Disulfide Arrangement and Chemical Modification of β-1,4-Endoglucanase E2 from *Thermomonospora fusca*†

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Received October 22, 1992; Revised Manuscript Received March 24, 1993

ABSTRACT: *Thermomonospora fusca* endoglucanase E2 contains six cysteine residues scattered along the protein sequence. Four of the cysteine residues were shown to participate in two disulfide bonds while the last two form a third disulfide bond. Neither full reduction of the disulfides nor complete carboxymethylation of all six cysteines totally destroys enzymatic activity, but the activity of the reduced enzyme is much lower than the native enzyme and the iodoacetamide-modified enzyme has very low activity. Reduction of only the accessible disulfides drastically decreases the enzyme's thermostability. One disulfide linkage joins Cys80 to Cys125, another joins Cys232 to Cys267, and the third joins Cys315 to Cys407. The first two bonds are similar to those in cellobiohydrolase II, which also belongs to cellulase family B (Rouvinen *et al.*, 1990; Lao *et al.*, 1991; Henrissat et al., 1989). Direct evidence for the involvement of carboxyl groups in catalysis by E2 was demonstrated by chemical modification with carbodiimide.

Our laboratory is studying the cellulolytic enzymes excreted by the organism *Thermomonospora fusca*. One of these enzymes, endoglucanase E2, has been purified from *T. fusca* supernatants and characterized (Calza *et al.*, 1985). This protein has a molecular weight of 42 000 and associates to form noncovalent dimers (McGinnis *et al.*, 1993). The E2 structural gene has been cloned and used to overproduce the enzyme in *Streptomyces lividans* (Ghangas & Wilson, 1988). E2 is strongly inhibited by Hg^{2+} (Calza *et al.*, 1985; Ghangas & Wilson, 1988). Heavy metals can inactivate enzymes by forming covalent salts with functional cysteines, histidines, or carboxyl groups (Dixon & Webb, 1964).

The amino acid sequence of *T. fusca* E2 deduced from the original DNA sequence showed that this enzyme contains 395 amino acids, including 4 cysteine residues (Lao *et al.*, 1991). However, further sequencing work shows that E2 contains six cysteine residues. In this paper, we show that E2 contains three disulfide bonds: one joining Cys80 to Cys125, another joining Cys232 to Cys267, and by elimination the third joins Cys315 to Cys407. Chemical modification of E2 using carbodiimide shows that carboxyl groups are involved in catalysis, suggesting that Hg^{2+} inhibits E2 by binding to active-site carboxyl groups.

MATERIALS AND METHODS

Materials. Dithiothreitol (DTT), iodoacetamide, guanidine hydrochloride, cyanogen bromide (CNBr), and (carboxymethyl)cellulose (sodium salt, low viscosity) (CMC) were all obtained from Sigma. Acetonitrile (HPLC grade) was from Burdick and Jackson Labs Inc., and trifluoroacetic acid (TFA) was from Fisher Scientific. The 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) used in these experiments was purchased from Pierce. (Hydroxyethyl)cellulose 300 (HEC) was obtained from Serva, and cellobiose was from ICN Biochemicals. Milli Q water was used in all HPLC solvents.

Enzyme Preparation. Cultures of *Streptomyces lividans* transformed by plasmid pGG85 carrying the *T. fusca* E2 gene were grown and harvested, and E2 was purified from the supernatant as previously described (Ghangas & Wilson, 1988; Walker *et al.*, 1992). The sample (~60 mg of protein) obtained from these procedures was run over a 30-mL Q-Sepharose (Pharmacia) column equilibrated with 10 mM Bis-Tris, pH 6.0. A wash of 150 mM NaCl eluted a large protein peak which contained CMC activity. These column fractions were assayed for purity by SDS–polyacrylamide gel electrophoresis, and those containing at least 95% E2 were pooled. Other contaminating proteins remained bound to the column. $E2_{cd}$, an N-terminal proteolytic derivative of E2, was purified as described in McGinnis *et al.* (1993).

Enzyme Assays. Cellulase assays were carried out as described previously (Wilson, 1988). The amount of reducing sugar produced was measured by the dinitrosalicyclic (DNS) procedure (Miller *et al.*, 1960). The reaction mix included the sample to be assayed, 0.05 M KP_i buffer, pH 6.0, and 0.02 mL of 2% CMC in a final volume of 0.40 mL. The samples were then incubated at 50 °C for 30 min. The reaction was stopped by addition of 1 mL of DNS reagent, the samples were boiled for 15 min and the OD at 600 nm was measured. CMC overlays were performed as described by Wilson (1988).

Temperature Stability Experiments. Two sets of tubes each containing 150 μL of a 0.04 mg/mL solution of purified E2 were incubated at various temperatures from 25 to 100 °C. One set of tubes contained 10 mM DTT. Aliquots were removed after 30 and 60 min and assayed for CMCase activity. All assays were done in triplicate.

