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Roles of the aromatic side chains in the binding of substrates, inhibitors, and cyclomalto-oligosaccharides to the glucoamylase from *Aspergillus niger* probed by perturbation difference spectroscopy, chemical modification, and mutagenesis*

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ABSTRACT

The roles of the aromatic side chains of the glucoamylase from *Aspergillus niger* in the binding of ligands, as determined by difference spectroscopy using four types of inhibitors (*a*) valienamine-derived, (*b*) 1-deoxynojirimycins, (*c*) D-glucono-1,5-lactone, and (*d*) maltitol, two types of disaccharide substrates (*a*) α-(1→4)-linked and (*b*) α-(1→6)-linked, and three cyclomalto-oligosaccharides (cyclodextrins, CDs) are discussed. An unusual change in absorbance from 300 to 310–320 nm, obtained only with the valienamine-derived inhibitors or when D-glucono-1,5-lactone and maltose are combined, is concluded to arise when subsite 2 is occupied in a transition-state-type of complex. The single mutations of two residues thought to be involved in binding, namely, Tyr116→Ala and Trp120→Phe, alter, but do not abolish this perturbation. The perturbations in the spectra also suggest that maltose and isomaltose have different modes of binding. The following K_d values (M) were determined: acarbose, $<6 \times 10^{-12}$; methyl acarviosinide, 1.6×10^{-6}; and the D-*gluco* and L-*ido* forms of hydrogenated acarbose, 1.4×10^{-8} and 5.2×10^{-6}, respectively. Therefore, both the valienamine moiety and the chain length of acarbose are important for tight binding. In contrast to the valienamine-derived inhibitors, none of the 1-deoxynojirimycin type protected glucoamylase against inactivating oxidation of tryptophanyl residues, although each had a K_d value of $\sim 4 \times 10^{-6}$M. There are two distinct carbohydrate-binding areas in glucoamylase, namely, the active site in the catalytic domain and a starch-granule-binding site in the C-terminal domain. The α-, β-, and γ-CDs have high affinity for the starch-binding domain and low affinity for the active site, whereas the reverse was found for acarbose.

INTRODUCTION

In recent years, the crystallography of proteins has disclosed structural details of the extensive participation of aromatic side chains in the binding of sugars[1–7]. Such sugar–protein interactions are monitored readily in solution by spectral shifts caused by a change in the microenvironment of the chromophores[8,9]. These shifts can be used to assess the type and number of the aromatic residues affected as well as the nature of the

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perturbing factor[10–15]. We now report on the application of difference spectroscopy to *Aspergillus niger* glucoamylase [GA; (1→4)-α-D-glucan glucohydrolase, EC 3.2.1.3] in order to quantitate the dissociation constants of various inhibitors and to characterise known functional amino acid side chains[16–22], using relevant mutants and derivatives of GA.

The three-dimensional structure of GA is not yet available, but kinetics have indicated that the active site is composed of seven consecutive binding subsites for glucosyl residues. Both (1→4)- and (1→6)-linked α-D-glucose can be released from the non-reducing ends of starch and related saccharides at a single catalytic site situated between subsites 1 and 2[22–25]. Several aromatic groups affected by binding have been identified by chemical modification of GA, including the essential Trp120 near subsite 4 (ref. 17); Trp212, Trp417, and Trp437 all associated with subsite 1 (ref. 18); and Trp170 that interacts specifically with isomaltose[21]. Furthermore, a binding role for Tyr116 can be postulated by comparison with an active-site sequence in the alpha-amylase Taka-amylase A[4,17,26]. Kinetic analysis of GA mutants showed that Trp120 is crucial for transition-state stabilisation[20] and Tyr116 is critical for hydrolysis of the glucoside bond in maltose[27]. A combination of differential labelling[28–30] and mutagenesis[31] identified Asp176 and Glu179 as the catalytic base and general acid, respectively, and Glu180 as critical for substrate binding at subsite 2. Additional studies of the GA mutants Trp120→Phe, Tyr116→Ala, and Asp176→Asn now reported improve the insight into the functional roles of these specific residues and provide a possible explanation for protein spectral perturbation observed in the 300–320-nm region[32]. Trp residues are also involved in a second binding site located in the C-terminal domain of GA1, which associates with starch granules, soluble polysaccharides, cyclomalto-oligosaccharides, or low molecular weight ligands[18,33–36]. In the present study, intrinsic aromatic reporter groups are exploited in order to characterise the interaction of different types of ligands with either the active site of GA or the second binding site situated in the C-terminal domain.

EXPERIMENTAL

Materials. — GA1 and GA2 from *Aspergillus niger*[37–40], GA1 derivatives containing either 6 or 7 oxindolalanyl residues[16], and the GA1 mutants Trp120→Phe and Asp176→Asn[20,31] were obtained as reported. The preparation of GA1 Tyr116→Ala will be described elsewhere[41]. The purity and molecular form of the GA preparations were confirmed by sodium dodecyl polyacrylamide gel electrophoresis (SDS–PAGE), using the "Phast"-system (Pharmacia, Uppsala, Sweden).

Methyl acarviosinide[42] (**4**, α,β-mixture, Bay o 4975) was a gift from Dr. F. Heiker (Bayer AG). Hydrogenated D-*gluco* (**2**) and L-*ido* (**3**) derivatives[43] of acarbose were prepared, using an improved procedure[44], by Professor K. Bock and Dr. M. Meldal (this laboratory). Acarbose (**1**, Bay g 5421), *N*-hydroxyethyl-1-deoxynojirimycin (**7**, miglitol, Bay m 1099), *N*-[2-(4-ethoxycarbonylphenoxy)ethyl]-1-deoxynojirimycin (**8**, emiglitate, Bay o 1248), *N*-methyl-1-deoxynojirimycin (**6**), and 1-deoxynojirimycin (**5**, mora-

noline) were gifts from Drs. E. Möller, D. Schmidt, and E. Truscheit. (Bayer AG). Maltitol, the D-glucose oxidase kit, and α-, β- and γ-cyclodextrin were obtained from Sigma, and the D-glucono-1,5-lactone and a glucose dehydrogenase kit were obtained from Merck.

