10
The Functions of 4-α-glucanotransferases and their use for the Production of Cyclic Glucans

TAKESHI TAKAHA[1] AND STEVEN M. SMITH[2*]

[1]*Biochemical Research Laboratories, Ezaki Glico Co Ltd., Utajima, Nishiyodogawaku, Osaka 555, Japan, and* [2]*Institute of Cell and Molecular Biology, University of Edinburgh, Mayfield Road, Edinburgh, EH9 3JH, UK.*

Introduction

α-1,4-D-glucans occur widely in nature in the form of glycogen and starch. Bacteria, lower eukaryotes and animals accumulate glycogen, whereas starch is characteristic of higher plants. While these two glucans are chemically the same, they differ in structure, particularly with respect to the number and organisation of the α-1,6-linked branches which they contain, and they differ in their molecular masses. Starch is comprised of two components: amylose which contains very few α-1,6-linked branches, and amylopectin which contains such branches at regular intervals (Hizukuri, 1986, 1996). Glycogen is typically more highly branched than amylopectin (Gunja-Smith *et al.*, 1970; Manners, 1991; Sandhya Rani *et al.*, 1992). These polymers serve as carbon stores which accumulate when glucose is plentiful and are utilised by the organism when needed. Since starch and glycogen are chemically the same, their metabolism, even in diverse organisms, is remarkably similar. This means that information obtained from studies of α-1,4-glucan metabolism in one organism can be of great value in trying to understand such metabolism in another. It also means that genes encoding enzymes of α-1,4-glucan metabolism can be transferred between very different organisms to modify glucan metabolism *in vivo*, or can be used in starch or glycogen processing. From an applied viewpoint, starch is of greatest importance because about 30 million tonnes is produced annually from plants, for food and

Abbreviations: 4αGTase, 4-α-glucanotransferase or 1,4-α-D-glucan:1,4-α-D-glucan, 4-α-D-glucanotransferase; AP, amylopectin; CA, cycloamylose; CA6, CA7, CA17 *etc.*, cycloamylose or cyclodextrins with 6, 7, 17 *etc.* glucose residues; CCD, cyclic cluster dextrin; CD, cyclodextrin; CDase, cyclodextrinase; CGTase, cyclodextrin glucanotransferase; D-enzyme, disproportionating enzyme; DP, degree of polymerisation; G1P, glucose-1-phosphate; G, G2, G3 *etc.*, glucose, maltose, maltotriose, *etc.*; GDE, glycogen debranching enzyme; P_i, inorganic phosphate; R, reducing end of 1,4-α-D-glucan.

*To whom correspondence may be addressed.

Biotechnology and Genetic Engineering Reviews – Vol. 16, April 1999
0264–8725/99/16/257–280 $20.00 + $0.00 © Intercept Ltd, P.O. Box 716, Andover, Hampshire SP10 1YG, UK

industrial uses (Lillford and Morrison, 1997). There is therefore great interest in trying to improve or modify starch production in plants, and in using enzymes from diverse organisms for starch processing.

Of all the enzymes involved in α-1,4-glucan metabolism, one group whose functions are more varied and less well understood than others, are the 4-α-glucanotransferases (1,4-α-D-glucan:1,4-α-D-glucan, 4-α-D-glucanotransferase; here abbreviated to 4αGTase). These enzymes break an α-1,4-link and transfer the resulting glucan moiety to an acceptor molecule through creation of a new α-1,4-link. Studies of the functions of these enzymes are particularly important not only for the understanding of their roles *in vivo*, but also because it has been discovered that they are able to synthesise cyclic glucans *in vitro*, and therefore have great potential for use in starch processing. The purposes of this review are:

- to draw together the available information concerning the functions of these enzymes in diverse organisms
- to compare the structures, relatedness and action patterns of these enzymes
- to introduce a unifying nomenclature
- to consider how they may be employed in starch processing
- to explain the structures and potential applications of the cyclic glucans which they can produce

The action of 4αGTases

The action of 4αGTases can be summarised by the following equation:

$$(\alpha\text{-}1,4\text{-glucan})_m + (\alpha\text{-}1,4\text{-glucan})_n \leftrightarrow (\alpha\text{-}1,4\text{-glucan})_{m-x} + (\alpha\text{-}1,4\text{-glucan})_{n+x}$$

This action is the inter-molecular glucan transfer reaction, it is readily reversible, and is often called the 'disproportionating reaction'. Such enzymes can potentially also catalyse an intra-molecular glucan transfer reaction, within a single linear glucan molecule, to create a cyclic glucan product, as follows:

$$(\alpha\text{-}1,4\text{-glucan})_m \leftrightarrow \text{cyclic}(\alpha\text{-}1,4\text{-glucan})_x + (\alpha\text{-}1,4\text{-glucan})_{m-x}$$

This reaction is also reversible, and is often referred to as the 'coupling reaction'

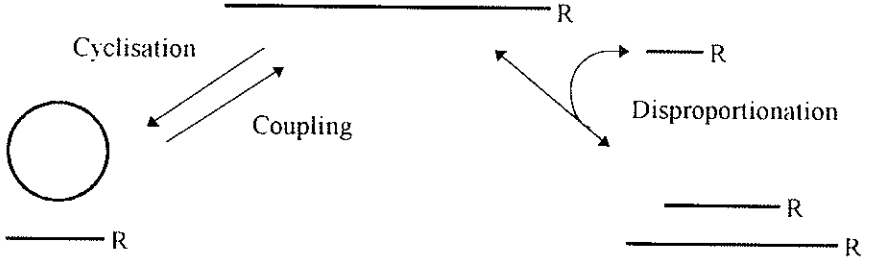


Figure 1. Reactions catalysed by 1,4-α-D-glucanotransferases (4αGTase) on 1,4-α-D-glucans. Cyclisation is achieved by 4αGTase attack a linear glucan (centre top) and transfer of the newly-formed reducing end (R) to the non-reducing end of itself, to form a cyclic molecule and a linear fragment. The reverse reaction is sometimes referred to as the Coupling Reaction. Disproportionation involves transfer from the linear donor glucan to an acceptor, which may be a glucan or glucose, to form two linear products of different size. The specificities of different types of 4αGTase are described in the text.

when the donor molecule is a cyclic glucan. These reactions are summarised diagrammatically in *Figure 1*. Since these reactions are reversible, the products of reaction of 4αGTases with glucan substrates are potentially varied and heterogeneous, particularly when complex glucan substrates (including amylopectin and glycogen) are also considered. However, the products are determined by the specificity of the different enzymes, the substrates employed, and the conditions of the reaction.

Groups within the 4αGTase family

There are three types of 4αGTase which can catalyse the glucan transfer reactions described above, and which have been extensively described and studied. These are:

1. Cyclodextrin glucanotransferase (CGTase) [EC 2.4.1.19]
2. Disproportionating enzyme (D-enzyme) or amylomaltase [EC 2.4.1.25]
3. Glycogen debranching enzyme (GDE) (amylo-1,6-glucosidase/4-α-glucanotransferase) [EC 3.2.1.33 + EC 2.4.1.25]

Unfortunately, the common names of these enzymes are either not informative or are misleading. Sometimes different names are used for the same enzyme, leading to potential confusion. It has also become apparent that there are fewer distinctions between the reaction specificities of these three enzyme types than previously thought. Furthermore, it has recently become clear that there are other 4αGTases which do not apparently belong to these three groups, based on their primary amino acid sequences and reaction specificities. Given the growing importance of these enzymes, it is necessary that the different types are carefully described for the benefit of future research. We propose that the three 4αGTases above should be referred to as Types I, II and III, respectively. In addition, there is a related but significantly different enzyme found in *Thermotoga maritima*, and another type from several thermophilic bacteria which has a completely unrelated primary structure. We propose that these should be Types IV and V, respectively. The charateristics of these five types will now be discussed.

Type I 4αGTase (CGTases)

DISTRIBUTION

This group of enzymes is well known for the synthesis of cyclodextrins (CDs), and most of the work on 4αGTase has been focused on this enzyme group. The enzyme has been reported in increasing numbers of bacteria and at least 20 genes for this enzyme have already been isolated (and more enzymes have been identified biochemically). The enzyme seems to be widespread in *Bacillus* species, since most reports are of CGTase genes from the Bacillaceae. Exceptions are two from *Klebsiella* (Binder *et al.*, 1986; Fiedler *et al.*, 1996) and two from *Themoanaerobacterium* (Bahl *et al.*, 1991; Jorgensen *et al.*, 1997). Recent results from genome sequencing projects also support this view, since no CGTase has been identified in the 15 completed genomes (including 10 Eubacteria, 4 Archaea and 1 Eukaryote). Given the large amount of work on this group of enzymes, the historical background of CDs and CGTases has already been comprehensively reviewed (French, 1957; Kobayashi, 1996) so will not

be covered here. Instead, we will briefly summarise some of their properties and discuss some of the most recent findings.

ENZYME ACTION

CGTase converts starch into a mixture of cyclic α-1,4-glucans (cyclodextrins, CD) with degree of polymerisation (DP) of 6, 7 or 8 (α-, β-, γ-CD, respectively), and residual dextrin. This is achieved by the intra-molecular glucan transfer (cyclisation) reaction. Since α-, β-, and γ-CDs have central cavities with distinct dimensions and different specificity for guest molecules (see below), the production of specific types of CD is desirable. The composition of CD produced is primarily determined by the type of enzyme employed, but can be influenced by additions (e.g. complexant or ethanol) to the reaction mixture (Rendleman, 1993; Mori *et al.*, 1995). The yield of CD depends on the enzyme employed, concentration of starch, and degree of hydrolysis of the starch substrate (Kitahata, 1995). Given the importance of CDs, product specificity (proportions of α-, β-, and γ-CDs) is one of the most important properties of this enzyme group. CGTases are often classified according to the major CD which they produce (for reviews see Schmid, 1989; Starnes, 1990; Kitahata, 1995; Kobayashi, 1996). The proportions of specific CDs produced can be changed by single amino acid substitutions in CGTase (Nakamura *et al.*, 1993; Nakamura *et al.*, 1994; Penninga *et al.*, 1995; Wind *et al.*, 1998).

Apart from the cyclisation reaction, Type I 4αGTases also catalyse disproportionation and coupling reactions as described in *Figure 1*. The final products from added substrate thus depend on the equilibrium of the three transferase reactions. These activities have been reviewed by Kitahata (1995). The smallest acceptor, smallest donor, and smallest glucan unit transferred were not determined in most cases, but glucose generally seems to be the smallest acceptor, and a glucose unit is the smallest transferred unit (*Table 1*). The smallest donor molecule might be different for each CGTase, since the smallest donor molecule was reported to be maltotetraose for the enzyme from *B. subtilis* No. 313 (Kato and Horikoshi, 1986), maltotriose for *B. macerans* and *B. ohbensis* enzymes, and maltose for the *B. megaterium* enzyme (Kitahata, 1995). CGTases also have hydrolytic activity, where the degree of hydrolytic activity relative to transferase activity, is variable between different CGTases (Kitahata and Okada, 1982).

