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Effect of modification of the tryptophan residues of cyclodextrin glucanotransferase with *N*-bromosuccinimide on the enzyme-catalysed hydrolysis (cleavage) of soluble starch and cyclomaltohexaose*,†

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ABSTRACT

Four tryptophan residues in cyclomalto-oligosaccharide (cycloamylose, cyclodextrin) glucanotransferase (CGTase) from *Bacillus stearothermophilus* were modified with *N*-bromosuccinimide (NBS), one of which ("Trp^{4*}") was markedly less reactive than the others. The modification of Trp^4 by NBS corresponded with inactivation of the CGTase-catalysed hydrolysis of cyclomaltohexaose (CG_6). Trp^4 was protected against NBS by glucose and the maltosaccharides G_2–G_4, which indicates Trp^4 to be located at the substrate binding site of CGTase.

INTRODUCTION

Cyclomalto-oligosaccharide (CG) glucanotransferase (CGTase, EC 2.4.1.19) catalyses some reactions, mainly (*a*) cyclisation (starch → CG_n), (*b*) coupling (CG_n + GX → G_nGX), (*c*) disproportionation (G_n → G_{n-1} + G_n + G_{n+1}), and (*d*) hydrolysis (CG_n → G_n). The mechanism of the functional differentiation by CGTase is of considerable interest because the enzyme is useful for the production of cyclomalto-oligosaccharides which have pharmaceutical and industrial applications. There have been numerous studies of the structure and function of the enzyme[1–4]. CGTase, which is well suited for the production of cyclomalto-oligosaccharides, has been isolated from *Bacillus stearothermophilus* and purified to give a single band in electrophoresis on agarose gel, and its primary and tertiary structures have been studied by a genetic

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technique[5] and X-ray crystallography[6], respectively. It was concluded that one Trp residue (Trp97), amongst other aromatic residues, is located at the active site.

Chemical modification with *N*-bromosuccinimide (NBS) of the Trp residues in amylase and lysozyme has been studied by difference absorption and fluorescence spectrophotometry[7–11]. For the glucoamylase from *Rhizopus niveus*, rate constants k for the modification of four Trp residues (Trp^1–Trp^4) with NBS were determined and Trp^3 was confirmed to be located at subsite 1. Trp^3 may be concerned with the binding of substrates, which produces changes in the u.v.-difference absorption and fluorescence intensity. Thus, modification with NBS is valuable for discrimination of Trp residues and for identifying their role in the enzyme-catalysed reaction[12].

We now report on the modification with NBS of the cyclodextrin glucanotransferase (CGTase) from *B. stearothermophilus*.

EXPERIMENTAL

Materials. — CGtase (EC 2.4.1.19) from *B. stearothermophilus* was isolated and purified as described elsewhere[13], and was confirmed to be homogeneous in disc electrophoresis and SDS-PAGE[14]. The concentration of the enzyme was determined spectrophotometrically, taking the absorption unit $A_{280}^{1\%}$ as 16.0 cm^{-1}, and the molecular weight as 75,459. Cyclomaltohexaose CG_6, maltose G_2, maltotriose G_3, maltotetraose G_4, and amylose (d.p. ~18) were purchased from Hayashibara Biochemical Laboratories Inc., Okayama. *N*-Bromosuccinimide (NBS) was recrystallised at least twice from water before use. The enzyme assay-kit, D-glucose oxidase and peroxidase, for the determination of glucose was purchased from Toyobo Biochemicals Co., Tokyo. 2-*p*-Toluidinylnaphthalene-6-sulfonic acid (TNS) was purchased from Sigma Chemical Co. and used without further purification.

