SIDE CHAIN REACTIVITIES OF GLUCOAMYLASE G2 FROM ASPERGILLUS NIGER EVALUATED BY GROUP-SPECIFIC CHEMICAL MODIFICATIONS

by

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Treatment of glucoamylase G2 with large excesses of different group specific reagents resulted in modification of 25% of the histidyl, 15% of the tyrosyl, 20–40% of the arginyl, 30–50% of the lysyl and none of the methionyl residues. The modified groups were not critical since the various derivatives possessed from 50% to 100% residual enzymatic activity and retained the thermostability. Carboxamidomethylation occurred specifically at His₂₅₄ with essentially no change of the kinetic parameters for hydrolysis of maltose and starch. Removal of the two *N*-linked sugar units by endoglycosidase H was similarly without effect on activity, thermostability and chemical reactivity of the histidyl residues. H⁺-titration revealed that glucoamylase G2 carries a lower net charge throughout the pH-range 3–11 than predicted from its amino acid composition.

1. INTRODUCTION

Glucoamylase G2 (1,4- α -D-glucan glucohydrolase, EC 3.2.1.3) from *Aspergillus niger* is a highly water-soluble glycoprotein. It comprises the 512 N-terminal residues of the 616 amino acid residues long G1 form and contains the *N*-glycosylated Asn₁₇₁ and Asn₃₉₅ as well as the highly *O*-glycosylated sequence Ser₄₄₁-Thr₅₁₁ (5, 43, 45). The two forms are equally active towards soluble substrates, but only G1 is able to degrade raw starch (47). A number of tryptophanyl residues and carboxyl groups involved in substrate binding and catalysis have been identified by chemical modification and sequencing (8, 9, 42, 46). The related enzymes,

α -amylase and α -glucosidase, in addition possess critical histidyl residues (2, 6, 11, 13, 14, 19, 23, 38), but modification of glucoamylase by diethyl pyrocarbonate (18, 31, 35) has only been found to decrease the affinity for soluble starch (18). In the light of the structural and functional similarities recognized recently for different starch-degrading enzymes (9, 20, 41), the role of histidyl and other side chains was reinvestigated in *A. niger* glucoamylase. The observed poor reactivity of groups normally exposed in native proteins motivated a characterization of the ionization behaviour of glucoamylase G2 by potentiometric and spectrophotometric titration.

Abbreviations: DEP = diethyl pyrocarbonate; EDTA = ethylene diaminetetraacetic acid; G1, G2 = glucoamylase G1 and G2, respectively (5, 43, 45, 47); RP-HPLC = reverse phase high pressure liquid chromatography; Tris = 2-amino-2(hydroxymethyl)-1,3-propanediol.

2. EXPERIMENTAL

2.1. Materials

Glucoamylase G2 was purified as previously described (47) from a commercial preparation from *A. niger* (gift from Novo Industries, Bagsvaerd, Denmark). Diethyl pyrocarbonate, iodoacetamide, iodoacetate, *O*-methylisourea, lactoperoxidase and trypsin were purchased from Sigma Chemical Co., St. Louis, MO; 2,3-butanedione and trinitrobenzene sulfonic acid were from Fluka AG, Buchs, Switzerland, phenylglyoxal and 2-vinylpyridine from Janssen Chimica, Beerse, Belgium, methyl acetimidate from Pierce Chemical Co., Rockford, IL, and succinic anhydride from BDH Ltd., Dorset, England. Endoglycosidase H (EC 3.2.1.96) was from Boehringer Mannheim, Mannheim, FRG. [¹⁴C]iodoacetamide [50-60 mCi/mmol] was supplied by Amersham International, Buckinghamshire, England. Maltose monohydrate, "System Glucose" kit and "Titrisol" 0.1 M NaOH were from Merck, Darmstadt, FRG. Acarbose was a generous gift from Drs. E. TRUSCHEIT, D. SCHMIDT and E. MÖLLER, Bayer AG, Wuppertal, FRG.

