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INFLUENCE OF ACARBOSE AND MALTOSE ON THE REACTIVITY OF INDIVIDUAL TRYPTOPHANYL RESIDUES IN GLUCOAMYLASE FROM ASPERGILLUS NIGER

by

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Tryptophanyl residues of A. niger glucoamylase G2 (EC 3.2.1.3) involved in substrate and inhibitor binding have been identified following N-bromosuccinimide (NBS) treatment in the presence and absence of protective ligands. Appropriate proteolytic cleavages of the glucoamylase derivatives enabled isolation of individual peptide fragments containing the 15 tryptophan positions and the extent of tryptophan oxidation was measured employing normal and 2nd derivative UV-spectrophotometry.

Trp-52, -141, -156, -228, -317, and -335 remained unoxidized while complete oxidation of Trp-6, -28, and -466 and partial oxidation of Trp-170 and -178 was observed after NBS-treatment whether ligands were added or not. Trp-212, -417, and -437 were partially converted in uncomplexed glucoamylase while the presence of either the substrate maltose or the inhibitor acarbose prevented the oxidation of these residues. Trp-120, required for maintaining the active catalytic site, was protected by acarbose only, and its oxidation did not prevent ligand binding. The functional roles of Trp-212, -417, -437, and -120 are discussed.

NBS-treatment of the acarbose-protected large form of glucoamylase, G1, destroyed its unique capacity to adsorb onto starch granules while catalytic activity was preserved towards soluble substrates. This effect could be correlated with the oxidation of Trp-590 and -615 located near the COOH-terminus.

The reactivities, probably reflecting the degree of solvent exposure, were also assessed for individual tyrosyl residues in G2.

The thermal stability of oxidized, catalytically active G1 and G2 was remarkably low as compared to the unmodified forms.

Abbreviations: G1 and G2 designate the larger and the smaller forms of glucoamylase from A. niger (29,43); CM- = S-carboxymethyl-; NBS = N-bromosuccinimide; nW_{ox} = number (n) of oxidized tryptophanyl residues per enzyme molecule; 2-pe- = 2-pyridylethyl-; RP-HPLC = reversed-phase high performance liquid chromatography; Tris = 2-amino-2(hydroxymethyl)- 1,3-propandiol; * signifies glycosylation.

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1. INTRODUCTION

Glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) releases glucose from the non-reducing ends of starch and related substances (32). Two forms of glucoamylase, termed G1 and G2, are produced by the black mold Aspergillus niger (20, 29). The amino acid sequence of the G2 form is identical to residues 1 through 512 (40) of the peptide chain of G1, which is 616 residues long (5, 42). This suggested G2 to be generated by limited proteolysis of G1 (12, 40). G1 is functionally distinct from G2 by its capacity to adsorb onto and to degrade starch granules (43, 45). Thus, the COOH-terminal segment of the G1 is likely to play an important role in the interaction with raw starch.

Fungal glucoamylases possess an extended substrate binding area with six or seven subsites accomodating the glucose residues of linear substrates and the catalytic site located between subsites 1 and 2 (13, 36). Substrate initially binds loosely to subsite 2 via the glucose residue at the non-reducing end, it then shifts to subsite 1 in a unimolecular rearrangement process to form a tight productive complex with concomitant quenching of tryptophan fluorescence and subsequent release of the terminal glucose residue (13, 26, 27).

In the A. niger glucoamylase-ligand complexes, one and two tryptophanyl residues appear to be in contact with bound maltose and acarbose, respectively (7). The first of these residues, which probably is located in subsite 1, was protected against NBS-oxidation by a variety of inhibitors and substrates, including soluble starch, whereas only acarbose protected two residues. The derivative having one protected tryptophan retained the ligand binding capacity, although it was catalytically inactive, while the derivative with two tryptophans protected remained active. The second tryptophanyl residue, which presumably is responsible for maintenance of the structural integrity of the active site, has recently been identified as Trp-120 in G2 (8) and is probably located in the fourth subsite (7, 8, 35).

In the present study the susceptibility of all tryptophanyl residues in glucoamylase G2 to NBS was determined in both the absence and presence of ligands. Three residues, not including Trp-120, appeared jointly to participate in ligand binding. They may be located in subsite 1. A study of the NBS-oxidation of G1 was also conducted to determine the role of tryptophanyl residues in raw starch-binding, and it was observed that oxidation significantly reduces the thermal stability of both G1 and G2.

2. MATERIALS

Glucoamylase G1 and G2 were purified as previously described from a commercial preparation of A. niger glucoamylase (AMG 200L) which was a gift from Novo Industries, Bagsvaerd, Denmark (43).

Acarbose (a pseudotetrasaccharide and potent inhibitor of glucoamylase (44)) was generously supplied by Drs. E. TRUSCHEIT and D. SCHMIDT (Bayer AG, Wuppertal, F.R.G.). Raw corn starch was a gift of Dr. B.S. ENEVOLDSEN (Department of Brewing Chemistry, Carlsberg Research Laboratory). Reagents, proteolytic enzymes, solvents, and chromatographic gels used for modification and fragmentation of proteins, sequence analysis and isolation of peptides/glycopeptides were obtained from the sources mentioned previously (7, 8, 40-42).

