Dimerization of *Thermomonospora fusca* β-1,4-Endoglucanase E2†

Kathleen McGinnis, Chris Kroupis, and David B. Wilson*

Section of Biochemistry, Molecular, and Cell Biology, Cornell University, Ithaca, New York 14853

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ABSTRACT: Unboiled *Thermomonospora fusca* endoglucanase E2 electrophoresed on SDS–polyacrylamide gels migrated in the range of 80–90 kDa, but when boiled it migrated in the 40–42-kDa range. Sedimentation equilibrium centrifugation as well as chemical cross-linking experiments confirmed that E2 is a dimer. The dimer was reversibly dissociated at low pH. The E2 dimer was stable up to 70 °C, but began to dissociate at this temperature after a 30–60-min incubation. A nondimerizing mutant was obtained using region-specific chemical mutagenesis. DNA sequencing of this mutant revealed a single base change that substituted Gly for Glu-263. Chemical modification of carboxylic acid residues in E2 disrupted the dimer interaction.

The thermophilic, filamentous, soil bacterium *Thermomonospora fusca* produces and excretes at least six cellulases. These enzymes are of particular interest due their high thermostability, making them useful for industrial applications (Margaritis & Merchant, 1986). Thus far, six *T. fusca* cellulases have been isolated, purified, and characterized (Calza et al., 1985; Wilson, 1988). These enzymes vary in their properties and enzymatic activities on various cellulolytic substrates. The genes encoding four of these proteins have been cloned and sequenced (Lao *et al.*, 1991; Wilson, 1992). The DNA sequence of endocellulase E2 codes for a protein containing 395 residues with a molecular weight of 42 000. During the course of our studies to determine the structure and enzymatic mechanism of this enzyme, we discovered that E2 had quaternary structure. The evidence presented here suggests that this protein normally exists as a noncovalent dimer.

MATERIALS AND METHODS

Materials. (Carboxymethyl)cellulose, low viscosity (CMC), dithiothreitol (DTT), sodium borohydride ($NaBH_4$), and trichloroacetic acid (TCA) were all purchased from Sigma. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) was obtained from Pierce and glutaraldehyde from Fisher.

Enzyme Preparation. All three proteins, E2, $E2_{cd}$, and E5, were purified from the culture supernatants of *Streptomyces lividans*, transformed with plasmids carrying either the *T. fusca* E2 or E5 gene, grown as described (Walker *et al.*, 1992). *T. fusca* E2 was purified according to established procedures (Walker *et al.*, 1992; McGinnis & Wilson, 1993). To prepare $E2_{cd}$ (a proteolytic product of E2), *S. lividans* TK24(pGG85) (Ghanges & Wilson, 1988) containing the whole E2 gene was grown in 200 mL of tryptic soy broth (TSB) containing 5 μg/mL thiostrepton (tsr) overnight at 28 °C. This culture was added to 8 L of TSB + tsr medium in a New Brunswick fementer, and the cells were allowed to grow for 2 days at 2 °C (very thick growth). The cells were concentrated using a 0.22-μm Durapore filter cassette (Millipore) to approximately 1 L, and transferred to 12 L of phosphate starvation media plus tsr. The culture was harvested by filtration after about 56 h when CMC overlays of SDS–PAGE gels (Wilson, 1988) showed a large portion of the activity was in the 30-kDa band rather than the 42-kDa band. Ammonium sulfate was added to the supernatant to a concentration of 0.6 M, and the resulting solution was loaded on a phenyl-Sepharose column following the protocol for *T. fusca* (Walker *et al.*, 1992). Gels run on the column fractions showed that approximately 70% of the active cellulase was $E2_{cd}$ with a molecular mass of 30 kDa. N-Terminal sequencing determined that the first 15 amino acids of the 30-kDa product were identical to those in the native protein (Lao *et al.*, 1991). The fractions containing $E2_{cd}$ and very little E2 were combined and further purified using a HAP column as described for E2 (Walker *et al.*, 1992). The HAP column eluate was equilibrated with 5 mM BisTris, pH 6, and loaded onto a Q-Sepharose column. A 1.8-L linear gradient from 0 to 150 mM NaCl was used to separate E2 and $E2_{cd}$. The final yield of $E2_{cd}$ was approximately 30 mg. E5 was prepared as previously described (Walker *et al.*, 1992).

