Carbohydrate Research, 206 (1990) 257–267
Elsevier Science Publishers B.V., Amsterdam

Studies of the mechanism of the cyclisation reaction catalysed by the wildtype and a truncated α-cyclodextrin glycosyltransferase from *Klebsiella pneumoniae* strain M 5 al, and the β-cyclodextrin glycosyltransferase from *Bacillus circulans* strain 8

Hans Bender
Institut für Organische Chemie und Biochemie der Universität Freiburg i.Br., Albertstr. 21, D-7800 Freiburg i. Br. (F.R.G.)

(Received December 4th, 1989; accepted for publication, February 13th, 1990)

ABSTRACT

The actions of the wildtype and a truncated α-cyclodextrin glycosyltransferase from *Klebsiella pneumoniae* strain M 5 al on malto-oligosaccharides showed no significant differences, and there was marked dependence of the kinetic parameters on the chain lengths of the substrate. The action of the β-cyclodextrin glycosyltransferase from *Bacillus circulans* was less dependent on the chain length of the substrate, but V_{max} of the initial cyclisation with the longer malto-oligosaccharides was only 28% of that determined for the enzyme of *K. pneumoniae*. The rate parameters suggested that the active site of each enzyme spans nine glucosyl residues, and that the catalytic sites are situated between subsites three and four for the *K. pneumoniae* enzymes and between subsites two and three for the *B. circulans* enzyme. The molecular binding affinities and the affinities of the 9th subsite were calculated from the rate parameters. The primary and tertiary structures of alpha-amylases and cyclodextrin glycosyltransferases are compared in the context of the reaction mechanism of the latter enzymes.

INTRODUCTION

Because of the technological importance of cyclodextrins (CDs, cyclomaltooligosaccharides) as clathrate-forming compounds, the cyclodextrin glycosyltransferases {(1→4)-α-D-glucan[(1→4)-α-D-glucopyranosyl]transferase (cyclising), EC 2.4.1.19; CGTase} are of special interest. Some 14 bacterial species[1,2] are known to produce α, β, and γ-CGTase. At least 10 CGTase genes have been cloned[3–10], and the sequences of the amino acids of the encoded polypeptides have been elucidated at the DNA level. The CGTases of the bacilli are homologous, with 50–70% of the amino acids identical, whereas the α-CGTase of *Klebsiella pneumoniae* strain M 5 al (Kp-α-CGTase) showed only ~30% homology with the enzymes of bacilli. Similarities in sequence between alpha-amylases {(1→4)-α-D-glucan[glucano]hydrolase, EC 3.2.1.1} and CGTases were found for at least four conserved regions, including the catalytically active amino acid residues of the alpha-amylases. The three-dimensional structure of the β-CGTase from *Bacillus circulans* strain 8 (Bc-β-CGTase) has been elucidated[11] at a resolution of 3.4 Å. The NH_2-terminal domains resemble closely the two known structures of alpha-amylases[12,13].

0008-6215/90/$03.50

X-Ray diffraction analyses of the alpha-amylases[12,13] and lysozyme[14] revealed that each active site forms a depression at the surface of the protein molecule, which is capable of accommodating 5–10 residues of the substrate, and may be regarded as consisting of the corresponding number of subsites. Each subsite interacts specifically with a substrate residue through hydrogen bonds, van der Waals forces, and hydrophobic interactions[15,16]. The catalytic site is situated between two of the subsites and the location depends on the type of enzyme. The interaction of the substrate with the subsites and the location of the catalytic site could be established by kinetic studies[17–21].

Some kinetic studies of the Kp-α-CGTase with malto-oligosaccharides up to G_8 and maltodextrin (average d.p. 19) have been performed[22]. Since both the affinity for the substrate and the maximum velocity of cyclisation were not higher for the maltodextrin, the active site was supposed to involve eight subsites, with the catalytic site situated between subsites two and three. Comparative kinetic studies are now reported for the cyclisation reactions mediated by the wildtype and a truncated Kp-α-CGTase and Bc-β-CGTase with malto-oligosaccharides (G_n) up to G_{13} as substrates.

EXPERIMENTAL

Enzymes. — Crude wildtype Kp-α-CGTase and the clone *Escherichia coli* DS 410 were kindly provided by Dr. G. Schmid (Consortium für Elektrochemische Industrie, Munich). *E. coli* DS 410 contains and expresses the plasmid-encoded Kp-α-CGTase gene that was shortened by a DNA stretch coding for the 90 COOH-terminal amino acid residues[23]. The clone was cultivated (10-L scale) in an ampicillin-LB-medium (0.8% of lactose) for 24 h at 35° and the CGTase was isolated from the homogenised cells (Laboratory Homogenizer, Manton–Gaulin, The Netherlands).

