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ARTICLE *in* BIOCHEMISTRY · FEBRUARY 1999

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## Comparing the Thermodynamic Stabilities of a Related Thermophilic and Mesophilic Enzyme<sup>†</sup>

Beth M. Beadle,<sup>‡</sup> Walter A. Baase,<sup>§</sup> David B. Wilson,<sup>||</sup> Neil R. Gilkes,<sup>⊥</sup> and Brian K. Shoichet<sup>\*‡</sup>

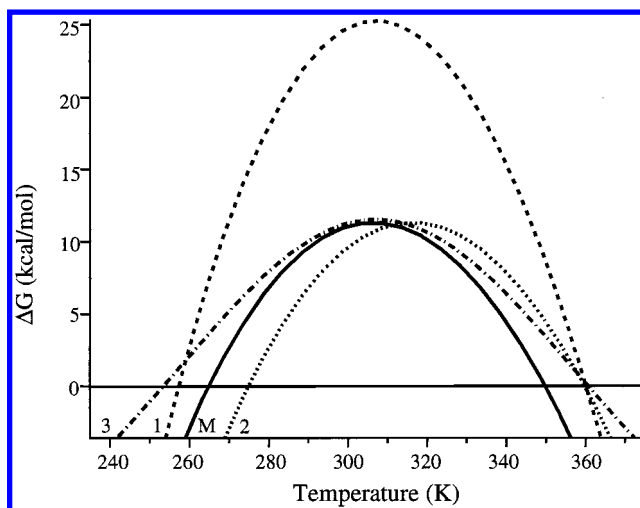
Department of Molecular Pharmacology & Biological Chemistry, Northwestern University, 303 East Chicago Avenue, Chicago, Illinois 60611-3008, Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403, Section of Biochemistry, Molecular & Cell Biology, Cornell University, Ithaca, New York 14853, and Department of Microbiology, University of British Columbia, Vancouver, Canada

Received October 19, 1998; Revised Manuscript Received December 9, 1998

**ABSTRACT:** Several models have been proposed to explain the high temperatures required to denature enzymes from thermophilic organisms; some involve greater maximum thermodynamic stability for the thermophile, and others do not. To test these models, we reversibly melted two analogous protein domains in a two-state manner. E2<sub>cd</sub> is the isolated catalytic domain of cellulase E2 from the thermophile *Thermomonospora fusca*. CenA<sub>P30</sub> is the analogous domain of the cellulase CenA from the mesophile *Cellulomonas fimi*. When reversibly denatured in a common buffer, the thermophilic enzyme E2<sub>cd</sub> had a temperature of melting ( $T_m$ ) of 72.2 °C, a van't Hoff enthalpy of unfolding ( $\Delta H_{VH}$ ) of 190 kcal/mol, and an entropy of unfolding ( $\Delta S_u$ ) of 0.55 kcal/(mol·K); the mesophilic enzyme CenA<sub>P30</sub> had a  $T_m$  of 56.4 °C, a  $\Delta H_{VH}$  of 107 kcal/mol, and a  $\Delta S_u$  of 0.32 kcal/(mol·K). The higher  $\Delta H_{VH}$  and  $\Delta S_u$  values for E2<sub>cd</sub> suggest that its free energy of unfolding ( $\Delta G_u$ ) has a steeper dependence on temperature at the  $T_m$  than CenA<sub>P30</sub>. This result supports models that predict a greater maximum thermodynamic stability for thermophilic enzymes than for their mesophilic counterparts. This was further explored by urea denaturation. Under reducing conditions at 30 °C, E2<sub>cd</sub> had a concentration of melting ( $C_m$ ) of 5.2 M and a  $\Delta G_u$  of 11.2 kcal/mol; CenA<sub>P30</sub> had a  $C_m$  of 2.6 M and a  $\Delta G_u$  of 4.3 kcal/mol. Under nonreducing conditions, the  $C_m$  and  $\Delta G_u$  of CenA<sub>P30</sub> were increased to 4.5 M and 10.8 kcal/mol at 30 °C; the  $C_m$  for E2<sub>cd</sub> was increased to at least 7.4 M at 32 °C. We were unable to determine a  $\Delta G_u$  value for E2<sub>cd</sub> under nonreducing conditions due to problems with reversibility. These data suggest that E2<sub>cd</sub> attains its greater thermal stability ( $\Delta T_m = 15.8$  °C) through a greater thermodynamic stability ( $\Delta\Delta G_u = 6.9$  kcal/mol) compared to its mesophilic analogue CenA<sub>P30</sub>.

The high stability to temperature (thermal stability) of enzymes from thermophilic organisms (thermophilic enzymes) has provoked considerable debate. Several reasons have been proposed to explain why thermophilic enzymes are more stable than their mesophilic analogues, including improved ionic (1–3), aromatic (2), hydrogen bonding, and solvophobic interactions (3). However, there is no general agreement that thermophilic enzymes are, in fact, thermodynamically more stable than their mesophilic analogues.

Three models have been proposed to explain the higher denaturation temperatures of thermophilic proteins (4–6) (Figure 1); each has a different thermodynamic consequence. Compared to the mesophilic enzyme (Figure 1; curve M), the thermophilic enzyme could be more thermodynamically stable throughout the temperature range, i.e., have a higher free energy of unfolding ( $\Delta G_u$ )<sup>1</sup> than the mesophilic enzyme at every temperature (Figure 1; curve 1). The parabolic dependence of  $\Delta G_u$  on temperature for proteins (7–11)



**FIGURE 1:** Three different models for the dependence of free energy on temperature of a thermophilic protein. Curve M (solid) depicts the relation of  $\Delta G_u$  to temperature for a typical mesophilic protein. In curve 1 (dashed), the thermophilic protein is stabilized across the temperature range and has a greater maximum stability (model 1). In curve 2 (dotted), the relationship between  $\Delta G_u$  and temperature is the same as that for the mesophilic enzyme, but the curve is shifted to a higher temperature (model 2). In curve 3 (dash-dot-dash patterned), the  $\Delta G_u$  of the thermophilic enzyme depends less on temperature and thus is 'flatter' (model 3).

<sup>†</sup>This work was partly supported by Grant MCB-9734484 from the NSF and by a starter grant from the Petroleum Research Fund of the American Chemical Society (both to B.K.S.).