SDS Gel Electrophoresis. SDS–polyacrylamide gels were run as described (Laemmli, 1970). Samples were either boiled or not boiled after addition of solubilization buffer (with or without DTT) prior to electrophoresis. Proteins were visualized by staining with Coomassie Brilliant Blue R-250. Molecular weight standards (Bethesda Research Labs prestained) consisted of myosin H-chain (M_w 200 000), phosphorylase B (M_w 97 000), bovine serum albumin (M_w 68 000), ovalbumin (M_w 43 000), carbonic anhydrase (M_w 29 000), β-lactoglobulin (M_w 18 000), and lysozyme (M_w 14 000).

Sedimentation Equilibrium. Purified E2 was dialyzed for 24 h at 4 °C vs 0.1 M KCl, pH 6.0, using animal membrane tubing. The protein concentration was measured after dialysis, and 120 μL of 2 mg/mL protein was loaded in one well of the cell with dialysate as the reference in the other well. The sample was centrifuged at 10 000 rpm for 24 h at 25 °C in a Beckman analytical ultracentrifuge, and the equilibrium protein concentration in the cell was determined using interference optics as described in Chervenka (1969). The partial specific volume of *T. fusca* E2 was calculated to be 0.72 from its amino acid composition (Cohn & Edsall, 1965).

Chemical Cross-Linking Experiments. E2 and $E2_{cd}$ were chemically cross-linked by modifying the procedures outlined in Jaenicke and Rudolph (1989). Glutaraldehyde (50% w/v) was added to 20 mL of 0.05 M KP_i (pH 6.0) buffer containing 50 μg of protein to give a final concentration of 2%. After a 12–24-h incubation at room temperature, 0.5 mL of freshly prepared 2 M $NaBH_4$/0.1 M NaOH was added to quench the

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* Correspondence should be addressed to this author.

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cross-linking reaction. This mixture was incubated for 20 min at room temperature. Then, 20 μL of 10% sodium deoxycholate in 0.1 M NaOH was added followed by 78% TCA (w/v) (0.25–0.50 mL) until the deoxycholate and protein precipitated out of the solution. More deoxycholate (20 μL) was added if no precipitate appeared after addition of 0.50 mL of TCA. The tubes were centrifuged for 20 min at 20000*g* to pellet the precipitated protein. The pellets were immediately redissolved in DTT-containing SDS–PAGE solubilization buffer, boiled, and electrophoresed.

Dissociation of the E2 Dimer. Dissociation of the E2 protein dimer at low pH and high temperature was accomplished according to methods adapted from published procedures (Hardy *et al.*, 1988; Luckey *et al.*, 1991). Six microliters of 0.1 M HCl was added to 20 μg of protein in 40 μL of 0.05 M KP_i, pH 6.0. At timed intervals, 6 μL was removed and added to 5 μL of SDS–PAGE solubilization buffer that did not contain reducing agent. After the last sample was removed, a stoichiometric amount of 0.1 M NaOH was immediately added, and 6-μL aliquots were removed at timed intervals after neutralization. These aliquots were dissolved in solubilization buffer without DTT, and all samples were electrophoresed on SDS–polyacrylamide gels without boiling. These experiments were carried out at room temperature and were repeated using $E2_{cd}$.

Dissociation of the E2 and $E2_{cd}$ dimers was also tested in buffered solutions at various pHs. E2 and $E2_{cd}$ were incubated in solutions of 1.0 M NaOAc at pH 4.0, 4.5, 5.0, and 5.5 and in 1.0 M KP_i at pH 6.0, 6.5, 7.5, 8.0, and 8.7, final protein concentration 0.1 mg/mL. These experiments were carried out at both 25 °C and 50 °C.

