Single-Molecule Imaging of Interaction between Dextran and Glucosyltransferase from *Streptococcus sobrinus*

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Of the glucosyltransferases (GTFs) from oral streptococci that catalyze glucosyl transfer from sucrose to growing glucan chains (17, 19), GTF I is known to produce water-insoluble glucans (WIGs) by synthesizing α-1,3-glucans. In the presence of dextrans (α-1,6-glucans), the glucosyl transfer is greatly accelerated, and numerous α-1,3-linked branches are formed on the dextrans, which results in the formation of WIG. Based on the structure of the WIGs formed, it has been shown elsewhere that branching points are made, on average, every 10 glucosyl residues along the α-1,6-linked chains (5). This suggests that GTF I travels from one branching point to another on the dextran chains. It has been well documented that the affinity of GTFs for dextrans is generally very high (our recent estimate of the binding constant of GTF I, 2 × 10^5 M^-1 [K. Kaseda, M. Mori, H. Komatsu, K. Fukui, and T. Kodama, unpublished data]). This property has been utilized for the purification of GTFs by dextran-affinity chromatography (20) and for targeting inhibitors of bacteria in dental plaque (18).

A challenging question then is how one can reconcile these apparently paradoxical properties of GTF I, having a high affinity for dextrans yet traveling efficiently around many branching points one after another. More specifically, how fast does GTF I alternate between dextran-attached and detached states and how does sucrose modulate the dynamics of the GTF I-dextran interaction?

To answer these questions, we have directly observed the interaction between GTF I and dextran at the single-molecule level. Recent advances in imaging technology have made it possible to observe single fluorescent dye molecules using low-background total internal reflection fluorescence microscopy (TIRFM) (9). This technique has been so far used to analyze molecular motors such as myosin (6, 12, 15), kinesin (2, 22, 25), and RNA polymerase (8) and has for the first time revealed some unique molecular behaviors of these enzymes while they are actually functioning.

One of the characteristic features shared by GTFs of oral streptococci is the absence of a cysteiny1 residue in the primary structure of GTF I. In this study, a cysteinyl residue has been introduced into the C-terminal region of a genetically truncated form of the enzyme from *Streptococcus sobrinus* 6715 (abbreviated as GTF † hereafter) (1) by genetic manipulation. Note that, in GTF †, the N-terminal 84 residues and the C-terminal 264 residues of GTF I are deleted but its enzymatic properties are fully retained (16). The sulphhydryl (SH) groups were also introduced into dextran by chemical modification. These SH-containing materials were then either fluorescently labeled with tetrathylrhodamine (TMR) for single-molecule imaging or biotinylated for fixation to the streptavidin-coated surface of the observation chamber for TIRFM.

A frameshift was inserted into the 3' end of the inserted DNA for GTF † in the plasmid pAB2 (1) by digestion with *XbaI* and then with *S1* nuclease, followed by ligation with *T4* ligase. Insertion of a new codon for cysteine into pAB2 was confirmed by DNA sequencing, from which the amino acid sequence at the C-terminal end of the deduced GTF † (abbreviated as GTF †-Cys) was purified according to the method previously described for GTF † (16). The reactivity of the cysteine residue in GTF †-Cys estimated with 5,5'-dithiobis-(2-nitrobenzoic acid) (4, 21) was 93% on the protein basis. GTF †-Cys (50 μM) was incubated with 150 μM TMR-5'-maleimide (Molecular Probes) in 20 mM sodium phosphate (pH 7.5) for 2 h at room temperature. After the addition of 10 mM dithiothreitol, the reaction mixture was filtered through a minicolumn of Toyopearl HW 40-F (Tosoh Corp., Tokyo, Ja-
Dextran was chemically modified by subjecting dextrans T500 (500 kDa) and T10 (10 kDa) from Amersham Pharmacia to mild oxidation with periodate (7, 14). The SH groups were then introduced by treatment with 500 μM 2-acetamido-4-mercaptobutanoic acid hydrazide (Molecular Probes) (11, 23, 24). The SH group-containing dextrans were then labeled with mercaptohexanol, followed by a phenol-sulfuric acid method (3). The average molar ratios of TMR to dextran were 0.5 and 4.0 for T10 and T500, respectively.

