Identification of a Histidyl Residue in the Active Center of Endoglucanase D from Clostridium thermocellum*

(Received for publication, February 14, 1991)

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Diethylpyrocarbonate modification of endoglucanase D from Clostridium thermocellum, cloned in Escherichia coli, resulted in a rapid but partial (maximally 70–80%) loss of activity. The second-order rate constant of inactivation proved to be exceptionally high (3210 m⁻¹ min⁻¹). A 3-fold reduction of the kcat and a 2-fold increase of the Km for 2'-chloro-4'-nitrophenyl β-cellubioside were observed.

Spectrophotometric analysis indicate the presence of one rapidly (k = 0.45 min⁻¹) and two slower (k = 0.23 min⁻¹) reacting histidyl residues. In the presence of 50 mM methyl β-cellulotrioside, the rate of inactivation was reduced 16-fold, and the kinetics of modification were compatible with the protection of 1 histidyl residue.

Since peptide analysis was inconclusive, identification of each of the 12 histidyl residues present in the endoglucanase D sequence was mutated into either Ala or Ser. Seven of the mutant enzymes had specific activities lower than 50% of the wild-type. Only in the case of the Ser-516 mutant, however, was the residual activity not affected by diethyl pyrocarbonate. These findings suggest an important functional or structural role for His-516 in the wild-type enzyme.

More than 50 genes from various organisms (fungi, bacteria, and plants) involved in cellulose or hemicellulose hydrolysis have been cloned and sequenced (Béguin, 1990). This has provided a wealth of information on the encoded endoglucanases (EC 3.2.1.4) and cellobiohydrolases (EC 3.2.1.91). Hydrophobic cluster analysis has indicated that these enzymes can be classified into six distinct structural families (Henrissat et al., 1989). As illustrated in the same study, alignments point to a limited number of conserved residues, likely to play an important role either in catalysis or in maintaining the enzymes’ structural integrity. Only occasionally have these residues been identified by chemical modification, site-directed mutagenesis, or x-ray diffraction studies. Chemical modification and spectrophotometric analysis have revealed critical tryptophan and carboxylic acid residues in Schizophyllum commune endoglucanase I (Clarke and Yaguchi, 1986, 1986; Clarke, 1987). Similarly, in cellobiohydrolase I from Trichoderma reesei an essential glutamic acid residue has been identified (Tomme and Claeyssens, 1989). For the core protein of cellobiohydrolase II, from the same organism, the three-dimensional structure has been determined recently by x-ray diffraction studies and catalytically important amino acid residues localized, using data collected with a ligand diffused into the crystal (Rouvinen et al., 1990). Essential glutamic acid residues in endoglucanases from various Bacillus sp. were studied recently by site-directed mutagenesis (Baird et al., 1990).

Clostridium thermocellum, a Gram-positive, thermophilic bacterium, produces a very active cellulase complex, termed “cellulosome” (Lamed et al., 1983). In this complex, 14–18 different components, many endowed with endoglucanase activity, have been identified. All other enzymes are associated to the corresponding cellulase families defined by Henrissat et al. (1989). Because it is easily purified from a hyperproducing clone and readily crystallizes (Jollif et al., 1986b and 1986c), endoglucanase D has been chosen for structural and functional studies. No information concerning critical or essential residues is available, neither for this nor for any other enzyme of the corresponding cellulase family E (Henrissat et al., 1989). We present evidence, based on chemical modification and site-directed mutagenesis research, that a histidyl residue plays an important role.

MATERIALS AND METHODS

Materials—Diethyl pyrocarbonate, (EtOCO)2O, tetranitromethane, 5,5'-dithiobis(2-nitrobenzoic acid), N-ethylmaleimide, and p-chloromercuribenzoate were purchased from Aldrich. Hydroxylamine hydrochloride was from Serva (Heidelberg, Germany). 125I-Labeled protein A was from Amersham (UK), and subtilisin was from Boehringer Mannheim. 2'-Chloro-4'-nitrophenyl β-cellubioside (CNPC) and methyl β-cellulotrioside were synthesized as described (Claeyssens, 1989). All other reagents and chemicals were analytical grade.