Difference spectroscopy. — Ligand-induced perturbation of absorption spectra was determined at 25° as described[11,16], using a Cary 219 spectrophotometer and double-chamber cuvettes with light paths of 4.375 mm. Sample and reference having GA (7–15μM, in 0.05M sodium acetate, pH 4.5) and buffer in either chamber were matched by scanning the range 240–350 nm at 0.05 nm/s, using a period of 10 s and a full recording scale of 0.05 absorbance unit. Ligand was added to the protein sample and the buffer reference, and the difference in absorbance (ΔA) between complexed and free GA was recorded. Due to slight baseline drift, the molar difference extinction coefficient ($\Delta\varepsilon$) was not calculated from ΔA measured to the baseline[11] but rather from the difference in ΔA for two adjacent extrema[45].

The dissociation constants, K_d, were determined as follows. Tight-binding ligands titrate GA at concentrations that produce a reliable ΔA value, and a plot of $\Delta\varepsilon$ *vs.* ligand concentration yields a straight line[46]. However, the K_d value is too low to be determined spectrophotometrically, but it may be estimated[46] from the relation $[E_o]/K_d > 10^3$, where $[E_o]$ is the initial concentration of enzyme (procedure I). The inhibition constant, K_i, for non-stoichiometric tight-binding ligands is equal to K_d if hydrolysis of the substrate in the presence of an equimolar amount of inhibitor does not increase exponentially with time and the ratio (a) of initial rates in the presence and absence of the inhibitor is essentially independent of the concentration of the substrate. This situation implies that neither displacement of the substrate nor dissociation of the enzyme–ligand complex, EL, occurs and K_i ($= K_d$) can be calculated[46] from $[I_o]/(1-a) = (K_i/a) + [E_o]$ (procedure II). Less-tight-binding ligands were added until a constant $\Delta\varepsilon$ was obtained ($= \Delta\varepsilon_{max}$) for a chosen peak-to-trough difference[45]. Assuming that $\Delta\varepsilon$ and the concentration of the complex are proportional, then [EL] ($= (\Delta\varepsilon/\Delta\varepsilon_{max}) \times [E_o]$), and the concentrations of the free enzyme [E] and the ligand [L] can be determined to calculate the dissociation constant K_d as follows: $K_d = [E][L]/[EL] = ([E_o]-[EL])([L_o]-[EL])/[EL]$, where $[L_o]$ is the initial concentration (procedure III). Finally, for loose-binding ligands, $[L] \approx [L_o]$, and K_d and $\Delta\varepsilon_{max}$ can be derived graphically according to $\Delta\varepsilon = \Delta\varepsilon_{max}[L_o]/(K_d + [L_o])$[14,47] (procedure IV). For α-, β-, and γ-CD, procedure III was used for the high-affinity site and procedure IV for the low-affinity site.

Modification of tryptophanyl residues. —The protection of Trp residues and enzyme activity by ligands against oxidation with *N*-bromosuccinimide was assessed as described[16,48]. To a solution of the GA1 inhibitor complex or GA1 [13μM, in 0.1M sodium acetate (pH 4.5), 1.0 mL in a cuvette of 10-mm light path] was added *N*-bromosuccinimide in increments, spectra were scanned after each increment, and the Trp contents were calculated[16] from the absorbance at 280 nm. When the decrease in absorbance stopped, the residual *N*-bromosuccinimide was destroyed with L-tryptophan (15mM, 50 μL), and the inhibitor was removed by dialysis (4°, overnight) against 1.6M Tris–HCl (pH 7.6) followed by 50mM sodium acetate (pH 4.3), prior to measurement[16] of the activity.

Assays. — Activity towards maltose (15mM, in 0.05M sodium acetate, pH 4.3) of GA treated with *N*-bromosuccinimide was determined at 25° by assay of the D-glucose liberated with glucose dehydrogenase[37]. Activity in the presence of the valienamine-derived inhibitors (**1–4**) was assayed after 30-min preincubation at 45°, using maltose as substrate (0.3–10mM, in 0.1M sodium acetate, pH 4.3) and D-glucose oxidase to detect the released D-glucose[20]. Hydrolysis of α-, β-, and γ-CD (0.75mM, in 0.1M sodium acetate, pH 4.3) by GA (10–14μM) at 25° was tested by assay of the D-glucose produced using glucose dehydrogenase[37]. Protein concentrations were measured either spectrophotometrically at 280 nm using the following values ε ($M^{-1} \cdot cm^{-1}$): GA1, 1.37 × 10^5; GA2[16], 1.09 × 10^5; Trp120→Phe GA1, 1.32 × 10^5; and Tyr116→Ala GA1, 1.35 × 10^5; or by amino acid analysis (*N*-bromosuccinimide-oxidized GA1; Asp176→Asn GA1)[16,38].