To visualize the binding of GTF' to dextran, GTF'-TMR was incubated with nonlabeled dextran T10 (20 mg/ml) and sucrose on incubation with GTF'-TMR (2 μM). Since the concentration of enzyme used in this experiment was sufficiently high to be detectable if bound to the WIG, this observation is consistent with the fact that GTF’ could be recovered in the supernatant when centrifuged at a low speed after a prolonged period of incubation with dextran and sucrose to form WIG. Thus, GTF’ appeared to have a very low affinity for the α-1,6-glucan chain with many α-1,3 branches formed by its own action.

The behavior of single molecules of GTF and dextran interacting with each other was then investigated by TIRFM, the details of which have been described elsewhere (9). In short (Fig. 1a), a beam of 4.4 mW from a frequency-doubled Nd: YAG laser (wavelength, 532 nm) was focused onto a specimen plane of 200 by 200 μm with a very high incident angle, which formed the evanescent field on the other side of the reflecting surface. When fluorescent molecules are trapped on the surface in the evanescent field, they are excited, and their images can be captured by a silicon-intensifier target tube (SIT) camera coupled to an image intensifier. Among a number of fluorescent molecules in the assay chamber, only those that have lost their motional freedom in the evanescent field are imaged, so that the background noise of fluorescence can be greatly reduced.

For dextran imaging (Fig. 1c), GTF'-biotin was first fixed on the streptavidin-biotinylated bovine serum albumin (BSA)-
coated surface of an assay chamber (13). The chamber was then filled with T500-TMR and washed with buffer A to remove unbound T500-TMR. GTF$^9$ imaging was performed in the same way using the T500-biotin-fixed chamber (Fig. 1b). Buffer A used in these observations was supplemented with 0.5% mercaptoethanol and an oxygen scavenger system (10).

In single-molecule imaging, it is necessary to determine how fast the fluorescent molecules undergo photobleaching. Thus, observations were first made on a glass surface thoroughly cleaned but without any coating (Fig. 2a and b). This method is based on the fact that the glass surface nonspecifically adsorbs various molecules rather strongly so that some remain trapped until photobleaching occurs. The time course of change in the intensity of the fluorescence spots recorded at the video rate was examined for those shown in Fig. 2a and b. Most of the GTF$^9$-TMR spots (>80%) were photobleached in a single step. A typical example is shown in Fig. 2c for the spot indicated by an arrow in Fig. 2a, which provides evidence that the spot corresponded to a single GTF$^9$-TMR molecule. However, the photobleaching of fluorescent spots for T500-TMR (Fig. 2b) took place mostly in two or three steps as exemplified in Fig. 2d. This result is consistent with the fact that T500 was labeled with several TMR molecules.

When T500-TMR was observed in the GTF$^9$-coated chamber, a number of fluorescent spots were continuously observed until photobleaching occurred, several tens of seconds to several minutes later (Fig. 3a). The extremely long duration on the surface (dwell time) of these spots is probably attributable to dextrans that could not escape from the many interacting GTF$^9$ molecules. This interpretation is justified by our recent observation, obtained by light-scattering titration of dextran with GTF$^9$, that single dextran molecules can bind a number of GTF$^9$ molecules and that the number bound is proportional to the molecular mass of dextran (Kaseda et al., unpublished data). Thus, the enzyme-binding unit of the dextran chain consists of approximately 15 glucosyl residues, so that a single T500 molecule that is free in solution could bind hundreds of GTF$^9$ molecules.

Apart from these spots with a long dwell time, a much smaller number of spots came in and out of sight in turn. This reflects the movement of dextran molecules associating with and then dissociating from the surface. This dynamic behavior of T500-TMR was more easily observed in the presence of an excess of nonlabeled dextran, which decreased the number of spots with a long dwell time by displacement (Fig. 3b). Figure 4a shows a histogram of dwell time estimated under such conditions with the presence of 40 nM nonlabeled T500, giving an apparent first-order dissociation rate of $3.0 \pm 0.8$ s$^{-1}$.
When the same assay was performed in the presence of sucrose, the dwell time of fluorescent spots was reduced as indicated by the histogram (Fig. 4b), which gave an increased dissociation rate of $5.3 \pm 1.0 \text{ s}^{-1}$. After 30 min, sufficient time to allow the formation of WIG, several extremely strong fluorescent spots were observed on the bottom surface of the observation chamber (data not shown). This suggests that chemically modified GTF\textsuperscript{9} actually synthesized WIG in the TIRFM observation system.

