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## Dynamic Light Scattering Evidence for a Ligand-Induced Motion between the Two Domains of Glucoamylase G1 of *Aspergillus niger* with Heterobivalent Substrate Analogues**

Nathalie Payre, Sylvain Cottaz, Claire Boisset, Redouane Borsali, Birte Svensson, Bernard Henrissat, and Hugues Driguez*

Glucoamylases (GAs) catalyze the hydrolytic release of β-D-glucose from the nonreducing ends of starch and related oligo- and polysaccharides. Most GAs possess a starch-binding domain (SBD) separated from the catalytic domain (CD) by a glycosylated peptide linker of variable length.[1] Removal of the SBD reduces the activity of GA from *Aspergillus niger* on insoluble starch but not on soluble substrates.[2] We have previously shown that $6^{II}$-thiopanose and its higher oligomers bind essentially to the SBD and modulate GA activity on starch.[3] This raised the possibility of an interaction between the CD and the SBD of GA, and these observations have suggested that a cooperativity of the two domains could be critical for optimal activity.[4] The only low resolution structural information available so far on the entire GA was obtained by scanning tunneling microscopy,[5] but the possible mobility of the two domains induced by substrate binding cannot be described by this technique. The three-dimensional structure of the CD of the GA from *Aspergillus awamori* X100 has been solved by X-ray crystallography,[6] while that of the SBD of GA isolated from *Aspergillus niger* has been recently determined by NMR spectroscopy.[7] Failure to crystallize the entire GA has repeatedly been observed and this is attributed to the inherent flexibility of the linker peptide that connects the two constitutive domains. Co-crystallization in the presence of a ligand targeted to both the CD and SBD may stabilize one conformer. Recently, closure of a flexible loop onto a substrate analogue has allowed the crystallization of a cellulase.[8]

Herein we describe the design and synthesis of high affinity probes that bind the CD and SBD of GA at the same time, and hence get new insights into the structure/activity relationships of GA. The structural variations of GA between free and bound states were monitored by quasi-elastic light

[*] Dr. H. Driguez, Dr. N. Payre, Dr. S. Cottaz, Dr. C. Boisset, Dr. R. Borsali
Centre de Recherches sur les Macromolécules Végétales (CERMAV-CNRS)
Affiliated with the Université Joseph Fourier, Grenoble
B.P. 53, F-38041 Grenoble cedex 9 (France)
Fax: (+33) 476-037-664
E-mail: hdriguez@cermav.cnrs.fr
Dr. B. Svensson
Department of Chemistry, Carlsberg Laboratory
Gamle Carlsberg Vej 10, DK-2500 Valby (Denmark)
Dr. B. Henrissat
Architecture et Fonction des Macromolécules Biologiques
CNRS-IFR1
31 Chemin Joseph Aiguier, F-13402 Marseille cedex 20 (France)

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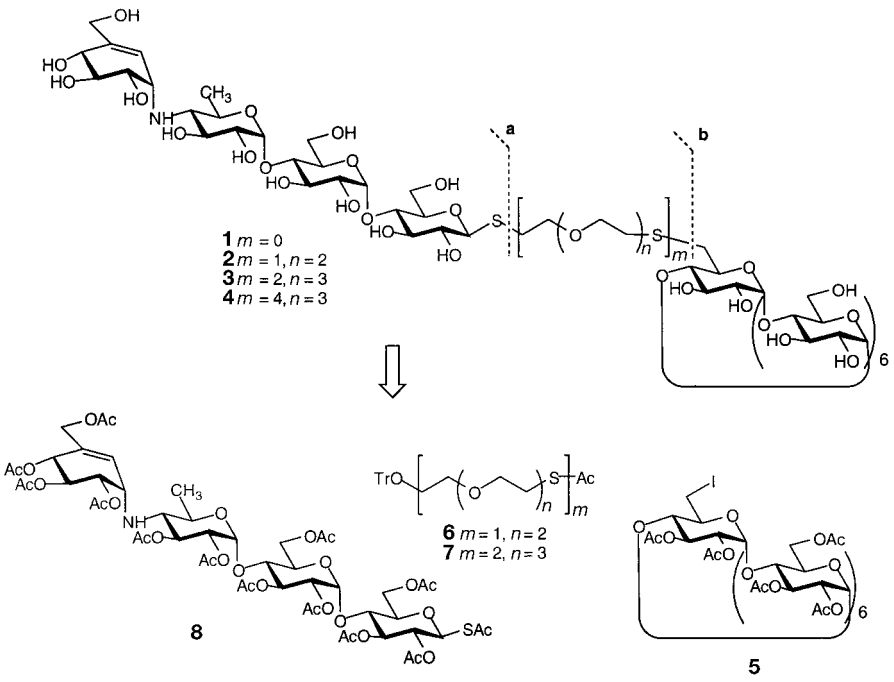
scattering, a technique that has recently been used with success for the determination of the hydrodynamic dimensions of a multidomain cellulase.[9]

