Identification of the Active Site Nucleophile in the Thermostable β-Glycosidase from the Archaeon *Sulfolobus solfataricus* Expressed in *Escherichia coli*†

F. Febbraio, R. Barone, S. D'Auria, M. Rossi,‡ and R. Nucci*

Institute of Protein Biochemistry and Enzymology, CNR, via Marconi 10, 80125 Naples, Italy

G. Piccialli and L. De Napoli

Department of Organic and Biological Chemistry, University of Naples, via Mezzocannone 16, 80134 Naples, Italy

S. Orrù and P. Pucci

Mass Spectrometry Service, CNR, and CEINGE Advanced Biothecnology, via Pansini 5, 80131 Naples, Italy

Received October 4, 1996; Revised Manuscript Received December 26, 1996⊗

ABSTRACT: *Sulfolobus solfataricus* β-glycosidase expressed in *Escherichia coli* was fully inactivated at 65 °C, according to pseudo-first-order kinetics, by [^{3}H]conduritol B epoxide (DL-1,2 anhydro-*myo*-inositol) synthesized as the active site directed inhibitor by a slight modification of Legler's procedure [Legler, G. (1977) *Methods Enzymol. 46*, 368−381]. The determination of kinetic constants for the inactivation showed that the process took place through the formation of a stabilized inhibitor−enzyme intermediate. Inactivation and reactivation studies suggested that the inhibitor−enzyme intermediate complex was formed more rapidly and hydrolyzed at a lower rate than it was for other glycosidases. Moreover, the stoichiometry of the binding, determined by electrospray mass spectrometric analysis, revealed that one molecule of the inhibitor was covalently bound to each enzyme subunit. The binding site for [^{3}H]conduritol B epoxide was identified by the isolation and partial sequence analysis of the radioactive peptide obtained by cyanogen bromide and pepsin digests. Electrospray tandem mass analysis of the labeled peptide showed that the inhibitor was covalently bound to E387. This result, in agreement with data obtained from sequence alignments of *S. solfataricus* β-glycosidase with other gluco- and galactosidases of the glycosyl hydrolase family 1 [Henrissat, B. (1991) *Biochem. J. 280*, 309−316], indicates that the conserved E387 is the nucleophilic amino acid residue in the active site of the enzyme.

As industrial biocatalysts, enzymes have long been in use in food processing and pharmaceutical areas. Some of the most common industrial enzymes and their applications include glycosidases, which, being responsible for the hydrolytic cleavage of glycosyl bonds, are utilized as catalysts of lactose hydrolysis in dairy products in the food industry (Cheetham, 1985; Ghose & Mattiasson, 1993).

In our laboratory, a β-galactosidase activity from the thermophilic archaeon *Sulfolobus solfataricus*, grown at 87 °C, pH 3.0 (De Rosa et al., 1980), was purified to homogeneity (Pisani et al., 1990). It was barely active up to 30 °C, showing its maximal enzyme activity over 95 °C, at pH 7.0. More recently, the β-galactosidase activity has been characterized as a glycosyl hydrolase, and in view of its wide substrate specificity and exoglucosidase activity, the enzyme has been classified as a β-glycosidase (Nucci et al., 1993). The gene coding for this enzyme has been cloned and expressed in both *Escherichia coli* (Cubellis et al., 1990) and *Saccharomyces cerevisiae* (Moracci et al., 1992). The recombinant enzymes have been purified to homogeneity and characterized, showing the same structural and functional features as the native enzyme (Moracci et al., 1995; D'Auria et al., 1996). Moreover, the native enzyme has been crystallized (Pearl et al., 1993), and its structure has been solved at 2.6 Å (Sanderson et al., 1996). Investigations on the effects of detergents on the functional and structural properties of *S. solfataricus* β-glycosidase have shown that sodium dodecyl sulfate (SDS)[1] activated the enzyme at room temperature and modified its substrate specificity. In fact, in the presence of SDS, the enzyme was unable to hydrolyze natural substrates, such as cellobiose and laminaribiose, while an activation (50%) was observed when synthetic substrates, such as 2-nitrophenyl β-D-galactopyranoside and 4-nitrophenyl β-D-glucopyranoside (pNPGlu), were utilized (Nucci et al., 1995).

Identification of the amino acid residues occurring at the active site in glycosidases is of considerable importance for structure/function or mutagenic studies as well as, when mutants are being designed, for providing enzymes with altered properties.

In an effort to further contribute to the structure/function studies of the glycosidases family, we performed a mechanistic characterization of the thermophilic β-glycosidase by

† Supported by Target Project "Ingegneria Genetica" (CNR Italy). Partially supported by EU Project on Extremophiles.

* To whom correspondence should be addressed.

‡ Also at the University of Naples.

⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1997.

[1] Abbreviations: [^{3}H]CBE, [2-^{3}H]-DL-1,2-anhydro-*myo*-inositol; CBE, DL-1,2-anhydro-*myo*-inositol; βgly, *Sulfolobus solfataricus* β-glycosidase expressed in *Escherichia coli*; CNBr, cyanogen bromide; *p*NPGlu, 4-nitrophenyl β-D-glucopyranoside; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; ESMS, electrospray mass spectrometry; MS/MS, tandem mass spectrometry; FABMS, fast atom bombardment mass spectrometry.

S0006-2960(96)02496-8 CCC: $14.00

using [2-^{3}H]conduritol B epoxide ([^{3}H]CBE) (Legler, 1977). This family of inhibitor molecules is widely used for identifying active site nucleophiles in glycosidases, as in bitter (Legler & Harder, 1978) and sweet almond β-glucosidases (Legler & Hasnain, 1970).