3. METHODS

3.1. Preparation of oxidized forms of glucoamylase

G1 and G2 were treated with NBS in the absence and presence of either acarbose (0.15 mM) or maltose (56 mM) as described (7). Ligands and excess reagents were removed by dialysis against 0.1 M-sodium acetate pH 4.3; the acarbose-enzyme complex was first dissociated by dialysis against 1.7 M-Tris-HCl pH 7.6. The enzyme derivatives were subsequently purified by affinity chromatography on acarbose-Sepharose (8). Preparations to be used for the identification of oxidized amino acid residues were finally dialyzed against water and lyophilized, while those designated for investigations of thermostability and raw starch-binding were dialysed against 0.1 M-sodium acetate pH 4.3 and 0.1 M-sodium acetate pH 3.6, respectively. These derivatives were either used immediately or stored at -18 °C.

3.2. Preparation and purification of peptide fragments

NBS-oxidized glucoamylase was reduced and carboxymethylated (14, 43) or 2-pyridylethylated (10, 41). Portions of 30-100 mg of CM-G2 were digested with trypsin followed by fractionation on Bio-Gel P-100 (8, 41). The peptide pools obtained were further purified by affinity chromatography on Con A-Sepharose (41) or by RP-HPLC (8, 22, 40, 41). 2-pe-G2 replaced CM-G2 for preparation of Asn(161)-Arg(194) and Ala(195)-Arg(241) in reasonable yields (41). These fragments were subfragmented with α-chymotrypsin and subsequently subjected to RP-HPLC (40). The large, highly glycosylated tryptic fragment T2-1 (40, 41) (0.9 mg × ml^{-1}) was digested with S. aureus V8 protease, using a substrate to enzyme mass ratio of 40:1 in 0.1 M-ammonium bicarbonate pH 7.8 at room temperature for 3 h, and the resulting fragments (5 mg) were separated by gel filtration on Bio-Gel P-60 (1.5 × 90 cm) in 0.2 M-ammonium bicarbonate. Tryptic fragments from the COOH-terminal region of G1 have been prepared as earlier published (41). The peptide nomenclature is as reported in refs. (40-42).

3.3. Raw starch-binding

The binding of G1 and the derivative G1-6W_{ox} to starch granules was measured according to SAHA and UEDA (33). Raw corn starch was washed with 0.1 M-sodium acetate pH 3.6 and aliquots (900 µl) containing 0.1 g of starch were added to enzyme solutions (100 µl) to give final protein concentrations of 0.02-1.2 mg × ml^{-1}. After gentle shaking for 20 min at 4 °C, the mixtures were centrifuged and the fraction of unadsorbed protein was determined from the UV-absorbance of the supernatant at 280 nm.

3.4. Thermostability

Aliquots from stock solutions of G1, G2 and the corresponding catalytically active, oxidized forms (100 µl), were added to preheated 0.1 M-sodium acetate pH 4.3 (900 µl, final protein concentration: 9 µM) and incubated for 5 min at different temperatures followed by quick freezing in dry-ice/ethanol. Later all samples were thawed, preequilibrated for 5 min at 25 °C and then 30 mM-maltose (1 ml) was added to measure the remaining activity (43). The progress of inactivation at constant temperatures within the range 50-70 °C was followed by analysis of enzymic activity in aliquots removed at appropriate time intervals. The apparent first order rate constant, k, was determined for the initial part of the inactivation and the activation energy, E_a, was calculated from the Arrhenius equation, $K = Aexp(-E_a/RT)$.

3.5. UV-Spectrophotometry

The extent of tryptophan oxidation in isolated tyrosine containing peptides could be determined with good precision from measurement of tryptophan contents by 2nd derivative spectrophotometry (8, 37). In fragments containing tryptophan and/or oxindolealanine but no tyrosine and in 2-pyridylethylated fragments the fraction of oxidized tryptophan, W_{ox}, was determined by a modification of the method of SPANDE and WITKOP (38) according to the equation:

$$W_{ox} = \frac{E_{unox} - E_{obs}}{E_{unox}} \times 1.31$$

where E_{unox} is the absorbance at 280 nm calculated for the corresponding unoxidized fragment from the concentration (determined by amino acid analysis) using $\varepsilon_{280} = 5500\ M^{-1} \times cm^{-1}$ for a tryptophanyl and $\varepsilon_{280} = 1450\ M^{-1} \times cm^{-1}$ for a tyrosyl residue. E_{obs} is the actual absorbance of the isolated fragment at 280 nm. The empirical factor 1.31 described by SPANDE and WITKOP (38) corrects for the oxindolealanine absorbance at 280 nm. In cases where tryptophan is absent (demonstrated by 2nd derivative spectroscopy) oxindolealanine was determined spectrophotometrically employing $\varepsilon_{250} = 6600\ M^{-1} \times cm^{-1}$ (8).

3.6. Analytical procedures

Concentrations of G1 and G2 were determined spectrophotometrically using $\varepsilon_{280} = 1.37 \times 10^5$ and $1.09 \times 10^5\ M^{-1} \times cm^{-1}$, respectively (7). Concentrations of the oxidized forms were determined by amino acid analysis. The enzymic activity was assayed by hydrolysis of maltose (15 mM) (43). Amino acid analysis of acid hydro-

lysates of peptides and protein derivatives was carried out as reported (41-43). NH_2-Terminal sequencing in the presence of Polybrene was performed using a Beckman 890 C Sequencer (15, 18). The phenylthiohydantoin-amino acids were analyzed by RP-HPLC (39). UV-spectra were recorded using a Cary 219 while a Varian 2200 spectrophotometer was employed for 2nd derivative UV-spectrophotometry.

