Identification of Glu-330 as the Catalytic Nucleophile of Candida albicans Exo-β-(1,3)-glucanase*

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The exo-β-(1,3)-glucanase from Candida albicans hydrolyzes cell wall β-glucans via a double-displacement mechanism involving a glycosyl enzyme intermediate. Reaction of the enzyme with 2',4'-dinitrophenyl-2-deoxy-2-fluoro-β-D-glucopyranoside resulted in the time-dependent inactivation of this enzyme via the accumulation of a 2-deoxy-2-fluoro-glycosyl-enzyme intermediate as monitored also by electrospray mass spectrometry. The catalytic competence of this intermediate is demonstrated by its reaction through hydrolysis ($k_{\text{react}} = 0.0019 \text{ min}^{-1}$) and by transglycosylation to benzyl thio-β-D-glucopyranoside ($k_{\text{react}} = 0.024 \text{ min}^{-1}$; $K_{\text{react}} = 56 \text{ mM}$). Peptic digestion of the labeled enzyme followed by tandem mass spectrometric analysis in the neutral loss mode allowed detection of two glycosylated active site peptides, the sequences of which were identified as NVAGEW and NVAGEWSAA. A crucial role for Glu-330 as the catalytic nucleophile is confirmed by site-directed mutagenesis at this site and kinetic analysis of the resultant mutant. The activity of the Glu-330-Cys mutant is reduced over 50,000-fold compared to the wild type enzyme. The glutamic acid, identified in the exoglucanase as Glu-330, is completely conserved in this family of enzymes and is hereby identified as the catalytic nucleophile.

The human pathogenic fungus Candida albicans secretes to the cell wall an exo-β-(1,3)-glucanase (Exg), which is a member of family 5 glycosidases (1). A number of these enzymes, including Exg, are also classified as family A glycans, a group that comprises mainly cellulases (β-1,4-endoglucanases) (2). This family of glycosyl hydrolases cleaves glycosidic bonds with net retention of configuration, the enzymes functioning via a proposed double-displacement mechanism, involving a covalent glycosyl-enzyme intermediate (Scheme 1) (3, 4).

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1 The abbreviations used are: Exg, Candida albicans exo-β-1,3-glucanase; BSA, bovine serum albumin; BTGlc, benzyl thio-β-D-glucopyranoside; 2ClDNPGlc, 2',4'-dinitrophenyl-2-deoxy-2-chloro-β-D-glucopyranoside; DNPGlc, 2,4-dinitrophenyl-β-D-glucopyranoside; 2FDNPGlc, 2',4'-dinitrophenyl-2-deoxy-2-fluoro-β-D-glucopyranoside; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; ESM, electrospray mass spectrometry.

The physiological role of Exg has not been clearly established, but it is undoubtedly involved in metabolism of cell wall β-1,3-glucan. It catalyzes the exolytic hydrolysis of polymers such as laminarin (β-1,3 linkages) and to a lesser extent pustulan (β-1,6 linkages) (5). Recently it has been shown that deletion of Saccharomyces cerevisiae EXG1, which encodes the major exoglucanase secreted by the yeast resulted in a marked resistance to killer toxin (6). This indicates that Exg1 is involved in the metabolism of β-1,6-linked glucan, the cell wall receptor for the toxin. In addition to catalyzing hydrolysis reactions, Exg of C. albicans also catalyzes the rapid transfer of glucosyl residues between suitable donor and acceptor molecules (7).

EXG, the gene for the C. albicans, encodes a protein consisting of a pre-pro sequence of 38 residues and a mature enzyme of 400 residues (8). It has 58% identity at the deduced amino acid sequence level with EXG1 of S. cerevisiae. A homology comparison of the deduced amino acid sequence of Exg with those of other related glycosidases reveals two absolutely conserved acidic residues, Glu-230 and Glu-330. Since the generally accepted mechanism involves two carboxyl-containing amino acid residues acting as an acid/base catalyst and a nucleophile (3, 4, 9), it is possible that these residues represent the catalytic pair of Exg. Indeed, in a previous study we showed that the point mutations (E230D and E230Q) decreased the catalytic activity 15,000- and 400-fold, respectively (10). However, an independent identification of the catalytic nucleophile by labeling studies is needed to distinguish the roles of these residues.