\* Corresponding author. E-mail: b-shoichet@nwu.edu.

<sup>‡</sup> Northwestern University.

<sup>§</sup> University of Oregon.

<sup>||</sup> Cornell University.

<sup>⊥</sup> University of British Columbia.

implies a greater absolute magnitude in the entropy and enthalpy of unfolding ( $\Delta S_u$  and  $\Delta H_u$ ) at the temperature of melting ( $T_m$ ). A second model predicts that the free energy profile of the thermophilic enzyme will be horizontally displaced to a higher temperature (Figure 1; curve 2). In this "shifted" model, the maximal values of  $\Delta S_u$ ,  $\Delta H_u$ , and  $\Delta G_u$  would be equal for the mesophilic and thermophilic proteins, but the maxima would occur at different temperatures. At higher temperatures, the thermophilic protein would be more stable; at lower temperatures, the mesophilic protein would be more stable. Finally, a third model predicts that the free energy profile of the thermophilic enzyme would be a flattened version of that of the mesophilic enzyme; the thermophilic enzyme would have a more shallow dependence of  $\Delta G_u$  on temperature (Figure 1; curve 3). In this "flattened" model, the maximal  $\Delta G_u$  would again be equal for the mesophilic enzyme and thermophilic enzyme; however, the lesser dependence on temperature would result in a lower magnitude of  $\Delta S_u$  and  $\Delta H_u$  at the  $T_m$  for the thermophilic enzyme compared to the mesophilic enzyme at its  $T_m$ . Several investigations have supported this "flattened" model (4, 5, 12, 13).

Experimental testing of these models has been hindered by difficulties in obtaining reversible, two-state temperature denaturation of pairs of homologous mesophilic and thermophilic proteins (3, 14–16). In particular, thermophilic enzymes often fail to refold upon cooling in temperature denaturation experiments (17, 18). Recently, reversible, two-state denaturation has been seen for several small proteins from hyperthermophilic organisms (12, 13). These proteins were observed to have stabilities within the range observed for mesophilic proteins, consistent with the "flattened" model of thermostability (Figure 1; curve 3). However, no direct comparison to mesophilic analogues was made, and there have been few studies that make such comparisons. In early work by Nojima et al., a thermophilic phosphoglycerate kinase was compared to a mesophilic analogue (4). The thermophilic enzyme was 6.5 kcal/mol more stable than the mesophilic enzyme at 25 °C, but the thermophilic enzyme displayed much lower  $\Delta S_u$  and  $\Delta C_p$  values. This behavior has aspects of both the "flattened" model (Figure 1; curve 3) and the increased stability model (Figure 1; curve 1), in that although the maximum thermodynamic stability is greater, the transition itself is much shallower (lower  $\Delta S_u$ ) (4). In a second study, Kanaya and Itaya compared the stability of a thermophilic RNase H to a mesophilic analogue (19). The thermophilic enzyme was 11.8 kcal/mol more stable at 25 °C than the mesophilic analogue, but it also had a larger magnitude  $\Delta S_u$  as compared the mesophilic RNase H. This behavior is consistent with the higher stability model showing a greater maximum stability and a steeper transition (Figure 1; curve 1). Very recently, Jaenicke and co-workers have found that phosphoglycerate kinase from *Thermotoga maritima* is stabilized by 20 kcal/mol at room temperature,

which is consistent with the higher stability model (Figure 1; curve 1) (20).

In this investigation, we compare the thermodynamic stabilities of the catalytic domains of two analogous cellulases ( $\beta$ -1,4-glucanases). The thermophilic catalytic domain, E2<sub>cd</sub>, is from *Thermomonospora fusca*; the mesophilic catalytic domain, CenA<sub>P30</sub>, is from *Cellulomonas fimi*. The catalytic domains were isolated by controlled proteolysis or genetic manipulation (21). They share 41% sequence identity (22), and both are classified in the glycosyl hydrolase family 6 (23). They have simple quaternary structures; the catalytic activity is localized within a single, isolatable domain of 30 kDa; they lack prosthetic groups and have no need for metal cofactors. Both enzymes have two disulfide bridges at equivalent positions (21).

## MATERIALS AND METHODS

(1) *Enzyme Preparation.* The isolated catalytic domain E2<sub>cd</sub> was expressed and purified as previously described (21, 24–26). The catalytic domain of CenA (CenA<sub>P30</sub>) was expressed and purified as previously described (27). Unless otherwise indicated, each enzyme was studied at a concentration of approximately 8  $\mu$ g/mL.

(2) *Buffer Preparation.* Each buffer was prepared using ACS reagent-grade potassium chloride (KCl) from Sigma and potassium phosphate (KPi) from Aldrich in deionized water (MilliQ). For several buffers, spectroscopic grade ethylene glycol from Sigma was also used. Ethylene glycol was found to improve the reversibility of folding for the mesophilic enzyme, presumably by reducing aggregation of the denatured state. The effects of small-molecule cosolvents on protein stability have been extensively studied (28). Ethylene glycol had no measurable stabilizing effect for either the mesophilic or the thermophilic enzyme. The pH of each buffer was adjusted by dropwise addition of 3 N HCl or 10 N NaOH from Aldrich.

Optimal thermal denaturation of E2<sub>cd</sub> took place in a 50 mM KPi, 100 mM KCl, pH 6.8, buffer. Optimal thermal denaturation of CenA<sub>P30</sub> took place in a 50 mM KPi, 800 mM KCl, 45% ethylene glycol, pH 6.8, buffer. The experiments that compared E2<sub>cd</sub> and CenA<sub>P30</sub> in a common buffer used a 50 mM KPi, 225 mM KCl, 11.25% ethylene glycol, pH 6.8, buffer.

For solvent denaturation, stock solutions of 10 M urea were made by addition of Ultrapure urea from Amresco to 50 mM KPi, 100 mM KCl, pH 6.8, buffer and readjustment of the pH to 6.8. These 10 M urea stocks were stored at –20 °C until used. Denaturation buffers were prepared by adding the appropriate amount of 10 M urea stock to 50 mM KPi, 100 mM KCl, pH 6.8, to produce a given concentration of urea; there was no change in the buffer or salt concentrations. For selected solvent denaturations, urea buffers were prepared as above with the addition of 25 mM dithiothreitol (DTT) to produce a concentration of 2.5 mM DTT. For renaturation, the sample was diluted 2-fold by addition of 50 mM KPi, 100 mM KCl, pH 6.8; reversibility was judged by the return of the folded CD signal at the appropriate wavelength.

