Identification of an Essential Tyrosyl Residue in the Binding Site of *Schizophyllum commune* Xylanase A†

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ABSTRACT: Ultraviolet difference spectroscopy studies with the *Schizophyllum commune* xylanase in the presence of inhibitors and substrates indicated the participation of one or more tyrosyl residues in the binding of substrates to xylanase. Chemical modification experiments with group-specific reagents in the absence and presence of substrates confirmed the essential role of a tyrosyl residue in substrate binding while discounting the participation of tryptophan. A fourth-derivative absorbance spectroscopic method was developed to facilitate the quantitation of modified tyrosyl and tryptophanyl residues. This analysis showed that two tyrosyl residues of the xylanase are modified by tetranitromethane in the absence of substrate with the concomitant loss of catalytic activity. Protection of the xylanase with xylooligosaccharides resulted in the nitration of only one residue, and such enzyme derivatives retained 94% catalytic activity. Differential modification of the xylanase with tetranitromethane generated an enzyme derivative with the characteristic absorbance at 428 nm of 3-nitrotyrosine. Amino acid analysis and N-terminal sequencing of peptides with strong absorbance at 428 nm isolated from the protease-digested modified enzyme by reverse-phase HPLC identified the essential residue as Tyr97. Alignment of the *S. commune* xylanase amino acid sequence with those of the 18 other known family G xylanases revealed that Tyr97 is a conserved aromatic residue, further suggesting its essential role in substrate binding.

Amino acids with aromatic side chains (Tyr and Trp) have been shown to play a prominent role in carbohydrate binding proteins in general (Vyas, 1991) for a variety of reasons, including the strong hydrogen-bonding characteristics of the phenolic hydroxyl of Tyr and the capacity of Trp and Tyr to act as docking residues. In this situation, the hydrophobic patch of a sugar, resulting from the disposition of the equatorial and axial hydroxyls to one side of the pyranose ring of a sugar monomer, aligns itself upon binding with the aromatic ring face to contribute to selectivity of fit of the substrate to the binding site of the enzyme (Quiocho, 1986). Tryptophan has been shown to be essential for substrate binding in most of the glycoside hydrolases studied to date, including lysozyme (Imoto *et al.*, 1972; Hayashi *et al.*, 1965), glucoamylases (Clarke & Svensson, 1984; Ohnishi & Hiromi, 1976), cellulases (Clarke, 1987; Clarke & Yaguchi, 1986), and xylanases (Deshpande *et al.*, 1990; Keskar *et al.*, 1989; Hoebler & Brillouet, 1984; Kubacková *et al.*, 1978). In other cases, such as various α- and β-amylases (Hoschke *et al.*, 1980; Connellan & Shaw, 1970), Tyr residues have been shown to play a more predominant role in substrate binding. With β-galactosidase, not only are aromatic residues involved in substrate binding, but a Tyr residue is thought to participate as the proton donor in the double-displacement mechanism of action (Ring & Huber, 1990).

The white-rot fungus (basidiomycete) *Schizophyllum commune* produces a full complement of cellulolytic and hemicellulolytic enzymes, including a very active xylanase (Jurášek & Paice, 1988). This and other xylanases (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) from different sources have recently attracted considerable attention in view of their potential in various industrial processes, including the biobleaching of pulp used in the manufacture of paper (Wong & Saddler, 1993). Xylanase A from *S. commune*, a family G glycosidase (Gilkes *et al.*, 1991), has been shown to catalyze the hydrolysis of xylan with the retention of anomeric configuration (Gebler *et al.*, 1992b). Chemical modification studies with carbodiimides have indicated that acidic residues are involved in catalysis (Bray & Clarke, 1990), and Glu78 was identified recently by differential chemical modification studies as the nucleophile involved in the double-displacement mechanism of action of the enzyme (Bray & Clarke, 1994). In addition, the action pattern of xylooligosaccharide hydrolysis by xylanase A suggests that the binding site cleft spans about seven xylose units (*i.e.*, seven subsites) and that the catalytic site is located asymmetrically within them (Bray & Clarke, 1992). In our continuing effort to characterize the structure and function relationship of xylanase A and to acquire thereby information for the protein engineering of the enzyme, we describe here investigations on the nature of the amino acid residues involved with the binding of substrate.

MATERIALS AND METHODS

Materials. *N*-Acetylimidazole (NAI),[1] *N*-acetyl-L-tryptophan ethyl ester (Ac-Trp-OEt), *N*-acetyl-L-tyrosine ethyl ester (Ac-Tyr-OEt), *N*-bromosuccinimide (NBS), guanidine-

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HCl (ultrapure), 2-hydroxy-5-nitrobenzyl bromide (HNB), iodoacetamide, oat spelts xylan, tetranitromethane [$C(NO_2)_4$] and D-xylose were purchased from Sigma Chemical Co. (St. Louis, MO). Pierce (Rockford, IL) supplied trifluoroacetic acid, while sequencing grade trypsin was obtained from Boehringer-Mannheim Canada (Laval, PQ). Bio-Rad (Richmond, CA) supplied AG 501-X8 ion-exchange resin, Bio-Gel P6-DG, sodium dodecyl sulfate, molecular mass markers, *N,N,N′,N′*-tetramethylethylenediamine, and methylenebis(acrylamide). All other chemicals used were purchased from Fisher Scientific (Toronto, ON) and were of analytical grade. NBS was recrystallized from water prior to use. Xylooligosaccharides and purified xylopentaose were prepared from oat spelts xylan and characterized by high-performance anion-exchange chromatography as previously described (Bray & Clarke, 1992).

Isolation and Purification of Xylanase A. S. commune strain Delmar (ATCC 38548) cultures were maintained at 4 °C on either plates or slants of potato dextrose agar (Difco Laboratories, Detriot MI). Medium for xylanase production was as described by Jurášek and Paice (1988), and the enzyme was purified to homogeneity from the ethanol precipitate of the crude culture filtrate by gel filtration and anion-exchange chromatography as previously described (Bray & Clarke, 1990) with one modification. Following the final chromatography of the xylanase on Bio-Gel P60, any residual pigment material was removed by chromatography on a 1 × 40 cm column of AG 501-X8 mixed-bed ion-exchange resin using water as eluent at a flow rate of 60 mL h^{-1}. The void volume of the column containing the xylanase was lyophilized and stored at −20 °C until required.