Studies of crystal structures have been carried out with five different CGTases (or mutant forms, in some cases), which include enzymes from alkalophilic *Bacillus* sp.1011 (Harata *et al.*, 1996), *Thermoanaerobacterium thermosulfurigenes* EM1 (Knegtel *et al.*, 1996; Wind *et al.*, 1998), *Bacillus circulans* strain 251 (Lawson *et al.*, 1994; Knegtel *et al.*, 1995; Strokopytov *et al.*, 1995, Strokopytov *et al.*, 1996), *B. stearothermophilus* (Kubota *et al.*, 1991, 1994) and *Bacillus circulans* strain 8 (Klein and Schultz, 1991; Klein *et al.*, 1992; Schmidt *et al.*, 1998). CGTases are structually homologous to α-amylases, whose crystal structures have also been determined (Matsuura *et al.*, 1984; Boel *et al.*, 1990; Qian *et al.*, 1993; Kadziola *et al.*, 1994; Machius *et al.*, 1995; Brayer *et al.*, 1995; Ramasubbu *et al.*, 1996; Morishita *et al.*, 1997). Both types of enzyme belong to the α-amylase super-family, all members of which contain a characteristic (β/α)8-barrel domain and four highly conserved regions (Svensson, 1994) (see *Figure 2*). Three acidic residues (Asp229, Glu257 and

Table 1. Characteristics of the activities of each type of 4αGTase

4aGTase	Strain	Smallest donor	Smallest acceptor	Smallest transferred unit	Disproportionated products	Cyclisation reaction	Smallest CA (DP)	References
Type I	*B.subtilis* 313	G4	nt	nt	G2,G3,Gn	+	8	Kato and Horikoshi (1986)
	B.ohbensis	G3	G	G	G,G2,G3,Gn	+	7	Kitahata (1995)
	B.macerans	G3	G	G	G,G2,G3,Gn	+	6	Kitahata (1995)
	B.megaterium	G2	G	G	G,G2,G3,Gn	+	6	Kitahata (1995)
Type II	Potato	G3	G	G2	G, G3,Gn	+	17	Takaha *et al.* (1993, 1996)
	Barley	G3	G	G2	G, G3,Gn	nt		Yoshio *et al.* (1986)
	Sweet potato	nt	nt	G2	G, G3,Gn	nt		Suganuma *et al.* (1991)
	C.butyricum	G3	G	G	G,G2,G3,Gn	nt		Goda *et al.* (1997)
	E.coli ML308	G3	G	G	G,G2,G3,Gn	nt		Palmer *et al.* (1976)
	E.coli ATCC3806	G2	G	G	G,G2,G3,Gn	nt		Kitahata *et al.* (1989a)
	E.coli K12	G2	G	G	G,G2,G3,Gn	+	17	Unpublished
	T.aquaticus	G2	G	G	G,G2,G3,Gn	+	22	Unpublished
Type III	Yeast	G4	G3	G2	G2,G3,Gn	nt		Tabata and Ide (1988)
Type IV	*T.maritima*	G4	G2	G2	G2,G3,Gn	nt		Liebl *et al.* (1992)
Type V	*T.litoralis*	G2	G	G	G,G2,G3,Gn	+	nt	Jeon *et al.* (1997)
	*Pyrococcus*KOD1	G2	G	G	G,G2,G3,Gn	nt		Tachibana *et al.* (1987)
Others	*S.mitis*	G2	G	G	G,G2,G3,Gn	nt		Walker (1966)
	S.bovis	G3	G	G	G,G2,G3,Gn	nt		Walker (1965)
	B.subtilis	G4	G4	G2	G2,G3,Gn	nt		Pazur and Okada (1968)
	S.mutans	G2	G	G	G,G2,G3,Gn	nt		Medda and Smith (1984)

nt = not tested. G, G1, G2, Gn = glucose, maltose, maltotriose, *etc.* Organism names are given in the text.

Species		Region 1		Region 2		Region 3		Region 4
Type I 4αGTase (CGTase)								
Bacillus circulans 251	129	NIKVIIDFAPNH	224	DGIRM**D**AVKH	253	FTFG**E**WFL	320	QVTFIDNH**D**
Bacillus sp. 1011	129	NIKVIIDFAPNH	224	DGIRV**D**AVKH	253	FTFG**E**WFL	320	QVTFIDNH**D**
Bacillus circulans 8	129	GIKIVIDFAPNH	224	DGIRV**D**AVKH	253	FTFG**E**WFL	320	QVTFIDNH**D**
Thermoanaerobacterium thermosulfurigenes	130	NIKVIIDFAPNH	225	DGIRL**D**AVKH	254	FTFG**E**WFL	321	MVTFIDNH**D**
Bacillus stearothermophilus	125	GIKVIIDFAPNH	220	DGIRM**D**AVKH	249	FTFG**E**WFL	316	QVTFIDNH**D**
Klebsiella pneumoniae	124	NMKLVLDYAPNH	118	DAIRI**D**AIKH	253	FFFG**E**WFG	325	QVVFMDNH**D**
Type II 4αGTase (Amylomaltase / D-enzyme)								
Potato	298	VGYHSADVWANK	368	DEFRI**D**HFRG	416	NIIA**E**DLG	465	QVVYTGTH**D**
Aquifex aeolicus	211	PSYSSADVWTNP	281	DFLRL**D**HFRG	329	PFIA**E**DLG	377	NVYYTSTH**D**
Synechocystis sp.	222	VAHDSADVWANP	293	DIVRI**D**HFRG	342	PIVA**E**DLG	383	AVVYTGTH**D**
Thermus aquaticus	218	VAEDSAEVWAHP	288	HLVRI**D**HFRG	336	PVLA**E**DLG	387	VVVYTGTH**D**
Streptococcus pneumoniae	220	VAEDSSDMWANP	290	DIVRI**D**HFRG	338	NIIA**E**DLG	387	SVMYTGTH**D**
Clostridium butyricum	209	IAQDSSDVWSNP	280	DILRI**D**HFRG	328	EIIA**E**DLG	377	CVAYTGTH**D**
Borrelia burgdorferi	232	IAYDSADVWAYQ	302	DIIKI**D**HFRG	350	KIWV**E**DFQ	399	CIVYTGSG**D**
Chlamydia psittaci	245	ISKDSCDVWYYR	309	SLYRL**D**HIVG	359	LPIG**E**DLG	409	SVTSLSTH**D**
Haemophilus influenzae	391	SSRGSADVWSDP	455	GVLRI**D**HVMG	504	LIIG**E**DLG	551	AYATIGTH**D**
Escherichia coli	379	VGTGGAETWCDR	443	GALRI**D**HVMS	492	MVIG**E**DLG	540	SMAVAATH**D**
Micobacterium tuberculosis	407	VHPNGADAWALQ	471	GAVRI**D**HIIG	520	VVVG**E**DLG	572	CLSSVTTH**D**
Type III 4αGTase (Glycogen debranching enzyme)								
Human muscle	192	NVICITDVVYNH	504	QGVRL**D**NCHS	534	YVVA**E**LFT	602	ALFMDITH**D**
Rabbit muscle	232	NVLCITDVVYNH	544	QGVRL**D**NCHS	574	YVVA**E**LFT	642	ALFMDITH**D**
Caenorhabditis elegans	354	NILTVQDVVWNH	670	HGLRI**D**NAHG	700	YVFA**E**LFT	768	GLFLDQSH**D**
Saccharomycescerevisiae	218	NMLSLTDIVFNH	530	DGFRI**D**NCHS	560	YVVA**E**LFS	662	ALFMDCTH**D**
Type IV 4αGTase								
Thermotoga maritima	83	GIKVVLDLPINH	190	DGFRF**D**AAKH	212	IFLA**E**IWA	270	PVNFTSNH**D**
α -Amylase								
Aspergillus oryzae	111	GMYLMVDVVANH b	200	DGLRI**D**TVKH c bb	226	YCIG**E**VLD cbbb	289	LGTFVENH**D** bc

Figure 2. Conserved regions in the 4αGTase family of enzymes and identification of four distinct types. The four conserved regions compared are those identified in the α-amylase super-family of enzymes. The same regions from one α-amylase (*Aspergillus oryzae*) are shown for comparison. Numbers denote amino acid positions in each polypeptide. Four types of related 4αGTase are distinguished, and a fifth unrelated type is shown in *Figure 4*. Proposed catalytic (c) and binding (b) sites, in *A. oryzae* α-amylase are indicated (Matsura *et al.*, 1984).

Asp328 – numbers from *B. circulans* strain 251) in conserved regions 2, 3 and 4, respectively, are present in all known α-amylase family enzymes (Svensson, 1994) and are believed to participate in catalysis. Their roles have been studied by mutagenesis in conjunction with crystallographic studies (Klein *et al.*, 1992; Nakamura *et al.*, 1992; Strokopytov *et al.*, 1995; Knegtel *et al.*, 1995) and Glu257 was identified to be the acid/base catalyst and Asp229 the catalytic nucleophile. Identification of Asp229 as a catalytic nucleophile and evidence for a covalent intermediate in CGTase was recently obtained (Mosi *et al.*, 1997).

PHYSIOLOGICAL ROLE

Utilisation of exogenous glucans (starch or glycogen) as a substrate for growth is achieved in some bacteria by secretion of amylases and CGTase. The CGTase can degrade the starch through cyclisation activity, creating CDs, but can also potentially then catalyse coupling reactions to linearise the CDs, and disproportionation reactions to change maltooligosaccharide chain lengths. Until recently, it was unknown if CDs could be imported into the cell, but studies of mutants of *Klebsiella oxytoca* suggest

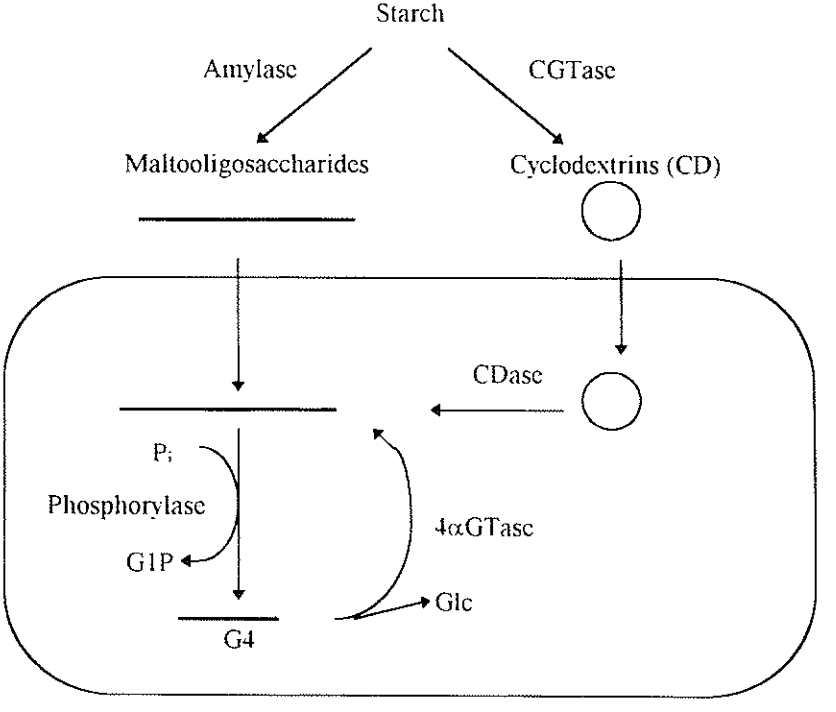


Figure 3. Roles of 4αGTases in glucan utilisation by bacteria. Breakdown of starch or other glucan is initiated by extracellular hydrolases such as amylase, or by CGTase, depending upon the species of bacterium. Maltooligosaccharides or cyclodextrins (CD) are imported by specific transporter proteins. Maltooligosaccharides are broken down by glucan phosphorylase, producing glucose-1-phosphate (G1P). Phosphorylase cannot break down molecules beyond maltotetraose (G4), so 4αGTase (amylomaltase or D-enzyme) catalyses a disproportionation reaction to create larger maltooligosaccharides and release glucose (Glc). In those bacteria which import CD, it is linearised by cyclodextrinase (CDase) and maltooligosaccharides metabolised by phopsphorylase and 4αGtase.