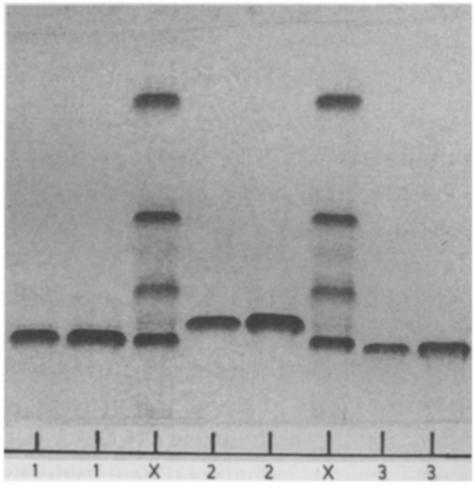


Fig. 1. SDS-PAGE of the wildtype (1) and truncated (2) Kp-α-CGTases and the Bc-β-CGTase (3); 5 and 10 μg of protein were subjected to electrophoresis on 12% gels at 80 mV and 20 mA for 2.5 h. The gels were stained with Coomassie Brilliant Blue: X, bovine serum albumin (M_r 68 000), albumin from chicken egg (43 000), carboanhydrase B (28 000), and myoglobin from horse (17 000).

The Bc-β-CGTase gene was isolated from a genomic library and mapped on a 3.4 kb fragment, that was cloned into the plasmid pTZ 18R, transformed, and expressed in *E. coli* JM 103 to yield[24] the clone pBC 22. The recombinant β-CGTase was isolated from homogenised cells of the clone pBC 22 that were grown (10-L scale) in an ampicillin-LB-medium for 26 h at 35°.

The crude homogenates were centrifuged (20 000 r.p.m., 1 h) and the CGTases were isolated from the supernatant solutions by affinity chromatography onto βCD covalently bound to Sepharose 6B (Pharmacia)[11].

Samples (5–8 μg) of protein were analysed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE). One protein band was revealed for each enzyme preparation (Fig. 1). The M_r of the CGTases, determined from the plots M_r (marker proteins) *vs.* $-\log R_F$, were 68 500 ± 600 (wildtype Kp-α-CGTase), 59 800 ± 400 (truncated Kp-α-CGTase), and 74 000 ± 500 (Bc-β-CGTase). The small errors reflect slight fluctuation of the R_F values.

The sequence of the amino acids of the wildtype Kp-α-CGTase has been elucidated on a DNA base[3]. The polypeptide contains 625 residues, corresponding to M_r 69 000. The truncated Kp-α-CGTase contains 535 residues, corresponding to M_r ~60 000. DNA sequencing yielded 684 residues for the recombinant Bc-β-CGTase, corresponding to M_r 74 416. Accordingly, the M_r values revealed by SDS-PAGE agreed well with those derived from DNA sequencing of the CGTase genes.

The recombinant Bc-β-CGTase showed the same specific activity, and the same pattern on SDS-PAGE and isoelectric focusing, as the extracellular enzyme of the parent bacillus. In order to ensure the predicted COOH-terminus (-Ser-Ile-Asn-Phe-Thr-COOH) of the truncated Kp-α-CGTase, the protein was digested with carboxypeptidase Y (peptidyl-L-amino acid hydrolase, EC 3.4.16.1)[25]. Analysis of the amino acids released yielded (mol%) Thr, Asn, Ser [one peak] (36.0), Phe (17.3), Ile (11.2), Gly (9.2), Ala (9.1), Leu (5.9), Val (5.9), and Tyr (5.4). As the sequence of the amino acids of the CGTase excluded any other interpretation for a polypeptide with M_r ~60 000, Thr-534 should be the COOH-terminal residue of the truncated enzyme, and some of the amino acids detected might have resulted from the action of endopeptidase impurities in the carboxypeptidase.

Substrates. — G_4 and G_5 were commercial materials (Boehringer), G_6–G_8 were prepared from cyclomaltohexaose (αCD), cyclomaltoheptaose (βCD), and cyclomaltooctaose (γCD), respectively, by hydrolysis (98°) with M maleic acid[26], and G_9–G_{13} were prepared[27] from digests of αCD and maltose with the wildtype Kp-α-CGTase.