The temperature stability of the E2 dimer was determined by incubating tubes containing a 0.5 mg/mL solution of E2 in 0.05 M KP_i, pH 6.0, at six temperatures from 25 to 100 °C. After 15, 30, and 60 min, 5-μL aliquots were removed, dissolved in gel electrophoresis buffer, and run on SDS–polyacrylamide gels.

Isolation and Characterization of Mutant CKDW8. Region-specific chemical mutagenesis as described in Kroupis (1991) was used to obtain mutant CKDW8. Single-stranded M13 DNA containing the E2 gene was treated with hydrazine and potassium permanganate, and reverse transcriptase was used to synthesize the other strand. A *Sac*II fragment of the E2 gene containing the catalytic domain was removed from the treated DNA. This fragment (0.9 kb) was recloned into the E2 gene insert present in plasmid pCK19 (a derivative of pUC 19) and transformed into DH5α. Mutants with altered CMCase activity in a colony screening assay (Teather & Wood, 1982) were purified by streaking on LBamp plates and rescreened for CMCase activity. CKDW8 produced a larger halo in the assay than the parent strain. The mutant E2 gene was sequenced according to methods outlined previously (Lao *et al.*, 1991). The cellulase activity of mutant CKDW8 in French-pressed extracts was determined on CMC using the method of Ghose (1987) as described in Walker *et al.* (1992).

Western Blot Procedure. Cells pellets from 1-mL cultures of *Escherichia coli* transformed with CKDW8 were dissolved in 150 μL of SDS–PAGE solubilization buffer. Samples (10 μL) were loaded on a 12% SDS–polyacrylamide gel, electrophoresed for 1 h at 100 V, and transferred overnight at 40 V to nitrocellulose filters using a large transfer apparatus (Towbin *et al.*, 1979). Purified proteins were prepared for Western blotting as described for SDS–PAGE. The filters were reacted with antisera raised against purified E2 and processed according to the methods described in the Bio-Rad Laboratories immune blot assay kit using alkaline phosphatase conjugated second antibody.

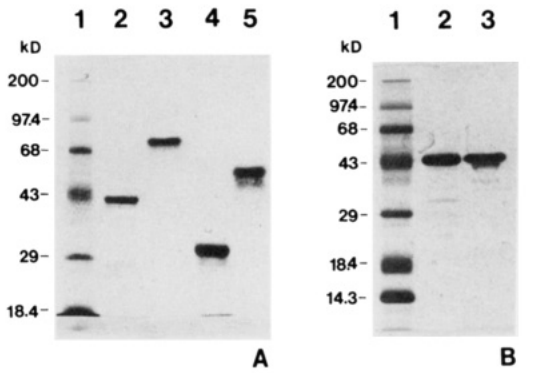

FIGURE 1: SDS–polyacrylamide gels of boiled and unboiled E2, $E2_{cd}$, and E5. Panel A: lane 2, E2 protein boiled for 2 min in solubilization buffer containing DTT; lane 3, E2, –DTT, unboiled; lane 4, $E2_{cd}$, –DTT, boiled; lane 5, $E2_{cd}$ –DTT, unboiled; lane 1, molecular mass standards as indicated. Panel B: lane 2, E5 (+DTT, boiled); lane 3, E5 (–DTT, unboiled); lane 1, molecular mass markers.

Carbodiimide Modification. E2 and $E2_{cd}$ were chemically modified using the water-soluble carbodiimide 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) (Pierce Chemical Co.). EDC was added to a final concentration of 20, 50, or 100 mM to solutions containing 0.2 mg/mL protein in 50 mM MES, pH 6.0. Reaction mixtures (50 μL) were mixed and incubated at 25 °C. After 1 h, solubilization buffer without DTT was added, and the samples were immediately electrophoresed on SDS–polyacrylamide gels (without boiling).

