Folding and Stability of Endoglucanase III, a Single-Domain Cellulase from *Trichoderma reesei*

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ABSTRACT: The reversible folding of an endoglucanase (EGIII) from the filamentous fungus *Trichoderma reesei* was investigated by activity, tryptophan fluorescence, and peptide CD measurements. Equilibrium stability was determined by urea denaturation at various pH and temperature values. Unfolding and refolding rates were measured over a range of urea concentrations. The data from the equilibrium and kinetic studies fit a simple two-state model, except at lower urea concentrations, where the folding kinetics indicate a transient intermediate. Unfolding is very slow, with a half-life of about 2 h in 8 M urea at pH 5.5 and 25 °C. Comparison of the urea dependence of the folding kinetics and equilibrium indicates the protein undergoes 93% of its total change in solvent exposure on going from the unfolded state to the transition state. Thus, the transition state is quite compact. The presence of dithiothreitol destabilized the protein by 7 kcal/mol, indicating the presence of an unusually strong disulfide linkage between the two cysteines in the molecule. Protein stability is dramatically reduced at alkaline pH values; this can be attributed to a titratable shift ($pK_a = 7.8$) in the slope of the urea dependence of unfolding.

Cellulases are a class of inducible extracellular enzymes produced by a variety of bacteria and fungi. The substrate for these enzymes, cellulose, is heterogeneous in supramolecular structure, and hence its degradation requires enzymes with widely differing properties and mechanisms of action. For example, endoglucanases preferentially hydrolyze the internal $\beta(1-4)$ linkages of amorphous cellulose, while exoglucanases, which act synergistically with endoglucanases, hydrolyze crystalline cellulose by cleavage of cellobiose from the chain ends (Begiun & Aubert, 1994). The treatment of cellulose has drawn the attention of not only biotechnologists interested in the hydrolysis of this abundant bioresource but is also of fundamental research interest, because of the diversity in the structure−function relationships between the multiple cellulases produced by an organism and its heterogeneous substrate (Begiun & Aubert, 1994).

Endoglucanase III (EGIII)[1] from the cellulolytic fungus *Trichoderma reesei* belongs to cellulase family H (Ward et al., 1993). It is a small protein (25 kDa), with a near-neutral p*I* (7.4). Unlike almost all other known endoglucanases, including the other endoglucanases from *T. reesei* (endoglucanases I, II, and V), EGIII does not have a cellulose binding domain or a linker region; it consists solely of a catalytic domain. This simplification makes EGIII an attractive model for studying cellulase folding and stability. EGIII has 56% identity in amino acid sequence with the endoglucanase from *Aspergillus acleatus*, an enzyme composed of three β-sheets and a small α-helix (Ooi et al., 1990). EGIII has 218 amino acid residues, with cysteines at positions 4 and 32. These residues form a disulfide linkage in the homologous enzyme from *A. acleatus* (Hata et al., 1994).

In order to enhance the utility of EGIII, either by chemical modification or by genetic engineering, a basic understanding of the molecular properties of the enzyme is important. Furthermore, because cellulases, including EGIII, are widely used in industrial applications where the lifetime under process conditions is an important issue, studies on the folding and stability of the protein are essential.

In this paper, we analyze the urea-mediated unfolding and refolding of this β-sheet protein. The data from both equilibrium and kinetic studies are in good agreement with each other and fit a simple two-state model. The urea dependence of the kinetics relative to the equilibrium indicates that, during unfolding, the enzyme undergoes only a 7% increase in solvent exposure on going from the native state to the transition state (normalized to the increase in solvent exposure for complete unfolding). We demonstrate that the enzyme has a disulfide linkage which provides about 7 kcal/mol in stability to the protein, and we show that EGIII stability is dramatically reduced at alkaline pH values, arising from a shift in the slope of the urea dependence of unfolding which fits a titration curve with a pK_a value of 7.8.

EXPERIMENTAL PROCEDURES

Materials

The cloning and expression of EGIII have been described (Ward et al., 1993). EGIII was purified by crystallization from culture media (H. Gros, Genencor International, Inc., unpublished results). Ultrapure urea was purchased from ICN Biochemicals. *o*-Nitrophenyl β-D-cellobioside was purchased from Sigma. All other chemicals and reagents used were of Analytical grade and were purchased from Sigma.