This paper is concerned with the inhibition kinetic studies of *S. solfataricus* β-glycosidase expressed in *E. coli* (βgly) by conduritol B epoxide (CBE), as well as with the identification of its active site nucleophile by mass spectrometry.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were of analytical grade. The conduritol B epoxide and *myo*-inositol were from Sigma. [2-^{3}H]-*myo*-Inositol was from Amersham. The HPLC chromatographic system Gold was from Beckman. ^{1}H NMR spectra were recorded on a Bruker WM 270 instrument (270 MHz).

[^{3}H]Conduritol B Epoxide Synthesis. (A) 1,2-O-Isopropylidene-myo-inositol. 2,2-Dimethoxypropane (0.55 mL) and *p*-toluenesulfonic acid (4 mg) in anhydrous dimethyl sulfoxide (1 mL) were added to the mixture of *myo*-inositol (200 mg, 1.1 mmol) and *myo*-[2-^{3}H]inositol (8.7×10^{-5} mmol). After 30 min at 100 °C the reaction was quenched by addition of triethylamine (0.05 mL) and ethanol (2 mL). The mixture was dried under reduced pressure, dissolved in 1 mL of H_2O/CH_3OH (8:2 v/v), and chromatographed on a reversed-phase C-18 column (Lobar Merck). The fractions eluted with 10 mL of H_2O/CH_3OH (8:2 v/v) yielded 180 mg of 1,2-*O*-isopropylidene-*myo*-inositol (75% yield). ^{1}H NMR data were in agreement with the values reported in the literature (Legler, 1977).

(B) 3,4,5,6-Tetra-O-acetyl-1,2-O-isopropylidene-myo-inositol. 1,2-*O*-Isopropylidene-*myo*-inositol (180 mg, 0.82 mmol), dissolved in pyridine (3 mL), was treated with acetic anhydride (2 mL). After 3 h at room temperature, 3 mL of CH_3OH was added, and the mixture was dried under reduced pressure. The residue was dissolved in $CHCl_3$ (15 mL) and washed three times with water (30 mL). The organic layer dried under reduced pressure provided almost pure 3,4,5,6-tetra-*O*-acetyl-1,2-*O*-isopropylidene-*myo*-inositol (294 mg, 93% yield). ^{1}H NMR (270 MHz, $CDCl_3$) δ: 5.50 (1H, dd), 5.32−5.17 (2H, complex signal), 5.03 (1H, dd) [H-3,4,5,6]; 2.09, 2.08, 2.04, 2.01 (3H each, $4CH_3CO$); 1.59 and 1.33 ($2CH_3$ isopropylidene).

The synthesis procedure from 1,4,5,6-tetra-*O*-acetyl-*myo*-inositol to conduritol B epoxide was performed as described by Legler (1977). At the end of the purification 36 mg of pure conduritol B epoxide, with a final yield of 18%, was obtained.

Protein Purification and Enzymatic Assay. βgly was overexpressed in *E. coli* and purified as described previously (Moracci et al., 1995). Protein concentration was determined using the ϵ_{280} of the protein (6.90×10^{-5} M^{-1} cm^{-1}) in 50 mM sodium phosphate buffer, pH 6.5, at 25 °C, or by Bio-Rad assay, using bovine serum albumin as standard (Bradford, 1976).

Assay for the glucosidase activity was carried out at 75 °C in a 1.0 mL final volume reaction mixture containing 50 mM sodium phosphate buffer, pH 6.5, and 4.0 mM *p*NPGlu ($K_M = 0.5$ mM), and the release rate of the 4-nitrophenolate was measured.

One enzymatic unit was defined as the enzyme amount catalyzing the hydrolysis of 1.0 μmol of substrate in 1 min at 75 °C, assuming an ϵ_{405} of 13×10^3 M^{-1} min^{-1} for the *p*NPGlu.

Inhibition Kinetics. βgly inactivation by CBE was monitored by incubating the enzyme in 50 mM sodium phosphate, pH 7.0, at 65 °C, in the presence of different CBE concentrations (0, 10, 20, 30, 40, 50, 70, 80 μM). Residual activity was determined at different times by assaying a 5 μL aliquot of the inactivation mixture for glucosidase activity.

The pseudo-first-order rate constant at each inhibitor concentration, k_{obs}, was determined by plotting the natural logarithm of the residual activity versus time. By replotting the reciprocal of the pseudo-first-order rate constants versus the reciprocal of the inhibitor concentrations, we found the values for the inactivation rate (k_i) and the equilibrium binding (K_i) constants.

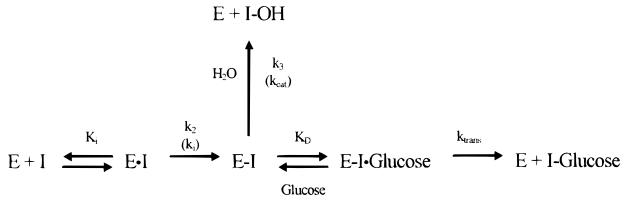
Protection against inhibition was performed by following the inactivation rate at 50 μM CBE in the presence of 0−0.05 and 0.1 M glucose (K_i = 96 mM; Pisani et al., 1990).

Reactivation of the inhibited enzyme was investigated by removing the excess of the inhibitor by a centrifugal microconcentrator (Centricon 30, Amicon, Inc., Beverly, MA) and by incubating it in 50 mM sodium phosphate buffer, pH 7.0 at 30 °C, in the absence and in the presence of 0.05 and 0.1 M glucose. Enzyme activity was assayed for over 300 h, using 4.0 mM *p*NPGlu as substrate.