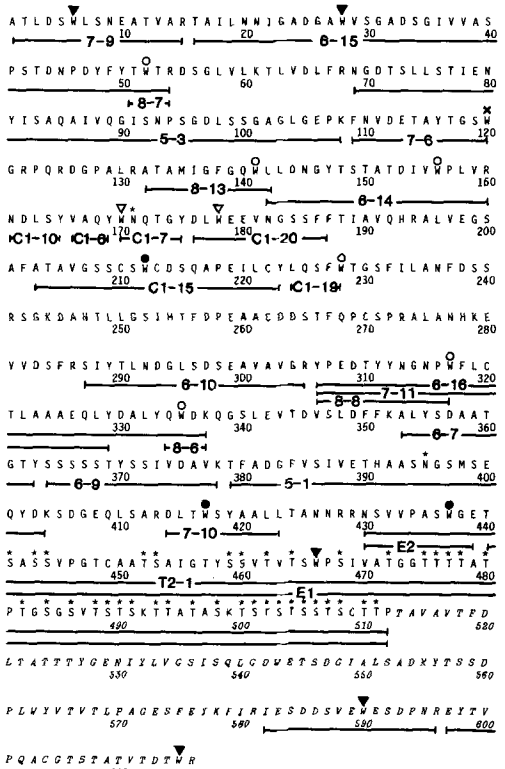

Figure 1. Reactivity of tryptophanyl residues in glucoamylase from A. niger. ▼ Indicates residues fully oxidized and ▽ residues partially oxidized irrespective of the presence of ligands. × Indicates the residue protected by acarbose only, ● residues protected both by maltose and acarbose, and ○ residues inaccessible to NBS-oxidation. Asterisks designate glycosylated positions. From the COOH-terminal segment specific to G1 (shown in italics) two tryptic fragments were prepared and purified as described (41). The glucoamylase sequence was reported in refs. 5, 40, and 42.

4. RESULTS

4.1. Identification of tryptophanyl and oxindolealanyl residues in NBS-treated glucoamylase

Glucoamylases G1 and G2 show very similar behaviour in interactions with inhibitors and soluble substrates (7, 30, 43). Since peptide fragments were not easily recovered from the region from Leu-521 to Lys-555 which is only present in G1 (see Figure 1) (42), the main study of the tryptophan reactivities was done on the smaller G2 molecule terminated by Pro-512 (40).

The isolated tryptophan containing peptides are indicated in the glucoamylase sequence in Figure 1. Their amino acid compositions, RP-HPLC retention times and NH_2-terminal sequence analyses are described in Table I. The strategy chosen for polypeptide cleavage ensured that the tryptophan positions were all in separate fragments. The RP-HPLC chromatograms published in ref. 40 were used as guide for isolation of short and medium size tryptic fragments, plus chymotryptic subfragments, which represented the total of residues 1 through 429 of G2 (40). The large COOH-terminal fragment, T2-1 = Asn(430)-Pro(512), was purified (40, 41) and subsequently digested by S. aureus V8 protease to the subfragments Asn(430)-Glu(439)(E2) and Thr(440)-Pro(512)(E1), containing Trp-437 and -466, respectively (Figure 1; Table I).

The oxidized peptides eluted during RP-HPLC with practically identical retention times as their tryptophan containing counterparts described elsewhere (40). Nevertheless, quantitative analysis of all tryptophan positions in derivatives of a protein as large as G2 is hardly feasible. Thus, RP-HPLC has the drawback that all peptides are not isolated in similar yields. Moreover, in practice the yields were difficult to estimate for certain G2 fragments because they, as earlier demonstrated, eluted at more than one position in gel filtration and/or in RP-HPLC (40). Furthermore, the high trypsin:substrate ratio needed for generation of exclusively soluble G2 fragments resulted in further degradation of most of the tryptic fragments comprising the tryptophan positions. Minor deviations also existed between the RP-HPLC patterns of frag-

ments from oxidized (not shown) and unoxidized G2 (40). Such differences appear to stem from spontaneous cleavage of peptide bonds containing the α-carbonyl group of oxindolealanine and from altered protease susceptibilities. Direct sequencing of oxidized G2 confirmed the first point for positions 6, 28, 120, and 466 in 10-30% of the protein molecules. Only oxindolealanine was found at these four positions (Tables I and II) both by spectrophotometric analysis of isolated tryptic fragments, which corresponded to fragments generated also from unoxidized G2 (40), and indirectly by sequencing following acid-promoted complete cleavage of peptide bonds involving oxindolealanine (Table II). In summary, based on the amount of CM- or 2-pe-G2 subjected to tryptic digestion, the G2 tryptophan positions were found in peptide fragments isolated in roughly estimated recoveries of 30-80%. The single exception was Trp-170 in Trp(170)-Tyr(175) (C1-7, Figure 1) which was obtained in only 10% yield. Substantial release of tryptophan/oxindolealanine probably had taken place from position 170, since, in accordance with hydrolysis of both peptide bonds in the tripeptide Tyr-Trp-Asn(171) (see Figure 1), Val(166)-Trp(170) was not detected and Asn(171)-Tyr(175) was recovered in a reasonable yield of 40%. In conclusion, we have been able to isolate the 15 tryptophanyl positions and we suggest, in spite of the complications mentioned above, that the contents of oxindolealanine and tryptophan in the analyzed peptides reliably reflect the extent of oxidation for the individual positions in the various derivatives of glucoamylase.