Protein Determination. Protein concentrations were determined by using the method in Bradford (1976) with bovine serum albumin as a standard.

RESULTS

Behavior of Nonboiled E2 on SDS–PAGE and Determination of Molecular Masses. When cellulase E2 was electrophoresed on an SDS–polyacrylamide gel without boiling, the protein migrated to a different position on the gel than E2 that was boiled before electrophoresis (Figures 1, panel A). These results were reproducible with different E2 preparations and at various protein concentrations. The apparent molecular mass of unboiled E2 calculated from a 10% SDS–polyacrylamide gel is 86 kDa. The molecular mass of boiled E2 corresponded to 42 kDa when calculated from its relative mobility in this same gel. Thus, unboiled E2 has twice the molecular mass of E2 calculated from the DNA sequence of its gene. Similar values were obtained from 8%, 10%, and 12% SDS–polyacrylamide gels. The molecular mass of E2 (boiled) varied between 40 and 45 kDa, while that of unboiled E2 varied between 80 and 90 kDa, with identical values always obtained for gels at the same acrylamide concentration. For comparison, *T. fusca* cellulase E5, which has a molecular mass of 46 000 (based on the DNA sequence of its gene), was treated in a similar manner (Figure 1, panel B). E5 migrated to the same position regardless of whether or not the protein was boiled before electrophoresis. Figure 1, panel A, also shows $E2_{cd}$, boiled or not boiled before electrophoresis. $E2_{cd}$ is a catalytically active proteolytic product of E2 missing approximately 100 amino acids from the carboxy terminus of E2. The missing C-terminal region of E2 was shown to contain the cellulose binding domain of the enzyme both from sequence comparisons (Lao *et al.*, 1991) and by binding experiments (Ghanges & Wilson, 1988).

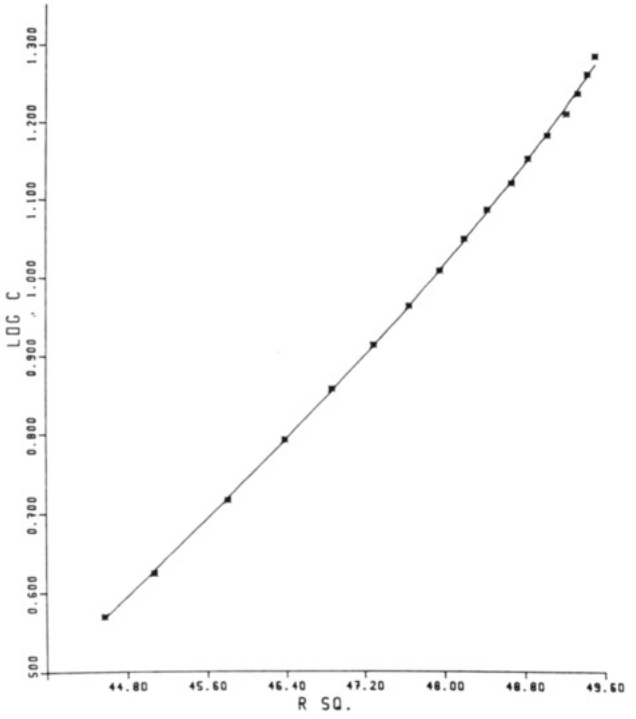

FIGURE 2: Sedimentation equilibrium centrifugation of E2. The experiment was run as described under Materials and Methods.