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[1] Abbreviations: EGIII, endoglucanase III from *Trichoderma reesei*; CD, circular dichroism; UV, ultraviolet; DSC, differential scanning calorimetry; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

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Methods

(1) Enzyme Assays. The activity of the enzyme was measured by an end point assay in microtiter plates, in 100 mM sodium acetate buffer, pH 5.5 at 40 °C. The concentration of the substrate *o*-nitrophenyl β-D-cellobioside was 10 mM in a total volume of 100 μL. The concentration of the enzyme was 40−80 μg/mL. The reaction was initiated by the addition of the substrate and was incubated at 40 °C for 10 min. The reaction was terminated with the addition of 100 μL of 50 mM glycine buffer, pH 10, and the absorbance at 405 nm was measured in a microtiter plate reader from Molecular Devices.

(2) Equilibrium Folding and Unfolding. (a) Fluorescence Measurements. All fluorescence measurements were made in a Perkin-Elmer LS50B luminescence spectrometer. Typically, for unfolding experiments, the enzyme was diluted into 100 mM sodium acetate buffer, pH 5.5 at 25 °C, containing different concentrations of urea (0−10 M) unless specified otherwise. The enzyme (0.3−0.5 μM) was unfolded by incubation for at least 16 h, and the fluorescence at 340 nm (excitation at 280 nm) was measured. For refolding experiments, EGIII (30−50 μM) was first unfolded in 8 M urea in 100 mM sodium acetate buffer, pH 5.5 at 25 °C, for at least 16 h and then refolded by diluting 100-fold into the same buffer containing various concentrations of urea (0−8 M). All urea solutions were fresh or were from frozen stocks.

The fluorescence data for the urea titrations were fit to the equation discussed below using nonlinear regression with the program GraFit (Erithacus Software). Denaturation is observed to be linearly dependent on the urea concentration:

$$\Delta G_{u-f} = \Delta G_{u-f}{}^{H_2O} - m_{u-f}[\text{urea}] = -RT \ln K_u \quad (1)$$

where ΔG_{u-f} is the free energy change upon unfolding in urea, $\Delta G_{u-f}{}^{H_2O}$ is the free energy of unfolding in water, m_{u-f} is a constant, and K_u is the equilibrium constant for unfolding where

$$K_u = \text{fraction unfolded/fraction native} \quad (2)$$

The above equations can be transformed to the following as explained by Clarke and Fersht (1993):

$$F = [a_N + b_N[\text{urea}] + (a_U + b_U[\text{urea}] \exp\{m_{U-F}([\text{urea}] - [\text{urea}]_{50\%})/RT\}]/[1 + \exp\{m_{U-F}([\text{urea}] - [\text{urea}]_{50\%})/RT\}] \quad (3)$$

where F is the observed fluorescence, a_N is the fluorescence of the native protein in water, b_N is the linear change in fluorescence of the native protein with the concentration of urea, a_U is the fluorescence of the unfolded protein in water, b_U is the linear change in fluorescence of the unfolded protein with the concentration of urea, m_{U-F} is the slope for ΔG_{U-F} vs [urea], $[\text{urea}]_{50\%}$ is the concentration of urea where 50% of the protein is denatured, R is the gas constant, and T is the absolute temperature. Fitting the fluorescence as a function of urea directly to this transformation provides values of $[\text{urea}]_{50\%}$ and m_{U-F} and their standard errors. $\Delta G_{u-f}{}^{H_2O}$ can then be calculated from eq 4:

$$\Delta G_{u-f}{}^{H_2O} = m_{u-f}[\text{urea}]_{50\%} \quad (4)$$

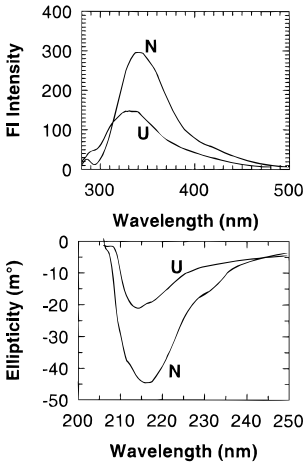

FIGURE 1: Top panel: Fluorescence spectra of native (N) and unfolded (U) endoglucanase III. Fluorescence intensity was measured with excitation at 280 nm in 100 mM sodium acetate buffer, pH 5.5 at 25 °C. The concentration of the enzyme was 0.3 μM. The spectrum of the unfolded enzyme was obtained after unfolding the enzyme overnight in 8 M urea. Bottom panel: Far-UV CD spectra of native (N) and unfolded (U) endoglucanase III. The spectra were measured in 100 mM sodium acetate buffer, pH 5.5 at 25 °C. The concentration of the enzyme was 50 μM. The spectrum of the unfolded enzyme was taken after unfolding the enzyme overnight in 8 M urea.

