EDTA-treated enzyme. Since Zn^{2+} is omnipresent in glassware, in plasticware, and in chemicals (even in highly pure chemicals) by the time we have extensively dialyzed the enzyme and added Ca^{2+}, we probably have added enough Zn^{2+} to contaminate the experiment. For this reason, none of our attempts to show the role of Zn^{2+} in *Pfu* α-amylase stability by adding between 0.01 and 500 μM Zn^{2+} to the EDTA-treated, Ca^{2+}-containing CCCCC enzyme indicated any effect of Zn^{2+} on *Pfu* α-amylase stability. Two lines of evidence, though, clearly demonstrate that Zn^{2+} is a stabilizing factor in *Pfu* α-amylase. First, mutants CCSCC and SSSSA contain less than 1 mol of Zn^{2+}/mol of enzyme, and these mutants do not bind Zn^{2+} as tightly as the wild-type enzyme does, since their Zn^{2+} is easily removed by EDTA at 20 °C (Table 1). Second, these two mutants are completely inactivated after less than 10 min at 115 °C, as opposed to CCCCC and SSCSA, which have half-lives of 43 and 26 min at 115 °C, respectively. The fact that DTT destabilizes *Pfu* α-amylase (probably through its Zn^{2+}-chelating properties) is another argument in favor of Zn^{2+} stabilizing *Pfu* α-amylase. Thus, it is our understanding that Cys165 is part of a Zn^{2+}-binding site, and that Cys165 and the Zn^{2+} ion are required for *Pfu* α-amylase's optimal thermostability. It would be interesting to determine whether the *P. kodakaraensis* and *T. profundus* α-amylases contain Zn^{2+}, and whether introducing the equivalent of Cys165 in these enzymes affects their Zn^{2+} content as well as their stability.

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SUPPORTING INFORMATION AVAILABLE

One figure showing the titration of zinc in *P. furiosus* α-amylase in the absence of His-Tag. This material is available free of charge via the Internet at http://pubs.acs.org.

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