Analytical Methods. Xylanase activity was routinely measured by the arsenomolybdate method of Nelson and Somogyi (Nelson, 1944; Somogyi, 1952) using 0.5% oat spelts xylan in 50 mM sodium acetate, pH 5.0, as recently described (Bray & Clarke, 1994). Concentrations of xylanase were determined by amino acid analysis using a Beckman System Gold amino acid analyzer [Beckman Instruments (Canada) Ltd., Mississauga, ON] assuming His + Lys = 8 residues (Oku *et al.*, 1993). Protein samples to be analyzed were hydrolyzed with 200 μL of 5.7 M HCl in sealed, evacuated tubes at 110 °C for 22 h. Total protein was alternatively determined using the method of Bradford (1976) using bovine serum albumin for standardization. Amino acid sequencing was performed with a MilliGen/Biosearch 6600 ProSequencer system with an On-Line PTH analysis system (Millipore, Mississauga, ON) according to the manufacturer's instructions. Peptide samples were covalently coupled to Sequelon-AA (aryl amine) membranes (Millipore) via their free carboxyl groups. SDS−PAGE was performed according to the method of Laemmli (1970) using 10.0% (w/v) polyacrylamide.

Difference UV Absorbance Spectroscopy. Difference absorption spectra of xylanase were obtained in the absence and presence of either xylose or a mixture of xylooligosaccharides using a Beckman DU-8 recording spectrophotometer at pH 6.0 and 20 °C. A 50 μM solution (490 μL) of xylanase A in 25 mM sodium acetate buffer, pH 6.0, was placed in one chamber of a dual-chamber cuvette, and an identical volume of either 1.5% xylo-oligomer solution or 50 mM xylose in the same buffer was placed in the other chamber of the cuvette. Following the acquisition and storage of a baseline scan from 310 to 250 nm, the chambers were gently mixed together and scans were taken repeatedly. To identify residues perturbed by the ligands, scans of the enzyme and ligand above were compared to scans of the model compounds *N*-acetyl-L-tryptophan ethyl ester (Ac-Trp-OEt) and *N*-acetyl-L-tyrosine ethyl ester (Ac-Tyr-OEt) perturbed by 40% dioxane [Δε values of maxima as a function of dioxane concentration are linear between 0% and at least 50% dioxane for these model compounds (Clarke & Yaguchi, 1986)]. The molar absorption coefficients of Ac-Trp-OEt and Ac-Tyr-OEt at 282 nm were taken as 5550 and 1340, respectively (Herskovits & Sorensen, 1968).

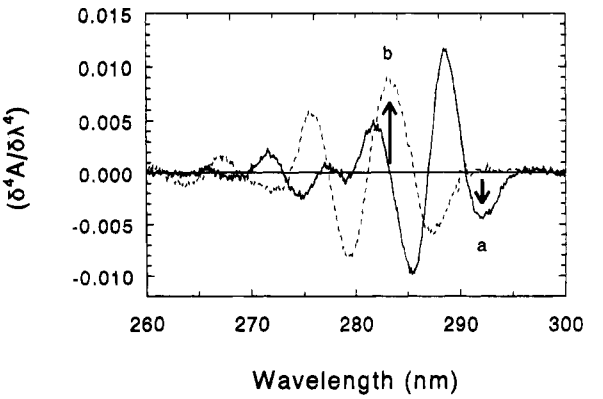

FIGURE 1: Quantitation of Trp and Tyr residues in proteins by fourth-derivative absorbance spectroscopy. Illustrated is the overlap of the fourth-derivative spectra of Ac-Trp-OEt (—) and Ac-Tyr-OEt (- - -) in 6 M Gdn showing (a) the Trp-unique trough at A_{292} and (b) the peak overlapping the two signatures at A_{282}. The measured amplitudes are indicated by arrows. Scans were performed at 120 nm min^{-1} with a fourth-derivative $\delta\lambda$ of 6.0 nm. [Adapted from Bray and Clarke (1994)].

Fourth-Derivative Spectroscopy. Fourth-derivative spectra ($\delta^4A/\delta\lambda^4$) were produced on a Beckman DU-70 spectrophotometer with a built-in derivative mode [based on the least-squares method of Savitsky and Golay (1964); $\delta\lambda$ = 6.0]. All samples for spectroscopic analysis were placed in 6 M guanidine-HCl (Gdn) in 50 mM sodium acetate, pH 5.0, to eliminate environmental heterogeneity of aromatic residues.

Quantitation of both Trp and Tyr residues in various enzyme derivatives was performed by the fourth-derivative UV spectroscopic method recently developed and described by us (Bray *et al.*, 1994). Briefly, the concentration of Trp is determined directly from the fourth-derivative spectrum of an enzyme sample by measuring the depth of the Trp-specific trough at 292 nm [$(\delta^4A/\delta\lambda^4)_{292}$] (Figure 1) and multiplying by the predetermined molar absorptivity coefficient, $(A_M{}^4)_{292}$, which under the conditions employed was 27.5. The number of Trp (N_{Trp}) present in a protein may then be estimated by the equation

$$N_{Trp} = (\delta^4A/\delta\lambda^4)_{292}(A_M{}^4)_{292}C_p{}^{-1} \quad (1)$$

where C_p is the protein concentration. The concentration of Tyr is calculated by first subtracting the contribution of Trp to the peak occurring at the overlap between the Tyr and Trp fourth-derivative signatures at 282 nm according to eq 2:

[1] Abbreviations: Ac-Trp-OEt, *N*-acetyl-L-tryptophan ethyl ester; Ac-Tyr-OEt, *N*-acetyl-L-tyrosine ethyl ester; $C(NO_2)_4$, tetranitromethane; Gdn, guanidine-HCl; HNB, 2-hydroxy-5-nitrobenzyl bromide; HPLC, high-performance liquid chromatography; NAI, *N*-acetylimidazole; NBS, *N*-bromosuccinimide.

$$(\delta^4A/\delta\lambda^4)_{282\text{Tyr}} = \{(\delta^4A/\delta\lambda^4)_{282}\text{total} - [(A_M{}^4)_{282\text{Trp}}N_{\text{Trp}}C_p{}^{-1}]\} \quad (2)$$

The sample is then repeatedly titrated with a known quantity of Ac-Tyr-OEt and corrected for Trp contribution after each addition using eq 2. A plot of $(\delta^4A/\delta\lambda^4)_{282\text{Tyr}}$ *vs* (concentration of Tyr added)/C_p yields the number of Tyr residues in the sample protein as its negative x-intercept.