(b) CD Measurements. CD experiments were carried out in an Aviv Model 62A DS circular dichroism spectrophotometer. The urea titration data were analyzed using GraFit with the equations discussed in the previous section. A 1-mm path length cell was used for the CD measurements.

(c) DSC Measurements. DSC measurements were carried out in a differential scanning calorimeter from MicroCal Inc. The protein concentration was 1 mg/mL. The data were analyzed using the software provided by MicroCal (version 2.1).

(3) Kinetics of Unfolding and Refolding. For unfolding experiments, EGIII was rapidly mixed with buffers (100 mM sodium acetate, pH 5.5 at 25 °C) containing different urea concentrations (5−9 M), and the change in fluorescence at 340 nm was monitored (excitation at 280 nm). The data were fit to a single-exponential equation using the program GraFit. Similarly, for refolding experiments, EGIII that had been unfolded in 8 M urea in 100 mM sodium acetate buffer, pH 5.5 at 25 °C, for at least 16 h was rapidly diluted into the same buffer containing different urea concentrations (2−5 M). The change in fluorescence was monitored at 340 nm (excitation at 280 nm). The kinetic traces were fit to a single-exponential equation.

RESULTS

(1) Reversibility of Urea-Mediated Activity Loss. Enzyme activity was measured by the hydrolysis of *o*-nitrophenyl β-D-cellobioside. The enzyme loses all activity in 8 M urea in 100 mM sodium acetate buffer, pH 5.5. However, refolding the enzyme by dilution into pH 5.5 sodium acetate buffer results in the recovery of over 90% of the activity. The activity of the native enzyme control was measured at the same final urea concentration (0.04 M) as the refolded enzyme.

(2) Equilibrium Folding and Unfolding. Figure 1 shows the fluorescence (top panel) and far-UV CD (bottom panel) spectra of the native and unfolded forms of EGIII. The

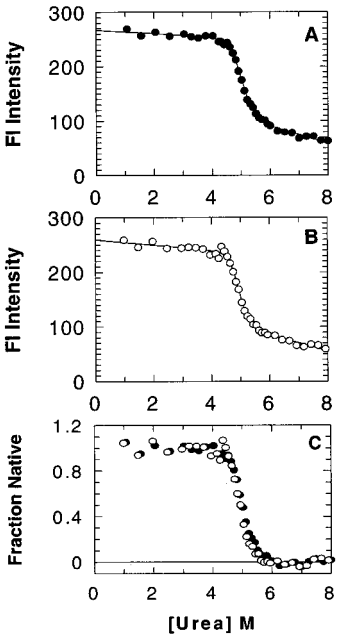

FIGURE 2: Urea dependence of the fluorescence intensity of endoglucanase III. The fluorescence intensity at 340 nm (excitation at 280 nm) was measured in 100 mM sodium acetate buffer, pH 5.5, at 25 °C. The concentration of the enzyme was 0.3 μM. The closed circles (panel A) are results obtained upon refolding the protein for at least 16 h after unfolding for at least 16 h in 8 M urea, and open circles (panel B) are results obtained upon unfolding the protein for at least 16 h. The solid line is the fit which is described under Methods. Panel C shows the data replotted as fraction native protein vs [urea], demonstrating reversibility and absence of hysteresis.

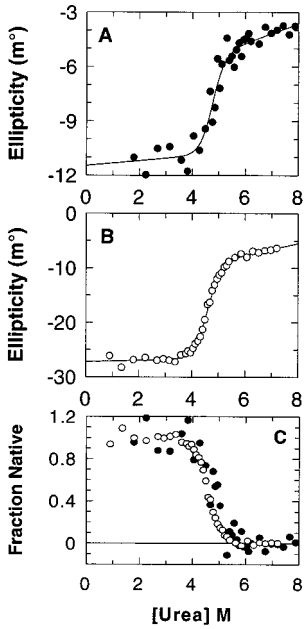

FIGURE 3: Urea dependence of the far-UV CD spectrum of endoglucanase III. The ellipticity at 222 nm was measured at 25 °C in the same buffer described in Figure 1. The concentration of the enzyme was 40 μM (unfolding) and 12.5 μM (refolding). Closed circles (Panel A) are results obtained upon refolding the protein for at least 16 h after unfolding for at least 16 h in 8 M urea, and the open circles (Panel B) are results obtained upon unfolding the protein for at least 16 h. The solid line is the fit which is described under Methods. The increased scatter in panel A is due to the lower protein concentration. Panel C shows the data replotted as fraction native vs [urea], demonstrating reversibility and absence of hysteresis.