The differing NBS-susceptibility of tryptophans in acarbose-protected G2 (G2-4W_{ox}), maltose-protected G2 (G2-5W_{ox}) and unprotected G2 (G2-6W_{ox}) identify the positions affected by the ligand binding. Earlier results implied that maltose protects a single tryptophanyl residue in A. niger glucoamylases against oxidation (7). In contrast, it is found in the present study that maltose has prevented the partial oxidation observed with uncomplexed G2 at three positions, viz. Trp-212, -417, and -437 (Table II). Acarbose also protected these three residues and in addition the essential Trp-120 (8). None of the ligands interfered with the oxidation of Trp-6, -28, -170, -178 and -466. Trp-52, -141, -156, -228, -317, and -335 remained unoxidized both in the presence and absence of ligands (Table II). The limited accessibility of Trp-170 and -178 (see Table II) could arise from the proximity of the carbohydrate unit on Asn-171 (41). The reactivity of other residues is probably dependent on the folding of the polypeptide chain. In the presence of 8 M-urea apparently 14 out of the 15 tryptophans in G2 undergo oxidation.

For glucoamylase G2 oxidized under non-denaturing conditions in the absence of ligands (G2-6W_{ox}, Table II) the fractions of oxindolealanine formed at the 9 susceptible tryptophan positions represent a total of 6 oxindolealanyl residues per molecule, which is in excellent agreement with the extent of oxidation calculated from the UV-spectrum of NBS-treated G2 (7, 38). The earlier reported contribution of oxidized tyrosyl residues (see 4.2) to spectral changes (25) was thus insignificant in the case of glucoamylase.

G1 has four more tryptophans than G2, located in the COOH-terminal segment (see Figure 1). NBS-treatment of G1, in the presence and absence of various protective ligands, resulted in a series of derivatives which contained two more oxindolealanyl residues in comparison to the corresponding derivatives of G2 (7). Investigations of the tryptic fragments from the COOH-terminal region of oxidized G1 revealed complete conversion to oxindolealanine at both positions 590 and 615 in acarbose-protected, enzymically active as well as in fully oxidized, inactive G1 (Figure 1 and Table I).

4.2. Identification of the oxidized tyrosyl residues in NBS-treated glucoamylase

The tyrosyl content was found by amino acid analysis of oxidized G1 and G2 to be reduced by 4 and by 3 residues, respectively. Apparently this loss of tyrosine was independent of ligands added prior to the NBS-treatment (7) and examination of peptides from the fully oxidized G2 (Table III) revealed the complete loss of a single specific residue together with minor losses for most of the tyrosyl residues. The tyrosine lost was identified as Tyr-329 by comparison of the

TABLE I

Amino acid compositions of tryptophan and oxindolealanine containing peptide fragments from oxidized glucoamylase

	Residues/peptide										
Peptide (n in G2-nW_{ox})	A(1)-R(15)[a] (6)	T(16)-Y(50)[a] (6)	T(16)-R(54)[a] (4)	T(51)-R(54)[a] (6)	F(109)-W(120)[a] (6)	A(132)-L(142)[a] (6)	L(142)-R(160)[a] (6)	W(170)-Y(175)[b] (6)	D(176)-F(187)[b] (6)	A(203)-Y(223)[b] (6)	A(203)-R(241)[b] (5)
Amino acid											
Aspartic acid	2.2 (2)	6.5 (7)	6.8 (7)	-	1.8 (2)	-	2.9 (3)	1.1 (1)	2.0 (2)	1.0 (1)	3.3 (3)
Threonine	1.9 (2)	1.2 (2)	3.3 (4)	1.8 (2)	1.7 (2)	0.9 (1)	2.6 (3)	1.0 (1)	-	0.9 (1)	1.9 (2)
Serine	1.7 (2)	4.1 (4)	4.2 (4)	-	1.3 (1)	-	1.2 (1)	-	1.8 (2)	3.1 (4)	6.2 (8)
Glutamic acid	1.0 (1)	-	-	-	0.9 (1)	1.2 (1)	-	0.9 (1)	2.0 (2)	1.8 (2)	3.5 (3)
Proline	-	1.8 (2)	1.6 (2)	-	-	-	0.7 (1)	-	-	0.8 (1)	1.3 (1)
Glycine	-	4.2 (4)	4.2 (4)	-	0.9 (1)	2.2 (2)	1.0 (1)	1.1 (1)	1.1 (1)	1.0 (1)	2.4 (2)
Alanine	2.8 (3)	5.0 (5)	4.8 (5)	-	1.0 (1)	1.9 (2)	1.4 (1)	-	-	2.5 (3)	3.9 (4)
Valine	1.0 (1)	2.6 (3)	3.0 (3)	-	0.8 (1)	-	1.3 (2)	-	1.0 (1)	0.8 (1)	1.0 (1)
Methionine	-	-	-	-	-	0.7 (1)	-	-	-	-	-
Isoleucine	-	2.4 (3)	2.5 (3)	-	-	0.9 (1)	0.6 (1)	-	-	0.9 (1)	1.9 (2)
Leucine	2.2 (2)	1.4 (1)	1.5 (1)	-	-	1.0 (1)	3.0 (3)	-	1.2 (1)	1.2 (1)	3.4 (3)
Tyrosine	-	1.7 (2)	1.7 (2)	-	0.8 (1)	-	0.5 (1)	0.9 (1)	-	0.7 (1)	0.8 (1)
Phenylalanine	-	0.9 (1)	0.8 (1)	-	0.7 (1)	1.0 (1)	-	-	1.6 (2)	-	2.9 (3)
Lysine	-	-	-	-	-	-	-	-	-	-	-
Arginine	0.8 (1)	-	0.8 (1)	1.0 (1)	-	-	0.8 (1)	-	-	-	0.9 (1)
Half-cystine	-	-	-	-	-	-	-	-	-	2.0[e](3)	1.2[e](3)
Tryptophan	0[h] (1)	0[gh] (1)	1.0[gh](2)	1.0[g](1)	0[h] (1)	1.0[g](1)	1.0[g](1)	0.4[gh](1)	0.7[g](1)	0.6[g](1)	2.0[g](2)
Oxindolealanine	1.0[g]	1.0[g]	-	-	1.0[g]	-	-	-	-	-	-
G2-4W_{ox}				-	0.9W;0.8Y			0.4W;1.0Y	0.8W	-	
G2-5W_{ox}				1.1W	0W;0.8Y			-	-	1.0W;0.7Y	
G2-6W_{ox}				-	-			-	-	-	
Bio-Gel P100[i]	7	6	5	8	7	8	6	1	1	1	1
RP-HPLC peak[k]	7-9	6-15	6-15	8-7	7-6	8-13	6-14	C1-7	C1-20	C1-15	C1-23
Elution conditions[l]	25.0	53[m]	52[m]	12.9	15.5	24.8	26.9	11.2	27.5	19.9	30
Peptide recovery[n] (%)	21	22	11	55	70	33	22	10	27	23	15
Cycles sequenced[o]	2	-	14	-	5	2	-	-	-	2	-
N-Terminal yield[p] (%)	62	-	74	-	55	60	-	-	-	25	-