RESULTS

*Interaction of GA with valienamine- (**1–4**) and 1-deoxynojirimycin-type (**5–8**) inhibitors.* — The spectral changes of GA1 induced by a 2–4-fold molar excess of each of the valienamine-derived inhibitors, methyl acarviosinide (**4**), acarbose (**1**), and the hydrogenated L-*ido* (**3**) and D-*gluco* (**2**) derivatives of acarbose, revealed typical features in the 280–300 nm region[11] and, except for the L-*ido* derivative (**3**), an unusual and dominating red shift at wavelengths longer than 300 nm (Fig. 1A). Saturation of GA1 by each of the 1-deoxynojirimycins **5–8** produced similar, but weaker spectral changes

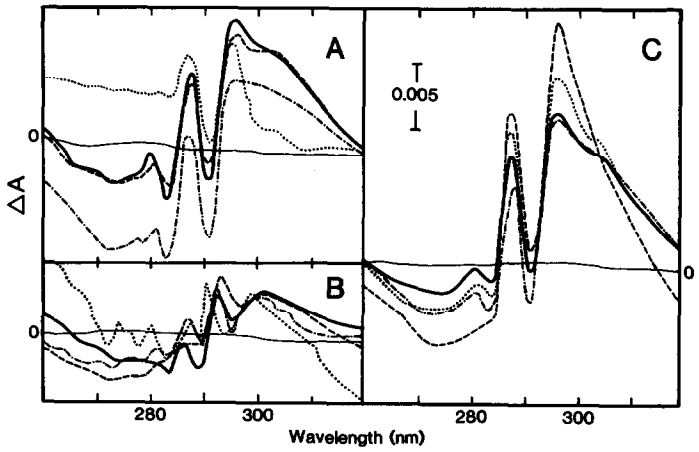
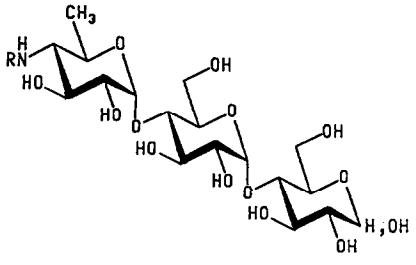
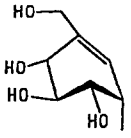
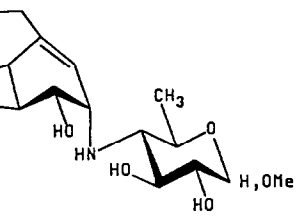


Fig. 1. Difference spectra induced by tight-binding inhibitors: **A** GA1 (13.9μM) and acarbose (**1**) (21.4μM) (—), GA1 (12.2μM) and methyl acarviosinide (**4**) (42.3μM) (---), GA1 (14.7μM) and the D-*gluco* derivative (**2**) (24.9μM) (–·–), GA1 (14.6μM) and the L-*ido* derivative (**3**) (31.5μM) (···); **B** GA1 (13.3μM) and 1-deoxynojirimycin (**5**) (480μM) (—), GA1 (12.1μM) and *N*-methyl-1-deoxynojirimycin (**6**) (450μM) (---), GA1 (13.5μM) and miglitol (**7**) (310μM) (–·–), GA1 (14.1μM) and emiglitate (**8**) (98μM) (···): **C** GA1 (14.3μM) and acarbose (14.6μM) (—) followed by acarbose (352μM) (···), GA1 (14.3μM) and the L-*ido* derivative (22μM) followed by acarbose (15μM) (---), GA1 (14.3μM) and acarbose (15μM) followed by the L-*ido* derivative (22μM) (–·–).

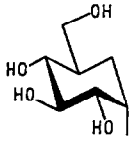
(Fig. 1B). An increase in perturbation resulting from higher concentrations of acarbose (**1**) observed with GA1 (Fig. 1C), but not GA2, indicates the binding of a second acarbose molecule to GA1. Addition of equimolar amounts of acarbose to GA1 previously complexed with the L-*ido* derivative (**3**) caused a larger perturbation than either GA1 complexed with excess of acarbose or GA1 complexed with the L-*ido* derivative (Fig. 1C). The reverse process, however, where an equimolar amount of the L-*ido* derivative was added to the acarbose–GA1 complex had little effect (Fig. 1C). This finding suggests that the L-*ido* derivative remains complexed at a second binding site when acarbose binds at the active site, but, when acarbose is already bound at the active site, the affinity for the L-*ido* derivative is much reduced.

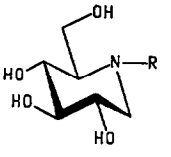


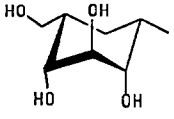
1 R =  (acarbose)



4 (methyl acarviosinide)

2 R =  (D-*gluco*)



3 R =  (L-*ido*)

5 R = H (1-deoxynojirimycin)

6 R = Me (*N*-methyl-1-deoxynojirimycin)

7 R = CH_2CH_2OH (miglitol)

8 R = $CH_2CH_2OC_6H_4$(p)COOEt (emiglitate)

Each of the four valienamine-type inhibitors (**1–4**) bound tightly to GA1 (Fig. 2). Because acarbose inhibits stoichiometrically at a GA concentration of 6×10^{-9}M, K_d was estimated as $< 6 \times 10^{-12}$M using procedure I. The complex is extremely stable as only 15% of the GA activity was recovered after exhaustive dialysis against either 0.05M sodium acetate (pH 4.5) or 0.01M Tris (pH 7.5), a competitive inhibitor[49]. However, in 1.7M Tris (pH 7.6)[17], complete dissociation occurs, presumably due to displacement by Tris rather than deprotonation of acarbose, since acarbose also binds tightly[30] to GA at pH 8.0. For the D-*gluco* derivative (**2**)–GA complex, neither dissociation nor displacement of the inhibitor by the substrate was observed (Fig. 3) and a K_d (= K_i) of 1.4×10^{-8}M was determined by procedure II. Methyl acarviosinide (**4**) and the L-*ido*