Table 1: Summary of Parameters and Their Standard Errors Derived from Analysis of Equilibrium and Kinetics of EGIII Folding and Unfolding

method	m (kcal mol^{-1} M^{-1})	$U_{50\%}$ (M)	$\Delta G_{u-f}^{H_2O}$ (kcal/mol)
fluorescence	2.38 ± 0.19	4.88 ± 0.02	11.61 ± 0.99
CD	2.44 ± 0.32	4.66 ± 0.04	11.37 ± 1.60
kinetics	2.54 ± 0.51	4.97 ± 0.99	12.65 ± 2.53

unfolded enzyme has an intact disulfide linkage (see below), and the fluorescence and CD spectra of the enzyme are consistent with the retention of some residual structure. The enzyme in the native state has a fluorescence emission maximum at 340 nm upon excitation at 280 nm. EGIII has nine tryptophan residues, and the transfer of the tryptophan side chains from relatively nonpolar environments in the native state to an aqueous environment upon unfolding results in the expected loss of fluorescence intensity. The trough at 217 nm in the CD spectrum and its relatively weak magnitude (mean residue ellipticity = -4000 deg·cm^2/dmol) are consistent with a predominance of β-sheet structure in EGIII (Yang et al., 1986).

Figure 2 shows the dependence of the fluorescence intensity at 340 nm (excitation 280 nm) on the concentration of urea. The fluorescence of the native and the denatured forms of the protein is linearly dependent on the concentration of urea, and the enzyme fluorescence undergoes a sigmoidal cooperative transition upon unfolding. The data were fit to eq 3 as discussed under *Methods*, and the ΔG for unfolding (11.6 kcal/mol) was calculated from eq 4 (Table 1). The data suggest a single transition and a simple two-state equilibrium between the native (N) and the unfolded (U) forms of the enzyme.

As protein fluorescence is only sensitive to the tryptophan environment, urea-mediated unfolding of the peptide backbone was monitored by far-UV CD. Consistent with the fluorescence data, the dependence of the ellipticity at 222 nm on the urea concentration (Figure 3) suggests a simple two-state equilibrium between the native and unfolded forms of the protein. Table 1 summarizes the good agreement for the values for $U_{50\%}$, m, and ΔG_{u-f} measured by fluorescence and CD.

(3) Kinetics of Folding and Unfolding. Unfolded or native EGIII was mixed in 100 mM sodium acetate buffer containing different urea concentrations (2−5 M for folding and 5−9 M for unfolding, respectively). The changes in fluorescence intensity at 340 nm (excitation at 280 nm) were monitored with time. The kinetic traces fit well to a single-exponential equation. The natural logarithms of the observed rate constants are plotted as a function of urea concentration in Figure 4 (top panel). The enzyme unfolds very slowly even at high urea concentrations (the half-life is 2 h in 8 M urea), and the rate of unfolding is only weakly dependent on the concentration of urea. However, refolding is relatively rapid, and the rate of folding is strongly dependent on the urea concentration. The linear portions of the observed rate constants in the folding and the unfolding pathway were fit as shown below:

$$\ln k_u = \ln k_u^{H_2O} + m_u[\text{urea}] \quad (5)$$

$$\ln k_f = \ln k_f^{H_2O} + m_f[\text{urea}] \quad (6)$$

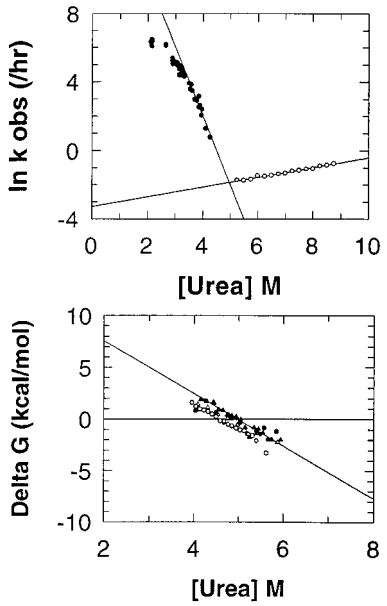

FIGURE 4: Top panel: Urea dependence of the first-order rate constants for the unfolding (open circles) and refolding (closed circles) of endoglucanase III. The rate constants were measured in 100 mM sodium acetate, pH 5.5, at 25 °C. The solid lines are linear fits of the data. Bottom panel: The solid line is ΔG_{u-f} calculated from the kinetic data, assuming a simple two-state equilibrium. Data from the transition region on the far-UV CD equilibrium unfolding (open circles) and refolding (filled circles) are plotted as well as fluorescence equilibrium data for unfolding (open triangles) and refolding (filled triangles).

where k_u is the observed rate constant for the unfolding reaction and k_f is the observed rate constant for the folding reaction. The ratio of the slopes for unfolding kinetics vs urea relative to equilibrium unfolding vs urea ($RT \times 0.28/2.36$) indicates the enzyme undergoes a 7% increase in solvent exposure on going from the native state to the transition state (normalized to the total increase in solvent exposure for unfolding) (Tanford, 1970). Thus, the transition state is very compact. The kinetic data suggest, consistent with the equilibrium data, that the native and the unfolded forms are the only significantly populated states, except at low urea concentrations where a transient intermediate is suggested by the deviation of the linear dependence of ln k_f on urea concentration (Figure 4, top panel).

ΔG_{u-f} was calculated from the linear fits of the kinetic data (Table 1) from eq 7:

$$\Delta G_{u-f} = -RT \ln (k_u/k_f) \quad (7)$$

Shown in Figure 4 (bottom panel, solid line) is the plot of ΔG_{u-f} as a function of urea concentration which results from the kinetic data. The data points in Figure 4 are ΔG_{u-f}-calculated (eqs 1 and 2) from the equilibrium folding and unfolding experiments measured by fluorescence and far-UV CD. This plot displays a reasonable concordance between data from the equilibrium and kinetic analysis, and strongly suggests a simple two-state equilibrium between the native and unfolded states in the transition region (4−6 M urea).

(4) Stability of EGIII. (A) Disulfide Linkage. EGIII was unfolded overnight at 25 °C in 100 mM sodium bicine buffer, pH 8.5, containing 7.5 M urea, 5 mM DTT, and 1 mM EDTA. The tube was flushed with argon to minimize air oxidation of the thiols. Refolding was carried out by diluting

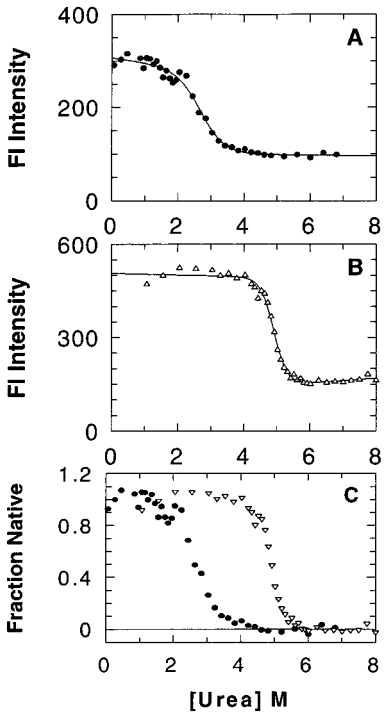

FIGURE 5: Equilibrium refolding transition for endoglucanase III with a reduced disulfide bond (closed circles, panel A) and with an intact disulfide bond (open triangles, panel B). Fluorescence intensity at 340 nm (excitation at 280 nm) was measured in 100 mM sodium acetate buffer, pH 5.5, 25 °C. The protein concentration was 0.7 μM. The concentration of EDTA was 1 mM, and DTT concentration was 500 μM (added only to the reduced enzyme). The solid line is the fit to the equation described under Methods. This was a different preparation of enzyme than was used in Figure 2, and it gave a different base line slope at high [urea]. $U_{50\%}$ was virtually unchanged in the absence of DTT, and ΔG_{u-f} and m differed by less than 20%. An increase in the ΔG_{u-f} value of about 7 kcal/mol was calculated for the stability provided by the disulfide linkage.

the unfolded enzyme into 100 mM sodium acetate buffer, pH 5.5, containing different concentrations of urea, 500 μM DTT, and 1 mM EDTA. The tubes were flushed with argon and incubated at 25 °C overnight. A control sample was prepared in parallel in the absence of DTT. The fluorescence emission at 340 nm (excitation at 280 nm) was measured. As shown in Figure 5, the reduction of the disulfide linkage results in the loss of about 7 kcal/mol in stability.