Dry samples (1 g) of carbohydrate were acetylated[28] in 1:1 pyridine–acetic anhydride (80 mL) for 90 min at 100°. A solution of each acetylated saccharide (150 mg) in acetonitrile (2 mL) was subjected to preparative reverse-phase h.p.l.c. on a column (3.2 x 25 cm) of C_{18}-Nucleosil (Knauer), using acetonitrile–water (63:37) at 9 mL/min (800 p.s.i., 60°) with refractometric detection. Each saccharide fraction was re-chromatographed, using the same conditions, and then *O*-deacetylated with 0.1M KOH in 3:1 methanol–toluene at room temperature for 1 h. Each mixture was neutralised with acetic acid, and the precipitate was collected by centrifugation, washed twice with

aqueous 96% ethanol, and vacuum-dried. The resulting malto-oligosaccharides were >96% pure (h.p.l.c.).

Analytical methods. — Total carbohydrate was determined with the anthrone reagent[29], reducing end groups with the Nelson reagent[30], and protein by the biuret method[31].

The initial rates of cyclisation were followed by an optical assay[32,33]. 0.07–5.0mM Solutions (3 mL) of substrate in 10mM Tris–HCl buffer (pH 6.8) that contained 3mM $CaCl_2$ and 46μM Methyl Orange were incubated with 1.0–10.0 μg of each CGTase at 30°. The decrease in absorbance at 510 nm, caused by complexing of the dye with the CD produced, was calibrated with free and CD-complexed Methyl Orange. The enzymic activity is expressed as katals (mol of CD/s), and the molar catalytic activity ($V/[E_o]$) as $kat.mol^{-1}$ of enzyme, where V and $[E_o]$ are the maximum velocity of cyclisation and the molar concentration of enzyme, respectively. The Michaelis constants (K_m) and V were determined[34] from double reciprocal $1/v_o$ *vs.* 1/[S] plots, where v_o and [S] are the initial rates of cyclisation and the concentration of substrate, respectively.

Subsite analysis. — The molecular binding affinities were calculated[18] according to equation *1*,

$$-\Delta G_p = RT(\ln K_{n,p}) + \Delta G_{mix}, \quad (1)$$

where $K_{n,p}$ ($= 1/K_m$) is the association constant of n-mer substrate for productive complex formation ($EG_{n,p}$), R and T are the gas constant and the absolute temperature, respectively, and $-\Delta G_{mix}$ ($= 10.04\ kJ.mol^{-1}$) is the contribution of the mixing entropy in water[35].

$K_{n,p}$ was calculated according to equation *2*,

$$K_{n,p} = |(V/[E_o])/K_m|/k_{int}, \quad (2)$$

where k_{int} is the true rate constant, assumed to be constant irrespective of the length of substrate, and the observed n-dependence of $V/[E_o]$ arises from the multiplicity of the binding modes of substrate[19].

Individual subsite affinities (A) were calculated[18,19,22] according to equation *3*,

$$-A_{(n+1)} = RT[\ln K_{(n+1),p} - \ln K_{n,p}], \quad (3)$$

where $K_{(n+1),p}$ and $-A_{(n+1)}$ are the association constant of the $n+1$-mer substrate and the affinity of the $n+1$th subsite, respectively.

Short-term digests. — These digests involved incubation for 1 min of 1.5mM solutions (1 mL) of G_8–G_{13} in 10mM sodium phosphate buffer severally with 2 μg/mL of wildtype and mutant Kp-α-CGTase and 3 μg/mL of Bc-β-CGTase at pH 6.8 and 30°. Each enzyme was inactivated by boiling (5 min), and each digest was concentrated to 1/10 volume. An aliquot (20 μL) of each concentrate was subjected to analytical h.p.l.c. on a column (3.9 x 300 mm) of μ-Bondapak-NH_2 (Waters), using acetonitrile–water (65:35) at 1.5 mL/min (1200 p.s.i., 30°) and refractometric detection. The carbohydrate content of the peaks was calculated by planimetry and calibrated using pure malto-oligosaccharides[36].

RESULTS AND DISCUSSION

The values of K_m and $V/[E_o]$ obtained for cyclisation by the wildtype and the truncated Kp-α-CGTase and the Bc-β-CGTase with the malto-oligosaccharides employed are summarised in Table I. Plots of log $1/K_m$, log $(V/[E_o])$, and log $|(V/[E_o]/K_m|$ indicated the dependence of the rate parameters on the chain length of the substrate (Fig. 2). The wildtype Kp-α-CGTase showed, on average, 1.2 times higher rates of

TABLE I

Rate parameters of cyclisation by the wildtype (Kp_w) and the truncated (Kp_{tr}) α-CGTase of *K. pneumoniae* and the β-CGTase (Bc) of *B. circulans* at pH 6.8 and 30°[a,b] with malto-oligosaccharides