Determination of the Active Site Nucleophile. βgly (31.87 nmol) was treated with [^{3}H]CBE (74 μL, 21.5 mM, specific activity 0.537 nCi/nmo) in 1.0 mL of 50 mM sodium phosphate buffer, pH 7.0, and incubated at 65 °C for 24 h; after incubation, the enzyme was 99% inactivated, while the control enzyme retained full activity. Unbound [^{3}H]CBE was removed by a centrifugal microconcentrator. Before being discarded, the filtered material was assayed and no protein was detected. The resulting radiolabeled enzyme was incubated with cyanogen bromide (CNBr) in 200 μL of 70% trifluoroacetic acid (TFA), using a 10-fold molar excess of reagent on methionine residues, for 18 h at room temperature in the dark. The CNBr reaction was stopped by adding 10 volumes of water, and the sample was lyophilized. The resulting peptide mixture was purified by reversed-phase HPLC on a Vydac C-18 column (218TP54, 250 mm × 4.6 mm, 5 μm, 300 Å pore size). The elution system consisted of 0.1% TFA (eluent A) and 95% acetonitrile, containing 0.07% TFA (eluent B); peptides were eluted by means of a nonlinear gradient of eluent B (from 20% to 35% in 15 min and from 35% to 60% in 33 min), at a flow rate of 1.0 mL/min; the elution profile was monitored at 220 nm. Fractions were collected and lyophilized. An aliquot of the radiolabeled CNBr peptidic fragments was digested with pepsin for 48 h at 37 °C in 20% acetic acid, pH 2.0, using an enzyme: substrate ratio of 1:50 (w/w). The reaction was stopped by raising the pH to 4.5 with 400 μL of 5.0 M sodium hydroxide. The sample was stored at 4 °C. The peptic digest was fractionated by a Vydac C-18 reversed-phase HPLC, using the solvent system described above and a linear gradient of eluent B, from 0% to 55% in 65 min, at a flow rate of 1.0 mL/min; the elution profile was monitored at 220 nm. Fractions were collected and stored at −20 °C.

A second aliquot of the CNBr-radiolabeled peptidic fragments was subdigested with endoproteinase Asp-N (Boehringer Mannheim, Mannheim, Germany) in 0.4%

Scheme 1



ammonium acetate, pH 6.5, for 18 h at 37 °C, using an enzyme:substrate ratio of 1:50 (w/w). The reaction was stopped by lyophilization.

The N-terminal sequence of the modified peptide was determined by using an Applied Biosystems 477A pulsed-liquid protein sequencer equipped with an Applied Biosystems 120A HPLC apparatus for Pth-amino acid identification.

Electrospray mass spectrometric (ESMS) and electrospray tandem mass spectrometric (MS/MS) analyses were performed on HPLC-purified samples by using a VG BioQ triple quadrupole mass spectrometer (Micromass, Manchester, U.K.). Aliquots (10 μL) of the protein and/or peptide solution were injected in the ion source at a flow rate of 10 μL/min; the mass spectrometer was scanned from *m/z* 500 to *m/z* 1800 at 10 s/scan, using a cone voltage of 40 V. Fragmentation experiments were carried out using argon as collision gas and a collision energy of 20−50 eV. Data were acquired and elaborated using the MassLynx program. Mass calibration was performed by means of the multiply charged ions from a separate injection of horse heart myoglobin (Sigma; average molecular mass 16951.5 Da); all masses are reported as average mass.

Fast atom bombardment mass (FABMS) spectra were recorded on a VG ZAB 2SE double-focusing mass spectrometer (Micromass, Manchester, U.K.) fitted with a VG cesium gun operating at 25 kV (2 μA). Samples were dissolved either in 5% acetic acid or in 0.1 M HCl, and 2 μL aliquots were loaded onto a glycerol-coated probe tip; thioglycerol was added just before the probe was inserted into the ion source. Spectra were recorded on ultraviolet-sensitive paper and manually counted; signals were assigned to the corresponding peptides within the enzyme sequence on the basis of their molecular masses with the aid of a suitable computer program (Pucci & Sepe, 1988).

RESULTS AND DISCUSSION

Kinetic Inhibition Studies. CBE is a more refined version of epoxide inactivators of glycosidases. It incorporates an endocyclic epoxide in a cyclical ring, which itself mimics the sugar ring. The reactivity of the epoxide group in a tetrahydroxycyclohexane system to acid-catalyzed and nucleophilic addition is greatly diminished by the electron-withdrawing effect of the neighboring hydroxyl groups. This inertness makes conduritol epoxides ideal k_{cat} inhibitors, as they are resistant to spontaneous hydration and react with proteins only if they are bound at a position that permits protonation of the epoxide oxygen atom by a neighboring acidic group. The specific reaction of a conduritol epoxide also requires a nucleophile for the formation of a covalent bond. Studies of the conduritol B epoxide have shown that it acts as an irreversible inhibitor of several glycosidases (Legler, 1990).

Incubation of βgly with CBE led to total enzyme inactivation, according to pseudo-first-order kinetics. Scheme 1 shows the kinetic inhibition reaction between the enzyme and CBE. The same inhibition mechanism has already been described for calf liver cytosolic β-glucosidase (Legler & Bieberich, 1988), rat liver glucosidase II (Alonso et al., 1993), *Agrobacterium faecalis* β-glucosidase (Street et al., 1992), *E. coli* β-galactosidase (Gebler et al., 1992; Mc Carter et al., 1992), and *Staphylococcus aureus* 6-phospho-β-galactosidase (Staedtler et al., 1995).