(B) Effect of pH. Figure 6 shows that EGIII dramatically loses stability at elevated pH values but remains folded in the absence of denaturant, at least up to pH 9.5. The loss in stability is due to a decrease in both the m value and $U_{50\%}$. Unfolding above pH 9.5 could not be analyzed due to a large decrease in m values. As the m value is thought to reflect the increase in solvent exposure upon unfolding (Tanford, 1970), this indicates the unfolded state is more compact at high pH, and/or the native enzyme is partially unfolded. However, peptide CD spectra of the native and unfolded protein were not altered from pH 5 to 9 (data not shown).

The decrease in stability can be fit to a titration curve with a pK_a of 7.5 (Figure 6, panel A). When ΔG_{u-f} is broken down into its parameters, it is found that $U_{50\%}$ decreases monotonically (Figure 6, panel B), while the effect of pH on the m value can be fit to a titration curve with a pK_a of 7.8 (Figure 6, panel C). This may indicate the ionization of a histidine residue. EGIII has two histidines, at positions 45 and 108. His-108 is conserved in EGIII and the

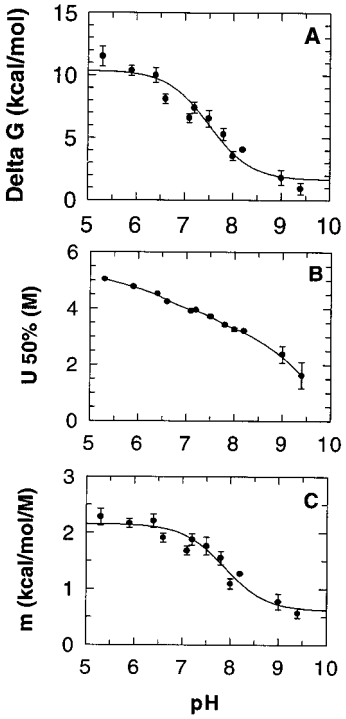

FIGURE 6: pH dependence of the equilibrium unfolding transition of endoglucanase III. 1 μM enzyme was unfolded at various urea concentrations at 25 °C in 25 mM sodium acetate (pH 5−6) or 25 mM ethylenediamine buffer (pH 6−10). The ionic strength in the buffers was adjusted to 100 mM with NaCl. The data were fit to the equation discussed under Methods. Panel A shows the calculated ΔG_{u-f} values plotted as a function of pH. The solid line is a titration curve fit to the data, yielding a pK_a value of 7.5. Panel B shows the concentration of the denaturant at which half the protein is unfolded ($U_{50\%}$) plotted as a function of pH. Panel C is the slope of ΔG_{u-f} vs urea concentration (*m* value) plotted as a function of pH. The solid line is a titration curve fit to the data, yielding a pK_a value of 7.8.

homologous endoglucanase from *Aspergillus acleatus* (Ward et al., 1993; Ooi et al., 1990). The tertiary structure of the endoglucanase from *A. acleatus* was studied by X-ray crystallography (2.3 Å resolution) and was found to be very similar to the xylanase from *Bacillus pumilus*, with which it shares 19% homology (Okada, 1991; Hata et al., 1994). The enzyme from *A. acleatus* has three β-sheets and a single α-helix. Sequence comparison with the published *B. pumilus* xylanase structure suggests that EGIII also has a single α-helix, and His-108 is located very close to its C-terminal (S. Wu, personal communication); perhaps the protonated form of His-108 is stabilized by the helix dipole, resulting in an elevated pK_a and decreased conformational stability of EGIII at alkaline pH. Why this putative deprotonation leads to a dramatic shift in *m* value is more difficult to speculate upon.

(C) Effect of Temperature. Figure 7 shows the loss of EGIII stability with increasing temperature. The melting temperature (T_m, where $\Delta G_{u-f} = 0$), measured by DSC, was 59.2 °C. The data from equilibrium urea unfolding at different temperatures and the DSC data were fit to the Gibbs−Helmholtz equation:

$$\Delta G_{u-f} = \Delta H_m(1 - T/T_m) - \Delta C_p[(T_m - T) + T \ln (T/T_m)] \quad (8)$$

where ΔH_m is the enthalpy change for unfolding at T_m, T is the absolute temperature, and ΔC_p is the change in heat capacity upon unfolding. We assume that ΔC_p is a constant over the temperature range of the experiment (Privalov, 1979). In addition:

$$\Delta S_m = \Delta H_m/T_m \quad (9)$$

where ΔS_m is the entropy change for unfolding at T_m. The thermodynamic values that gave the best fit to the data are given in Table 2. The predicted temperature for optimal stability is about 10 °C. Due to irreversible unfolding of EGIII at higher temperatures, urea titrations above 45 °C were not carried out.