It is well known that acarbose is a potent inhibitor of GA and that two molecules of $\beta$-cyclodextrin may mimic amylose binding on two sites of the SBD but cannot fit into the pocket-shaped active site.[10] These observations indicate that an amylose chain that interacts with the two constitutive domains of GA could be mimicked by an acarbose molecule tethered to a cyclodextrin molecule through flexible spacers of different lengths.

A few years ago we developed an efficient strategy for the synthesis of $\beta$-cyclodextrins that were substituted at the 6-position with a thioglucosyl moiety.[11] The X-ray structure of a cyclodextrin glycosyltransferase complexed with one of these molecules was recently solved, and indicated that the presence of a thioglucosidic linkage did not hinder the binding to the protein.[12] These results encouraged us to connect linkers to acarbose and $\beta$-cyclodextrin through sulfur linkages.

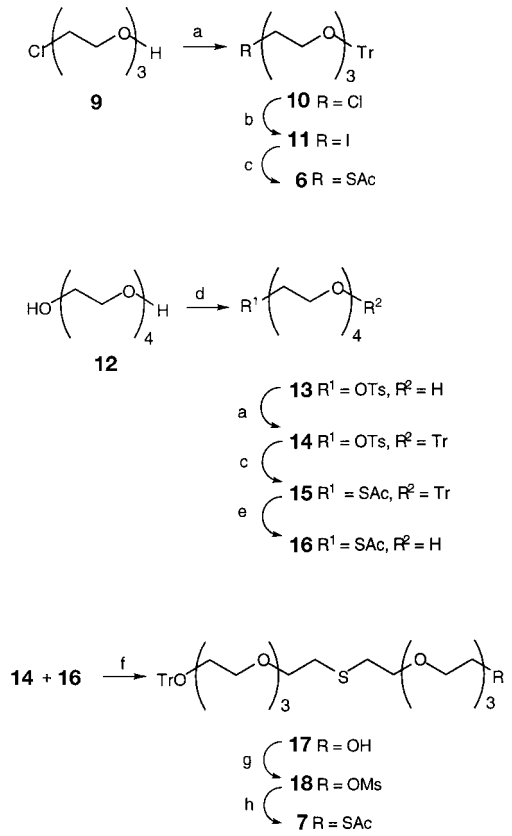



Scheme 1. Retrosynthetic pathway of the bifunctional ligands **1**–**4.**

Scheme 1 presents the retro-synthetic analysis on which the synthesis was based. Disconnection at the indicated bonds led to the key building blocks **5**–**8** for the synthesis of **1**–**3**. The longest probe **4** was best prepared by elongation of the linker **7** already coupled to $\beta$-cyclodextrin. To allow coupling with 6-deoxy-6-iodo-$\beta$-cyclodextrin **5** the bifunctional spacers **6** and **7** had to carry a thiol function and a protected hydroxyl group that could be easily converted into a thiophilic group for the establishment of the thioglycosidic linkage with *S*-acetylacarbose (**8**).

Protection of the hydroxyl groups of commercially available monochlorotriethylene glycol **9** by tritylation (trityl = Tr = triphenylmethyl), followed by halogen exchange and nucleophilic displacement of iodine with potassium thioacetate, afforded the first spacer-arm **6** in 33% overall yield (Scheme 2). Tetraethylene glycol **12** was treated with tosyl chloride and the resulting monotosylate **13** was tritylated to give **14.** Substitution of the tosylate with thioacetate and cleavage of the trityl group gave **16**, which was coupled with **14** after S-deacetylation (→**17**). Mesylation (mesyl=Ms = methanesulfonyl) of **17** gave **18**, and substitution of the mesylate group with thioacetate gave the bifunctional spacer **7** in 22% overall yield (from **12**).