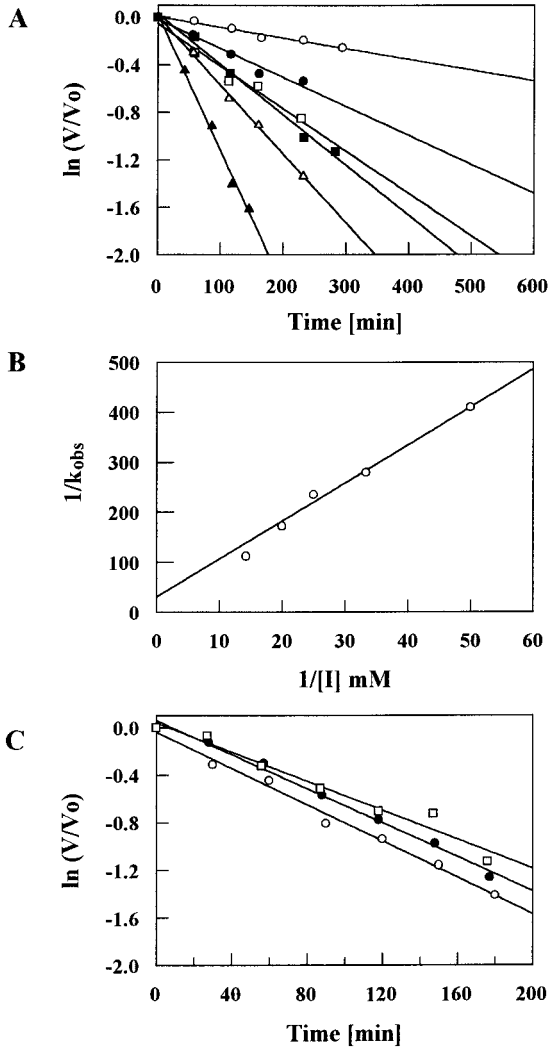


FIGURE 1: Inactivation of βgly by conduritol B epoxide. (A) The enzyme (250 μg/mL) was incubated with different concentrations of CBE at 65 °C in 50 mM sodium phosphate: (○) 10, (●) 20, (□) 30, (■) 40, (△) 50, and (▲) 70 μM. (B) Reciprocal plot of the slope from (A) against inhibitor concentration (mM). (C) Inhibition by 50 μM CBE in the presence of different glucose concentrations: (○) 0, (●) 50, and (□) 100 mM.

According to Scheme 1, the inhibition rate constant k_i corresponds to the glycosylation constant k_2 whereas k_3 represents the deglycosylation constant k_{cat}. Since CBE acts as an irreversible inhibitor, the rate constant k_{cat} is very small. The inactivation constant, k_{obs}, for each inhibitor concentration was obtained by plotting the natural logarithm of the remaining enzyme activity versus time (Figure 1A). A double-reciprocal replot of the pseudo-first-order rate constants versus inhibitor concentration (Figure 1B) yielded an inactivation rate constant k_i of 32.78×10^{-3} min^{-1} $\pm$ 2.51×10^{-3} and a dissociation constant K_i of 0.25 $\pm$ 0.018 mM. The constants were calculated from intersections of both the *x*-axis and the *y*-axis, using the Grafit software (Leatherbarrow, 1990).

The k_i/K_i ratio calculated for the inactivation kinetics of βgly by CBE was 139.4 min^{-1} M^{-1}. Similar inhibition investigations on lysosomal β-glucosidase from calf spleen (Liedtke & Legler, 1988) and β-glucosidase B from sweet almonds (Legler & Hasnain, 1970) showed k_i/K_i values of 360 and 76 min^{-1} M^{-1}, respectively. These results are quite different from those described above for βgly but similar to those obtained for the β-glucosidases from *Aspergillus wentii* (150 min^{-1} M^{-1}) (Legler, 1968) and *Aspergillus orizae* (130 min^{-1} M^{-1}) (Legler & Omar Osama, 1968). The lower value found for βgly with respect to the purified calf spleen lysosomal β-glucosidase could be due to intrinsic structural rigidity of the thermophilic enzyme at the incubation temperature of the inhibition kinetics.

Incubation of the enzyme with CBE in the presence of glucose (0.05 and 0.1 M) reduced the apparent rate constant for inactivation (k_{obs}) from 7.64×10^{-3} $min^{-1} \pm 0.30 \times 10^{-3}$ to 7.18×10^{-3} $min^{-1} \pm 0.28 \times 10^{-3}$ and to 6.13×10^{-3} $min^{-1} \pm 0.51 \times 10^{-3}$, respectively (Figure 1C), giving the expected rate reduction for a competitive mechanism. The protection from the inactivation, using a competitive inhibitor, provided evidence that the inactivation of βgly occurred at the active site.

The CBE-inactivated enzyme incubated for several days in the absence or in the presence of 50 and 100 mM glucose did not show any recovery of activity, thus suggesting both that the rate constant for the trans-glycosylation reaction k_{trans} is very small and that the deglycosylation reaction rate k_{cat} for this inhibitor is smaller than that for other inhibitor−enzyme complexes (Staedtler et al., 1995; Street et al., 1992).

These results show that the inhibitor−enzyme intermediate is highly stable, probably for the presence of an OH group at the C6 position of the CBE ring. In fact, as described by Withers et al. (1988), the inhibitors that possess a CH_2OH group at the C6 position, such as the 2-deoxy-2-halo-β-D-glycosyl derivatives, increase the ratio k_i/K_i but reduce the stability of the inhibitor−enzyme intermediate. This suggests a crucial role of the residue at the C6 position in the inhibitor−enzyme interaction. In fact, βgly is not able to hydrolyze the disaccharides which are phosphorylated on position 6 of the nonreducing end sugar, unlike some members of glycosyl hydrolase family 1. Moreover, the failed identification of glucuronic and galacturonic acid as substrates or inhibitors (Nucci et al., 1993) indicates the possible repulsive interaction with the charged 2-carboxylate group of these sugars.

