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The Purification and Characterization of an Extremely Thermostable α-Amylase from the Hyperthermophilic Archaebacterium *Pyrococcus furiosus**

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The α-amylase from *Pyrococcus furiosus*, a hyperthermophilic archaebacterium, has been purified to homogeneity. The enzyme is a homodimer with a subunit molecular mass of 66 kDa. The isoelectric point is 4.3. The enzyme displays optimal activity, with substantial thermal stability, at 100 °C, with the onset of activity at approximately 40 °C. Unlike mesophilic α-amylases there is no dependence on Ca^{2+} for activity or thermostability. The enzyme displays a broad range of substrate specificity, with the capacity to hydrolyze carbohydrates as simple as maltotriose. No subtrate binding occurs below the temperature threshold of activity, and a decrease in K_m accompanies an increase in temperature. Except for a decrease in Asp and an increase in Glu, the amino acid composition does not confirm previously defined trends in thermal adaption. Fourth derivative UV spectroscopy and intrinsic fluorescence measurements detected no temperature-dependent structural reorganization. Hydrogen exchange results indicate that the molecule is rigid, with only a slight increase in conformational flexibility at elevated temperature. Scanning microcalorimetry detected no considerable change in the heat capacity function, at the pH of optimal activity, within the temperature range in which activity is induced. The heat absorption peak due to denaturation, under these conditions, occurred within the temperature range of 90–120 °C. When the pH was increased, a change in the shape of the heat absorption peak was observed, which when analyzed thermodynamically shows that the process of heat denaturation is complex and includes at least three stages, indicating that the protein structure consists of three domains. At temperatures below 90 °C no excess heat absorption or change in the CD spectra were observed which could be associated with the cooperative conformational transition of the protein. According to the thermodynamic characteristics of the heat denaturation, the cold denaturation of this protein can be expected only at −3 °C. Therefore, the observed inactivation of this enzyme is not caused by the cooperative change of its tertiary structure. It can be associated only with the gradual changes of protein domain interaction.

The discovery of hyperthermophilic archaebacteria has provided a valuable tool for the analysis of protein stability. The intrinsic thermal stability of the enzymes isolated from these sources makes it possible to study the molecular mechanisms governing structure and function in a system adapted for elevated temperatures. The thermostability exhibited by these enzymes is maintained without any components unique to thermophiles, suggesting that the increase in molecular stability is accomplished through the same stereochemical interactions found in their mesophilic counterparts. The characteristic range of activity observed in hyperthermophilic enzymes tends to parallel growth temperature, there being little or no activity at temperatures which would be optimal for their mesophilic counterparts. Through analysis of these enzymes it should be possible to determine the stabilizing interactions by which the enzymes maintain activity at extreme temperatures.

α-Amylases are a favorable choice for comparison of mesophilic and thermophilic enzymes due to the wealth of data currently available about this enzyme family. The industrial importance of this enzyme also makes it a popular subject for study. α-Amylases have been purified from a variety of species spanning the range of thermostability from mesophiles (Takagi *et al.*, 1971), moderate thermophiles (Antranikian, 1989; Glymph and Stutzenberger, 1977; Hasegawa *et al.*, 1976) to hyperthermophiles (Koch *et al.*, 1991; Schumann *et al.*, 1991).

Pyrococcus furiosus is an anaerobic marine heterotroph with an optimal growth temperature of 100 °C, isolated by Fiala and Stetter (1986) from solfataric mud off the coast of Vulcano island, Italy. α-Amylase activity has been reported in the cell homogenate and growth medium of *P. furiosus* (Brown *et al.*, 1990; Koch *et al.*, 1990) but purification was not completed. In this report we present data on the purification of an α-amylase from *P. furiosus* and on a number of its physical, thermodynamic, and catalytic properties.

MATERIALS AND METHODS

Chemicals and Reagents—All chemicals were of reagent grade or better and were obtained from the following sources: Sigma, Aldrich Chemical Co., Fisher, or J. T. Baker Chemical Co. Yeast extract, Tryptone, and Bacto-agar were obtained from Difco. Q-Sepharose anion exchange resin was from Pharmacia LKB Biotechnology Inc. All gases were obtained from Bay State Liquid Nitrogen Inc. (Baltimore, MD).

Bacterial Strains and Culture Conditions—All of the cultures of *P. furiosus* used in these experiments employed strain DSM 3638, originally obtained from Deutsch Sammlung von Mikroorganismen, Braunschweig, Germany. Bacteria were grown on a complex medium modified from that previously described by Blumentals *et al.* (1990).

Standard Enzyme Assay—The dextrinizing activity of the α-amy-

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lase was determined using a modification of the assay of Manning and Campbell (1961). 20 μl each of sample, 1% soluble starch, and 100 mM sodium phosphate, pH 7.0, were incubated at 92 °C for 10 min, and the reaction was terminated by cooling in ice water. Color was developed by the addition of 15 μl of an iodine solution (4% KI, 1.25% iodine), and an additional 1 ml of distilled water was added to each sample to dilute the color of the sample to a measurable range at 600 nm. One unit of α-amylase activity was defined as the amount of protein which hydrolyzed 1 mg of starch/min.

SDS-PAGE[1]—Electrophoresis under denaturing conditions was carried out on a Pharmacia PhastSystem, using preprepared 8–25% gradient gels (lot QK12892) and SDS-buffer strips (lot QL13090). Separation was carried out using the optimized program detailed in the PhastSystem separations technique file no. 110 as found in the systems manual.

Native PAGE—Native gel electrophoresis was carried out according to the method of Laemmli (1970), excluding the presence of SDS. Gels were prepared at 8%, molecular weights were based on the comparative migration of β-amylase, bovine serum albumin, and carbonic anhydrase.

Standard techniques were utilized for Coomassie Blue staining. Gels were silver stained using a modification of the method of Morrissey (1981).

Activity staining was accomplished by the incorporation of starch into the acrylamide matrix of the resolving gel. When preparing the gels, as described above, 0.05% soluble starch was used in place of distilled water when the gels were cast. To observe the thermophilic amylase activity following electrophoresis the spacers and the gel left between the glass plates were sealed with Saran Wrap to prevent desiccation. The gel, between the plates, was then incubated for 30 min at 98 °C, then stained with iodine solution (see above). The band containing the α-amylase appeared as a clear area in the blue background of the gel. Following this procedure visualization with Coomassie Blue was carried out.