Reduction and Carboxymethylation. Samples of E2 and $E2_{cd}$ were reduced and carboxymethylated either in 0.5 M Tris, pH 8.5 (nondenatured), or in 6 M guanidine hydrochloride/0.5 M Tris pH 8.5 (denatured). The proteins were present at 1 mg/mL, and DTT was added to 10 mM final concentration. After incubation at 37 °C for 3 h, the tubes were cooled on ice, and iodoacetamide was added to a final concentration of 20 mM. The samples were incubated on ice in the dark for 2 h. Guanidine hydrochloride and excess reagents were removed by Amicon ultrafiltration using a 30- or 10-kDa MW cutoff filter (Millipore PTGC). The reduction and carboxymethylation reactions were adapted from methods described by Mayes (1984).

Cyanogen Bromide Cleavage. Aliquots of purified protein were lyophilized and dissolved in 6 M guanidine hydrochloride/

† This work was supported by Grant FG02-84-ER13233 from the U.S. Department of Energy.

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0.2 M HCl to a final protein concentration of 5–10 mg/mL. A 50-fold molar excess (with respect to methionine content) of cyanogen bromide was added in solid form to 0.1–1.0 mL of protein solution (Gross, 1967; Villa et al., 1989). This reaction mix was incubated for 18–24 h in the dark at room temperature. Excess reagents and solvents were then removed from the cleavage products by lyophilization after addition of 1 mL of distilled water. The peptides were washed once more with 1 mL of distilled water and lyophilized. The dried peptide mix was stored at −80 °C.

Reverse-Phase High-Pressure Liquid Chromatography (RP-HPLC) of CNBr Peptide Fragments. Peptides were dissolved in 0.1% TFA (trifluoroacetic acid) and filtered through a 0.45-μm nylon filter (Lida Manufacturing Corp.). Peptides (25–100 μg) in 100 μL were injected into a 25 cm × 4.6 mm C_8 300-Å Aquapore column (Brownlee Laboratories) equilibrated with 95% solvent A (0.1% TFA in H_2O and 5% solvent B (0.1% TFA/90% acetonitrile in H_2O). Peptides were eluted at 1 mL/min with a linear gradient of 5–60% solvent B over 50 min, and effluents were monitored at 210 or 214 nm.

Disulfide Analysis of Peptide Fragments. E2 CNBr peptides generated under nonreduced conditions were run on an HPLC system equipped with a "disulfide analyzer" (Thannhauser et al., 1987) under the conditions described above. Most of the effluent was monitored at 214 nm, while a portion was diverted and assayed for disulfide content as described by Thannhauser *et al.* (1987) using the reagent disodium 2-nitro-5-thiosulfobenzoate (NTSB). The peaks obtained at 412 nm were compared to those obtained at 214 nm to determine which peaks contained disulfide bonds. These samples were collected for analysis.

Amino Acid Analysis. Amino acid analysis was performed on the chemically modified E2 samples and E2 CNBr generated peptide fractions collected from the RP-HPLC column. The samples were hydrolyzed in 6 N HCl for 95 min at 150 °C, and the hydrolysates were fractionated on a Waters Pico Tag analyzer.

Chemical Modification with EDC. The procedures used for carboxyl group modification were adapted from methods described by Hurst *et al.* (1977) and Clarke and Yaguchi (1985).

Reaction of E2 and $E2_{cd}$ with the water-soluble carbodiimide EDC was carried out at pH 6.0. Solutions (200 μL) containing 50 μg of enzyme in 50 mM MES, pH 6.0, were treated with various concentrations of EDC and incubated at 25 °C. At timed intervals, 20-μL aliquots were removed from each reaction mixture and diluted to 500 μL with 100 mM sodium acetate, pH 5.5, to quench the reaction. Aliquots of these diluted samples were assayed for residual activity.

Reactions were also carried out in the presence of cellobiose or (hydroxyethyl)cellulose. Cellobiose (1–25 mM final concentration) or HEC (0.01–0.5% final concentration) was included in 100-μL reaction mixtures containing 25 μg of enzyme in 50 mM MES, pH 6.0, and 25 mM EDC. Aliquots (10 μL) of the reaction mixtures were removed at specific times after incubation at 25 °C and added to 240 μL of 100 mM sodium acetate, pH 5.5. Each sample was assayed for activity, and the percent residual activity was calculated.

SDS Electrophoresis. SDS–polyacrylamide gel electrophoresis was carried out in polyacrylamide slab gels as previously described (Laemmli, 1970). Samples were dissolved in dye mix with and without DTT and boiled 2 min before electrophoresis. The proteins or peptides were visualized with Coomassie Brilliant Blue R-250 or silver-stained using the method of Oakley *et al.* (1980).