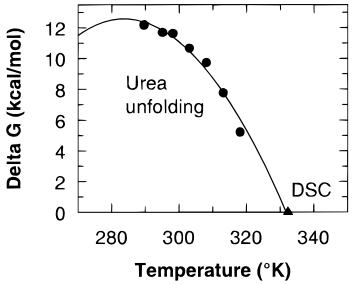

FIGURE 7: Temperature dependence of endoglucanase III stability. 1 μM enzyme was unfolded at various urea concentations in 100 mM sodium acetate, pH 5.5, buffer at 25, 30, 35, 40, and 45 °C. The data were fit to the equation discussed under Methods. ΔG_{u-f} was calculated from the data and is shown plotted as a function of temperature. The filled circles are data obtained from urea-mediated unfolding, and the filled triangle indicates the melting temperature obtained from DSC. The solid line is the fit to eq 9, yielding the thermodynamic parameters given in Table 2.

Table 2: Summary of Thermodynamic Parameters for EGIII Unfolding Derived from DSC and Urea-Mediated Unfolding (cf. Figure 7)

parameter	value
ΔH_m	169 kcal/mol
ΔS_m	0.51 kcal mol^{-1} K^{-1}
ΔC_p	3.2 kcal mol^{-1} K^{-1}
T_m	59.0 °C

DISCUSSION

Cellulases are an important and ubiquitous class of enzymes, and fundamental studies on their biophysical properties contribute to our knowledge of biodegradation of cellulose, a major world resource. In addition, systematic studies on the folding and stability of these enzymes are important, because the industrial applications in which they are used often require them to function under extremes of pH and temperature. Although several workers have studied the irreversible inactivation of cellulases at higher temperatures (Baker et al., 1992; Dominguez et al., 1992), little research has been published on the reversible folding of this class of enzymes; this is the focus of the studies presented in this paper.

The two major endoglucanases from *Trichoderma reesei* are endoglucanase I (EGI) and endoglucanase II (EGII). In addition to their catalytic domains, EGI and EGII also possess cellulose binding domains and linker regions which add complexity to folding studies. EGIII is a small endoglucanase and has no linker or cellulose binding domain (Ward et al., 1993). Thermal unfolding of EGIII, similar to EGI, EGII, and cellobiohydrolases (Jimenez et al., 1995;

Dominguez et al., 1992; Baker et al., 1992), results in irreversible aggregation of the protein. However, urea-mediated unfolding of EGIII is reversible (>90%) as monitored by activity, fluorescence, and peptide CD measurements. Under the conditions of these studies, the folded protein is about 12 kcal/mol more stable than the unfolded state (Table 1). This value is well within the common range for globular proteins (Schellman, 1987).

The loss of EGIII activity (hydrolysis of *o*-nitrophenyl β-D-cellobioside) occurs at much lower concentrations of urea ($U_{50\%} = 1$ M) than protein unfolding as monitored by tryptophan fluorescence or peptide CD. This result is similar to observations regarding *T. reesei* cellobiohydrolase I made by Woodward et al. (1990a,b). The authors showed that guanidinium chloride is a competitive inhibitor of cellobiohydrolase I and hypothesized that an interaction between the positively charged guanidinium group and the active site glutamate residue results in this inhibition. Complete unfolding of cellobiohydrolase I requires a significantly higher concentration (4 M) of guanidinium chloride (Woodward et al., 1990a). The underlying mechanisms for EGIII's differences in sensitivity to urea with regard to activity and conformational stability are still unknown. However, EGIII clearly retains secondary and tertiary structure above 1 M urea, when it has lost activity (cf. Figures 2 and 3).