Chemical Modification of Trp. To assess the possible role of Trp residues in the enzymatic function of xylanase A, the specific Trp-modifying reagents *N*-bromosuccinimide (NBS) and 2-hydroxy-5-nitrobenzyl bromide (HNB) were used.

(A) NBS. Oxidation of the 3-methylindole group of Trp to 3-methyloxindole with NBS was routinely performed by titrating 0.5 mL of a 5.1−10.2 μM solution of enzyme in 25 mM sodium acetate, pH 4.8 or 5.5, with 10-μL aliquots of 0.7 mM NBS (freshly made up in the same buffer) at 20 °C. At each titration point, a 10-μL aliquot was removed from the reaction, quenched in 40 μL of 15 mM L-Trp in 50 mM sodium acetate, pH 6.0, and assayed for residual activity. The titration was repeated until absorbance rose significantly above initial levels (see Results) at 280 nm. Numbers of Trp oxidized were calculated using both the normal absorbance method of Spande and Witkop (1967) and the fourth-derivative UV absorbance method described above.

(B) HNB. Modification of xylanase A with HNB was performed essentially as described in the method of Poulos and Price (1971). A 5.1 μM xylanase solution (200 μL) in 25 mM sodium acetate, pH 5.5, was treated with 10 μL of a 50 mM solution of HNB (in dry acetone). The reaction solution was mixed with a vortex mixer and allowed to react for 5 min at 20 °C. A 10-μL aliquot was then removed from the reaction mixture, diluted into 40 μL of 25 mM sodium acetate, pH 6.0, and assayed for residual activity. This titration was repeated six times. To assess the effectiveness of HNB under various pH conditions, 5 μL of a 20.5 μM xylanase solution was added to 45 μL of 50 mM sodium acetate, pH 4.7, 5.7, or 6.5. After the reaction mixtures had been treated with 5 μL of a 200 mM solution of HNB in dry acetone and incubated at 20 °C for 10 min, 50 μL of each was removed and assayed directly for residual activity.

Chemical Modification of Tyr. (A) N-Acetylimidazole. *O*-Acetyl-Tyr derivatives of xylanase A were generated using *N*-acetylimidazole (NAI) as a modifying reagent according to the method of Riordan and Vallee (1972). An enzyme solution (2−5 μM) in 100 μL of 10 mM potassium phosphate buffer, pH 6.5, was modified by adding dry NAI directly to the reaction tube and incubating at 20 °C for 50−135 min. Aliquots (10 μL) were removed at time intervals, diluted into 50 μL of 50 mM sodium acetate buffer, pH 5.0, and then assayed for residual activity.

(B) Tetranitromethane. Nitration of Tyr residues was performed using tetranitromethane [$C(NO_2)_4$] as described by Sokolovsky *et al.* (1966). Aliquots (20 μL) of a 20 mM stock solution of $C(NO_2)_4$ in 95% ethanol were added to 0.8-mL samples of xylanase A (48 μM) in 100 mM Tris-HCl, pH 8.0, in a cuvette and incubated at 25 °C for 300 min. To modify the xylanase in a greater excess of $C(NO_2)_4$ (approximately 100-fold over enzyme concentration), reactions were performed as above except 20 μL of a 60 mM stock of $C(NO_2)_4$ was added to 0.8 mL of a 24.05 μM enzyme solution. The number of modified Tyr residues was estimated spectrophotometrically using the ϵ_{428} value of 4100 M^{-1} cm^{-1} for 3-nitrotyrosine (Sokolovsky *et al.*, 1966). At appropriate intervals, 10 μL of the reaction mixture was removed, diluted in 40 μL of 100 mM sodium acetate buffer, pH 5.0, and assayed for activity by the DNSA assay as described. Control solutions were identical except for the addition of $C(NO_2)_4$.

Identification of a Functional Tyr Residue in Xylanase A. The conditions for nitration of xylanase A with $C(NO_2)_4$ were essentially as described above. An initial modification with $C(NO_2)_4$ was performed by adding 10 μL of a 50 mM solution of $C(NO_2)_4$ to 0.8 mL of a 28 μM solution of enzyme in 100 mM Tris·HCl, pH 8.0, preincubated for 0.5 h at 20 °C with 1.5% xylopentaose. The progress of the reaction was followed by monitoring absorbance at 428 nm until no further change was observed. To follow activity loss of the enzyme, 10-μL aliquots were taken periodically, diluted into 40 μL of 200 mM sodium acetate, pH 6.0, and assayed for residual xylanase activity. The sample was then separated from protecting ligand and reaction byproducts by gel filtration chromatography on a 1 × 25 cm column of Bio-Gel P6-DG using water as eluent. The collected fractions containing protein were lyophilized. Approximately one-third of the protein sample was carboxamidomethylated according to the method of Stone and Williams (1993) and then digested at 37 °C for 1 h with 6 μg of trypsin. The remainder of the protein sample was remodified with $C(NO_2)_4$ as above but without added ligand and then lyophilized, redissolved in 200 μL of buffer, and digested with trypsin as above except that 12 μg of protease was added. These proteolytic digests of the nitrated xylanase A were subjected to reverse-phase HPLC on a 4.6 × 25 mm Ultrasphere ODS column (Beckman), previously equilibrated with 0.1% trifluoroacetic acid. Separation of peptides was achieved by application of a linear gradient to 35% acetonitrile in 0.1% trifluoroacetic acid over 80 min at a flow rate of 0.6 mL min^{-1}. Eluents from reverse-phase HPLC were monitored at 215 nm and at either 428 or 350 nm. Peptides with absorbances at 428 (350) nm were pooled, lyophilized, and rechromatographed to apparent homogeneity before they were subjected to recleavage with trypsin and rechromatography, followed by amino acid and sequencing analysis.