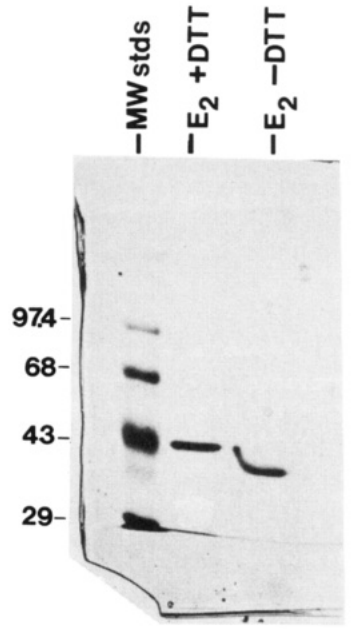

FIGURE 1: SDS–polyacrylamide gel of cellulase E2 with (lane 2) and without (lane 3) DTT in the solubilization buffer before electrophoresis. Lane 1 contains the indicated molecular weight ($\times 10^{-3}$) markers.

Protein Determination. Protein concentrations were determined by the Bradford procedure using bovine serum albumin as a standard (Bradford, 1976).

RESULTS

Determination of the Disulfide Content of E2. SDS–polyacrylamide gel electrophoresis showed that E2 has a different mobility when dithiothreitol (DTT) is present in the solubilization buffer (Figure 1). The native protein has a higher relative mobility due to its more compact structure. This indicated the presence of at least one disulfide bond.

Addition of DTT to the native protein reduced some but not all of the disulfide bonds. This sample was then treated with iodoacetamide to carboxymethylate the cysteines involved. When the native protein was denatured in 6 M guanidine hydrochloride and then treated with DTT and iodoacetamide, all disulfide bonds were broken, and all the cysteine residues were modified. As a control, the protein was denatured and reacted with iodoacetamide without reductant, and no cysteine residues were modified.

The differences in the mobilities on SDS–PAGE of native E2, partially carboxymethylated E2 (E2 D/I), and fully carboxymethylated E2 (E2 G/D/I) are shown in Figure 2. Without DTT in the solubilization buffer (panel A), these three samples had increasingly lower relative mobilities. In the presence of DTT, all three had identical mobilities since all are fully reduced (panel A). Panel B in Figure 2 shows the same results but with samples run side by side to illustrate the differences in their respective mobilities with and without DTT. The difference in mobility between nonreduced E2 D/I compared to its reduced form was half of the difference seen between the reduced and nonreduced native E2. This shows that there is still at least one disulfide intact in E2 D/I. There was no difference in the relative mobilities of the E2 G/D/I reduced and nonreduced forms. Since all disulfides in this sample were reduced and carboxymethylated, addition of DTT should have no effect on mobility.

Amino acid analysis of the E2 sample denatured, reduced, and iodoacetamide-treated (E2 G/D/I) confirmed that only (carboxymethyl)cysteine was present. The DTT- and io-

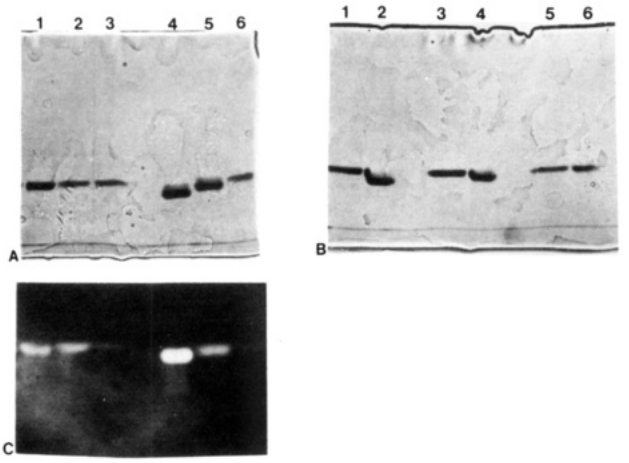

FIGURE 2: (Panel A) Native E2: lanes 1 and 4, partially carboxymethylated E2 (E2 D/I); lanes 2 and 5, fully carboxymethylated E2 (E2 G/D/I); lanes 3 and 6, run on SDS–polyacrylamide gels with DTT (lanes 1–3) and without DTT (lanes 4–6) in the solubilization buffer. (Panel B) Same as panel A except lanes 1 and 2, E2; lanes 3 and 4, E2 D/I; and lanes 5 and 6, E2 G/D/I. Lanes 1, 3, and 5 contain DTT in the solubilization buffer. (Panel C) CMC overlay of the same samples as in panel A; this overlay was incubated 30 min at 50 °C before being stained as described in Wilson (1988).

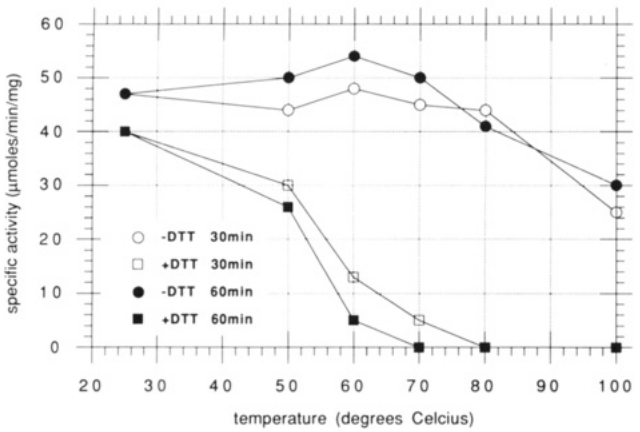

FIGURE 3: Effect of various temperatures on the specific activity of reduced and nonreduced cellulase E2. After incubation at the indicated temperatures for 30 or 60 min, the samples were assayed for activity for 30 min at 50 °C as described under Materials and Methods.

doacetamide-treated nondenatured protein (E2 D/I) contained both cysteine and (carboxymethyl)cysteine. No (carboxymethyl)cysteine was found in the control sample (E2 G/I) as expected if the protein contained no free sulfhydryl groups.