Isoelectric Focusing—The isoelectric point was determined by isoelectric focusing with the Pharmacia PhastSystem electrophoresis apparatus, using the manufacturers optimized method. A preprepared pH 3–9 isoelectric focusing gel (lot QM 13303) was used. Calibration was accomplished using a Pharmacia isoelectric focusing calibration kit.

Purification of α-Amylase from P. furiosus—Cells were harvested from 20 liters of growth medium at 7,000 × *g* for 10 min, the supernatant decanted, and the pellet collected. The cells were resuspended in a final volume of 15 ml of 50 mM sodium phosphate, pH 5.5, and subsequently disrupted by sonication. The cytosolic fraction of the lysate was collected after ultracentrifugation. All subsequent purification procedures were carried out at room temperature; protein solutions were stored at 4 °C.

The cytosolic fraction was applied, in sodium phosphate buffer, to a Q-Sepharose anion exchange column in three consecutive stages, twice at pH 5.5 followed by once at pH 10.3. Elution was carried out using linear sodium chloride gradients.

Purification was completed by electroelution from native PAGE with the Bio-Rad model 491 Prep Cell, using conditions recommended by the manufacturer. Amylase-containing fractions were detected by activity and screened, using silver stained native PAGE, for purity. The active fractions, shown to be pure within the level of resolution of the silver stain, were pooled as a final product.

Protein Quantization—Protein concentration was determined using the Bio-Rad microprotein determination assay, following the manufacturer's specifications, with bovine serum albumin as a standard. Using concentration data obtained with the above method, extinction coefficients were calculated at 254 and 280 giving values of 0.883 and 1.717, respectively.

Size Exclusion Chromatography—The apparent molecular weight of the purified enzyme, under various conditions, was determined on a precalibrated Superose 12 column using Pharmacia fast protein liquid chromatography.

Chelation of Divalent Cation and Determination of Free Ca^{2+} Concentration—A solution of purified *P. furiosus* α-amylase was depleted of divalent cations by passing it through a Bio-Rad Chelex 0.25-mm syringe filter. Free Ca^{2+} concentration of the filtered sample was determined by fura-2 fluorescence following the technique of Grynkiewicz *et al.* (1985).

Substrate Binding—Enzyme binding of substrate was quantitated by the adsorption of protein in solution to insoluble starch. 50 mg of starch were suspended in 200 μl of a 180 μg/ml solution of purified α-amylase and incubated at the desired temperature for 15 min. The substrate was sedimented by centrifugation in a microcentrifuge, and the supernatant activity was compared with control values to determine the quantity of protein bound.

Substrate Specificity—Substrate specificity of the enzyme was studied using purified amylase at a concentration of 350 μg/ml in a standard activity assay as described above with a variety of polysaccharide substrates. Incubations were carried out at 92 °C for 15 min. The pattern of hydrolysis was examined by thin-layer chromatography using the method of Hansen (1975).

Analytical Ultracentrifugation—Analytical ultracentrifugation was carried out using a Beckman Instruments model E analytical ultracentrifuge with a scanning adsorption optical system interfaced to an acquisition computer by means of a 12-bit Metrabyte DAS-8 analog to digital board. Scanning was in the rapid scan mode; 90,000 data acquisitions were made in the 18 s required for the scan. These were averaged in groups of 100 and the actual data density was 425 of these averaged points/cm of radius in the centrifuge cell (Lewis, 1992) Initial data conversion and editing was accomplished using software specifically written for this purpose. Further editing and data analysis by mathematical modeling using non-linear least-squares curve fitting were performed using MLAB (Civilized Software, Bethesda, MD) operating on the acquisition computer.

Hydrogen Exchange—A stock solution of tritiated water was obtained from DuPont-NEN with a specific activity of 1 mC/ml (lot 1258-250). 50 μl of tritium stock were added to 100 μl of purified α-amylase at 100 μg/ml in 50 mM sodium phosphate, pH 7.5. The samples were placed in microcentrifuge tubes and incubated at 24 and 95 °C. At time points of 2 and 24 h, the protein was separated from the tritiated water by size exclusion, using disposable NAP-5 columns.

To determine the rate of hydrogen exchange out of the protein, a 0.5-ml aliquot of the protein solution, following size exclusion, was incubated at either 24 or 95 °C for 2 h, and the size exclusion process was repeated.

Samples were prepared from the void volume when analyzing the exchange rate into the protein and both void and inclusion volumes when analyzing the exchange rate out of the protein. To prepare suitable samples, 50 μl of sample were added to 3 ml of Opti-Fluor liquid scintillation fluid (Packard Instrument Co.). Counting was carried out, for 1-min intervals, in an LKB 1212 Rackbeta liquid scintillation counter.

Fourth Derivative UV Spectrophotometry—Fourth derivative UV spectra were obtained using a Beckman DU-70 spectrophotometer equipped with a thermally jacketed stage. Absorption readings were taken every 0.5 nm from 240 to 350 nm. The fourth derivatives were calculated numerically with the $\Delta\lambda$ being 12, the results interpreted on a scale of 0.01 to −0.01 absorbance units. The sample used for spectrum generation contained 350 μg/ml purified amylase in 50 mM Hepes buffer, pH 7.0.

Fluorescence Spectroscopy—Temperature effects on fluorescence intensity and fluorescence emission spectra were recorded on an SLM 8000 spectrofluorometer. Excitation wavelength used was 290 nm and fluorescence intensity was measured at 350 nm. The band wavelength was 8 nm. The sample utilized was identical to that used in fourth derivative measurements.

Amino Acid Analysis—Protein samples were hydrolyzed in constant boiling HCl (no. 24309 Pierce Chemical Co.) containing 0.1% phenol at 110 °C for 24 h. Amino acids were analyzed using a Waters high performance liquid chromatography system and Pico-Tag™ derivitization on a 3.9 × 300 mm Pico-Tag™ column (no. 10950 Waters, Division of Millipore). Postcolumn detection was carried out at 254 nm on a model 440 Waters detector.

