Polysaccharide Branching

DOI: 10.1002/ange.201305132

Nature's Dendrimer: Characterizing Amylopectin as a Multivalent Host**

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Molecular structure defines the chemical and physical properties of species ranging from small molecules to very large biopolymers like polysaccharides. Detailed structural information on polysaccharides is very difficult to obtain, and they are therefore often described either schematically or as "averaged" structures, obscuring the molecular detail. Herein, we present the development of a small supramolecular probe that yields detailed structural features of the starch polysaccharide, amylopectin.

Starch is composed of two polysaccharides, amylose and amylopectin. Both are complex α-glucans whose molecular structures and capacities for supramolecular interactions are strongly linked to their utility as energy storage units in plants, ingredients in foods, raw material for paper, adhesive and bioethanol production,[1] and as structural or functional components for glyconanotechnology.[2] Amylose is an essentially linear polymer made up of α(1-4)-linked glucopyranose units and can, through supramolecular wrapping, solubilize carbon nanotubes[3] and insulate and confer chirality onto molecular wires.[4] Amylopectin is a highly branched polysaccharide composed of segments of linear α(1-4)-linked glucopyranose units joined at branching points via α(1-6) glycosidic linkages to give a structure that resembles a dendrimer (Figure 1 a).[5] Amylopectin is capable of binding hydrophobic guests in aqueous solution,[6] and yet its potential as a multivalent host (for example, for molecular transport and drug delivery or in enzyme responsive materials) has received little attention,[7] possibly owing to the difficulty of predicting and characterizing the molecular recognition of this poorly-defined polydisperse branched polymer with molecular weights ranging from 10^5 to 10^8 g mol^{-1}.[1]

The generally accepted model for the structure of amylopectin is that linear segments or branches, with a degree of polymerization (DP) of six to approximately 20, are arranged together in clusters (Figure 1 a).[1] These branches are linked together through longer linear branches spanning out radially from a single reducing-end (hemiacetal) glucose. The clustering of branches is responsible for the semicrystalline nature of the polymer, and in the solid state, pairs of adjacent branches at the non-reducing ends of the polymer self-assemble to form left-handed parallel double helices. The physicochemical properties of different starches are influenced by variations in the number, length, and distribution of these branches.[8]

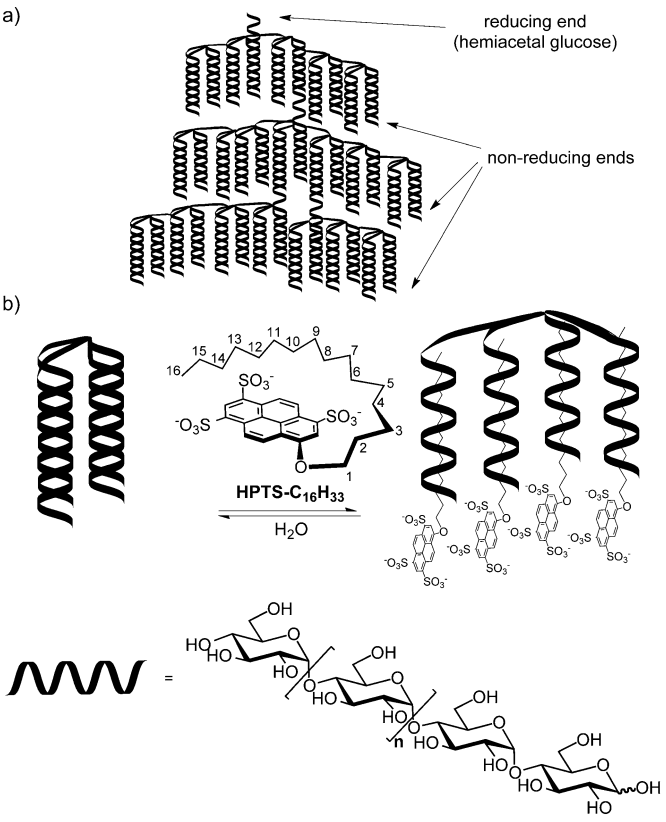

Figure 1. a) Cluster model for the structure of amylopectin. Branches at the non-reducing ends of the dendrimer-like polysaccharide self-assemble into left-handed double helices. b) Binding of the amphiphilic probe HPTS-$C_{16}H_{33}$ to the non-reducing-end branches of amylopectin in left-handed single helices.