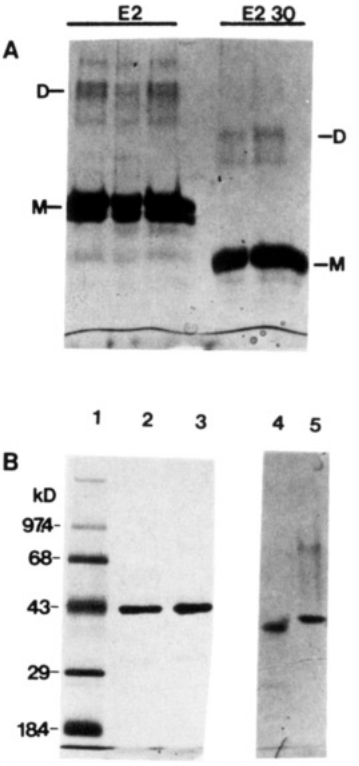

FIGURE 3: Panel A: Coomassie-stained SDS–polyacrylamide gel of chemically cross-linked E2 and $E2_{cd}$ (M = monomer, D = dimer). Both samples were solubilized with DTT present and boiled. Panel B: lane 2, E2 D/I (–DTT, unboiled); lane 3, E2 D/I (+DTT, unboiled); lane 4, E2 (–DTT, boiled); lane 5, E2 (+DTT, unboiled); lane 1, molecular mass markers as shown. [E2 D/I = partially carboxymethylated E2 as described in McGinnis and Wilson (1993)].

Unboiled $E2_{cd}$ also had a lower relative mobility than its boiled counterpart. The molecular masses of these two species were calculated from several different gels. The apparent molecular mass of unboiled $E2_{cd}$ was consistently between 60 and 70 kDa, while the boiled protein migrated to a position between 30 and 35 kDa, depending on the acrylamide concentration that was used. This evidence suggests that both E2 and $E2_{cd}$ exist as noncovalent dimers.

Sedimentation Equilibrium Experiment. In order to confirm these results, sedimentation equilibrium centrifugation was performed on homogeneous E2. The plot of ln concentration vs (distance)^2 (Figure 2) showed an obvious positive deviation, indicating the presence of associating monomers (Freifelder, 1982). This upward curvature is a result of higher molecular mass species (dimers) sedimenting at a faster rate than lower molecular mass species (monomers) in the sample and accumulating at the bottom of the cell. The weight-average molecular mass, 52 kDa, calculated from the data also suggests that there is a mixture of monomer and dimer in the sample. The M_z/M_w value was 1.45. For monomeric proteins, this value is equal to or very close to 1.0. An M_z/M_w ratio greater than 1.0 is characteristic of oligomeric proteins (Cantor & Schimmel, 1988).

Cross-Linking of E2. Chemical cross-linking experiments were performed on E2 and $E2_{cd}$ to further substantiate these findings. Both E2 and $E2_{cd}$ were cross-linked in the presence of glutaraldehyde at pH 6.0. As shown in Figure 3, panel A, after SDS–polyacrylamide gel electrophoresis, two major bands appeared in both the E2 and $E2_{cd}$ cross-linked samples which correspond to the monomer and dimer forms of each protein. Varying the protein concentration over a 100-fold range did not change the amount of cross-linked dimer. These results further confirm that cellulase E2 behaves as a dimeric protein.

Effect of Disulfide Reduction on the E2 Dimer. E2 contains six cysteine residues participating in three disulfide bonds (McGinnis & Wilson, 1993). Figure 3, panel B, lane 5 shows that with DTT present the E2 dimers dissociate even if the sample is not boiled before SDS–polyacrylamide gel electrophoresis; thus, reduction of the disulfide bridges disrupts the dimer. Two of these disulfide bonds were found to be exposed and can be reduced upon addition of DTT to $E2_{cd}$ or E2 (McGinnis & Wilson, 1993). When reduced E2 is treated with iodoacetamide, a partially carboxymethylated protein with one disulfide still intact is produced. The other disulfide bond is buried and is only susceptible to reduction when the protein is denatured. Partially carboxymethylated E2 also runs as a monomer when the samples are not boiled before electrophoresis with or without DTT present (Figure 3, panel B, lanes 2 and 3). Therefore, reduction of only the exposed disulfides dissociates E2.

pH and Temperature Stability of the E2 Dimer. Dissociation of E2 was achieved by addition of 0.1 M HCl (final pH of approximately 3.0) (Figure 4). The dimers reassociated rapidly after neutralization with a stoichiometric amount of 0.1 M NaOH. The same results were obtained when these experiments were performed on $E2_{cd}$ (Figure 5).