Cystine content was determined by performic acid oxidation and quantitization of cysteic acid.

Protein Sequence Analysis—Purified enzyme was digested with cyanogen bromide and, following reduction and pyridylethylation, with trypsin, using a modification of the methodology of Stone *et al.* (1989). The resulting fragments were then separated by gradient elution from 100% water containing 0.1% (v/v) trifluoroacetic acid to 70% acetonitrile containing 0.1% (v/v) trifluoroacetic acid on an Aquapore RP-300 reverse phase narrow bore column (0.2 cm × 25 cm), utilizing a Dionex Al-450 BioLC system.

Amino acid sequence analysis was performed on a Porton Instruments model 2020 off-line sequenator using standard program no. 1. Phenylthiohydantoin-derivative analysis was carried out on a Beck-

[1] The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; MES, 4-morpholineethanesulfonic acid.

man System Gold system using a modified sodium acetate gradient program and a Hewlett-Packard narrow bore C-18 column.

Scanning Microcalorimetry—Calorimetric measurements were completed using a DASM-4 scanning microcalorimeter, allowing the heating of aqueous solutions, under excess pressure, to 130 °C. Measurements were carried out with a heating rate of 1 °C/min and at a protein concentration of 1 mg/ml. The partial specific heat capacity of the protein solution was determined as described previously (Privalov and Potekhin, 1986), using the partial specific volume of the amylase $V = 0.751$ as calculated from the amino acid sequence (Makhatadze *et al.*, 1990). The heat capacity function of the α-amylase was analyzed using the sequential procedure of Freier and Biltonen (1978).

Circular Dichroism—Circular dichroism spectra were measured in the range of 190–360 nm at various temperatures with a Jasco-710 spectropolarimeter using a cell with a path length from 0.5 to 10 mm. The protein concentration was maintained at 0.68 mg/ml in 50 mM potassium phosphate, pH 7.0. To obtain the temperature dependence of the characteristics at a fixed wavelength, the cells were thermostated over the temperature range from 2 to 90 °C.

Effect of Temperature on Absorbance at 280 nm—Light absorption spectra in the range of 240–370 nm were measured with a Hitachi-200 spectrophotometer. The optical cells were thermostated with an accuracy of ±0.1 °C in the temperature range from 2 to 90 °C using a Neslab water bath. The temperature dependence of the absorption at 280 nm was measured by continuous heating with a constant rate of 1.0 °C/min. The protein concentration in the solution examined was 0.6 mg/ml.

Protease Susceptibility—The protease susceptibility of purified *P. furiosus* α-amylase was determined at 22, 37, and 50 °C. Equal volumes of 1.5 mg/ml amylase and 5 mg/ml thermolysin in 250 mM MES, 20 mM $CaCl_2$, pH 6.5, were incubated for 200 minutes in the presence and absence of 2 M urea. To provide a non-thermophilic digestion control, samples were prepared in parallel substituting ovalbumin for the *P. furiosus* amylase. The extent of proteolytic digestion was determined by separating equal volumes of sample on an 8% native PAGE gel. Intact protein was visualized using Coomassie Blue stain and quantitated with a Molecular Dynamics model 300 computing densitometer utilizing ImageQuest 3.15 software (Sunnyvale, CA).

Effect of Urea on Temperature-dependent Enzyme Activity—The effect of various concentrations of urea on the temperature dependent activation of the amylase was determined using a modification of the standard activity assay. Urea solutions were added to the assay mixture to bring the final urea concentration to either 0.1, 0.5, 1, or 2 M. The activity of these samples was then determined at 60, 79, and 92 °C following the standard protocol.

Effect of Temperature on Buffer pH—The pH values of the buffers used were calibrated for accuracy at the temperatures used for the respective measurements.

RESULTS

Purification of α-Amylase—To purify the amylase, crude cell supernatant was applied, then eluted from three successive ion exchange columns (see "Materials and Methods"). The apparent molecular weight and the relative purity of the protein were monitored throughout the purification process using native PAGE with activity staining in conjunction with Coomassie staining. Following the third ion exchange column the obtainable separation on native PAGE of the proteins remaining in solution made electroelution a viable option for production of purified protein.

The isoelectric point of the enzyme, as determined by isoelectric focusing, was found to be approximately pI 4.3.

Physiochemical Properties—The purified enzyme displays optimal activity at 100 °C with an onset of activity at approximately 40 °C and a substantial loss of activity at 120 °C. Within the temperature range of *P. furiosus* growth, the purified amylase exhibits activity at the level of 80% of optimum or higher (Fig. 1*A*). The thermostability of activity at the temperature of optimum activity (100 °C) was found to be relatively constant over the interval tested (Fig. 1*B*). The pH optimum, determined at the optimal temperature, was found in a pH range from 6.5 to 7.5 with a rapid decline in activity as pH moved to either extreme (Fig. 1*C*).

The molecular mass and subunit composition of the purified enzyme were determined by size exclusion chromatography under various conditions, analytical ultracentrifugation, and both native and SDS-polyacrylamide gel electrophoresis. Evaluated within a range of pH from 7.0 to 10.3, the apparent molecular mass of the protein as determined by gel filtration was found to be 157 ± 15 kDa. An increase in the ionic strength up to 1 M had no significant effect on the apparent molecular mass as determined by size exclusion. For a summary of electrophoretic analysis see Fig. 2. When analyzed using native polyacrylamide gel electrophoresis the apparent molecular mass was 129 kDa, regardless of sample heating, based on comparison with the migration of β-amylase, bovine serum albumin, and carbonic anhydrase. Electrophoresis in the presence of 8 M urea yields a shift in molecular mass to 66 kDa, with a slight shift in molecular mass associated with denaturation when the sample is heated. This suggests that the protein is a homogenous dimer which is dissociated in the presence of 8 M urea, the individual subunits of which are not completely denatured until heated. SDS-polyacrylamide gel electrophoresis, when performed without heating the sample prior to loading, yields results identical to those observed under native conditions. When the sample was heated in the presence of SDS thermal breakdown was observed, the extent of which was dependent on the duration of boiling (data not shown). This thermal instability in the presence of SDS is similar to that which has been observed with other thermophilic enzymes from hyperthermophilic archaebacteria (Pihl and Maier, 1991).