In aqueous solution, in the presence of suitable hydrophobic guests, linear α(1-4)-linked glucose oligosaccharides form left-handed single helices. The guests are bound in the hydrophobic channel at the center of the helix[9] in a binding mode that is reminiscent of inclusion complexes with cyclodextrins.[10] We previously reported that similar helical binding sites can form in branched starch polysaccharides in solution.[11] Studies have indicated that amylopectin binds lipids and surfactants with non-cooperative Langmuir-type binding.[6a] It is hypothesized that the hydrophobic tails of lipids and surfactants bind in the external non-reducing-end

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[**] We thank Dr. Sebastian Meier and Dr. Michael Pittelkow for helpful discussions. We acknowledge the Danish Instrument Center for NMR Spectroscopy of Biological Macromolecules at the Carlsberg Laboratory, where the 800 MHz spectra were recorded.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201305132.

branches of amylopectin.[12] In lipid-amylose complexes in the solid state the polysaccharide is typically wound around the hydrophobic tail of the lipid with six glucose units per turn,[6] and recent modeling studies have suggested that a DP of 13 should be sufficiently long to bind the lipid glycerolmonooleate in two oligosaccharide turns.[13]

We recently described an amphiphilic NMR probe, HPTS-$C_{16}H_{33}$, that undergoes a conformational change upon binding to α(1-4)-linked glucose oligosaccharides and may be used to qualitatively assess the presence of hydrophobic binding sites in starch polysaccharides (Figure 1b).[11] Herein, we show how the binding of multiple guests to amylopectin is similar to the multiple non-cooperative binding of ligands to a dendrimer and may be modeled as such to determine binding affinities and the number of binding sites on the polysaccharide. We relate these findings to the molecular structure of amylopectin, deduce the minimum branch length required to create suitably stable helical binding sites, and show that binding to non-reducing-end branches is stronger than to internal branches. The molecular recognition of HPTS-$C_{16}H_{33}$, is hereby used as a method to study the molecular structure of amylopectin, by identifying and characterizing binding sites for hydrophobic guests.

The binding of HPTS-$C_{16}H_{33}$ to amylopectin from potato results in significant changes in the ^{1}H NMR spectrum of the amphiphile (Figure 2). Unbound, the aliphatic tail folds over the top of the pyranine moiety (as a consequence of the hydrophobic effect), the methyl and methylene protons encounter the aromatic ring current and this results in upfield-shifted signals in the ^{1}H NMR spectrum of the probe.[14] Upon binding to amylopectin, the amphiphile unfolds and the aliphatic proton signals shift downfield.

The change in chemical shift of the H16 proton of HPTS-$C_{16}H_{33}$ upon titration with amylopectin was monitored to obtain a binding isotherm (Figure 3; Supporting Information, Figure S3). The average molecular weight of the amylopectin was estimated from its translational self-diffusion coefficient obtained by DOSY NMR to be approximately 280000 g mol^{-1}, which corresponds to a degree of polymerization on the order of 1700.[11] The data were fitted to a dendrimer binding model in which there are an unknown number of similar binding sites (n) of similar binding strength

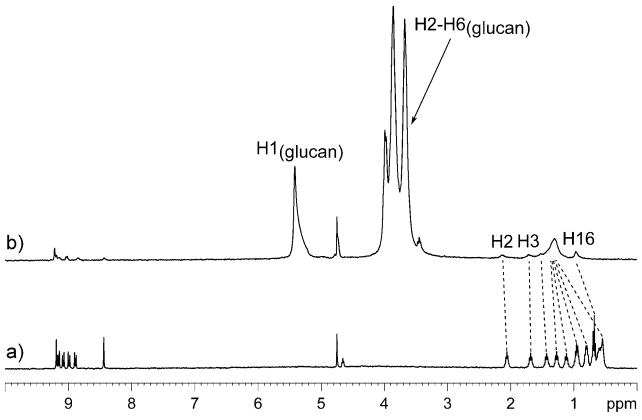

Figure 2. ^{1}H NMR spectra of HPTS-$C_{16}H_{33}$ (0.5 mM) in D_2O in the a) absence and b) presence of amylopectin (5 mg mL^{-1}).