Peptide (n in G2-nW_{ox})	L(224)-W(228)[b] (6)	Y(306)-Y(329)[a] (6)	Y(306)-K(337)[a] (6)	Q(334)-K(337)[a] (6)	D(414)-L(423)[a] (6)	N(430)-E(439)[c] (4)	N(430)-P(512)[a] (4)	T(440)-P(512)[c] (6)	I(582)-R(596)[d] (G1-8W_{ox})	E(597)-R(616)[d] (G1-8W_{ox})
Amino acid										
Aspartic acid	-	3.1 (3)	4.5 (5)	1.1 (1)	1.0 (1)	0.9 (1)	1.1 (1)	-	3.7 (4)	1.3 (1)
Threonine	-	1.7 (2)	2.0 (2)	-	0.9 (1)	-	22.2(24)	22.8(24)	-	4.8 (6)
Serine	0.9 (1)	-	-	-	0.9 (1)	2.2 (2)	16.6(20)	14.3(18)	2.9 (3)	1.2 (1)
Glutamic acid	1.0 (1)	2.5 (3)	4.0 (4)	1.2 (1)	-	1.0 (1)	1.3 (1)	-	2.9 (3)	2.0 (2)
Proline	-	1.5 (2)	2.2 (2)	-	-	0.8 (1)	4.5 (5)	3.6 (4)	1.0 (1)	1.0 (1)
Glycine	-	1.4 (1)	1.3 (1)	-	-	1.1 (1)	7.0 (7)	5.6 (6)	-	1.1 (1)
Alanine	-	2.8 (3)	4.0 (4)	-	1.8 (2)	1.2 (1)	8.5 (9)	6.9 (8)	-	2.0 (2)
Valine	-	-	-	-	-	1.7 (2)	5.5 (7)	3.7 (5)	1.1 (1)	1.2 (2)
Methionine	-	-	-	-	-	-	-	-	-	-
Isoleucine	-	-	-	-	-	-	1.6 (2)	1.1 (2)	1.0 (1)	-
Leucine	1.0 (1)	2.9 (3)	4.4 (4)	-	2.8 (3)	-	-	-	-	-
Tyrosine	-	2.6 (4)	3.6 (5)	-	1.0 (1)	-	1.0 (1)	1.0 (1)	-	0.8 (1)
Phenylalanine	0.9 (1)	1.0 (1)	0.9 (1)	-	-	-	-	-	-	-
Lysine	-	-	0.9 (1)	0.9 (1)	-	-	2.0 (2)	2.2 (2)	-	-
Arginine	-	-	-	-	-	-	-	-	0.9 (1)	0.8 (1)
Half-cystine	-	0.3[f](1)	0.4[f](1)	-	-	-	1.3[f] (2)	1.0[f](2)	-	0.5[f](1)
Tryptophan	1.0[g](1)	1.0[h](1)	2.0[h](2)	1.0[g](1)	0.9[h](1)	1.0[g](1)	1.1[gh](2)	0[h] (1)	0[h] (1)	0.2[h](1)
Oxindolealanine	-	-	-	-	-	-	-	1.0[g]	0.9[g]	0.8[g]
G2-4W_{ox}	1.0W			1.0W;0.9Y		-	-	0.W;0.9Y		
G2-5W_{ox}	1.0W			1.0W;0.9Y		0.9W	0.9W;1.0Y	-		
G2-6W_{ox}	-			-		-	0.4W;1.0Y	-		
Bio-Gel P100[i]	1	7	6	8	7	E-2[j]	2	E-1[j]	7	7
RP-HPLC peak[k]	C1-19	7-11	6-16	8-6	7-10	-	-	-	-	-
Elution conditions[l]	22.3	31.0	32.5	12.6	28.1	-	-	-	10.6	13.4
Peptide recovery[n] (%)	80	30	14	42	55	-	-	-	-	-
Cycles sequenced[o]	-	-	3	-	3	-	20	-	-	-
N-Terminal yield[p] (%)	-	-	100	-	35	-	80	-	-	-