Substrate (G_n)	K_m (M x 10^{-4}) *Bc*	*Kp_w*	*Kp_{tr}*	$V/[E_o]$ (*kat. mol*$^{-1}$) *Bc*	*Kp_w*	*Kp_{tr}*
4	8 (±0.3)	43 (±0.6)	45 (±0.6)	42 (±1.8)	22 (±1.5)	18 (±0.8)
5	6 (±0.2)	25 (±0.4)	27 (±0.8)	61 (±1.5)	46 (±0.7)	38 (±1.2)
6	4 (±0.4)	16 (±0.5)	17 (±0.3)	72 (±1.8)	68 (±0.9)	55 (±1.2)
7	3 (±0.2)	8 (±0.3)	9 (±0.4)	74 (±0.9)	92 (±1.6)	76 (±2.1)
8	2 (±0.3)	2.5 (±0.2)	2.8 (±0.18)	77 (±1.3)	239 (±1.2)	200 (±1.7)
9	1.1 (±0.06)	1.4 (±0.1)	1.6 (±0.08)	80 (±2.2)	287 (±1.9)	240 (±1.6)
10	1.1 (±0.1)	1.4 (±0.12)	1.6 (±0.1)	80 (±1.6)	287 (±2.1)	240 (±2.1)
11	1.1 (±0.08)	1.4 (±0.06)	1.6 (±0.08)	80 (±2.1)	287 (±1.6)	240 (±2.4)
12	1.1 (±0.09)	1.4 (±0.1)	1.6 (±0.1)	80 (±1.9)	287 (±1.2)	240 (±1.8)
13	1.1 (±0.06)	1.4 (±0.08)	1.6 (±0.09)	80 (±1.6)	287 (±2.0)	240 (±1.5)

[a] Initial cyclisation was followed in 10mM Tris–HCl buffer (3mM $CaCl_2$), using an optical assay (see Experimental). The rate parameters were determined from double reciprocal $1/v_o$ *vs.* 1/[S] plots. [b] The standard deviations were listed in brackets[37].

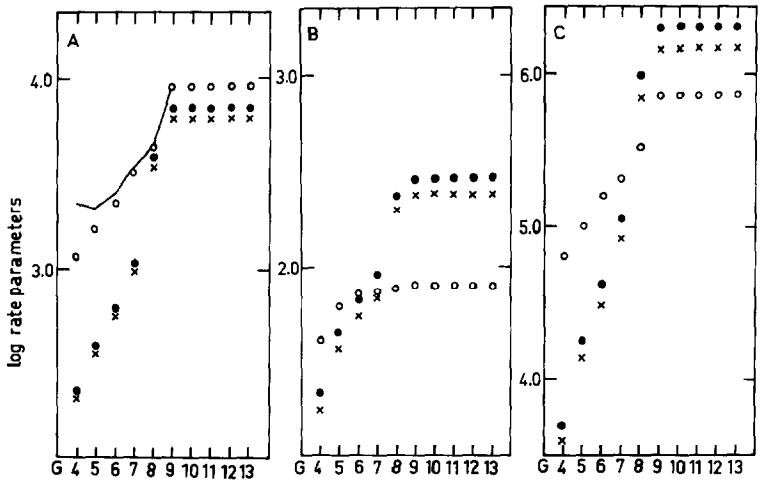


Fig. 2. Dependence of the rate parameters experimentally obtained for the initital cyclisation on the chain length of the substrate: • and x, wildtype and truncated Kp-α-CGTases, respectively; o, Bc-β-CGTase; A, log $1/K_m$; B, log $(V/[E_o])$; C, log$|(V/[E_o])/K_m|$. Line in A, values calculated for the Bc-β-CGTase according to equation *3*.

cyclisation and 1.04–1.14 times higher affinities for the substrates than the truncated enzyme, *i.e.*, the differences are negligible. Each CGTase exhibited marked dependence of V_{max} and K_m on the chain length of the substrate. The maximum velocity of cyclisation occurred with G_9, but the plots showed a marked break between G_7 and G_8. The rate parameters determined for the Bc-β-CGTase were less dependent on the chain length of the substrate and there was a pronounced affinity even for the shorter substrates. However, the V_{max} of cyclisation with G_9 was only 28% of that for the Kp-enzymes. There was a marked break between G_8 and G_9 in the plots of log $1/K_m$ and log $|(V/[E_o])/K_m|$.