the existence of a specific CD uptake system (Fiedler *et al.*, 1996; Pajatsch *et al.*, 1998). Imported CDs are apparently then hydrolysed by a cytoplasmic cyclodextrinase (Feederle *et al.*, 1996) and further metabolised by a phosphorylase-dependent pathway of a type found in many bacteria (*Figure 3*). This phosphorylase-dependent pathway makes use of a Type II 4αGTase (see below). Presumably, the evolution of such a CD synthesis and uptake mechanism provides a competitive advantage over other bacteria which cannot import CDs.

Type II 4αGTase (D-enzyme and amylomaltase)

DISTRIBUTION AND RELATEDNESS

Amylomaltase was first found in *Escherichia coli* as a maltose-inducible enzyme (Monod and Torriani, 1948). The amylomaltase gene has been cloned from *E. coli* (Pugsley and Dubrevil, 1988), *Streptococcus pneumoniae* (Lacks *et al.*, 1982), *Clostridium butyricum* (Goda *et al.*, 1997), *Chlamidia* (Hsia *et al.*, 1997) and *Thermus aquaticus* (Terada *et al.*, unpublished), but further homologous genes have been identified in the genomes of *Haemophilus influenzae* (Fleischmann *et al.*, 1995), *Aquifex aeolicus* (Deckert *et al.*, 1998), *Borelia* sp. (Fraser *et al*, 1997), *Mycobacterium tuberoculosis* (Cole *et al.*, 1998) and *Synechosystis* sp. (Kaneko *et al.*, 1996). It is clearly a widely-distributed enzyme, but probably not ubiquitous since some bacterial genome projects have failed to reveal an obvious amylomaltase gene.

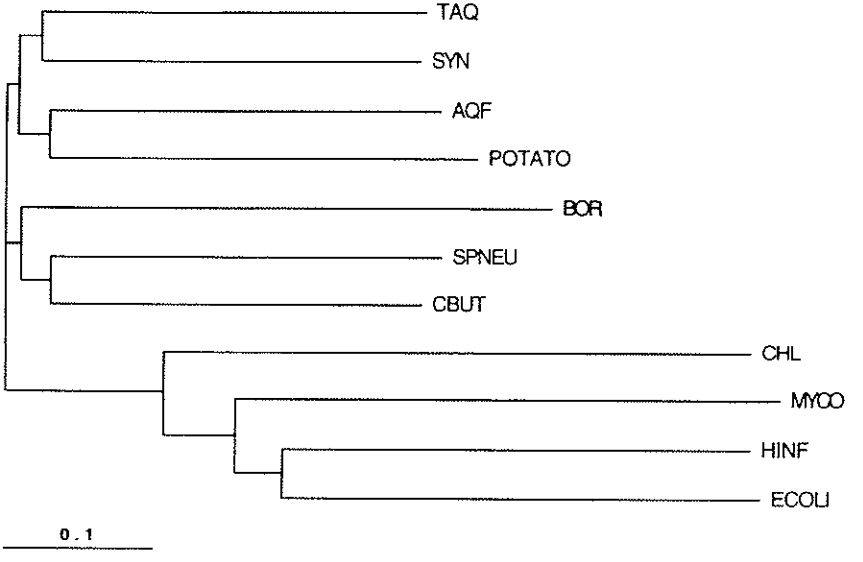


Figure 4. Phylogenetic relationships of Type II 4αGTases. Comparisons of amino acid sequences (all deduced from nucleic acid sequences) for 11 enzymes using the 'Phylip' programme indicate that they fall into at least two distinct groups. At this time it is not possible to distinguish the activities of these groups because enzyme activities have not been fully characterised in most cases. TAQ, *Thermus aquaticus*; SYN, *Synechosystis* sp.; AQF, *Aquifex aeolicus*; POTATO, *Solanum tuberosum*; BOR, *Borelia* sp.; SPNEU, *Strepotococcus pneumoniae*; CBUT, *Clostridium butyricum*; CHL, *Chlamidia* sp.; MYCO, *Mycobacterium tuberculosis*; HINF, *Haemophilus influenzae*; ECOLI, *Escherichia coli*.

A similar 4αGTase is also present in plants and is called disproportionating enzyme (D-enzyme). D-enzyme was first found in potato tubers (Peat *et al.*, 1956) but has since been found in carrot roots (Manners and Rowe, 1969), tomato fruits (Manners and Rowe, 1969), germinated barley seeds (Yoshio *et al.*, 1986), sweet potato tubers (Suganuma *et al.*, 1991), spinach leaves (Okita *et al.*, 1979), pea leaves (Kakefuda *et al.*, 1986) and *Arabidopsis* leaves (Lin and Preiss, 1988). A cDNA for D-enzyme was isolated from RNA of potato tubers (Takaha *et al.*, 1993), where the similarity of plant D-enzyme and bacterial amylomaltase was first confirmed at the molecular level. The phylogenetic tree of nearly all available (eleven) amylomaltase and D-enzyme amino acid sequences shows that these enzymes may be further divided into two subgroups in which sequence homolgy is very high within each subgroup, but less between subgroups (*Figure 4*). The first subgroup comprises enzymes from *E. coli*, *Mycobacterium*, *Chlamidia* and *H. influenzae*, and the second comprises the other amylomaltases and potato D-enzyme. It is too premature to formally propose two subgroups since more sequence analysis may provide further information, and the reaction specificity of only a few of these enzymes is currently available.

It has been suggested that D-enzyme (amylomaltase) is a member of the α-amylase super-family of enzymes (Heinrich *et al.*, 1994; Svensson, 1994). Alignments of these enzymes clearly indicates the presence of the four conserved regions characteristic of the α-amylase super-family of enzymes (*Figure 2*). We also note that the motifs RIDH(FRG), and EDLG in regions 2 and 3, are conserved within this enzyme group (Type II 4αGTase). However, not all of the 7 'invariant' amino acid residues found in these four conserved regions (Svensson, 1994) are found in Type II 4αGTases. In particular, the His residue in region 1 is completely replaced.

ENZYME ACTION

Plant D-enzyme has been partially purified from potato tubers (Jones and Whelan, 1969), germinating barley seeds (Yoshio *et al.*, 1986), *Arabidopsis* leaves (Lin and Preiss, 1988) and sweet potato tubers (Suganuma *et al.*, 1991), and its activity on maltooligosaccharides was investigated. These studies demonstrated that D-enzyme catalyses a disproportionation reaction on maltooligosaccharides in which a glucan moiety is transferred from one α-1,4-glucan molecule to another, or to glucose. The results of these studies indicated that D-enzymes of plants have common reaction characteristics. The smallest donor molecule is maltotriose, the smallest acceptor molecule is glucose, the smallest transferred glucan is a maltose unit, and glucosyl transfer never occurs (*Table 1*). It should also be noted that maltose is not produced during D-enzyme reaction on any substrate, because there are two 'forbidden linkages' in maltooligosaccharides larger than maltotetraose: the non-reducing end linkage and the bond penultimate to the reducing end (Jones and Whelan, 1969).

A limited number of bacterial amylomaltases have been subjected to biochemical analysis of their action. Analysis of amylomaltases from *E. coli* (Palmer *et al.*, 1976; Kitahata *et al.*, 1989a; Kitahata *et al.*, 1989b) and *C. butyricum* (Goda *et al.*, 1997) suggests that they catalyse a similar disproprtionation reaction to plant D-enzymes, but there are some differences. The major difference is that the bacterial amylomaltases catalyse a glucosyl transfer (*Table 1*). Additionally, maltose can act as a donor molecule for the enzyme from *E. coli* strain ATCC380 (Kitahata, 1989a), although the

rate with maltose is much lower than that for maltotriose (our unpublished work). However, the work of Palmer *et al.* (1976) with *E. coli* strain ML308 and that of Goda *et al.* (1997) with *C. butyricum* indicates that maltose is not a donor.

PHYSIOLOGICAL ROLE

In *E. coli*, amylomaltase is part of a maltooligosaccharide transport and utilisation system, which includes maltodextrin phosphorylase and maltose transport proteins (Schwartz, 1987). The role of amylomaltase is apparently to convert short maltooligosaccharides into longer chains upon which glucan phosphorylase can act (*Figure 3*). This phosphorylase, like that in plants, degrades maltooligosaccharides to maltotetraose, but no further. The genes for amylomaltase and glucan phosphorylase constitute the *malPQ* operon. A similar operon structure was also found in *S. pneumoniae* (Lacks *et al.*, 1982), *K. pneumoniae* (Bloch *et al.*, 1986) and *C. butyricum* (Goda *et al.*, 1997), so the function of these amylomaltases is expected to be the same as the *E. coli* enzyme. On the other hand, the genes for amylomaltase found in the genomes of *H. influenzae* (Fleischmann *et al.*, 1995) and *A. aeolicus* (Deckert *et al.*, 1998), are part of the glycogen operon, which include genes of glycogen synthesis and degradation. Furthermore, these organisms do not have the genes homologous to *E. coli malE, malF, malG* which are involved in the transport of maltooligosaccharides into the cytoplasm. All these observations suggest that in *H. influenzae* and *A. aeolicus*, amylomaltase may not be involved in exogenous maltooligosaccharide utilisation, but is involved in glycogen metabolism. Thus, the physiological role of amylomaltase may be different in each organism, but these differences do not correlate with the enzyme subgroups identified above (*Figure 4*).

The physiological role of D-enzyme in plants is not clear, but it is a plastidic enzyme and is assumed to be involved in starch metabolism. It is not required for the accumulation of starch or creation of starch structure, so it is likely to be involved in starch turnover (Takaha *et al.*, 1998a). The long-held view is that D-enzyme converts small maltooligosaccharides into larger ones which serve as substrates for breakdown by starch phosphorylase, analogous to the maltooligosaccharide utilisation system of *E. coli* (Schwartz, 1987). This view is consistent with the observed preference of D-enzyme for maltooligossaccharide substrates and the observed effects of antisense inhibition in transgenic potato (Takaha *et al.*, 1998a). However, other possible functions should not be excluded, since D-enzyme can also act upon high molecular weight starch molecules (see below).

