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The Raw Starch Binding Domain of Cyclodextrin Glycosyltransferase from *Bacillus circulans* Strain 251*

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The E-domain of cyclodextrin glycosyltransferase (CGTase) (EC 2.4.1.19) from *Bacillus circulans* strain 251 is a putative raw starch binding domain. Analysis of the maltose-dependent CGTase crystal structure revealed that each enzyme molecule contained three maltose molecules, situated at contact points between protein molecules. Two of these maltoses were bound to specific sites in the E-domain, the third maltose was bound at the C-domain.

To delineate the roles in raw starch binding and cyclization reaction kinetics of the two maltose binding sites in the E-domain, we replaced Trp-616 and Trp-662 of maltose binding site 1 and Tyr-633 of maltose binding site 2 by alanines using site-directed mutagenesis. Purified mutant CGTases were characterized with respect to raw starch binding and cyclization reaction kinetics on both soluble and raw starch. The results show that maltose binding site 1 is most important for raw starch binding, whereas maltose binding site 2 is involved in guiding linear starch chains into the active site. β-Cyclodextrin causes product inhibition by interfering with catalysis in the active site and the function of maltose binding site 2 in the E-domain.

CGTase mutants in the E-domain maltose binding site 1 could no longer be crystallized as maltose-dependent monomers. Instead, the W616A mutant CGTase protein was successfully crystallized as a carbohydrate-independent dimer; its structure has been refined to 2.2 Å resolution. The three-dimensional structure shows that, within the error limits, neither the absence of carbohydrates nor the W616A mutation caused significant further conformational changes. The modified starch binding and cyclization kinetic properties observed with the mutant CGTase proteins thus can be directly related to the amino acid replacements.

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The atomic coordinates and structure factors (code ITCM) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

§ Contributed equally to this work.

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Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) converts raw starch into cyclodextrins, which are cyclic oligomers of (1→4) linked glucose residues (1, 2). Cyclodextrins can form inclusion complexes with small hydrophobic molecules (3) and are increasingly used in industrial and research applications (4). The cgf gene of *Bacillus circulans* strain 251 has been cloned and sequenced, and the crystal structure of the CGTase protein has been determined at 2.0 Å resolution (1, 5). The protein consists of a single polypeptide chain of 686 amino acid residues, as in other known CGTase structures (6–8), five domains (A–E) can be recognized. The three N-terminal domains (A–C) have structural similarity with the three α-amylase domains. Domain E contains a raw starch binding motif (5, 9, 10) (see Fig. 1).

In glucoamylases, several Trp residues (Trp-590, Trp-615) in the domain containing this motif were shown to be essential for degradation of raw starch (11–13). This domain also has a high affinity for β-cyclodextrin (14, 15), the binding of which inhibits starch hydrolysis (16). Also in CGTases, a starch binding site separate from the active site is present (17), and fusion of the E-domain of the *Bacillus macerans* CGTase to β-galactosidase demonstrated that it can function as a starch binding domain (18). CGTase is sensitive to product inhibition by cyclodextrin, which severely limits yields in industrial production processes (19). Recently, we showed that cyclodextrins bind at the CGTase E-domain (20). This suggested that cyclodextrins may interfere with starch binding.

Here we report a detailed analysis of raw starch binding and cyclization reaction kinetics of wild-type and mutant CGTase enzymes with a modified E-domain. Evidence is presented that the E-domain functions in binding of raw starch and guiding of linear starch chains into the active site.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids—*Escherichia coli***

MC1061 (MhskR mcrB araD139 Δ[araABC-leu]7679 lacX74 galU galK rpsL thi) (21) was used for recombinant DNA manipulations and site-directed mutagenesis. E. coli DH5α (F′EndA1 hsdS17 supE44 thi1 recA1 gyrA [Nal] relA1 [lacZYA-argF] U196 (o80lacΔ3 [lacZ]M15)] (22) was used for the production of monomeric supercoiled plasmid DNA for sequencing. CGTase (mutant) proteins were produced with the α-amylase and protease negative *Bacillus subtilis* strain DB104A (amy nprB nprE18 aprA3) (23). Plasmid pKM1 (Ap′ Km′ ColE1) (24) was digested with HinII. The fragment containing the kanamycin resistance marker was ligated with the largest fragment from plasmid pDP66S (25) containing the *B. circulans* strain 251 cgf gene digested with HindIII and XbaI (made blunt with Klenow polymerase). The resulting CGTase protein expression shuttle vector pDP66K, with the cgf gene under control of the erythromycin-inducible p32 promoter (26), was transformed to E. coli MC1061 under selection for erythromycin and kanamycin resistance (see Fig. 2). This plasmid was considerably more stable than plasmid pDP66S, both in *E. coli* and in *B. subtilis*. DNA manipulations and calcium chloride transformation of