Further information was obtained by h.p.l.c. of short-term digests (Table II). The typical αCD, βCD, and γCD ratios for digests of potato starch with Bc-β-CGTase were 1:7.3:1.8. However, digestion of G_8 yielded αCD as the main cyclic product, indicating

TABLE II

Cyclic and linear products of short-term digests of G_8–G_{13} with the Bc-β-CGTase[a]

Substrate	*CDs (μmol/L)*			*Molar ratio*	*Linear products (μmol/L)*						*Molar ratio*
	α	β	γ		G_2	G_3	G_4	G_5	G_6	G_7	CD/G_2–G_7
Potato starch[b]	39	281	69	1:7.2:1.8	–	–	–	–	–	–	–
G_8	154	125	78	1:0.8:0.5	150	50	42	38	30	8	1.12
G_9	77	230	78	1:3.0:1.0	150	51	40	30	30	15	1.22
G_{10}	59	264	60	1:4.5:1.0	145	60	45	36	34	15	1.14
G_{11}	51	268	64	1:5.3:1.3	138	58	50	45	42	12	1.11
G_{12}	50	273	82	1:5.5:1.6	131	43	45	50	60	20	1.16
G_{13}	50	283	91	1:5.7:1.8	140	40	50	50	56	20	1.19

[a] 1.5mM Substrate in 10mM sodium phosphate buffer (1 mL) was incubated with 3 μg/mL of CGTase at pH 6.8 for 1 min at 30°; ~34% of the substrate reacted. [b] The concentration of potato starch was 10 g/L.

TABLE III

Cyclic and linear products of short-term digests of G_8–G_{13} with the truncated Kp-α-CGTase[a]

Substrate	*CDs (μmol/L)*			*Molar ratio*	*Linear products (μmol/L)*						*Molar ratio*
	α	β	γ		G_2	G_3	G_4	G_5	G_6	G_7	CD/G_2–G_7
Potato starch[b]	380	20	15	1:0.05:0.04	–	–	–	–	–	–	–
G_8	380	12	8	1:0.03:0.02	*290*[c]	50	8	42	–	–	1.02
G_9	390	25	18	1:0.06:0.05	110	*190*	6	10	9	20	1.26
G_{10}	392	28	22	1:0.07:0.06	80	75	*180*	10	8	6	1.23
G_{11}	390	30	40	1:0.08:0.1	90	80	30	*185*	10	7	1.24
G_{12}	390	30	40	1:0.08:0.1	100	73	10	12	*175*	8	1.22
G_{13}	395	32	42	1:0.08:0.1	110	60	8	10	15	*180*	1.22

[a] 1.5mM Substrate in 10mM sodium phosphate buffer (1 mL) was incubated with 2 μg/mL of CGTase at pH 6.8 for 1 min at 30°; ~37% of the substrate reacted. [b] The concentration of potato starch was 10 g/L. [c] The numbers in italics are the concentrations of the products of the reaction $G_n \rightarrow G_{(n-6)}$ + αCD.

the influence of the chain length of the substrate on the size of the macrocycle produced. The amounts of βCD relative to the other CDs increased with increase in chain length. The main linear product was maltose and only traces of glucose were detected. The main cyclic product of Kp-α-CGTase was αCD (Table III; the wildtype enzyme showed similar behaviour; data not reported). Depending on the chain length of the substrate, marked amounts of the linear products of the reaction $G_n \rightarrow G_{(n-6)} + \alpha CD$ were found (Table III, italicised numbers). For example, G_8 yielded mainly maltose and αCD. Since equimolar concentrations should be formed, the differences observed might be caused by disproportionation, whereby the longer chains formed were not detected by h.p.l.c. Glucose was a minor product of the short-term digests.

The increase in $V/[E_o]$ and $1/K_m$ up to G_9 observed for the wildtype and truncated Kp-α-CGTase pointed to a length of the active site of 9 glucose residues. As judged from the main initial products revealed by h.p.l.c., the part of the active site to the right of the catalytic site in Schemes 1 and 2 should be 6 subsites in length, *i.e.*, the catalytic site is likely to be situated between subsites 3 and 4, numbered from the reducing end (∅) of the substrate. Since only small amounts of G_3 and G_5 were formed from G_8 initially (see Table III), G_8 (which is the smallest substrate that is cyclised directly[22]) should bind to subsites 2–9 preferentially (Scheme 1). The larger amounts of maltose that were detected in each digest do not conflict with a catalytic site situated between subsites 3 and 4. Maltose is a poor substrate for the CGTases[36] and will accumulate if the binding mode of substrate is that supposed for G_8.

```
  ∅–0–0–0–0–0–0–0–0   a, j = 2 (EG8,p); b, j = 1 (EG8,q)
∅–0–0–0–0–0–0–0–0     a, j = 3 (EG8,q); b, j = 2 (EG8,p)
1  2↑3↑4  5  6  7  8  9 (subsites)
     b  a
```

Scheme 1. Binding modes (j) of G_8 at the active site of CGTases: a and b are the putative positions of the catalytic sites of the Kp- and Bc-CGTases, respectively, and ∅ is the reducing residue of G_8; p and q are the enzyme–substrate complexes productive and nonproductive for cyclisation.