Type III 4αGTase (GDE)

DISTRIBUTION AND STRUCTURE

This is a bifunctional enzyme with amylo-1,6-glucosidase and 4αGTase activities. It appears to be present only in those Eukaryotes which synthesise glycogen (therefore excluding higher plants). The enzyme has been isolated, and primary sequence determined, from human muscle (Yang *et al.*, 1992) and rabbit muscle (Liu *et al.*, 1993), and gene sequences found in the yeast *Saccharomyces cereviseae* (Bussey *et al.*, 1997) and in *Caenorhabditis elegans* (Wilson *et al.*, 1998). Alignment of amino

acid sequences shows that they are similar enzymes and have the four conserved regions characteristic of the α-amylase super-family (Svensson, 1994). The three invariant acidic residues present in regions 2, 3 and 4 of all known α-amylase super-family enzymes (Svensson, 1994) are also found in Type III 4αGTases, and therefore are expected to participate in catalysis (*Figure 2*). Recently, Asp549 of the rabbit enzyme was identified as the catalytic nucleophile (Braun *et al.*, 1996) in support of this expectation.

PHYSIOLOGICAL ROLE AND ENZYME ACTION

Glycogen phosphorylase is the major enzyme responsible for attacking glycogen during its breakdown in Eukaryotes. This enzyme can sequentially remove glucose units (as glucose-1-phosphate) from the non-reducing ends of branches, but stops when branch lengths are reduced to four residues. At this point, the 4αGTase activity of GDE, catalyses maltotriosyl transfer from the shortened branch to another non-reducing end on the glycogen molecule (thereby creating a new substrate for phosphorylase) which then allows removal of the remaining α-1,6-linked glucosyl residue by the glucosidase activity of GDE. In this way, glycogen is completely debranched and degraded to glucose-1-phosphate (for review see Nelson *et al.*, 1979).

It has been suggested that the enzyme catalyses the two different reactions by means of two different active sites (Nelson *et al.*, 1979). This has more recently been supported by two new pieces of evidence. Firstly, the transferase reaction occurs with net retention of anomeric configuration, but the glucosidase reaction occurs with net inversion of anomeric configuration (Liu *et al.*, 1991). This indicates that the two reactions operate by means of different catalytic mechanisms. Secondly, transferase activity can be irreversibly inactivated by a water-soluble carbodiimide in the presence of amines, without affecting glucosidase activity (Liu *et al.*, 1991).

Yeast and mammalian (rabbit) enzymes catalyse similar reactions (Tabata and Hizukuri, 1992; Tabata *et al.*, 1995) but may have differences in substrate preference (Lee and Carter, 1973). In particular, both enzymes will act on phosphorylase-limit dextrin, but while native glycogen and amylopectin are good substrates for the yeast enzyme, they are not for the rabbit enzyme. The disproportionation (transferase) reaction on linear maltooligosaccharides (G4 to G7) has been extensively studied with the yeast enzyme (Tabata and Ide, 1988). The results show that the smallest donor is maltotetraose, the smallest acceptor is maltotriose, and smallest unit transferred is the maltosyl unit. The rate is higher with a longer substrate, where maltotriosyl units are preferentially transferred. Maltose is the smallest product and glucose is not produced. Glucose may serve as an acceptor for the rabbit enzyme (Brown and Illingworth, 1962). There is no information available on the biochemical properties of the *C. elegans* enzyme.

Type IV 4αGTase

The fourth group includes the 4αGTase found in the hyper-thermophilic bacterium *Thermotoga maritima* MSB8 (Liebl *et al.*, 1992). The enzyme catalyses the disproportionation of maltooligosaccharides and gives products including maltose, but no glucose. Analysis with maltooligosaccharides concluded that the smallest

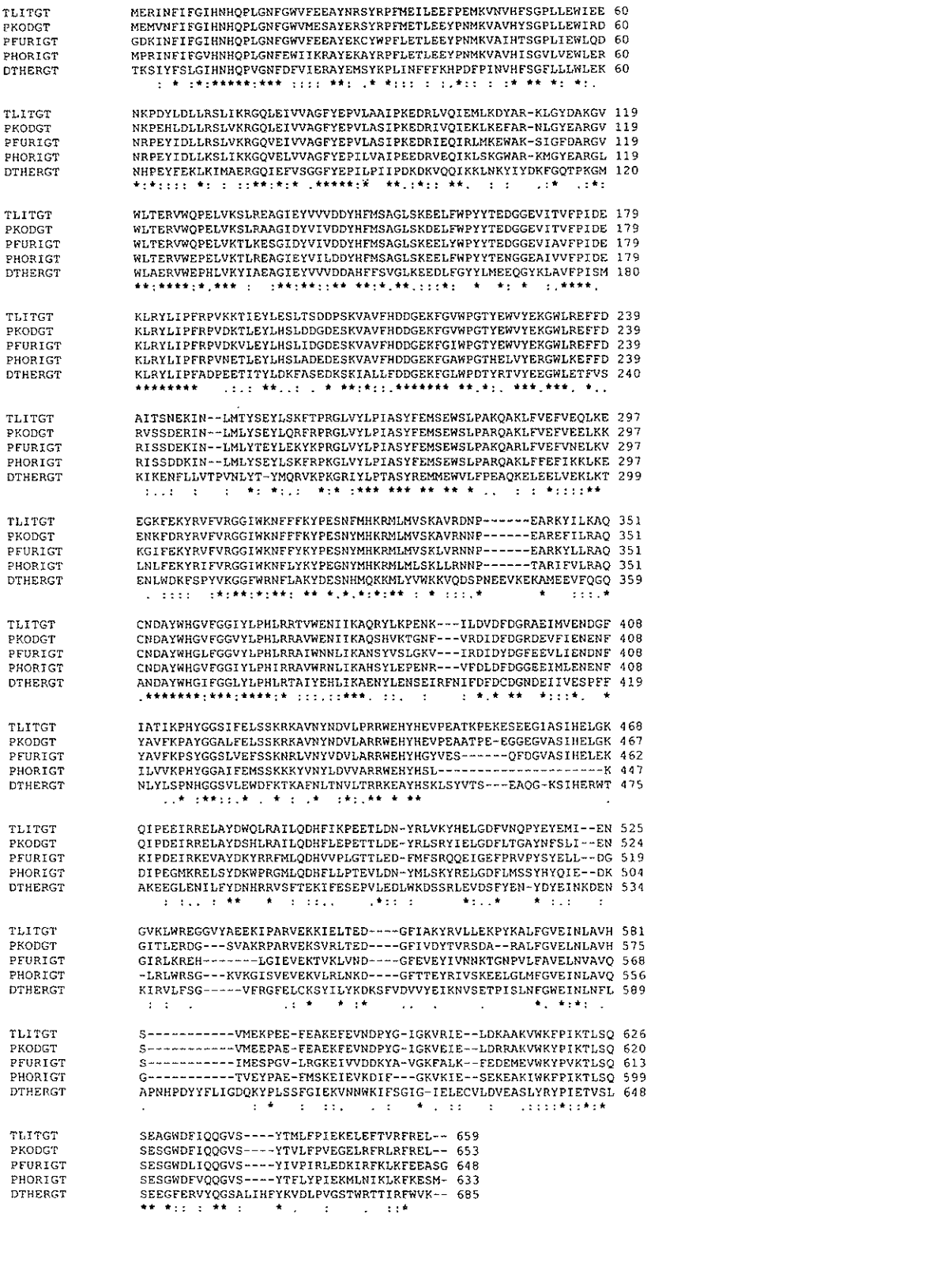
donor is maltotetraose, the smallest acceptor is maltose, and the smallest glucan unit to be transferred by this enzyme is a maltosyl unit (Liebl *et al.*, 1992). Such characteristics of this enzyme are most similar to the transferase action of Type III 4αGTase, but different to any of the other 4αGTases described above (*Table 1*). The enzyme is also reported to act on high molecular weight amylose, soluble starch and amylopectin, and to decrease the iodine colour without increasing reducing power (Liebl *et al.*, 1992). The gene for the enzyme was cloned (Liebl *et al.*, 1992) and sequenced, but does not show overall similarity to bacterial amylomaltases and potato D-enzyme (Heinrich *et al.*, 1994), but shows the highest similarity to α-amylase (*AmyC*) of *Dictyoglomus thermophilum* (Horinouchi *et al.*, 1988), and α-amylase of *Bacillus megaterium* (Metz *et al.*, 1988). From these results, the authors concluded that this 4αGTase is the first member of a new 4αGTase, which is more closely related to α-amylase, α-glucosidase and CGTase than to other 4αGTases (Heinrich *et al.*, 1994).

Type V 4αGTases

Type V 4αGTases were recently found independently in the hyper-thermophilic Archaea, *Thermococcus litoralis* (Jeon *et al.*, 1997) and in *Pyrococcus* sp. KOD1 (Tachibana *et al.*, 1997). These enzymes have similar properties. They both catalyse the disproportionation of maltooligosaccharides (G2 and larger) and produce a series of maltooligosaccharides, including maltose and glucose. These enzymes also transfer glucan chains from starch (soluble starch, amylose) to the acceptor glucose, and produce a series of maltooligosaccharides including maltose. All this information indicates that the smallest donor for these two enzymes is maltose, the smallest acceptor is glucose, and the smallest glucan unit to be transferred is the glucosyl unit (*Table 1*). Therefore, these enzymes are more similar to the amylomaltase of *E. coli* than to the plant D-enzyme. The genes for these 4αGTases were cloned and sequenced (Jeon *et al.*, 1997; Tachibana *et al.*, 1997). Surprisingly, they do not show overall similarity to the 4αGTases described above, but show significant similarity to α-amylase of the hyper-thermophilic Archaeon *Pyrococcus furiosus* (Laderman *et al.*, 1993a, 1993b) and α-amylase (*AmyA*) of the hyper-thermophilic bacterium *Dictyoglomus thermophilum* (Fukusumi *et al.*, 1988). Although these latter two enzymes were reported as α-amylases, they are likely to be 4αGTases. In particular, for the *P. furiosus* enzyme, the presence of disproportionation activity on maltooligosaccharides was clearly documented (Laderman *et al.*, 1993a) and both enzymes are able to decrease the ability of starch to form a blue complex with iodine, as found for 4αGTases of *T. litoralis* and *Pyrococcus*. sp. KOD1 (Laderman *et al.*, 1993a; Fukusumi *et al.*, 1988).