(3) *CD Measurements.* CD experiments were carried out in a Jasco J-715 spectropolarimeter with a Jasco PTC-348WI peltier-effect temperature controller. Quartz cells with a 1 cm path length from Hellma, Inc., were used for all

<sup>1</sup> Abbreviations: E2<sub>cd</sub>, *T. fusca* endoglucanase 2 catalytic domain; E2, *T. fusca* endoglucanase 2; CenA<sub>P30</sub>, *C. fimi* cellobiohydrolase A catalytic domain;  $T_m$ , temperature of melting;  $\Delta H_{VH}$ , van't Hoff enthalpy of unfolding;  $\Delta S_u$ , entropy of unfolding;  $\Delta G_u$ , free energy of unfolding;  $C_m$ , concentration of melting;  $\Delta C_p$ , change in constant-pressure heat capacity; CD, circular dichroism; UV, ultraviolet; KPi, potassium phosphate; KCl, potassium chloride; DTT, dithiothreitol.

experiments. For thermal denaturations, an in-cell temperature probe and stirbar were used.

Thermal denaturations were analyzed using the program EXAM (29) to calculate all  $T_m$  and  $\Delta H_{VH}$  values. Each figure shows a single denaturation experiment; however, all stability measurements reflect an average of at least two, and typically three, independent experiments. The exceptions are the slow renaturations of E2<sub>cd</sub> and CenA<sub>P30</sub>, which were done once. Error estimates are indicated in the tables. In the analyses, the change in heat capacity upon denaturation ( $\Delta C_p$ ) for each enzyme was set to 3.8 kcal/(mol•K) for each enzyme. This is consistent with the  $\Delta C_p$  values determined experimentally for other enzyme systems (12, 13, 30) and with theoretical considerations, based on the size and percent hydrophobicity of these enzymes (31). We note that the  $\Delta H_{VH}$  and  $T_m$  values are affected only slightly by varying  $\Delta C_p$  between 0 and 4.8 kcal/(mol•K), although the fits to the data for the CenA<sub>P30</sub> melts deteriorate with  $\Delta C_p$  values above 3.8 kcal/(mol•K). Solvent denaturations were analyzed using the program ORIGIN from Microcal, Inc. (32).

Samples were monitored for helical content by CD in the far-UV region at 223 or 228 nm at an enzyme concentration of approximately 8  $\mu$ g/mL, and for tertiary structure by CD in the near-UV region at 285 nm at an enzyme concentration of approximately 32  $\mu$ g/mL. Thermal melting was performed at ramp rates of 1 and 2 °C/min; denaturation behavior did not vary with the ramp rate when rates were less than 4 °C/min (data not shown). Reversibility was judged using three criteria: the return of the original CD signal upon quick cooling, the path of renaturation as determined upon slow cooling, and comparison of the CD spectra of the enzyme prior to denaturation and then after renaturation.

(4) *Fluorescence Measurements.* Fluorescence experiments were performed in a Jasco J-715 spectropolarimeter with a peltier-effect temperature controller and an excitation fluorescence attachment. This allowed us to monitor thermal behavior by CD and fluorescence simultaneously. Excitation spectra were used to choose a wavelength that allowed for simultaneous monitoring of denaturation by fluorescence and CD. E2<sub>cd</sub> samples were excited at 226.5 nm; CenA<sub>P30</sub> samples were excited at 228 nm. Thermal melting was performed as indicated for the CD measurements. Reversibility was judged by the return of the fluorescence signal upon quick cooling.

(5) *Solvent Denaturations.* E2<sub>cd</sub> and CenA<sub>P30</sub> were denatured with urea in 50 mM KP<sub>i</sub>, 100 mM KCl, 2.5 mM DTT, pH 6.8, buffer at 30 °C. CenA<sub>P30</sub> was also denatured under the same conditions in the absence of DTT. Separate buffer samples were prepared for each urea concentration in Eppendorf tubes. Enzyme was added, and the samples were incubated at 30 °C. The CD signal of the enzyme was measured at 223 nm. Data were plotted in ORIGIN and fit using sigmoidal analysis to provide a concentration of melting ( $C_m$ ) and values of the folded and unfolded base lines (32). Error estimates are listed in the tables. The equilibrium constant of the unfolding reaction ( $K$ ) was determined from the measured CD value compared to the baseline values as the ratio of unfolded protein to folded protein at a given concentration of urea. The  $\Delta G_u$  value for each concentration of urea was calculated using