No corrections were made for incomplete release or destruction of amino acids in the 24 h hydrolysates. a) Generated by tryptic digestion of CM-G2-nW_{ox}; b) chymotryptic subfragment of the tryptic fragments N(161)-R(194) or A(195)-R(241) from 2-pe-G2-nW_{ox}; c) obtained by S. aureus V8 protease digestion of the glycosylated region, N(430)-P(512); d) tryptic fragment from the COOH-terminal part of G1-8W_{ox} (41); e) as 2-pe-cysteine; f) as CM-cysteine; g) determined by UV-spectrophotometry (see 3.5); h) determined by 2nd derivative UV-spectrophotometry (see 3.5); i) elution position (pool number) in Bio-Gel P-100 chromatography (see ref.41); j) pool number in Bio-Gel P-60 chromatography (see 3.2); k) peak nomenclature in G2 fingerprints (see ref. 40); l) 1-propanol (vol.pct.) at the elution position from J.T. Baker Wide-Pore C_{18} column; m) CH_3CN (vol.pct.) at the elution position from Waters Radial-Pak C_{18} column; n) yield of fragment based on the amount of alkylated G2 employed for digestion. The yields of additional fragments containing the same tryptophan position are not included; o) number of cycles identified by automated sequencing; p) NH_2-terminal phenylthiohydantion-amino acid/peptide (molar ratio).

Table II. Effect of acarbose and maltose on the reactivity of tryptophans in G2 to NBS

Position in the sequence	Fraction of tryptophan intact			Experimental evidence
	G2-4W_{ox} (acarbose)	G2-5W_{ox} (maltose)	G2-6W_{ox} (no ligand)	
6	0	(0)	0	4W_{ox} (a,b); 6W_{ox} (a,b,c)
28	0	(0)	0	4W_{ox} (a,b,d); 6W_{ox} (b,c)
52	1.0	1.0	1.0	4W_{ox} (d,e); 5W_{ox} and 6W_{ox} (c)
120	1.0	0	0	4W_{ox} and 5W_{ox} (c,d); 6W_{ox}(b,c,d)
141	(1.0)	(1.0)	1.0	6W_{ox} (c)
156	(1.0)	(1.0)	1.0	6W_{ox} (c)
170	0.4	(0.4)	0.4	4W_{ox} and 6W_{ox}(c)
178	0.8	(–)	0.7	4W_{ox} and 6W_{ox} (c)
212	(1.0)	1.0	0.6	5W_{ox} (c); 6W_{ox} (c,d)
228	1.0	1.0	1.0	4W_{ox}, 5W_{ox} and 6W_{ox} (c)
317	(1.0)	(1.0)	1.0	6W_{ox} (c,d)
335	(1.0)	(1.0)	1.0	6W_{ox} (c)
417	1.0	1.0	0.9	4W_{ox}, 5W_{ox} and 6W_{ox} (c,d)
437	1.0	0.9	0.4	4W_{ox}, 5W_{ox} and 6W_{ox} (a,c,d)
466	0	0	0	4W_{ox} (b,d); 5W_{ox} (c,d); 6W_{ox} (b,c,d)

The values in parentheses were deduced from results obtained for G2 oxidized to a lower and higher degree. Peptides comprising the positions are further described in Table I and indicated in the sequence shown in Figure 1.
a) Automated sequencing of the position in question (see Table I); b) cleavage of the peptide bond containing the α-carbonyl group of oxindolealanine as detected by direct sequencing of the G2 derivative after redissolution in 70% trifluoroacetic acid; c) UV-spectrophotometry (see Table I); d) 2nd derivative UV-spectrophotometry (see Table I); e) COOH-terminal sequencing utilizing carboxypeptidases B and Y in combination (42).

Table III. Recovery of tyrosine in peptide fragments from NBS-oxidized G2

Fragment	Tyrosine(s) in the sequence	Tyrosine(s) recovered
Thr(16)-Tyr(50)	2	1.9
Asn(69)-Lys(108)	1	1.0
Phe(109)-Trp(120)	1	0.8
Leu(142)-Arg(160)	1	0.5
Asn(161)-Tyr(165)	1	0.5
Val(166)-Tyr(169)	1	0.9
Asn(171)-Tyr(175)	1	0.9
Ala(203)-Tyr(223)	1	0.8
Ser(287)-Arg(305)	1	1.0
Tyr(306)-Trp(317)	3	2.6
Tyr(306)-Tyr(329)	4	2.7
Tyr(306)-Lys(337)	5	3.6
Ala(353)-Tyr(363)	2	1.7
Ser(364)-Lys(378)	1	1.0
Thr(379)-Lys(404)	1	0.9
Asp(414)-Leu(423)	1	0.7
Asn(430)-Pro(512)	1	1.0

The fragments have been prepared from G2-6W_{ox} and the tyrosine contents were determined by amino acid analysis. The fragments are indicated in Figure 1.

tyrosine contents in the peptides Tyr(306)-Tyr(317), Tyr(306)-Tyr(329), and Tyr(306)-Lys(337) (Table III).