Enzyme Purification—The soluble cytoplasmic fraction from E. coli TG1(pCT603) cultures was processed according to Jollif et al. (1986b). The supernatant (containing 20–50 mg of protein), obtained after the heat-treatment step, was applied on an affinity column (1 × 10 cm), carrying p-aminoenzyl 1-thio-β-cellubioside coupled to Sepharose 4B (Tomme et al., 1988), and the bound endoglucanase D was eluted with 10 mM cellobiose. The combined fractions were dialyzed against double-distilled water, concentrated to approx.

1 The abbreviations used are: CNPC, 2'-chloro-4'-nitrophenyl β-cellubioside; PC buffer, 50 mM sodium phosphate/citric acid buffer, pH 6.4; SDS, sodium dodecyl sulfate; kb, kilobase(s).
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mately 10 mg/ml by ultrafiltration (PM-10 membrane, Amicon) and stored at -20 °C.

The homogeneity of the preparation was checked by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and isoelectric focusing (van Tilbeurgh and Claeyssens, 1985). The enzyme's absorption coefficient (ε = 3200 M⁻¹·cm⁻¹) was calculated (Boehringer, 1970; van Tilbeurgh and Claeyssens, 1985) from its Tryp and Tyr content (Jollif et al., 1969a).

Enzyme Assay—Endoglucanase D activity was measured, at the temperatures specified, in PC buffer using 2 mM CNPC as substrate (Claeyssens, 1989). The release of 2-chloro-4-nitrophenol was monitored continuously at 405 nm (ε = 1.6 × 10⁵ M⁻¹·cm⁻¹). Enzyme Activity—Endoglucanase D (3 µM in PC buffer), incubated at 23 °C in the absence or presence of a competitive ligand (50 mM methyl β-cellobiose), was treated with freshly prepared (EtOCO)₂O solution (0.08-1.2 mM) made up in ice-cold ethanol. The concentration of the histidine reagent in the commercial stock solution was determined spectrophotometrically at 242 nm (ε = 2500 M⁻¹·cm⁻¹) (Ovadi et al., 1967). The final ethanol concentration did not exceed 2% (v/v) and did affect neither the activity nor the stability of the enzyme. From the enzyme-modifier mixtures 5-µl samples were quenched at various time intervals in 400 µl of ice-cold PC buffer containing 1.5 mM N-acytylemimazole. Residual activity, expressed as percentage of that of unmodified enzyme, was then measured after addition of 400 µl of 2 mM CNPC as described above.

N-Ethoxycarbonylation of histidyl residues was reversed by incubating the modified enzyme at 37 °C with neutral hydroxyamine (final concentration, 50 mM) (Burnstein et al., 1974).

Modification of Amino-acids—Endoglucanase D (30-40 µM) in PC buffer were treated for 10 min with 2 mM (EtOCO)₂O (23 °C), in the presence or absence of a ligand, and inactivation was followed as described above. After adding 90 µl of 0.1 M Tris/HCl buffer, pH 8.0, containing 5 µM guanidine HCl and 3.5 µM of subtilisin to the modified protein samples, the mixtures were incubated for another 9 min at 33 °C. The resulting peptides (-150 µg) were analyzed by reversed-phase liquid chromatography (Waters chromatographic system) on a C4-Vydac 214 TP-54 column (0.46 x 25 cm) using a linear gradient of 0-70% acetonitrile in 0.05% trifluoroacetic acid at a flow rate of 1.5 ml/min and monitoring at 214 and 247 nm with a diode array detector (220 nm band-pass filter). By comparing the 242 nm tracings of proteolytes of untreated samples with those where modification was reversed (2 µM hydroxyamine, 2 min, 33 °C), peptide fractions containing N-ethoxyacarbonylated His were identified.

Amino-terminal sequencing (10 cycles) of isolated peptides was performed according to the procedure described by Hewick et al. (1981) (Dr. Strosberg, Institut Pasteur, Paris).