Ultracentrifugal equilibrium at 10,000 rpm and 20 °C for 113 h yielded a value for the molecular weight of 130,500. There was no evidence of heterogeneity or non-ideality. From the standard error estimates returned in the fitting procedure and from preliminary studies of the standard error using the bootstrap technique (Efron 1982) an error estimate of ± 550 seems probable. To determine the effect of denaturants and metal ions on the activity of *P. furiosus* α-amylase activity, stained native PAGE, or *in vitro* activity assays were utilized. Analysis by native PAGE in which samples were heated for 15 min in the presence of either 5 mM EDTA, 2% β-mercaptoethanol, or 1% SDS indicated that EDTA and β-mercaptoethanol, at these concentrations, had no significant effect on activity while, with 1% SDS, the loss of activity was almost complete. These results could not be repeated using a standard activity assay due to interference caused by the aformentioned compounds.

The extent of activity loss induced by the denaturants, urea and guanidine HCl, at 1 M concentrations, was determined using a standard activity assay. At 98 °C the residual activity in the presence of these denaturants was 86.6 and 73% for urea and guanidine HCl, respectively.

The effect of free Ca^{2+} and other metal ions on amylase activity was determined using an enzyme solution depleted of free calcium and other divalent cations to a level below 100 nM. Activity was then measured in the presence of the divalent cations Ca^{2+}, Co^{2+}, Cr^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, and Zn^{2+} at different concentrations. With the exception of Ca^{2+}, the addition of all the metal ions tested caused enzyme inhibition. The addition of free calcium caused a slight stabilization of the enzyme, the extent of which was constant over the range of concentrations tested.

The ability of the purified amylase to bind substrate was assessed at a number of temperatures below the range of enzymatic activity. When measured as a function of activity

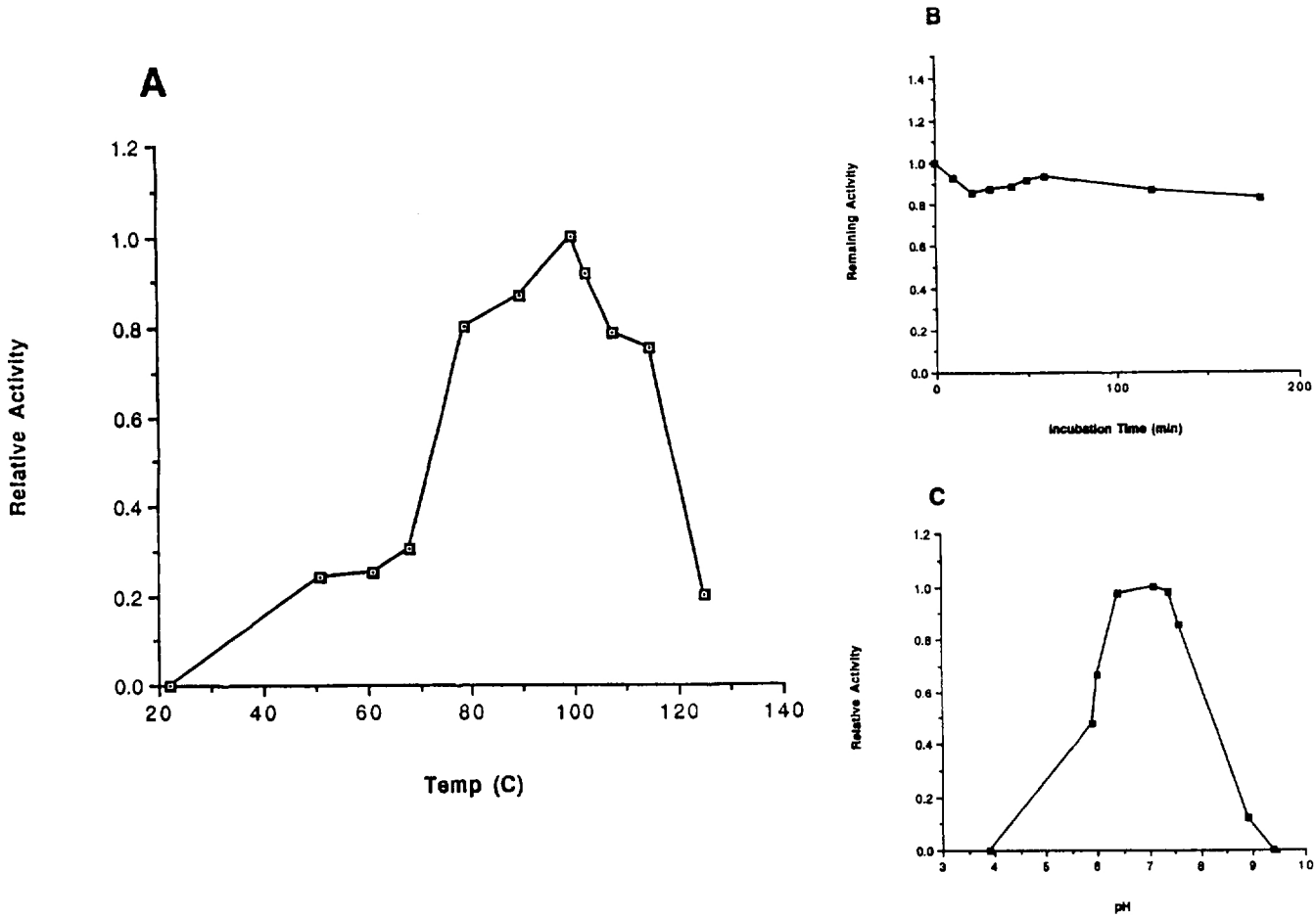

FIG. 1. **Characteristics of *P. furiosus* amylase activity.** *A*, effect of temperature on amylase activity. Activity was assayed at different temperatures using the standard activity assay. Incubation was for 10 min at pH 7.0. *B*, thermostability of enzymatic activity. The amylase samples were incubated at 100 °C for various time intervals, then the relative activity was determined and compared with a non-incubated sample using the standard activity assay. *C*, effect of pH on amylase activity. The relative enzyme activity was determined at varying pH using the standard activity assay (pH was maintained using 100 mM sodium phosphate adjusted to the appropriate value).