The application of 2-deoxy-2-fluoro-glycosides in identifying active site nucleophiles has proven successful in a number of retaining glycosidases and glycanases (11). These compounds function as mechanism-based inactivators, trapping the enzyme by the accumulation of a relatively stable but viable 2-deoxy-2-fluoro-glycosyl-enzyme intermediate. This labeling technique has been made more powerful with the advent of electrospray tandem mass spectrometry, circumventing the need for radiolabels in the identification and isolation of the labeled residue. Analysis of the proteolytic digest of a labeled enzyme by mass spectrometry has allowed for the identification of the catalytic nucleophiles of Bacillus subtilis xylanase (9) and human glucocerebrosidase (12).

In this study we report the use of 2',4'-dinitrophenyl-2-deoxy-2-fluoro-β-D-glucopyranoside to inactivate the exo-(1,3)-β-glucanase and the use of this reagent to identify the catalytic nucleophile. In addition we describe the mutation of this residue and report the kinetic studies that confirm its key role in catalysis.

EXPERIMENTAL PROCEDURES

General Procedures—Buffer chemicals and other reagents were obtained from Sigma unless otherwise noted. The substrate 2,4-dinitro-
inhibitor 2 synthesized following the general procedure described below. The reported data (15, 16). Elemental analysis was as listed below. 4% (v/v) HCl/methanol (15). Crystallization from methanol/diethyl

...fagments were recovered from agarose gels using a Geneclean II kit used for molecular cloning, transformation, and electrophoresis. DNA ...a 1.4-kilobase pair Pst I and HindIII fragment, fractionated on agarose gels and recovered using the Geneclean II kit. The digested products were then ligated into pBluescript KS and cloned into pGB4, pFOX2, and pFOX3, and the fragments were sequenced fully in both directions to confirm the integrity of pGB4 inserted into pGB4.

**PCR Mutagenesis—**Primary PCR was performed in a 20-μl reaction mixture containing 1.5 mM MgCl₂, 50 μM each of dATP, dCTP, dGTP, dTTP, 2.5 units of Taq DNA polymerase, 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 200 μM each of the appropriate mutagenic oligonucleotides, and either pFOX2 or pFOX3 as the template. The T7 and T3 oligonucleotide primers for pBluescript were used as the outside primers in both the primary and secondary PCR reactions. Reaction mixtures were subjected to an initial cycle of 95°C for 60 s, 55°C for 30 s, and 72°C for 90 s. Thirty cycles of PCR were then performed. The PCR cycle was 15 s at 95°C, 30 s at 55°C, and 90 s at 72°C. Following the final cycle, the tubes were held at 72°C for 5 min. The reaction products were then size-fractionated by agarose gel electrophoresis, and desired bands were band-stabbed into secondary reaction mixtures. Products of desired bands were then size-fractionated by agarose gel electrophoresis, and the final cycle, the tubes were held at 72°C for 5 min. The reaction products were then size-fractionated by agarose gel electrophoresis, and desired bands were band-stabbed into secondary reaction mixtures.

**Expression of Exoglucanase in S. cerevisiae—**Wild type fragments of...
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By fitting to Equation 1.

0.6M (NH₄)₂SO₄ in 50 mM sodium phosphate buffer, pH 7.0. The column Superose HR5/5 column (Pharmacia Biotech Inc.) pre-equilibrated with

inactivator concentration (2,4-dinitrophenolate release. Pseudo-first order rate constants at each

5.6. Active fractions (at the end of the gradient) were pooled and

fractation at 384 nm. One unit of enzyme activity produces 1

0.6–0.0M (NH₄)₂SO₄ in phosphate buffer over 30 min. Fractions (1 ml)

conditions in the presence of various concentrations of acceptor, BTPGlc (0–88 mM). Reactivation was monitored by removal of aliquots (10 µl) at appropriate time intervals and assaying as described above. Any activity loss due to denaturation of the enzyme was corrected for by a control experiment involving incubation of enzyme with no inactivator. The observed reactivation rate constant, \( k_{\text{react}} \), for each reaction was determined from the slope of the plots of ln(1/F) versus time. The rate constant (\( k_{\text{react}} \)) and disassociation constant (\( K_{\text{diss}} \)) for the reactivation were determined from a reciprocal plot of \( k_{\text{react}} \) versus acceptor concentrations.

Labeling of Exo-1,3-β-glucanase—Exg (20 µl, 2.0 mg/ml, in 100 mM sodium acetate, pH 5.6, buffer) was incubated with 2FDNPGlc (2 µl, 20 mM) at 37°C for 2 h. Complete inactivation was confirmed by assaying with DNPGlcs as described earlier. This mixture was immediately used for intact protein experiments or digested with pepsin as described below.