2.2. Analytical procedures

Concentrations of glucoamylase G2 were determined spectrophotometrically employing $\epsilon_{280} = 1.09 \cdot 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (8). Amino acid and glucosamine contents were analyzed as earlier described (43-45) and 1-carboxymethyl-histidine, which coeluted with proline, was quantified using the ninhydrin colour yield at 570 nm for serine (10). Peptide sequencing and identification of phenylthiohydantoins were performed on an Applied Biosystems model 470A gas-phase sequencer and a Hewlett-Packard HPLC chromatograph, model 1084B equipped with a NovapakTM C₁₈ column (40).

Glucoamylase activity (47) was determined with maltose (15 mM) or starch (2%) as substrate in 0.1 M sodium acetate pH 4.3 at 25 °C and 37 °C, respectively. k_{cat} and K_m were derived from Eadie-plots of activities against maltose (0.5-4 mM) and soluble starch (0.01-0.8%) at the same temperatures. The thermostability was assessed essentially as described (42) at an enzyme concentration of 0.9 μM . Radioactivity was mea-

sured with an LKB 1219 Rackbeta liquid scintillation counter using Dimilume-30 (United Technologies Packard) as scintillant.

Perturbation of the UV spectrum of glucoamylase G2 (8) obtained by addition of ligand in water (5-10 μl) to a Millipore filtered protein solution was recorded at a scan rate of 0.1 nm $\cdot$ s⁻¹ using a Cary Model 219 spectrophotometer and double-chamber cuvettes.

2.3. Chemical modifications

G2 (1 mg $\cdot$ ml⁻¹ in 0.1 M sodium acetate pH 5.0 or 0.1 M sodium phosphate either pH 6.0 or pH 7.0) was reacted with DEP (0.5-20 mM) for 40-60 min at room temperature (24). The formation of ethoxyformyl-histidine was followed spectrophotometrically at 242 nm using $\Delta\epsilon = 3,200 \text{ cm}^{-1} \cdot \text{mol}^{-1}$ (34). Alternatively, G2 (1 mg $\cdot$ ml⁻¹ in 0.1 M sodium acetate pH 4.3 or 0.1 M sodium phosphate pH 7.0) was incubated with 0.1 M of iodoacetamide (or iodoacetic acid) for 5-6 days at 30 °C in the dark (10, 16). The control contained sodium azide (0.05%). After removal of excess reagent by either dialysis or gel filtration the enzyme was isolated by affinity chromatography on acarbose-Sepharose (9). G2 (3 mg $\cdot$ ml⁻¹ in 0.1 M sodium acetate pH 5) was labelled with [¹⁴C]iodoacetamide (0.1 M, 690 dpm $\cdot$ nmol⁻¹) as above.

G2 (1 mg $\cdot$ ml⁻¹) was iodinated either by the lactoperoxidase reaction at pH 5.0 in 0.1 M sodium acetate (25, 26) or by a 15 fold molar excess of I₂/I⁻ in 0.1 M sodium phosphate, 1 mM KI pH 8.0 at 0 °C (3), followed by dialysis. For nitration (37) G2 (5 mg $\cdot$ ml⁻¹ in 0.2 M sodium borate pH 8.2) was incubated with tetranitromethane (25 mM) at room temperature for 2 h, the derivative was purified by gel filtration followed by affinity chromatography (9). All these reactions took place in the dark.

To modify arginine equal volumes of G2 (1.8 mg $\cdot$ ml⁻¹ in 0.2 M sodium phosphate pH 8) and phenylglyoxal (0.32 M) were mixed (48) and incubated for 1.5 h at 30 °C followed by dialysis. Alternatively, G2 (2 mg $\cdot$ ml⁻¹, in 0.1 M sodium borate pH 8.0) was treated with 2,3-butanedione (0.1 M) (30). The activity was measured after 20 h and the remaining arginine was determined by amino acid analysis on aliquots acidified imme-

diately after removal (30).