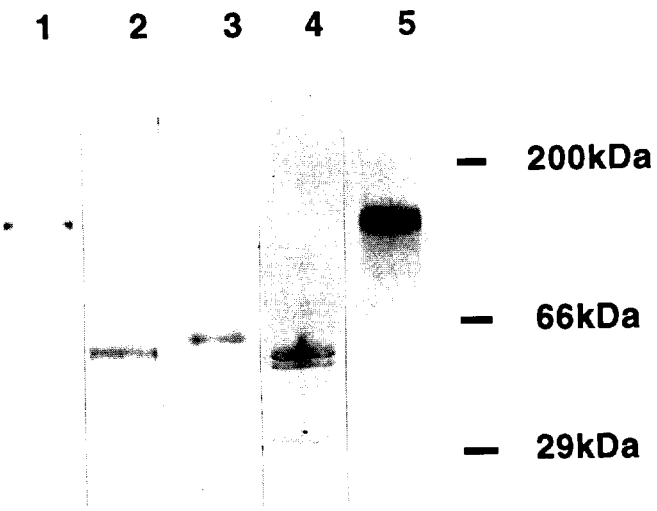

FIG. 2. **Electrophoretic characterization of *P. furiosus* amylase.** *Lanes*: *1*, Native polyacrylamide electrophoresis; *2* and *3*, electrophoresis in the presence of 8 M urea with and without sample heating respectively; *4* and *5*, SDS-polyacrylamide electrophoresis with and without sample heating, respectively. Molecular masses for native PAGE are approximated.

bound to insoluble substrate, a loss of less than 4% of the total enzymatic activity was found at 4, 21, and 37 °C.

Kinetic experiments were carried out using the standard activity assay at a variety of temperatures. The pH was maintained at 7.0 corresponding to the enzymatic activity maximum. Values for k_m and V_{max} were obtained from Lineweaver-Burk plots. At 65 and 75 °C similar results, 6.84 and 6.85 mg/ml, respectively, were obtained for k_m. When the temperature was increased to 91 °C the k_m decreased approximately 50% to 3.69 mg/ml. The values obtained for V_{max} dropped from 37.45 to 20.0 mg/ml/mg of enzyme with this increase in temperature.

Substrate Specificity—The enzymatic activity of the purified amylase digested starch to the level of glucose and maltose in addition to a mixture of polysaccharides, a majority of which were maltotetraose (G4), maltopentaose (G5), and maltohexaose (G6). In the presence of shorter polysaccharides the enzyme displays equilibrium-dependent product formation favoring the previously enumerated polysaccharides (G4, G5, and G6). With maltose as a substrate there is negligible enzymatic activity, limited to the production of a small quantity of maltotetraose and maltohexaose. The amylase cleaves maltotriose to glucose and maltose, in addition to formation of G4, G5, and G6. Patterns similar to those obtained with maltotriose were obtained when maltohexaose and maltoheptaose were used as substrate. The evidence suggests that,

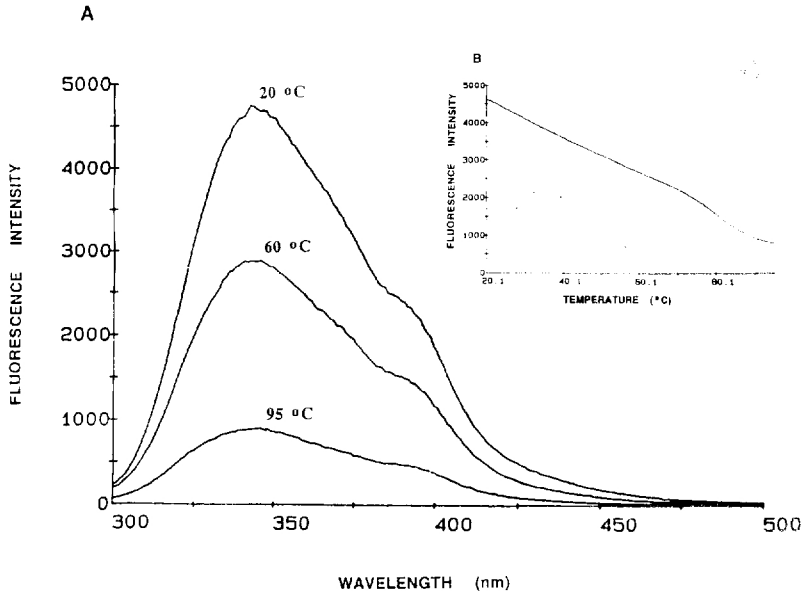

FIG. 3. **Effect of temperature on the intrinsic fluorescence of the purified amylase.** *A*, comparison of emission spectra at various temperatures. Excitation wavelength was 290 nm, the band wavelength, 8 nm. *B*, fluorescent intensity as a function of temperature. Excitation wavelength at 290, emission at 350 nm, with a band wavelength of 8 nm.

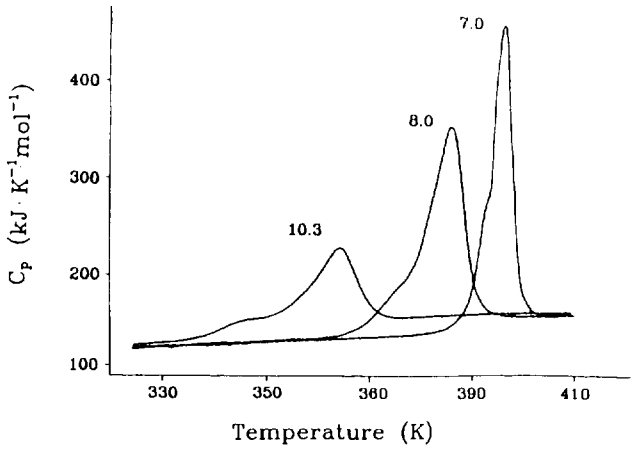

FIG. 4. **Temperature dependence of the partial heat capacity of the *P. furiosus* α-amylase in solutions at different pH values (indicated on the *curves*).** The scanning rate was 1.0 °C/min with a protein concentration of 1 mg/ml.

although maltotriose can be cleaved by the enzyme, the final equilibrium also mirrors the reverse reaction, producing longer polysaccharides.