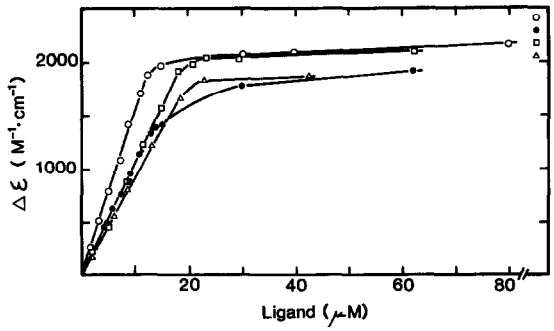


Fig. 2. The $\Delta\varepsilon_{295-291}$ values from the difference obtained with GA1 (12.2–14.6μM) by the addition of acarbose (**1**) (○), methyl acarviosinide (**4**) (△), and the D-*gluco* derivative (**2**) (□), and $\Delta\varepsilon_{294-299}$ for the L-*ido* derivative (**3**) (●). The respective final concentrations were: 750, 320, 125, and 480μM.

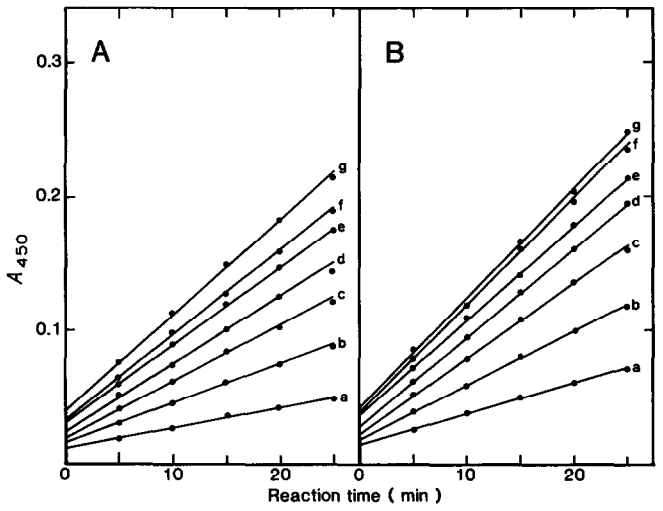


Fig. 3. Activity of GA1 (6×10^{-9}M) towards maltose (a, 0.3; b, 0.6; c, 1.0; d, 1.5; e, 2.0; f, 2.5; and g, 3.5mM) in the presence (**A**) and absence (**B**) of the D-*gluco* derivative (**2**) (6×10^{-9}M). The A_{450} values represent the amount of D-glucose released.

TABLE I

Dissociation constants[a] for acarbose and related compounds interacting with GA1

Inhibitor	K_d (M)	*Inhibitor*	K_d (M)
Acarbose (**1**)	$<6 \times 10^{-12}$ (I)	1-Deoxynojirimycin (**5**)	2.1×10^{-6} (III)
Methyl acarviosinide (**4**)	1.6×10^{-6} (III)	*N*-Methyl-1-deoxynojirimycin (**6**)	2.0×10^{-6} (III)
D-*gluco* derivative (**2**)	1.4×10^{-8} (II)	Miglitol (**7**)	5.9×10^{-6} (III)
L-*ido* derivative (**3**)	5.3×10^{-6} (III)	Emiglitate (**8**)	4.0×10^{-6} (III)

[a] K_d was obtained by procedures I–III as indicated (see Experimental).

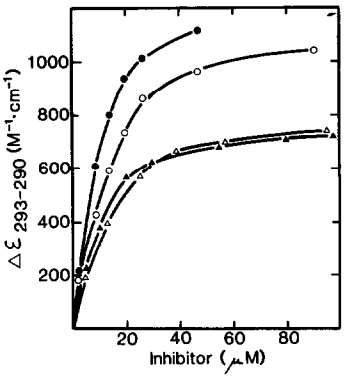


Fig. 4. The $\Delta\varepsilon_{293-290}$ values from difference spectra obtained with GA1 (12–15μM) by the addition of 1-deoxynojirimycin (**5**) (●), *N*-methyl-1-deoxynojirimycin (**6**) (○), miglitol (**7**) (△), and emiglitate (**8**) (▲).

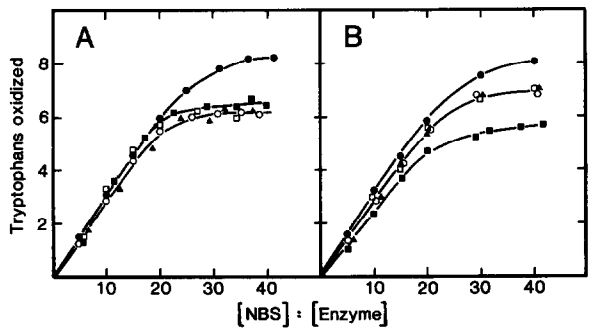


Fig. 5. Oxidation of tryptophanyl residues in GA1 by *N*-bromosuccinimide: **A** GA1 (13μM) alone (●) or in the presence of 0.15mM acarbose (**1**) (○), methyl acarviosinide (**4**) (▲), the D-*gluco* derivative (**2**) (□), or the L-*ido* derivative (**3**) (■); **B** GA1 (12μM) alone (●) or in the presence of 1.5mM 1-deoxynojirimycin (**5**) (○), *N*-methyl-1-deoxynojirimycin (**6**) (▲), miglitol (**7**) (□), or 0.3mM emiglitate (**8**) (■).

derivative (**3**) bound more weakly with a K_d of 1.6×10^{-6} and 5.2×10^{-6}M, respectively, as determined using procedure III. Methyl acarviosinide (**4**), a pseudodisaccharide, perturbed the GA spectrum in a manner identical to that of acarbose (**1**) despite its much higher K_d value (Fig. 1A). The four 1-deoxynojirimycins **5–8**, representing monosaccharide analogues, had similar K_d values ranging from 2.0×10^{-6} to 5.9×10^{-6}M as determined by procedure III (Table I, Fig. 4), which agrees with the K_d 1.6×10^{-5}M reported[50] for 1-deoxynojirimycin (**5**) and *Rhizopus* GA.