Imaging of GTF\textsuperscript{9}-TMR was also done in the presence of nonlabeled GTF\textsuperscript{9} (1 \text{ mM}). In the absence of sucrose, the majority of the fluorescent spots remained on the surface momentarily. A histogram of dwell time for these spots showed the dissociation rate to be $9.2 \pm 1.7 \text{ s}^{-1}$ (Fig. 5a). In the presence of sucrose (Fig. 5b), the histogram as a whole was shifted to shorter dwell times, indicating a higher dissociation rate of $13.3 \pm 1.5 \text{ s}^{-1}$. The slight discrepancy in the dissociation rates estimated from dextran and GTF\textsuperscript{9} imagings could be accounted for by a larger contribution of the fluorescent spots with a long dwell time in the dextran observations (see above). By contrast, the fluorescent spots with a long dwell time were hardly detected in the GTF\textsuperscript{9} observation, which is consistent with the fact that a GTF\textsuperscript{9} molecule can interact with only a single dextran molecule at a time. Thus, the rate estimated from the GTF\textsuperscript{9} imaging is more likely to correspond to that for a single GTF\textsuperscript{9} molecule dissociating from the interacting dextran.

The present work using TIRFM clearly shows a dynamic interaction between GTF\textsuperscript{9} and dextran at the single-molecule level, repeating association with and then dissociation from each other. The apparent dissociation rate estimated from the histogram of dwell time in the absence of sucrose is in good agreement with that estimated by a stopped-flow method to monitor light-scattering change accompanying the GTF\textsuperscript{9}-dextran interaction (Kaseda et al., unpublished data). In addition, GTF\textsuperscript{9} and dextran dissociate from each other more easily in the presence of sucrose, even before the dextran has been fully decorated with \(\alpha\)-1,3 branches. It is worth emphasizing that the sucrose effect was not known before and is still difficult, if not impossible, to show by other methods.
How can the effect of sucrose be explained? It is conceivable that the branched structure formed by GTF\textsuperscript{9} itself facilitates dissociation. In fact, the rate of glucosyl transfer to dextran by GTF\textsuperscript{9}-TMR was estimated to be $\approx 25$ s\textsuperscript{-1}, much higher than the dissociation rate observed here. Thus, the enzyme would have sufficient time to make a new $\alpha$-1,3 branch or to elongate preformed branches once it had bound to dextran in the presence of sucrose. As the $\alpha$-1,3 branch elongates, the affinity of the enzyme for the directly interacting portion of the $\alpha$-1,6 chain would be reduced. Alternatively, the affinity for dextran may be reduced at a certain step or steps of the GTF\textsuperscript{9} catalytic cycle, which would facilitate the dissociation of the enzyme by thermal agitation. Our recent structural study by electron microscopy indicates that GTF\textsuperscript{9} consists of a spherical head and a smaller spherical tail (16): the former corresponds to the N-terminal catalytic domain responsible for sucrose splitting and glucosyl transfer and the latter corresponds to the C-terminal dextran-binding domain. In addition, the catalytic domain shows a very low affinity for dextran. Thus, it would be of great interest to investigate how the head and tail of the GTF\textsuperscript{9} molecule cooperate to make the $\alpha$-1,3 branches while the tail is holding a dextran as the glucosyl acceptor. Although further studies are required to test these hypotheses, the sucrose-induced affinity change could be at least a part of the mechanism by which GTF\textsuperscript{9} travels from one branching point to another to form many branches along the $\alpha$-1,6-glucan chain.

Finally, to the best of our knowledge, the present work is the first application of the single-molecule assay to protein-polysaccharide interactions. Although a single catalytic event, as in the case of motor proteins (6, 25), cannot yet be observed, the experimental method described here should greatly facilitate the study of other related protein systems.

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