Primary amino groups were succinylated at room temperature by addition of succinic anhydride as 7 portions over 60 min to G2 (1 mg · ml⁻¹ in 0.1 M sodium phosphate pH 8) to maintain a 700 fold molar excess (7). The extent of modification was estimated by the trinitrobenzene sulfonic acid method (15) from the decrease in adsorbance at 420 nm compared to a control. The extent of *O*-acylation was not assessed. The amino groups of G2 were also reacted with methyl acetimidate (0.04 M and 3 mg · ml⁻¹ of protein in 0.1 M sodium borate pH 8.0 and 9.0) for 1 h (33), KCNO (0.24 M and 0.8 mg · ml⁻¹ of protein in 0.2 M sodium borate pH 8.0) (39) at 30 °C, and *O*-methylisourea (0.4 M and protein, 3 mg · ml⁻¹, in the pH range 8.5-10.5) at 4 °C for 23 h (21, 29).

In an attempt to oxidize methionines G2 (1.0 mg · ml⁻¹ in 0.5 mM EDTA, 0.1 M sodium citrate pH 3.2) was treated with H₂O₂ (0.74 M) for 3 h at 30 °C. After dialysis against the same buffer containing MnO₂ and then water (32) methionine contents were determined (17, 27).

Enzymatically active *N*-bromosuccinimide oxidized or carbodiimide modified G2 were prepared in the presence of acarbose as earlier described (8, 9, 42, 46).

2.4. Enzymatic deglycosylation

G2 (2.5 mg · ml⁻¹ in 0.1 M sodium citrate pH 5.0, 0.1% sodium azide) was incubated with endoglycosidase H (0.1 units · ml⁻¹) (50) for 48 h at 37 °C. The released carbohydrate was removed by gel filtration on Bio-Gel P-30 in 5 mM sodium acetate pH 4.3.

2.5. Preparation and purification of peptide fragments

[¹⁴C]carboxamidomethyl-G2 prepared at pH 5.0 (see 2.3) or deglycosylated G2 were 2-pyridylethylated and digested with trypsin (43). The resulting fragments were separated on a Bio-Gel P-100 column followed by RP-HPLC using a Wide Pore C₁₈ column (J.T. Baker Research Laboratories, Phillipsburg, NJ) (43, 44). Selected peptides were rechromatographed on a Vydac 218 TPb column (43) eluted isocrat-

ically at 13% 1-propanol in 0.1% trifluoroacetic acid for 20 min followed by a linear gradient up to 22% 1-propanol over 110 min.

2.6. Titration

Potentiometric titrations (49) were carried out using a Radiometer autoburette ABU 13, a PHM 64 pH meter and a GK 2321 C electrode (Radiometer, Denmark) in a water-jacketed vessel at 25 °C under nitrogen. The G2 sample was exhaustively dialyzed against distilled water, freeze-dried and dissolved at a concentration of 5 mg · ml⁻¹ in distilled water. After Millipore filtration solid KCl was added to 0.1 M. A sample of 8 ml was adjusted to pH 3.0 by 0.1 M HCl and titrated with 0.1 M NaOH (Titrisol). KCl was added to the last dialysate which was used for solvent titration. The number of protons dissociated from G2 at a given pH was calculated from the difference between the uptake of base by the sample and solvent solutions.

Spectrophotometric titration (12) in 0.1 M KCl was performed by adding increments of either 3 M or 6 M KOH (0.1-200 µl) to 2.3 ml of G2 (1 mg · ml⁻¹). pH was measured as above and difference spectra recorded with a Cary Model 219 spectrophotometer at a scan rate of 0.2 nm · s⁻¹. A sample of denatured G2, prepared by boiling in water for 2 min, was cleared by centrifugation and solid urea and KCl were added to the supernatant followed by dilution.