This paper is available on line at http://www-jbc.stanford.edu/jbc/
E. coli strains were as described (27). Transformation of B. subtilis was performed according to Bron (28).

Site-directed Mutagenesis—Mutations were introduced with a PCR1 method using VENT-DNA polymerase (New England Biolabs Inc., Beverly, MA). The first PCR reaction was carried out with a mutagenesis primer for the coding strand plus a primer 960–1000 bp upstream on the coding strand. The product of the last primer for the coding strand plus a primer 910–1050 bp downstream on the coding strand. The 910-1050-bp reaction product was subsequently used as primer in a second PCR reaction together with a primer carrying (mutant) CGTase (6

Table I

<table>
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<th>Cell dimensions (P21):</th>
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<th>b (Å)</th>
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* For molecule A and B, respectively.

Proteinase K digestion was performed on partially hydrolyzed potato starch, with an average degree of polymerization of 50 (Paselli SA2; AVEBE, Foxhol, The Netherlands), was used as a substrate. At regular time intervals, samples were taken, and the amount of β-cyclodextrin formed was determined based on its ability to form a stable colorless inclusion complex with phenolphthalein (31). One unit of activity is defined as the amount of enzyme able to produce 1 μmol of β-cyclodextrin/min.

Enzyme Assays—β-Cyclodextrin forming activity was determined by incubating appropriately diluted enzyme (0.1–0.2 units of activity) for 2–4 min at 50 °C. A 5% solution of partially hydrolyzed potato starch, with an average degree of polymerization of 50 (Paselli SA2; AVEBE, Foxhol, The Netherlands), was used as a substrate. At regular time intervals, samples were taken, and the amount of β-cyclodextrin formed was determined based on its ability to form a stable colorless inclusion complex with phenolphthalein (31). One unit of activity is defined as the amount of enzyme able to produce 1 μmol of β-cyclodextrin/min.

Raw Starch Binding Properties—Raw starch binding properties were studied by incubating pure (mutant) CGTase (6 μg/ml final concentration) with increasing amounts (0–10%) of granular potato starch (AVEBE) at 4 °C for 1 h in the presence and absence of 0.1 mM of β-cyclodextrin (equilibrium was reached within 10 min). CGTase protein bound to the starch granules was spun down at 4 °C for 1 min at 10,000 × g, and the remaining cyclization activity in the supernatant was measured as described above.

Kinetic studies were performed by measuring β-cyclodextrin forming activity of (mutant) CGTase enzymes (final concentration 0.6 μg/ml) with increasing amounts (0–10%) of granular potato starch (AVEBE) at 4 °C for 1 h in the presence and absence of 0.1 mM of β-cyclodextrin (equilibrium was reached within 10 min). CGTase protein bound to the starch granules was spun down at 4 °C for 1 min at 10,000 × g, and the remaining cyclization activity in the supernatant was measured as described above.

Plasmid pDP66K carrying the correct restriction site was primed to DB104A with Plasmid pDP66K, carrying wild-type or mutant cgt genes, was grown in a 5-liter flask, containing 1 liter of medium with 2% tryptone, 0.5% yeast extract, 1% sodium chloride, and 1% casamino acids, pH 7.0, with 10 μg/ml erthyromycin and 5 μg/ml kanamycin, to a final optical density at 600 nm of 4.5 (for approximately 36 h). Under these conditions, high extracellular CGTase levels were obtained reproducibly, allowing purification to homogeneity of up to 25 mg of CGTase.

The abbreviations used are: PCR, polymerase chain reaction; bp, base pairs; MBS, maltose binding sites.