$$\Delta G_u^\circ = -RT \ln K \quad (1)$$

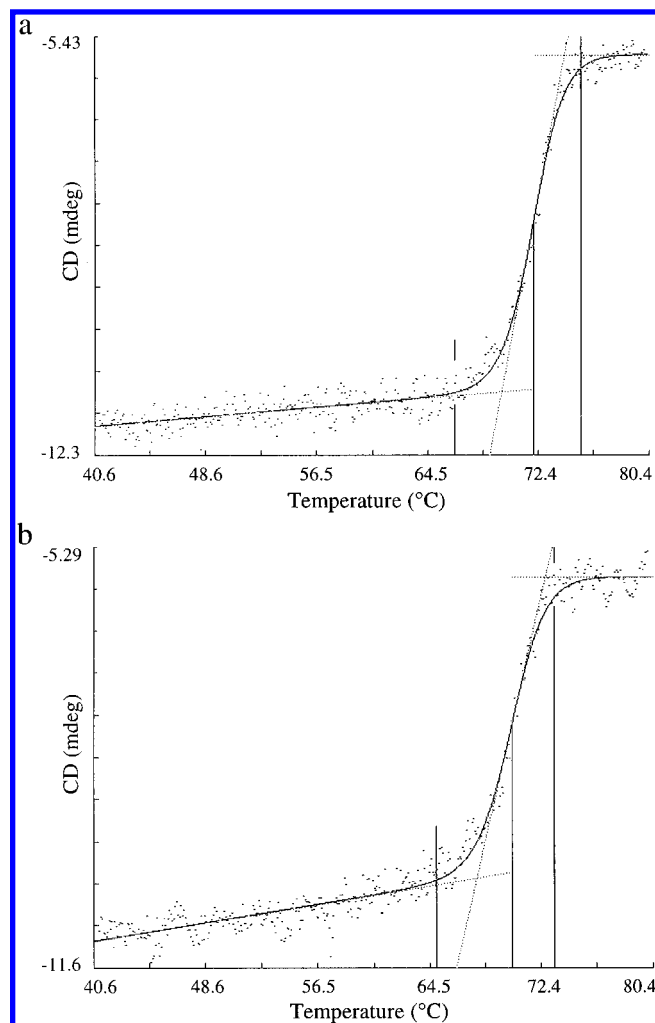


FIGURE 2: Thermal denaturation (a) and renaturation (b) curves of E2<sub>cd</sub> in 50 mM KP<sub>i</sub>, 100 mM KCl, pH 6.8, as monitored by CD at 223 nm and as fit by the two-state analysis program EXAM (29). CD signal (mdeg) on the y-axis; temperature (°C) on the X-axis. For both the melt and the “demelt”, the rate of temperature change was 2 °C/min. (a) For thermal denaturation, the average  $T_m$  is 72.3 °C; the average  $\Delta H_{VH}$  is 223 kcal/mol. (b) For thermal renaturation, the  $T_m$  is 70.3 °C; the  $\Delta H_{VH}$  is 192 kcal/mol.

Table 1: Thermodynamic Quantities Determined by Melting of the Thermophilic and Mesophilic Enzymes

	buffer	technique	$T_m$ (°C)	$\Delta H_{VH}$ (kcal/mol)	$\Delta S$ [kcal/(mol•K)]
E2 <sub>cd</sub>	a	far-UV CD	72.3 ± 0.3	223 ± 22	0.65 ± 0.05
	a	near-UV CD	71.4 ± 1.5	261 ± 25	0.76 ± 0.07
	a	fluorescence	72.6 ± 0.1	216 ± 14	0.63 ± 0.03
CenA <sub>P30</sub>	b	far-UV CD	49.9 ± 0.6	84.2 ± 16	0.26 ± 0.04
	b	fluorescence	50.1 ± 0.4	90.2 ± 5.5	0.28 ± 0.02
E2 <sub>cd</sub>	c	far-UV CD	72.2 ± 0.2	190 ± 14	0.55 ± 0.04
CenA <sub>P30</sub>	c	far-UV CD	56.4 ± 0.3	107 ± 3.1	0.32 ± 0.01

<sup>a</sup> Buffer is 50 mM KP<sub>i</sub>, 100 mM KCl, pH 6.8. <sup>b</sup> Buffer is 50 mM KP<sub>i</sub>, 800 mM KCl, 45% ethylene glycol, pH 6.8. <sup>c</sup> Buffer is 50 mM KP<sub>i</sub>, 225 mM KCl, 11.25% ethylene glycol, pH 6.8.

The linear dependence of  $\Delta G_u$  on urea concentration (M) was used to determine the change in  $\Delta G$  with urea concentration, the ‘ $m$ -value’. Extrapolation back to zero molar urea gives the  $\Delta G_u^{H_2O}$  under nondenaturing conditions (33):

$$\Delta G_u^{H_2O} = m \cdot C_m \quad (2)$$



Attempts were made to denature each enzyme in the absence of DTT with the same procedure. In this case, E2<sub>cd</sub> was incubated for 7 days at 32 °C and CenA<sub>P30</sub> for 24 h at 30 °C and analyzed as before.

## RESULTS

**Reversible Denaturation and Two-State Behavior of E2<sub>cd</sub>.** E2<sub>cd</sub> was reversibly denatured by temperature (Figure 2a) and analyzed by far-UV CD. The melting behavior was fit using a two-state analysis program (29) to give an average  $T_m$  of 72.3 °C and an average  $\Delta H_{VH}$  of 223 kcal/mol (Table 1). After immediate cooling of the sample, 95–100% of the folded CD signal returned. The CD spectrum (210–300 nm) of the renatured sample closely resembled that of the unmelted native enzyme (not shown). Alternatively, the enzyme could be renatured by cooling the sample at the same rate as it had been heated; in this case, the renaturation  $T_m$  was 70.3 °C, 2.0 °C lower than the denaturation  $T_m$  for this particular experiment (Figure 2b). The  $\Delta H_{VH}$  of renaturation was calculated to be 192 kcal/mol, 31 kcal/mol lower than the denaturation  $\Delta H_{VH}$ .

To determine whether E2<sub>cd</sub> denatures in a two-state manner, the enzyme was studied using three different techniques. In addition to thermal denaturation at 223 nm (Figure 2), thermal denaturation of E2<sub>cd</sub> was monitored by near-UV CD at 285 nm (not shown). Using this measure of tertiary structure, the  $T_m$  was 71.4 °C and the  $\Delta H_{VH}$  261 kcal/mol (Table 1). Thermal denaturation was also monitored using fluorescence excitation at 226.5 nm; the average  $T_m$  was 72.6 °C, and the average  $\Delta H_{VH}$  was 216 kcal/mol (not shown) (Table 1). These data suggest that E2<sub>cd</sub> denatures in a good approximation to the reversible, two-state ideal.

**Reversible Denaturation and Two-State Behavior of CenA<sub>P30</sub>.** CenA<sub>P30</sub> was reversibly denatured by temperature with an average  $T_m$  of 49.9 °C and an average  $\Delta H_{VH}$  of 84.2 kcal/mol as measured by far-UV CD (Figure 3a) (Table 1). After immediate cooling of the sample, 100% of the original folded CD signal returned. The CD spectrum (210–300 nm) of the renatured sample closely resembled that of the native enzyme (not shown). On slow cooling, CenA<sub>P30</sub> renatured with a  $T_m$  of 50.4 °C and a  $\Delta H_{VH}$  of 67.0 kcal/mol (Figure 3b). In these experiments, where the protein is cooled slowly from the unfolded state back through the folding transition, the unfolded baselines were limited to minimize the time the protein was unfolded at high temperature, thus maximizing refolding. Despite the short upper baselines, the thermodynamic values determined from these experiments were consistent with those determined with more extensive upper baselines.