Enzymatic Activity of Reduced and Carboxymethylated E2. Enzyme assays showed that reduction of the exposed disulfide decreased enzymatic activity by half and reduction of all disulfides or carboxymethylation of all six cysteines resulted in an 80–90% loss in activity. A CMC overlay was also used to detect enzymatic activity. The CMC overlay in Figure 2, panel C, shows that native E2 –DTT had the most activity, native E2 +DTT and the partially carboxymethylated enzyme (E2 D/I) had about half the activity of native E2 –DTT, and fully carboxymethylated enzyme (E2 G/D/I), though still active, retained only a small fraction of the native activity. These results suggest that the disulfide linkages must be important but not essential in maintaining the active-site structure.

The catalytically active proteolytic derivative of E2, $E2_{cd}$, is missing approximately 100 amino acids from its C-terminus (Ghanges & Wilson, 1988). $E2_{cd}$ contains two disulfides and retains full activity on CMC. It also loses activity upon

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                                                  MetSerProArgProLeuArgAlaLeuLeuGly

    AlaAlaAlaAlaAlaLeuValSerAlaAlaAlaLeuAlaPheProSerGlnAlaAlaAla

  1 AsnAspSerProPheTyrValAsnProAsnMetSerSerAlaGluTrpValArgAsnAsn
    PEPTIDE 1---------------------->  PEPTIDE 2----------------

 21 ProAsnAspProArgThrProValIleArgAspArgIleAlaSerValProGlnGlyThr
    ------------------------------------------------------------

 41 TrpPheAlaHisHisAsnProGlyGlnIleThrGlyGlnValAspAlaLeuMetSerAla
    ----------------------------------------------------->  PEP 3-

 61 AlaGlnAlaAlaGlyLysIleProIleLeuValValTyrAsnAlaProGlyArgAspCys
    ------------------------------------------------------------80

 81 GlyAsnHisSerSerGlyGlyAlaProSerHisSerAlaTyrArgSerTrpIleAspGlu
    ------------------------------------------------------------

101 PheAlaAlaGlyLeuLysAsnArgProAlaTyrIleIleValGluProAspLeuIleSer
    ------------------------------------------------------------

121 LeuMetSerSerCysMetGlnHisValGlnGlnGluValLeuGluThrMetAlaTyrAla
    ----> PEP 4-125->  PEPTIDE 5---------------------->  PEP 6---

141 GlyLysAlaLeuLysAlaGlySerSerGlnAlaArgIleTyrPheAspAlaGlyHisSer
    ------------------------------------------------------------

161 AlaTrpHisSerProAlaGlnMetAlaSerTrpLeuGlnGlnAlaAspIleSerAsnSer
    ---------------------->  PEPTIDE 7-------------------------

181 AlaHisGlyIleAlaThrAsnThrSerAsnTyrArgTrpThrAlaAspGluValAlaTyr
    ------------------------------------------------------------

201 AlaLysAlaValLeuSerAlaIleGlyAsnProSerLeuArgAlaValIleAspThrSer
    ------------------------------------------------------------

221 ArgAsnGlyAsnGlyProAlaGlyAsnGluTrpCysAspProSerGlyArgAlaIleGly
    ---------------------------------232------------------------

241 ThrProSerThrThrAsnThrGlyAspProMetIleAspAlaPheLeuTrpIleLysLeu
    ------------------------------>  PEPTIDE 8-----------------

261 ProGlyGluAlaAspGlyCysIleAlaGlyAlaGlyGlnPheValProGlnAlaAlaTyr
    ------------------267-----------------------------------

281 GluMetAlaIleAlaAlaGlyGlyThrAsnProAsnProAsnProAsnProThrProThr
    ---->  PEPTIDE 9-------------------------------------------

301 ProThrProThrProThrProProProGlySerSerGlyAlaCysThrAlaThrTyrThr
    ----------------------------------------315--------------

321 IleAlaAsnGluTrpAsnAspGlyPheGlnAlaThrValThrValThrAlaAsnGlnAsn
    ------------------------------------------------------------

341 IleThrGlyTrpThrValThrTrpThrPheThrAspGlyGlnThrIleThrAsnAlaTrp
    ------------------------------------------------------------

361 AsnAlaAspValSerThrSerGlySerSerValThrAlaArgAsnValGlyHisAsnGly
    ------------------------------------------------------------