The Hill equation than the Michaelis-Menten equation, indicating that some form of cooperativity is involved both in the cyclization reaction and raw starch binding kinetics. To determine the degree of cooperativity, the data from these kinetic and binding studies were fitted with an equation equivalent to the Hill equation (Equation 1) (32), using the Sigma Plot program (Jandel Scientific). Plotting x versus [S] yields a sigmoidal curve (primary plot).

\[
x = \frac{X_{\text{max}} \cdot [S]^n}{K_{v_{50}} + [S]^n}
\]

(Eq. 1)

In this equation, [S] is the substrate concentration; K_v_{50} is equivalent to the dissociation constant; h is the Hill constant, indicating the degree of cooperativity involved; x is for the binding studies where b is the fraction of protein bound to the raw starch, and for the kinetic studies where v is the velocity of the reaction; X_{max} is for the binding studies where B_{max} is the maximal fraction of protein bound to the raw starch, and for the kinetic studies where V_{max} is the maximal velocity of the reaction.

Equation 1 can be rewritten as follows.

\[
\log \left( \frac{x}{X_{\text{max}} - x} \right) = h \cdot \log [S] - h \cdot \log K_{v_{50}}
\]

(Eq. 2)
By plotting \( \log \left( \frac{x}{X_{\text{max}}^2} \right) \) versus \( \log [S] \) (Hill plot), a straight line is obtained (secondary plot). In this plot, the slope of the line gives the Hill constant \( h \), the y intercept is \( -h \log K_{50} \) and the x intercept is \( \log K_{50} \). The values for the inhibitory effect of \( \beta \)-cyclodextrin on kinetics of the cyclization reaction on Paselli SA2 were calculated using the following equations (32).

For competitive inhibition

\[
K_{i1} = \frac{[I]}{K_{50i}} - 1
\]  
(Eq. 3)

For non-competitive inhibition

\[
K_{i2} = \frac{[I]}{V_{\text{max}i}}
\]  
(Eq. 4)

In Equation 3, \( K_{50i} \) is the apparent \( K_{50} \) when \( \beta \)-cyclodextrin is added, and \( K_{i1} \) is the concentration \( \beta \)-cyclodextrin at which the apparent \( K_{50} \) is 2\( K_{50} \). In Equation 4, \( V_{\text{max}i} \) is the apparent \( V_{\text{max}} \) when \( \beta \)-cyclodextrin is added and \( K_{i2} \) is the concentration of \( \beta \)-cyclodextrin at which the apparent \( V_{\text{max}} \) is 1/20 \( V_{\text{max}} \).

**Crystallization and Data Collection**—Attempts to crystallize the W616A mutant CGTase under similar conditions as the wild type (5) resulted in phase separation in the hanging drops. Therefore, a Sparse matrix (33) (48 experiments) was set up using hanging drops with a protein concentration of 8 mg/ml in 10 mM sodium acetate buffer, pH 5.5, at room temperature. Five conditions, all containing PEG8000 at pH 5.5–7.3, resulted in crystalline material. Refinement of these conditions showed that the biggest crystals could be grown from 9–12% (w/v) PEG8000 at pH 6.8 to 7.2. Many crystals were intergrown plates or twinned needles, but a small number of them were rod-shaped single crystals belonging to the monoclinic space group \( P_2_1 \) with cell dimensions \( a = 73.7 \) Å, \( b = 84.8 \) Å, \( c = 118.3 \) Å, and \( \beta = 107.0^\circ \). X-ray diffraction data were collected on a MacScience Dip2000K image plate system and processed with XDS (34) yielding a data set of 50,563 unique reflections with an \( R_{\text{sym}} \) of 8.3% and 71.3% completeness to 2.2 Å resolution.

**Molecular Replacement**—Molecular replacement was carried out with the native CGTase structure (without maltoses or solvent and with residue Trp-616 replaced by Ala) as the search model. The rotation and translation functions (35, 36) were solved with diffraction data in the 8.0–4.0 Å resolution range. Two unique solutions were obtained at 9r, indicating the presence of a dimer in the asymmetric unit. The relative \( y \) coordinates of the two solutions were determined with the program BRUTE (37). The two solutions correspond to a rotation of the search model over \( \alpha = 77.5^\circ, \beta = 140.0^\circ, \gamma = 150.0^\circ \) and a translation in fractional coordinates of 0.13, 0.00, and 0.24 for the first solution and a rotation of \( \alpha = 105.0^\circ, \beta = 100.0^\circ, \gamma = 355.0^\circ \) and a translation of 0.44, 0.44, and 0.03 for the second solution.