The alignment of the four enzymes described above, together with the homologous sequence recently identified in the genome of *Pyrococcus horikoshii* (Kawasaki *et al.*, 1998), is shown in *Figure 5*. These five enzymes are highly homologous, but lack the

Figure 5. Comparisons of deduced polypeptide sequences from Type V 4αGTases. The complete amino acid sequences are shown for five enzymes. Identical amino acids are denoted by asterisks and conserved amino acids by dots. There appears to be no similarity with the sequences of the other four types of 4αGTase shown in *Figure 2*. (TLITGT: *Thermococcus litoralis*; PKODGT: *Pyrococcus* sp. KOD1; PFURIGT: *Pyrococcus furiosus*; PHORIGT: *Pyrococcus horikoshii*; DTHERGT: *Dictyoglomus thermophilum*).



four highly conserved sequences found in enzymes of the α-amylase super-family, so form a new group of 4αGTase. Further investigations of these enzymes in terms of their structure and mechanism of action will be very interesting, since they are the first example of 4αGTases which might work by a mechanism different to the one commonly employed by α-amylase-family enzymes. Furthermore, the enzymes belonging to this new group are expected to have industrial applications, since they have optimum temperatures for activity of 90 to 100°C (Laderman *et al.*, 1993a; Jeon *et al.*, 1997; Tachibana *et al.*, 1997; Fukusumi *et al.*, 1988).

Other 4αGTases

Several additional glucanotransferase activities have been identified biochemicaly (in some cases as amylomaltase) in several bacterial species, including *Streptococcus bovis* (Walker, 1965), *Streptococcus mitis* (Walker, 1966), *Bacillus subtilis* (Pazur and Okada, 1968), *Pseudomonas stutzeri* (Schmidt and John, 1979) and *Streptococcus mutans* (Medda and Smith, 1984). Since the primary sequences for these enzymes are not available, we do not know to which group each enzyme belongs. The properties of these enzymes are summarised in *Table 1*.

Synthesis of cycloglucans by 4αGTases

DISCOVERY AND SYNTHESIS OF CYCLOAMYLOSE

Studies aimed at determining whether potato D-enzyme can act upon high molecular weight glucans, led to the discovery that this enzyme can catalyse an intra-molecular transglycosylation reaction on synthetic amylose, to produce cycloamylose (CA) of high DP (Takaha *et al.*, 1996). In the early stages of the reaction with amylose AS-320, CAs with several hundred glucoses were produced, and as the reaction progressed, the mean product size tended towards a DP of about 90, but the yield of CA reached more than 95% (Takaha *et al.*, 1996). D-enzyme apparently breaks an internal α-1,4-bond within the amylose molecule, then transfers the newly-formed reducing end to the non-reducing end of itself, to create the CA molecule. This reaction is readily reversible, because the large CAs produced initially, are converted to smaller CAs as the reaction proceeds (*Figure 6*). The high yield of CA indicates that hydrolytic activity is very low. The smallest product is CA with 17 glucoses (CA17). CAs have properties which suggest that they may be of applied value (see below).

This observation led to examination of the possibility that bacterial Type II enzymes may also catalyse the formation of CA from amylose. 4αGTases from *E. coli* and *Thermus aquaticus* have subsequently been shown to produce CA (Terada *et al.*, unpublished). Similar to potato D-enzyme action, the initial products are larger than the final products. The smallest product of the *E. coli* enzyme is also CA17, but that from *T. aquaticus* is CA22 (Terada *et al.*, unpublished). The yield of CA from the *E. coli* enzyme is less than that of the potato enzyme, which is attributable to its higher hydrolytic activity, as revealed by the increase in reducing power during the reaction (*Figure 7*).

Subsequently, it has been demonstrated that Type V 4αGTases can produce

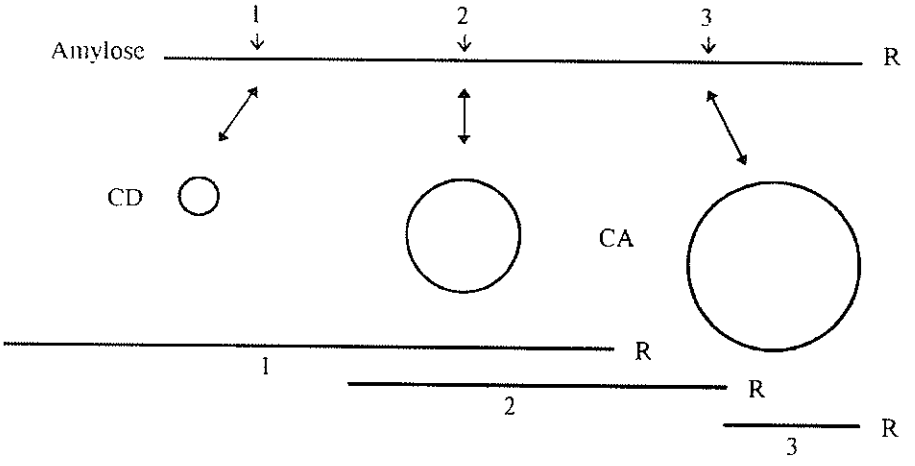


Figure 6. Cycloglucan synthesis from high molecular weight amylose. 4αGTase is able to attack within the linear amylose molecule (where R is the reducing end) and to create a cyclic product by intramolecular transglycosylation. Smaller linear products are also created in these reactions. In those cases studied, the first cyclic products of such a reaction are cycloamyloses (CA) with a high degree of polymerisation (>50). As the reaction proceeds, smaller cycloglucans are produced through further coupling and cyclisation reactions. The end-products of CGTase (Type I 4αGTase) activity are cyclodextrins (CD) whereas those of Type II 4αGTases are cycloamyloses (CA).

cycloglucans. The enzymes from *T. litoralis* and *Pyrococcus* sp. KOD1 were incubated with amylose and soluble starch, respectively, without an additional acceptor molecule. In both cases the ability to form a blue complex with iodine decreased, but there was little increase in reducing power. The products from the reaction using the *T. litoralis* enzyme contained cyclic α-1,4-glucans indicating that the enzyme catalyses the cyclisation of amylose (Jeon *et al.*, 1997). The formation of such cyclic glucans by Type IV enzymes has not been demonstrated, but is suggested by the observation that such enzymes can reduce the ability of amylose to form a blue complex with iodine, without increasing reducing power (Liebl *et al.*, 1992). It is not known if Type III 4αGTases can produce cyclic glucans from amylose.

These discoveries led to a re-examination of the action of Type I 4αGTases on amylose, and the discovery that this enzyme from two *Bacillus* species also preferentially produced large CAs early in the reaction with amylose (Terada *et al.*, 1997). As the reaction proceeded, the characteristic α-, β- and γ-cyclodextrins (CA6, 7 and 8) were produced. This was a remarkable finding since CAs with 6, 7 or 8 glucoses had been thought to be the only products of CGTase activity since their discovery in 1903 (Szejtli, 1988). This observation therefore demands a re-evaluation of the mode of action of Type I 4αGTases on amylose.

The discovery of CA synthesis with a range of 4αGTases raises important questions about substrate specificity and conformation. It appears that each of these enzymes can accommodate the amylose molecule in its active site and can catalyse the cyclisation reaction. Presumably, the properties of the linear amylose dictate that the initial cleavage and glucanotransferase reaction will produce a CA molecule of high DP. Subsequently, such large CA molecules must be accommodated again by the enzyme

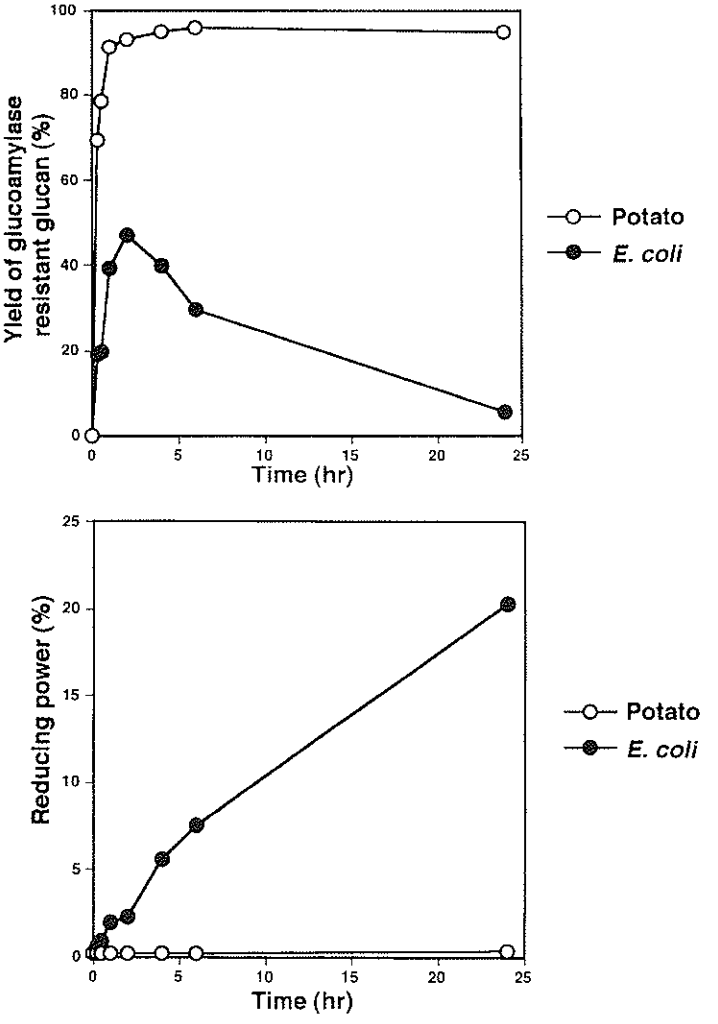


Figure 7. Cycloamylose production from linear amylose and hydrolytic activity of Type II 4αGTases of potato and *E. coli*. Synthetic amylose (AS-320) was incubated with potato and *E. coli* enzymes. The yield of glucoamylose-resistant products (cycloglucans) was determined (upper panel) and the change in reducing power assayed (lower panel) during the reaction (Takaha *et al.*, 1996).

and a further glucanotransferase reaction takes place to create a smaller CA. Somehow we have to be able to explain how each enzyme determines the final product size. In the extreme cases, potato D-enzyme preferentially produces CA90 while CGTases produces CA7 and CA8. Significant progress has been made with the determination of some CA structures (see below) but we are still remarkably ignorant of substrate conformations and the structures of enzyme-substrate complexes.