381 ThrLeuSerGlnGlyAlaSerThrGluPheGlyPheValGlySerLysGlyAsnSerAsn
    ------------------------------------------------------------

401 SerValProThrLeuThrCysAlaAlaSer*
    ------------------407----------
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FIGURE 4: Amino acid sequences of E2 as predicted by the DNA sequence (Lao *et al.*, 1991). The N-terminal sequence of native E2 is underlined and is preceded by a 46 amino acid long signal peptide. Cysteine residues are numbered starting from the native N-terminal residue. The eight methionine residues are underlined, and the nine E2 CNBr peptides are shown.

addition of DTT or reduction and carboxymethylation. Differential reduction and carboxymethylation of $E2_{cd}$ showed that both disulfides in this truncated protein are exposed (both disulfides could be reduced and modified with iodoacetamide without denaturing the protein).

Temperature Stability of Reduced and Nonreduced E2. E2 was incubated with and without DTT for 30 and 60 min at temperatures from 25 to 100 °C. Addition of DTT to native E2 only reduces the accessible disulfides, and Figure 3 shows the effect of reduction of the thermostability of E2. The specific activity of E2 –DTT remains between 45 and 55 units/mg from 25 to 70 °C, peaking slightly at 60 °C. The activity decreases slightly at 80 °C. Even after 60 min at 100 °C, the enzyme still retains more than half of its original activity. When E2 was incubated at these same temperatures

Table I: Predicted Amino Acid Composition of E2 CNBr Peptides 3 and 4 and the Amino Acid Composition of the RP-HPLC Fraction of Disulfide-Containing Peak 1 from the –DTT Peptide Map (See Figure 5A)

	amino acid composition (mol %)	
	peptide 3+4	disulfide peak 1
Asx	8.8	8.4
Glx	4.4	5.7
Ser	13.2	12.9
Gly	8.8	12.0
His	2.9	1.9
Arg	4.4	4.7
Thr	0	2.7
Ala	14.7	14.1
Pro	7.4	7.8
Tyr	4.4	4.1
Val	4.4	4.1
Met[a]	2.9	0
Cys	2.9	0.4
Ile	8.8	7.5
Leu	5.9	6.2
Phe	1.5	1.7
Lys	2.9	2.5
Trp	1.5	
Hser[a]		1.4

[a] Tryptophan is destroyed in the analysis, and methionine is converted to homoserine by CNBr cleavage.

with DTT present, the specific activity decreased dramatically from 50 to 70 °C, and there was complete loss of activity after incubation at 80 and 100 °C. The specific activity after 60-min incubation at 60 °C of E2 +DTT was only 5 units/mg as compared to 55 units/mg for E2–DTT. Thus, the accessible disulfides play an important role in the temperature stability of this enzyme.

CNBr Cleavage-Preserving Disulfides. In order to determine the location of the disulfides in E2, the protein was cleaved with CNBr under conditions which left the disulfide bonds intact (Villa, 1989). This involves using 6 M guanidine hydrochloride rather than formic acid as the denaturant. The molecular weights ($\times 10^{-3}$) of the peptides expected from CNBr cleavage of E2 under conditions where the disulfide bonds are reduced are 1.1, 4.7, 6.4, 0.4, 1.1, 3.1, 8.3, 3.1, and 11.3 from the N-terminus (Figure 4). These peptides are designated 1–9. The six cysteines in the enzyme are located on five separate CNBr peptides: 3, 4, 7, 8, and 9.

Identifying Disulfide-Linked Peptides. The peptides obtained from CNBr cleavage were separated by RP-HPLC using a C8 column. The peptide maps shown in Figure 5 were obtained using a gradient of 5–60% solvent B over a period of 50 min at a flow rate of 1 mL/min. Two disulfide-containing peaks (peaks 1 and 2) were identified using the HPLC system equipped with a disulfide analyzer. These were the only peaks that gave absorbance at 412 nm after reaction with NTSB. Because of the large size and hydrophilic character of peptide 9, it probably did not elute from the column or was never dissolved and was removed by filtration.

Position of the Disulfide Bridges in E2. The two peaks containing disulfide bonds were collected and electrophoresed on a 17% SDS gel with and without DTT. Peak 1 contained a peptide with an apparent molecular weight (MW) of 6000–7000 when DTT was absent, and the MW decreased slightly when DTT was present. The only pair of cysteine-containing peptides that would give this MW are 3 (6400) and 4 (400). Peak 2 contained a peptide that migrated at approximatley 12 kDa (–DTT), and that gave a band of ~9 kDa and one at the front near the 3-kDa marker upon addition of DTT. These results indicated that disulfide peak 2 contained CNBr peptide 7 (MW 8300) joined to peptide 8 (MW 3100). No other combinations of the four cysteine-containing peptides could yield these results.