Site-directed Mutagenesis—Mutagenic oligonucleotides were prepared using a Milligen/Biosearch DNA synthesizer. His codons were mutated to Ala or Ser, depending on the likelihood of second-site hybridization as determined by computer search. The mismatched nucleotides were flanked on each side by 6-10 bases (total length, 15-20mers). Mutants of His-492, and His-516 were constructed according to the phosphorothioate strategy of Taylor et al. (1985), using the mutagenesis kit of Amersham (UK). The pCTH600 template was obtained by recloning, at the HincII site of M13mp8 (Messing and Vieira, 1982), a 1.7-kb HincII fragment which encodes the catalytic domain of endoglucanase D (Chauvaux et al., 1990). The coding sequence was obtained according to the strategy of Kunkel et al. (1987); i.e. the mutagen was selected by transformation of a mut5 strain. A mutagenesis kit was used according to the instructions of the supplier (Bio-Rad). Since the C2J236 dut 'ung' host does not carry the amber suppressor required for growth of M13mp8, the 1.7-kb fragment containing the catalytic site of endoglucanase D was recloned in M13mp19 (Yanisch-Perron et al., 1985). The coding sequence of the gene was fused in frame with lacZ at the level of the HindIII site of the polylinker.

Mutations were identified by sequencing (Sanger et al., 1977) and, for all mutants showing less than 50% of the wild-type activity, the entire, 1.7-kb fragment was checked for other mutations.

Preparation of Crude Extracts from Clones Expressing Mutant Proteins—3 ml of a 100-fold diluted overnight culture of E. coli TG1 (Wain-Hobson et al., 1985) (absorbance at 600 nm, -0.05) in 2XLB medium (2% tryptone, 1% yeast extract, 1% NaCl) was infected with a fresh plaque of recombinant phage. After incubating the culture with vigorous aeration for 5 h at 37 °C, the cells were centrifuged and the supernatant was kept as phage stock. 500 µl of the latter were used to infect 50 ml of a 100-fold diluted overnight culture of E. coli TG1 in 2XLB medium. After 4 h of growth under aeration at 37 °C and centrifugation, cells were harvested, resuspended in 10 ml of PC buffer, and disrupted by sonication. The extracts were cleared by centrifugation (5 min; 3000 × g) and kept in aliquots at -20 °C.

Immunoblotting Assay of Endoglucanase D—Enzyme concentrations in crude extracts of strains expressing mutant genes were estimated from Western blots. 40 µg of protein from each crude extract, containing 20-120 ng of antigen, was loaded on a 10% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose (Towbin et al., 1979), and the blot was probed with anti-endoglucanase D antisera preadsorbed with crude extract of E. coli TG1(pUC6) (GrBpinet et al., 1988), followed by 125I-labeled protein A. Bands located by autoradiography were cut out, weighed, and counted by γ-ray scintillation. Values were corrected for nonspecifically bound radioactivity by subtracting the number of counts per mg of nitrocellulose detected in a weighed sample from a blank area of the blot. The actual amount of endoglucanase D antigen was estimated from a standard curve obtained with purified protein.

Modification of Mutant Proteins with (EtOCO)₂O—Protein concentration in the crude extracts (measured according to Siedmak and Grossberg, 1977, using bovine serum albumin as a standard) was adjusted with PC buffer to 1.56 mg/ml, and (EtOCO)₂O was added to a final concentration of 5.5 mM. After incubating at room temperature for 0-8 min, 100-µl samples were withdrawn and quenched by diluting into 400 µl of PC buffer containing 2.5 mM N-acytylemimazole. The activity was determined as described above.