To investigate two-state behavior, thermal denaturation of CenA<sub>P30</sub> was followed by fluorescence excitation at 228 nm (not shown); the average  $T_m$  was 50.1 °C, and the average  $\Delta H_{VH}$  was 90.2 kcal/mol (Table 1). Like E2<sub>cd</sub>, CenA<sub>P30</sub> demonstrates reversible, two-state thermal denaturation.

**Thermal Denaturation: Comparison of E2<sub>cd</sub> and CenA<sub>P30</sub>.** The two enzymes, E2<sub>cd</sub> and CenA<sub>P30</sub>, were reversibly denatured by temperature in a common buffer to allow direct comparison between the thermodynamic variables.

Under these conditions, E2<sub>cd</sub> melted at an average  $T_m$  of 72.2 °C with a  $\Delta H_{VH}$  of 190 kcal/mol (Figure 4) (Table 1). Upon quick cooling, E2<sub>cd</sub> recovered 95–100% of its initial CD signal. Upon slow renaturation at the same temperature

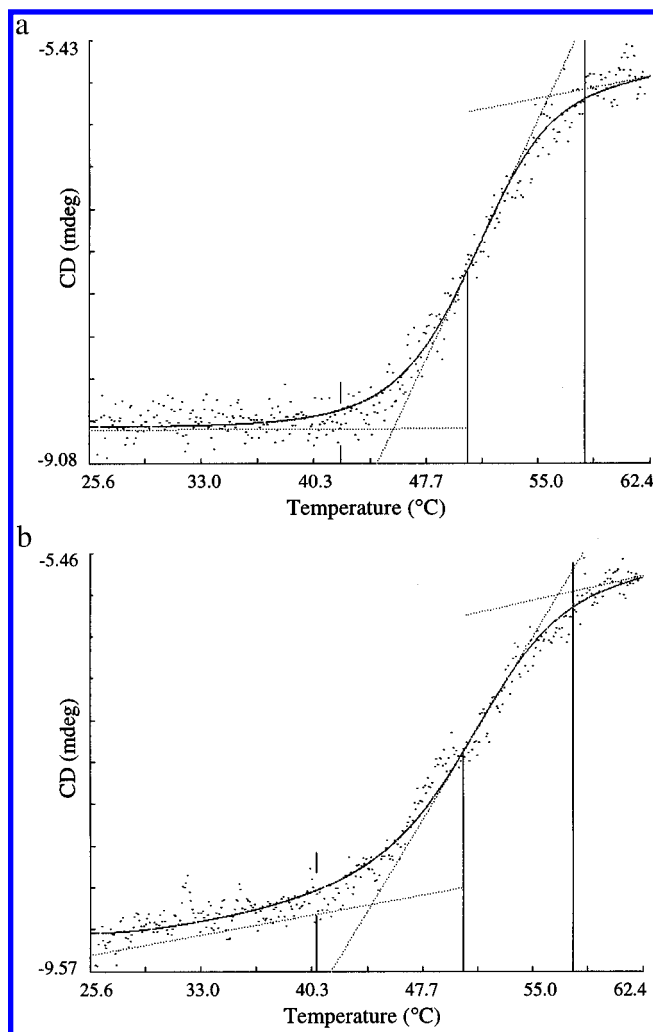


FIGURE 3: Thermal denaturation (a) and renaturation (b) of CenA<sub>P30</sub> in 50 mM KPi, 800 mM KCl, 45% ethylene glycol, pH 6.8, monitored by CD at 223 nm with a ramp rate of 2 °C/min. Both were analyzed as two-state (29). (a) For thermal denaturation, the average  $T_m$  is 49.9 °C; the  $\Delta H_{VH}$  is 84.2 kcal/mol. (b) For thermal renaturation, the  $T_m$  is 50.4 °C; the  $\Delta H_{VH}$  is 67.0 kcal/mol.

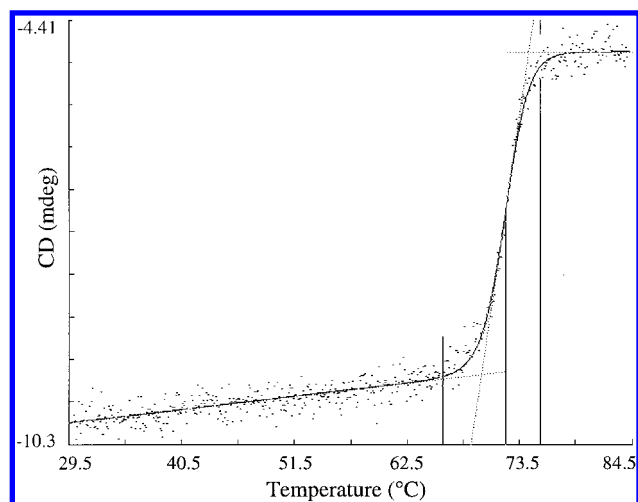


FIGURE 4: Thermal denaturation of E2<sub>cd</sub> in 50 mM KPi, 225 mM KCl, 11.25% ethylene glycol monitored by CD at 223 nm with a ramp rate of 2 °C/min. The thermal denaturation was analyzed as two-state (29). The average  $T_m$  is 72.2 °C, and the average  $\Delta H_{VH}$  is 190 kcal/mol.

ramp as the original denaturation, the  $T_m$  was 68.8 °C, and the  $\Delta H_{VH}$  was 176 kcal/mol (not shown).