Mass Spectrometry—Mass spectra were recorded using PE-Sciex API III (neutral loss experiments) and PE-Sciex API 300 (LC/MS and MS/MS experiments) triple quadrupole mass spectrometers (Sciex, Thornhill, Ontario, Canada), each equipped with an ion spray ion source. Peptides were separated by reverse phase HPLC on an Ultrafast Microprotein Analyzer (Michrom BioResources Inc., Pleasanton, CA) directly interfaced with the mass spectrometer. In each of the MS experiments, the proteolytic digest was loaded onto a C18 column (Relasiil, 1 × 150 mm), then eluted with a gradient of 0–60% solvent B over 10 min, followed by 100% B over 2 min at a flow rate of 50 µl/min (solvent A: 0.05% trifluoroacetic acid, 2% acetonitrile in water; solvent B: 0.045% trifluoroacetic acid, 80% acetonitrile in water). A post-column splitter was present in all experiments, splitting off 80% of the sample into a fraction collector and sending 20% into the mass spectrometer. Spectra were obtained in either the single-quadrupole scan mode (LC/ MS), the tandem MS neutral loss mode, or the tandem MS daughter scan mode (LC/MS/MS) using the settings described in each experiment.

Stoichiometry of Incorporation of Inactivator by ESMS—Exg (4 µg, native or 2FGlc-labeled) was introduced into the mass spectrometer through a microbore PLRP-5 column (1 × 50 mm) on a Microm HPLC system. The quadrupole mass analyzer of an API 300 Triple Quadrupole Mass Spectrometer was scanned over a m/z range of 300-2500 Da, with a step size of 0.5 Da and a dwell time of 1 ms/step. The ion source voltage was set at 4.8 kV, and the orifice energy was 50 V. The molecular weights of the glucanase species were determined by using the deconvolution software, Multiview 1.1, supplied by Sciex.

Proteolytic digest—Exg (20 µl, native or labeled) 2.0 mg/ml was mixed with 100 mM phosphate buffer, pH 2 (40 µl), and pepsin (20 µl, 0.2 mg/ml in 100 mM phosphate buffer, pH 2). Mixtures were incubated at room temperature for 90 min, and then immediately analyzed by ESMS or frozen until required.

ESMS Analysis of the Proteolytic Digest—The single-quadrupole mode (LC/MS) conditions used were identical to those described earlier for analysis of the intact protein. In the neutral loss scanning mode, MS/MS spectra were obtained on the API III by searching for the mass loss of m/z 165, corresponding to the loss of the 2FGlc label from a peptide ion in the singly charged state. Thus, scan range: m/z 200-1800; step size: 0.5; dwell time: 1 ms/step; ion source voltage: 5 kV; orifice energy: 80; RE1 = 117; DM1 = 0.05; R1 = 10 V; R2 = -40 V; RE3 = 120; DM3 = 0.10; collision gas (90% argon, 10% N₂) thickness (CGT): 3.0 × 10⁻⁸ molecules/cm². To maximize the sensitivity of neutral loss detection, the resolution (RE and DM) is normally compromised without generating artifact neutral loss peaks.

The MS/MS daughter ion spectrum was obtained in the single-quadrupole daughter scan mode by selectively introducing the m/z 839 (or 1068.5) peptide from the first quadrupole (Q1) into the collision cell (Q2) and observing the daughter ions in the third quadrupole (Q3). Thus, Q1 was locked on m/z 839; Q3 scan range: 100–900; step size: 0.5; dwell time: 1 ms; ion source voltage: 5.5 kV; orifice energy: 50; RNG = 400 V; Q0 = -10; RO1 = -14 V; RO2 = -55 V; RO3 = -63; CAD = 5 (collision gas = N₂).

Aminolysis of the 2FGlc-labeled Peptide Digest—To a sample (20 µl, 0.5 mg/ml) of 2FGlc-labeled digest was added concentrated ammonium hydroxide (5 µl). The mixture was incubated for 15 min at 50°C, acidified with 50% trifluoroacetic acid, and analyzed by ESMS using the same conditions as for the intact protein.
RESULTS AND DISCUSSION

Inactivation Kinetics—Incubation of C. albicans Exg with 2FDNPGLc resulted in time-dependent inactivation of the enzyme. Data were consistent with the simple kinetic scheme shown below in which inactivation is a consequence of the accumulation of the glycosyl-enzyme intermediate E-I (Scheme II).