The rate parameters determined for Bc-β-CGTase also indicated the length of the active site to be 9 glucose residues. The marked amount of αCD and maltose produced from G_8 indicated that direct cyclisation occurred. Because of the high rate of disproportionation relative to that of cyclisation, the location of the catalytic site could not be derived from the h.p.l.c. data. Since maltose was the main linear product, the catalytic site might be situated between subsites 2 and 3.

Accordingly, the differences between α- and β-CGTases could arise from the location of the catalytic sites. However, the active sites are not specific for one ring size of the macrocyclic product, as shown by the slow formation of CDs other than those produced initially, depending on the type of enzyme.

Assuming that the equilibrium

$$E + G_n \overset{K_{n,p}}{\rightleftharpoons} EG_{n,p} \; (n = 9)$$

is established rapidly, then the molecular binding affinities can be obtained from

TABLE IV

Kinetic and thermodynamic parameters of enzyme–substrate complexes productive for cyclisation from malto-oligosaccharides

Substrate	k_o ($x\ 10^{-3}$)[a]			k_o/K_m			$K_{n,p}$ ($x\ 10^3$)[b]			$-A_i$ ($kJ.mol^{-1}$)[c]		
	Bc[d]	Kp_w[e]	Kp_{tr}[f]	*Bc*	Kp_w	Kp_{tr}	*Bc*	Kp_w	Kp_{tr}	*Bc*	Kp_w	Kp_{tr}
G_6	13.9	14.7	–	31.6	9.2	–	2.53	2.63	–	–	–	–
G_7	13.4	10.9	–	44.7	13.6	–	3.57	3.89	–	0.90	–	–
G_8	13.0	4.2	5.0	56.5	16.8	17.9	4.52	4.80	4.25	0.60	–	–
G_9	12.5	3.5	4.2	113.6	25.0	26.3	9.09	7.14	6.25	1.76	1.0	0.97

[a] $1/(V/[E_o])$, $s.mol^{-1}$. [b] Calculated according to equation *2* where $k_{int} = k_o(G_9)$. [c] Calculated according to equation *3*, A_i affinity of the i^{th} subsite. [d] Bc-β-CGTase. [e,f] Wildtype and truncated Kp-α-CGTases.

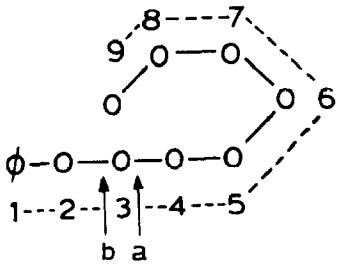
equation *1*. The values of $-\Delta G_p$ were −32.9 (Bc-β-CGTase), −32.3 (wildtype Kp-α-CGTase), and −32.0 $kJ.mol^{-1}$ (truncated Kp-α-CGTase). These values are comparable to that obtained for an alkalophilic *Bacillus* sp.[38].

If G_8 is a substrate for direct cyclisation, the affinities of the 9th subsites can be evaluated. $1/K_m$ (G_8) will not give the value of $K_{8,p}$, since EG_n-complexes might be formed that do not result in cyclisation ($EG_{n,q}$) in at least one binding mode j (Scheme 1). H.p.l.c. of the short-term digests indicated that the binding mode j = 2 was predominant. Thus, the true value of $K_{8,p}$ can be calculated according to equation *2*, where k_o (G_9) = k_{int}. The affinities of the 9th subsites obtained by equation *3* are summarised in Table IV.

Only the longer (enzyme-bound?) products of disproportionation of substrates $< G_8$ could serve for cyclisation. The values of $K_{7,p}$ and $K_{6,p}$ obtained from equation *3* for Bc-β-CGTase were near those determined experimentally (Fig. 2A). Thus, the formation of disproportionation products, suitable for cyclisation in a productive binding mode, was not rate-limiting. The approximate affinities of subsites 7 and 8 of Bc-β-CGTase are listed in Table IV. If the overall binding affinity is −32.9 $kJ.mol^{-1}$, the highest affinities must be among subsites 1–6.