Amino Acid Composition—The results obtained from amino acid analysis did not show any significant differences from the compositions of the enzyme of other species with markedly differing stabilities (Ihara *et al.*, 1985a; Koch *et al.*, 1991; Melasniemi 1988; Yang *et al.*, 1983; Yuuki *et al.*, 1985).

Protein Sequence Analysis—Eighteen amino acid residues were obtained from the sequencing of the N terminus of the purified protein: G/M-D-K-I-N-F-I-F-G-I-H-N-H-Q-P-L-G-N. Following digestion a segment of internal protein sequence was purified. This fragment had the N-terminal sequence: T-L-N-D-M-R-Q-E-Y-Y-F-K. These sequences were used for the preparation of degenerate oligonucleotide probes and are being utilized currently for the isolation of the *P. furiosus* α-amylase gene.

Hydrogen Exchange—Due to the nature of the gel filtration column utilized, the counts incorporated with "fast," "intermediate," and "slow" exchange rates were included in the protein bound fraction. Native protein at ambient and active temperature, 24 and 94 °C, respectively, displayed a level of incorporation much lower than that observed in urea denatured samples, indicating a low availibility of hydrogen atoms for exchange in the native folded conformation. A slight increase in bound tritium was observed when the temperature was maintained at 94 °C. When the rate of out-exchange was analyzed, at ambient and 94 °C, the exchange was found to be virtually complete after 2 h. Tritium in exchange at 94 °C followed by out-exchange at ambient temperature resulted in the trapping of 13% of the total counts. This may also be attributed to a temperature dependent increase in the accessibility of exchangeable hydrogens.

Fourth Derivative UV Spectrophotometry—A series of fourth derivative spectra were generated at temperatures ranging between 26 and 85 °C. Over this range there was a gradual reversible red shift, which was temperature-dependent. The shift is insignificant in magnitude and is linear as a function of temperature. This change in spectrum is thought to be a solvent effect rather than an indication of a change in the environment of the constituent aromatic amino acids in the protein.

Intrinsic Fluorescence Measurements—Fluorescence emission of α-amylase from *P. furiosus* at 20 °C exhibits a maximum at 345 nm. This maximum indicates that the tryptophan environments at this temperature are relatively polar (Teale 1960). When the spectrum was monitored over a range of temperatures there was no shift in the wavelength of the emission maximum (Fig. 3*A*). The maintenance of a constant emission maximum suggests that the tryptophan residues remain in the polar environment independent of temperature. When the fluorescence intensity was examined as a function of temperature it displayed a gradual decrease with a minor transition at approximately 65 °C (Fig 2*B*). This transition is indicative of a minor transfer of one or more tryptophan residues to a more polar environment (Ingham *et al.*, 1984). The smooth decrease in intensity with temperature reflects increased quenching due to greater thermal motion (Galley and Edelman, 1964). When the intrinsic fluorescence characteristics are considered in concert, it appears that the tryptophan residues in the enzyme are maintained in a polar environment and may shift slightly to a more polar environment at higher temperature, this shift being insufficient to be detected as a shift in emission maximum.

Scanning Microcalorimetry—Fig. 4 shows the temperature

dependence of the α-amylase partial heat capacity in solution at various pH values. The extensive heat absorption peak due to heat denaturation of the α-amylase at neutral pH is observed within the temperature range of 90–120 °C. This process is accompanied by an increase in the heat capacity of the protein of 0.35 $J \cdot K^{-1}g^{-1}$. The heat capacity of the native α-amylase in the pH range from 6.5 to 10.3 at 25 °C is 1.42 $J \cdot K^{-1}g^{-1}$. This is similar to the value obtained for many globular proteins (Privalov, 1979). The heat capacity increases linearly to 1.71 $J \cdot K^{-1}g^{-1}$ at 80 °C. The protein in the denatured state, following the denaturation transition, displays a heat capacity at 130 °C of 2.07 $J \cdot K^{-1}g^{-1}$. This closely approximates the heat capacity of the denatured state of the α-amylase (2.35 $J \cdot K^{-1}g^{-1}$) as calculated, based on the assumption that the polypeptide chain is completely unfolded (Makhatadze *et al.*, 1990).

Variation in pH leads to changes in the shape and temperature of the calorimetrically observed melting profile. Increasing the pH to 10.3 leads to significant destabilization and exhibits a change in the shape of the heat absorption peak, indicating that the α-amylase denaturation is not a two-state process under these conditions. The thermodynamic analyses of the excess heat capacity function of the enzyme shows that this process is complex and includes at least three stages which correspond to the unfolding of three cooperative domains (Fig. 5). At pH 10.3 the molecule maintains the same domain organization as at neutral pH, where it displays maximal enzyme activity. This suggests that the disappearance of enzymatic activity in alkaline pH is not due to denaturation, but appears to be the result of the pH dependent titration of some basic amino acids in the active site. The observed decrease in the denaturational enthalpy is caused by its trivial temperature dependence.

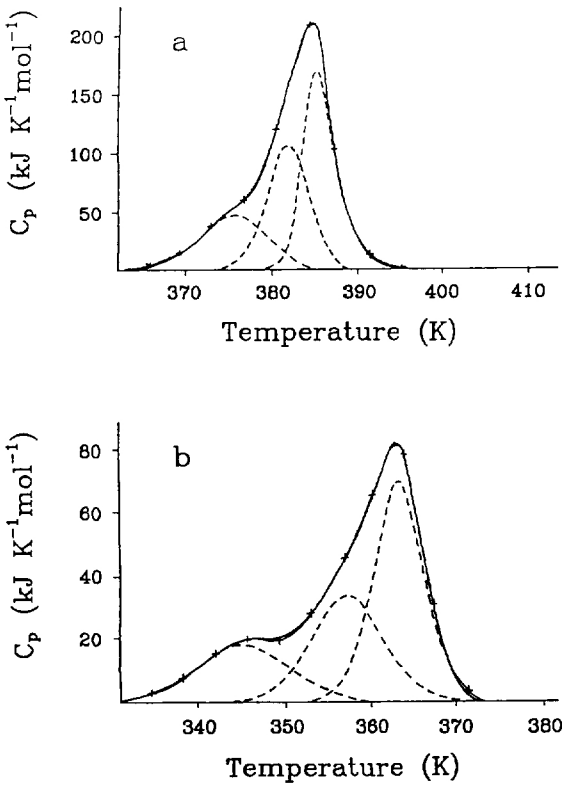

FIG. 5. **Deconvolution of the excess heat capacity function of the α-amylase.** *A*, *P. furiosus* amylase prepared in 50 mM KH_2PO_4, pH 8.0. *B*, *P. furiosus* amylase prepared in 50 mM Na_2CO_3, pH 10.3. *Crosses* indicate the functions calculated using the transition parameters obtained by deconvolution analysis.