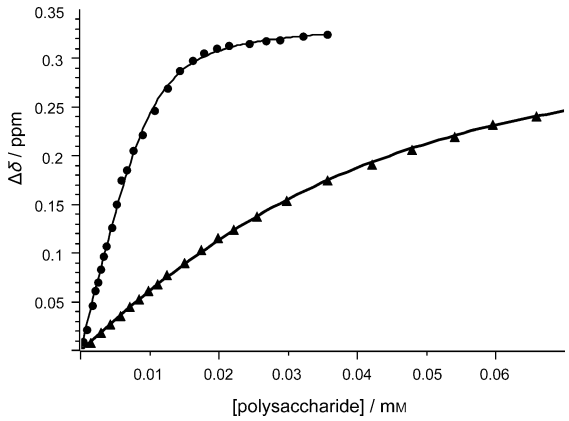

Figure 3. Fitted binding isotherms for the interactions of HPTS-$C_{16}H_{33}$ with amylopectin (●) and its β-limit dextrin (▲).

(K_a; see Equation (1), where $[G]_0$ and $[D]_0$ are the total concentrations of guest (HPTS-$C_{16}H_{33}$) and polysaccharide, respectively).[15] An association constant (K_a) of $(2\times10^4\pm3\times10^3)$ M^{-1} was obtained for the individual binding interactions, $\Delta\delta_{max}$ was measured as 0.338 ± 0.003 ppm and the number of binding sites per molecule (n) was determined to be 49 ± 1.

$$\Delta\delta_{obs}=\frac{\Delta\delta_{max}}{2}\left\{\left(1+\frac{1}{K_a[G]_o}+\frac{n[D]_o}{[G]_o}\right)-\sqrt{\left(1+\frac{1}{K_a[G]_o}+\frac{n[D]_o}{[G]_o}\right)^2-\frac{4n[D]_o}{[G]_o}}\right\} \quad (1)$$

To assess the validity of this multiple non-cooperative guest binding model for amylopectin we sought an alternative means to quantify the number of guests that can bind. Generally, the number of lipids bound to a polymer when the binding sites are saturated may be estimated from the difference between the critical micelle concentration (ΔCMC) of the lipid in the absence (CMC) and in the presence (CMC*) of a known concentration of the polymer.[16] The CMCs of HPTS-$C_{16}H_{33}$ in aqueous solution in the absence and presence of amylopectin (10 mg mL^{-1}, 0.036 mM) were estimated from the dilution curves obtained by plotting the change in chemical shifts of H2 in the ^{1}H NMR spectrum of HPTS-$C_{16}H_{33}$ upon dilution (Figure 4; Supporting Information, Figures S5,S6). ΔCMC was estimated to be approximately 2 ± 0.5 mM, which corresponds to approximately 55 ± 15 HPTS-$C_{16}H_{33}$ molecules bound per amylopectin molecule. This value is consistent with the results obtained by fitting of the binding isotherm to Equation (1) (49 amphiphiles bound to amylopectin).

Titration with our amphiphilic probe can give an estimate of the number of binding sites on an amylopectin molecule, but to be able to relate such a finding to the molecular structure of the amylopectin, we needed to first have a measure of the size of the binding sites, that is, the length of the helix in terms of DP. The number of branches in an amylopectin molecule of known molecular weight may be calculated from the percentage of α(1-6)-linked glucose units determined using ^{1}H NMR spectroscopy (Supporting Information, Figure S1). For this sample, we calculated 5% branching, which for a polysaccharide with 1700 glucose

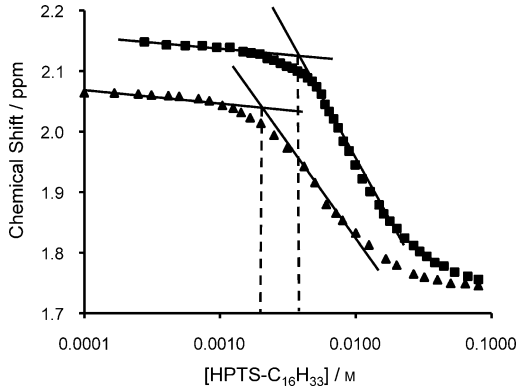

Figure 4. HPTS-$C_{16}H_{33}$ dilution curves monitoring the change in chemical shift of H2 in the presence (■) and absence (▲) of amylopectin (10 mg mL^{-1}); critical micelle concentrations are estimated from the intersection of tangents to the curves as indicated.

units, equates to 85 branches. Making the assumption that only one amphiphile can bind per branch and because only 49 binding sites were identified on the amylopectin, we calculated that approximately 40% of the branches do not strongly bind HPTS-$C_{16}H_{33}$. It was expected that there would be a minimum branch length required for strong binding of the amphiphile to take place[12c] and we therefore inferred that 40% of the branches must be too short to bind the guests.