Modification of the Trp residues of CGTase with NBS. — Equal volumes (1.5 mL) of the NBS and 1.0–5.0 μM CGTase solutions were mixed (conditions: pH 7.4, 0.01 M Tris buffer, and 25.0°). The modification by NBS was observed as follows: (*a*) The reaction mixture (3.0 mL) was put into a cuvette in a Hitachi 850 fluorescence spectrophotometer and the change in fluorescence intensity ΔF was monitored using excitation and emission wavelengths of 280 and 340 nm, respectively. The concentrations of NBS were varied over a wide range and are represented by the ratio [NBS]/[CGTase]. (*b*) Aliquots (0.4 mL) of the reaction mixture at appropriate intervals were each mixed with the free L-tryptophan solution (1.6 mL) in order to stop the reaction with NBS; the final concentration of tryptophan was usually set to the ratio [Trp]/[NBS] = 100. The excess of NBS was rapidly (< 1 s) captured by the added tryptophan. The residual CGTase-catalysed hydrolytic and cyclising activities were determined by the assay described below.

Assay of the CGTase-catalysed hydrolytic activity. — Time curves of the CGTase-catalysed hydrolysis of soluble starch (SS) and cyclomaltohexaose (CG_6) were obtained at pH 5.5 and 37.0° as follows. (*a*) The solutions of the enzyme (1.0 mL) and CG_6 (4.0

mL) were mixed, aliquots (0.4 mL) were taken at intervals, and each was heated to ~ 100° for 10 min in order to stop the reaction after the addition of 0.02M acetate buffer (pH 4.5, 1.5 mL). The product (maltohexaose G_6) was treated with a solution of glucoamylase (0.1 mL, 10 mg/mL, in 0.02M acetate buffer, pH 4.5) at 37° for 30 min and converted completely into glucose, which was determined by the enzyme assay (1.0 mL); the solution was kept at 37° for 5 min and A_{500} was measured. (*b*) Solutions of the enzyme (1.5 mL) and SS (10 mL) were mixed, aliquots (1.0 mL) were taken at intervals, each was treated with 0.02M H_2SO_4 (2 mL) in order to stop the reaction, 0.1M I_2 (0.1 mL) was added, and A_{600} was determined for the iodine–starch complex. The slope of the time curve gives the initial velocity of the CGTase-catalysed reaction.

The CGTase-catalysed synthesis of CG_n from amylose was also determined using the fluorescent reagent TNS, which complexes with CG_n with a consequent change in the fluorescence spectrum. 610μM TNS (0.05 mL), aqueous 20–0.005% amylose (0.95 mL), and 0.023μM CGTase (0.3 mL) were mixed at 25° in a cuvette (the pH of the mixture was 5.5). The fluorescence intensity at 468 nm by excitation at 366 nm was monitored, and the initial velocities were obtained from the time curves.

The Michaelis constant K_m and the molar activity k_o were determinated from the experimental data by using an EPSON 286V PC and the software "Bio-Graph" of KYOTO SOFT Co.

U.v.-difference absorption spectrophotometry of the modification of CGTase by NBS. — The spectra were recorded for solutions at pH 4.5 and 25.0° with a Shimadzu UV-240 spectrophotometer equipped with tandem quartz cells. The difference absorption at 280 nm (ΔA_{280}) was determined from the spectra, as described[15,16]. The number of Trp residues modified was calculated on the basis of ΔA_{280} per mol of enzyme ($\Delta\varepsilon_{280}$), using as a standard the value of $\Delta\varepsilon_{280}$ of 3,000 determined with *N*-acetyltryptophan ethyl ester (ATE).

RESULTS AND DISCUSSION

Modification of CGTase by NBS. — The modification of the Trp residues of CGTase with NBS was monitored by fluorescence spectrophotometry. A typical time curve (Fig. 1a) for [NBS]/[CGTase] = 100 shows that the rate of reaction was low with an apparent rate constant (k_{app}) of 0.50 min^{-1}.

A fixed [CGTase] was used and [NBS] was varied. The fluorescence intensities, *F*(%), were evaluated from these curves by extrapolation to 0 and 5 min. The results are summarised in Fig. 1(b), which shows that the Trp residue modified by NBS carried ~ 46% (change in *F*, ΔF) of the total fluorescence intensity of the enzyme.

The modification of CGTase was also monitored by difference spectrophotometry, as for the fluorescence spectrophotometric study. The decrease in ΔA_{280} was monitored and $\Delta\varepsilon_{280}$ was evaluated as described[17]. The results (Fig. 2) indicate that four Trp residues (Trp^1–Trp^4) out of a total of 14 in CGTase were modified by NBS, three of which reacted at low [NBS] and one (Trp^4) at high [NBS]. Thus, Trp^4 can be discriminated and may be located at an inner part of, or a cleft in, the enzyme molecule.