RESULTS

Difference Spectroscopy. Figure 2 shows the difference UV absorbance spectrum of xylanase A mixed with 0.75% xylo-oligomers compared with the scans of Ac-Trp-OEt and Ac-Tyr-OEt perturbed with dioxane. The model compounds were not affected by the presence of xylo-oligomers (results not shown), indicating that the profiles in Figure 2 were not a result of nonspecific interactions. The difference scan of xylanase in the presence of 50 mM xylose (data not shown) is virtually identical to the baseline scan shown in Figure 2d, suggesting that the monomer, which is a very weak competitive inhibitor of xylanases (Dekker & Richards, 1975), does not bind sufficiently close to the functional aromatic residues to cause a red shift. However, the difference absorption spectrum of xylanase induced by the xylo-oligomer mixture (Figure 2a), which is rapidly broken down to xylobiose and xylotriose by the enzyme (Bray & Clarke, 1992), resembled the well-known double-peaked Tyr pertubation spectrum (Figure 2b) with peaks near 280, 283, and 285 nm (Donovan, 1969). Comparison of these two

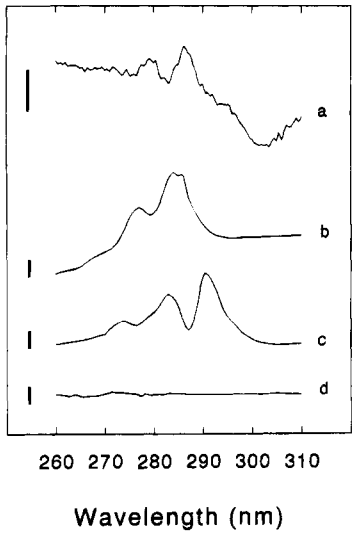

FIGURE 2: Difference ultraviolet absorbance spectra of xylanase A and model compounds. Difference ultraviolet absorbance spectra of (a) xylanase A (50 μM) perturbed with 0.75% xylooligomers and (b) Ac-Tyr-OEt and (c) Ac-Trp-OEt, each perturbed with 40% dioxane, were obtained with a Beckman DU-8 single-beam recording spectrophotometer at pH 6.0 (25 mM sodium acetate) and 20 °C. The baseline scan is shown as profile d. The black bars each represent 0.01 absorbance unit.

spectra revealed a small red shift in the absorption spectrum of the xylanase−substrate complex relative to that of the Tyr model compound, suggesting the participation of the perturbed xylanase Tyr residue(s) in additional hydrogen bonding (Strickland *et al.*, 1972). No significant changes to the scan were observed over the time monitored, indicating that the ratio of xylo-oligomers was relatively unchanged following initial hydrolysis by the xylanase following mixing, at least with respect to the red shift of the binding site Tyr residue(s). Though Trp may be present in the binding site, it does not appear to be perturbed by ligand under the experimental conditions.

Chemical Modification of Trp Residues. NBS was used to probe the xylanase A binding site for Trp residues potentially involved in enzymatic function. Ordinarily, the indole chromophore of Trp, which absorbs strongly at 280 nm, is converted to oxindole by NBS (Spande & Witkop, 1967), enabling quantitation of Trp through progressive decrease of absorbance of the protein sample at 280 nm. Figure 3 shows typical reactions of xylanase A with NBS at pH 5.5 in both the absence and presence of various ligands. In the absence of added ligand, the characteristic drop in 280-nm absorbance is followed by a rise in absorbance at this wavelength which is accompanied by a concomitant rapid drop of xylanase activity to less than 10% of the original activity. Before the rise in A_{280}, the number of Trp residues modified by NBS was calculated to be 2.91 ± 0.14 residues (at an [NBS]:[xylanase] ratio of 16). The rise of absorbance at 280 nm beyond this point renders quantification of Trp by the standard spectrophotometric method of Spande & Witkop (1967) impossible. This NBS-induced rise in absorbance at 280 nm was likely due to the modification of one or more Tyr residues since NBS oxidation of the model compound Ac-Tyr-OEt leads to increases in A_{280} by a factor of 2.97 and in A_{250} by a factor of approximately 14 (Bray *et al.*, 1994; Ohnishi, *et al.*, 1980).

The rise in A_{280} at higher NBS:xylanase concentrations was also observed when the enzyme was pretreated with

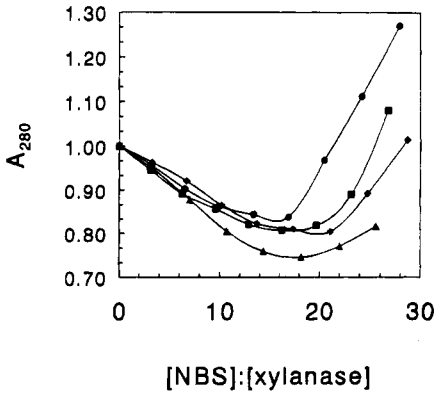

FIGURE 3: NBS oxidation of xylanase A. Enzyme (10 μM) in 50 mM sodium acetate buffer, pH 5.5, was treated with 0.7 mM NBS as indicated in the absence (●) or the presence of (■) 1.2% xylobiose or (◆) 0.3% or (▲) 1.2% xylopentaose. Following 2 min of incubation at 25 °C with each addition, the absorbance at 280 nm was determined. The absorbance data presented has been normalized by equating the original absorbance of each reaction to 1.0.

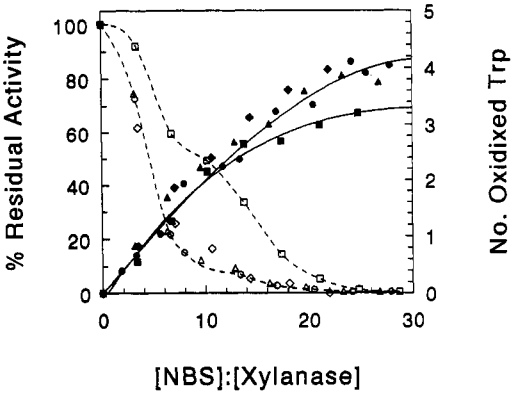

FIGURE 4: Quantitation of *S. commune* xylanase A Trp residues oxidized by NBS. Enzyme (5−10 μM) in 50 mM sodium acetate buffer, pH 5.5, was treated with 0.7 mM NBS as indicated in the absence (●, ○) or the presence of (▲, △) 1.2% xylobiose or (◆, ◇) 0.3% or (■, □) 1.2% xylopentaose. After each addition, the number of residual Trp was determined by fourth-derivative spectroscopy (closed symbols, solid lines), and residual catalytic activity of 10-μL samples, quenched in 1 mM Trp, was determined using soluble oat-spelts xylan in 50 mM sodium acetate buffer, pH 5.0, as substrate (open symbols, dashed lines).

various ligands, although their presence did appear to decrease the extent of this reaction. In particular, the presence of 1.25% xylooligosaccharides [predominantly xylopentaose, xylohexaose, and xyloheptaose (Bray & Clarke, 1990)] did provide significant protection of the Tyr residue(s) responsible for the absorbance increase.