The four valienamine-derived inhibitors **1–4** prevented the oxidation of approximately two Trp residues in GA1 (Fig. 5A); three conferred retention of 70% of the activity, whereas, with the L-*ido* derivative (**3**), only 40% was retained. In contrast, none of the 1-deoxynojirimycins **5–8** hindered inactivation despite their high affinity and protection of either one or two Trp residues (Fig. 5B).

Disaccharide substrates and weak inhibitors. — The difference spectra induced by

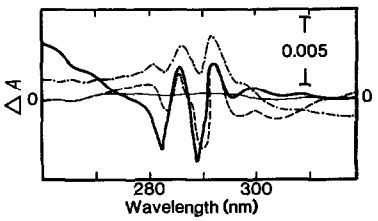


Fig. 6. Difference spectra for GA1 induced by disaccharide substrates: GA1 (12.9μM) and methyl β-maltoside (25mM) (—), GA1 (13.9μM) and maltose (15mM) (---), GA1 (13.1μM) and isomaltose (48mM) (-·-).

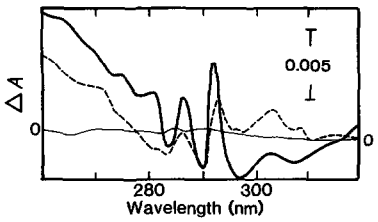


Fig. 7. Difference spectra for GA1 induced by loose-binding inhibitors: GA1 (13.1μM) and D-glucono-1,5-lactone (21mM) (---), GA1 (13.6μM) and maltitol (15mM) (—).

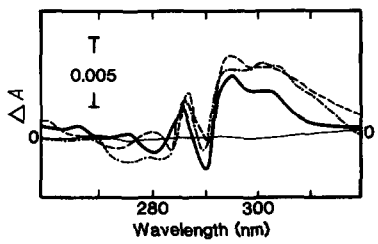


Fig. 8. Difference spectra for GA1 (8.0μM) induced by methyl acarviosinide (**4**) (80μM) (-·-), acarbose (**1**) (0.15mM) (---), and D-glucono-1,5-lactone (21mM) plus maltose (50mM) (—).

isomaltose and maltose possess characteristic, equally intense peaks near 286 and 292 nm, but maltose produced a trough and isomaltose a shoulder near 300 nm (Fig. 6). This variation may reflect the specific interaction of Trp170 with isomaltose[21] due to the different modes of binding of the two substrates. Both the substrate methyl β-maltoside (Fig. 6) and the competitive inhibitor maltitol[49] (Fig. 7) yielded a distinct, larger minimum near 282 nm. Maltitol also produced sharp peaks at 286 and 292 nm, and a deep trough near 300 nm.

D-Glucono-1,5-lactone, a transition-state analogue[25] and mixed-type inhibitor that binds at subsite 1[23–24], with a reported K_d of 1×10^{-3}M[16,22], perturbed the absorbance (Fig. 7) in a manner similar to that of the 1-deoxynojirimycins **5–8** (see Fig. 1B). The proposed mechanism of action of GA allows a ternary complex to be formed with D-glucono-1,5-lactone bound at subsite 1 of GA and maltose at subsites 2 and 3[50–52].

Since the resulting perturbation-difference spectrum from this combination (Fig. 8) lacked a minimum near 297 nm and extended to wavelengths longer than 300 nm, it resembles most closely those of the acarbose (**1**) and methyl acarviosinide (**4**) complexes, and differs from those of both of the corresponding individual binary complexes (*cf.* Figs. 6 and 7).

GA1 derivatives and mutants. — Trp120→Phe GA1 displayed a decreased intensity of the acarbose-induced peaks near 287 and 295 nm, and a large trough at 302 nm with D-glucono-1,5-lactone or maltose (Fig. 9). GA with seven oxidized tryptophanyl residues, including[17] Trp120, yielded a maltose-perturbed spectrum similar to that of the Trp120→Phe mutant (Fig. 9C), whereas the enzymically active GA, where only six Trps, excluding Trp120, are oxidised[17], did not give rise to the maltose perturbation trough at 302 nm (Fig. 9C). The binding affinities for the two *N*-bromosuccinimide-oxidized GA1 forms were comparable to those of the unmodified enzyme[17]. Thus,

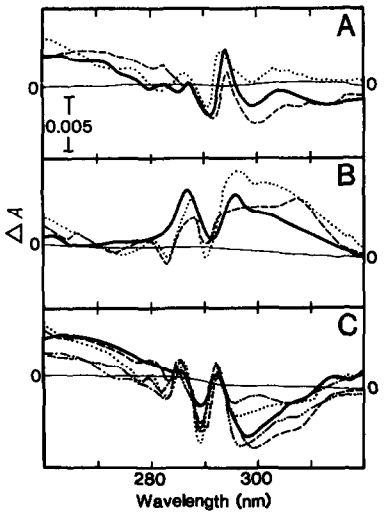


Fig. 9. Difference spectra for GA1 mutants induced by **A** D-glucono-1,5-lactone (21mM) for Trp120→Phe (---; 7.8μM), Tyr116→Ala (—; 8.5μM), and wild-type (···; 7.9μM); **B** acarbose (**1**) (0.15mM) for Trp120→Phe (---; 7,4μM), Tyr116→Ala (—; 7.4μM), and wild- type (···; 8.0μM); **C** maltose (24 or 50mM) for Trp120→Phe (---; 9.7μM), Tyr116→Ala (—; 8.0μM), wild-type (···; 8.0μM), and GA1 with six (8.0μM) (–·–) or seven (9.3μM) (···–···) oxidized Trp residues, respectively.

