ACS Catalysis

Research Article

pubs.acs.org/acscatalysis

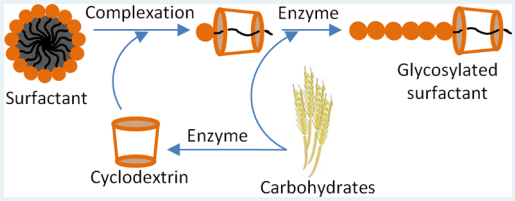
Hydrophobic Complexation Promotes Enzymatic Surfactant Synthesis from Alkyl Glucoside/Cyclodextrin Mixtures

Tim Börner,* Kevin Roger,† and Patrick Adlercreutz‡

Department of Biotechnology, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden

Supporting Information

ABSTRACT: The unique ability of cyclodextrin glycosyltransferase to form and utilize the cyclic maltooligosaccharide cyclodextrin (CD) makes this enzyme an attractive catalyst for the synthesis of alkyl glycosides. Here, we characterize the sugar headgroup elongation of alkyl glucosides (acceptor) via two transglycosylation reactions from either a linear (maltohexose) or a cyclic (CD) glycosyl donor. Inclusion complex formation overcomes both poor substrate solubility and aggregation. We have used pure alkyl glucosides and αCD as model compounds. The complex between CD and alkyl glucoside was efficiently used as a substrate. Kinetic and thermodynamic measurements allow the prediction of the optimal synthesis conditions. This optimum corresponds to the transition between a donor-limiting and an acceptor-limiting regime. The resulting rational design should lead to the practical development of a cost-efficient industrial synthesis. Our findings with respect to the importance of complexation should also readily apply to other enzymatic systems.



KEYWORDS: *alkylglycoside, glucanotransferase, cyclodextrin, inclusion complex, self-assembly*

1. INTRODUCTION

Alkyl glycosides (AGlys) are biobased, nonionic surfactants composed of a hydrophobic alkyl chain and a hydrophilic sugar headgroup derived from renewable resources. AGlys possess interesting physicochemical properties, combining biodegradability with chemical stability, and are found, for instance, in detergents, cosmetics, and protein extraction applications.[1] Industrially, the synthesis of such carbohydrate-based surfactants relies entirely on chemical methods (Fischer glycosylation) involving condensation between a carbohydrate and a fatty alcohol.[2] Despite using renewable raw materials, chemical methods producing a long sugar head and anomerically pure AGlys involve, in general, inherently circuitous and expensive protection and deprotection steps,[3,4] which decreases the attractiveness of the process. Complex mixtures of oligomeric species composed of alkyl mono-, di-, tri-, and oligoglucopyranosides with C_8−C_{16} long tails are typically obtained.

Alternatively, enzymatic syntheses of AGlys represent an attractive path to produce anomerically pure AGlys with a longer carbohydrate part. In principle, two methodologies could be exploited for the enzymatic glycosylation: reverse hydrolysis (thermodynamically controlled approach) and transglycosylation (kinetically controlled approach). So far, reverse hydrolysis catalyzed by glycosidases is limited to substrates of moderate size.[5] Indeed, efficient synthesis is prevented by miscibility problems arising from substrate mixtures of longer alcohols and sugars.[6]

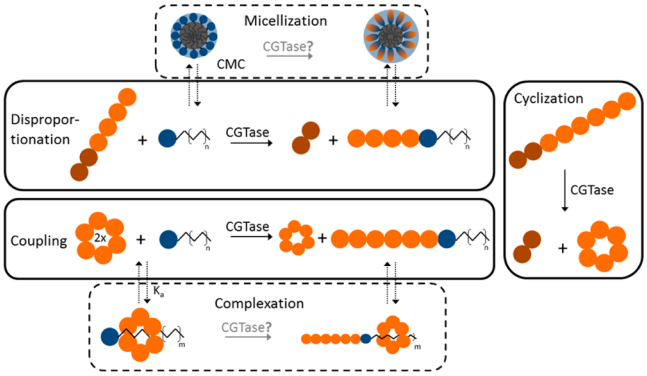
An effective method to synthesize AGlys containing long sugar and alkyl chain may be the transglycosylation by action of the cyclodextrin glucanotransferase (CGTase),[7] a member of the hydrolase family 13.[8] This enzyme has the unique ability of producing and utilizing cyclodextrins (CDs). The cyclic maltooligosaccharides, consisting of 6 (α), 7 (β), or 8 (ω) glucose units linked via 1,4 α-glycosidic linkages, are formed through an intramolecular transglycosylation (cyclization) reaction from long and linear maltooligosaccharide chains, for example, starch as a natural substrate. The opposite of cyclization is the coupling reaction in which a CD molecule (glycosyl donor) is first cut (linearization) and then transferred entirely onto an acceptor molecule. This transfer represents an intermolecular transglycosylation reaction with a bound glycosyl intermediate (substrate-enzyme complex) as transfer group. The coupling reaction proceeds via a ternary complex mechanism[9] because both substrates can bind simultaneously and in random order. In the third reaction type, disproportionation, rearrangement between two linear maltooligosaccharides (donor and acceptor) is achieved by cleaving the linear sugar donor, thereby forming the transfer but also a leaving group (coproduct). After the liberation of the coproduct, the acceptor binds and becomes transglycosylated by the glycosyl intermediate. Thus, disproportionation proceeds via a substituted-enzyme mechanism.[10] Scheme 1 schematically summarizes the major reactions catalyzed by CGTase. Natively, pure hydrolysis activity of CGTase is only minor.[9]

CGTase can usually be used to elongate the sugar headgroups of alkyl glucosides (AGlu's) by exploiting the coupling[11] and disproportionation reaction, respectively, using either cyclic or linear sugars as donor molecules. In both approaches, using AGlu as the acceptor substrate, a complex system is obtained as a result of aggregate- and complex-

Received: February 13, 2014
Revised: May 22, 2014
Published: June 23, 2014

   dx.doi.org/10.1021/cs500192q | ACS Catal. 2014, 4, 2623−2634

Scheme 1. Three Reaction Catalyzed by CGTase: Disproportionation, Coupling and Cyclization (solid lined boxes)[a]



[a]Non-enzymatic processes between the substrate molecules are shown in dashed boxes. At the critical micelle concentration (CMC) the alkyl glucoside/glycoside surfactant molecules (blue/blue-orange circle(s) with black chain) aggregate and form micelles, whereas complexation occurs when surfactants are complexed by cyclodextrin (orange ring). In the scheme: each circle represents one glucose unit connected to another via an *O*-glycosidic bond.

forming substrates, respectively. Because AGlu's are surfactants, they self-assemble (micelle formation) in aqueous solution above the critical micelle concentration (CMC). Enzymatically catalyzed synthesis is often hampered by self-assembling substrates because of decreased monomer availability.[12] The addition of organic solvent is frequently employed with the purpose of increasing the monomer availability of poorly dissolved and aggregating substrates, respectively. However, enzyme stability and activity often suffer from the presence of organic solvents, with the consequence of enzyme inactivation.[13] An attractive alternative among reaction additives, enhancing the dissolved substrate form, is the use of CDs in enzymatic reactions.[14] Because of their nature, CDs are benign to enzymes and the environment. The principle behind substrate solubilization with CDs is based on inclusion complexation between the guest molecule (substrate) and the CD.[15] As a drawback, the complexed substrate may become inert for enzymatic catalysis. Both decrease and increase in enzymatic conversion have been reported when using a CD as the solubilizing additive.[15−19]

Complex formation between a guest molecule and CDs has been studied intensively.[20−23] There are many applications in which complexation is exploited: for example, aroma complexation and slow release, stabilization of flavors, for odor removal and perfume carrier, in pharmaceuticals for drug protection, in membrane protein science, and many more.[24−26] The complexation between CDs and AGlu's has also been intensively studied in the field of physical chemistry.[27] Among those, Casu and co-workers[28] provided one of the earlier studies on formation, structure, and properties of CD−AGlu complexes (CD:AGlu). As a result of the hydrophobic cavity of the conical cylinder, nonpolar guest molecules can be encapsulated by CDs. Several interactions are involved to form such a "host−guest" inclusion complex. All of them are weak interactions, strong enough for complexation (with water displacement by the guest molecule in the cavity as the main driving force), but also weak enough to allow the release of the guest molecule.[20] Thus, complexation is a dynamic equilibrium process established between dissociated and associated species expressed by the equilibrium association constant, K_A. For AGlu's with long alkyl chains, complexes with stoichiometry larger than one were detected.[29] In addition to creating different substrate species, in free and complexed forms, complexation stands in direct competition to micelle formation, and it was thus found that in a CD/AGlu aqueous system, the CMC is shifted (equilibrium shift) directly dependent on the amount of CD added.[27] In the presence of CDs the critical association concentration (CAC) is then determining micelle formation.

So far, mainly alkyl maltosides have been used as starting material because they possess better solubility and reactivity than AGlu's. Promising results were obtained by Zehentgruber at al.[30] in elongating the maltose headgroup of dodecyl-β-D-maltoside (C12G2) by applying an excess amount of αCD. This system combines the complexation process with CD as solubilization agent and being an actual substrate. Nonetheless, because of the process of complexation, different molecular (substrate) species are formed, and their reactivity is unknown. For industrial applications, AGlu's are much more attractive because they constitute a less expensive precursor as they are more easily synthesized. In addition, an excess amount of CDs as donor needs to be avoided if not even replaced by less expensive alternatives. With this article, we provide a rational design for AGly synthesis with quantitative analysis of mechanisms and kinetics, which condenses into a model predicting optimal synthesis conditions. In addition, because CGTase was found to accept guest−CD complexes as substrate, this system could be applied to transglycosylate different hydrophobic compounds.