Thus, the inactivation followed the expected pseudo-first order kinetics as seen in Fig. 1a. Values for the inactivation rate constant ($k_i = 0.042 \pm 0.002 \text{ min}^{-1}$) and the dissociation rate constant ($K_i = 1.49 \pm 0.14 \text{ mM}$) were determined by direct fit to the kinetic expression as described under “Experimental Procedures.” A double-reciprocal plot of the pseudo-first order rate constants versus inhibitor concentration is shown in Fig. 1c. Comparison of the Michaelis-Menten kinetic parameter for the substrate DNPGlc ($K_m = 2.2 \pm 0.2 \text{ mM}$) with the $K_i$ value for 2FDNPGLc indicates that replacement of the 2-hydroxyl by fluorine does not significantly affect ground state binding of the inactivator to the enzyme.

Interestingly, no inactivation was observed for the 2-chloro analog of the inhibitor (2C1DNPGlc), as previously demonstrated with other glycosyl hydrolases (26, 27). Further, 2C1DNPGlc did not function as a substrate, no detectable hydrolysis over background being observed. 2C1DNPGlc was then tested as a competitive inhibitor against DNPGlc hydrolysis, and an approximate $K_i$ of $0.7 \pm 0.2 \text{ mM}$ was determined. These results indicated that the 2C1DNPGlc can clearly bind, but the 2-chlorine prohibits not only turnover, but even formation of the glycosyl-enzyme intermediate.

Incubation of Exg with 2FDNPGLc in the presence of a competitive inhibitor, BTGlc, resulted in reduction of the apparent inactivation rate constant ($k_{obs}$) from 0.025 \pm 0.001 min$^{-1}$ to 0.0053 \pm 0.0006 min$^{-1}$ (Fig. 1b). These results indicate that the inactivator is active site-directed, and by analogy with results from $\beta$-glucosidase (28, 29), this indicates that inactivation is a consequence of the stabilization and trapping of the normal intermediate in catalysis.

Reactivation of Inactivated Exg—Evidence for the catalytic competence of the 2-deoxy-2-fluoro-glycosyl-enzyme intermediate was obtained by measuring the rates of spontaneous reactivation upon incubation in buffer at 37 °C after removal of excess inactivator. The regain of activity (due to regeneration of free enzyme) followed a first order process, giving a reactivation rate constant of $k_{react} = 0.0019 \text{ min}^{-1}$, corresponding to a $t_{1/2}$ of 526 min. The rate of reactivation was increased upon addition of BTGlc (a non-hydrolyzable glucoside) to the reaction mixture. This suggested that, as seen previously (9, 12, 30), the sugar presumably facilitates turnover of the intermediate via transglycosylation. The reactivation process followed pseudo-first order kinetics, and the rate was dependent on the BTGlc concentration in a saturable manner (Fig. 2a). A double-reciprocal replot of $k_{obs}$ versus reactivation concentrator yielded the kinetic parameters for the reactivation process of $k_{react} (0.024 \pm 0.002 \text{ min}^{-1}$ and $K_{react} (56.3 \pm 9.5 \text{ mM})$ (Fig. 2b).

Stoichiometry of Incorporation of Inactivator—The mass of native Exg was determined by ESMS to be 45,758 \pm 6 Da (expected mass of 45,755 Da from the amino acid sequence). After inactivation of the enzyme with 2FDNPGLc, a sample was analyzed by ESMS, and a new peak at 45,921 \pm 4 Da was observed in the mass spectrum. The mass difference between the labeled and unlabeled glucanase is therefore 163 \pm 10 Da, equal within experimental error to that of the 2FGlc label (165 Da), indicating a 1:1 stoichiometry of inactivation.
Identification of the Labeled Active Site Peptide by ESMS—