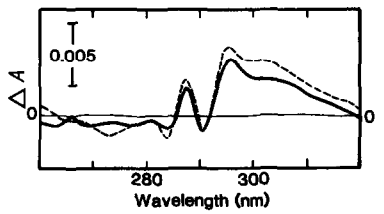


Fig. 10. Difference spectra for the GA1 mutant Asp176→Asn (7.5μM) induced by 0.15 or 2.5mM acarbose (**1**) (—), and GA1 (8.0μM) and 0.15mM acarbose (---).

counteracting-ligand-induced spectral effects are likely to occur near 300 nm, first a large red shift corresponding to a conformational change of Trp120 and second a similar blue shift caused by other Trp residues.

The Tyr116→Ala perturbation spectra also differed from that of wild-type GA. The characteristic trough at 282 nm was lacking with the three ligands tested (Fig. 9A–C), possibly due to the loss of the phenol chromophore of Tyr116. Moreover, Tyr116 seems to influence one or more Trp residues, since perturbation throughout the 295–320 nm region is decreased for Tyr116→Ala GA. Finally, the perturbation of the proposed catalytic base mutant Asp176→Asn GA induced[31] by acarbose (**1**), retained the wild-type shape, although the intensity was slightly reduced (Fig. 10).

Interaction of cyclomalto-oligosaccharides (cyclodextrins, CDs) at a second binding site. — Typical tryptophan spectral perturbations with red shifts of decreasing intensities at 286, 294, and 303 nm were produced by complexation of GA1 with cyclomalto-hexaose (αCD), -heptaose (βCD), and -octaose (γCD) (Fig. 11A). Tyr residue(s) were also affected, as apparent from the 278–281 nm peak (Fig. 11A,a). These features are clearly different from those obtained with the inhibitory ligands and suggest

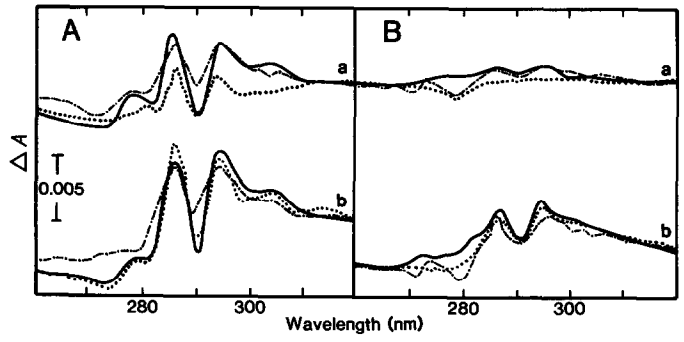


Fig. 11. Difference spectra for GA1 and GA2 induced by cyclodextrins: **A** GA1 (a, 12.9μM; b, 12.6μM) and αCD (a, 104μM; b, 3.3mM), βCD (a, 145μM; b, 1.5mM), γCD (a, 99μM; b, 2.0mM); **B** GA2 (a, 13.1μM; b, 12.6μM) and αCD (a, 104μM; b, 2.1mM), βCD (a, 145μM; b, 3.0mM), γCD (a, 108μM; b, 2.0mM); αCD (···), βCD (—), γCD (–·–).

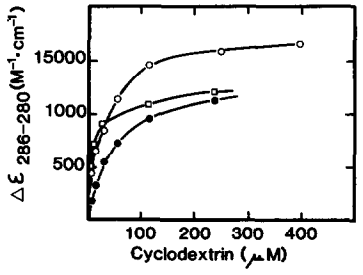


Fig. 12. The $\Delta\varepsilon_{286-280}$ values from the difference spectra obtained with GA1 (7.8–8.5μM) by addition of αCD (●) or βCD (○), and with GA1 Trp120→Phe (8.0μM) by αCD (□).

TABLE II

Dissociation constants[a] for cyclodextrin binding to *A. niger* glucoamylase 1 and 2

Ligand	*High affinity*		*Low affinity*			
	GA1 (III)		GA1 (IV)		GA2 (IV)	
	K_d (M)	$\Delta\varepsilon_{286-280}$ ($M^{-1} \cdot cm^{-1}$)	K_d (M)	$\Delta\varepsilon_{295-291}$ ($M^{-1} \cdot cm^{-1}$)	K_d (M)	$\Delta\varepsilon_{295-291}$ ($M^{-1} \cdot cm^{-1}$)
αCD	3.5×10^{-5}	1200	1.3×10^{-4}	1350	3.9×10^{-4}	960
βCD	1.9×10^{-5}	1600	6.7×10^{-5}	1780	6.9×10^{-4}	710
γCD	–	–	–	–	5.1×10^{-4}	1070

[a] K_d was calculated by procedures III and IV as indicated (see Experimental).

that the CDs bind at a second site distinct from the active site of GA1. K_d values of 3.5×10^{-5} and 1.9×10^{-5} M were measured for α- and β-CD, respectively (Fig. 12; Table II), in reasonable agreement with the K_d of $3.0–3.7 \times 10^{-5}$ M reported for αCD and 5.6×10^{-6} M for βCD binding to GA1[33,34]. Due to a slight hydrolytic activity of GA1 also noted[53] earlier for γCD, K_d was not determined for this compound. Under the conditions used in the present study, GA2, which lacks the starch-binding tail found at the C-terminal region of GA1, did not cleave γCD. The larger K_d values of the CDs obtained