The S-deacetylation of compounds **6** or **7** gave the corresponding sodium thiolates, which were coupled, without characterization, with 6-deoxy-6-iodo-cyclodextrin **5** prepared as already described[11] (Scheme 3). The branched cyclodextrins **19** and **20** were obtained in 76 and 64% yield, respectively. Detritylation, followed by mesylation and displacement of the mesyl group with iodine gave the




Scheme 2. Preparation of the bifunctional spacers **6**–**7**. a) $Ph_3CCl$, $CH_2Cl_2$, 4-DMAP, $NEt_3$, RT, 20 h, **10**: 70%, **14**: 51%; b) NaI (1.1 equiv), DMF, 100 °C, 3 h, 75%; c) KSAc (6 equiv), DMF, 100 °C, 2 h, **6**: 63%, **15**: 87%; d) TsCl (1 equiv), pyridine, 0 °C →RT, 12 h, 52%; e) $HBF_4$ in $H_2O$, $CH_3CN$, RT, 1 h, 95%; f) 1. **16**, MeONa (1.3 equiv), MeOH, 0 °C, 2 h, argon; 2. **14** (0.6 equiv), DMF, 45 °C, RT, argon, 73%; g) $MeSO_3Cl$, pyridine, 0 °C →RT, 84%; h) KSAc, DMF, RT, 2 h, argon, 87%. 4-DMAP = dimethylaminopyridine.

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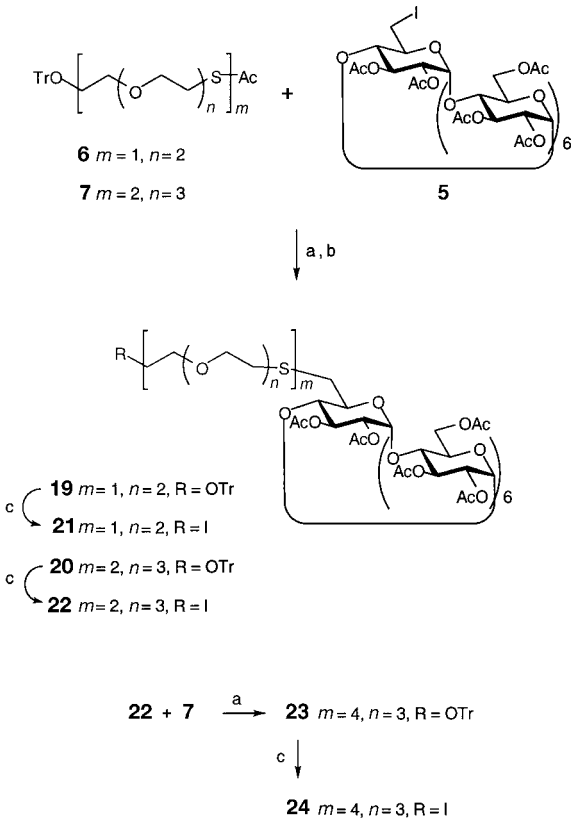



Scheme 3. Preparation of branched cyclodextrins **21**, **22**, and **24**. a) **6**, **7**, **22**, MeONa (10 equiv), 1 h, 0 °C, argon; b) 1. DMF, RT, 12 h, argon; 2. $Ac_2O$, pyridine, 4-DMAP, 60 °C, 12 h, **19**: 76 %, **20**: 64 %, **23**: 70 %; c) 1. $HBF_4$ in $H_2O$, $CH_3CN$, RT, 20 min; 2. $MeSO_3Cl$, pyridine, 0 °C → RT, 12 h; 3. NaI, DMF, 100 °C, 4 h, **21**: 51 %, **22**: 40 %, **24**: 43 %.