3. RESULTS

3.1. Ligand binding to glucoamylase at pH 8.0

The glucoamylase from *A. niger* displays maximum activity near pH 4 and very low activity above pH 6. UV-spectral perturbation (Fig. 1A), however, allowed estimation of K_{diss} for the substrate maltose at pH 8.0 from a Scatchard plot (Fig. 1B), although this procedure is not strictly correct to apply because the ligand can occupy a number of both non-equivalent and overlapping sites (1, 28). The value of $K_{\text{diss}} = 2.4$ mM was in reasonable agreement with a K_m of about 1 mM for maltose determined at pH 4.3 (46), the peak shapes in the 280-300 nm range (Fig. 2) were also very similar in difference

Figure 1. A) Difference absorption spectrum of G2 (13 μ M) induced at pH 8.0 (0.1 M sodium phosphate) by 12 mM maltose (—) and at pH 5.0 (0.05 M sodium acetate) by 15 mM maltose (---)

B) A Scatchard plot based on the change in absorbance at 245 nm of G2 (13 μ M) induced by the presence of 5 different concentrations of maltose (0.51–3.00 mM) at pH 8.0. $\bar{v}$ is the fractal change of the spectrophotometric signal and $[L]$ the concentration of free ligand in the solution.

spectra induced by maltose at pH 5.0 and 8.0, respectively (Fig. 1A). The observed pH-dependence of the intensity of the perturbation presumably reflects that maltose is consumed by the enzyme at the lower pH value. Moreover, proportionality was observed at both pH values between the amount of added acarbose and the resulting absorbance differences (Fig. 2A and B) indicating a tight binding even at pH 8.0. K_{diss} , however, could not be calculated as above due to

the low concentrations of free acarbose. Taken together, these results suggest that no gross conformational change of glucoamylase takes place between pH 4 and 8. Hence, valid information on structure/function relationships might also be gained from chemical modification experiments performed at pH 8.0.

3.2. Chemical modifications of glucoamylase G2

3.2.1. Histidyl residues

DEP substituted only one out of four histidines in i) G2, ii) a destabilized but enzymatically active G2 treated with *N*-bromosuccinimide to contain four oxindolealanyl residues (8, 42) or iii) *N*-deglycosylated G2 prepared as described in 2.4. In these three cases no effect on activity towards either maltose or soluble starch was observed (data not shown). All four histidyl residues easily underwent modification in heat denatured G2.

Iodoacetamide similarly alkylated one histidyl residue at pH values 4.3 (Table I), 5.0 and

Figure 2. Difference absorption spectra of G2 (A: 12.8 μ M. B: 13.1 μ M) induced at A) pH 5.0 (0.1 M sodium acetate) by acarbose: a) 4.5 μ M, b) 8.9 μ M, c) 13.3 μ M, d) 150 μ M and B) pH 8.0 (0.1 M sodium phosphate) by acarbose: a) 2.2 μ M, b) 4.5 μ M, c) 6.7 μ M, d) 8.8 μ M, e) 150 μ M. There was no further change in spectrophotometric signal upon addition of more ligand.

Table I. Carboxamidomethylation and carboxymethylation of G2

Reaction mixture	Histidine (mol/mol)	Specific activity (%)
Iodoacetamide	2.8	89
Iodoacetamide + acarbose	2.8	95
Iodoacetate	3.4	97
Iodoacetate + acarbose	3.2	99
Control	3.8	100
Control + acarbose	3.7	105

The modification was performed at pH 4.3 (see 2.3). The acarbose concentration was 0.5 mg · ml⁻¹. Acarbose and excess reagents were removed by dialysis (9) and the remaining histidine content measured by amino acid analysis. Enzyme activity was assayed using maltose.

7.0 regardless of the presence of the tight-binding inhibitor acarbose. The derivatives were purified on acarbose-Sepharose (9) and had essentially retained K_m and k_{cat} towards maltose and soluble starch (Table II). Modification at pH 4.3 generated about 10%, and at pH 7.0 about 30%, inactivated and probably denatured enzyme derivative, which was not retained by acarbose-Sepharose and contained approx. 1.2 carboxamidomethyl equivalents.