ACTION OF 4αGTASES ON AMYLOPECTIN *IN VITRO*

Following the discovery that potato D-enzyme can catalyse a cyclisation reaction on

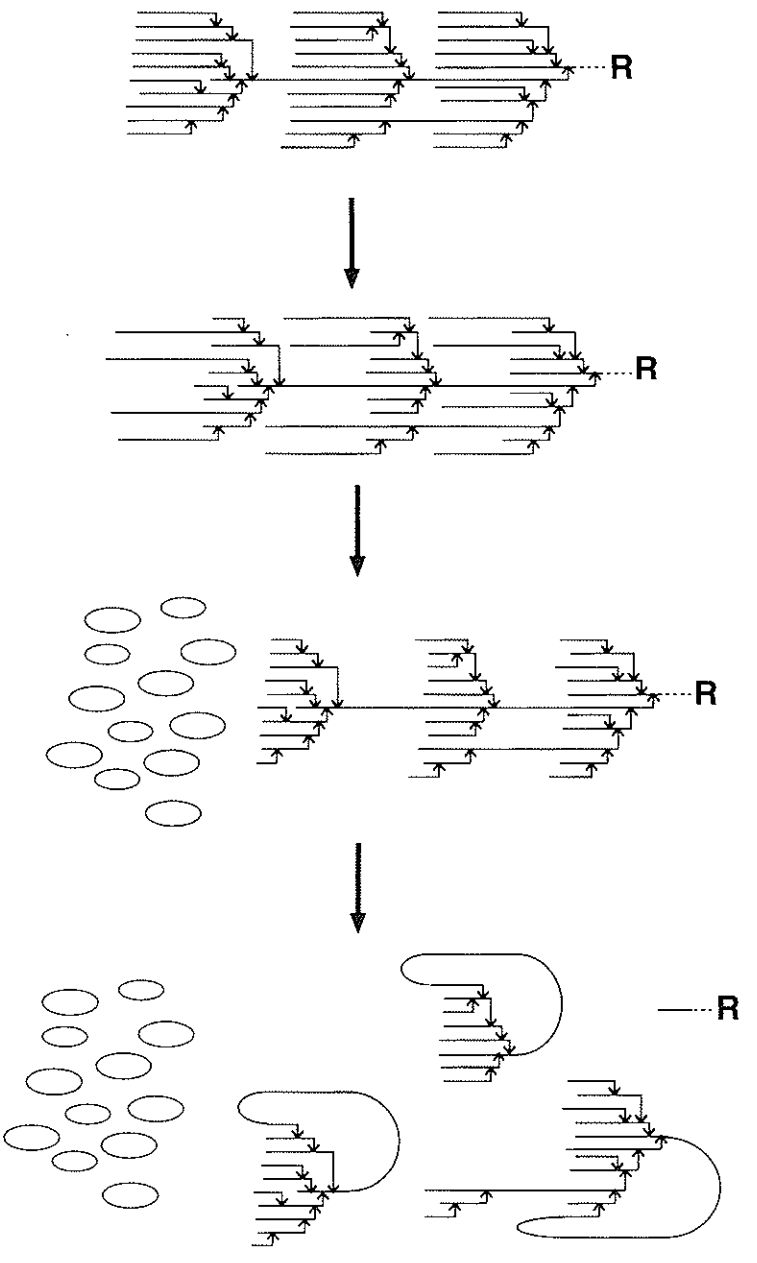


Figure 8. Action of potato Type II 4αGTase on amylopectin. 4αGTase first attacks outer chains and creates longer branches through disproportionation activity. Next, cycloglucans are produced from the lengthened side chains. Cycloamylose accounts for most of these products but some molecules can also contain α-1,6-links (not shown). 4αGTase next attacks between cluster units and catalyses another intramolecular transglycosylation reaction to create cyclic cluster dextrins.

amylose, its action on amylopectin (AP) was investigated (Takaha *et al.*, 1998b). It was found that D-enzyme can degrade AP to products of smaller size, which are largely glucoamylase-resistant. Analysis of these products showed that D-enzyme catalyses cyclisation reactions on the outer chains of AP to produce CA molecules, and other cycloglucans which also contain α-1,6-links. These cycloglucans are larger than the outer chains of the AP molecule, implying that D-enzyme first carried out disproportionation reactions to produce longer side chains, before catalysing cyclisation reactions. The way in which such structures are produced is shown in *Figure 8*. One result of this activity is that the AP side chains become shortened. Subsequently, D-enzyme attacks glucan chains between AP cluster units and catalyses intra-cluster cyclisation reactions which separate cluster units from each other (Takaha *et al.*, 1998b). The product of this reaction is called cyclic cluster dextrin (CCD). Type I 4αGTases can catalyse formation of cyclodextrins and CCD from AP (Terada *et al.*, unpublished results) but other 4αGTases have not apparently been tested.

It is also of note that branching enzyme (EC 2.4.1.18; 1,4-α-D-glucan:1,4-α-D-glucan 6-α-D-[1,4-glucano]-transferase) can catalyse cyclisation reactions on AP to produce cyclic cluster units. In this case, the enzyme does not necessarily attack outer chains, but cleaves the glucan chain between clusters and then forms an intra-cluster α-1,6-link, producing a long-chain CCD (Takata *et al.*, 1996, 1997). CCDs are of potential applied value. They are highly soluble in water and have a low propensity for retrogradation. Starch pastes containing CCD have low viscosity and high transparency (Takata *et al.*, 1997).

STRUCTURES AND PROPERTIES OF CYCLOAMYLOSES

Cyclodextrins are annular molecules of 6, 7 or 8 glucoses, the internal cavity of which is hydrophobic, while the outer surface is hydrophilic. They are therefore soluble in aqueous solutions, but can accommodate hydrophobic guest molecules in the central cavity with high specificity (Szejtli, 1988). Such inclusion complexes can change the solubility, reactivity or stability of guest molecules. They have been the subject of much research over many decades, and have found many applications in the food, chemical and pharmaceutical industries. For example, they provide the basis for formulations used in pesticides, cosmetics and drug delivery, and can change the flavour or odour of compounds. The high specificity of guest molecule inclusion is reflected in their ability to discriminate isomers of the same compound. Some chemically-modified cyclodextrins also possess catalytic activity (Szejtli, 1988). The central cavities of α-, β- and γ-cyclodextrins have different dimensions, allowing them to accommodate different guest molecules. The discovery of the much larger CAs immediately suggested that if they could also accommodate guest molecules, they would be of different sizes or properties to those of cyclodextrins and could therefore have new applications.

CA molecules of defined size were purified by high performance liquid chromatography using an ODS (C18) column, for structural analysis by NMR and X-ray crystallography (Jacob *et al.*, 1998; Saenger *et al.*, 1998). The first aim was to determine how the structure of the CA molecule changes when the number of glucoses is increased progressively to more than 8 (γ-cyclodextrin). As the macrocycle increases

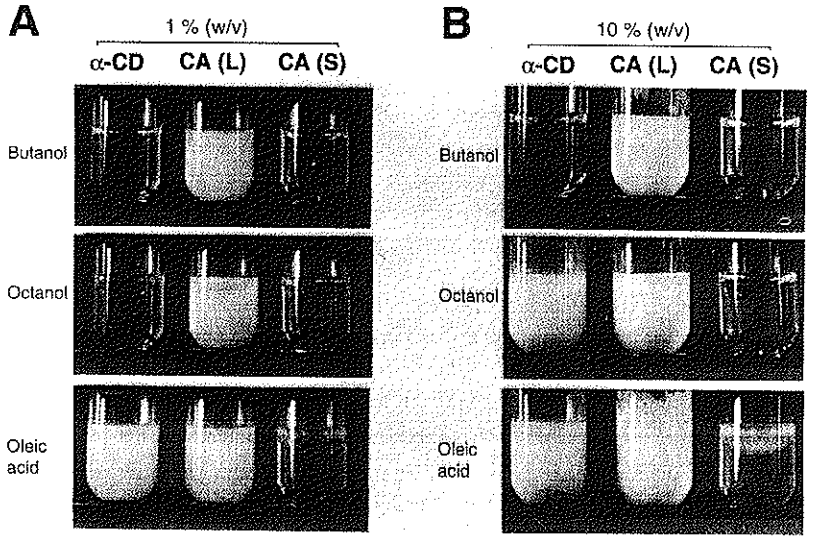


Figure 9. Formation of inclusion complexes between cycloamyloses and guest molecules. Aqueous solutions (1% and 10% [w/v]) of α-CD (CA6) and CA were prepared. In one case the CA was comprised of a mixture of large molecules (L) with DP greater than 50. In the other case the CA was a mixture of smaller molecules (S) with DP between 17 and 50. To 2 ml of these solutions was added about 0.2 ml of butanol, octanol or oleic acid, then mixed. A precipitate indicates that the alcohol or fatty acid has formed an inclusion complex with the cycloglucan.

from 8 to 9 glucoses, the molecule apparently experiences steric strain which results in distortion of the previously annular molecule into one which is boat-shaped. Molecules with 10 and 14 glucoses could be crystallised and their structures were determined. Remarkably, when the molecule increases in size beyond 9 glucoses, the steric strain is relieved by 180° rotations about two diammetrically-opposed glucosidic bonds to create a novel hydrogen bonding pattern. Such rotations create two 'band-flip' motifs in the molecule, which is now described as 'butterfly-shaped'. The CA14 molecule also contains the 'band-flip' motifs and adopts a similar conformation. The central cavities of these CA molecules are likely to be too small to accommodate guest molecules. CA26 was also crystallised and its structure solved. This molecule is comprised of two antiparallel single helices of 12 glucoses each, connected by the 'band-flip' motifs at each end. The helices which have 6 glucoses per turn, contain central cavities with similar diameter to that of α-cyclodextrin, but are much longer (Saenger *et al.*, 1998). This suggests that such CAs could potentially accommodate guest molecules. This possibility has been tested by mixing CA with alcohols and fatty acids (*Figure 9*). It is clear that butanol, octanol and oleic acid can form inclusion complexes with a CA mixture containing molecules with DP greater than 50. The high solubility, non-reducing character and ability to form inclusion complexes suggest that CA has great potential for future applications.

Future prospects

4αGTases are important in α-1,4-glucan metabolism in organisms from all kingdoms. Furthermore, they are of applied value for the production of cycloglucans and for starch processing. There are several aspects which require more research in order to better understand their functions, and to exploit these enzymes. Only the structures of Type I enzymes have been determined at atomic resolution. Structural determination of other 4αGTases will be required in order to understand the reaction specificities of these enzymes, and to explain how they produce distinct cycloglucan products. Rational design of enzymes to produce defined products can then be initiated. A particular aim for starch processing will be to obtain thermostable enzymes with low hydrolytic activity and high reaction specificity. This aim may be achieved both by protein engineering, and by searching natural sources for new enzymes. The evolutionary relationships of different 4αGTases are of interest, but the origin of Type V enzymes is particularly fascinating, and studies of their properties should provide valuable new information. Systems for the preparation of cycloamyloses and cyclodextrins are required so that their properties and potential applications can be effectively investigated.

References

BAHL, H., BURCHHARDT, G., SPREINAT, A., HAECKEL, K., WIENECKE, A., SCHMIDT, B. AND ANTRANIKIAN, G. (1991). *Applied Environmental Microbiology* **57**, 1554–1559.

BINDER, F., HUBER, O. AND BOCK, A. (1986). *Gene* **47**, 269–277.

BLOCH, M.A. AND RAIBAUD, O. (1986). *Journal of Bacteriology* **168**, 1220–1227.

BOEL, E., BRANDY, L., BRZOZOWSKI, A.M., DEREWENDA, Z., DODSON, G.G., JENSEN, V.J., PETERSEN, S.B., SWIFT, H., THIM, L. AND WOLDIKE, H.F. (1990). *Biochemistry* **29**, 6244–6249.

BRAUN, C., LINDHORST, T., MADSEN, N.B. AND WITHERS, S. (1996). *Biochemistry* **35**, 5458–5463.

BRAYER, G.D., LUO, Y. AND WITHERS, S.G. (1995). *Protein Science* **4**, 1730–1742.