The highly conserved regions characteristic of alpha-amylases, including the catalytically active residues and the residues responsible for binding substrate and Ca^{2+}, are found also for CGTases in corresponding positions[3,6,39]. This finding implies similar first steps in the actions of these enzymes, namely, protonation of a glucosidic bond of the substrate and attack at C-1 by the carboxyl group of Asp and/or Glu of the enzyme. For alpha-amylases, the enzyme–substrate complex glycosylates water, *i.e.*, hydrolysis occurs[17,40], whereas, for CGTases, a site in the substrate is glycosylated. The cyclisation observed with the shorter malto-oligosaccharides indicates that a helical configuration of the substrate is not a prerequisite[41] since, at least up to G_{10}, helical configurations scarcely exist in aqueous solution[42]. Rather, the active site of the CGTases is curved in a way that allows cyclisation to occur (Scheme 2).

From X-ray diffraction data[11], the folding of the chain of Bc-β-CGTase could be



Scheme 2. Putative curvature of the active site of the CGTases suitable for cyclisation. The symbols are the same as in Scheme 1.

considered as similar to that in alpha-amylase but with two additional domains, *i.e.*, the polypeptide chain of the CGTase has been subdivided into 5 domains*. The active site, which has the form of a broad depression, is situated at the COOH terminals of the β-strands of a $(\beta\alpha)_8$-barrel built up by domains A and B (A, residues 1-NH_2–140 and 195–403 + 8 residues; B, residues 140–195 + 2 residues). The domains C (residues 403–492 + 3 residues) and D (residues 492–570 + 2 residues) are distant from the active site and cannot contribute efficiently to catalysis. Indeed, less homology is noticed in these regions. Domain E (residues 570–664 + 5 residues), which shows rather high homology between all CGTases and also some homology with glucoamylases {(1→4)-α-D-glucan[gluco]hydrolase, EC 3.2.1.3} (ref. 43) is situated near the depression. The COOH-terminal region may be involved in catalysis[6] and in binding the substrate[43]. Thus, a CGTase of the alkalophilic *Bacillus* sp. strain 1011 deleted by 10–13 COOH-terminal residues showed altered CD/linear product and αCD/βCD ratios, and a reduced stability in the alkaline pH range[44]. Although not absolutely comparable, the kinetic parameters obtained for the truncated Kp-α-CGTase conflict with these results. At least for this enzyme, the COOH-terminal region is not involved significantly in catalysis.

The Kp-α-CGTase had markedly higher rates of cyclisation with the longer-chain substrates than had Bc-β-CGTase. The alignment of the primary structures revealed that Kp-α-CGTase differed from the bacilli CGTases in two respects, namely, the region of the polypeptide (83 residues) corresponding to domain D of Bc-β-CGTase is deleted, and a stretch of 21 residues is inserted near region 4, common to both CGTases and alpha-amylases.

Essentially, the differences between CGTases and alpha-amylases are restricted to domains A and B. However, refinement of the three-dimensional structure of the CGTases and their enzyme–substrate complexes is necessary for elucidation of the exact mechanism of the transfer reactions.

* 664 Amino acid residues can be fitted into the electron density map. DNA sequencing of the CGTase gene yielded 684 residues. The refinement of the three-dimensional structure (unpublished results) showed that the remaining 20 residues were scattered over the polypeptide chain.

ACKNOWLEDGMENTS

Dr. G. Schmid is thanked for provision of the clone *E. coli* DS 410, and Dr. E. Schiltz for his help in the determination of the COOH terminus of the truncated CGTase.