2. EXPERIMENTAL SECTION

Bacillus macerans cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) was kindly provided by Amano Enzyme Europe Ltd. (Oxfordshire, UK). *N*-Octyl-β-D-glucoside (C8G1), *N*-*n*-decyl- (C10G1), and *n*-dodecyl- (C12G1) were obtained from Anatrace Inc. (Maumee, OH); and α-cyclodextrin, αCD, (CAVAMAX W6) was kindly provided by Wacker Chemie AG (Stuttgart, Germany). Maltohexaose, G6, (purity >90%) was purchased from Sigma-Aldrich Co. (Sweden). All other chemicals were of pro-analysis grade from VWR International (Stockholm, Sweden) if not otherwise stated.

2.1. Enzymatic Reactions. Enzymatic reactions were performed in 4.5 mL glass vials with 10 mM sodium citrate buffer, pH 5.2, containing 2 mM $CaCl_2$ placed in a ThermoMixer (HCL Biotech, Bovenden, Germany) set to 60 °C and 750 rpm. Stock solutions of the substrates were prepared in the reaction buffer. α-Cyclodextrin solutions were boiled at 100 °C and kept at 60 °C in the thermoshaker to ensure solubility during use. The donor (αCD) and the acceptor (AGlu) concentrations were set by using stock solutions of αCD and AGlu. First, the respective amount of αCD was added to the buffer, and second, the respective amount of C8G1, to obtain a homogeneous mixture. The reaction mixture was equilibrated for 10 min at 60 °C in a thermoshaker.

The reaction was started by adding the enzyme solution (0.1−2 μg/mL). Samples were withdrawn at respective time intervals with a Hamilton syringe and diluted in 5 mM sodium hydroxide solution (quenching the enzyme reaction). Reaction products were subsequently analyzed by UHPLC or HPAEC-PAD (high-performance anion-exchange chromatography, pulsed amperometric detector). For the disproportionation reaction between G6 and other substrates (AGlu's, αCD, G6)

dx.doi.org/10.1021/cs500192q | ACS Catal. 2014, 4, 2623−2634

the same procedure was applied, but by boiling the substrate solution prior to the reaction. The specific transglycosylation activity (U/mg, disproportionation or coupling) was estimated by the amount (micromoles) of primary product (for example, octyl-β-D-pentaoside, C8G5, octyl-β-D-heptaoside, C8G7, or sugars) formed over time (minutes) per amount (milligrams) of enzyme used.

2.2. UHPLC and HPAEC-PAD Analysis. Alkyl glucosides and their elongated alkyl glycosides (AGly's), transglycosylation products, were analyzed using a Dionex Ultimate 300 UHPLC (ultra high pressure) system provided with a C-18 reverse phase column (Acclaim RSCL 120 Å, 2.2 μm, 150 × 2.1 mm) connected to a charged aerosol detector (CAD) (Dionex, ESA Corona ultra CAD). The autosampler and column compartment were set to 40 °C, and the post column cooler, to 30 °C. For sample elution, the initial conditions were composed of 25% (v/v) acetonitrile and 75% acetic acid (aq., 0.1%). Injected samples were eluted by a linear gradient of 25−95% acetonitrile within 3 min, followed by 2 min holding time at 95/5 (v/v) acetonitrile/0.1% acetic acid, 1 min linear gradient back to initial conditions; and finally, 5 min reconditioning of the column with the initial conditions of the mobile phase. The injection volume was set to 5 μL. Peak analysis was performed using Chromeleon 7 software from Dionex (Thermo Scientific, Sweden). Because the primary product C8G7 was not commercially available, a standard curve for C8G7 was made by in-house production and purification.

Sugar analysis was performed on a HPAEC ICS 5000 DC Dionex system (Thermo Fischer Scientific Inc.) equipped with a pulsed amperometric detector (PAD with a gold working electrode (Ag/AgCl as reference electrode) and an AS-AP auto sampler. All eluents were degassed by flushing with helium and kept constantly under helium pressure. An anion-exchange 250 × 3 mm CarboPac PA 200 column was used at 30 °C, and the auto sampler temperature was set to 10 °C to suppress possible residual enzyme activity and product decomposition. The sample injection volume was set to 10 μL. Eluent A was pure Milli-Q water, eluent B consisted of 200 mM sodium hydroxide and 400 mM sodium acetate, and eluent C was 200 mM sodium hydroxide. The initial mobile phase was composed of 50% A, 5% B, and 45% C (resulting in pH 13.0) at a flow of 0.5 mL/min. Injection samples were eluted by a linear gradient of B from 5% to 50% and C from 45% to 0% within 20 min, followed by 5 min isocratic mode and back to initial conditions within 1 min, followed by 14 min reconditioning. Peaks were analyzed by Chromeleon 7 software. Identification of the different carbohydrates was done on the basis of maltose (G2), maltotriose (G3), maltotetraose (G4), maltohexaose (G6), and α-cyclodextrin (αCD) standards. Longer maltooligosaccharides were identified by their regular elution pattern with respect to the standards.

2.3. Isothermic Titration Calorimetry (ITC). Isothermic titration calorimetry was used to determine the association constant between octyl-β-D-glucoside (C8G1) and α-cyclodextrin (αCD). The calorimetry measurements were performed at 60 °C with a VP-ITC microcalorimeter from MicroCal (USA) consisting of a 1.463 mL sample cell and an injector module with a total syringe volume of ∼295 μL. Under optimal conditions, this instrument has a high sensitivity and is capable of detecting in the nanowatts range. After the input of the experimental parameters (concentration, temperature, injection volume, amount of injections, etc.) the entire experiment takes place under computer control. All used solutions were filtered and degassed prior the ITC measurements. For the measurements, the sample cell was loaded with a buffered C8G1 solution (10 mM sodium citrate buffer, pH 5.2) having a concentration of 1.2, 0.5, and 0.25 mM, and a buffered 8, 10, and 20 mM CD solution was titrated into the sample cell. The ITC experiments consisted thereof of four independent measurements covering the molar ratio (CD/C8G1) of 0−1.37, 0−4, 0−8.5, and 0−10.2 by having an initial concentration ratio (syringe/cell, mM/mM) of 8/1.2, 10/0.5, 10/0.25, and 20/0.5, respectively. The four different sets of concentrations provided four ranges of molar ratios between CD and C8G1, to increase precision of the modeling procedure and parameter determination. Origin software (Origin 7 SR4; OriginLab Corporation, Northampton, MA) was then used to analyze the ITC data using two fitting models to calculate association constant (K_A), enthalpy (ΔH), and entropy (ΔS). The one-site binding model was used mainly to fit the experimental data, assuming a complex stoichiometry of 1. The sequential binding model was used to estimate possible 2:1 complex formation. The dilution heat of injection was subtracted from the total signal as well as the blank run using only buffer. The monomeric (αCD and S) and complexed (αCD:S) species can be expressed with the following scheme:

$$\alpha\mathrm{CD} + \mathrm{S} \overset{K_A}{\rightleftharpoons} \alpha\mathrm{CD{:}S}$$

The association (equilibrium) constant, K_A, of the complex between αCD and surfactant (S) is then expressed as the concentration ratio between complex [αCD:S] and the monomers [αCD] and [S].

$$\frac{K_A}{c_0} = \frac{[\alpha\mathrm{CD{:}S}]}{[\alpha\mathrm{CD}]\cdot[\mathrm{S}]}$$

Using the general convention, the standard concentration, c_0, is taken as 1 mol·L^{-1}, and the constant, K_A, divided by c_0 is then expressed in liters per mole (M^{-1}) in coherence with other studies.[27]

3. RESULTS AND DISCUSSION

3.1. Alkyl Glycoside Formation through Disproportionation or Coupling. When using cyclodextrin glucanotransferase (CGTase), the elongation of the glycosyl headgroup of alkyl glucosides (AGlus's) can be achieved by exploiting the transglycosylation activity of this enzyme. Elongation of AGlus's is seen as the enzymatically catalyzed transfer reaction (transglycosylation) adding glycosyl residues from a sugar donor to the acceptor molecule, forming alkyl glycosides (AGly's). In principle, the two reactions, disproportionation and coupling, can be used to transglycosylate AGlu's, utilizing either linear or cyclic (CD) oligosaccharides as donor substrate. However, the catalytic rate of the two mechanisms may be different. In addition, because the two donor substrates have a very different structure, the binding mode of each one at the active site of CGTase may also differ. This would cause divergent elongation patterns, that is, a polydisperse product, between disproportionation and the coupling reaction. Whether AGlus's constitute a reactive substrate for CGTase has not been previously observed. The natural acceptor substrate, with highest affinity (K_m), is optimally composed of two to four glucose units.[31] Using alkyl maltosides with up to 12-carbon-atom-long chains was shown to be utilized by CGTase as the acceptor, together with linear sugar donors.[32] For AGlus's, so far, only the complexed form has been studied

dx.doi.org/10.1021/cs500192q | ACS Catal. 2014, 4, 2623−2634

in transglycosylation reactions using CGTase to form AGlys.[11] We thus investigated the two possibilities to elongate the sugar headgroup of octyl-β-D-glucoside (C8G1) by the two transglycosylation reactions, using either α-cyclodextrin (αCD) or maltohexaose (G6) as the glycosyl donor.