E2 exists as a dimer between pH 6.0 and 8.7 (Table I). The dimer begins to dissociate slightly at pH 5.5; at pH 5.0, equal amounts of monomer and dimer were observed; by pH 4.5, the subunits are mostly dissociated, and the dimer becomes completely dissociated at pH 4.0. These experiments were run at 50 °C for 30 min to mimic cellulase assay conditions, but the same results were observed at room temperature or if the protein concentration was increased 2-fold to 0.2 mg/mL. Table I also shows that identical results were obtained with $E2_{cd}$.

The stability of the dimer was tested at various temperatures as shown in Figure 6. The dimer was stable from 25 to 70 °C after a 15-min incubation, but was almost completely

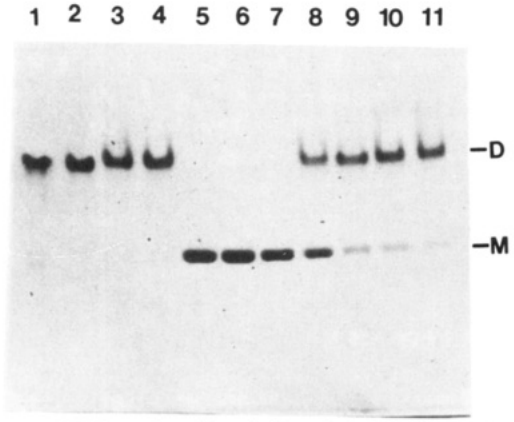

FIGURE 4: pH-dependent dissociation of the E2 dimer. A solution of E2 was acidified, and samples were removed 0.1 min (lane 5), 2 min (lane 6), and 5 min (lane 7) after addition of 0.1 M HCl. Five minutes after acidification, an equivalent amount of 0.1 M NaOH was added, and aliquots were taken at 0.1 min (lane 8), 2 min (lane 9), 5 min (lane 10), and 10 min (lane 11) after neutralization. Lanes 1–4 show E2 dimers before the addition of 0.1 M HCl. All samples were run without DTT, unboiled.

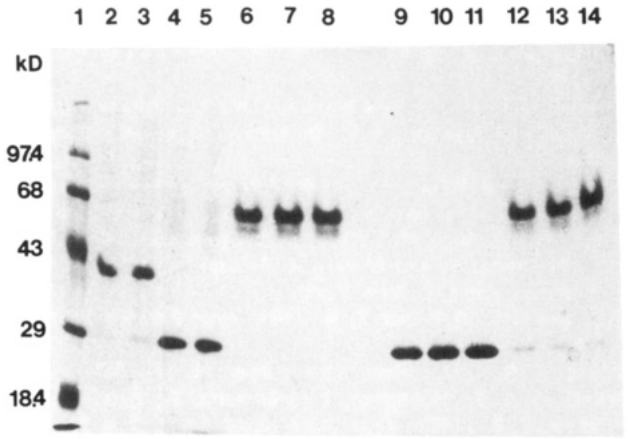

FIGURE 5: pH-dependent dissociation of the $E2_{cd}$ dimer. A solution of $E2_{cd}$ was acidified with 0.1 M HCl, and aliquots were removed after 0.1-min (lane 9), 2-min (lane 10), and 5-min (lane 11) incubation. Five minues after acidification, an equivalent amount of 0.1 M NaOH was added, and samples were taken 0.1 min (lane 12), 2 min (lane 13), and 5 min (lane 14) after neutralization. Lane 1 is molecular mass standards as indicated; lanes 2 and 3 are E2 (boiled, +DTT); lanes 4 and 5 are $E2_{cd}$ (boiled, +DTT); lanes 6–8 are $E2_{cd}$ dimer before addition of 0.1 M HCl. All samples lacked DTT and were not boiled unless otherwise indicated.