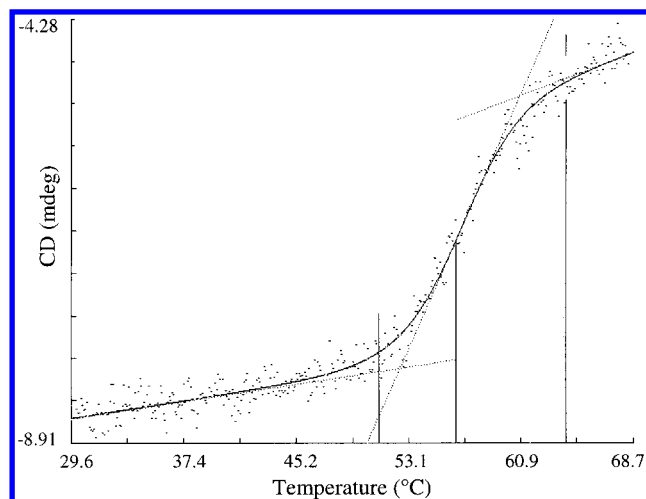


FIGURE 5: Thermal denaturation of CenAP<sub>30</sub> in 50 mM KP<sub>i</sub>, 225 mM KCl, 11.25% ethylene glycol monitored by CD at 223 nm with a ramp rate of 2 °C/min. The thermal denaturation was analyzed as two-state (29). The average  $T_m$  is 56.4 °C, and the average  $\Delta H_{VH}$  is 107 kcal/mol.

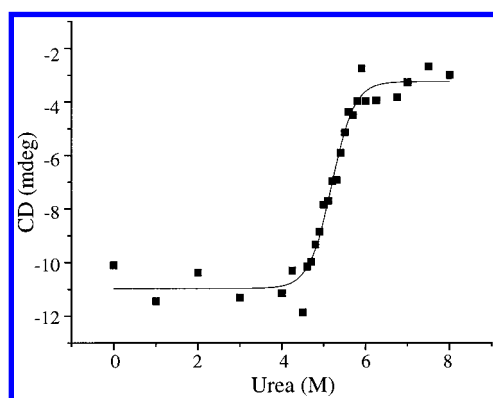


FIGURE 6: Solvent denaturation of E2<sub>cd</sub> under reducing conditions. Individual samples of enzyme were denatured in a series of concentrations of urea in 50 mM KP<sub>i</sub>, 100 mM KCl, 2.5 mM DTT, pH 6.8, at 30 °C. The  $C_m$  is 5.2 M; the  $m$ -value is 2.2 kcal/(mol•M); the  $\Delta G_u$  is 11.2 kcal/mol.

In the common buffer, CenAP<sub>30</sub> melted with an average  $T_m$  of 56.4 °C and an average  $\Delta H_{VH}$  of 107 kcal/mol (Figure 5) (Table 1). Upon quick cooling, CenAP<sub>30</sub> typically recovered 75–80% of its initial CD signal. Upon slow renaturation at the same temperature ramp as the original denaturation, the  $T_m$  was 54.6 °C, and the  $\Delta H_{VH}$  was 101 kcal/mol (not shown).

In this common buffer, two-state behavior deteriorated somewhat compared to the behavior of each enzyme in its “ideal” buffer. The behavior was nevertheless judged to be a close enough to two-state to allow meaningful thermodynamic comparison. All analyses assume two-state behavior of both enzymes in the common buffer.

**Solvent Denaturation: Comparison of E2<sub>cd</sub> and CenAP<sub>30</sub>.** Under reducing conditions, E2<sub>cd</sub> and CenAP<sub>30</sub> were incubated for 24 h in various concentrations of urea at 30 °C. For E2<sub>cd</sub>, the  $C_m$  was 5.2 M urea, and the  $m$ -value was 2.2 kcal/(mol•M);  $\Delta G_u$  was 11.2 kcal/mol (Figure 6) (Table 2). The denaturation was reversible with representative samples exhibiting 100% return of signal throughout the transition. For CenAP<sub>30</sub>, the  $C_m$  was 2.6 M, and the  $m$ -value was 1.7 kcal/(mol•M);  $\Delta G_u$  was 4.3 kcal/mol (Figure 7) (Table 2). The denaturation was reversible.

Table 2: Thermodynamic Quantities Determined by Solvent Denaturation of CenAP<sub>30</sub> and E2<sub>cd</sub> in 50 mM KP<sub>i</sub>, 100 mM KCl, pH 6.8, and Various Concentrations of Urea

	temp (°C)	$C_m$ (M)	$m$ -value [kcal/(mol•M)]	$\Delta G_u$ (kcal/mol)
CenAP <sub>30</sub> with DTT	30	$2.6 \pm 0.1$	$1.7 \pm 0.1$	$4.3 \pm 0.6$
CenAP <sub>30</sub> native	30	$4.5 \pm 0.3$	$2.4 \pm 0.5$	$10.8 \pm 3$
E2 <sub>cd</sub> with DTT	30	$5.2 \pm 0.1$	$2.2 \pm 0.1$	$11.2 \pm 1$
E2 <sub>cd</sub> native	32	$7.4 \pm 0.2$	ND <sup>a</sup>	ND

<sup>a</sup> Not determined.

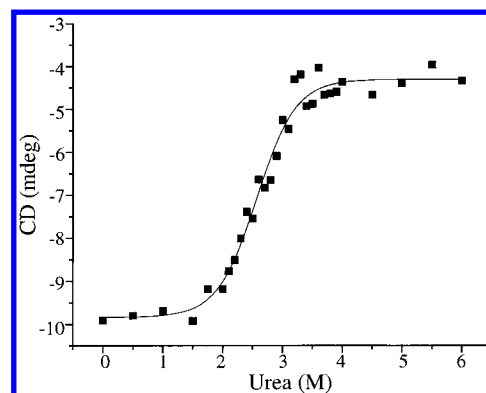


FIGURE 7: Solvent denaturation of CenAP<sub>30</sub> under reducing conditions. Individual samples of enzyme were denatured in a series of concentrations of urea in 50 mM KP<sub>i</sub>, 100 mM KCl, 2.5 mM DTT, pH 6.8, at 30 °C. The  $C_m$  is 2.6 M; the  $m$ -value is 1.7 kcal/(mol•M); the  $\Delta G_u$  is 4.3 kcal/mol.

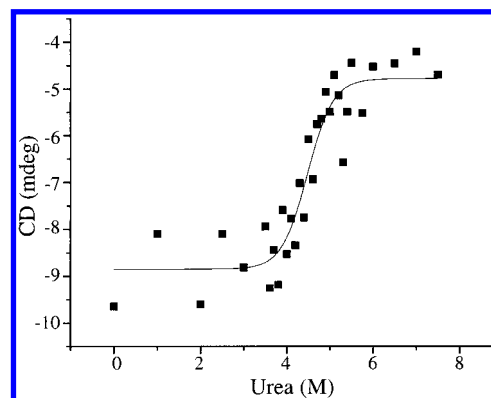


FIGURE 8: Solvent denaturation of CenAP<sub>30</sub> from native state. Individual samples of enzyme were denatured in a series of concentrations of urea in 50 mM KP<sub>i</sub>, 100 mM KCl, pH 6.8, at 30 °C. The  $C_m$  is 4.5 M; the  $m$ -value is 2.4 kcal/(mol•M); the  $\Delta G_u$  is 10.8 kcal/mol.