**Structure Refinement**—Both molecules were subjected to rigid body refinement with TNT (38), followed by alternating manual adjustments with O (39) and refinement with TNT. Free \( R \)-factors (40) were calculated using 5% of the data. The final model has crystallographic and free \( R \)-factors of 19.3 and 25.0%, respectively, and good stereochemistry (see Table I). The final model has been deposited at the protein data bank (41) (entry code 1TCM).

**RESULTS AND DISCUSSION**

**Structures of Maltose Binding Sites in the E-domain**—In previous studies, we identified two maltose binding sites (MBS)
in the E-domain of \textit{B. circulans} strain 251 CGTase (5). MBS1 includes Trp-616 and Trp-662, which bind a maltose unit through van der Waals contacts of their indole groups with the glucose rings. Direct hydrogen bonds with the side groups of Lys-651 and Asn-667 and water-mediated hydrogen bonds with the main chain carbonyl oxygen atoms of Trp-616 and Glu-663 further enhance maltose binding (Fig. 1) (5). In MBS2, Tyr-633 forms van der Waals contacts with a glucose residue. Direct hydrogen bonds with the side chains of Thr-598, Asn-627, and Gln-628, the main chain carbonyl oxygen atoms of Ala-599 and Gly-601, and one water-mediated hydrogen bond with Asn-603 complete maltose binding (Fig. 1) (5). MBS2 is located near a groove leading to the active site (Fig. 3), indicating that its function may be a combination of starch binding and guiding the substrate into the groove leading to the active site. A third MBS, located at the C-domain, has also been identified (5). The distances between the different MBS sites measured over the surface of the protein are from MBS1 to MBS2, 30Å; from MBS1 to MBS3, 60Å; and from MBS2 to MBS3, 52Å (5Å).

Recently, we showed that cyclodextrins bind at MBS1 and MBS2 but not (or less) at MBS3 (Fig. 3) (20). The functions of MBS1 and MBS2 in the E-domain were studied further by replacing the residues responsible for hydrophobic interactions with sugar residues (Trp-616, Tyr-633, and Trp-662) by alanine residues. The biochemical and structural properties of these mutant CGTase proteins are reported below.

\textbf{MBS1 (Trp-616, Trp-662) Is Most Important for the Raw Starch Binding Capacity—}The wild-type protein has a high affinity toward raw starch, and at high substrate concentration, virtually all of the enzyme is bound to the raw starch (Fig. 4(A1) and Table II). Especially, the W616A/W662A mutations in MBS1 clearly affected raw starch binding, resulting in highly decreased affinities as shown by the strongly enhanced $K_{50}$ (dissociation constant) values (Fig. 4(B) and Table II). The Y633A mutation in MBS2 also resulted in a strong increase of the $K_{50}$ value (Fig. 4(C) and Table II). The W616A and W616A/W662A mutations also resulted in a decreased binding capacity indicated by the strongly reduced $B_{\text{max}}$ (maximal fraction of bound protein) values (Fig. 4(B1) and Table II). The Y633A mutation had much less effect on $B_{\text{max}}$ values (Fig. 4(C1) and Table II). From this, we conclude that MBS1 (Trp-616, Trp-662) is of crucial importance for raw starch binding, while MBS2 (Tyr-633) especially improves the binding affinity for raw starch and only makes a relatively small contribution to the binding capacity.

The Hill factor $h$ (the degree of cooperativity) for raw starch binding is 1.7 for the wild-type CGTase, indicating that at least two sites are involved in binding (Table II). The Hill factors have severely decreased in mutants W616A and W616A/W662A, demonstrating that in wild-type CGTase MBS1 is one of the sites contributing to cooperative binding. Mutant Y633A has an intermediate Hill factor; cooperativity in raw starch binding thus was less affected in this mutant. This suggests that one or more sites other than MBS2 cooperate with MBS1 in raw starch binding in wild-type CGTase. This is in agreement with the residual starch binding occurring in the W616A/W662A mutant. Conceivably, these alternative sites are the...
active site cleft and/or MBS3 in the C-domain. Preliminary results with an MBS3 mutant indicate that this site is indeed involved in raw starch binding (not shown).