Peptic digestion of the 2FGlc-labeled enzyme resulted in a mixture of peptides that was separated by reverse phase HPLC, using the mass spectrometer as a detector. When scanned in LC/MS mode, a large number of peaks, arising from every peptide in the digestion, was observed (Fig. 3a). The peptides bearing the 2FGlc label were identified in a second experiment by using the tandem mass spectrometer in neutral loss mode. In this mode the ions are subjected to limited fragmentation by an inert gas in a collision cell. The ester linkage between the inactivator and the peptide is one of the more labile bonds present and would be expected to undergo facile homolytic cleavage. The loss of a neutral sugar of known mass (165 Da) allows for the quadrupoles Q1–Q3 to be scanned in a linked mode, detecting only ions differing in mass by loss of the label. When the spectrometer was scanned in the neutral loss tandem MS/MS mode, a search for the mass loss \( m/z \) 165 (corresponding to the loss of 2FGlc label) resulted in the detection of two peaks at 16.4 and 16.8 min not present in the unlabeled, control digest MS/MS experiment (Fig. 3b). The background signals that are present in the profile are due to unlabeled peptides undergoing equivalent neutral losses to that of the label (165 Da), most likely in this case loss of a phenylalanine residue as is shown in the HPLC profile of Fig. 3c, where a sample of unlabeled enzyme was subjected to an identical analysis. The two peptides of mass 839 Da and 1068.5 Da correspond to unlabeled peptide fragments of 674 Da (839 – 165) and 903.5 Da (1068.5 – 165). Computer analysis of the amino acid sequence of ExG (22) revealed 9 and 11 respective candidate peptides for the masses 674 and 903.5 ± 1 Da. However, since the suspect peptide sequences should contain either an aspartate or glutamate residue (known nucleophiles of retaining glycosyl hydrolases) and since this residue should be present in both mass groups, elimination of all but two candidate peptides for each mass was possible. Only a single glutamic acid residue, Glu-330, is present in the following possible peptides: WNAGE (325–330) or NVAGEW (326–331) for mass 674 ± 1 Da and NVAGWE (326–334) or AGEWSAALT (328–336) for mass 903.5 ± 1 Da. Since all the possible sequences are overlapping, Glu-330 can be tentatively assigned as the active site nucleophile for ExG.

Further evidence was obtained by the determination of the complete amino acid sequence of the labeled peptides by ESMS/MS experiments. Daughter ion scans (Fig. 4) of the peptide of 839 Da subjected to collision-induced fragmentation resulted in the loss of the covalent inactivator, generating the unlabeled peptide (675 Da). Further fragmentation of this peptide was observed, giving rise to peaks at 471, 342, 285, and 214 Da corresponding to respective losses of W, EW, GEW, and AGEW. A parallel series of “y” (labeled) fragments are also observed. The sequence NVAGWE is therefore predicted. To confirm this, analysis of the predicted “b” and “y” fragmentation pattern for the other plausible sequence, WNVAGE, showed poor correlation to the experimental results. Similar MS/MS experiments were carried out with peptide of mass 1068.5 Da (data not shown), predicting that the corresponding sequence is NVAGWEWSA.

Covalent attachment of 2FGlc to Glu-330 through an ester linkage was confirmed by treatment of the labeled peptide digest with ammonium hydroxide. After treatment, the labeled peptide (839 Da) was replaced by two new peptides having molecular weights of 674 (m/z 675, MH+) and 673 (m/z 674, MH+). This would indicate that the labeled glutamate reacted by both amidolysis and hydrolysis, generating Glu-330 and the free acid Glu-330, thereby implicating the catalytic nucleophile to be Glu-330.

Sequence Analysis—As reported earlier (30), family 5 enzymes can be further divided into five “subfamilies” within which alignments are considerably improved. Several new enzymes have since been added to this family, including a number of exo-1,3-β-glucanases, which form another subfamily within which there is a high degree (>50%) of homology across the entire protein. The N-terminal region of this subfamily shows
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### TABLE I

| Species | Laminarin | MUG |
|---------|-----------|-----|
|         | Units/g | Kₘ | Units/mg | Kₘ |
| Wild type, native | 110 | 3.8 | 28 | 2.0 |
| Wild type, recombinant | 110 | 3.5 | 23 | 2.0 |
| E230Q | 0.40 | 2.6 | 0.2 | ND* |
| E330Q | 0.0023 | 2.9 | 0.0003 | ND* |
| Q530E | 119 | 2.9 | 32 | 1.9 |

* ND, not determined.

**Fig. 5. Alignment of region containing the proposed catalytic nucleophile glutamate labeled by 2FDNPGlc in selected family 5 glycosyl hydrolases.**

The exo-1,3-β-glucanase enzyme from C. albicans (Exg), GenBank accession no. Z46867; yeast (S. cerevisiae) endoglucanase by EGZ, GenBank accession no. Z46868; Paracoccidioides brasiliensis (P. brasiliensis) endoglucanase by EGZ, GenBank accession no. Z46870; Agaricus bisporus (A. bisporus) endoglucanase by EGZ, GenBank accession no. Z46871; Acidothermus cellulolyticus (A. cellulolyticus) endoglucanase by EGZ, GenBank accession no. Z46872; Pichia agarica (P. agarica) endoglucanase by EGZ, GenBank accession no. Z46873; and Debaryomyces occidentalis (D. occidentalis) endoglucanase by EGZ, GenBank accession no. Z46874. These sequences are shown in bold.

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**Table I**

Activities of various Exg species

The various Exg species were purified and assayed with laminarin and methylumbelliferyl-β-glucoside (MUG) as described under “Experimental Procedures.”
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