3.2.1.1. Localization of [¹⁴C]carboxamidomethyl-histidine

Tryptic fragments of [¹⁴C]carboxamidomethylated G2 were separated on Bio-Gel P-100 (Fig. 3). Radiolabelled fragments were recovered in pool B in approx. 60% yield and subjected to RP-HPLC (Fig. 4). Rechromatography (43) of the radioactive peak 1 in Figure 4 resolved Asp₂₄₅-Arg₂₇₃ and Thr₃₇₉-Lys₄₀₄. Both sequences contain histidine, but only Asp₂₄₅-Arg₂₇₃ was radioactive (50% recovery) (Table III). It carried a stoichiometric amount of [¹⁴C]carboxamidomethyl-group and sequencing confirmed the absence of His₂₅₄. 1-carboxymethyl-histidine was identified after hydrolysis (10), while neither 3-carboxymethyl- nor 1,3-dicarboxymethyl-histidine were detected. Subfragmentation by chymotrypsin (43) and RP-HPLC of material in peak A in Figure 3 failed to identify further modified groups.

3.2.2. Reactivity of other side chains

Various reagents in large excesses were employed to modify lysyl, arginyl, tyrosyl, and methionyl residues, but only the carboxyl groups and tryptophanyl residues as found earlier seemed essential for activity and thermostability (Table IV) (8, 9, 42, 46).

Iodination or nitration reduced the activity by 40-50%. The inactivation due to lactoperoxi-

Table II. The kinetic parameters of carboxamidomethylated G2

Enzyme sample		Substrate			
Modification condition	Loss of Histidine (mol/mol)	Maltose		Starch	
		k_{cat} (s ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	K_m (%)
pH 4.3	0.7	2.5	1.2	56	0.10
pH 7.0	0.8	2.1	0.66	58	0.13
Control	-	2.2	0.95	52	0.07

k_{cat} is expressed in number of glucosyl bonds split per enzyme molecule per second. Assay conditions are described in section 2.2.

Figure 3. Separation of tryptic fragments (5.4 mg) from [¹⁴C]-carboxamidomethyl-2-pyridylethyl G2 on Bio-Gel P-100. A₂₂₀ (-----), A₂₈₀ (—) and radioactivity (.....).

Figure 4. RP-HPLC of 60% of pool B (Fig. 3). Essentially all radioactivity that was eluted (19,300 dpm, 70% yield) was present in peak 1.

dase-catalyzed iodination was prevented in the presence of acarbose. The incorporated iodine was not determined quantitatively, but only 3 of the 21 tyrosines in G2 underwent nitration which normally is the more efficient of the methods. Thus unusually many tyrosyl groups in G2 behave as buried.

Only 3 amino groups were succinylated and 4 were carbamylated. Despite the larger fraction of unprotonated amino groups available at pH 10, still only 5 lysyl residues reacted with *O*-methylisourea at that pH or with methyl acetimidate at pH 9 (Table IV). Neither the two methionines (Table IV) nor the single free thiol group (B. SVENSSON, unpublished results) reacted in the native enzyme.

Endoglycosidase H released the two sugar moieties on Asn₁₇₁ and Asn₃₉₅ as indicated by the loss of 2 glucosamine residues and verified in the case of Asn₃₉₅ by lack of neutral carbohydrate in the isolated fragment Thr₃₇₉-Lys₄₀₄ (data not shown). The deglycosylation neither affected the activity towards maltose and starch nor the thermostability. Moreover, treatment of *N*-deglycosylated G2 with either DEP at pH 6 or iodoacetamide at pH 5 resulted in fully active derivatives having a single modified histidyl residue.

3.3. Titration of glucoamylase G2

The low chemical reactivity in G2 of lysyl, histidyl, and tyrosyl side chains with expected

surface-location motivated investigations of their degree of dissociation by potentiometric and spectrophotometric titration, respectively. Increasing the pH from 3 to 11 dissociates 45–47 protons (Fig. 5). This number can be compared with a theoretical number of 61 calculated from a theoretical titration curve based on the amino acid composition (43, 45) and published intrinsic ionization constants of the side chains (4) and

Table III. Amino acid composition of the [¹⁴C]-carboxamidomethylated peptide, Asp₂₄₅-Asp₂₇₃^{a)}

Aspartic acid	4.9 (5)
Threonine	2.9 (3)
Serine	2.9 (3)
Glutamic acid	2.3 (2)
Proline	3.4 (3)
Glycine	1.5 (1)
Alanine	3.0 (3)
Isoleucine	0.9 (1)
Leucine	2.2 (2)
Phenylalanine	2.0 (2)
Histidine	<0.1 (1)
Arginine	0.8 (1)
2-Pyridylethylcysteine	0.8 (2)
1-Carboxymethylhistidine ^{b)}	0.8

^{a)} The composition obtained from the amino sequence (5, 43, 45) is given in parenthesis.