Table I: Dissociation of the E2 Dimer at Various pHs[a]

	pH								
	4.0	4.5	5.0	5.5	6.0	6.5	7.5	8.0	8.7
E2	M	M*/D	M/D	M/D*	D	D	D	D	D
$E2_{cd}$	M	M*/D	M/D	M/D*	D	D	D	D	D

[a] The distribution of dimer and monomer was determined from SDS–PAGE. The protein was incubated in a buffered solution at each pH as indicated above for 30 min at 50 °C. Samples were then dissolved in solubilization buffer minus DTT and electrophoresed without boiling. M, monomer; D, dimer; M/D, equal amounts of monomer and dimer; M*/D, predominantly monomer; M/D*, predominantly dimer.

dissociated at 80 °C. After a 30-min incubation, the dimer began to dissociate at 70 °C with an equal mixture of monomer and dimer present. After a 60-min incubation, more of the dimer was dissociated into monomers at 70 °C, but the dimer was still completely stable up to 60 °C.

Nondimerizing E2 Mutant. When unboiled extracts of the cellulase E2 mutant CKDW8 were analyzed on a Western blot, the mutant protein was found to run as a monomer instead of the dimer found in unboiled E2 and the mixed population of monomer and dimer found in the parent *E. coli* extract (Figure 7). The molecular mass of the unboiled mutant protein was the same as that of boiled E2. CKDW8 was sequenced, and a single base change was identified (Kroupis, 1991). This change results in a substitution of Gly for Glu-263. The change

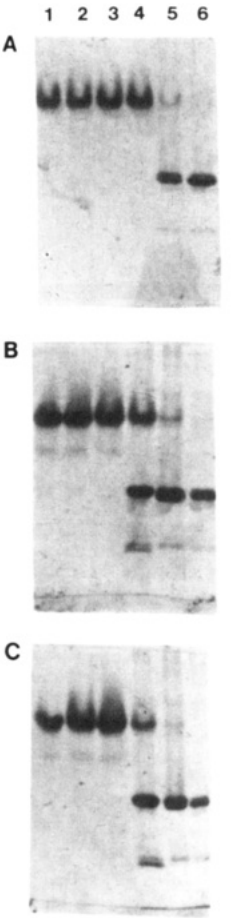

FIGURE 6: Stability of the E2 dimer at various temperatures. Samples of E2 were incubated at 25 °C (lane 1), 50 °C (lane 2), 60 °C (lane 3), 70 °C (lane 4), 80 °C (lane 5), and 100 °C (lane 6). Aliquots were removed after 15 min (panel A), 30 min (panel B), and 60 min (panel C) and immediately dissolved in solubilization buffer without reducing agent and subjected to SDS–PAGE. Samples were not boiled.

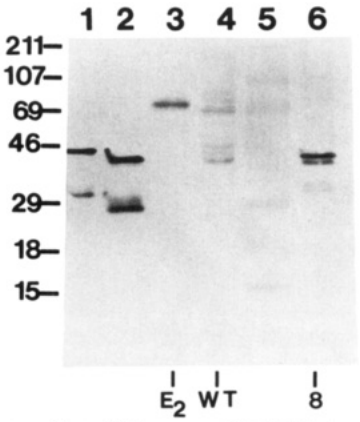

FIGURE 7: Western blot of E2 mutant CKDW8. Lane 1, boiled E2 (+DTT); lane 2, boiled E2 (–DTT); lane 3, unboiled E2 (–DTT); lane 4, unboiled wild type, –DTT (from *E. coli* extracts); lane 6, unboiled CKDW8 (–DTT). The proteins were transferred from an SDS–polyacrylamide gel onto a nitrocellulose filter. The filters were then reacted with anti-E2 antibody prepared by affinity purification from E2 coupled to Bio-Rad affi-gel 15 (Kroupis, 1991).