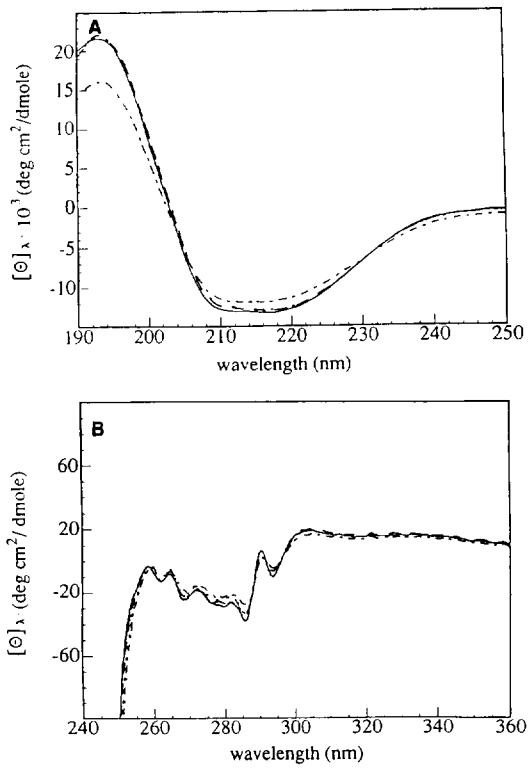

FIG. 6. **Circular dichroism spectrum of the *P. furiosus* α-amylase at various temperatures.** The protein was analyzed at a concentration of 0.68 mg/ml, in KH_2PO_4 at pH 7.0. *A*, The CD spectra of the α-amylase in the far ultraviolet at 2 °C, ——; 25 °C, – –; 50 °C, - - - -; 80 °C, — – —. *B*, CD spectra of the α-amylase in the near ultraviolet at 2 °C, ——; 25 °C, – –; 50 °C, - - - -; 80 °C, — – —.

In the temperature range of 5–60 °C the heat capacity function does not change considerably: it increases linearly with a slope of $5.5 \cdot 10^{-3}$ $J \cdot K^{-1}g^{-1}$. The value of the heat capacity in this temperature range, 1.42–1.65 $J \cdot K^{-1}g^{-1}$, indicates that the molecule does not have regions (domains) displaying a significant degree of unfolding.

One possible explanation for the difference between the observed and calculated values of heat capacity in the denatured state may be an aggregation effect observed with the α-amylase at extremely high temperature. This aggregation may also be the reason for the irreversibility of the α-amylase heat denaturation.

Circular Dichroism—The CD spectra of the α-amylase in the near and far UV region are presented in Fig. 6. There is no significant change in the CD spectra of the amylase in the temperature range from 2 to 80 °C. Since the ellipticity in the near UV region is caused by asymmetry in the aromatic amino acid environment we can conclude that the slight differences in the spectra reflect only a temperature dependence of this parameter, without any significant changes due to conformational transitions (Fig. 4*A*). Similar behavior was observed in the ellipticity in the far UV region (Fig 4*B*).

Effect of Temperature on Absorbance at 280 nm—The reversible temperature inactivation of the α-amylase is not accompanied by concurrent changes in absorbance at 280 nm, in direct contrast with results observed with another thermophilic protein from *P. furiosus*.[2] It was observed that the absorption at this wavelength decreases linearly with increas-

[2] F. T. Robb, H. Klump, J. Park, and M. W. W. Adams, manuscript submitted for publication.

ing temperature, up to 95 °C, without any cooperative changes within this temperature range.

Protease Susceptibility—The control digests containing ovalbumin displayed a temperature dependent increase in proteolytic digestion, the presence of 2 M urea accentuating this effect. In contrast, the *P. furiosus* amylase displays a moderate susceptibility to digestion at ambient temperature, but displayed only a slight temperature dependent increase in digestion.

Effect of Urea on Temperature-dependent Enzyme Activity—When the temperature dependent activation of the enzyme was analyzed in the presence of various quantities of urea a concentration dependent decrease in activity was observed. No significant inhibition of activity was noted at low urea concentration, the variation remaining within the limits of experimental error. At a urea concentration of 2 M there was a significant decrease in activity at all temperatures investigated. Nevertheless, a substantial portion of the overall enzyme activity remained intact.

DISCUSSION

The amylase from *P. furiosus* displays a temperature optimum of activity similar to that observed with the enzyme purified from the hyperthermophilic archaebacterium *Pyrococcus woesei*. The enzymes differ markedly in that the purification of the protein from *P. woesei* involves the capacity of the enzyme to bind substrate at ambient temperatures (Koch *et al.*, 1991). Analysis of the capacity of the *P. furiosus* amylase to bind substrate as a function of temperature indicates that no significant binding occurs at temperatures below that required for enzyme activation.

In contrast to the amylase isolated from *P. woesei*, the enzyme described herein displays a capacity for recognition of substrate with a lower degree of polymerization. Substrate specificity determination indicates that glucose polymers as short as maltotriose can serve as substrate. Surprisingly, under the conditions of the assay, an equilibrium condition was established favoring the production of maltotetraose, maltopentaose, and maltohexaose.

The thermal stability of the purified amylase was found to exceed that reported from *P. woesei*, while the pH optimum for activity was shifted towards a more neutral value. It can therefore be assumed that, although both the enzymes isolated from hyperthermophilic microorganisms have a similar temperature optimum, they differ in many general characteristics.