For both reactions, transglycosylation products were observed with a difference in the product pattern (Figure 1).

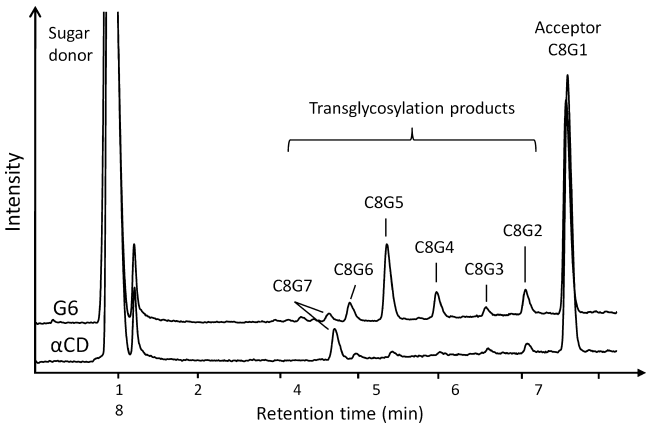


Figure 1. Number of glycosyl residues transferred onto C8G1 acceptor represented by the different transglycosylation products from the disproportionation (top) and coupling (bottom) reaction between G6 and C8G1 as well as αCD and C8G1, respectively. Similar substrate concentrations for each reaction were chosen, 20 mM donor and 5 mM acceptor substrate. The reaction time for the CGTase Amano (1 μg/mL) was 20 min before the reaction was quenched and analyzed.

Interestingly, CGTase was able to convert the monomeric C8G1 into octyl glycosides, revealing that catalytic binding occurred. Substituting C8G1 with decyl-β-D-glucoside (C10G1) also resulted in transglycosylation (see Figure S1 in the Supporting Information (SI)). As we are showing in section 3.3 below, even dodecyl-β-D-glucoside (C12G1) was used as the acceptor, which also confirmed that longer-chained AGlus's are accepted by CGTase, despite the lack of a maltose headgroup. In addition, CGTase not only transglycosylated monomeric C8G1 via disproportionation using the linear donor G6, it also appeared to convert more than via the coupling reaction in the presence of complexed C8G1 together with αCD as the glycosyl donor and complexing agent (compare the peak height in Figure 1).

The primary coupling product was found to be octyl-β-D-heptaoside (C8G7) accompanied by minor coproducts, which resulted from the conversion of the primary product in subsequent disproportionation reactions. In the primary coupling step, all six glucose residues of αCD were transferred onto C8G1, with no release of a complementary product (leaving group). In a similar study, when CGTase was exposed to higher αCD and acceptor concentrations, secondary and ternary coupling but less disproportionation products were observed.[7,11] In the case of C8G1 elongation by disproportionation, several transglycosylation products were formed with octyl-β-D-pentaoside (C8G5) as the predominant octyl glycoside. The formation of multiple transglycosylation products for disproportionation can be caused by consecutive reaction steps using the primary product as donor and, in addition, by multiple binding events of the donor molecule (G6) at the active site. That C8G5 was, in fact, the primary disproportionation product was confirmed through comparison with the product pattern when G6 was used as a single substrate, acting as donor and acceptor simultaneously.

In this experiment, all possible primary sugar products originated from two G6 substrate molecules. At an early stage of the reaction, multiple product combinations depended mainly on the binding mode of G6 at the donor and acceptor site. As shown in Figure 2A, the main transglycosylation product found was maltodecanose (G10). In addition to G10, almost equal amounts of maltose (G2) were formed. To realize G10 formation, the donor G6 had to bind in such a manner that a maltotetraose:enzyme complex (E-G4) was formed with G2 as the leaving group. After this first hydrolysis step with E-G4 formation, acceptor binding (G6) and its subsequent transglycosylation (G4 transfer) took place, resulting in G10 release. Hence, in the transglycosylation of C8G1 together with G6, a G4 residue was transferred onto C8G1, resulting in C8G5 as the primary product and G2 as the leaving group. Because other initial transglycosylation products were shown to be

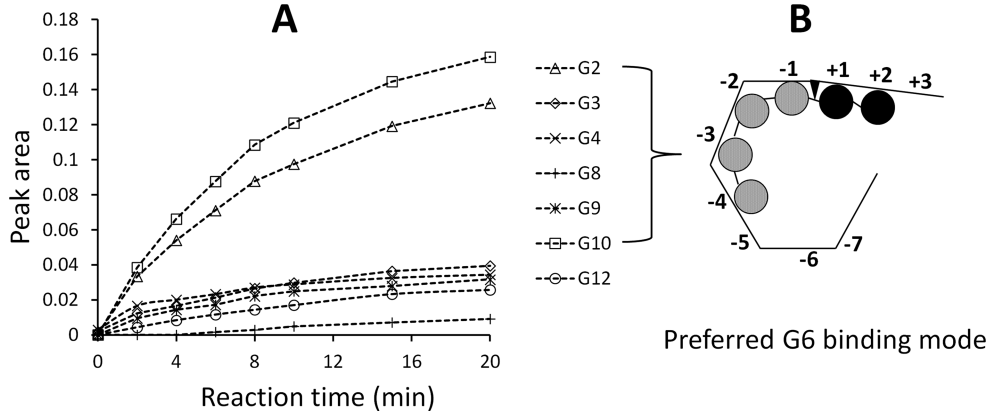


Figure 2. (A) Conversion of G6 into different disproportionation products with maltodecanose (G10) and maltose (G2) as their main transglycosylation product pair. (B) The preferred binding mode of G6 as donor alongside the donor and acceptor binding site of CGTase is schematically illustrated. The active site pocket of CGTase is reported to be constructed of a donor binding site, containing seven subsites (−1 to −7), and an acceptor binding site, consisting of at least two subsites (+1 and +2). The cleavage of the bound donor substrate occurs between subsite −1 and acceptor subsite +1.[33,34] The resulting chain length of the glycosyl−enzyme intermediate (gray circles) and leaving group (black circles) thus depends on how the donor binds alongside the two binding sites.

dx.doi.org/10.1021/cs500192q | ACS Catal. 2014, 4, 2623−2634

minor (Figure 2A), the predominant binding form of G6 alongside the donor and acceptor site (Figure 2B) to form an E-G4 complex and a G2 leaving group was defined as the preferred binding mode.

Apart from the preferred binding mode, only a few other glycosyl−intermediate transfers were found. For the single substrate reaction with G6, different combinations between primary product and leaving group, such as maltononaose (G9) together with maltotriose (G3) and maltooctaose (G8) together with G4, were also detected, as shown in Figure 2A. Surprising, however, was the formation of maltododecanose (G12). Its creation from the condensation between two G6 molecules would require the action of reverse hydrolysis, which is very unlikely to occur in aqueous solution. A possible explanation can be the occurrence of two consecutive reactions with G10 as an alternative donor. It is worth mentioning that the G2 leaving group acts as a competitive acceptor substrate in CGTase-catalyzed reactions.[31] Especially with a progressing reaction time, G2 may participate as an acceptor in consecutive reaction steps in addition to G6. However, the initial formation of G3 and G4 indicated, rather, the presence of alternative binding modes instead of the participation of primary products (G10 and G2) in consecutive reaction steps. Nonetheless, although alternative binding modes of G6 were found possible, the preferred binding of G6 in the active site of CGTase was to transfer a G4-glycosyl intermediate onto the G6 acceptor, which is schematically illustrated in Figure 2B. Other transglycosylation products besides C8G5 seen in Figure 1 were thus likely caused by both alternative donor binding and consecutive disproportionation steps. On the contrary, the coproducts in the coupling reaction, which do not possess a number of sugar residues originating from αCD (plus six) addition could be formed only through consecutive disproportionation of the primary product C8G7. For αCD, only a G6-glycosyl intermediate can be formed, since alternative binding of CD does not occur. Because CD is a cyclic substrate, a leaving group cannot be formed through a single cleavage site, and rotation will have no influence on the length of the cleaved intermediate or on the binding mode.

3.2. Nonaggregated Donor and Acceptor Reactivity.

As discussed above, the linear sugar substrate G6 simultaneously acts as the glycosyl donor and acceptor. Consequently, in the presence of another acceptor, such as the alkyl glucoside C8G1, both types of acceptors will compete for the same acceptor subsite. To evaluate the acceptor quality of C8G1 compared with G6, we measured the transglycosylation activity in the presence and absence of C8G1. In the absence of C8G1, the formation of G10 over time yielded a total disproportionation rate of 110.0 U/mg of CGTase, whereas by addition of equal amounts of C8G1, the G6 disproportionation rate decreased only to ∼82% (90.7 U/mg) (Figure 3). This means that G6 features roughly 5-fold higher acceptor reactivity than the C8G1. In other words, using G6 as the donor substrate in AGly synthesis, only a fraction of it participates in the elongation of the AGlu sugar headgroup; most of it will form oligosaccharides, such as G10, instead.