We have shown that EGIII unfolds very slowly, with a half-life of 2 h in 8 M urea at 25 °C. This is not without precedent; for example, the RNA binding protein, ROP, has also been shown to unfold and refold slowly and takes at least 48 h to completely unfold (Munson et al., 1994). Interestingly, the proteins are very dissimilar in structure; ROP is a four-helix bundle, and EGIII has predominantly β-sheet structure [based on its high homology to the endoglucanase from *Aspergillus acleatus*, an enzyme composed of three β-sheets and a small α-helix (Ooi et al., 1990)]. This demonstrates that both α-helical and β-sheet proteins can have extremely slow unfolding kinetics. The slow rate of EGIII unfolding is only weakly dependent on the urea concentration (see Figure 4). However, the rate of refolding is relatively rapid, and this rate is strongly dependent on the urea concentration. The great disparity in dependence of the rates on urea concentration indicates the transition state undergoes only a small fractional increase in solvent exposure upon unfolding and thus is very close to the native state in solvent exposure.

At all urea concentrations, the kinetic data for unfolding and refolding fit well to a single exponential. The single exponential phase displayed by the refolding data indicates that the four prolines of EGIII do not form incorrect imide isomers in the unfolded, disulfide-bonded form (or X-Pro imide isomerization is not rate-limiting). The logarithm of the rate of refolding at urea concentrations below 3 M deviates from linearity with respect to urea concentration. Because only one kinetic phase is observed, the most likely interpretation is that a transient folding intermediate becomes populated at these urea concentrations, and the observed rate is its conversion to the native form. This is analogous to the behavior observed with barnase, for example, where the existence of a kinetic intermediate has been confirmed by site-directed mutagenesis (Matouschek et al., 1990) and NMR spectroscopy (Bycroft et al., 1990).

Given the extracellular nature of EGIII and its homology with the endoglucanase from *Aspergillus acleatus*, we expected that the two cysteines in EGIII would exist as a disulfide bridge, as they do in the *Aspergillus* enzyme (Hata et al., 1994). We confirmed this by reducing EGIII and using urea denaturation to measure the resulting destabilization (Figure 5). The disulfide linkage provides a remarkable degree of stabilization to the protein—about 7 kcal/mol. This is considerably more stabilization than the 3.6 kcal/mol calculated for the statistical effect on the entropy of the unfolded state brought about by cross-linking the protein [$\Delta S_{\text{conformational}} = 2.1 + (3/2)R \ln n$, where n is the number of residues between the cross-linked side chains (Pace et al., 1988)]. This purely entropic mechanism of disulfide stabilization has been called into question by Doig and Williams (1991), who argue that the entropic destabilization brought about by cross-linking the unfolded state is more than offset by the entropic cost of a diminished hydrophobic effect. Doig and Williams showed that the effect of a disulfide actually arises from enthalpic destabilization of the unfolded state. It is reasonable to conclude that the high degree of stabilization of EGIII provided by its disulfide is probably due to a combination of entropic and enthalpic destabilization of the unfolded state and enthalpic stabilization of the native state. EGIII represents a potentially valuable model for protein engineering, structural, and thermodynamic studies to understand disulfide stabilization.

Trichoderma reesei is an acidophilic fungus, and the cellulases it secretes have evolved to function optimally in low-pH environments. This is evident in the loss of EGIII stability at alkaline pH (Figure 6). This is due to a decrease in both the m value and $U_{50\%}$. The change in $\Delta G_{u-f}^{H_2O}$ with pH fits reasonably well to a titration curve with a pK_a of 7.5, and the stability changes by almost 10 kcal/mol. This reflects a titration of the m value, because $U_{50\%}$ decreases monotonically. This suggests deprotonation of a histidine leads to destabilization of the native state by creating a smaller difference in solvent exposure between the native and unfolded states. Indeed, the urea titration curves become too shallow for analysis beyond pH 9.4. However, inspection of the fluorescence and far-UV CD spectra of the native and denatured states of the protein revealed no significant differences between pH 5 and 9. In addition, the native enzyme showed no binding of ANS (anilinonaphthalenesulfonic acid) at either pH 5.5 or pH 10, suggesting the absence of molten globule character at elevated pH (data not shown). Thus, any pH-dependent conformational changes, although implied by the urea titrations, have yet to be observed directly.

The temperature dependence of urea denaturation quantifies the decrease in stability of EGIII with increasing temperature and predicts maximum stability at 10 °C at pH 5.5. Fitting the data to the Gibbs−Helmholtz equation yields reasonable values for ΔH_m, ΔS_m, and ΔC_p (Table 2), lending additional support to the two-state nature and reversibility of EGIII unfolding.

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