RESULTS

Inactivation Kinetics and Specificity of Modification—Endoglucanase D (3-4 µM) was rapidly inactivated by excess (EtOCO)₂O (0.08-1.2 mM) and pseudo-first-order kinetics applied for the early part of the inactivation curves (Fig. 1A). However, even in the presence of high initial modifier concentrations and after prolonged incubations, the N-ethoxyacyrbonylated enzyme retained 20-30% of its activity. An intermediate enzyme (EtOCO)₂O−protein was probably not formed (Fig. 1B), since the pseudo-first-order rate constants (kobs) depended linearly on the modifier concentration. The second-order rate constant for inactivation (3210 min⁻¹·M⁻¹) exceeded most published values (e.g. Lundblad and Noyes, 1984; Van Grysperre et al., 1988).

Treatment of modified endoglucanase D with 50 mM hydroxyamine resulted in complete reactivation of the enzyme within 20 min (Fig. 2B). Since this reagent is known to reverse N-ethoxyacyrbonylation of modified Tyr and His residues only (Burnstein et al., 1974), reactivation excludes the possible involvement of Cys or Lys residues (Melchior and Fahrney, 1970; Miles, 1977). Incubation of endoglucanase D with tetranitromethane, in 5- or 10-fold molar excess over the total amount of Tyr, did not affect enzymatic activity. Furthermore, under native conditions and using several specific reagents (mercury compounds, dithio derivatives, or maleimides), no inactivation due to the modification of thiol groups was observed.²

² P. Tomme, S. Chauvaux, P. Béguin, J. Millet, J.-P. Aubert, and M. Claeyssens, unpublished data.
Specific reaction was confirmed by difference spectra analysis of native and modified endoglucanase D (Fig. 3). The absorbance maximum at 240-243 nm, characteristic for N-ethoxycarbonylated His, disappeared upon hydroxylamine treatment (Ovadi et al., 1967). A discrete minimum apparent at 290 nm could be due to the perturbation of a Trp or Tyr residue in the proximity of a modified His (De Boeck et al., 1984).

Number of Modified His Residues and Protection by Ligands—Spectrophotometric analysis allowed quantitation of the modification reaction (Ovadi et al., 1967; Shina and Brewer, 1985). Under the conditions used (Fig. 2A), endoglucanase D, incubated in the absence of inhibitor, was converted to a form with 30% residual activity. The pseudo-first-order constant of inactivation was calculated as 0.45 min⁻¹. During the first 10 min, the kinetics of modification paralleled inactivation (Fig. 2A) and were compatible with the reaction of 3 residues. Slow reaction of a fourth residue became apparent after longer incubation times (>30 min) but caused no further activity loss. Assuming that the modification of the first residue occurred at the initial inactivation rate, 0.45 min⁻¹, curve-fitting applied to all experimental data (Fig. 2A) yielded an average rate constant of 0.23 min⁻¹ for the other 2 His residues.

In the presence of methyl β-cellotrioside, inactivation was 16-fold reduced (k = 0.028 min⁻¹). The degree of modification was compatible with the protection of 1 His residue (Fig. 2A). Similarly as above, data could be fitted assuming 1 His residue to react with a pseudo-first-order rate constant of 0.028 min⁻¹ and the other 2 residues at 0.17 min⁻¹.

Catalytic Properties of Modified Endoglucanase D—The catalytic parameters for CNPC hydrolysis determined for modified endoglucanase D are reported in Table I. Compared to the values for intact enzyme, Kₘ was increased 2-fold and kₐct was three times lower. Since there is only partial activity loss and changes appear in both parameters, this suggests the presence of homogeneously modified endoglucanase D with reduced catalytic efficiency. The effect of the modification on the activation energy (ΔG = 4.6 kJ/mol) could be typical for the loss of an uncharged hydrogen bond in the transition state complex in the modified enzyme (Fersht, 1987).