To determine the distribution of different length branches in the amylopectin sample, the polysaccharide was treated with hydrolytic enzymes, isoamylase from *Pseudonomas sp.* (E.C. 3.2.1.68) and pullulanase from *Krebsiella planticola* (E.C. 3.2.1.41), which cleave α(1-6) glycosidic linkages (Figure 5, left).[17] The resulting linear fragments were fluorescently labeled at their reducing-end glucose (hemiacetal) by way of reductive amination with 2-aminobenzamide and then analyzed by UPLC/MS (Supporting Information, Figure S7). Figure 6a shows the branch length distribution and cumulative molar fraction of amylopectin branches of increasing length; it can be seen that the shortest 40% of the branches have DP ≤ 12. It was therefore deduced that only branches with DP ≥ 12 are capable of binding HPTS-$C_{16}H_{33}$. Because one turn of a single α-glucan helix requires six glucose units, binding of HPTS-$C_{16}H_{33}$ would appear to require at least two turns of the oligosaccharide, which is in agreement with recent modeling studies.[13]

Complexation of non-reducing-end branches of amylopectin with HPTS-$C_{16}H_{33}$ might be expected to inhibit the action of *exo*-acting hydrolytic enzymes on the polysaccharide. β-Amylase sequentially hydrolyzes maltosyl disaccharides from the non-reducing ends of α-glucans until α(1-6)-linked branch points are reached, leaving one, two, or three glucose units at the non-reducing-end side of the branch point (Figure 5, right).[16] The resulting structure is called the β-limit dextrin. We hypothesized that because HPTS-$C_{16}H_{33}$ binds preferentially to amylopectin branches with DP ≥ 12, then treatment of amylopectin with β-amylase in the presence of HPTS-$C_{16}H_{33}$ should result in a partially hydrolyzed amylopectin with an increased proportion of branches with DP ≥ 12. Amylopectin (5 mg mL^{-1}) was therefore treated with β-amylase from barley (E.C. 3.2.1.2) in the presence and

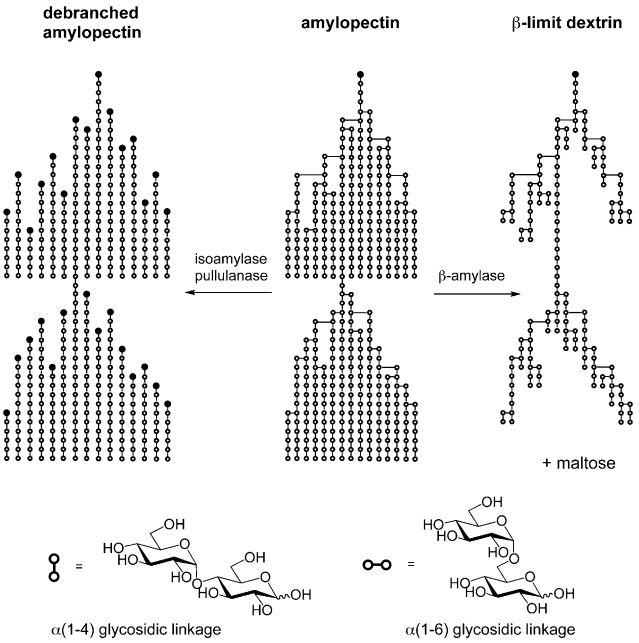

Figure 5. The action of hydrolytic enzymes on amylopectin: isoamylase and pullulanase cleave α(1-6) glycosidic linkages (left); β-amylase cleaves maltosyl disaccharides from the non-reducing ends of amylopectin until an α(1-6) linkage is reached, generating the β-limit dextrin (right). Reducing-end (hemiacetal) glucose molecules are shown by filled circles.