β-Cyclodextrin Competitively Inhibits Raw Starch Binding at MBS1—Under cyclodextrin production conditions, B. circulans strain 251 CGTase suffers from product inhibition (19). A crystallographic analysis of cyclodextrin binding to CGTase revealed that cyclodextrin is bound at MBS1 and MBS2 (20) in the E-domain (Fig. 3), but not at MBS3 in the C-domain, or in the active site. We have now observed that the presence of β-cyclodextrin indeed affected the raw starch binding properties of the wild-type CGTase (Fig. 4(A) and Table II). Addition of β-cyclodextrin resulted in an unchanged raw starch binding capacity (indicated by $B_{\text{max}}$) but in an increased $K_{\text{50}}$ (dissociation constant) value for the wild-type CGTase, reflecting a decreased affinity for raw starch. β-Cyclodextrin thus competitively inhibits raw starch binding by the wild-type enzyme. Almost the same competitive effect was observed for mutant Y633A (Fig. 4(C) and Table II), indicating that with an impaired MBS2, the effect of β-cyclodextrin on raw starch binding had not changed. This again illustrates that MBS2 does not contribute substantially to raw starch binding. The effect of β-cyclodextrin on the binding of raw starch by mutants W616A and W616A/W662A, however, was completely different. Here the $K_{\text{50}}$ values did not change upon addition of β-cyclodextrin (Fig. 4(B2) and Table II), while the raw starch binding capacity (indicated by $B_{\text{max}}$) was severely decreased (Fig. 4(B1) and Table II). β-Cyclodextrin, thus, non-competitively inhibits raw starch binding by CGTase mutants with an impaired MBS1. It follows that in the wild-type CGTase, binding competition between raw starch and β-cyclodextrin takes place at MBS1.

MBS2 (Tyr-633) Is Most Important for Guiding Linear Starch Chains into the Active Site—The raw starch binding studies show that MBS1 is important for the binding capacity, while MBS2 enhances the affinity, suggesting that these sites have distinct functions. Kinetic studies of the cyclization activity of (mutant) CGTase proteins with soluble starch (Paselli SA2) as substrate were performed to further elucidate these functions (Fig. 5 and Table III). Mutations in MBS1 and MBS2 had relatively minor effects on the $V_{\text{max}}$ (maximal velocity of the reaction) values. The mutations W616A/W662A in MBS1 resulted in a doubling of the $K_{\text{50}}$ (dissociation constant) value (Table III), indicating that binding of the substrate at MBS1 on the E-domain enhances the affinity of the enzyme for the substrate. Although MBS2 does not strongly contribute to raw starch binding capacity, mutation Y633A resulted in an even more increased $K_{\text{50}}$ value (Table III), indicating a strongly reduced affinity for the substrate. This, together with the fact that MBS2 is situated at the beginning of the groove leading to the active site (Fig. 3), suggests a role for MBS2 in guiding the substrate to the active site. The cooperativity factor $h$ decreased to 1 for all mutants, indicating complete loss of cooperativity (Table III). This implies that both MBS1 and MBS2 are crucial for the cooperativity observed in cyclization reaction kinetics on Paselli SA2. In the absence of MBS1, the affinity of MBS2 for the substrate is very low (Table II), causing a reduced efficiency of this MBS2; furthermore, MBS2 and the active site cleft can be regarded as one binding site for a high molecular substrate. In the absence of MBS2 the substrate will still bind to MBS1, but it is not efficiently guided to the active site.

β-Cyclodextrin Inhibits the Cyclization Reaction at the Active Site and by Interfering with MBS2—β-Cyclodextrin reduces the capacity of CGTase to bind raw starch (Table II). Also, the cyclization reaction with soluble starch is inhibited by β-cyclodextrin (Table III). This inhibitory effect on the cyclization reaction was investigated further to see whether it involves the maltose binding sites. For the wild-type enzyme, the presence of β-cyclodextrin resulted in clearly decreased $V_{\text{max}}$ and increased $K_{\text{50}}$ values (Fig. 5(A) and Table III). Inhibition of cyclization activity by β-cyclodextrin, therefore, appears to be of a mixed type (32). This type of inhibition can be divided into an effect on the $K_{\text{50}}$ normally observed for competitive inhibition, yielding $K_{i1}$, and an effect on the $V_{\text{max}}$ normally observed for non-competitive inhibition, yielding $K_{i2}$ (Table III). So in the wild type, β-cyclodextrin interferes competitively with the cyclization reaction at the active site and non-competitively elsewhere on the enzyme. The W616A and W616A/W662A mutants showed the same type of inhibition as the wild type, with $K_{i}$
Mutations in the E-domain of CGTase