To quantitate the number of Trp and Tyr residues oxidized by NBS during the complete course of the reaction, we developed a fourth-derivative spectrometric method for protein analysis which was shown to be unaffected by either the production of oxindolealanine or the oxidation of Tyr (Bray *et al.*, 1994). Application of this method indicated complete inactivation of xylanase A by NBS at pH 5.5 concurrent with the modification of four of the eight Trp residues present in the enzyme (Figure 4). Fourth-derivative spectroscopy of the NBS-treated xylanase also revealed that 0.97 Tyr residue was oxidized. Analysis by SDS−PAGE (data not shown) confirmed that this NBS-oxidized xylanase derivative appeared as a single band at 22.5 kDa (the approximate molecular mass of xylanase A), precluding the possibility that activity loss was associated with peptide bond

cleavage. Similar results were obtained when the xylanase was preincubated with either 1.2% xylobiose or 0.3% xylopentaose. However, the inclusion of 1.2% xylopentaose prevented the oxidation of one Trp, but while the rate of inactivation was slightly slower compared to that of unprotected enzyme, such xylanase derivatives were still devoid of catalytic activity (Figure 4).

To further probe the role of Trp in xylanase A function, a series of modification reactions were performed with another reagent relatively specific for Trp, HNB. The activity loss of xylanase A when treated with HNB was seen to vary with pH, with a 10.0% loss of residual activity seen at pH 6.5, a 14.7% loss at pH 5.7, and a 25.7% loss at pH 4.7. Extended modification at pH 5.7 indicated that the residual activity leveled off at approximately 75.0% of the original activity. Taken together with the results of the NBS modification, these results suggest that Trp residues may be necessary for stability or structure of the enzyme and may play a minor role in substrate binding, but they do not appear to be essential for activity in xylanase A.

Chemical Modification of Tyr Residues. The results obtained from both the difference spectroscopy studies and the fourth-derivative analysis of NBS modifications strongly suggest that an essential Tyr may be located in a binding subsite of xylanase A. To confirm this and to locate and identify the Tyr residue(s) in the enzyme, chemical modification with the specific Tyr-modifying reagents NAI and $C(NO_2)_4$ was pursued. Reaction of the enzyme at pH 6.5 (phosphate buffer) with 60 mM NAI for an extended period of time (135 min) at 25 °C resulted in the loss of approximately 35% of the original catalytic activity. When the reaction was performed in Tris buffer, enzyme activity initially dropped but then recovered, indicating possible hydrolysis of the acetylated Tyr (Riordan & Vallee, 1967). Whereas these losses of activity were significant, they did not reflect the modification of an essential Tyr residue. Similar conclusions were drawn from initial studies with $C(NO_2)_4$. The use of a 14-fold molar excess of $C(NO_2)_4$ over protein has previously indicated that approximately 0.7 Tyr is modified over a reaction time of 340 min with a concomitant residual activity loss of only 16% (Bray & Clarke, 1990). However, when reacted with >100-fold excess of reagent, approximately 2 Tyr are modified and activity is reduced to less than 5% of the original after 360 min (Figure 5). In the presence of 2.8% xylo-oligomers, 0.7 Tyr residue was nitrated during the same time period, and the enzyme retained greater than 80% of its original catalytic activity. Thus, one Tyr residue that appears essential for activity is protected by ligand from $C(NO_2)_4$ modification. Controls of $C(NO_2)_4$ incubated with xylo-oligomers under the above experimental conditions indicated that $C(NO_2)_4$ does not produce significant background absorbance at 428 nm in the presence of ligand alone.

Identification of the Essential Tyr Residue. To determine the location and possibly the identity of the putative functional Tyr, the enzyme was treated with an excess of $C(NO_2)_4$ in the presence of 2.8% xylo-oligomers. The protecting ligand was removed by chromatography over Bio-Gel P6-DG, and the resulting sample was divided into two aliquots; one was reduced and alkylated prior to tryptic digestion, while the second was remodified with $C(NO_2)_4$ in the absence of added ligand. In the presence of 2.8% xylo-oligomers in the initial modification with $C(NO_2)_4$, one Tyr was nitrated, while no activity loss could be detected

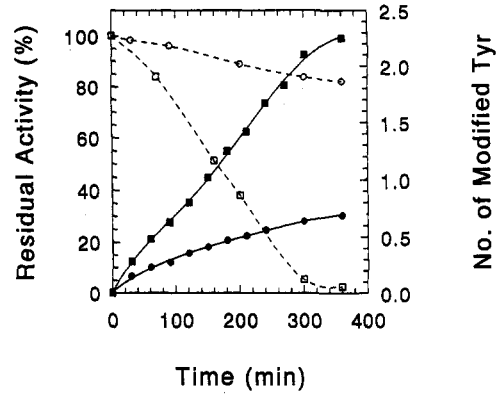

FIGURE 5: Tetranitromethane modification of xylanase A. Xylanase A (24 μM) in 100 mM Tris·HCl, pH 8.0, in the (■, □) absence or the presence (●, ○) of 2.8% xylo-oligomers was treated with a 100-fold excess of $C(NO_2)_4$ over protein at 25 °C. The number of Tyr residues modified/molecule (closed symbols) was estimated spectrophotometrically using the ϵ_{248} value of 4100 M^{-1} cm^{-1} for 3-nitrotyrosine. Residual catalytic activity (open symbols) was determined and expressed as a percentage of a control.

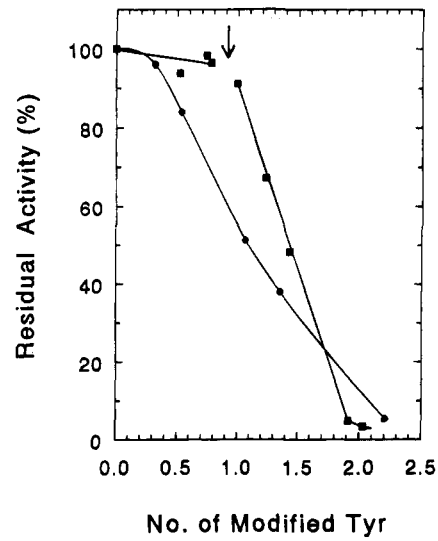

FIGURE 6: Preparation of tetranitromethane-modified xylanase A. Xylanase A (28 μM) in 100 mM Tris·HCl, pH 8.0, in the presence of 2.8% xylopentaose (w/v) was treated with a 100-fold excess of $C(NO_2)_4$ over protein. The progress of the reaction was followed by monitoring both the number of Tyr modified and the loss of catalytic activity as described in the caption to Figure 5. The sample was subjected to gel filtration chromatography on Bio-Gel P6-DG to remove ligand and byproducts. One-third of the protein was prepared for proteolyis (reduction and carboxamidomethylation) and peptide mapping, while the remainder was remodified with $C(NO_2)_4$ (arrow) as above but without added ligand. The inactivated and modified xylanase was also prepared for proteolysis and peptide mapping. As a control for the $C(NO_2)_4$ modification, a sample of xylanase A without prior addition of ligand (●) was treated with $C(NO_2)_4$ as described above.