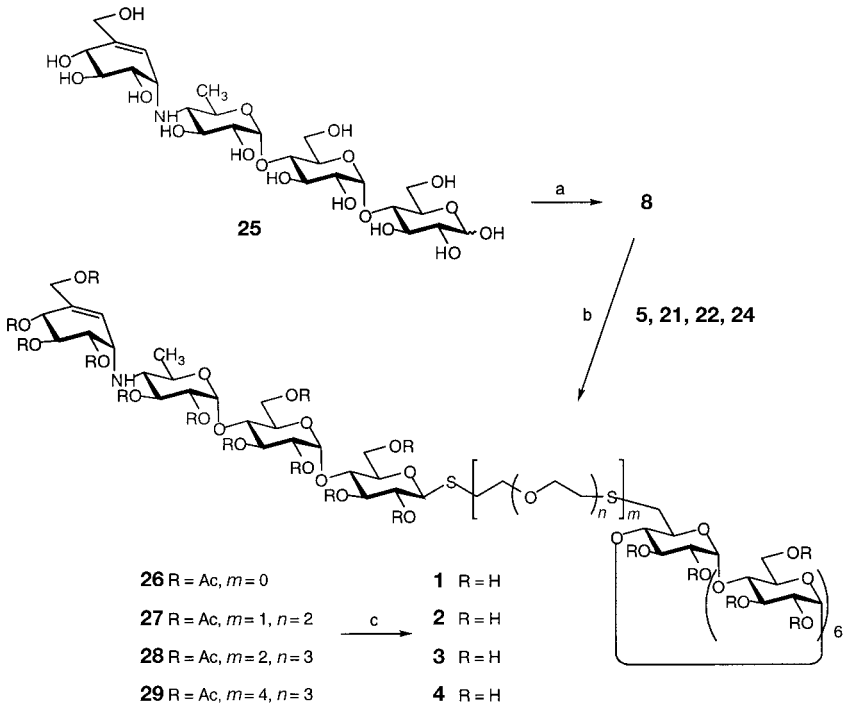
corresponding iodo compounds **21** and **22** in overall yields of 51 and 40 %, respectively. This stepwise protocol gave a higher yield than the direct OH → I substitution.[13] Coupling of the iodo derivative **22** with the thiolate arising from S-deacetylation of **7**, as already described, gave compound **23** in 70 % yield. Transformation of **23** into **24** was obtained by sequential reactions as described for the synthesis of **21** and **22**. The longest synthon **24** was obtained in an overall yield of 43 % starting from **22**.

To synthesize donor **8**, acarbose (**25**) was first acetylated, then treated with HBr/AcOH,[14] and the corresponding bromide was transformed into **8** in 63 % overall yield (Scheme 4).

Coupling of the acarbose derivative **8** with acceptors **5**, **21**, **22**, and **24** under the conditions of von Itzstein et al.[15] led to the acetylated target molecules **26**–**29** in yields of 63, 88, 83, and 74 %, respectively. The final products **1**–**4** were obtained by O-deacetylation and were purified by reversed-phase chromatography on a C-18 column.

The thermodynamic parameters of ligand binding to GA, as well as to the CD and SBD of GA, have been studied previously by isothermal titration calorimetry.[16] The $\Delta H^0$ values of binding of the four heterobidentate ligands were, within experimental error, equal to the sum of the $\Delta H^0$ values of the binding of free acarbose and $\beta$-cyclodextrin to the CD and SBD, respectively. These results showed that the catalytic and starch binding sites of GA are in close proximity in solution and this suggests considerable flexibility of the linker region.

Here the hydrodynamic dimensions of GA and the CD were determined by quasi elastic light scattering experiments. In accord with their three-dimensional structures the isolated SBD and CD were approximated as spheres, while the dumbbell two-domain GA was approximated as an ellipsoid whose minor semi-axis corresponded to the hydrodynamic radius of the largest individual component, for example, the CD. The experiments were performed at different angles and several protein concentrations (0.3 – 1.1 mg mL$^{-1}$). The autocorrelation function was represented by a single exponential decay for all concentrations and scattering angles. A very small contribution from a slow process has been identified at high concentration, which was attributed to the presence of minor protein aggregates. Relaxation times were estimated from the correlation functions, and the variation of the frequencies versus the scattering angles allowed the determination of the translational diffusion coefficients $D_T(c)$ at different concentrations. The values of the diffusion coefficients at infinite dilution $D_T^0 = D_T(c)_{c\to 0}$, which represents the single particle property, were converted into their hydrodynamic radii (modified Stokes – Einstein relation). Neither the GA or CD systems showed any concentration dependence on the diffusion coefficient (Figure 1).




Scheme 4. Preparation of ligands **1**–**4**. a) 1. $Ac_2O$, pyridine, RT, 24 h; 2. $CH_2Cl_2$, HBr/AcOH, −10 °C → 0 °C, 1 h; 3. KSAc, DMF, RT, 18 h, 63 %; b) $Et_2NH$, DMF, RT, 4 h, **26**: 63 %, **27**: 88 %, **28**: 83 %, **29**: 74 %; c) MeONa, MeOH, RT, 1 h, then $NH_4OH$ (1M), RT, 12 h, **1**: 78 %, **2**: 74 %, **3**: 91 %, **4**: 74 %.