BROWN, D.H. AND ILLINGWORTH, B. (1962). *Proceedings of the National Academy of Sciences USA.* **48**, 1783–1787.

BUSSEY, H., STORMS, R.K., AHMED, A., ALBERMANN, K., ALLEN, E., ANSORGE, W., ARAUJO, R., APARICIO, A., BARRELL, B., BADCOCK, K., BENES,V., BOTSTEIN, D., BOWMAN,S., BRUCKNER, M., CARPENTER, J., CHERRY, J.M., CHUNG, E., CHURCHER, C., COSTER, F., DAVIS, K., DAVIS, R.W., DIETRICH, F.S., DELIUS, H., DIPAOLO, T., DUBOIS, E., DUSTERHOFT, A., DUNCAN, M., FLOETH, M., FORTIN, N., FRIESEN, J.D., FRITZ, C., GOFFEAU, A., HALL, J., HEBLING, U., HEUMANN, K., HILBERT, H., HILLIER, L., HUNICKE-SMITH, S., HYMAN, R., JOHNSTON, M., KALMAN, S., KLEINE, K., KOMP, C., KURDI, O., LASHKARI, D., LEW, H., LIN, A., LIN, D., LOUIS, E.J., MARATHE, R., MESSENGUY, F., MEWES, H.W., MIRTIPATI, S., MOESTL, D., MULLER-AUER, S., NAMATH, A., NENTWICH, U., OEFNER, P., PEARSON, D., PETEL, F.X., POHL, T.M., PURNELLE, D., SCHAFER, M., SCHARFE, M., SCHERENS, B., SCHRAMM, S., SCHROEDER, M., SDICU, A.M., TETTELIN, H., URRESTARAZU, L.A., USHINSKY, S., VIERENDEELS, F., VISSERS, S., VOSS, H., WALSH, S.V., WAMBUTT, R., WANG, Y., WEDLER, E., WEDLER, H., WINNETT, E., ZHONG, W.W., ZOLLNER, A., VO, D.H. AND HANI, J. (1997). *Nature* **387**, (6632 Suppl), 103–105.

COLE, S.T., BROSCH, R., PARKHILL, J., GARNIER, T., CHURCHER, C., HARRIS, D., GORDON, S.V., EIGLMEIER, K., GAS, S., BARRY III, C.E., TEKAIA, F., BADCOCK, K., BASHAM, D., BROWN, D., CHILLINGWORTH, T., CONNOR, R., DAVIES, R., DEVLIN, K., FELTWELL, T., GENTLES, S., HAMLIN, N., HOLROYD, S., HORNSBY, T., JAGELS, K., KROGH, A., MCLEAN, J., MOULE, S., MURPHY, L., OLIVER, S., OSBORNE, J., QUAIL, M.A., RAJANDREAM, M.A., ROGERS, J., RUTTER, S., SEEGER, K., SKELTON, S., SQUARES, S., SQARES, R., SULSTON, J.E., TAYLOR, K., WHITEHEAD, S. AND BARRELL, B.G. (1998). *Nature* **393**, 537–544.

DECKERT, G., WARREN, P.V., GAASTERLAND, T., YOUNG, W.G., LENOX, A.L., GRAHAM, D.E., OVERBEEK, R., SNEAD, M.A., KELLER, M., AUJAY, M., HUBER, R., FELDMAN, R.A., SHORT, J.M., OLSON, G.J. AND SWANSON, R.V. (1998). *Nature* **392**, 353–358.

FEEDERLE, R., PAJATSCH, M., KREMMER, E. AND BOCK, A. (1996). *Archives of Microbiology* **165**, 206–212.

FIEDLER, G., PAJATSCH, M. AND BOCK, A. (1996). *Journal of Molecular Biology* **256**, 279–291.

FLEISCHMANN, R.D., ADAMS, M.D., WHITE, O., CLAYTON, R.A., KIRKNESS, E.F., KERLAVAGE, A.R., BULT, C.J., TOMB, J-F., DOUGHERTY, B.A., MERRICK, J.M., MCKENNEY, K., SUTTON, G., FITZHUGH, W., FIELDS, C.A., GOCAYNE, J.D., SCOTT, J.D., SHIRLEY, R., LIU, L-I., GLODEK, A., KELLEY, J.M., WEIDMAN, J.F., PHILLIPS, C.A., SPRIGGS, T., HEDBLOM, E., COTTON, M.D., UTTERBACK, T.R., HANNA, M.C., NGUYEN, D.T., SAUDEK, D.M., BRANDON, R.C., FINE, L.D., FRITCHMAN, J.L., FUHRMANN, J.L., GEOGHAGEN, N.S.M., GNEHM, C.L., MCDONALD, L.A., SMALL, K.V., FRASER, C.M., SMITH, H.O. AND VENTER, J.C (1995). *Science* **269**, 496–512.

FRASER, C.M., CASJENS, S., HUANG, W.M., SUTTON, G.G., CLAYTON, R.A., LATHIGRA, R., WHITE, O., KETCHUM, K.A., DODSON, R., HICKEY, E.K., GWINN, M., DOUGHERTY, B., TOMB, J.-F., FLEISCHMANN, R.D., RICHARDSON, D., PETERSON, J., KERLAVAGE, A.R., QUACKENBUSH, J., SALZBERG, S., HANSON, M., VAN-VUGT, R., PALMER, N., ADAMS, M.D., GOCAYNE, J.D., WEIDMAN, J., UTTERBACK, T., WATTHEY, L., MCDONALD, L., ARTIACH, P., BOWMAN, C., GARLAND, S., FUJII, C., COTTON, M.D., HORST, K., ROBERTS, K., HATCH, B., SMITH, H.O. AND VENTER, J.C. (1997). *Nature* **390**, 580–586.

FRENCH, D. (1957). *Advances in Carbohydate Chemistry* **12**, 189–260.

FUKUSUMI, S., KAMIZONO, A., HORINOUCHI, S. AND BEPPU, T (1988). *European Journal of Biochemistry* **174**, 15–21.

GODA, S.K., EISSA, O., AKHTAR, M. AND MINTON, N.P. (1997). *Microbiology* **143**, 3287–3294.

GUNJA-SMITH, Z., MARSHALL, J.J., MERCIER, C., SMITH, E.E. AND WHELAN, W.J. (1970). *FEBS Letters* **12**, 101–104.

HARATA, K., HAGA, K., NAKAMURA, A., AOYAGI, M. AND YAMANE, K. (1996). *Acta Cryst.* **D52**, 1136–1145.

HEINRICH, P., HUBER, W. AND LIEBL,W. (1994). *Syst. Applied. Microbiology* **17**, 297–305.

HIZUKURI, S. (1986). *Carbohydrate Research* **147**, 342–347.

HIZUKURI, S. (1996). Starch: Analytical aspects. In *Carbohydrates in Food*. Ed. A. Eliasson, pp 347–429. New York: Marcel Dekker, Incorporated.

HORINOUCHI, S., FUKUSUMI, S., OHSHIMA, T. AND BEPPU, T. (1988). *European Journal of Biochemistry* **176**, 243–253.

HSIA, R.C., PANNEKOEK, Y., INGEROWSKI, E. AND BAVOIL, P.M. (1997). *Molecular Microbiology* **25**, 351–359.

JACOB, J., GESSLER, K., HOFFMANN, D., SANBE, H., KOIZUMI, K., SMITH, S.M., TAKAHA, T. AND SAENGER, W. (1998). *Angewandte Chemie International Edition* **37**, 606–609.

JEON, B.S., TAGUCHI, H., SAKAI, H., OHSHIMA, T., WAKAGI, T. AND MATSUZAWA, H. (1997). *European Journal of Biochemistry* **248**, 171–178.

JONES, G. AND WHELAN, W. J. (1969). *Carbohydate Research* **9**, 483–490.

JORGENSEN, S.T., TANGNEY, M., STARNES, R.L., AMEMIYA, K. AND JORGENSEN, P.L. (1997). *Biotechnology Letters* **19**, 1027–1031.

KADZIOLA, A., ABE, J., SVENSSON, B. AND HASER, R. (1994). *Journal of Molecular Biology* **239**, 104–121.

KAKEFUDA, G., DUKE, S.H. AND HOSTAK, M.S. (1986). *Planta* **168**, 175–182.

KANEKO, T., SATO, S., KOTANI, H., TANAKA, A., ASAMIZU, E., NAKAMURA, Y., MIYAJIMA, N., HIROSAWA, M., SUGIURA, M., SASAMOTO, S., KIMURA, T., HOSOUCHI, T., MATSUNO, A., MURAKI, A., NAKAZAKI, N., NARUO, K., OKUMURA, S., SHIMPO, S., TAKEUCHI, C., WADA, T., WATANABE, A., YAMADA, M., YASUDA, M. AND TABATA, S. (1996). *DNA Research* **3**, 109–136.

KATO, T. AND HORIKOSHI, K. (1986). *Journal of the Japanese Society of Starch Science* **33**, 137–143.

KITAHATA, S. (1995). In *Enzyme Chemistry and Molecular Biology of Amylases and Related Enzymes*. Ed. The Amylase Research Society of Japan, pp 6–17. Boca Raton (Florida): CRC Press.

KITAHATA, S. AND OKADA, S. (1982). *Journal of the Japanese Society of Starch Science* **29**, 13–18.

KITAHATA, S., MURAKAMI, H. AND OKADA S. (1989a). *Agricultural Biological Chemistry* **53**, 2653–2659.

KITAHATA, S., MURAKAMI, H., SONE, Y. AND MISAKI, A. (1989b). *Agricultural Biological Chemistry* **53**, 2661–2666.

KLEIN, C. AND SCHULZ, G.E. (1991). *Journal of Molecular Biology* **217**, 737–750.

KLEIN, C., HOLLENDER, J., BENDER, H. AND SCHULZ, G.E. (1992). *Biochemistry* **31**, 8740–8746.

KNEGTEL, R.M., STROKOPYTOV, B., PENNINGA, D., FABER, O.G., ROZEBOOM, H.J., KALK, K.H., DIJKHUIZEN, L. AND DIJKSTRA, B.W. (1995). *Journal of Biological Chemistry* **270**, 29256–29264.

KNEGTEL, R.M., WIND, R.D., ROZEBOOM, H.J., KALK, K.H., BUITELAAR, R.M., DIJKHUIZEN, L. AND DIJKSTRA, B.W. (1996). *Journal of Molecular Biology* **256**, 611–622.

KOBAYASHI, S. (1996). In *Enzymes for Carbohydrate Engineering*. Eds. K.H. Park, J.F. Robyt, and Y.-D. Choi, pp 23–41. Amsterdam: Elsevier Science.

KUBOTA, M., MATSUURA, Y., SAKAI, S. AND KATSUBE, Y. (1991). *Denpun Kagaku* **38**, 141–146.

KUBOTA, M., MATSUURA, Y., SAKAI, S. AND KATSUBE, Y. (1994). *Oyo Toshitu Kagaku* **41**, 245–253.

LACKS, S.A., DUNN, J.J. AND GREENBERG, B. (1982). *Cell* **31**, 327–336.