Table III

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<tr>
<th>(Mutant) protein</th>
<th>$V_{\text{max}}$ (units/mg)</th>
<th>$K_{50}$</th>
<th>$h$</th>
<th>$K_{i1}$</th>
<th>$K_{i2}$</th>
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<td>Y633A</td>
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<td>$27.8 \pm 9.7$</td>
</tr>
</tbody>
</table>

* $K_{i1}$ represents competitive inhibition.
* $K_{i2}$ represents non-competitive inhibition.
* Fitting of these data was performed using the Michaelis-Menten equation ($h = 1$).

Table IV

<table>
<thead>
<tr>
<th>(Mutant) protein</th>
<th>$V_{\text{max}}$ (units/mg)</th>
<th>$K_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>$15.3 \pm 0.5$</td>
<td>$18.4 \pm 1.3$</td>
</tr>
<tr>
<td>W616A</td>
<td>$14.4 \pm 2.1$</td>
<td>$43.3 \pm 11.4$</td>
</tr>
<tr>
<td>W616A/W662A</td>
<td>$(15.3) (\epsilon)$</td>
<td>$(42.5) (\epsilon)$</td>
</tr>
<tr>
<td>Y633A</td>
<td>$16.0 \pm 0.5$</td>
<td>$42.5 \pm 2.7$</td>
</tr>
</tbody>
</table>

Values for $\beta$-cyclodextrin, which did not differ significantly from the wild-type values (Table III). Upon addition of $\beta$-cyclodextrin, the Y633A $V_{\text{max}}$, however, was less affected, resulting in a higher $K_{50}$, while its $K_{i1}$ value increased more, resulting in a lower $K_{i1}$ (Fig. 5(C) and Table III). This indicates that in wild-type CGTase, non-competitive inhibition is mainly caused by interference of $\beta$-cyclodextrin with the function of MBS2. A $\beta$-cyclodextrin bound to this site may not only prevent starch binding but also block the groove leading to the active site (Fig. 3) (5, 20). The more pronounced competitive type of inhibition of the cyclization reaction in the Y633A mutant is probably a direct effect of the decreased non-competitive inhibition; the relatively higher $V_{\text{max}}$ upon addition of $\beta$-cyclodextrin compared with the wild-type and other mutant enzymes, automatically results in a higher $K_{50}$.

The raw starch binding studies showed that MBS1 (Trp-616, Trp-662) is crucial for raw starch binding, while the kinetic studies of cyclization activity on Paselli SA2 clarified that MBS2 (Tyr-633) has a function in guiding linear starch chains into the active site.

The Functions of MBS1 and MBS2 Are Equally Important for Cyclization Activity with Raw Starch—The cyclization activity kinetics was also studied with raw starch as substrate (Fig. 6 and Table IV). Mutations W616A (MBS1) and Y633A (MBS2) resulted in increased $K_{50}$ (dissociation constant) values, while $V_{\text{max}}$ (maximal velocity of the reaction) values remained unaffected (Table IV). The kinetic parameters of the W616A/W662A mutant could not be measured accurately because of a shift in the curve (Fig. 6); most likely, its $V_{\text{max}}$ approaches those of wild type and the W616A and Y633A CGTases, and its $K_{50}$ value approaches those of mutants W616A and Y633A. The results nevertheless clearly indicate that the affinity toward raw starch in the cyclization reaction is much higher when both MBS1 and MBS2 are present (Fig. 6). The Y633A mutation resulted in a similarly reduced affinity for raw starch (Table IV) and Paselli SA2 (Table III) when studying the cyclization reaction kinetics. As discussed above, loss of MBS2 converts CGTase into a protein that is less efficient in guiding the starch chain to the active site, while loss of MBS1 results in a reduced ability to bind to raw starch granules. For the conversion of raw starch into cyclodextrins, both these features of the two maltose binding sites are equally important. At the surface of starch granules, chains of varying lengths may protrude into the medium. Binding of CGTase protein to a starch granule will result in an increased local concentration of substrate for the enzyme. Loss of binding, therefore, results in a decrease in the effective substrate concentration, and thus in an increased $K_{50}$ value. Fig. 6 shows that, up to a concentration of 35% raw starch, mutations in both MBS1 and MBS2 appear to have similar effects. At higher raw starch concentrations, however, the cyclization activity of the W616A/W662A mutant (MBS1) shifts toward the level displayed by wild type. This shift in the curve suggests that MBS1 is functionally replaced by another binding site. Conceivably, this site is MBS3 in the C-domain, also involved in raw starch binding. This effect may only be visible at high raw starch concentrations because of the relatively long distance between MBS3 and MBS2, which might cause a high Hill factor value (Fig. 6(B), (hypothetical dotted line)). Starch chains of sufficient length may be scarce on the surface of the starch granule, resulting in a high $K_{50}$ value (Fig. 6(A), (hypothetical dotted curve)).