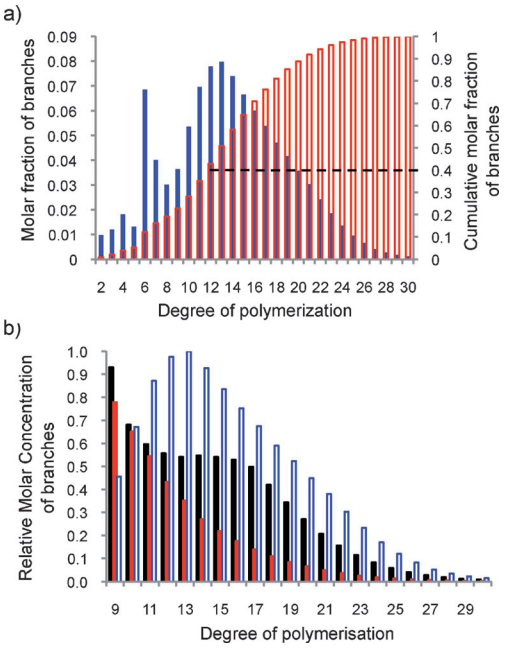

Figure 6. a) Distribution of branch lengths making up the amylopectin sample (blue) and the cumulative molar fraction of branches of increasing length (red, unfilled). b) Comparison of branch lengths in native amylopectin (blue, unfilled) with amylopectin that has been treated with β-amylase in the presence (black) and absence (red) of HPTS-$C_{16}H_{33}$. Data obtained by UPLC/MS analysis of fluorescently labeled enzymatically debranched samples.

absence of one equivalent per binding site of HPTS-$C_{16}H_{33}$ (0.88 mM). The solutions were then boiled for two minutes to inactivate the β-amylase, and methyl-β-cyclodextrin, which has a strong affinity for lipids, was added in excess to sequester and mask the detergent properties of the HPTS-$C_{16}H_{33}$. The polysaccharides were then enzymatically debranched, and the resulting linear oligosaccharides were fluorescently labeled and analyzed by UPLC/MS (Supporting Information, Figure S8). Figure 6b shows the distribution of branch lengths in the two samples in comparison with the branch distribution in the native amylopectin. In keeping with our predictions, the presence of HPTS-$C_{16}H_{33}$ inhibited the action of β-amylase specifically on branches with DP $\geq$ 12, leading to a relative increase in the concentration of these oligomers in the debranched sample compared with the amylopectin treated with β-amylase in the absence of complexing HPTS-$C_{16}H_{33}$.

It has been suggested that lipids and surfactants bind preferentially in the external non-reducing-end branches of amylopectin.[12] To test this theory, we used our dendrimer binding model to compare the binding of HPTS-$C_{16}H_{33}$ to a sample of amylopectin with binding to its β-limit dextrin (Figure 5). Amylopectin was hydrolyzed exhaustively with β-amylase, converting 70% of the polysaccharide material to maltose, as determined by integration of the relevant peaks in the ^{1}H NMR spectrum (see Supporting Information, Section S3 for details). The molecular weight of the resulting β-limit dextrin was therefore estimated to be 84000 g mol^{-1}. A solution of HPTS-$C_{16}H_{33}$ (0.5 mM) in D_2O was titrated with a solution of the β-limit dextrin and maltose mixture (total sugar concentration 50 mg mL^{-1}, therefore 15 mg mL^{-1} β-limit dextrin (0.18 mM)). The change in chemical shift of H16 on the amphiphile was monitored and the binding isotherm was fitted to the dendrimer binding model with $\Delta\delta$ set at 0.338 ppm (Figure 3; Supporting Information, Figure S4). An association constant (K_a) of $(4\times10^3 \pm 1.5\times10^2)$ M^{-1} was determined for the binding of HPTS-$C_{16}H_{33}$ to 15 ± 1 binding sites. Binding to the β-limit dextrin was significantly weaker than to the native amylopectin (4×10^3 M^{-1} as compared with 2×10^4 M^{-1}), which supports the theory that lipid and surfactant binding takes place preferentially in the external non-reducing-end branches of the polymer. We suggest that binding to the internal branches or polymer backbone was weaker, even when made accessible by hydrolysis of the external branches, because the presence of branch points impedes the formation of left-handed helices in linear sections of the polymer backbone.

In conclusion, we have shown that it is possible to quantitatively characterize the interaction between amylopectin and hydrophobic guests by applying a multiple non-cooperative binding model. Knowledge of the number, location and size of binding sites not only provides essential information for the development of nanodevices and materials that use amylopectin as a multivalent host, it provides an assessment of the branching pattern in the amylopectin sample. We suggest that an extended range of hydrophobic probes could allow for direct quantification of non-reducing-end branches of different lengths in amylopectins. Such methods to investigate amylopectin structure would facilitate the study of the structural variation between amylopectins from different sources and how these variations correlate with the physicochemical properties of the starches, information which could aid in the selection or bioengineering of plants producing starches with desirable properties.

Received: June 14, 2013
Published online: September 2, 2013

Keywords: carbohydrates · hydrophobic effect · polysaccharides · starches · supramolecular chemistry

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