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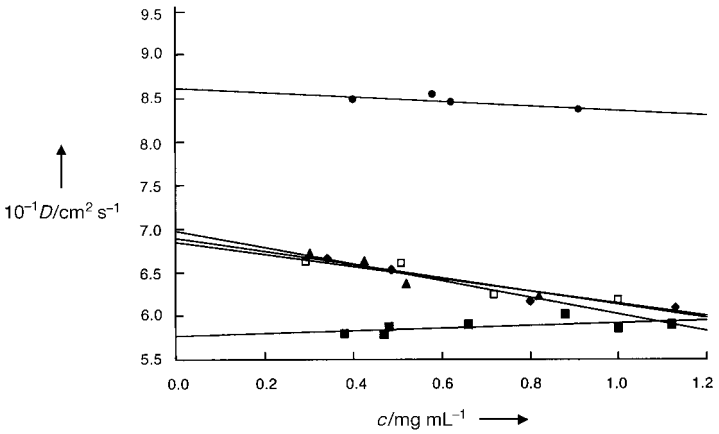


Figure 1. Variation of the translational diffusion coefficient $D_T(c)$ as a function of concentration $c$ of GA (■), CD (●), GA + **1** (◆), GA + **2** (▲), and GA + **3** (□).

The extrapolated values at infinite dilution gave hydrodynamic sizes of 78.5 Å (major semi-axis) and 30 Å (semi-axis), for the GA and CD, respectively. These values are in good agreement with those obtained previously with other techniques (141 and 60–65 Å for the major and minor axis of GA, respectively).[5, 6] The diffusion coefficients for **1**, **2**, and **3** in the presence of GA show a decrease as a function of concentration (Figure 1).

The three systems gave roughly the same concentration dependence within experimental errors. The associated diffusion coefficients decrease with the protein concentration and the extrapolated value at infinite dilution gave a hydrodynamic size of 62 Å (major semi-axis). However a value of 72.5 Å was found with the longer probe **4**. These values are intermediate between systems with a CD and GA only; and is in fact expected if the ligand somehow decreases the size of the GA system. The negative value of the slope of $D_T$ versus $c$ shows that there is a strong attraction between the particles and reflects the thermodynamics of the system; in general the value of the slope is pH dependent. It appears from these results that the presence of the bound bifunctional ligands stabilizes a more compact conformation of GA. If this motion of the two domains also occurs upon binding of the natural substrate it may achieve the processivity of this exo-enzyme: GA would bind to the polymer through its SBD and the motion of the CD would allow the cleavage of several glucose moieties at the nonreducing end of the same glucan chain before the release of the enzyme. This new concept is under investigation by using mutants of glucoamylase with linkers of various lengths.

*Experimental Section*

All compounds were homogeneous according to elemental analysis or HR-MS and $^1$H and $^{13}$C NMR spectra.

Dynamic light scattering experiments: Sample preparation, equipment, and data analysis are essentially the same as described in ref. [9], except that sodium acetate buffer (50 mM, pH 4.2) and a temperature of 27 °C were used. Equimolar ratios of ligands and enzyme were utilized.

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## Metal-Free Haloperoxidases: Fact or Artifact?

Ole Kirk* and Lars Sparre Conrad

Haloperoxidases are enzymes that catalyze the oxidation of a halide ion ($X^-$) to the corresponding hypohalous acid [Eq. (1)]. The hypohalous acid produced can further react with different nucleophilic acceptors to form a diversity of halogenated compounds.[1, 2] Different classes of haloperox-

$$H_2O_2 + X^- + H^+ \overset{\text{haloperoxidase}}{\rightleftharpoons} HOX + H_2O \qquad (1)$$

[*] Dr. O. Kirk
Department of Bio-Organic Chemistry
Enzyme Research, Novo Nordisk A/S
Novo Alle, DK-2880 Bagsvaerd (Denmark)
Fax: (+45) 4442-3206
E-mail: oki@novo.dk
Dr. L. Sparre Conrad
Department of Formulation Design
Enzyme Development and Application, Novo Nordisk A/S
Novo Alle, 2880 Bagsvaerd (Denmark)

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