Structural Analyses. HPLC-purified native and CBE-modified βgly were analyzed by ESMS. Figure 2 shows the corresponding ES spectra transformed on a real mass scale. The molecular mass of untreated βgly was measured as 56690.3 ± 4.2 Da, corresponding to the expected value of a single βgly subunit (Figure 2A), whereas the CBE-treated protein displays a molecular mass of 56861.6 ± 4.7 Da (Figure 2B). The mass difference between these two values was calculated as 171.4 Da, which is consistent with the covalent attachment of a single CBE molecule per βgly subunit.

The identification of the βgly active site residue bound to the inhibitor was achieved by using a combination of mass spectrometric procedures with radioactivity measurements.

The synthesis of [2-^{3}H]conduritol B epoxide was performed starting from a mixture of *myo*-inositol/*myo*-[2-^{3}H]-inositol in a 1 to 3.6×10^{-5} molar ratio, with an overall yield of 18%. The structures of the synthesized compounds were confirmed by ^{1}H NMR analyses (Legler, 1977). The specific activity of the final compound was calculated to be 0.537 nCi/nM.

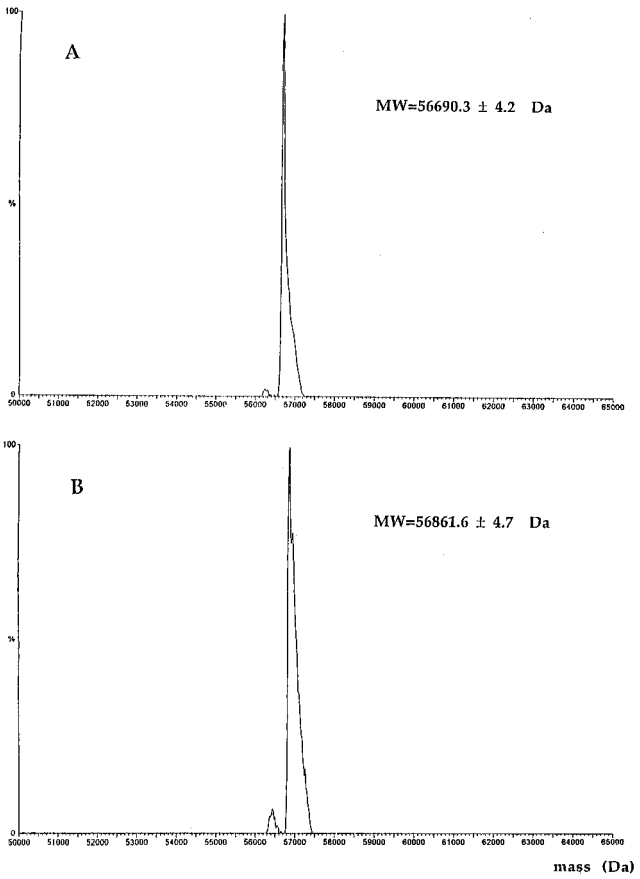


FIGURE 2: Transformed electrospray mass spectra of the native (panel A) and inhibited (panel B) βgly subunit. The multiply charged ion spectra are transformed on a real mass scale.

A rapid screening of the entire βgly structure was achieved by submitting [^{3}H]CBE-treated βgly to CNBr hydrolysis, followed by HPLC separation on a Vydac C-18 column. All the fractions collected were submitted to radioactivity measurements and ESMS analysis. The fraction eluted at 25.4 min gave positive results when examined for radioactivity. The relative ESMS spectrum showed the presence of two components; on the basis of the accurate mass measurement, the major species was identified as the C-terminal fragment 440−489 (molecular mass 5849.3 ± 0.7 Da), whereas the minor component showed a molecular mass of 6587.5 ± 0.1 Da, which did not correspond to any expected CNBr peptide. The mass value of this fragment was assigned to the CNBr peptide 384−439 covalently linked to a single [^{3}H]CBE molecule. This assignment was confirmed by N-terminal sequencing of the chromatographic peak, thus indicating the occurrence of the active site residue within this protein region.

An aliquot of the [^{3}H]CBE peptide was subdigested with pepsin, and the resulting peptide mixture was fractionated by C-18 RP-HPLC; fractions were assayed for radioactivity and analyzed by ESMS. A single radiolabeled fraction was detected, showing a mass value of 1443.8 ± 0.1 Da (Figure 3A), which was tentatively assigned to the peptide 384−395 containing the covalent modification. The N-terminal sequence of this fraction reported in the insert to Figure 3A