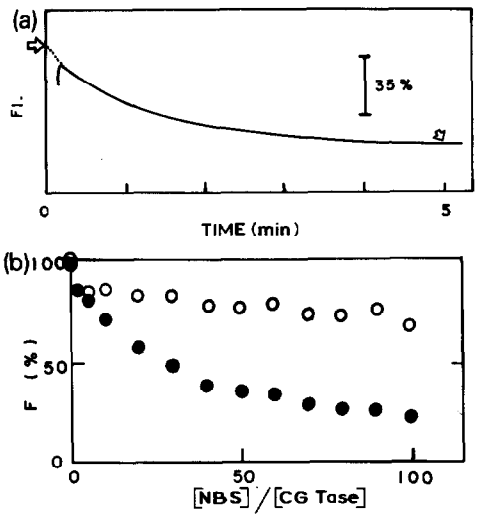

Fig. 1. Dependency of ΔF on the modification of CGTase by NBS. (*a*) [CGTase] = 0.125μM at pH 7.4 and 25.0°, and [NBS]/[CGTase] = 100. (*b*) ΔF by extrapolation to zero time (○) and 5 min (●) [indicated by the arrows in (*a*)].

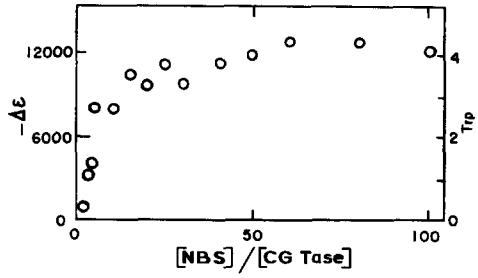

Fig. 2. Dependency of $-\Delta\varepsilon_{280}$ on [NBS]; [CGTase] 12.5μM, pH 7.4, 25.0°.

Assuming that four Trp residues are oxidised also at the 100-fold lower [NBS], then these results suggest that Trp^4 is responsible for the change in fluorescence intensity ΔF. The other three Trp residues cause little change (13% at most) in fluorescence. Reaction at Tyr was excluded since the u.v.-difference spectrum due to modification of Tyr was not observed.

Effect of modification by NBS on CGTase-catalysed hydrolyses and cyclisations. — The effect of modification of CGTase by NBS was observed for the hydrolyses of soluble starch (SS) and cyclomaltohexaose (CG_6), and the results are shown in Fig. 3, where the ratio [NBS]/[CGTase] was 100. The rate of hydrolysis of CG_6 was reduced as the Trp^4 residue was modified by NBS, and k_{app} for the inactivation was 0.33 min^{-1} (*cf.* 0.5 min^{-1} for the rate of modification by NBS described above). These findings suggest that Trp^4 is located at the active site of the enzyme and plays a decisive role in the enzyme-catalysed

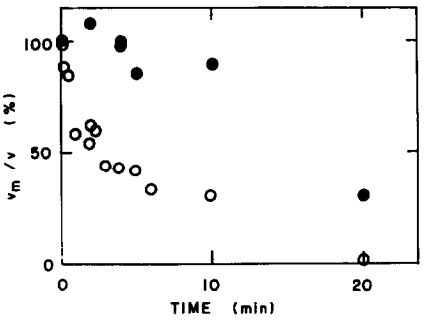

Fig. 3. Effect of modification by NBS on the activity of CGTase on cyclomaltohexaose (CG_6) and soluble starch (SS): [NBS]/[CGTase] = 100, [CGTase] = 0.125μM, pH 7.4, 25°; CG_6 (○), [CGTase] = 0.043μM, [CG_6] = 12 mM; SS (●), [CGTase] = 0.074μM, [SS] = 0.03%, pH 5.5, 25.0°; v_m and v are the initial velocities of the modified and intact preparations of CGTase, respectively.