Crystal Structure of the W616A Mutant CGTase—In wild-type CGTase, the maltose that is bound to MBS1 is involved in crystal packing contacts (5). Replacement of Trp-616 by Ala reduced the affinity for binding of carbohydrate chains at this site. This has resulted in a novel maltose-independent crystal...
form. The W616A mutant CGTase crystallizes as a dimer with the mutated MBS1 of each monomer face to face (Fig. 7). Dynamic light scattering measurements of the protein dissolved in 10 mg/ml α-cyclodextrin and 10 mM sodium acetate, pH 5.5, showed, however, that the protein is monomeric in solution (results not shown) and that, therefore, the dimeric form is a crystallographic artifact. The two protein molecules in the dimer, called A and B, respectively, are related to each other by a rotation of 170° resulting in different local environments of the two molecules. The largest changes in the protein backbone conformation occur at residues A336–A338, A665–A659, B40–B43, B89–B91, B598–B603, and B655–B659, all of which are located at the surface of the protein, near the dimer interface or crystal packing contacts. The dimer interface is at regions near crystal contacts and the corresponding aromatic residues have been indicated. The W616A structure deviates only from the wild-type enzyme structure is drawn in thin lines. The maltose binding site, probably MBS3 (with Trp-413, in the C-domain) and a major contribution from an additional binding site, probably MBS2 (with Tyr-633) and a minor contribution from MBS2 (with Trp-616, Trp-662) is required for efficient binding to raw starch granules with a minor contribution from MBS2 (with Tyr-633) and a major contribution from an additional binding site, probably MBS3 (with Trp-413, in the C-domain).

**TABLE V**

Inter-molecular hydrogen bonds and van der Waals interactions of the W616A CGTase mutant

For van der Waals interactions, only the shortest distance between two residues is listed. Symmetry operation 1 denotes contacts within the dimer.

<table>
<thead>
<tr>
<th>Atom 1</th>
<th>Atom 2</th>
<th>Distance (Å)</th>
<th>Symop</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp A662-C</td>
<td>Trp B662-C</td>
<td>3.0</td>
<td>1</td>
</tr>
<tr>
<td>Phe A39-C</td>
<td>Tyr A301-C</td>
<td>3.6</td>
<td>2</td>
</tr>
<tr>
<td>Gly A41-C</td>
<td>Tyr A301-C</td>
<td>3.5</td>
<td>2</td>
</tr>
<tr>
<td>Asp B147-C</td>
<td>Val B265-C</td>
<td>3.5</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Atom 1</th>
<th>Atom 2</th>
<th>Distance (Å)</th>
<th>Symop</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala A616-O</td>
<td>Trp B662-Nr1</td>
<td>3.0</td>
<td>1</td>
</tr>
<tr>
<td>Asp A617-O</td>
<td>Asp B617-O</td>
<td>3.0</td>
<td>1</td>
</tr>
<tr>
<td>Trp A662-N</td>
<td>Gly B665-O</td>
<td>2.7</td>
<td>1</td>
</tr>
<tr>
<td>Trp A662-O</td>
<td>Gly B665-N</td>
<td>3.3</td>
<td>1</td>
</tr>
<tr>
<td>Asp A147-O</td>
<td>Tyr A492-OH</td>
<td>3.0</td>
<td>4</td>
</tr>
<tr>
<td>Ser A205-Oy</td>
<td>Gin B453-O</td>
<td>3.4</td>
<td>4</td>
</tr>
<tr>
<td>Thr A206-Oy1</td>
<td>Gin B453-Ne2</td>
<td>2.8</td>
<td>4</td>
</tr>
<tr>
<td>Asn A336-O</td>
<td>Ser A658-Oy</td>
<td>2.9</td>
<td>2</td>
</tr>
<tr>
<td>Ala A557-N</td>
<td>Ser B90-Oy</td>
<td>3.3</td>
<td>5</td>
</tr>
<tr>
<td>Asn A681-O</td>
<td>Cys B400-Sy</td>
<td>2.8</td>
<td>4</td>
</tr>
<tr>
<td>Pro A686-O</td>
<td>Lys B128-Nf</td>
<td>3.3</td>
<td>4</td>
</tr>
<tr>
<td>Tyr B301-OH</td>
<td>Gin B632-Or1</td>
<td>3.1</td>
<td>5</td>
</tr>
<tr>
<td>Asn B416-O</td>
<td>Gin B632-Ne2</td>
<td>3.3</td>
<td>5</td>
</tr>
<tr>
<td>Asn B416-Ns2</td>
<td>Gin B632-Or1</td>
<td>2.8</td>
<td>5</td>
</tr>
</tbody>
</table>