^{b)} The radioactivity content of the peptide is 720 dpm · nmol⁻¹ which corresponds to equimolar incorporation of carboxamidomethyl group.

Table IV. Chemical modification of glucoamylase G2 from *A. niger*

Reagent (conditions)	Target residue (in G2)	Number of modified residues	Residual activity (%)	t _m (°C)
Diethyl pyrocarbonate (15 mM, pH 5-7)	His (4)	1	102 (105)	n.d.
Iodoacetamide (0.10 M, pH 4-7)		1	89-64	68
Iodoacetic acid (0.10 M, pH 4-7)		0.4-0.6	97-93	n.d.
Tetranitromethane (25 mM, pH 8)	Tyr (21)	3	60 (82)	64
Triiodide (I ₃ ⁻) (0.25 mM, pH 8)		n.d.	56	n.d.
Lactoperoxidase ^{a)} + acarbose (0.1 mg · ml ⁻¹)		n.d. n.d.	50 (50) 93	n.d. n.d.
Potassium cyanate (0.24 M, pH 8)	Lys (10) α-NH ₂ (1)	4	70	n.d.
Succinic anhydride (7×10 mM, pH 8)		3 ^{d)}	88	66
O-Methylisourea (0.4 M, pH 10)		5	85	68
Methyl acetimidate (0.04 M, pH 9)		5	90	68
H ₂ O ₂ (0.74 M, pH 3)	Met (2)	0	94	n.d.
Phenylglyoxal (0.16 M, pH 8)	Arg (15)	3	109	n.d.
2,3-Butanedione (0.10 M, pH 7-8)		6	80	66
N-Bromosuccinimide ^{b)} (pH 4)	Trp (15)	6	0	
+ acarbose		4	80	52
1-Ethyl-3-(4-azonia-4,4- dimethylpentyl)carbodi- imide ^{c)} (0.10 M, pH 6)	Asp, Glu (53) α-COOH (1)	16	0	
+ acarbose		12	68	62

The conditions for chemical modifications and analyses are described in section 2.3. The residual activity was measured with maltose as substrate, values within parenthesis are for starch hydrolysis.

t_m = the temperature where glucoamylase loses 50% of the initial activity under the conditions employed (see section 2.2). t_m of G2 is 67 °C (42).

^{a)} lactoperoxidase (90 nM), iodide (0.36 mM), H₂O₂ (5×0.02 mM), pH 5.0.

^{b)} and ^{c)} are described in refs 9, 42 and 46.

^{d)} The contribution from α-NH₂ substitution was not assessed.

Figure 5. Potentiometric titration curve of G2 in 0.1 M KCl. The theoretical curve calculated from $\bar{z}_H = +3$ to $Z_H = -45$ using $\omega = 0.025$ (see 2.6) and the curve through the experimental points (4 different experiments) calculated using $\omega = 0.070$ are shown.

peptide termini (36) with the number of SH groups set to one (B. SVENSSON, unpublished data). The relation ($\bar{Z}$, pH) where $\bar{Z}$ is the mean net charge on the protein was solved graphically (data not shown) for a number of $\bar{Z}$ values setting:

$$\bar{Z} = Z_{\max} - \sum \frac{n_i K_{\text{int}} \cdot e^{2\omega\bar{Z}}}{[H^+] + K_{\text{int}} \cdot e^{2\omega\bar{Z}}}$$

i.e. setting $K = K_{\text{int}} \cdot e^{2\omega\bar{Z}}$ (49)

The theoretical curve (Fig. 5) was computed with an electrostatic interaction factor, ω , of 0.025 calculated with the assumption that the charges were uniformly distributed on a spherical molecule of 36 Å diameter (22). The reference point of zero net charge was set at the calculated isoionic point, pH 4.1, which agrees well with the value of pH 4.2 determined experimentally by isoelectric focusing including known reference proteins (47). G2 was stable at pH 3 for at least 2 h, but had lost 25% activity after 2 h at pH 11.