TABLE I

Kinetic parameters (±10%) for the CGTase-catalysed hydrolysis[a] of CG_6

Enzyme	K_m (mM)	k_0 (min^{-1})	k_0/K_m ($mM^{-1}.min^{-1}$)
Modified	1.8	910	510
Intact	0.56	2,300	4,100

[a] [NBS]/[CGTase] = 300 for 1 min, [CGTase] = 0.2μM, pH 7.4, 25.0°; CGTase-catalysed reaction, pH 5.5, 37.0° (see Experimental).

hydrolysis of CG_6. It has been suggested[6] that Trp97 is located at subsite 3 of the enzyme.

The effect of modification of CGTase by NBS on the hydrolysis of SS was very different from that for CG_6. Within 10 min, the remaining activity v_m/v was still ~90%. Moreover, in the initial part of the time course, v_m/v was ~110% (activation). Hence, the kinetic parameters, the Michaelis constant K_m and the molar activity k_0, for the action of the enzyme on CG_6 were evaluated for both the modified ([NBS]/[E] = 300, 1 min) and intact enzymes as summarised in Table I. The value of k_0/K_m for the action of the modified enzyme on CG_6 was ~10% of that of the intact enzyme, whereas the remaining activity v_m/v for SS was 88–95%.

Using various [NBS]/[CGTase] ratios, the residual activities (time courses) for SS and CG_6 were examined at pH 7.4 and 25°. The residual activity for CG_6 at [NBS]/[CGTase] = 300 for 1 min was confirmed to be almost the same as the value at [NBS]/[CGTase] = 100 for 5 min. At present, the difference in the effects of modification by NBS on the hydrolysis of SS and CG_6 by CGTase cannot be explained. The structure of the substrate carbohydrate may affect the interaction with the subsite, possibly subsite 3 (ref. 6), in which Trp^4 is located. The binding of substrates depends on the structure of the subsite[18,19], and hence modification of the subsite may change the binding mode(s).

TABLE II

Kinetic parameters of the CGTase-catalysed synthesis of CG_n from amylose (d.p. ~ 18)

Enzyme	K_m (%)	Relative V_{max} (%)[b]
Modified[a]	0.033 ±0.003	98
Intact	0.019 ±0.010	100

[a] [NBS]/[CGTase] = 300 for 1 min, [CGTase] = 0.2 μM, pH 7.4, 25.0°; CGTase-catalysed synthesis: [CGTase] = 0.13–0.27μM, pH 5.3, 25° (see Experimental). [b] Product is a mixture of CG_6–CG_8.

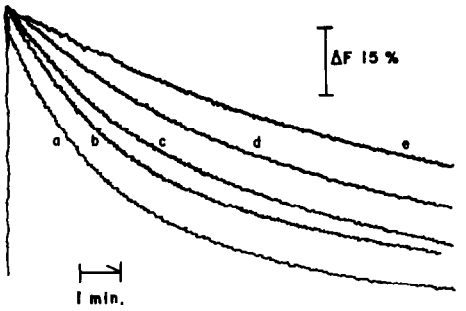

Fig. 4. Time courses of the modification of CGTase with NBS in the presence of glucose (G) determined by CGTase fluorescence spectrophotometry: [NBS]/[CGTase] = 100, [CGTase] = 0.2μM, pH 6.8, 19.0°; [G] (a) 0, (b) 60, (c) 120, (d) 180, and (e) 240mM. The fluorescence intensity is assigned an arbitrary value.

The production of cyclomalto-oligosaccharides (CG_n) from amylose (d.p. ~ 17), catalysed by the modified and intact CGTase, was examined using fluorescence spectrophotometry with TNS as a probe. The enzyme catalyses mainly the production of cyclomaltoheptaose (CG_7) but some CG_6 and cyclomaltooctaose CG_8 are also produced. The increase in fluorescence intensity at 468 nm was observed with production of CG_n because of the formation of inclusion complexes. CGTase also converts amylose into malto-oligosaccharides (G_n), but, by using the fluorescence technique, the production of CG_n can be observed. Based on the time curves, the initial velocities and the kinetic parameters in Table II were evaluated; V_{max} is expressed as a relative value, because the product cannot be specified as CG_6, CG_7, or CG_8. The findings show that the modification by NBS has little effect on the synthesis of CG_n from amylose.