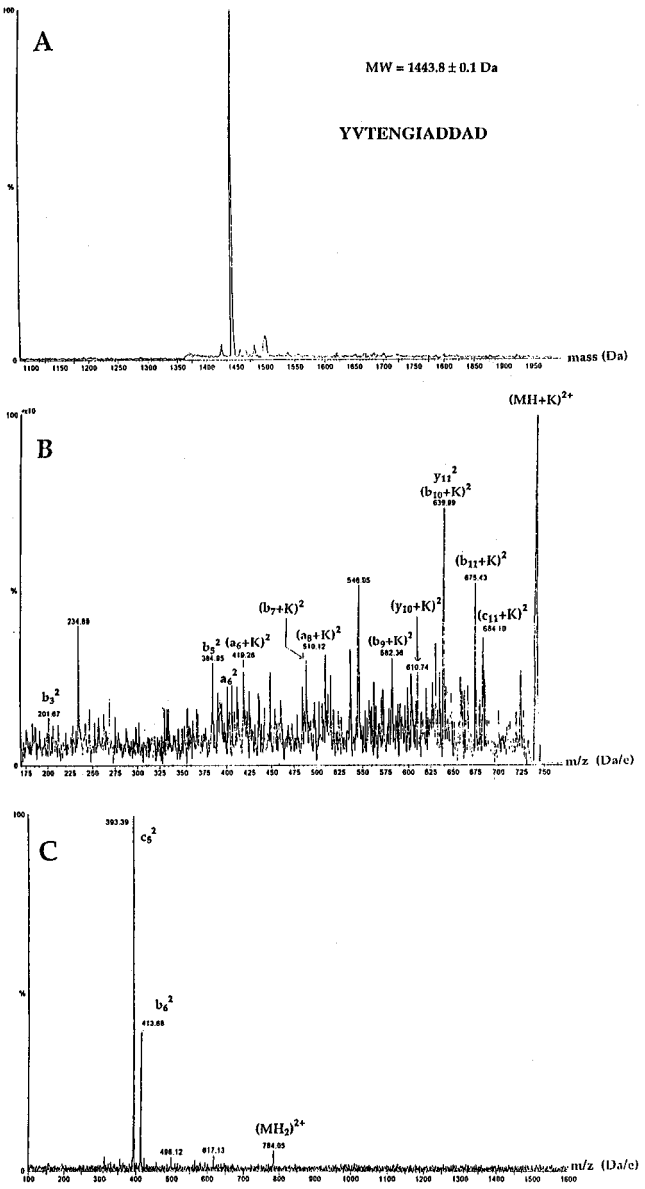


FIGURE 3: (A) Transformed electrospray mass spectrum of the modified peptide (384−395). (B) ESMS/MS spectrum of the doubly charged ion corresponding to the potassium salt of the modified peptide. (C) Daughter ion spectrum obtained by ESMS/MS of the doubly charged ion corresponding to the esterified modified peptide. For details see the text.

confirmed the assignment. The peptide 384−395 contains four putative catalytic sites, namely, E387, D392, D393, and D395, each of which might bind [^{3}H]CBE. The sequence analysis, in fact, could not discriminate among these putative active site residues, since the acetal bond between the protein and the inhibitor is base-labile and the modifying group is then released under the Edman conditions. However, D395 was recognized and cleaved by pepsin, indicating that this residue is very likely unmodified.

In order to distinguish among the three active site candidates, the covalently modified peptide was submitted to different ESMS/MS experiments. Figure 3B shows the daughter ion spectrum of the doubly charged ion at *m/z* 741.4, corresponding to the potassium salt of the modified peptide. The fragmentation process yielded mainly doubly charged fragment ions of the b series, although some fragments belonging to the a, y, and c series were also observed. The analysis of the daughter ion spectrum indicated that D392 and D393 are unmodified and suggested E387 as the residue covalently bound to [^{3}H]CBE.

This finding was confirmed by ESMS/MS analysis of the modified peptide following esterification of the free carboxylic acid with propanol. The tandem mass spectrum of the doubly charged ion at *m/z* 784.1 performed at a much higher collision energy was dominated by two strong signals at *m/z* 393.4 and 413.7 Da, as shown in Figure 3C. Both signals were interpreted as doubly charged fragment ions belonging to the c and b series, c_5^{2+} and b_6^{2+}, respectively. These fragments correspond to the sequences Y-V-T-E-N and Y-V-T-E-N-G, respectively, both carrying the modified group and containing a single acidic residue, E387.

An independent confirmation of these results was achieved by a complementary approach. A second aliquot of the CNBr-modified peptide 384−439 was subdigested with endoproteinase AspN, and the resulting peptide mixture was directly analyzed by FABMS. The FAB spectrum showed the occurrence of a mass signal at *m/z* 1030, which was assigned to peptide 384−391 containing the covalent modification. Since this peptide contains a single acidic residue, E387 was again identified as the βgly active site residue specifically modified by the [^{3}H]CBE inhibitor.

The MS/MS experiments show that the inhibitor−enzyme intermediate is not easily hydrolyzed during the fragmentation process, as happens in the case of the xylanase from *Bacillus subtilis* inhibited by 2-deoxy-2-fluoro-β-D-glycosyl (Withers & Aebersold, 1995), suggesting a higher bond stability of the CBE−enzyme complex.

Sequence Alignment. The βgly sequence was searched for homology against the NCBI protein data bank using the BLAST (Altschul et al., 1990) network service and the SWISS-PROT data bank using the FASTA (Pearson & Lipman, 1988) program. Sequence alignments were performed by the program of multialignment PILEUP (default setting) and when necessary by a word-processing program. The amino acid sequence of βgly derived from the *S. solfataricus lac*S gene (Cubellis et al., 1990) has classified this enzyme as a member of the glycosyl hydrolase family 1 along with related archaeal and bacterial β-glycosidases, 6-phospho-β-gluco- and galactosidases, cyanogenic β-glycosidases, plant thioglucosidases, and mammalian gut lactases (Henrissat, 1991; Henrissat & Bairoch, 1993).

Figure 4 shows the sequence alignments of *S. solfataricus* β-glycosidase with *A. faecalis* β-glucosidase (Wakarchuk et al., 1988), *S. aureus* 6-phospho-β-galactosidase (Breidt & Stewart, 1987), *Lactococcus lactis* 6-phospho-β-galactosidase (De Vos et al., 1990), and other glucosidases and 6-phospho-β-glucosidases from archaea and bacteria.