REFERENCES

1 H. Bender, in A. Mizrahi (Ed.), *Advances in Biotechnological Processes*, Vol. 6, Liss, New York, 1986, pp. 31–71.
2 J. Szejtli, *Cyclodextrin Technology*, Kluwer, Dordrecht, 1988.
3 F. Binder, O. Huber, and A. Böck, *Gene*, 47 (1986) 269–277.
4 T. Sugimoto, M. Kubota, and S. Sakai, Fr. Demande, FR 2,574,081 (1986); *Chem. Abstr.*, 107 (1987) 234835f.
5 T. Takano, M. Fukuda, M. Monma, S. Kobayashi, K. Kainuma, and K. Yamane, *J. Bacteriol.*, 166 (1986) 1118–1122.
6 K. Kimura, S. Kataoka, Y. Ishii, T. Takano, and K. Yamane, *J. Bacteriol.*, 169 (1987) 4399–4402.
7 K. Kimura, T. Takano, and K. Yamane, *Appl. Microbiol.*, 26 (1987) 149–153.
8 T. Kaneko, T. Hamamoto, and K. Horikoshi, *J. Gen. Microbiol.*, 134 (1988) 97–105.
9 K. Horikoshi, in O. Huber and J. Szejtli (Eds.), *Proc. Int. Symp. Cyclodextrins*, Vol. 4, Kluwer, Dordrecht, 1988, pp. 7–19.
10 G. Schmid, A. Engelbrecht, and D. Schmid, ref. 9, pp. 71–77.
11 B. E. Hofmann, H. Bender, and G. E. Schulz, *J. Mol. Biol.*, 209 (1989) 793–800.
12 Y. Matsuura, M. Kusunoki, W. Harada, and M. Kakudo, *J. Biochem.*, 95 (1984) 697–702.
13 G. Buisson, R. Haser, E. Duée, and F. Payan, *EMBO J.*, 6 (1987) 3909–3916.
14 D. C. Philips, *Proc. Natl. Acad. Sci. U.S.A.*, 57 (1967) 484–495.
15 D. M. Chipman and N. Sharon, *Science*, 165 (1969) 454–465.
16 K. Bock and H. Pederson, *FEMS Symp.*, 31 (1986) 173–182.
17 D. French, *MTP Int. Rev. Sci. Org. Chem., Ser. 1*, 5 (1975) 267–335.
18 Y. Nitta, M. Mizushima, K. Hiromi, and S. Ono, *J. Biochem. (Tokyo)*, 69 (1971) 567–576.
19 K. Hiromi, Y. Nitta, C. Numata, and S. Ono, *Biochim. Biophys. Acta*, 302 (1973) 362–375.
20 J. F. Robyt and D. French, *J. Biol. Chem.*, 245 (1970) 3917–3927.
21 J. D. Allan and J. A. Thoma, *Biochemistry*, 17 (1978) 2338–2344.
22 H. Bender, ref. 9, pp. 19–26.
23 G. Schmid, *Trends Biotechnol.*, 7 (1989) 244–248.
24 L. Nitschke, K. Heeger, H. Bender, and G. E. Schulz, *Appl. Microbiol.*, (1990), in press.
25 H. M. Lee and J. F. Riordan, *Biochem. Biophys. Res. Commun.*, 85 (1978) 1135–1142.
26 A. Yazaki, Jpn. Pat. 86,191,690 (1986); *Chem. Abstr.*, 106 (1987) 35038c.
27 H. Bender, *Carbohydr. Res.*, 65 (1978) 85–97.
28 G. B. Wells and R. L. Lester, *Anal. Biochem.*, 97 (1979) 184–190.
29 F. J. Viles and L. Silverman, *Anal. Chem.*, 21 (1949) 950–953.
30 N. Nelson, *J. Biol. Chem.*, 153 (1944) 375–380.
31 G. Beisenherz, H. J. Boltze, T. Bücher, T. Czok, K. H. Garbade, E. Meyer-Arendt, and G. Pfleiderer, *Z. Naturforsch., Teil B*, 8 (1953) 555–577.
32 J. P. Landert, E. Flaschel, and A. Renken, in J. Szejtli (Ed.), *Proc. Int. Symp. Cyclodextrins*, Vol. 1, Akadémiai Kiadó, Budapest, 1982, pp. 89–94.
33 M. J. Makela and T. K. Korpela, *J. Biochem. Biophys. Methods*, 15 (1988) 307–318.
34 H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, 56 (1934) 658–666.
35 W. Kauzmann, *Adv. Protein Chem.*, 14 (1959) 33–35.
36 H. Bender, *Carbohydr. Res.*, 117 (1983) 1–11.
37 W. L. Masterton and E. J. Slowinski, *Mathematical Preparations for General Chemistry*, Verlag Chemie, Weinheim, 1983.
38 M. Makela, P. Mattson, M. E. Schinina, and T. Korpela, *Biotechnol. Appl. Biochem.*, 10 (1988) 414–427.
39 E. A. MacGregor and B. Svensson, *Biochem. J.*, 259 (1989) 145–152.
40 B. Y. Tao, P. J. Reilly, and J. F. Robyt, *Biochim. Biophys. Acta*, 995 (1989) 214–220.

41 H. Bender, *Carbohydr. Res.*, 135 (1985) 291–302.
42 J. A. Thoma and D. French, *J. Phys. Chem.*, 65 (1961) 1825–1828.
43 B. Svensson, *FEBS Lett.*, 230 (1988) 72–76.
44 K. Kimura, S. Kataoka, A. Nakamara, T. Takano, S. Kobayashi, and K. Yamane, *Biochem. Biophys. Res. Commun.*, 161 (1989) 1273–1279.