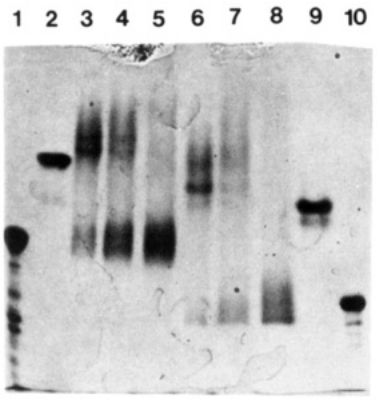

FIGURE 8: SDS–PAGE showing the effect of EDC on E2 and $E2_{cd}$. Lane 1, E2 (boiled, +DTT); lane 2, E2 (unboiled, –DTT); lane 3, E2 (20 mM EDC); lane 4, E2 (50 mM EDC); lane 5, E2 (100 mM EDC); lane 6, $E2_{cd}$ (20 mM EDC); lane 7, $E2_{cd}$ (50 mM EDC); lane 8, $E2_{cd}$ (100 mM EDC); lane 9, $E2_{cd}$ (unboiled, –DTT); lane 10, $E2_{cd}$ (boiled, +DTT). The EDC-modified samples were dissolved in solubilization buffer without DTT and not boiled.

in the mutant was verified by *Hph*I digestion of the mutant plasmid; since this mutation abolishes one restriction site, the digestion patterns are different. The cellulase activity of the mutant enzyme was twice that of the wild type in the overlay assay, but similar to that of the wild type in the liquid assay (14 nmol min^{-1} mL^{-1} for CKDW8 vs 13 nmol min^{-1} mL^{-1} for wild type).

Chemical Modification of Carboxylic Groups. Since a gluatmic acid residue was found to participate in the interaction of the subunits, modification of carboxylic groups with EDC should lower dimer stability. Figure 8 shows that EDC modification affects the stability of the E2 dimer. After 1-h incubation in 20 mM EDC, there is a slight dissociation of the E2 dimer. In 50 mM EDC, a mixture of dimer and monomer was observed, while after 1 h in 100 mM, the dimer was completely dissociated. The same results were found with $E2_{cd}$.

DISCUSSION

The dimeric nature of E2 was shown by SDS gel electrophoresis, cross-linking, and sedimentation equilibrium. The dimers were noncovalent as they could dissociate even when the disulfide bonds were maintained. Dimerization was unaffected by the removal of the cellulose binding domain of E2, showing it is due to interactions of catalytic domain residues. Dissociation of E2 was caused by heating above 70 °C or pH values below 5.0 or by reduction of two disulfide bonds.

The DNA sequence of CKDW8 revealed only a single base change which resulted in the substitution of Glu-263 by Gly. This single amino acid change disrupts the interaction between the monomers so that only the monomeric form of E2 is found in this mutant. The mutation results in 1 less negative charge at neutral pH, so that the mutant enzyme has 12 negative charges instead of 13 at pH 7. This was confirmed as a CMC overlay of the mutant run on an isoelectric focusing gel shows a slight p*I* shift of the CKDW8 protein toward neutrality compared to wild type (data not shown). The participation of a carboxylic group in the monomer association was substantiated by the fact that EDC modification of E2 resulted in dissociation. Most excreted proteins are monomeric, but examples of secreted oligomeric proteins include the bacterial enterotoxin secretion by *Vibrio cholerae* (Hirst & Holmgren, 1987) and the pertussis toxin from *Bordatella pertussis* (Nicosia & Rappuoli, 1987). Thus far, there is no evidence for other free oligomeric cellulases; however, little information is known about the structure of most cellulolytic enzymes. The CKDW8 mutant enzyme has wild-type activity on soluble CMC so that the CMCase activity of E2 is not dependent on its quaternary structure.

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