Unlike its mesophilic counterparts, the presence of free calcium does not appear to have a stabilizing effect on the enzyme. Calcium has been shown to be essential for the activity of a number of amylases obtained from mesophilic sources (Hsiu *et al.*, 1964; Vallee *et al.*, 1959) and has been found to increase the thermostability of the enzyme isolated from a strain of *Bacillus subtilis* (Moseley and Keay, 1970). As the temperature of optimal activity increases it appears that the requirement for free calcium decreases. The amylases from *Clostridium thermosulfurogenes* EM1, with a temperature optimum of 70 °C (Bahl *et al.*, 1991), and *Thermatoga maritima*, with a 95 °C temperature optimum (Schumann *et al.*, 1991), in addition to those isolated from the *Pyrococcus* genus, maintain activity despite the absence of free calcium.

Evaluation of apparent molecular mass utilizing size exclusion, native gel electrophoresis, and analytical ultracentrifugation, yielded consistent results in the neighborhood of 130 kDa. Upon incubation under extreme denaturing conditions a shift in molecular mass to 66 kDa was noted indicating a dimeric quaternary structure. The presence of a single N-terminal sequence indicates a homodimer. The rigorous means necessary to disrupt the quaternary structure of the protein are a testament to the inherent stability of the molecule. Complete dissociation of the dimer requires heat in addition to denaturant. The thermal destruction of the protein, as a result of boiling in SDS, implies an instability which may be a characteristic of enzymes isolated from hyperthermophiles. The hydrogenase isolated from *Pyrodictium brockii* displays a similar lability when boiled in SDS (Pihl and Maier, 1991) but will dissociate into its monomeric components without heating, thus displaying a less stable quaternary structure. It is possible that the breakdown of the protein is due to trace quantities of a thermostable proteinase which damages the protein as it unfolds in hot denaturant. This would necessitate that the contaminating enzyme remain active in 1% SDS while inactivated by 8 M urea. Heating in 1% SDS results in fragmentation, whereas heating in 8 M urea results in subunit dissociation but no degradation.

Both the characterization of optimal activity parameters (thermal stability and activity as a function of temperature) and the extreme conditions under which native quaternary structure is still maintained suggest an enzyme of substantial structural stability. In an attempt to explain the increase in thermal stability the amino acid compositions of a sampling of enzymes with a wide range of temperature optimum have been compared. From this comparison trends in amino acid exchanges which accompany increased thermostability were deduced (Argos *et al.*, 1979). Comparing the amino acid composition of various amylases, described previously, with the results obtained from *P. furiosus* amylase it is obvious that these trends are only partially applicable. The most significant exchange is Asp to Glu, whereas the other preferred changes, Gly to Ala, Ser to Ala, Ser to Thr, and Lys to Arg were not noted. Without additional information regarding the tertiary structure of the enzyme it is not possible to assign significance to the observed changes in amino acid composition.

When considering the structural features necessary to produce an enzyme which possesses long term thermal stability one must adapt what is known about the requirements for mesophilic stability to the problems inherent in a high temperature system. For an enzyme to exist in a functional state it must maintain a conformation appropriate for the recognition of ligand while maintaining the flexibility to allow for structural adjustment during binding and release of substrates, products, or other modulating factors (Wrba *et al.*, 1990). These two conflicting requirements must be maintained regardless of the temperature of optimal activity, the general result of which is the marginal free energy of stabilization under physiological conditions (Baldwin and Eisenberg 1987). Thermophilic enzymes must be maintain this balance of stability and flexibility while compensating for the additional molecular motion provided by elevated temperatures.

Kinetic analysis of starch hydrolysis, as a function of temperature, indicates a 50% decrease in the dissociation constant as the temperature is raised from 75 °C to 91 °C. This indicates a structural shift in the enzyme since the affinity of the enzyme for substrate seems to increase as a function of temperature. Enzymatic activity at high temperature suggests two possible models for this balance of physical characteristics. First, temperature drives a structural reorganization, modifying the active center, to create an environment which can recognize substrate. Second, the enzyme is maintained in a rigid structure at low temperature but requires increased temperature to provide the molecular flexibility for enzymatic activity. Both models propose a shift in the structure at the active center, the difference being that one is a concerted

change to create a new quaternary or tertiary structure, while the other is an increase in flexibility within a preexisting structure.

In the temperature range of 5–60 °C, the heat capacity function does not change considerably: it increases linearly with what is usually interpreted as a trivial increase in heat capacity (Privalov, 1979), indicating that the molecule does not have regions (domains) displaying a significant degree of unfolding until the commencement of the main process of heat denaturation.

From the results it is possible to conclude that the loss of α-amylase activity, induced by lowering the temperature below 60 °C, cannot be attributed to cold denaturation. This loss of tertiary structure, indicative of cold denaturation, is usually a cooperative process associated with a large enthalpy and entropy change. The profile of the temperature dependence of the α-amylase activity displays a maximum at 95–100 °C and decreases above and below this temperature range. It is clear that the mechanism of enzyme inactivation is different is these two cases. Inactivation above 100 °C is caused by protein denaturation, while the inactivation below 60 °C cannot be considered as denaturation with regard to the disruption of the tertiary structure. The temperature of the real cold denaturation of the amylase can be calculated as shown previously (Privalov *et al.*, 1986), and was found, in first approximation, to be approximately 270 K (−3 °C).

Fluorescence emission spectra displayed no shift in the emission maximum as a function of temperature, although when fluorescence intensity was examined as a function of temperature a minor transition was noted. Thus, from analysis based on the characteristics of constituent aromatic amino acids, only a slight shift of one or more tryptophan residues to a more polar environment parallels the onset of activity when the enzyme is heated to 100 °C.

The results obtained from scanning microcalorimetry, CD spectra analysis, fourth derivative UV spectroscopy, and protease suceptibility of the *P. furiosus* α-amylase suggest that the protein has a stable rigid tertiary structure within the temperature range of 5–90 °C. There were no detectable conformational changes, under native conditions, within the range of temperature at which the onset of enzyme activity is observed. Hydrogen exchange results indicate that there is a slight increase in molecular flexibility with increased temperature. However, the concentration dependent decrease in activity in the presence of urea suggests that the thermal activation of the enzyme cannot be attributed to this increased flexibility. It is likely that the thermal activation of the enzyme is due to slight changes in interdomain distances.

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