(Figure 6). Upon removal of ligand and remodification with $C(NO_2)_4$, a second Tyr was modified with the concomitant loss of >95% catalytic activity. Figure 7A shows a reverse-phase HPLC profile of the reduced and alkylated xylanase A after remodification with $C(NO_2)_4$. The peptides showing strong 350-nm absorbances were rehydrolyzed with trypsin in an attempt to create smaller peptides, but no significant change was noticed in the subsequent mobility of the peptides on reverse-phase chromatography. A typical profile showing the two peptides with absorbance at 350 nm after redigestion with trypsin is shown in Figure 7B. It was apparent from amino acid analysis, however, that prior to redigestion

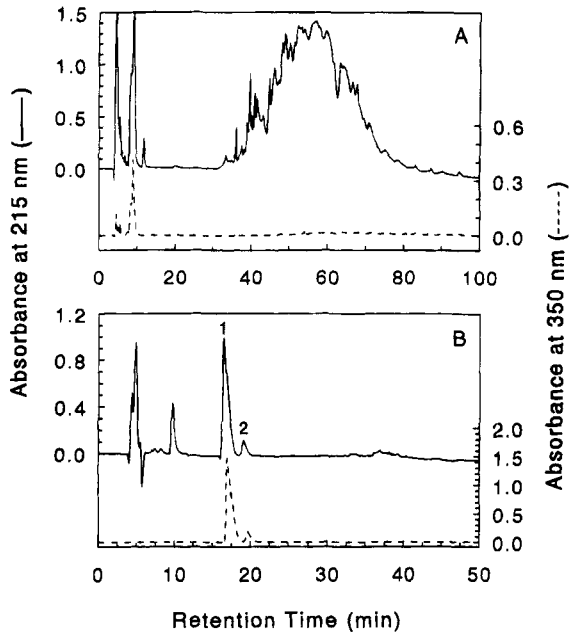

FIGURE 7: Reverse-phase HPLC separation of tryptic peptides of tetranitromethane-modified xylanase A. (A) Reduced and carboxamidomethylated xylanase, previously treated with $C(NO_2)_4$, was digested at 37 °C with trypsin for 1 h, and the resulting peptides were separated by reverse-phase HPLC (Beckman Ultrasphere ODS, 4.6 × 25 mm). The column was eluted with a linear gradient of 0−35% (v/v) acetonitrile in 0.1% trifluoroacetic acid over 80 min at a flow rate of 0.6 mL min^{-1}. The effluent was monitored at both 215 and 350 nm. Peptides with absorbances at 350 nm were pooled and lyophilized. (B) Rechromatography of isolated peptides to apparent homogeneity by reverse-phase HPLC as above, except with application of a linear gradient of 0−10% (v/v) acetonitrile in 0.1% trifluoroacetic acid over 60 min.

Table 1: Amino Acid Composition of Peptides from Tryptic Digests of $C(NO_2)_4$-Nitrated Xylanase A[a]

	peptide 1				peptide 2	
	B		A			
amino acid	pmol	ratio[b]	pmol	ratio[b]	pmol	ratio[b]
aspartic acid	339	2.37 (1)[c]	35.2	1.46 (1)	415	1.07 (1)
threonine	35.8	0.25 (0)	4.8	0.02 (0)	39.7	0.10 (0)
serine	247	1.73 (4)	88.4	3.67 (4)	1340	3.47 (4)
glutamic acid	360	2.52 (0)	1.3	0.05 (0)	726	1.87 (0)
glycine	1054	7.37 (1)	29.2	1.21 (1)	904	2.33 (1)
alanine	382	2.67 (2)	45.3	1.88 (2)	590	1.53 (2)
valine	n.d.[d]	0 (0)	4.1	0.17	n.d.	0 (0)
methionine	n.d.	0 (0)	n.d.	0 (0)	n.d.	0 (0)
isoleucine	22.6	0.15 (0)	n.d.	0 (0)	n.d.	0 (0)
leucine	28.2	0.19 (0)	n.d.	0 (0)	n.d.	0 (0)
tyrosine	82.9	0.58 (2)	2.6	0.11 (2)	303	0.78 (1)
phenylalanine	n.d.	0 (0)	n.d.	0 (0)	n.d.	0 (0)
lysine	143	1.00 (1)	24.1	1.00 (1)	387	1.00 (1)
histidine	n.d.	0 (1)	17.1	0.71 (1)	189	0.49 (1)
arginine	8.6	0.06 (0)	n.d.	0 (0)	n.d.	0 (0)

[a] Samples of peptide 1, before (B) and after (A) rechromatography, and peptide 2 were hydrolyzed in 200 μL of 5.7 M HCl for 24 h, *in vacuo*. Amino acid analysis was performed by cation-exchange chromatography using a Beckman System Gold amino acid analyzer. [b] Based on lysine = 1.0. [c] Values in brackets represent expected amino acid ratios based on composition of identified peptide. [d] n.d., none detected.

peptide 1 was contaminated with other peptides (Table 1), possibly as a result of its proximity to the void volume of the column during elution. Amino acid analysis revealed that the peptides with high 350-nm absorbances (peptides 1 and 2 of Figure 7B) were core peptides, the more predominant peptide 1 corresponding to Tyr94−Arg123 and peptide 2 being one residue shorter than peptide 1, corresponding to Gly95−Arg123. Nitrotyrosine was visible in amino acid analyses of both peptides (eluting after Phe on the HPLC profile). Sequencing studies performed on duplicate samples confirmed the identities of the $C(NO_2)_4$-modified peptides. An N-terminal sequence of Tyr-Gly-Ser-(Tyr-NO_2) was obtained for peptide 1, and Gly-Ser-(Tyr-NO_2) was obtained as the N-terminal sequence of peptide 2. These data indicate that the functional Tyr modified by $C(NO_2)_4$ and protected by substrate in xylanase A is Tyr97, a highly conserved aromatic residue in the family G carbohydrases (Figure 8). Peptides corresponding to Tyr94−Arg123 and Gly95−Arg123 isolated from the digests of the protein modified by $C(NO_2)_4$ in the presence of substrate did not show absorbance at 350 nm.