When the donor quality of αCD and G6 was evaluated in a similar manner, the disproportionation reaction was strongly favored over the coupling reaction, revealing that the cyclic donor was much less reactive (18%) than the linear G6 donor (Figure 3). Thus, in the presence of αCD, the G10 formation decreased from 110.0 to 89.7 U/mg, and the complementary G12 formation amounted to 20.3 U/mg. Although different

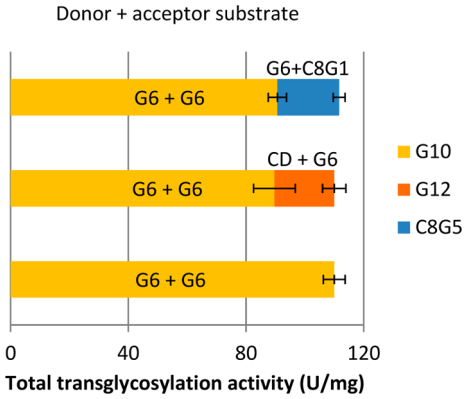


Figure 3. Estimation of the donor and acceptor performance for the different substrates αCD, G6, and C8G1 with 20 mM G6, αCD, and C8G1, respectively. Single G6 reaction (G6 + G6) represents the maximum disproportionation rate (G10 formation, yellow bars). Donor competition between disproportionation and coupling was achieved by adding equimolar amounts of αCD to the G6 substrate (CD + G6); G10 became reduced through formation of G12 (red bar). In addition to having a good donor quality, G6 also dominated as the acceptor in the presence of C8G1 (G6 + C8G1). Only a little formation of AGly (C8G5) was observed (blue bar).

catalytic rates have been reported for coupling and disproportionation,[9,31,35] the conducted competition experiments were solely dependent on the affinity of the donor substrates to the donor subsite of CGTase.

The conclusion of this quantitative study on nonaggregated donor and acceptor reactivity is that the G6 donor constituted a rather strong, competitive acceptor substrate, thereby decreasing the C8G5 formation rate and yield. Alternatively, the coupling reaction using αCD offers the advantage of elongating AGlu without coproduct formation. However, considering the pure donor performance, linear sugars (e.g., G6) are more reactive than cyclic ones (e.g., αCD). The requirement of excess CD in the coupling reaction remains a significant drawback for an economic process. In addition, G6 is a nonattractive donor substrate because it is too expensive for industrial application. A less expensive sugar material such as maltodextrins may be an attractive alternative to utilize disproportionation more efficiently. From our disproportionation studies with G6 (Figure 2), we can deduce that a longer sugar chain increases the donor potential, whereas the acceptor potential should decrease. Hence, on the basis of reasoning that a long sugar chain preferably binds alongside donor and acceptor sites, maltooligosaccharides (maltodextrins) should act as a donor substrate. In addition, the AGlu concentration could be raised to compensate for the poor acceptor quality of AGlu in disproportionation reactions. The feasibility and consequences of using AGlu in excess is discussed in the following section, 3.3.

3.3. Disproportionation: Aggregation, Solubility and Complexation of AGlu in Disproportionation Reactions.

In enzyme-catalyzed reactions, the monomer availability of the substrate(s) is an essential factor for efficient conversion. Only a few enzymes are known to utilize aggregates (for example, micelles) as substrate or even to be activated by interfaces. Within the class of hydrolases, lipases and phosphorylases are well-known to adsorb to interfaces,[36] but also glycosidases such as cellulases, are assumed to show interfacial activity.[37]

In Figure 4, the C8G1 acceptor concentration was increased to compensate for the competitive effect of G6 toward the

dx.doi.org/10.1021/cs500192q | ACS Catal. 2014, 4, 2623−2634

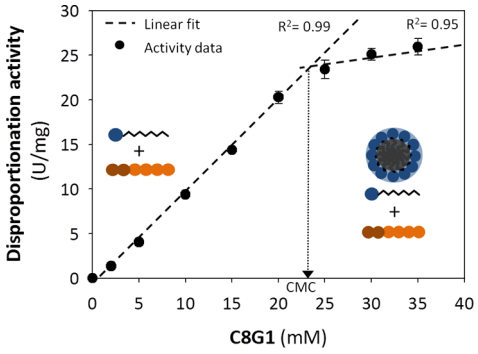


Figure 4. Effect of monomeric and aggregated acceptor substrate (octyl-β-D-glucoside, C8G1) on the disproportionation activity of CGTase. Maltohexaose (G6, depicted as orange circle chain) served as the donor substrate at a constant concentration of 20 mM. The critical micelle concentration for C8G1 (circle with zigzag chain) is ~22 mM[37,38] and represents the point at which micelle formation (large circle with C8G1) occurs; matching the intersection point of the two linear regressions for monomeric (R^2 = 0.99) and mixed (R^2 = 0.95) substrate species. When the C8G1 concentration above the CMC was increased, a stagnation of the disproportionation activity was observed (visible as a large change in slope between the two linear fittings).

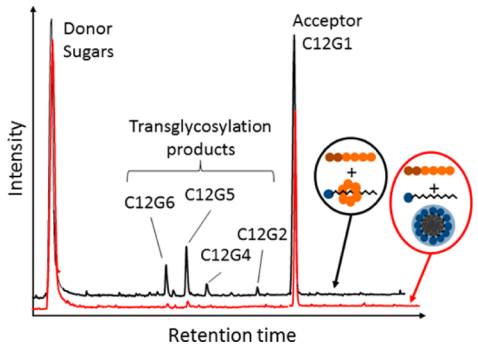


Figure 5. Increased product formation of CGTase in the presence of complexed acceptor (top chromatogram) compared with aggregated (bottom chromatogram) acceptor species. The CMC (0.02 mM) of dodecyl-β-D-glucoside (C12G1) was shifted by addition of α-cyclodextrin (αCD). The bottom chromatogram represents a reaction solution composed of 5 mM C12G1 and 20 mM maltohexaose (G6, depicted as chain); hardly any product could be detected. The addition of equimolar concentration (5 mM) of αCD shifted the CMC to ~5 mM (CAC), and in this case, reactive complexes (top chromatogram) were produced. For the set conditions, complexes between αCD and C12G1 were expected to have a stoichiometry of 1.

acceptor site. The reaction rate increased linearly until the CMC (~22 mM) of C8G1[38,39] was reached. The reaction rate was clearly affected most probably as a result of the formation of micelles, which caused the monomer concentration to remain constant and the conversion of C8G1 to stagnate. It has been reported that hydrophilic enzymes, such as proteases and glucosidases, do not adsorb to surfactant aggregates and, therefore, do not accept the micelle form as the substrate.[40,41]

Another parameter for the monomer availability in an enzymatic reaction is the solubility of the substrate in that phase in which the enzyme is active. The CMC and solubility of alkyl glucosides decreases drastically with a increase in the alkyl chain length,[42] whereas the change in the sugar headgroup has only minor effects on the CMC.[43] For example, the CMC of C10G1 (~2 mM) is ~10-times lower than that of C8G1. Each ethyl extension of the alkyl chain leads to roughly 10-fold decrease in CMC, and approximately the same behavior is observed for the solubility.[43] For long-chained AGlus's, not only the low CMC but also the solubility will limit their monomer concentration and, thus, their availability as acceptor in AGly synthesis. Indeed, when using dodecyl-β-D-glucoside (C12G1) as an acceptor together with G6 in the disproportionation reaction, hardly any transglycosylation product could be detected (Figure 5, red line). The addition of 5 mM C12G1 (with a CMC of 0.02 mM) to aqueous buffer resulted in the formation of an emulsion at 60 °C owing to the poor solubility. Nonetheless, the few monomers present were converted into the corresponding dodecyl glycosides, clearly detectable by HPLC (Figure 5). Again, this confirms, as stated above, that the single glucose residue and bare hydrocarbon chain does not hinder binding of long alkyl chain glucosides at the acceptor site of CGTase.

We then followed the approach of CD addition to form inclusion complexes together with the surfactant, preventing micellization and forming instead pseudomonomeric substrate species. The simple addition of an equal amount of αCD to the C12G1/G6/water emulsion caused the system to become homogeneous, representing the solubilization of the surfactant. As shown in Figure 5 (black line), the soluble complex was then more efficiently converted by the enzyme using G6 as the donor. Interestingly, the typical AGly product pattern as seen for monomeric C8G1 together with G6 (discussed above) also appeared for the complexed C12G1 acceptor. This indicated that the complex can be used as an acceptor substrate but not as a donor. Sugar headgroup elongation via αCD would predominantly form C12G7, which was found to be produced very little in the presence of αCD and a complexed acceptor together with G6 (Figure 5).

Providing a good solubility, the complexed acceptor would allow a much higher concentration for the reaction compared with the complexed surfactant. For realizing a cost-effective process, including an inexpensive (linear) donor and complexed acceptor substrate, the characterization of the complex together with its formation and utilization by the enzyme is required. On the basis of these results, we continued to investigate the complex as the acceptor substrate. A simpler system with only one initial donor and acceptor substrate, however, was experimentally suitable. The use of the coupling reaction between αCD and C8G1 appeared to be the optimal model system for this approach.