Under nonreducing conditions, the CenAP<sub>30</sub> was incubated for 24 h in various concentrations of urea at 30 °C. The  $C_m$  was 4.5 M, the  $m$ -value was 2.4 kcal/(mol•M), and the  $\Delta G_u$  was 10.8 kcal/mol (Figure 8) (Table 2). The denaturation was fully reversible. After 7 days, E2<sub>cd</sub> had not reached equilibrium under these conditions. Under nonreducing conditions at 32 °C, E2<sub>cd</sub> had an apparent  $C_m$  of 7.4 M; however, the denaturations were not fully reversible, so an  $m$ -value and  $\Delta G_u$  could not be reliably calculated.

In an attempt to observe cold denaturation, E2<sub>cd</sub> was incubated at −12 °C, both in phosphate buffer with 45% ethylene glycol and in 3 M guanidinium hydrochloride. After 2 weeks, E2<sub>cd</sub> gave no indication of denaturation in either condition.

## DISCUSSION

*Comparing the Stabilities of a Thermophilic and Mesophilic Enzyme Pair.* Of the three models that explain the temperature stability of thermophilic enzymes (Figure 1), the one with the most support in the literature predicts that thermophilic enzymes will have a flattened stability curve relative to mesophilic enzymes (Figure 1; curve 3) (5, 12, 13). This model suggests that thermophilic enzymes are not thermodynamically more stable than mesophilic enzymes, but rather that their free energy of folding is less sensitive to temperature. This model predicts that at the  $T_m$ , the dependence of  $\Delta G_u$  on the temperature for the mesophilic enzyme will be greater than that of the thermophilic enzyme. The rate of change of  $\Delta G_u$  with temperature at the  $T_m$  is  $\Delta S_u$  (10). Thus, this "flattened" model predicts that the  $\Delta S_u$  for the thermophilic enzyme will be less than that of the mesophilic enzyme.

Comparing the thermal denaturations of E2<sub>cd</sub> to CenA<sub>P30</sub>, we found the opposite to be true. The  $\Delta S_u$  of the thermophilic enzyme E2<sub>cd</sub> was much higher than that of the mesophilic enzyme CenA<sub>P30</sub> [550 cal/(mol•K) for E2<sub>cd</sub> vs 320 cal/(mol•K) for CenA<sub>P30</sub>]. Indeed, the  $\Delta S_u$  of E2<sub>cd</sub> was high compared to the values observed for many mesophilic proteins. This result is inconsistent with the "flattened" model (Figure 1; curve 3), and also the "shifted" model (Figure 1; curve 2). As well, E2<sub>cd</sub> failed to denature at cold temperatures (−12 °C), even with the addition of 3 M guanidinium hydrochloride. Both observations are consistent with the higher stability model (Figure 1; curve 1).

To compare the stabilities at a common temperature, we investigated the stability of the enzymes to urea. At 30 °C, under reducing conditions where one or both disulfides present in both enzymes were reduced, the stability of the thermophilic enzyme E2<sub>cd</sub> was 11.2 kcal/mol compared to the stability measured for CenA<sub>P30</sub>, 4.3 kcal/mol. This is consistent with the thermal denaturation experiments, where we found the thermophilic enzyme denatured at a higher temperature and with a steeper dependence on temperature (larger magnitude of  $\Delta S_u$ ) than the mesophilic enzyme. Under *nonreducing* conditions at 30 °C, only the denaturation of CenA<sub>P30</sub> was fully reversible, giving a stability of 10.8 kcal/mol.

The disulfide bonds are in equivalent positions in the two enzymes (21), and we assume that their presence or absence has the same thermodynamic effect in both enzymes. Thus, we use the three  $\Delta G_u$  values that were determined at 30 °C to predict the  $\Delta G_u$  of the thermophilic enzyme at 30 °C. The  $\Delta\Delta G_u$  between E2<sub>cd</sub> and CenA<sub>P30</sub> with DTT is 6.9 kcal/mol; the  $\Delta\Delta G_u$  between the CenA<sub>P30</sub> with DTT and without DTT is 6.5 kcal/mol. If the disulfides have the same stabilizing effect on E2<sub>cd</sub> as on CenA<sub>P30</sub>, then the  $\Delta G_u$  for E2<sub>cd</sub> under native conditions can be extrapolated to be 17.7 kcal/mol. Clearly this value is an extrapolation not a measurement and must be interpreted cautiously. Still, it is consistent with the high  $C_m$  of E2<sub>cd</sub>, 7.4 M, determined under nonreducing conditions at 32 °C. The combination of thermal and solvent denaturations of E2<sub>cd</sub> and CenA<sub>P30</sub> suggests that the thermophilic enzyme derives its greater thermal stability from a greater thermodynamic stability.

As a technical aside, we were surprised by the slow rate of unfolding of the thermophilic enzyme in urea. At 25 °C,

E2<sub>cd</sub> took 24 days to equilibrate; at 32 °C, it required 7 days. This behavior may reflect the relatively high stability of E2<sub>cd</sub> and its size (30 kDa). Thermophilic and hyperthermophilic enzymes that are unusually stable and are the same size or larger than E2<sub>cd</sub> may pose unanticipated kinetic barriers to thermodynamic study by solvent denaturation. Conversely, they should be amenable to study by temperature denaturation, where the rates of unfolding are much faster. As a theoretical consideration, we note that a higher overall stability of a thermophilic enzyme, and a larger magnitude  $\Delta S_u$ , may have important implications for the "folding funnels" of thermophilic versus mesophilic enzymes, and for the degree of frustration that they experience on folding at various temperatures.