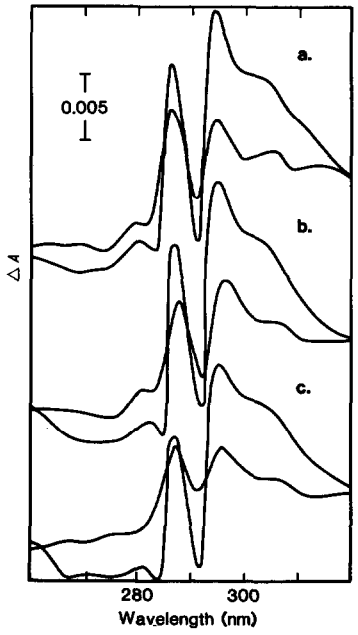


Fig. 13. Effects of addition of acarbose (1) (270–285 μM) to the complexes described in Fig. 11 A,b: a, αCD; b, βCD; c, γCD. The upper curves are difference spectra induced by the CD in combination with acarbose.

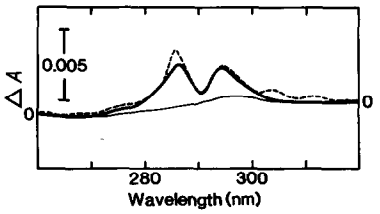


Fig. 14. Difference spectra for the GA1 mutant Trp120→Phe (7.95μM) (—) and GA1 (8.02μM) (---) with αCD (241μM).

with GA2, 3.9–6.9 × 10^{-4}M (Fig. 11B; Table II), indicate that the CDs have an affinity for the active site which is lower than that for the starch-granule-binding site in GA1, as reported[54] earlier for inhibition of GAs from *Rhizopus niveus* and *Chalara paradoxa* by the various CDs.

In the present study, the K_d values estimated for the binding of CDs to the active site in GA1 are 3–10-fold lower than those for GA2 (Table II), suggesting co-operation between the two sugar-binding areas present in GA1. Addition of acarbose (1) raised ΔA in the CD–GA1 (Fig. 13), but not in CD–GA2, complexes (data not shown), supporting the view that two distinct binding sites exist in GA1. Since Trp120 is believed to be important for ligand-induced conformational changes in the active site[20], a preliminary investigation of the possible co-operation between these two binding areas was attempted using Trp120→Phe GA1. The K_d for binding of the αCD at the high-affinity site of Trp120→Phe was estimated to be 7.5 × 10^{-6}M, which is 5-fold lower than for wild-type enzyme (Fig. 13). Further addition of αCD suppressed the maxima at 286, 303, and 310 nm for Trp120→Phe relative to wild-type GA1 (Fig. 14), whereas the peak height at 298 nm was retained. This finding suggests that the Trp residues that generate the 298 nm peak are not influenced by Trp120, and that the aromatic groups that generate the 286, 303, and 310 nm peaks, when both binding sites are occupied, are influenced by Trp120. These findings indicate that certain spectral perturbations induced in GA1 and hence the role of aromatic groups in ligand bindings are specific for CDs.

DISCUSSION

Like many glycosidases, GA is strongly inhibited[55] by hexose derivatives and oligosaccharide structural analogues having an NH group adjacent to C-1. In the present approach, such tight-binding inhibitors have been used, in conjunction with mutants and derivatives of GA, in order to examine the roles of aromatic side chains in the mechanism of binding.

The ligand-induced-perturbation changes in the spectra of GA in the region 280–300 nm are characteristic of the red-shifted absorbance bands of indolyl and phenol rings[10,11] due to their transfer from a solvent-accessible location to a less-polar environment upon binding[56–58], perhaps involving hydrogen bonding of the indole NH

group[11,59]. The 290–295 nm peak reflects Trp perturbations. Assuming a $\Delta\varepsilon$ of 1600 $M^{-1} \cdot cm^{-1}$ (ref. 11) for each Trp perturbed, the 1-deoxynojirimycin-based inhibitors (**5–8**) affected approximately one, and the valienamine-type (**1–4**) approximately two, Trp residues in excellent agreement with their protection of Trp in GA against oxidation. The peak at 285–288 nm may originate from both Trp and Tyr, whereas the small peak at 279–282 nm should reflect an influence on Tyr[10,11]. Genetic substitution of the functional Trp120, Tyr116, and Asp176[20,31] led to assignment of the peaks at or near 287 and 298 nm to Trp 120, and the peak at 282 but not at 287 nm to Tyr116. The peak at 298 nm is associated with the formation of transition-state-like complexes, as produced by acarbose (**1**) and methyl acarviosinide, but not maltose or isomaltose.