3.4. Complex Characterization by ITC. Previous studies have indicated that CD and AGly can form complexes and that complexation influences the kinetics of CGTase-catalyzed reactions involving AGly and CD substrates.[30] To elucidate these mechanisms in detail, the formation of inclusion complexes between αCD and C8G1 was studied separately under the conditions used in the enzymatic synthesis reactions, by isothermal titration calorimetry (ITC). The results showed that the 1:1 complex (αCD:C8G1) was the dominating species. The association constant (K_A) was 972 ± 6 M^{-1} (see SI, Figure S2 and Table S1), which is somewhat lower than that reported in the literature (1700−3680 M^{-1}).[23,29,44] This difference was expected to be caused by using altered experimental conditions and techniques, respectively. Previous measurements by others were mainly conducted in pure water or different buffers at 25 °C. Here, we used an aqueous buffer system and a much higher temperature (60 °C) to simulate the conditions for the enzymatic reactions. Because association constants between

dx.doi.org/10.1021/cs500192q | ACS Catal. 2014, 4, 2623−2634

CDs and other hydrophobic guest molecules have been shown to decrease considerably with increasing temperature,[45] the higher temperature is a likely explanation for the discrepancy. At 25 °C, the formation of a 2:1 complex was reported to occur to some extent (K_A/c_0= 64 M^{-1}),[46] but in our experiments at 60 °C, it was not found significant. The affinity constant determines the quantitative relationship between the free and complexed substrate species at equilibrium. For the following kinetic measurements, we used the determined K_A value and the assumption of 1:1 complexes for the calculation of each substrate species and for the general interpretation of the data.

3.5. Effect of Complexation on CGTase Catalysis. *3.5.1. Coupling Reaction: What Is a Good Donor?* In the following kinetic study, we characterized the αCD/C8G1/CGTase system including the determination of substrate species reactivity. From the ITC experiment, the thermodynamic and structural parameters of the complex were extracted and enabled us to quantify each substrate species at any ratio of the two added substrates: total αCD and C8G1 (see Tables S2−4 in the SI). The possible substrate species formed are the free, noncomplexed form of αCD and C8G1 and the inclusion complex of both donor and acceptor.

To investigate the donor influence on the coupling activity, the αCD concentration was varied at a constant C8G1 acceptor concentration (50 mM). As we can observe from Figure 6A, the coupling activity featured a non-Michaelis−Menten dependency on the donor concentration. Taking the species' concentrations (αCD(free), C8G1(free), and complex) into account, the initial "lag phase" of the sigmoidal activity curve correlated well with the lack of αCD(free) donor. This suggests that the process of complexation was consuming the added αCD as long as the donor concentration was below that of the surfactant acceptor. As soon as all surfactant tails were saturated with αCD, no more complexes could be formed, and the αCD(free) concentration increased together with the coupling activity. In other words, just below a total donor to acceptor ratio (αCD/C8G1) of 1/1, corresponding to total 40 mM αCD and 50 mM C8G1, complexation depleted the reactive (free) αCD donor. Our calculations showed that only about 7% of the total αCD remained free (see Table S2 in SI). Approaching an equal ratio of donor and acceptor, the αCD(free) concentration increased drastically and correlated well with the coupling activity (Figure 6A). In contrast, the complexed form of αCD apparently could not serve as donor substrate. The poor donor performance of the complex can be explained by considering the binding interactions of αCD at the donor subsite of CGTase. For donor binding in addition to certain donor and acceptor residues, a single tyrosine residue (Tyr195) interacts with the inner cavity of the αCD molecule,[47] resembling a αCD:TYR complex. If one compares the affinity constant for αCD:C8G1 with that of αCD and phenylalanine (PHE), which is similar to tyrosine, the surfactant complex is ∼80-times stronger than the amino acid complex αCD:PHE.[48] Hence, the surfactant complex αCD:C8G1 will not dissociate to exchange the thermodynamically more favorable alkyl glucoside guest molecule for the amino acid tyrosine.

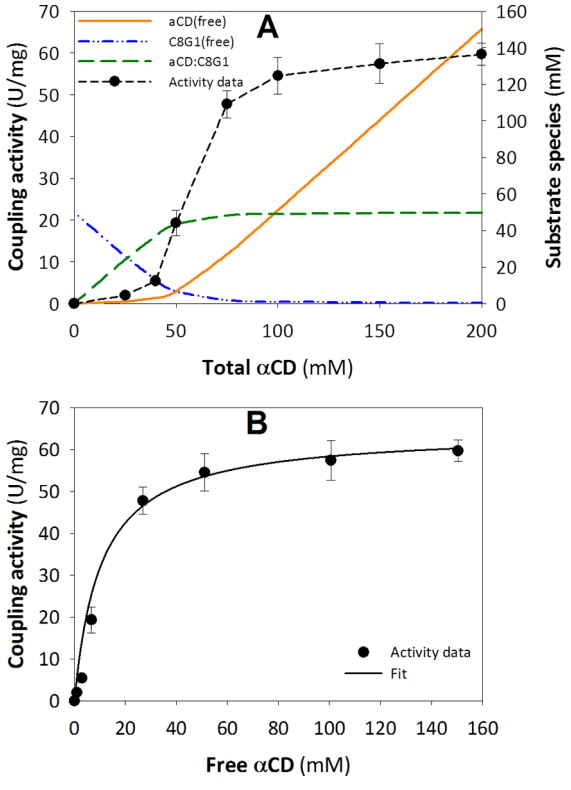


Figure 6. Importance of the noncomplexed αCD donor (αCDfree) on the coupling reaction. (A) Varying the total αCD concentration at constant C8G1 (50 mM) caused a change in substrate species (αCD(free); C8G1(free); complex αCD:C8G1) through complexation. (B) When coupling activity was plotted against the calculated αCD(free) concentration, a typical Michaelis−Menten behavior was observed, and fitting (R^2 = 0.996) resulted in apparent parameters, with K_m = 10.3 ± 2.2 mM and V = 64.4 ± 2.2 U/mg.

The dependency of the coupling activity on free donor was clarified by plotting the activity against the calculated αCD(free) concentration (Figure 6B). A typical Michaelis−Menten behavior was observed, and apparent kinetic parameters for αCD(free) were estimated.

3.5.2. Complexed Acceptor Binding As Rate-Limiting Step. Although free C8G1 was shown (section 3.3) to constitute a good acceptor, its aggregated form (micelles) did not contribute to the reaction (Figure 4). In the coupling reaction, however, most of the C8G1 was present as a complex with αCD. Consequently, the utilization of the free C8G1 acceptor in the coupling reaction seemed unlikely because it was present only at low total αCD/C8G1 ratios, at which αCD(free) constituted the limiting substrate. Despite its acceptor potential, C8G1(free) and its contribution to the coupling activity (Figure 6A) can be assumed to be negligible. Hence, the only available acceptor in the coupling reaction between αCD and C8G1 represented the αCD:C8G1 complex. As discussed above, because of the high association constant of the complex, the release of αCD(free) and its participation in the catalysis as donor does not occur; consequently, C8G1 also remained complexed. We also can exclude that the CGTase binding sites feature a more thermodynamically favorable environment (higher binding constant) for the C8G1 molecule than the hydrophobic αCD cavity. Otherwise, complex dissociation at the active site could spontaneously occur, and consequently, complexation would not affect the free donor availability that drastically, which we have shown to be the case. For these reasons, we propose that the entire complex binds at the acceptor subsite, and the complexed C8G1 becomes as such elongated. That CGTase is able to use surfactants and complexes as acceptor substrate suggests a loosely defined substrate specificity of the acceptor binding site, which offers a high potential for the transglycosylation of other compounds.

dx.doi.org/10.1021/cs500192q | ACS Catal. 2014, 4, 2623−2634

For estimating the complexed acceptor performance, the αCD(free) concentration was kept constant at 100 mM and the concentration of total C8G1 was increased (Figure 7A). This

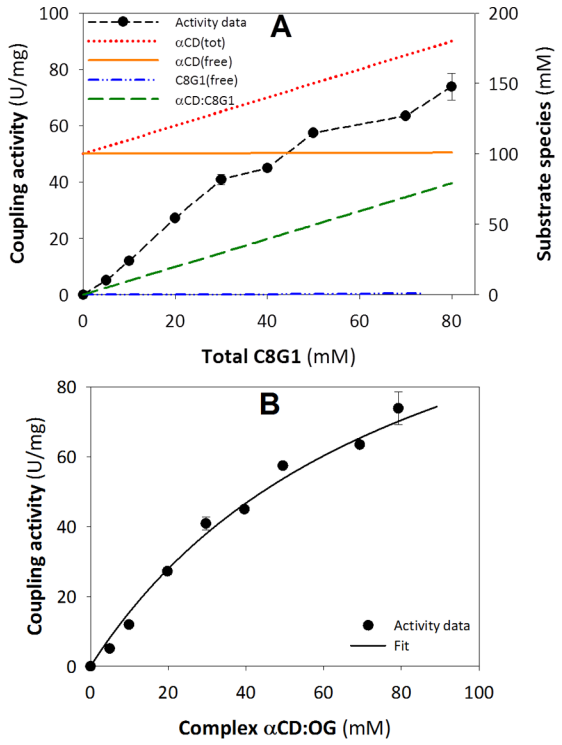


Figure 7. Performance of complexed acceptor (αCD:C8G1) in the coupling reaction at constant αCD(free) concentrations (100 mM). (A) By varying the total octyl glucoside (C8G1) concentration, the coupling activity depends only on the αCD:C8G1 concentration. (B) When coupling activity was plotted against the calculated complex (αCD:C8G1) concentration, a typical Michaelis–Menten behavior was observed, and fitting (R^2 = 0.991) resulted in apparent parameters, with K_m = 82.6 ± 16.0 mM and V = 142.2 ± 16.5 U/mg.

approach enabled a maximal possible coupling activity of 73.8 U/mg at 180/80 (mM/mM) total αCD/C8G1 ratio; corresponding to an αCD(free)/complex ratio of ∼100/80 (compare Figure 7A,B). Unfortunately, because of the complex solubility limit, precipitations occurred above 80 mM, which prevented a further enrichment in soluble acceptor.