*Symop, Symmetry operations are 1 = X, Y, Z; 2 = 1 − X, Y + 0.5, 1 − Z; 3 = 1 − X, Y − 0.5, −Z; 4 = X − 1, Y, Z, 5 = 1 − X, Y + 0.5, −Z.

Our data suggest the following functions for the maltose binding sites in the *B. circulans* strain 251 CGTase E-domain. MBS1 (with Trp-616, Trp-662) is required for efficient binding to raw starch granules with a minor contribution from MBS2 (with Tyr-633) and a major contribution from an additional binding site, probably MBS3 (with Trp-413, in the C-domain). A β-cyclodextrin bound at MBS1 competitively inhibits binding of CGTase to raw starch granules. The cyclization reaction kinetics with soluble starch indicated that MBS2 has an important role in guiding linear starch chains to the active site. For wild-type CGTase, a mixed type of inhibition by β-cyclodextrin was observed. Competitive inhibition takes place at the active site, while non-competitive inhibition results from interference of β-cyclodextrin with substrate binding at MBS2. β-Cyclodextrin bound at MBS2 may block the groove leading to

**CONCLUSIONS**

Our data suggest the following functions for the maltose binding sites in the *B. circulans* strain 251 CGTase E-domain. MBS1 (with Trp-616, Trp-662) is required for efficient binding to raw starch granules with a minor contribution from MBS2 (with Tyr-633) and a major contribution from an additional binding site, probably MBS3 (with Trp-413, in the C-domain). A β-cyclodextrin bound at MBS1 competitively inhibits binding of CGTase to raw starch granules. The cyclization reaction kinetics with soluble starch indicated that MBS2 has an important role in guiding linear starch chains to the active site. For wild-type CGTase, a mixed type of inhibition by β-cyclodextrin was observed. Competitive inhibition takes place at the active site, while non-competitive inhibition results from interference of β-cyclodextrin with substrate binding at MBS2. β-Cyclodextrin bound at MBS2 may block the groove leading to
Mutations in the E-domain of CGTase

![Electron density in 2|F| - |F|2 maps contoured at 1σ for residues A614–A618 and B614–B618, labeled A and B, respectively. Residues adjacent to the mutated tryptophan have been indicated. The replacement of Trp-616 by Ala is confirmed by the electron density in this region.](image)

fig. 8. Electron density in $2|F|_2 - |F|^2$ maps contoured at 1σ for residues A614–A618 and B614–B618, labeled A and B, respectively. Residues adjacent to the mutated tryptophan have been indicated. The replacement of Trp-616 by Ala is confirmed by the electron density in this region.

the active site. The cyclization reaction kinetics with raw starch revealed that these different roles of MBS1 and MBS2 are equally important for degradation of raw starch.

Structural analysis of the W616A mutant CGTase revealed only minor changes compared with the wild-type enzyme. The modified starch binding and cyclization kinetic properties observed with the mutant CGTase proteins thus can be directly related to the amino acid replacements.

The data obtained on the functions of the CGTase E-domain in raw starch binding have important implications for our understanding of this process in glucoamylase and other members of the α-amylase family (9–12, 16, 43–45).

REFERENCES