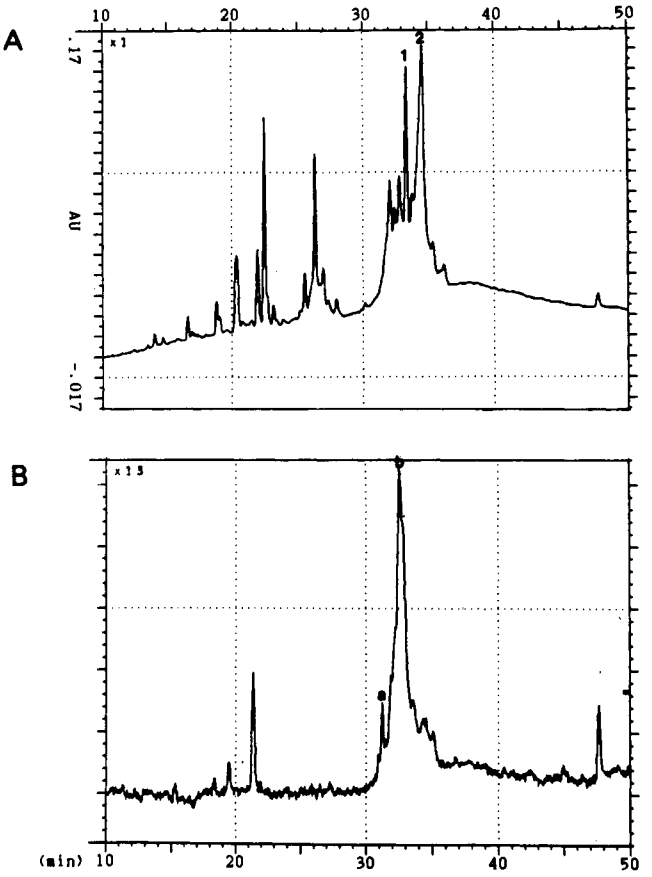

FIGURE 5: RP-HPLC CNBr peptide maps of E2. The absorbance at 210 nm was monitored over the 50-min gradient. (Panel A) Peptide map of E2 CNBr fragments obtained under nonreducing conditions. Peaks labeled 1 and 2 are the disulfide-containing peptides as determined by using the HPLC system connected to the disulfide analyzer. (Panel B) HPLC trace of disulfide-containing peak 2 which was collected and reduced with DTT. The unlabeled peaks in panel B are artifacts.

Table II: Predicted Amino Acid Composition of CNBr Peptides 7 and 8 Compared to the Amino Acid Composition of Peaks a and b (See Figure 5B) Obtained from RP-HPLC of Peak 2 from the –DTT Peptide Map after Reduction with DTT

	amino acid composition (mol %)			
	peptide 8	peak a	peptide 7	peak b
Asx	6.5	2.7	14.5	8.0
Glx	12.9	7.1	6.0	4.5
Ser	0	3.4	10.8	7.9
Gly	12.9	20.5	9.6	12.9
His	0	0	1.2	1.0
Arg	0	0	4.8	4.1
Thr	0	0	9.6	6.3
Ala	19.3	14.5	15.7	15.1
Pro	6.5	6.0	6.0	7.0
Tyr	3.2		2.4	10.7[b]
Val	3.2	2.9	3.6	3.2
Met[a]	3.2	0	1.2	0
Cys	3.2	0.0	1.2	0
Ile	9.7	7.6	6.0	8.2
Leu	6.5	4.8	2.4	7.4
Phe	6.5	4.8	1.2	1.1
Lys	3.2	1.9	1.2	1.5
Trp	3.2		3.6	
Hser[a]		0		0

[a] Tryptophan is destroyed in analysis, and methionine is converted to homoserine by CNBr cleavage. [b] Tyrosine was high in the blank.

Amino acid analysis of peak 1 confirmed that this peak contained peptides 3 and 4 (Table I). The amino acid

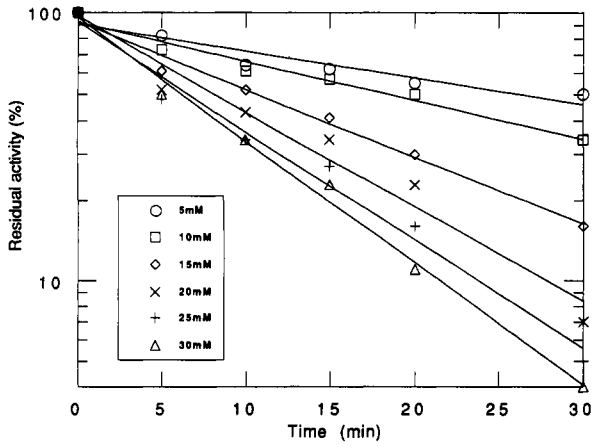

FIGURE 6: Inactivation of E2 with EDC. The log of percent residual activity is plotted as a function of time at various concentrations of EDC. Final EDC concentrations are indicated in the inset.