Modification of Trp[4] with NBS in the presence and absence of glucose and maltosaccharides. — The modification of Trp^4 in the active site of CGTase by NBS was carried out in the presence and absence of glucose (G) and maltosaccharides (G_2–G_4). Fig. 4 shows that the presence of G impedes the modification by NBS as found for lysozyme[20]. Similar effects were obtained when G_2, G_3, and G_4 were used. These findings indicate Trp^4 to be located at the active site. In fact, based on the X-ray crystallographic data[6] for the crystalline CGTase–maltose complex, it has been suggested that one Trp residue is located at the maltose binding site[6]; it was inferred that this residue could be Trp97 located at substite 3 of the enzyme.

Thus, it is concluded that Trp^4 is located at the substrate binding site of CGTase and is intimately related to the hydrolysis of CG_6. It is possible that Trp^4 is Trp97 at subsite 3.

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REFERENCES

1 D. French, M. L. Levine, E. Norberg, P. Nordin, J. H. Pazur, and G. M. Wild, *J. Am. Chem. Soc.*, 76 (1954) 2387–2390; D. French, *Adv. Carbohydr. Chem.*, 12 (1957) 189–260.
2 S. Kobayashi, K. Kainuma, and D. French, *J. Jpn. Soc. Starch. Sci.*, 30 (1983) 62–68.
3 E. G. Hehre, K. Mizokami, and S. Kitahata, *J. Jpn. Soc. Starch Sci.*, 30 (1983) 76–82.
4 S. Kitahata, *Handbook of Amylases and Related Enzymes*, Pergamon, Tokyo, 1988, pp. 154–163.
5 S. Sakai, M. Kubota, K. Yamamoto, T. Nakae, K. Toriguoye, O. Ando, and T. Suguimoto, *J. Jpn. Soc. Starch Sci.* 34 (1987) 140–147.
6 M. Kubota, Y. Matsuura, S. Sakai, and Y. Katsube, *Nippon Nogei Kagaku Kaishi*, 64 (1990) 413.
7 M. Ohnishi, *Kagaku To Seibutsu*, 21 (1983) 502–512.
8 M. Ohnishi, M. Taniguchi, and K. Hiromi, *Biochim. Biophys. Acta*, 744 (1983) 64–70.
9 M. Ohnishi, *J. Jpn. Soc. Starch Sci.*, 34 (1987) 58–65.
10 M. Ohnishi, T. Kawaguishi, and K. Hiromi, *Arch. Biochem. Biophys.*, 272 (1989) 46–51.
11 M. Ohnishi, Y. Nakamura, M. Murata Nakai, and K. Hiromi, *Carbohydr. Res.*, 97 (1990) 237–244.
12 M. Ohnishi and K. Hiromi, *Anal. Sci. (Tokyo)*, 1 (1985) 89–90.
13 S. Kitahata and S. Okada, *J. Jpn. Soc. Starch Sci.*, 29 (1982) 7–12.
14 M. Kubota, B. Mikami, Y. Tsujisaka, and Y. Morita, *J. Biochem. (Tokyo)*, 104 (1988) 12–13.
15 M. Ohnishi, T. Kawaguishi, T. Abe, and K. Hiromi, *J. Biochem. (Tokyo)*, 87 (1980) 173–179.
16 T. Kawaguishi, M. Ohnishi, and K. Hiromi, *IARC Reports (Kyoto)*, 2 (1976) 5–10.
17 M. Ohnishi and K. Hiromi, *J. Biochem. (Tokyo)*, 79 (1976) 11–16.
18 M. Ohnishi, in M. Nakamura, M. Ohnishi, Y. Sakano, and H. Taniguchi (Eds.), *Amylase: Approach to Biotechnology*, Japan Scientific Societies Press, Tokyo, 1989, pp. 21–54.
19 M. Ohnishi, *Introduction to Enzyme Science*, Keigaku Shuppan, Tokyo, 1988, pp. 149–160.
20 K. Hiromi, T. Kawaguishi, and M. Ohnishi, *J. Biochem. (Tokyo)*, 81 (1977) 1583–1586.