Fitting the activity data from Figure 7A as a function of complex concentrations, an apparent K_m value of 82.6 ± 16.0 mM for the complex and theoretical (solubility limited) maximum coupling activity value of 142.2 ± 16.5 U/mg were estimated. The rather high apparent K_m value of the complex indicated weak acceptor interactions toward the acceptor subsite.

Although the complex featured no donor quality, its αCD part may still interact with the maltose binding sites of CGTase. The maltose binding sites play an important role in guiding the substrates (oligosaccharides and CD) into the active site.[49] Free and complexed αCD may compete for the maltose binding sites, which could be one factor contributing to the rather high apparent K_m values of αCD(free) and complex determined. In the literature, K_m values for free CD are usually determined with 1–5 mM.[9,31,35] For the complex, however, its bulky and non-native acceptor structure was certainly decisive for its interactions with the enzyme.

To identify the relative reactivity of the complex, we compared the transglycosylation activity of CGTase in the presence and absence of the complexed acceptor. For the coupling reaction, complexation always occurs, but exploiting the disproportionation reaction complexation can be initiated by simple addition of αCD to a G6/C8G1 mixture forming 1:1 complexes (αCD:C8G1). Results of this approach are shown in Table 1. Without αCD, the transglycosylation activity amounted to 12.2 U/mg, but as soon as the free acceptor was converted into a complex, the activity decreased drastically to only 4.1 U/mg. Because the catalytic sugar residue has not been altered during complexation, the effect on the rate constant of the reaction (k_{cat}) might be only minor. We assume the binding event of the complexed acceptor is the rate -limiting step. This assumption becomes supported by the fact that the disproportionation and coupling reaction of CGTase exhibit a very similar velocity for the complexed acceptor C8G1 (see Table 1). The type of donor, linear or cyclic, and the glycosyl transfer step seemed thus not to be rate-determining.

For the free αCD donor, we estimated a specificity constant (k_{cat}/K_m) ∼4-fold higher than for the complexed acceptor (see Table S5 SI). In both reactions, disproportionation and coupling, activity values (around 4 U/mg) for the complexed acceptor are ∼3-fold smaller than for the noncomplexed form. These observations suggest a difference in specificity constant factor of 3 between the free and complexed C8G1 acceptor, which is affected mainly by a change in the Michaelis–Menten constant (K_m). This in fact indicates that the complex featured the overall limiting substrate species in both the coupling and disproportionation reactions. Nonetheless, the complexed C8G1 offered significantly higher maximal conversion rates than the monomer form (free C8G1) because its solubility is constant and thus available over a much wider concentration range. The low complex reactivity can simply be counteracted by increasing its concentration. Doubling its concentration increased the transglycosylation activity about 2-fold (Table 1).

3.5.3. Acceptor and Donor Limiting Regime. Because of the intrinsic process of inclusion complex formation between CD and alkyl glucosides, the reaction system composed of αCD, C8G1, and CGTase is constituted of free and complexed

Table 1. Comparison between Free and Complexed Acceptor Species for the Two Transglycosylation Reactions Catalyzed by CGTase[a]

reaction	coupling		disproportionation	
donor + acceptor [mM/mM]	αCD + (αCD:C8G1) [20/10]	G6 + C8G1 [20/10]	G6 + (αCD:C8G1) [20/(10/10)]	G6 + (αCD:C8G1) [20/(20/20)]
transgly. activity (U/mg)	4.0 ± 0.7	12.2 ± 0.4	4.1 ± 0.5	9.1 ± 0.4

[a]For both reaction mechanisms, coupling and disproportionation, the acceptor complex (αCD:C8G1) resembled the limiting substrate species. The specific transglycosylation activity is based on the formation of transglycosylated surfactant acceptor; side reactions forming pure sugars, as in disproportionation, were excluded. Numbers in square brackets indicate the total donor to acceptor ratio (free or complexed). Standard deviation estimated from N = 3.

dx.doi.org/10.1021/cs500192q | ACS Catal. 2014, 4, 2623–2634

substrates. In the coupling reaction, we identified two substrate limiting regimes, depending on the ratio of total αCD to C8G1. When varying the total C8G1 concentration at constant total αCD, a coupling activity curve exhibiting a bell shape (Figure 8) was obtained. Because αCD and C8G1 form complexes with

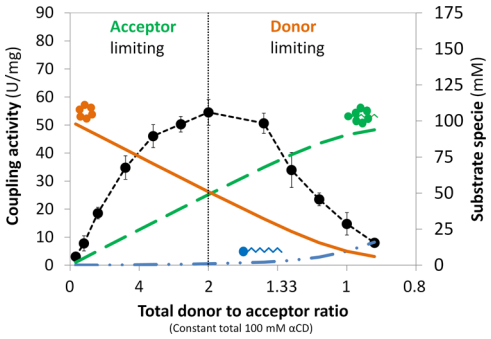


Figure 8. Effect of different donor-to-acceptor substrate ratios on the coupling activity of CGTase. The maximum coupling activity was reached at 2/1 total ratio, respectively, an equal donor (free αCD)-to-acceptor (αCD/C8G1) ratio. The stoichiometry of the complex (αCD:C8G1 of 1:1) determined the optimal substrate ratio. Each side of the bell-shaped activity curve was limited by either too little acceptor complex (acceptor-limiting regime) or free donor (donor-limiting regime), respectively. The change in substrate species was achieved by varying the total C8G1 concentration and keeping the total αCD concentration constant.

a stoichiometry of only 1:1, the amount of C8G1 added to the system sequestrates equal amounts of free αCD, which is required for maximum reaction rates. As can be seen in Figure 8, the optimum substrate conditions were found at a total αCD/C8G1 ratio of 2/1. This also corresponded to the actual available substrate species ratio of 1/1 between αCD(free) and the complex. The region at higher total donor-to-acceptor ratios, in which sufficient donor is available but the reaction suffers from too little acceptor, was ascribed as the acceptor-limiting regime. When the coupling activity is suppressed because of αCD(free) depletion, we refer to the donor-limiting regime. The donor-limiting regime is obtained when too much of the C8G1 surfactant is present, which results in total donor/acceptor ratios lower than 2/1.

The rapid decrease in the coupling activity in the donor-limiting regime indicated that in addition to the high K_m values (αCD and complex), other competitive events between the free and complexed substrate form must appear. Complexation itself is a process in equilibrium between association and dissociation. This means each complex has a certain lifetime before dissociation and new complex formation occurs. It was shown by Haller et al.[46,50] that the monomer dissociation rate of the αCD:C8G1 complex is similar to that of monomer exchange of C8G1 micelles on the order of 10^5–10^7 s^{-1}. In contrast, previously determined rate constants (k_{cat}) for the coupling and disproportionation reaction are in the range 200–1500 s^{-1}.[9,31,35] The enzymatic reaction is thus several orders of magnitude slower than the process of complexation. When assuming a lifetime of a few microseconds for the αCD:C8G1 complex, which is also simular to micelles,[51] a single C8G1 guest molecule could complex with a few other αCD(free) molecules during a single substrate binding event and catalytic turnover. In other words, within that timeframe, the monomer exchange is so fast that a single surfactant molecule could "jump" from one complex to a few other αCD(free) molecules, thereby altering the binding ability as donor through complexation. This would decrease the apparent concentration of αCD(free), since a noncomplexed αCD molecule interacting with the enzyme could be complexed before it has bound and reacted as free donor. The fast monomer exchange of the complex may decrease the probability of αCD(free) entering and binding at the CGTase donor subsite. Of course, this rapid exchange of guest molecules happens in both donor and acceptor regimes. At high donor/acceptor ratios, however, there will still be sufficient αCD(free) remaining, whereas at low ratios containing higher surfactant concentrations, the guest molecule exchange becomes essentially more dominant. This increased temporary "occupation" of αCD(free) would further contribute to lowering the αCD(free) donor availability. Hence, the dynamic process of associating and dissociating complexes not only forms new substrate species but also may alter substrate species already or about to interact with the enzyme, especially at low donor/acceptor ratios (donor limiting regime). A reaction system composed of complexing compounds, thus, constantly changes the properties of the substrates from free to complexed and, thereby, their kinetic parameters (e.g., K_m).

In principle, both donor- and acceptor-limiting regimes can be applied to any suitable surfactant guest molecule used in the coupling reaction together with CDs. The actual determinants of the CD/guest/CGTase system are (i) the area available for inclusion complexation (stoichiometry), such as the hydrophobic tail of surfactants, and (ii) the association constant of the complex. The only requirement for a reactive complex is that the guest molecule reach the catalytic substrate site of the enzyme. Obviously, the prerequisite is that the guest molecule features a suitable sugar moiety to be transglycosylated. However, if very long chain AGly's or ligands with multiple binding areas are to be synthesized, the formation of complexes with a stoichiometry larger than 1 causes the optimum substrate composition to be shifted toward higher donor/acceptor ratios. Consequently, an excess of CD addition is required for sufficient CD(free), and together with large complexes, precipitation is expected to occur at much lower concentrations compared with the αCD/C8G1 system. This constraint, however, can be reduced by exploiting complexation only as a process to generate soluble and reactive acceptor complexes and disproportionation as transglycosylation reaction to elongate the glycosyl headgroup. Knowing the association constant, multicomplex formation (stoichiometry larger than 1) can be avoided by minimal CD supply. The addition of CD as a pure solubilization agent and exploiting disproportionation as transglycosylation reaction for the synthesis of AGly's is demonstrated in Figure 5 (see section 3.3).