According to the sequence alignments, βgly E387 covalently bound to [^{3}H]CBE is equivalent to the active site E359 in *A. faecalis* (Withers et al., 1990) and E375 in *S. aureus* (Staedtler et al., 1995) and *L. lactis* (Wiesmann et al., 1995) identified through site-directed mutagenesis or by covalent bond with suicide inhibitors.

This paper points out that E387 is involved in βgly catalysis by covalent modification with a suicide inhibitor. In addition, this amino acid residue has very recently been localized in the enzyme active site through X-ray crystallographic determination of the 3-D structure (Sanderson et al., 1996). When a β(1−4)-linked oligoglucose chain is modeled in the active site, with the nonreducing end directed toward the closed end, the nonreducing end sugar and the

FIGURE 4: Amino acid sequences of βgly, *S. shibatae* β-glycosidase, *S. solfataricus* β-glucosidase strain P1, *P. furiosus* β-glucosidase, *L. lactis* 6-phospho-β-galactosidase, *S. aureus* 6-phospho-β-galactosidase, *E. chrysanthemi* 6-phospho-β-glucosidase, *E. coli* 6-phospho-β-glucosidase, *B. subtilis* 6-phospho-β-glucosidase, *T. brockii* β-glucosidase, *C. thermocellum* β-glucosidase A, *T. maritima* β-glucosidase A, and *A. faecalis* β-glucosidase are aligned. The highly conserved amino acid residues are indicated by an asterisk; the active site nucleophiles of the enzymes from *A. faecalis*, *S. aureus*, and *L. lactis* are underlined.

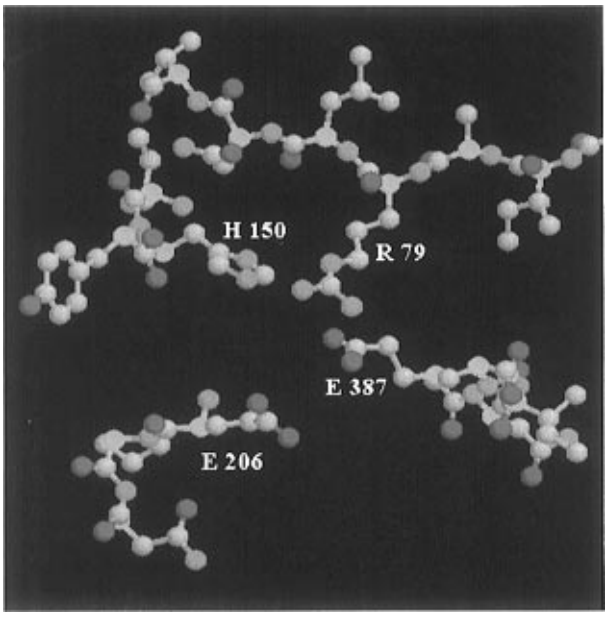


FIGURE 5: Location of the nucleophile (E387) and the putative acid/base catalyst (E206 or H150) with the spatial proximity of the E387 nucleophile to the R79 residue in the βgly active site at 2.6 Å resolution. The colors of the atoms are those conventionally used by chemists: C = light gray; O = red; N = light blue; S = yellow. The Cαs are shown in cyan.

scissile glycosidic bond are in close proximity to several residues, A15, R79, H150, W151, N205, E206, N320, Y322, E87, and W433 (Sanderson et al., 1996). In particular, the totally conserved residues, H150 and E206 (Figure 4), near E387 (Figure 5) may play a crucial role as the general acid/base residue catalyzing the protonation of the glycosidic oxygen in a retaining configuration of the catalytic mechanism that it has been proposed for enzymes of the glycosyl hydrolase family 1, to which βgly has been assigned.

As shown by the X-ray crystallographic analysis, the guanidinium head group of R79 is hydrogen bonded to the carboxylate of E387 (Figure 5) and probably serves to stabilize a negative charge and thereby enhance its nucleophilicity. The H150 is well positioned to act as the general base for the second stage of the hydrolysis reaction (Figure 5), activating a water molecule to attack the covalent glycosyl−enzyme intermediate. On the other hand, the roles of both general acid in the first step and general base in the second step can be attributed to E206 (Figure 5). However, for a definitive description of the roles of E206 and H150 further studies are needed, and CBE offers us the opportunity to investigate the catalytic mechanism of βgly by the crystallographic analysis of the inhibitor−enzyme complex.

ACKNOWLEDGMENT

We thank Professor Laurence H. Pearl for the availaibility of crystallographic coordinates and Mr. Carlo Vaccaro, Mr. Vito Carratore, and Mrs. Nunzia Vespa for technical assistance.

REFERENCES

Alonso, J. M., Santa-Cecilia, A., & Calvo, P. (1993) *Eur. J. Biochem. 215*, 37−42.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990) *J. Mol. Biol. 215*, 403−410.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.

Breidt, F., Jr., & Stewart, G. C. (1987) *Appl. Environ. Microbiol. 53*, 969−973.

Cheetham, P. S. J. (1985) in *Handbook of Enzyme Biotechnology* (Wiseman, A., Ed.) Part B, 2nd ed., Chapter 3, Ellis Horward, Sussex, England.

Cubellis, M. V., Rozzo, C., Montecucchi, P., & Rossi, M. (1990) *Gene 94*, 89−94.

D'Auria, S., Morana, A., Febbraio, F., Vaccaro, C., De Rosa, M., & Nucci, R. (1996) *Protein Expression Purif. 7*, 299−308.