## CONCLUSIONS

One hypothesis explaining the high temperature stability of thermophilic proteins suggests that they are not more stable than their mesophilic counterparts, rather that their free energy of folding is less sensitive to temperature; i.e., their free energy profile is flattened compared to the mesophilic enzymes (Figure 1; curve 3). From a theoretical standpoint, this hypothesis is consistent with the observation that substitutions on protein surfaces typically have a small effect on protein stability. Thermophilic enzymes could gain temperature stability by a series of small adaptations, no one of which is itself key and which, in aggregate, lead not to a thermodynamically more stable protein, but merely to one that is less sensitive to temperature (5). Experimentally, this hypothesis is supported by recent results with small DNA binding proteins isolated from hyperthermophilic organisms (12, 13). Although these proteins were observed to denature at very high temperatures, their thermodynamic stabilities fell within the range expected of mesophilic proteins. Direct comparison of a thermophilic and mesophilic phosphoglycerate kinase also is consistent with the "flattened" model, although these results are not clear-cut (4).

Our results suggest that higher temperature stability can also arise because of higher thermodynamic stability. This is consistent with the results of Kanaya and co-workers, who found that the thermophilic RNase H was globally more stable than its mesophilic analogue (19), and with recent work of Jaenicke and co-workers studying phosphoglycerate kinase (20). Both sets of results are consistent with a greater overall thermodynamic stability; neither fits the "flattened" model of thermophilic enzyme stability. More broadly, our results are consistent with the ideas discussed in earlier studies (5, 6, 12, 13). Proteins tolerate many substitutions, and enzymes may achieve higher stability to temperature by a number of strategies. Each of the three models (Figure 1) may be used in nature. At present, there is experimental evidence for two of them.

## ACKNOWLEDGMENT

B.K.S. and W.A.B. thank Brian Matthews, in whose laboratory this work was begun. We are grateful to Andreas Matouschek for critiquing the manuscript and the Keck Biophysics Facility for generous use of its equipment.

## REFERENCES

1. Perutz, M. F., and Raidt, H. (1975) *Nature* 255, 256–259.

2. Teplyakov, A. V., Kuranov, I. P., Harutyunyan, E. H., and Vainshtein, B. K. (1990) *J. Mol. Biol.* 214, 261–279.
3. Davies, G. J., Gamblin, S. J., Littlechild, J. A., and Watson, H. C. (1993) *Proteins: Struct., Funct., Genet.* 15, 283–289.
4. Nojima, H., Ikai, A., Oshima, T., and Noda, H. (1977) *J. Mol. Biol.* 116, 429–442.
5. Jaenicke, R. (1993) in *Biocatalyst Design for Stability and Specificity* (Himmel, M. E., and Georgiou, G., Eds.) pp 53–66, American Chemical Society, Washington, DC.
6. Rees, D. C., and Adams, M. W. (1995) *Structure* 3, 251–254.
7. Brandts, J. F. (1964) *J. Am. Chem. Soc.* 86, 4291–4301.
8. Pace, C. N. (1975) *CRC Crit. Rev. Biochem.* 3, 1.
9. Privalov, P. L. (1979) *Adv. Protein Chem.* 33, 167–241.
10. Becktel, W. J., and Schellman, J. A. (1987) *Biopolymers* 26, 1859–1877.
11. Chen, B. L., and Schellman, J. A. (1989) *Biochemistry* 28, 685–691.
12. Knapp, S., Karshikoff, A., Berndt, K. D., Christova, P., Atanasov, B., and Ladenstein, R. (1996) *J. Mol. Biol.* 264, 1132–1144.
13. McCrary, B. S., Edmondson, S. P., and Shriver, J. W. (1996) *J. Mol. Biol.* 264, 784–805.
14. Kimura, S., Nakamura, H., Hashimoto, T., Oobatake, M., and Kanaya, S. (1992) *J. Biol. Chem.* 267, 21535–21542.
15. Laderman, K. A., Davis, B. R., Krutzsch, H. C., Lewis, M. S., Griko, Y. V., Privalov, P. L., and Anfinsen, C. B. (1993) *J. Biol. Chem.*, 24394–24401.
16. Chen, C. H., Roth, L. G., MacColl, R., and Berns, D. S. (1994) *Biophys. Chem.* 50, 3213–3221.
17. Rehder, V., and Jaenicke, R. (1992) *J. Biol. Chem.* 267, 10999–11006.
18. Wrba, A., Schweiger, A., Schultes, V., Jaenicke, R., and Zavodsky, P. (1990) *Biochemistry* 29, 7584–7592.
19. Kanaya, S., and Itaya, M. (1992) *J. Biol. Chem.* 267, 10184–10192.
20. Grattinger, M., Dankesreiter, A., Schurig, H., and Jaenicke, R. (1998) *J. Mol. Biol.* 280, 525–533.
21. McGinnis, K., and Wilson, D. B. (1993) *Biochemistry* 32, 8151–8156.
22. Spezio, M., Wilson, D. B., and Karplus, P. A. (1993) *Biochemistry* 32, 9906–9916.
23. Tomme, P., Warren, R. A., and Gilkes, N. R. (1995) *Adv. Microb. Physiol.* 37, 1–81.
24. Walker, L. P., Wilson, D. B., Irwin, D. C., and McQuire, G. M. (1992) *Biotechnol. Bioeng.* 40, 1019–1026.
25. Irwin, D. C., Spezio, M., Walker, L. P., and Wilson, D. B. (1993) *Biotechnol. Bioeng.* 42, 1002–1013.
26. McGinnis, K., Kroupis, C., and Wilson, D. B. (1993) *Biochemistry* 32, 8146–8150.
27. Gilkes, N. R., Warren, R. A., Miller, R. C., Jr., and Kilburn, D. G. (1988) *J. Biol. Chem.* 263, 10401–10407.
28. Gekko, K., and Timasheff, S. N. (1981) *Biochemistry* 20, 4667–4676.
29. Kirchhoff, W. H. (1993) NIST, Gaithersburg, MD.
30. Privalov, P. L., and Khechinashvili, N. N. (1974) *J. Mol. Biol.* 86, 665–684.
31. Dill, K., Alonso, D., and Hutchinson, K. (1989) *Biochemistry* 28, 5439–5449.
32. Microcal Software, Inc. (1995) Microcal Software, Inc., Northhampton.
33. Tanford, C. (1970) *Adv Protein Chem* 24, 1–95.

BI9824902