Certain ligands induced changes of GA in the region 300–320 nm. This effect is unusual for protein difference spectra[8–11,56–60] and suggests an involvement of several aromatic side chains. Transfer of energy between closely situated aromatic rings, such as the cluster of Trp residues proposed[18] in *A. niger* GA, probably plays a role in this unusual spectral perturbation[15]. Since Trp120 is involved in transition-state stabilisation[20], based on the wild-type and Trp120→Phe GA difference spectra, the ligand-induced maximum at 302 nm is correlated with a presumed change in the conformation of Trp120 triggered by a transition-state analogue bound in subsite 1. When one or more subsites beyond subsite 1 are also occupied, the plateau in this type of complex in the region 300–320 nm is obtained. This effect is seen in the spectra from the acarbose and methyl acarviosinide complexes and also in the ternary complex with D-gluco-no-1,5-lactone and maltose. GA loses activity when Trp120 is either oxidized by *N*-bromosuccinimide[16,17] or replaced by mutagenesis[20]. Because both the pseudo-di- and -tetrasaccharides, methyl acarviosinide (**4**) and acarbose (**1**), respectively, protect against inactivating oxidation by *N*-bromosuccinimide, Trp120 is not accessible in either binary complex. Therefore, either each of these inhibitors elicits a change in conformation to shield Trp120, or Trp120 is not located at the fourth subsite as proposed[17] in the studies using only acarbose. The almost identical difference spectra for the complexes of GA with acarbose and methyl acarviosinide suggest that aromatic groups are not perturbed by the binding of ligands in subsites 3 and 4. Thus, the long-wavelength plateau in the perturbation spectra may be correlated[22] with the specific occupation of the high-affinity subsite 2 in a transition-state complex. A large trough near 300 nm in the difference spectra induced by maltose distinguished Trp120→Phe from the wild-type GA. A similar trough reported[61] for the complex of maltose and wild-type *Rhizopus* GA indicates a variation of the function of aromatic groups in GAs from different sources, noticed also by others[19], despite five conserved Trp residues[62]. The large decrease in the perturbation at longer wavelengths seen with Tyr116→Ala GA is more likely to be due to an effect on the interacting aromatic groups than to the loss of the phenol side chain. In the conformation of a peptide segment in Taka-amylase A, which is related structurally and functionally to the Tyr116/Trp120-region in GA[4,26], the corresponding Tyr79 and Trp83 groups are juxtaposed on either side of the binding cleft[4], permitting interactions when complexes are formed. The interplay of Trp residues upon binding has been indicated by difference spectroscopy in

lysozyme[63], the periplasmic maltodextrin-binding protein from *E. coli*[7,15] and tryptophan–indole lyase[64]. In some earlier studies, spectral effects above 300 nm have been correlated with alterations in the microenvironmental charge that affect a discrete Trp absorption band[12,13,32,56–60]. A positive $\Delta\varepsilon$ was proposed to indicate that either a negative group has approached, or a positive group is removed from, the chromophore and *vice versa*[11,12,57]. In lysozyme, this effect was explained[32] by the influence of the carboxylate group of the catalytic Glu35 on Trp108. For GA, however, acarbose exerted[30] almost identical effects on the spectrum at pH 8 and pH 5.0, and substitution of the proposed GA base catalyst, Asp176 by Asn only marginally reduced ΔA throughout the difference spectrum. Thus, the carboxylate of Asp176 seems not to be the cause of the unusual spectral perturbation.

The planar structures assumed for the C-5–N environment in acarbose (**1**) and methyl acarviosinide (**4**) and the C-1 environment in D-glucono-1,5-lactone are not required for binding to subsite 1, as demonstrated by experiments with the D-*gluco* derivative (**2**) of hydrogenated acarbose or the 1-deoxynojirimycins (**5–8**). Therefore, it is proposed that the interaction of a protonated nitrogen and a carboxylate group is more important for the formation of complexes of GA with the present inhibitors. Since the pK_a of the nitrogen in acarbose[55] is ~ 5 and the unprotonated form retains[30] high affinity for GA, there may be a mechanism-based protonation of the NH group by the general acid catalyst, Glu179.

Certain molecular forms of fungal GAs can adsorb onto raw starch at a second binding site located in a terminal structural domain which is ~ 100 amino acid residues long[33,34,37,65] and recurs in several amylolytic enzymes[35]. The structure of one of these enzymes, cyclodextrin glucanotransferase from *B. circulans*, has been solved by X-ray diffraction analysis[66], and aromatic residues were shown to be involved in this raw-starch binding site[18]. CDs compete with raw starch or soluble malto-oligosaccharides with more than 11–13 glucose residues[33,34] for binding to GA1. The absorption spectra for the complexes of GA1 with CDs indicate that association occurs with considerably lower affinity for the active site than for the starch-binding site. Our experiments suggest that GA1 can complex with acarbose at the active site, and CD at a second site. The L-*ido* derivative (**3**) may also bind at the second site, since it adopts a mode of binding different from that of the other valienamine-derived inhibitors as reflected in the spectral perturbations and K_d values, and also because binding of acarbose to the high-affinity site caused perturbation additional to that for either of the single complexes of GA1 with the L-*ido* derivative (**3**) or acarbose (**1**). An interaction of the two binding sites is proposed because equimolar amounts of the L-*ido* derivative (**3**) will occupy only the second binding site when acarbose is not already bound to the active site. Further evidence for this interaction is reflected in the hydrolysis of γCD, since GA1, but not GA2, had slight activity, suggesting that the second site has to be present as a prerequisite for cleavage of γCD glucosyl bonds.

Thus, the important functional roles of GA Tyr116 and Trp120 have been illustrated by ligand-induced spectral perturbations obtained for relevant GA mutants. A peak at 298 nm attributable to Trp120 characterises occupation of subsite 1 in

GA-transition-state complexes, whereas the unusual features in the difference spectra in the region 300–320 nm are attributed to interaction of aromatic groups when subsite 2 in these complexes is occupied. Both the valienamine ring and the chain length of acarbose contributed to its extremely high affinity for the active site of GA. The role of a basic nitrogen in the formation of tight complexes with subsite 1 of GA is demonstrated by the binding of both acarbose (**1**) and 1-deoxynojirimycin (**5**), a structural analogue of D-glucose much preferred by GA over the planar inhibitor and transition-state analogue D-glucono-1,5-lactone. Finally, the formation of ternary complexes of GA1 containing either cyclodextrin and acarbose, or the L-*ido* derivative and acarbose, indicates two different binding sites, one in the catalytic domain and the second in the starch-binding domain. Co-ordinated binding of ligands at these sites suggests that interactions of the two sites may be important in the hydrolysis of certain long-chain substrates.

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