LADERMAN, K.A., DAVIS, B.R., KRUTZSCH, H.C., LEWIS, M.S., GRIKO, Y.V., PRIVALOV, P.L. AND ANFINSEN, C.B. (1993a). *Journal of Biological Chemistry* **268**, 24394–24401.

LADERMAN, K.A., ASADA, K., UEMORI, T., MUKAI, H., TAGUCHI, Y., KATO, I. AND ANFINSEN, C.B. (1993b). *Journal of Biological Chemistry* **268**, 24402–24407.

LAWSON, C.L., VAN MONTFORT, R., STROKOPYTOV, B., ROZEBOOM, H.J., KALK, K.H., DE VRIES, G.E., PENNINGA, D., DIJKHUIZEN, L. AND DIJKSTRA, B.W. (1994). *Journal of Molecular Biology* **236**, 590–600.

LEE, E.Y.C. AND CARTER, J.H. (1973). *Archives of Biochemistry and Biophysics* **154**, 636–641.

LIEBL, W., FEIL, R., GABELSBERGER, J., KELLERMANN, J. AND SCHLEIFER, K.H. (1992). *European Journal of Biochemistry* **207**, 81–88.

LILLFORD, P.J. AND MORRISON, A. (1997). In *Starch: structure and functionality*. Eds. P.J. Frazier, P. Richmond, and A.M. Donald, pp 1–8. Cambridge: The Royal Society of Chemistry.

LIN, T. P. AND PREISS, J. (1988). *Plant Physiology* **86**, 260–265.

LIU, W., MADSEN, N.B., BRAUN, C. AND WITHERS, S. (1991). *Biochemistry* **30**, 1419–1424.

LIU, W., DE CASTRO, M.L., TAKRAMA, J., BILOUS, P.T., VINAYAGAMOORTHY, T., MADSEN, N.B. AND BLEACKLEY, R.C. (1993). *Archives of Biochemistry and Biophysics* **306**, 232–239.

MACHIUS, M., WIEGAND, G. AND HUBER, R. (1995). *Journal of Molecular Biology* **246**, 545–559.

MANNERS, D.J. (1991). *Carbohydrate Polymers* **16**, 37–82.

MANNERS, D.J. AND ROWE, K. L. (1969). *Carbohydrate Research* **9**, 441–450.

MATSUURA, Y., KUSUNIKI, M., HARADA, W. AND KAKUDO, M. (1984). *Journal of Biochemistry* (Tokyo) **95**, 697–702.

MEDDA, S. AND SMITH, E.E. (1984). *Analytical Biochemistry* **138**, 354–359.

METZ, R.J., ALLEN, L.N., CAO, T.M. AND ZEMAN, N.W. (1988). *Nucleic Acids Research* **16**, 5203.

MONOD, J. AND TORRIANI, A.M. (1948). *Compt. Rend.* **227**, 240.

MORI, S., GOTO, M., MASE, T., MATSUURA, A., OYA, T. AND KITAHATA, S (1995). *Bioscience, Biotechnology and Biochemistry* **59**, 1012–1015.

MORISHITA, Y., HASEGAWA, K., MATSUURA, Y., KATSUBE, Y., KUBOTA, M. AND SAKAI, S. (1997). *Journal of Molecular Biology* **267**, 661–672.

MOSI, R., HE, S., UITDEHAAG, J., DIJKSTRA, B.W. AND WITHERS, S.G. (1997). *Biochemistry* **36**, 9927–9934.

NAKAMURA, A., HAGA, K., OGAWA, S., KUWANO, S., KIMURA, K. AND YAMANE, K. (1992). *FEBS Letters* **296**, 37–40.

NAKAMURA, A., HAGA, K. AND YAMANE, K. (1993). *Biochemistry* **32**, 6624–6631.

NAKAMURA, A., HAGA, K. AND YAMANE, K. (1994). *Biochemistry* **33**, 9929–9936.

NELSON, T.E., WHITE, R.C. AND GILLARD, B.K. (1979). *American Chemical Society Symposium Series* **88**, 131–162.

OKITA, T.W., GREENBERG, E., KUHN, D.N. AND PREISS, J. (1979). *Plant Physiology* **64**, 187–192.

PAJATSCH, M., GERHART, M., PEIST, R., HORLACHER, R., BOOS, W. AND BOCK, A. (1998). *Journal of Bacteriology* **180**, 2630–2635.

PALMER, T.N., RYMAN, B.E. AND WHELAN, W.J. (1976). *European Journal of Biochemistry* **69**, 105–115.

PAZUR, J.H. AND OKADA, S. (1968). *Journal of Biological Chemistry* **243**, 4732–4738.

PEAT, S., WHELAN, W.J AND REES, W.R. (1956). *Journal of the Chemical Society*, 44–53.

PENNINGA, D., STROKOPYTOV, B., ROZEBOOM, H.J., LAWSON, C.L., DIJKSTRA, B.W., BERGSMA, J. AND DIJKHUIZEN, L. (1995). *Biochemistry* **34**, 3368–3376.

PUGSLEY, A.P. AND DUBREVIL, C. (1988). *Molecular Microbiology* **2**, 473–479.

QIAN, M., HASER, R. AND PAYAN, F. (1993). *Journal of Molecular Biology* **231**, 785–799.

RAMASUBBU, N., PALOTH, V., LUO, Y., BRAYER, G.D. AND LEVINE, M.J. (1996). *Acta Crystallogr.* **D52**, 435–446.

RENDLEMAN, JUNIOR, J.A (1993). *Carbohydrate Research* **247**, 223–237.

SAENGER, W., JACOB, J., GESSLER, K., STEINER, T., HOFFMANN, D., SANBE, H., KOIZUMI, K., SMITH, S.M. AND TAKAHA, T. (1998). *Chemical Reviews* **98**, 1787–1802.

SANDHYA RANI, M.R., SHIBANUMA, K. AND HIZUKURI, S. (1992). *Carbohydrate Research* **227**, 183–194.

SCHMID, G. (1989). *Trends in Biotechnology* **7**, 244–248.

SCHMIDT, J. AND JOHN, M. (1979). *Biochimica Biophysica Acta* **566**, 100–114.

SCHMIDT, A.K., COTTAZ, S., DRIGUEZ, H. AND SCHULZ, G.E. (1998). *Biochemistry* **37**, 5909–5915.

SCHWARTZ, M. (1987). In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*. Ed. F.C. Neidhardt, pp 1482–1502. Washington DC: American Society for Microbiology.

STARNES, R.L. (1990). *Cereal Food World* **35**, 1091–1099.

STROKOPYTOV, B., PENNINGA, D., ROZEBOOM, H.J., KALK, K.H., DIJKHUIZEN, L. AND DIJKSTRA, B.W. (1995). *Biochemistry* **34**, 2234–2240.

STROKOPYTOV, B., KNEGTEL, R.M., PENNINGA, D., ROZEBOOM, H.J., KALK, K.H., DIJKHUIZEN, L. AND DIJKSTRA, B.W. (1996). *Biochemistry* **35**, 4241–4249.

SUGANUMA, T., SETOGUCHI, S., FUJIMOTO, S. AND NAGAHAMA, T. (1991). *Carbohydrate Research* **212**, 201–212.

SVENSSON, B. (1994). *Plant Molecular Biology* **25**, 141–157.

SZEJTLI, J. (1988). *Cyclodextrin Technology*. The Netherlands: Kluwer Academic Publishers, Dordrecht.

TABATA, S. AND HIZUKURI, S. (1992). *European Journal of Biochemistry* **206**, 345–348.

TABATA, S. AND IDE, T. (1988). *Carbohydrate Research* **176**, 245–251.

TABATA, S., NAKAYAMA, A. AND HIZUKURI, S. (1995). *Oyo Toshitu Kagaku* **42**, 193–202.

TACHIBANA, Y., FUJIWARA, S., TAKAGI, M. AND IMANAKA, T (1997). *Journal of Fermentation Bioengineering* **83**, 540–548.

TAKAHA, T., YANASE, M., OKADA, S. AND SMITH, S.M. (1993). *Journal of Biological Chemistry* **268**, 1391–1396.

TAKAHA, T., YANASE, M., TAKATA, H., OKADA, S. AND SMITH, S.M. (1996). *Journal of Biological Chemistry* **271**, 2902–2908.

TAKAHA, T., CRITCHLEY, J., OKADA, S. AND SMITH S.M. (1998a). *Planta* **205**, 445–451.

TAKAHA, T., YANASE, M., TAKATA, H., OKADA, S. AND SMITH, S.M. (1998b). *Biochemical and Biophysical Research Communications* **247**, 493–497.

TAKATA, H., TAKAHA, T., OKADA, S., HIZUKURI, S., TAKAGI, M. AND IMANAKA, T. (1996). *Carbohydrate Research* **295**, 91–101.

TAKATA, H., TAKAHA, T., NAKAMURA, H., FUJII, K., OKADA, S., TAKAGI, M. AND IMANAKA, T. (1997). *Journal of Fermentation Bioengineering* **84**, 119–123.

TERADA, Y., YANASE, M., TAKATA, H., TAKAHA, T. AND OKADA, S. (1997). *Journal of Biological Chemistry* **272**, 15729–15733.

WALKER, G.J. (1965). *Biochemical Journal* **94**, 299–308.

WALKER, G.J. (1966). *Biochemical Journal* **101**, 861–872.

WILSON, R., AINSCOUGH, R., ANDERSON, K., BAYNES, C., BERKS, M., BONFIELD, J., BURTON, J., CONNELL, M., COPSEY, T., COOPER, J., COULSON, A., CRAXTON, M., DEAR, S., DU, Z., DURBIN, R., FAVELLO, A., FULTON, L., GARDNER, A., GREEN, P., HAWKINS, T., HILLIER, L., JIER, M., JOHNSTON, L., JONES, M., KERSHAW, J., KIRSTEN, J., LAISTER, N., LATREILLE, P., LIGHTNING, J., LLOYD, C., MCMURRAY, A., MORTIMORE, B., O'CALLAGHAN, M., PARSONS, J., PERCY, C., RIFKEN, L., ROOPRA, A., SAUNDERS, D., SHOWNKEEN, R., SMALDON, N., SMITH, A., SONNHAMMER, E., STADEN, R., SULSTON, J., THIERRY-MIEG, J., THOMAS, K., VAUDIN, M., VAUGHAN, K., WATERSTON, R., WATSON, A., WEINSTOCK, L., WILKINSON-SPROAT, J. AND WOHLDMAN, P. (1998). *Nature* **368**, 32–38.

WIND, R.D., BUITELAAR, R.M. AND DIJKHUIZEN, L. (1998). *European Journal of Biochemistry* **253**, 598–605.

YANG, B.Z., DING, J.H., ENGHILD, J.J., BAO, Y. AND CHEN, Y.T. (1992). *Journal of Biological Chemistry* **267**, 9294–9299.

YOSHIO, N., MAEDA, I., TANIGUCHI, H. AND NAKAMURA, M. (1986). *Journal of the Japanese Society of Starch Science* **33**, 244–252.