As mentioned earlier, the concept and findings overcome and explain the limiting factors, such as excess CD addition and poor acceptor quality of long alkyl chained glucosides. Both cases can be ascribed to either the acceptor- or donor-limiting regime. Consequently, the rational design presented in this article may be directly applied to develop an industrially feasible process for AGly synthesis from AGlus's using CD alone or together with linear sugars. Furthermore, the concept of limiting regimes on the basis of complexation through CD addition should be applicable to other systems. The general applicability of our rational design approach to other enzymatic systems with respect to exploiting complexation for solubilization purposes is discussed in the following section (3.6).

dx.doi.org/10.1021/cs500192q | ACS Catal. 2014, 4, 2623–2634

3.6. General Applicability of Limiting Regimes. For enzymatic reactions, which involve CD addition to increase substrate concentration, there should exist a region (comparable to the acceptor-limiting regime in Figure 8) where the reaction benefits from the substrate solubilization through complexation upon CD addition, illustrated in Figure 9 as the substrate-/solubility-limited regime. After a given threshold (in Figure 9), however, complexation will affect or even inhibit the enzymatic reaction, and the complexation limited regime is reached (comparable to the donor-limiting regime in Figure 8). Especially for other reaction systems not utilizing CD as an actual substrate, complexation usually causes enzyme inhibition.[18,52,53] However, some enzymes, because of their active site architecture, may utilize the complex in a manner similar to what we have reported in this article for CGTase. Thus, complexation may lead to increased pseudomonomer availability by forming reactive complexes (scenario 1 in Figure 9) and thereby changing kinetic parameters. López-Nicolás and co-workers reported that the lipooxygenase catalyzed reaction resulted in an overall higher conversion upon CD addition.[19] Compared with our experiments, they did not cover CD-to-substrate (polyunsaturated fatty acids) ratios systematically and studied only a few ratios of different concentrations. Nonetheless, the trend was similar to our results, that complexation affected the Michealis−Menten parameters, presumably through increased K_m values. In the study of lipase-catalyzed hydrolysis of *p*-nitrophenol esters, Otero et al.[16] compared CD addition to acetonitrile and concluded that the enzymatic parameters were not affected substantially in the presence of CD. One has to mention here that two effects influence the enzyme kinetics: one is the complexation itself, affecting the K_m of the substrate by altering the structure and eliminating inhibiting compounds,[53] and the other is increased substrate solubility and monomer availability, respectively, which eventually should result in higher conversions rates (shown as a combination of scenarios 1 and 2 in Figure 9).

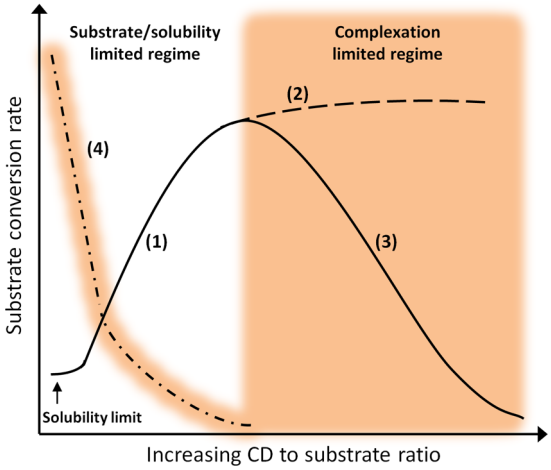


Figure 9. Effect of substrate solubilization through complex formation upon CD addition for enzyme-catalyzed reactions. Four scenarios related to complexation are illustrated at constant substrate concentration above solubility. Enzymatic substrate conversion can be improved when (1) complexation forms a soluble and reactive complex. The substrate conversion rate increases (2) until enzyme saturation occurs. Toward large CD-to-substrate ratios (3), complexation can inhibit the reaction. Immediate inhibition and a negative effect is seen (4) when the complex constitutes a nonreactive substrate. For examples and explanations, see the text.

In addition to CGTase, lipases seemed to accept complexed substrates well because CD addition has been repeatedly reported to serve as a conversion promoter for transesterification and hydrolysis reactions.[15,16,54,55] A similar bell-shaped activity behavior dependent on CD addition (comparable to scenarios 1 and 3 in Figure 9) was described by Ávila-González et al. for the transacylation of butyryl propranolol (BPP) having a maximal conversion rate at a modified-βCD/BPP ratio of around 70.[56,57] Because complexation data were not collected, it can only be assumed that either multiple complex formation occurred (BPP may have three possible complexation sites) or the complex had a rather low association constant to cause the drastic shift to very high modified-βCD/BPP ratios. The latter case could be attributed to the substrate-/solubility-limited regime because CD needs to be added above equimolar quantities to form complexes (maximum of bell curve, solid line Figure 9).

An inhibiting effect on the CD/substrate ratio was reported by Truppo et al.[18] when the solubility of bridged bicyclic ketones was increased using βCD. In contrast to refs 56 and 57, optimal reaction conditions were found at an equimolar CD-to-substrate ratio, and increasing the CD/substrate ratio led to decreased activity. As in our presented CGTase case, acceptor limitation occurred as a result of complexation. the ketoreductase used by Truppo et al.[18] was inhibited by either the presence of free βCD or by the complexation-inhibiting regime, indicating that the complex itself was not a reactive substrate (compare Figure 9, scenario 4). Another possibility may be the formation of multiple complexes, which fully cover the catalytic residues of the substrate, whereas for a complex with stoichiometry of 1, the residues were still accessible by the enzyme. Because of the lack of data in Truppo et al.,[18] the CD effect is not assigned unambiguously to either scenario 4 or the complexation-limited regime in Figure 9. The examples discussed clearly show that the characterization of substrate complexation would reveal the nature of the enzyme kinetics by defining the substrate/solubility and complexation limiting regimes. This, in turn, may promote the reaction design and facilitating reaction optimization.

As a summary, when CD is applied solely as a solubilization agent and not accepted as a substrate by the enzyme, the complex stoichiometry, monomer substrate solubility, inhibiting effect of free CD, and acceptance of the guest−CD complex determines the outcome of the reaction. This culminates in the two general phenomena of substrate/solubility and complexation-limiting regime, as illustrated in Figure 9.

4. CONCLUSION

The enzymatic synthesis of alkyl glycosides from alkyl glucosides/αCD mixtures catalyzed by CGTase is heavily influenced by the formation of inclusion complexes between substrates. Combining kinetic studies of substrate complexation and enzyme reactions resulted in full characterization of the system and substrate reactivities. Monomeric AGlus's served as the reactive acceptor, whereas their aggregates (micelles) constituted very poor substrates. Complexing a AGlus with CD created a less reactive acceptor substrate, as shown by an increased K_m value. Altogether, because of substrate complexation, two limiting regimes were observed for the enzymatic reaction kinetics: donor-limiting at low CD-to-surfactant ratios and acceptor-limiting at high CD-to-surfactant ratios. The

dx.doi.org/10.1021/cs500192q | ACS Catal. 2014, 4, 2623−2634

optimum conversion rate depends mainly on the stoichiometry of the complex formed. At higher complex stoichiometries, more CD would be needed to obtain optimal conditions at a 1:1 ratio between free donor and complexed acceptor.

For CGTase, high disproportionation rates were determined for the monomeric AGlu form, whereas for the complexed AGlu species, both coupling and disproportionation were found equally fast. We concluded that complexation presents a process competitive with the enzymatic reaction with the overall limiting step of enzyme (CD:AGlu) complex formation. The strong association constant between AGlu and CD shifted their CMC's and solubilized the long alkyl chain glucosides. It also prevented the complexed CD molecule from acting as a free donor substrate, which enables minimal addition of αCD solely as a complexation agent. This is advantageous when using a glycosyl donor other than CD. The (CD:AGlu) complex can be used only as an acceptor substrate. The systematic analysis and characterization of the AGlu/CD/CGTase system resulted in a rational design for AGly synthesis, facilitating the prediction of optimal conditions for various AGlus substrates.

Starting from AGlus's instead of maltosides and linear sugar donor material should have a dramatic impact on making AGly synthesis industrially competitive. In addition, if CD is exploited as complexation agent in solely stoichiometric amounts, it can be recycled after product purification. This will therefore contribute to an efficient biocatalysis process. Furthermore, the impact of complexation on enzymatic reactions is found in other systems, which readily extends the scope of this study.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: tim.borner@biotek.lu.se.

Present Addresses

†Kevin Roger, Dept. of Physical Chemistry, Lund University, SE-22241 Lund, Sweden. E-mail: kevin.roger@fkem1.lu.se.

‡Patrick Adlercreutz, Dept. of Biotechnology, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden. E-mail: patrick.adlercreutz@biotek.lu.se.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This project was supported by the Swedish Research Council (VR).

■ REFERENCES

(1) Hill, K.; von Rybinski, W.; Stoll, G. *Alkyl Polyglycosides*; Wiley-VCH Verlag GmbH: Weinheim, 2008; p 71−131.