4.3. Effect of NBS-treatment on raw starch-adsorption

When G1 was modified by NBS in the presence of acarbose the resulting derivative had 6 oxindolealanyl residues and retained approx. 80% of the original catalytic activity towards maltose (7) but the affinity for raw starch was lost. Thus less than 0.05 mg of G1-6W_{ox} adsorbed, in contrast to 0.8 mg of G1, onto 0.1 g of starch (Figure 2). Provided the tryptophans in the acarbose complexes of G1 and G2 have similar susceptibility to NBS the lack of raw starch-binding capacity can be correlated with oxidation of two additional residues, Trp-590 and -615 (see 4.1), near the COOH-terminus of G1 (Figure 1).

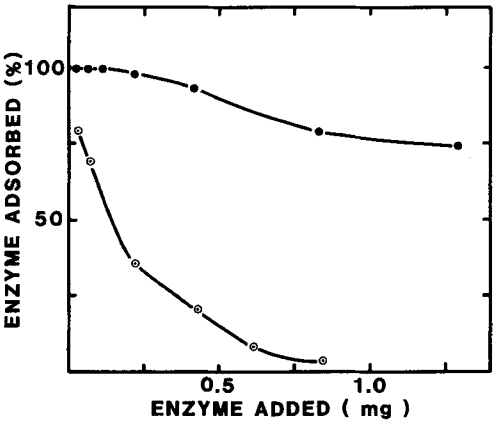

Figure 2. Raw starch-adsorption of glucoamylase G1 (-●-) and oxidized, catalytically active G1-6W_{ox} (-⊙-). The amount of protein bound was calculated by subtraction of the unadsorbed protein from the total amount added to raw starch suspended in 0.1 M-sodium acetate pH 3.6.

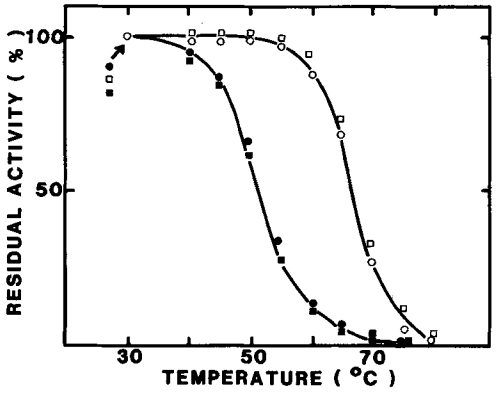

Figure 3. Thermostability of G1 (○) and G2 (□) and the corresponding catalytically active derivatives, G1-6W_{ox} (●) and G2-4W_{ox} (■). The enzymes (9 μM) were incubated at varying temperatures in 0.1 M-sodium acetate pH 4.3 for 5 min and the residual activity determined using maltose as substrate.

4.4. Thermal stability of NBS-treated G1 and G2

The catalytically active G1-6W_{ox} and G2-4W_{ox} were both inactivated considerably faster than G1 and G2 when incubated at temperatures in the range 50-70 °C (Figure 3). The apparent first order rate constants and the calculated activation energies for the denaturation are compared for the unmodified enzymes and their active, oxidized forms (Table IV). The oxidation was found, in spite of retained catalytic activity (7), to reduce the thermal stability of the glucoamylases drastically.

5. DISCUSSION

Previously Trp-120 in glucoamylase G2 from A. niger was shown to be an essential residue, responsible for maintaining the functional integrity of the active site, although its oxidation did not hinder ligand binding to the enzyme (7, 8). Both these experiments and independent studies on the identical enzyme from A. awamori (24, 35) indicated the essential tryptophan to be located at some distance from the catalytic site, possibly in the fourth substrate binding subsite. Figure 4 shows the structural model of the active site of glucoamylase introduced by

Table IV. Effect of NBS-oxidation on the thermostability of glucoamylase

Enzyme	Rate of inactivation, k ($10^3 \times min^{-1}$)	Activation energy, E_a ($kJ \times mol^{-1}$)
G1-6W_{ox}	260	110
G2-4W_{ox}	690	90
G1	6.7	280
G2	5.7	260

The apparent first order rate constant for inactivation, k, was calculated from the loss of enzymic activity at 55 °C during the initial 5-10 min of incubation at a protein concentration of 12 μM in 0.1 M-sodium acetate pH 4.3. The activation energy, E_a, was calculated from the Arrhenius equation using k-values determined at 50, 55, 60, 65, and 70 °C.

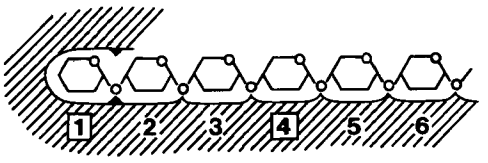

Figure 4. Tentative model of the active site of glucoamylase. The wedges denote the catalytic site; a productive malto-oligosaccharide enzyme-complex is indicated (13, 27). Three and one tryptophanyl residues are suggested to be located in or near subsites 1 and 4, respectively (the present work and ref. 8).