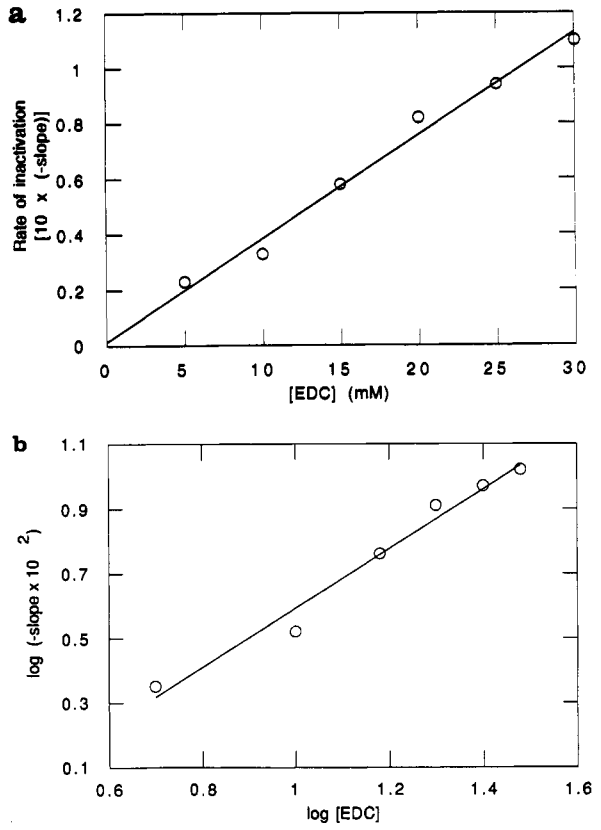

FIGURE 7: Kinetic analysis of E2 inactivation by EDC. (a) Rate of inactivation versus EDC concentration. The rate of inactivation at each concentration was derived from the slopes of the data in Figure 6. (b) The order of the reaction with respect to carbodiimide concentration is determined by plotting the data of Figure panel a in logarithmic form.

composition of peak 1 does not match the amino acid composition of any other combination of Cys-containing peptides. Peak 2 was collected, dried, reduced (as described under Materials and Methods), and rechromatographed under the same running conditions (Figure 5, panel B). As expected, the retention time of the new peaks is different from the retention time of peak 2 since this large disulfide-linked peptide was reduced to give two smaller peptides. The two peaks obtained from this run (peaks a and b) were collected, and their amino acid compositions were determined (Table II). Peak a corresponds to peptide 8 and peak b to peptide 7. The amino acid composition of these peaks showed the best fit with these cysteine-containing peptides. This confirms that disulfide-containing peak 2 consisted of peptides 7 and 8 in

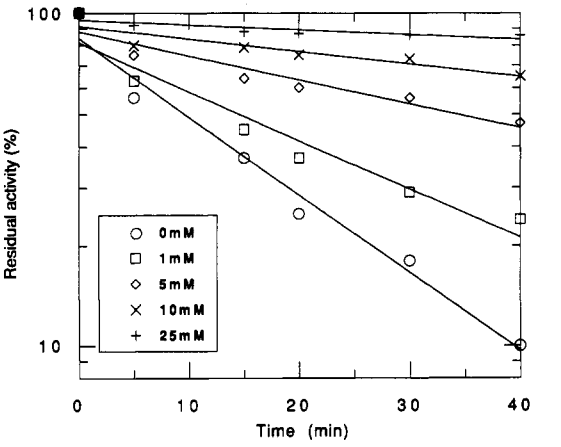

FIGURE 8: Protection of E2 from EDC inactivation by cellobiose. The data were plotted as in Figure 6. Concentrations of cellobiose are identified in the inset. The concentration of EDC was constant at 25 mM.

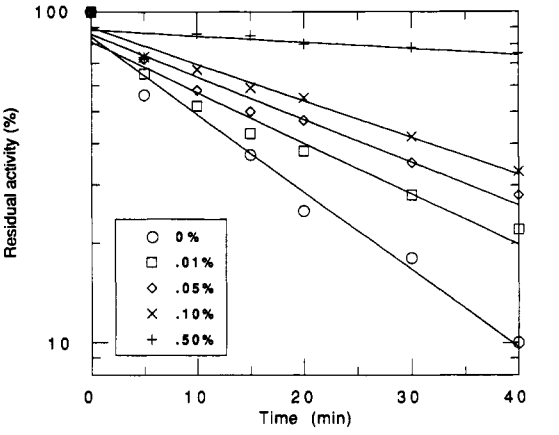

FIGURE 9: Protection of E2 from EDC inactivation by (hydroxyethyl)-cellulose. The data obtained were plotted as in Figure 6. Concentrations of HEC are shown in the inset, while the concentration of EDC was 25 mM.

disulfide linkage. Therefore, one disulfide bond joins Cys80 to Cys125, another joins Cys232 to Cys267, and the third must join Cys315 to Cys407.

Inactivation of E2 Activity with EDC. Semilogarithmic plots of residual activity as a function of time of inactivation of E2 at various concentrations of EDC are linear (Figure 6). This indicates that the inactivation process follows pseudo-first-order kinetics. The rate of inactivation, determined from the slopes of plots of residual activity vs EDC concentration, showed that the inactivation was first order with respect to carbodiimide concentration (Figure 7a). Analysis by the method of Levy *et al.* (1963) yielded a slope of 1 (Figure 7b). This indicated that 1 mol of EDC reacts with 1 mol of E2 during inactivation.