Figure 6. Spectrophotometric titration of G2 in 0.1 M KCl. The theoretical curve (solid line) was calculated using the $\bar{Z}$, pH values obtained as described in section 3.3, an ω of 0.025, an intrinsic pK value of tyrosine of 10.07 (4) and a $\Delta\epsilon_{295}$ of 2,330 (12). Experimental points are from different titrations of native G2 in 0.1 M KCl (Δ , ∇ , $\bullet$, $\circ$) or of heat denatured G2 in 6 M urea and 0.1 M KCl ($\square$, $\blacksquare$).

Heat denaturation in urea exposes groups that are buried in the native conformation as shown in section 3.2. Consistent with this, spectrophotometric titrations indicated the average apparent pK_a of the tyrosyl residues in native, but not in heat denatured G2 to be approx. half a unit higher than the theoretical value (Fig. 6).

4. DISCUSSION

In addition to tryptophanyl and carboxyl groups, tyrosyl and basic residues participate in substrate binding in Taka-amylase A (23) and pancreatic α -amylase (6), which both are related to glucoamylase (9, 41). However, in G2 only few of the lysyl, arginyl, histidyl and tyrosyl side chains were susceptible to chemical modification, the largest effect being 40-50% decrease of the activity caused by tyrosine modification. Only a small effect was obtained by modification of other side chains and the reported increased resistance of other enzymes to thermal unfolding due to guanidination or acetamidation of ϵ -amino groups (29, 33) was not observed in these G2 derivatives.

Modification of histidyl residues was of special interest, because a short sequence near His₃₉₁ in *A. niger* glucoamylase shows similarity with regions containing the substrate-binding His₂₉₆ in Taka-amylase A and His₂₉₉ in porcine pancreatic α -amylase, respectively (6, 20, 41). However, His₃₉₁ in G2 even after deglycosylation of Asn₃₉₅ seems to remain unmodified in the presence of high concentrations of iodoacetamide. The reactive group was His₂₅₄ and its modification has no significance for catalysis similar to the modification of a single, unidentified histidyl residue by DEP. The carboxamidomethyl-His₂₅₄ derivative retained thermostability. Similarly, in related glucoamylases, only a single exposed histidyl residue was found (31, 35). This is in conflict with a reported modification of 3 histidines by DEP in the same enzyme leading to reduced affinity for soluble starch (18), thus it remains an open question whether histidines other than His₂₅₄ might play a role in the mechanism of action.

The difference between the experimental and the theoretical titration curve in Figure 5 sug-

gests that approx. 15 groups fewer than expected titrate between pH 3 and 11. This might be partially due to an unusually strong interaction between some of the ionizable residues, since a determination of ω from a plot of $\text{pH} - \log \frac{\alpha}{1-\alpha}$ vs. $\bar{Z}$, where α is the fraction of the carboxylic acids that has lost protons, (22) indicated a value of 0.0705. A curve based on this ω is included in Figure 5 but, as it can be seen, this titration curve is also inconsistent with the experimental curve. Such behaviour might be explained from incorrectness of the used model, viz. that the ionizable side chains are located in clusters instead of being uniformly distributed over a sphere, or, that some of the ionizable residues are engaged in salt-bridges which drastically displace their pH-values. Due to the instability of the molecule at both high and low pH we have not been able to investigate this matter further. However, the results taken together indicate a low reactivity of the side chains of glucoamylase which might be connected with the high number of carbohydrate substituents which could also explain the high solubility of G2 in water. The occupancy of a large surface area by carbohydrates could sterically shield many side chains from chemical reagents and at the same time provide a microenvironment of dielectric constant intermediary to that of a hydrophobic interior of a protein and of water that still permits salt bridge formation, but destabilizes charges.

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