HIROMI and his colleagues (13, 27). Trp-120 belongs to a region of the molecule which shows sequence homology with a glucoamylase from Saccharomyces diastaticus (46). In addition, Trp-52, -170, and -178 of glucoamylase from A. niger are conserved in the corresponding yeast enzyme (41, 46), which points to them as possible candidates in the search for the tryptophanyl residue tentatively associated with subsite 1 and protected by a variety of substrates and inhibitors (7, 13). The results of the present study, however, indicated none of them to be in this site since, whether ligands were present or not, Trp-52 was inaccessible to NBS-oxidation while Trp-170 (adjacent to the glycosylated Asn-171 and therefore unlikely to be located in subsite 1 of an exoglucanase) and -178 were converted to oxindolealanine in 60% and 30% yield, respectively. In contrast, three other residues, identified as Trp-212, -417, and -437, were protected from NBS-oxidation both by maltose and by acarbose. In preparations of fully oxidized G2, which are devoid of ligand binding ability, these three positions are modified to an extent of approx. 40%, 10%, and 60% respectively. Thus, their side chains would not be freely accessible, reflecting perhaps that this part of the substrate binding site forms the end of a pocket or crevice. Since the substrate maltose binds in a productive mode, i.e. in subsites 1 and 2 according to the model in Figure 4, it is likely that Trp-212, -417, and -437 are located in close proximity to one or both of these subsites. Oxidation experiments in the presence of glucose and gluconolactone are consistent with this proposal. Thus glucose, a competitive inhibitor which binds in subsite 2 (27), does not provide any protection from NBS-oxidation whereas gluconolactone, considered to be a transition state analogue located in subsite 1 (13), affords protection and secures an enzyme derivative that, while catalytically inactive, is still able to bind substrates and inhibitors (7).

If Trp-212, -417, and -437 are clustered in subsite 1 it is conceivable that oxidation of one or two side chains sterically hinders the oxidation of the remaining one(s), perhaps through conformational changes. That the formation of oxindolealanine can cause local conformational changes in a substrate binding cleft has been observed with hen-egg white lysozyme. In this case the specific oxidation of Trp-62 induced a shift of the three-dimensional structure which blocked subsite C of the active site cleft such that productive binding is prevented (4). Oxidation of Trp-108 in lysozyme similarly induced a conformational change near the catalytic residue Glu-35 (3, 11).

While the current proposals are consistent with the various observations recorded in this and other studies (7, 13, 16), they do not eliminate the possibility of an alternative explanation. Indeed, the porcine pancreatic α-amylase is known to have an additional sugar binding site located on the surface outside of the active site area (28). Similarly, a second substrate binding region was implicated from studies of barley malt α-amylase subsites in amylose hydrolysis (21). Thus, the three protected glucoamylase G2 residues, Trp-212, -417 and -437, perhaps belong to different ligand binding sites rather than being located in the active site region. Finally, we cannot exclude the possibility that the three tryptophanyl residues, or one or two of them, are also required for catalysis to occur, since derivatives have not been obtained in which Trp-212, -417, and -437 are oxidized while the essential Trp-120 remained intact.

Only the larger form of A. niger glucoamylase, G1, adsorbs onto and digests raw starch (43, 45). This unique capacity is presumably associated with a second binding site on the surface of the enzyme distinct from the active site and probably involves the COOH-terminal segment specific for G1. When G1 was modified by NBS

in the presence of acarbose the raw starch-binding ability was lost in an otherwise catalytically active derivative. Out of the four tryptophans in the COOH-terminal segment we have shown that the two nearest to the COOH-terminus, i.e. Trp-590 and -615, were converted to oxindolealanine. A connection between structural elements from this region in G1 and the affinity for raw starch is therefore strongly suggested and the isolation from A. niger culture liquid of a raw starch-binding inhibitory factor which has a size and amino acid composition similar to the COOH-terminal segment of G1 (34) lends support to this proposal. Further studies are needed to elucidate whether other parts of the G1 molecule participate in the raw starch-binding.

The oxidations caused a destabilization of glucoamylase in the temperature range 50-70 °C. Similarly, NBS-oxidized semi-alkaline proteinase from Aspergillus melleus showed decreased thermal stability (19). A detailed characterization of the inactivated glucoamylases was not attempted in the present study. However, it was found that cleavage of peptide bonds containing the α-carbonyl group of oxindolealanine residues occurred to some degree in the derivatives. Perhaps elevated temperatures, like acidic conditions, facilitated scission of these labile peptide bonds with subsequent unfolding of the protein. Thermal stability of proteins has also been attributed to the presence of salt bridges between charged amino acid residues as well as to hydrophobic interactions among non-polar residues and it has recently been shown that aromatic-aromatic interactions play a major role in the stabilization of protein tertiary structure (6, 31). Furthermore, tryptophanyl residues are known to initiate and stabilize α-helices (1). It is not surprising, therefore, if oxidation of the very hydrophobic tryptophan (9) to the more polar oxindolealanine caused disruption of stabilizing structural elements. In summary, mild oxidation of glucoamylase in the presence of acarbose provides a less thermostable, catalytically active form, but whether the main cause of the inactivation was the chemical modification of certain residues or the peptide backbone cleavage, preceded perhaps by a scrambling of the tertiary structure or by aggregation (2, 17), cannot be decided at the moment.

The present work suggests that Trp-212, -417, and -437 in the glucoamylase from A. niger are jointly associated with ligand binding in subsite 1 while Trp-120, also present in glucoamylase from S. diastaticus (46) and probably present in Taka-amylase A from A. oryzae (8, 23), belongs to the fourth substrate binding subsite (8).

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