DISCUSSION

This study has shown that inactivation of *S. commune* xylanase A can be achieved by the nitration of a Tyr residue. By differential labeling in combination with peptide mapping, Tyr97 has been identified as an essential residue at the active site of this enzyme. Tyr97 is a highly conserved aromatic amino acid residue among the 19 known amino acid sequences of the family G glycosidases. This residue is in close proximity to Glu87 (Oku *et al.*, 1993), the amino acid which was previously identified as the catalytic nucleophile in the mechanism of action of the enzyme (Bray & Clarke, 1994). Secondary structure predictions indicate that Tyr97 appears at the C-terminal end of the β-sheet containing the catalytic Glu (Bray & Clarke, 1994). The X-ray crystal structure of this xylanase is unknown, but the enzyme−substrate complex of another family G xylanase, the *Bacillus circulans* xylanase, has recently been determined to 1.8-Å resolution using a catalytically incompetent mutant (Wakarchuk *et al.*, 1994). These studies suggest that, rather than the homologous Tyr88, two other Tyr residues, Tyr69 and Tyr80 (homologous to Tyr78 and Tyr89, respectively, in *S. commune* xylanase A), function in substrate binding. However, xylotetraose was used to form the enzyme−substrate complex in the X-ray crystallographic studies, whereas in our current experiments, protection from both $C(NO_2)_4$ nitration and NBS oxidation was provided only by xylopentaose and higher xylooligosaccharides. These substrates are rapidly hydrolyzed to xylobiose and xylotriose (Bray & Clarke, 1992), and studies on the action pattern of xylooligosaccharide hydrolysis predict that these ligands would predominantly occupy subsites −II, −III, and −IV (Bray, 1993). It is thus tempting to speculate that one of these subsites comprises in part Tyr97. With the X-ray crystallographic studies involving a catalytically incompetent enzyme mutant, the xylotetraose was shown to span the catalytic site and thereby occupy subsites −II, −I, I, and II. Thus, it would appear that Tyr97 (and presumably Tyr88 of the *B. circulans* enzyme) is located in a binding subsite further removed from the catalytic carboxyl groups (acid−base catalyst and nucleophile-stabilizing anion), presumably subsite −III or −IV. This view is further supported by the UV absorbance spectra induced by xylose and xylo-oligomers.

The characteristic absorption spectrum obtained for xylanase A by perturbation with xylo-oligomers indicated that the perturbed Tyr residue(s) located in the binding site cleft becomes more hydrophobic. The small red shift of the maxima at approximately 280 and 287 nm relative to the