De Rosa, M., Gambacorta, A., Nicolaus, B., Buonocore, V., & Poerio, E. (1980) *Biotechnol. Lett. 2*, 29−34.

De Vos, W. M., Boerrigter, I., Van Rooyen, R. J., Reiche, B., & Hengstenberg, W. (1990) *J. Biol. Chem. 265*, 22554−22560.

El Hassouni, M., Henrissat, B., Chippaux, M., & Barras, F. (1992) *J. Bacteriol. 174*, 765−777.

Gebler, J. C., Aebersold, R., & Withers, S. G. (1992) *J. Biol. Chem. 267*, 11126−11130.

Ghose, S., & Mattiasson, B. (1993) in *Thermostability of enzymes* (Gupta, M. N., Ed.) Narosa Publishing House, New Delphy, India.

Graebnitz, F., Seiss, M., Ruecknagel, K. P., & Staudenbauer, W. L. (1991) *Eur. J. Biochem. 200*, 301−309.

Hall, B. G., & Xu, L. (1992) *Mol. Biol. Evol. 9*, 688−706.

Henrissat, B. (1991) *Biochem. J. 280*, 309−316.

Henrissat, B., & Bairoch, A. (1993) *Biochem. J. 293*, 781−788.

Leatherbarrow, R. J. (1990) *Grafit Version 2.0*, Erithacus Software Ltd., Staines.

Legler, G. (1968) *Z. Physiol. Chem. 349*, 767−774.

Legler, G. (1977) *Methods Enzymol. 46*, 368−381.

Legler, G. (1990) *Adv. Carbohydr. Chem. Biochem. 48*, 319−385.

Legler, G., & Omar Osama, L. M. (1968) *Z. Physiol. Chem. 349*, 1488−1492..

Legler, G., & Hasnain, S. N. (1970) *Z. Physiol. Chem. 351*, 25−31.

Legler, G., & Harder, A. (1978) *Biochim. Biophys. Acta 524*, 102−108.

Legler, G., & Bierberich, E. (1988) *Arch. Biochem. Biophys. 260*, 437−442.

Liebl, W., Gabelsberger, J., & Schleifer, K. H. (1994) *Mol. Gen. Genet. 242*, 111−115.

Liedtke, H., & Legler, G. (1988) in *Lipid Storage Disorders* (Salvayre, R., Douste-Blazy, L., & Gatt, S., Eds.) pp 353−358, Plenum Press, New York.

Little, S., Cartwright, P., Campbell, C., Prenneta, A., Mcchesney, J., Mountain, A., & Robinson, M. (1989) *Nucleic Acids Res. 17*, 7980−7980.

Mc Carter, J. D., Adam, M. J., & Withers, S. G. (1992) *Biochem. J. 286*, 721−727.

Moracci, M., La Volpe, A., Pulitzer, J. F., Rossi, M., & Ciaramella, M. (1992) *J. Bacteriol. 174*, 873−882.

Moracci, M., Nucci, R., Febbraio, F., Vaccaro, C., Vespa, N., La Cara, F., & Rossi, M. (1995) *Enzyme Microb. Technol. 17*, 992−997.

Nucci, R., Moracci, M., Vaccaro, C., Vespa, N., & Rossi, M. (1993) *Biotechnol. Appl. Biochem. 17*, 239−250.

Nucci, R., D'Auria, S., Febbraio, F., Vaccaro, C., Morana, A., De Rosa, M., & Rossi, M. (1995) *Biotechnol. Appl. Biochem. 21*, 265−274.

Pearl, L. H., Hemmings, A. M., Nucci, R., & Rossi, M. (1993) *J. Mol. Biol. 229*, 561−563.

Pearson, W. R., & Lipman, D. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2444−2448.

Pisani, F. M., Rella, R., Raia, C. A., Rozzo, C., Nucci, R., Gambacorta, A., De Rosa, M., & Rossi, M. (1990) *Eur. J. Biochem. 187*, 321−328.

Pucci, P., & Sepe, C. (1988) *Biomed. Environ. Mass Spectrom. 17*, 287.

Sanderson, I., Aguilar, C., Moracci, M., Ciaramella, M., Nucci, R., Rossi, M., & Pearl, L. H. (1997) *J. Mol. Biol.* (in press).

Staedtler, P., Hoenig, S., Frank, R., Withers, S. G., & Hengstenberg, W. (1995) *Eur. J. Biochem. 232*, 658−663.

Street, I. P., Kempton, J. B., & Withers, S. G. (1992) *Biochemistry 31*, 9970−9978.

Wakarchuk, W. W., Greenberg, N. M., Kilburn, D. G., Miller, R. C., Jr., & Warren, R. A. G. (1988) *J. Bacteriol. 170*, 301−307.

Wiesmann, C., Beste, G., Hengstenberg, W., & Schulz, G. E. (1995) *Structure 3*, 961−968.
Wilfried, G. B., Voorhorst, W. G., Eggen, R. I., Luesink, E. J., & De Vos, W. M. (1995) *J. Bacteriol. 177, 7105*−7111.
Withers, S. G., & Aebersold, R. (1995) *Protein Sci. 4*, 361−372.
Withers, S. G., Rupitz, K., & Street, I. P. (1988) *J. Biol. Chem. 263*, 7929−7932.
Withers, S. G., Warren, R. A. J., Street, I. P., Rupitz, K., Kempton, J. B., & Aebersold, R. (1990) *J. Am. Chem. Soc. 112*, 5887−5889.
Zhang, J., & Aronson, A. I. (1994) *Gene 140*, 85-90.

BI962496W