(2) Fischer, E. *Ber. Dtsch. Chem. Ges.* **1893**, *26*, 2400−2412.

(3) Igarashi, K. *Adv. Carbohydr. Chem. Biochem.* **1977**, *34*, 243−283.

(4) Von Rybinski, W.; Hill, K. *Angew. Chem., Int. Ed.* **1998**, *37*, 1328−1345.

(5) Van Rantwijk, F.; Woudenberg-van Oosterom, M.; Sheldon, R. A. *J. Mol. Catal. B: Enzym.* **1999**, *6*, 511−532.

(6) Turner, P.; Svensson, D.; Adlercreutz, P.; Karlsson, E. N. *J. Biotechnol.* **2007**, *130*, 67−74.

(7) Svensson, D.; Ulvenlund, S.; Adlercreutz, P. *Biotechnol. Bioeng.* **2009**, *104*, 854−861.

(8) Henrissat, B. *Biochem. J.* **1991**, *1*, 309−316.

(9) Nakamura, A.; Haga, K.; Yamane, K. *FEBS Lett.* **1994**, *337*, 66−70.

(10) Van der Veen, B. A.; van Alebeek, G.-J. W. M.; Uitdehaag, J. C. M.; Dijkstra, B. W.; Dijkhuizen, L. *Eur. J. Biochem.* **2000**, *267*, 658−665.

(11) Svensson, D.; Ulvenlund, S.; Adlercreutz, P. *Green Chem.* **2009**, *11* (8), 1222−1226.

(12) Chopineau, J.; Lesieur, S.; Carion-Taravella, B.; Ollivon, M. *Biochimie* **1998**, *80* (5−6), 421−435.

(13) Klibanov, A. M. *Trends Biotechnol.* **1997**, *15* (3), 97−101.

(14) Villalonga, R.; Cao, R.; Fragoso, A. *Chem. Rev.* **2007**, *107* (7), 3088−3116.

(15) Comménil, P.; Belingheri, L.; Sancholle, M.; Dehorter, B. *Lipids* **1995**, *30* (4), 351−356.

(16) Otero, C.; Cruzado, C.; Ballesteros, A. *Appl. Biochem. Biotechnol.* **1991**, *27* (2), 185−194.

(17) Yeates, C. A.; Krieg, H. M.; Breytenbach, J. C. *Enzyme Microb. Technol.* **2007**, *40* (2), 228−235.

(18) Truppo, M. D.; Kim, J.; Brower, M.; Madin, A.; Sturr, M. G.; Moore, J. C. *J. Mol. Catal. B: Enzym.* **2006**, *38* (3−6), 158−162.

(19) López-Nicolás, J. M.; Bru, R.; García-Carmona, F. *Biochim. Biophys. Acta* **1997**, *1347* (2−3), 140−150.

(20) Szejtli, J. *Chem. Rev.* **1998**, *98* (5), 1743−1754.

(21) Connors, K. A. *Chem. Rev.* **1997**, *97* (5), 1325−1358.

(22) Noriaki, F.; Seiji, I.; Saburo, N. *Pure Appl. Chem.* **2008**, *80* (7), 1511−1524.

(23) Bernat, V.; Ringard-Lefebvre, C.; Le Bas, G.; Perly, B.; Djedaini-Pilard, F.; Lesieur, S. *Langmuir* **2008**, *24* (7), 3140−3149.

(24) Davis, M. E.; Brewster, M. E. *Nat. Rev. Drug Discovery* **2004**, *3* (12), 1023−1035.

(25) Astray, G.; Gonzalez-Barreiro, C.; Mejuto, J. C.; Rial-Otero, R.; Simal-Gándara, J. *Food Hydrocolloids* **2009**, *23* (7), 1631−1640.

(26) Singh, M.; Sharma, R.; Banerjee, U. C. *Biotechnol. Adv.* **2002**, *20* (5−6), 341−359.

(27) Valente, A. J. M.; Söderman, O. *Adv. Colloid Interface Sci.* **2014**, *205*156176.

(28) Casu, B.; Grenni, A.; Naggi, A.; Torri, G.; Virtuani, M.; Focher, B. *Carbohydr. Res.* **1990**, *200* (0), 101−109.

(29) Valente, A. J. M.; Nilsson, M.; Söderman, O. *J. Colloid Interface Sci.* **2005**, *281* (1), 218−224.

(30) Zehentgruber, D.; Lundemo, P.; Svensson, D.; Adlercreutz, P. *J. Biotechnol.* **2011**, *155* (2), 232−235.

(31) Leemhuis, H.; Dijkstra, B. W.; Dijkhuizen, L. *Eur. J. Biochem.* **2003**, *270* (1), 155−162.

(32) Zhao, H.; Naito, H.; Ueda, Y.; Okada, K.; Sadagane, K.; Izumi, M.; Nakajima, S.; Baba, N. *Biosci., Biotechnol., Biochem.* **2008**, *72*, 3006−3010.

(33) Davis, G.; Wilson, K.; Henrissat, B. *Biochem. J.* **1997**, *15*, 557.

(34) Knegtel, R. M. A.; Wind, R. D.; Rozeboom, H. J.; Kalk, K. H.; Buitelaar, R. M.; Dijkhuizen, L.; Dijkstra, B. W. *J. Mol. Biol.* **1996**, *256*, 611.

(35) Leemhuis, H.; Uitdehaag, J. C. M.; Rozeboom, H. J.; Dijkstra, B. W.; Dijkhuizen, L. *J. Biol. Chem.* **2002**, *277* (2), 1113−1119.

(36) Panaiotov, I.; Ivanova, M.; Verger, R. *Curr. Opin. Colloid Interface Sci.* **1997**, *2* (5), 517−525.

(37) Lee, S. B.; Shin, H. S.; Ryu, D. D. Y.; Mandels, M. *Biotechnol. Bioeng.* **1982**, *24* (10), 2137−2153.

(38) Le Maire, M.; Champeil, P.; Møller, J. V. *Biochim. Biophys. Acta* **2000**, *1508* (1−2), 86−111.

(39) Brennerhenaff, C.; Valdor, J. F.; Plusquellec, D.; Wroblewski, H. *Anal. Biochem.* **1993**, *212* (1), 117−127.

(40) Lundahl, P.; Mascher, E.; Kameyama, K.; Takagi, T. *J. Chromatogr., A* **1990**, *518* (0), 111−121.

(41) Chopineau, J.; Lesieur, S.; Carion-Taravella, B.; Ollivon, M. *Biochimie* **1998**, *80* (5−6), 421−435.

(42) Lopez, O.; Cocera, M.; Parra, J. L.; de la Maza, A. *Colloids Surf., A* **2001**, *193* (1), 221−229.
(43) Iglauer, S.; Wu, Y.; Shuler, P.; Tang, Y.; Goddard, W. A. *Tenside, Surfactants, Deterg.* **2010**, *47*, 87−97.
(44) Piñeiro, Á.; Banquy, X.; Pérez-Casas, S.; Tovar, E.; García, A.; Villa, A.; Amigo, A.; Mark, A. E.; Costas, M. *J. Phys. Chem. B* **2007**, *111* (17), 4383−4392.
(45) Illapakurthy, A. C.; Wyandt, C. M.; Stodghill, S. P. *Eur. J. Pharm. Biopharm.* **2005**, *59* (2), 325−332.
(46) Haller, J.; Kaatze, U. *J. Phys. Chem. B* **2009**, *113* (7), 1940−1947.
(47) Schmidt, A. K.; Cottaz, S.; Driguez, H.; Schulz, G. E. *Biochemistry* **1998**, *37* (17), 5909−5915.
(48) Chokchainarong, S.; Fennema, O. R.; Connors, K. A. *Carbohydr. Res.* **1992**, *232* (1), 161−168.
(49) Penninga, D.; van der Veen, B. A.; Knegtel, R. M. A.; van Hijum, S. A. F. T.; Rozeboom, H. J.; Kalk, K. H.; Dijkstra, B. W.; Dijkhuizen, L. *J. Biol. Chem.* **1996**, *271* (51), 32777−32784.
(50) Haller, J. *Ph.D. Dissertation*; Georg-August-University, Göttingen, Germany, 2008; p 115.
(51) Wennerström, H.; Evans, D. *The Colloidal Domain: Where Physics, Chemistry, Biology, and Technology Meet*, 2nd ed.; Wiley-VCH, New York, 1999; pp 305.
(52) Dünnwald, T.; Demir, A. S.; Siegert, P.; Pohl, M.; Müller, M. *Eur. J. Org. Chem.* **2000**, 2161−2170.
(53) Nieder, V.; Kutzer, M.; Kren, V.; Gallego, R. G.; Kamerling, J. P.; Elling, L. *Enzyme Microb. Technol.* **2004**, *34*, 407−414.
(54) Ghanem, A. *Org. Biomol. Chem.* **2003**, *1*, 1282−1291.
(55) Shin, G.-S.; Lee, K.-W.; Kim, T.-K.; Shin, H.-D.; Lee, Y.-H. *J. Mol. Catal., B: Enzym.* **2005**, *33*, 93−98.
(56) Ávila-González, R.; Pérez-Gilabert, M.; García-Carmona, F. *J. Biosci. Bioeng.* **2005**, *100*, 423−428.
(57) Ávila-González, R.; Pérez-Gilabert, M.; López-López, M. A.; García-Carmona, F. *Biotechnol. Prog.* **2005**, *21*, 338−342.