Substrate Protection against Inactivation of E2 by EDC. Cellobiose acts as competitive inhibitor and inhibits cellulases through feedback inhibition while (hydroxyethyl)cellulose is a soluble substrate. Rapid EDC inactivation was prevented if cellobiose or (hydroxyethyl)cellulose was included in the reaction mixtures. The semilogarithmic plots obtained from the percent residual activity as a function of time with cellobiose or (hydroxyethyl)cellulose present during reaction of E2 with EDC are shown in Figures 8 and 9. Reaction of E2 with 25 mM EDC in the absence of cellobiose results in a rapid decrease in CMC activity over time, whereas with cellobiose (1–25 mM) in the reaction mix, the rate of inactivation decreases with increasing cellobiose concentration (Figure 8); 1 mM cellobiose gave little protection while 25 mM gave strong

protection. (Hydroxyethyl)cellulose also protected from inactivation (Figure 9). As the concentration of HEC in the reaction mixtures increased from 0.01 to 0.5%, the rate of inactivation decreased.

The data obtained for carbodiimide inactivation and substrate protection for $E2_{cd}$ yielded the same results found with E2. The semilogarithmic plots of percent residual activity vs time at various concentrations of inhibitor were also linear. Kinetic analysis using the methods employed for E2 showed that 1 mol of EDC reacts with 1 mol of $E2_{cd}$. Addition of either cellobiose or HEC to reaction mixtures containing $E2_{cd}$ and 25 mM EDC showed a decrease in the rate of inactivation that was identical to that seen with E2. The semilogarithmic plots obtained from these data showed that as the concentration of cellobiose or HEC increased, the rate of inactivation of $E2_{cd}$ by EDC decreased.

DISCUSSION

The easiest way to determine whether a protein contains disulfide bonds is to compare its electrophoretic mobility in polyacrylamide gels with and without reduction (Creighton, 1984). Nonreduced E2 has a greater mobility than reduced E2; therefore, at least one disulfide was present. The differential reduction and carboxymethylation experiments show that all the cysteine residues form disulfide bonds. One of these disulfides joins Cys80 to Cys125, another joins Cys232 to Cys267, and the third joins Cys215 to Cys407. Two of these disulfides appear to be accessible, since addition of DTT without prior denaturation of $E2_{cd}$ reduced both disulfides, while the other disulfide present only in E2 is inaccessible. The thermostability of E2 decreases drastically upon reduction of the accessible disulfides. Furthermore, reduction of the disulfides and/or modification of the resulting cystines also affects activity. Therefore, it appears that these two disulfides stabilize the catalytic domain. Since disulfide bonds are known to serve a stabilizing role in extracellular proteins, they have been engineered into proteins in order to increase their thermostability (Wetzel *et al.*, 1988; Matsumura *et al.*, 1989).

On the basis of a computer analysis of the amino acid sequences of several cellulases from various organisms, E2 belong to cellulase family B which includes cellobiohydrolase II (CBH II) from *Trichoderma reesei* (Lao *et al.*, 1991; Wilson, 1992; Henrissat *et al.*, 1989). The three-dimensional structure recently reported for this enzyme shows four cysteines in the catalytic domain that participate in two disulfide bonds (Rouvinen *et al.*, 1990). Sequence alignments among members of the family B indicate that these disulfides are expected to be conserved throughout the family (Rouvinen *et al.*, 1990). The positions of the two disulfides in $E2_{cd}$ align with those of CBH II.

Since there are no free sulfhydryl groups present, the inhibition of activity by Hg^{2+} is not due to reaction with an active-site cysteine. In addition, modification of histidine residues in E2 resulted in no loss of enzymatic activity. The data presented here support the involvement of carboxyl groups in catalysis. The simplest interpretation is that inactivation of E2 and $E2_{cd}$ by EDC is due to modification of at least one essential carboxyl group in the catalytic domain. Furthermore, since the presence of cellobiose or (hydroxyethyl)cellulose prevents the carbodiimide from reacting with and inactivating E2, it appears that these molecules are blocking the active site and protecting functionally important carboxyl groups. Evidence for carboxyl group participation in catalysis has been reported for several other cellulases (Hurst *et al.*, 1977; Clarke & Yaguchi, 1985; Baird *et al.*, 1990; Rouvinen *et al.*, 1990). Tomme and Claeyssens (1989) determined that carboxyl groups were involved in catalysis in both cellobiohydrolase I (CBH I) and endoglucanase I (EG I) of *T. reesei* by chemical modification. Subsequent site-directed mutagenesis of conserved glutamic acid residues showed that substitution of Glu127 with glutamine resulted in complete loss of EG I activity (Mitsuichi *et al.*, 1990).

ACKNOWLEDGMENT

We thank Ted Thannhauser for his help in the peptide separation and disulfide assays.

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