Identification of Modified His Residues by Peptide Mapping—Proteolysates of endoglucanase D, modified in the presence of methyl β-cellotrioside, were analyzed by reversed-phase liquid chromatography (Tomme and Claeyssens, 1989). Since comparison of the chromatograms did not lead to the detection of specifically labeled fractions, differential peptide mapping was attempted using proteolysates before and after hydroxylamine treatment (Fig. 4, A and B). This led to the identification of three peptides in accordance with the number of modified His residues (see above). The partial sequences determined were compatible with modification of residue His-286 in peptide I (ThrNFGGFIMPEN(EHD)²⁸⁷) and of His-65 in II (⁶⁶NAALDAISHV⁹⁶⁹). Peptide III was probably blocked as it...
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**Fig. 3.** Protein difference spectra for modification of endoglucanase D with (EtOCO)₂O. Endoglucanase D (3-4 μM) was treated with (EtOCO)₂O (0.22 mM) in 50 mM PC buffer, pH 6.4 (25 °C), and difference spectra were recorded with a double-beam spectrophotometer at several time intervals: 0 (0), 7 (1), 13 (2), 18 (3), and 30 (4) min.

**Fig. 4.** Reversed-phase liquid chromatography of modified endoglucanase D proteolysates before (A) and after (B) treatment with hydroxylamine. Endoglucanase D was modified and processed as described under "Materials and Methods." Peptides I, II, and III were collected for further amino acid sequence analysis.

**TABLE I**

| Enzyme   | Residual activity (%) | Kₓ (μM) | kₐ (10⁴ s⁻¹) | kₓ/kₐ (M⁻¹ s⁻¹) | ΔG* (kJ/mol) |
|----------|-----------------------|---------|---------------|-----------------|--------------|
| Intact   | 100                   | 98      | 7.9           | 8.0             | -28.0        |
| Modified | 25                    | 204     | 2.5           | 1.2             | -23.4        |

*ΔG* = −RT ln (kₓ/kₐ).

**DISCUSSION**

All the evidence presented above point to the presence of a His residue in the catalytic center of endoglucanase D from *C. thermocellum*. His-specific modification of endoglucanase D with (EtOCO)₂O resulted in 70-80% loss of activity. Inactivation of the enzyme was prevented in the presence of methyl β-cellotrioside, suggestive for modification of a critical histidine within the active center. The identification of His-516 as the critical residue was based on site-directed mutagenesis studies. The specific activity of the His-516 → Ser mutants Ala-197 and Ser-516 were thermolabile, but at 30 °C both enzymes were stable and their activity could be compared with that of the wild-type.

Since the identity of the (EtOCO)₂O-sensitive histidyl residue in the native protein could not unequivocally be established by these activity assays, the inactivation of the various mutated enzymes by the same reagent was investigated. Indeed, the enzyme mutated at the critical target should be resistant to the reagent. Contrary to the wild-type, and to all other His mutants (data not shown), only the activity of the enzyme bearing the His-516 → Ser mutation was not affected by (EtOCO)₂O (Fig. 5).

The results of the mutagenesis experiments are gathered in Table II. Mutations of His-65, His-167, His-170, His-187, His-269 did not result in significant loss of activity relative to isogenic wild-type constructions. However, the residual activity of proteins mutated in His-174, His-197, His-222, His-286, His-445, His-492, and His-516 ranged from 2 to 54%.

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The exact role of His-516 remains to be defined. It does not seem absolutely essential for catalysis, since the mutant is still enzymatically active and changes in kinetic parameters for a substrate such as CNPC are small. It could tentatively be suggested that in the wild-type enzyme this histidyl residue is implicated in the formation of a salt bridge or hydrogen bond to the substrate transition state (Fersht, 1987). Alternatively, the lower thermostability of the Ser-516 mutant could point to a role in the maintenance of an active enzyme conformation. The presence of critical carboxylic acid groups implicated in the formation of a salt bridge or hydrogen bond to the substrate transition state (Fersht, 1987).
Acknowledgments—P. T. is indebted to the Belgian Institut voor Wetenschappelyk Onderzoek in Nijverheid en Landbouw and the Commission of European Communities for grants. M. C. thanks the Belgian National Fonds voor Wetenschappelyk Onderzoek for financial support.

Note Added in Proof—X-ray diffraction analysis of endoglucanase D co-crystallized with o-iodobenzyl-1-thio-β-cellobioside confirmed the presence of His-516 within the enzyme’s active center.

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