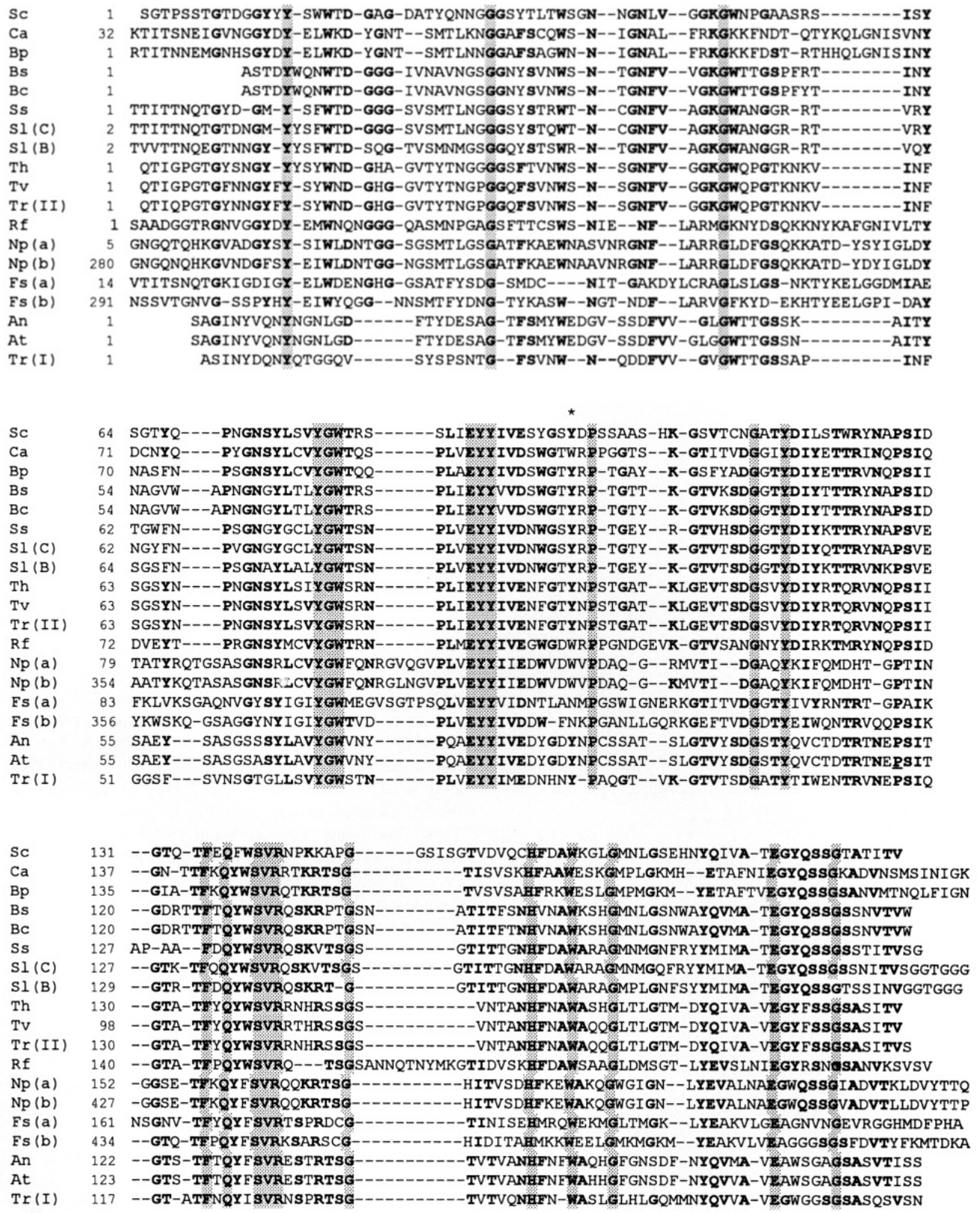

FIGURE 8: Alignment of known amino acid sequences of family G glycosidases. The substrate-binding Tyr97 of *S. commune* xylanase A (Sc) is identified by the asterisk. Residues in bold type indicate homology among at least 10 of the 19 aligned sequences, while shaded residues identify complete conservation. The sequences correspond to the xylanases from the following: Ca, *Clostridium acetobutylicum* (Zappe *et al.*, 1990); Bp, *Bacillus pumilus* (Fukasaki *et al.*, 1984); Bs, *Bacillus subtilis* (Paice *et al.*, 1986); Bc, *Bacillus circulans* (Yang *et al.*, 1988); Ss, *Streptomyces* sp. 36a (Nagashima *et al.*, 1989); Sl(C) and Sl(B), *Streptomyces lividans* xylanases C and B, respectively (Shareck *et al.*, 1991); Th, *Trichoderma harzianum* (Yaguchi *et al.*, 1992b); Tv, *Trichoderma viride* (Yaguchi *et al.*, 1992a); Rf, *Ruminococcus flavefaciens*;[2] Np(a) and Np(b), *Neocallimastix patriciarum*, domains A and B, respectively (Gilbert *et al.*, 1992); Fs(a) and Fs(b), *Fibrobacter succinogenes* S85, domains A and B, respectively (Paradis *et al.*, 1993); An, *Aspergillus niger awamori* (Maat *et al.*, 1992); At, *Aspergillus tubigensis* (de Graff *et al.*, 1992); Tr(I) and Tr(II), *Trichoderma reesei* xylanases I and II, respectively (Torronnen *et al.*, 1992).

Tyr model compound (maxima at 275, 283, and 286 nm), in addition to the reduction of the band at 283 nm, may reflect the formation of new hydrogen bonds involving the Tyr residue(s) (Strickland *et al.*, 1972). Alternatively, the

formation of the enzyme−substrate complex may disrupt the intrinsic hydrogen-bonding pattern involving the phenolic hydroxyl (Wetlaufer, 1962). The apparent trough centered around 300 nm, rather than a peak, precludes an increase in polarizability of the Tyr residue(s) (Strickland *et al.*, 1972). Xylose appears neither to interact directly with the Tyr residue nor to induce sufficient conformational changes to perturb it, suggesting that the monomer binds in a subsite some distance from the Tyr residue.

That Tyr97 is likely positioned in a subsite not directly adjacent to the catalytic acid residues precludes the possibility that it plays a more active role in the mechanism of substrate hydrolysis, as has been suggested for Tyr503 of *Escherichia coli lacZ* β-galactosidase, Tyr48 of *Aspergillus niger* glucoamylase, Tyr298 of *Agrobacterium faecalis* β-glucosidase, or Tyr80 of *B. circulans* xylanase. In the former case, Tyr503 has been identified as the proton donor (acid−base catalyst) in the S_N2 mechanism (Ring & Huber, 1990), although this has recently been disputed (Gebler *et al.*, 1992a). Tyr48 of the *A. niger* glucoamylase is situated near the endocyclic oxygen of a substrate glucopyranosyl residue bound to subsite 1 and has been proposed to stabilize the formation of the oxocarbonium ion transition-state complex (Stoffer *et al.*, 1994). Tyr298 of *A. faecalis* β-glucosidase has been postulated to modulate the pK_a of the catalytic nucleophile, Glu170 (Wang & Withers, 1994). With *B. circulans* xylanase, Tyr80 appears to hydrogen bond to Glu172, the putative acid−base catalyst, and thereby contribute to its correct positioning for catalysis (Wakarchuk *et al.*, 1994). While this latter role cannot be excluded for Tyr97, it is more likely that this residue plays a critical function in positioning the substrate within the binding cleft of *S. commune* xylanase through stacking interactions and/or hydrogen bonding. The inductive effect of a substituent nitro group *ortho* to the phenolic hydroxyl would lower the pK_a of the hydroxyl from approximately 10.3 to 7.3, resulting in the nitrated Tyr being partially ionized at physiological pH (Lundblad & Noyes, 1984). This effect may critically disrupt function of a binding residue in the tight confines of the binding cleft.

NAI did not prove to be an effective reagent for the inactivation of xylanase A, which may reflect the environment of Tyr97. NAI is quite polar and has been shown to preferentially modify exposed surface Tyr residues (Myers & Glazer, 1971), whereas the relatively nonpolar $C(NO_2)_4$ typically reacts with buried residues. The observations made with these two reagents would thus be consistent with Tyr97 being located in the relatively hydrophobic environment of a substrate binding cleft. The partial loss of activity associated with NAI may simply be a result of modification of exposed Tyr residues, causing subtle conformational changes and/or steric interactions that impede the approach of substrate.

Attempts to oxidize Trp residues in xylanase A with NBS resulted in a rise in the 280-nm absorbance of the sample, which has been seen to occur upon modification of Tyr (Ohnishi *et al.*, 1980). By application for the first time of the recently developed fourth-derivative spectroscopic method for Trp and Tyr quantitation, this complex situation was resolved. The process of xylanase A inactivation with NBS appeared to occur by the slow modification of approximately three Trp followed by both the oxidation of a further Trp and one Tyr and a rapid activity loss that is enhanced by raising the pH, a condition known to decrease the specificity of NBS in terms of its reaction with other amino acids (Inokuchi *et al.*, 1982). This evidence, together with the relative lack of activity loss in xylanase A modified with HNB, suggests that Trp does not play an essential role in the catalytic processes of xylanase A. Nevertheless, one Trp residue can be protected from oxidation, indicating that it may also participate in stacking interactions with substrate. Attempts have been initiated to identify this Trp, which may prove to be the highly conserved Trp18 since, with the *B. circulans* xylanase, a stacking interaction of the homologous Trp9 with the face of the xylose ring occurs in the substrate−enzyme complex (Wakarchuk *et al.*, 1994).

[2] EMBL database accession number Z11127 (J. Zhang and H. J. Flint).

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