

Biochemical properties and structural features of the thermostable maltodextrin transglycosidases from *Thermotoga maritima*

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Maltosyltransferase (MTase) is an extremely thermostable enzyme which, based on its primary structure, is classified into glycoside hydrolase family 13. The enzyme is a non-hydrolytic transglycosidase (maltodextrin glycosyltransferase, MGTase) which catalyses the transfer of maltosyl units from α -1,4-linked glucans or malto-oligosaccharides to other α -1,4-linked glucans, malto-oligosaccharides or glucose. MTase represents the first exo-MGTase known. To date, the only organism known to produce a starch-converting enzyme with this unique reaction chemistry is the hyperthermophilic bacterium *Thermotoga maritima*, a strictly anaerobic heterotroph with a maximum growth temperature of 90 °C. In addition to MTase, *T. maritima* possesses a second MGTase, 4- α -glucanotransferase (GTase), also a member of the glycoside hydrolase family 13. In contrast to MTase, GTase displays a broad transfer specificity. Recently, crystals of recombinant MTase and GTase have been obtained by the hanging-drop vapor-diffusion method, and the crystal structures of MTase and its complex with maltose have been determined at 2.4 and 2.1 Å resolution, respectively. In this communication, the enzymatic characteristics of MTase and GTase are reviewed, and structural features, possibly of importance for the unique transfer specificity and thermostability of MTase, are discussed.

Key words: *Thermotoga maritima*, transglycosidase, maltosyltransferase, 4- α -glucanotransferase, amylase, crystal structure, transfer specificity.

Abbreviations: AmyA, α -amylase; glycoside hydrolase family, GHF; GTase, 4- α -glucanotransferase; MGTase, maltodextrin glycosyltransferase; MTase, maltosyltransferase.

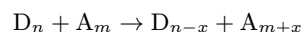
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Amylolytic enzymes of *Thermotoga maritima*

Phylogenetic analyses based on 16S rRNA sequence data indicate that hyperthermophilic microorganisms (i. e. those with an optimal growth temperature of 80 °C or higher) represent the deepest and shortest branches in the domains *Archaea* and *Bacteria* (STETTER, 1999), suggesting that they could have retained some archaic characteristics. The biomolecules, cell structures and metabolism of these organisms are adapted to withstand extremes of temperature (STERNER & LIEBL, 2001). Most of the approximately 70 species of hyperthermophiles described to date are archaea. *Thermotoga maritima* is one of very few hyperthermophilic bacteria currently known and represents a model organism for the study of this group of organisms. This species is a strictly anaerobic obligate heterotroph with the ability to utilize fermentatively various organic compounds, including some polysaccharides like xylan or starch (LIEBL et al., 1996; LIEBL, 1999; 2001). A number of *T. maritima* genes and the corresponding enzymes (putatively) involved in starch breakdown and utilization or starch or maltodextrins conversion have been characterized in the past few years (see Table 1). In addition to the enzymes listed in Table 1, further genes for putative amylolytic enzymes have been identified in the genome sequence of *T. maritima* (NELSON et al., 1999), but information about the properties of these proteins is not available yet. Also, some information is available for certain amylolytic enzymes from *T. neapolitana* (BEREZINA et al., 1999), a hyperthermophilic species closely related to *T. maritima*.

Biochemical properties of the *T. maritima* maltodextrin glycosyltransferases

Maltodextrin glycosyltransferases (MGTases) are enzymes that transfer segments of malto-oligosaccharides or α -1,4-linked glucans (Donors) to other α -1,4-linked glucans, malto-oligosaccharides, or glucose (Accceptors), according to the general scheme:



T. maritima strain MSB8 contains genes for two distinct MGTases, i. e. 4- α -glucanotransferase (GTase) and maltosyltransferase (MTase). Both genes have been cloned, expressed in *E. coli*, and sequenced (HEINRICH et al., 1994; MEISSNER & LIEBL, 1998), and the corresponding enzymes have been characterized (LIEBL et al., 1992; HUBER & LIEBL, 1994; MEISSNER & LIEBL, 1998). The MGTases differ with respect to various properties, like size, pH optimum, calcium dependence, resistance to thermoinactivation, etc. (Tab. 2).

One of the most striking differences between MTase and GTase lies in their transfer specificities. GTase is a MGTase with a broad transfer specificity, similar to MGTases found in many bacteria and archaea, e. g. in *E. coli* and species of diverse genera like *Streptococcus*, *Bacillus*, *Clostridium*, *Haemophilus*, *Thermus*, *Thermococcus*, etc. These enzymes play a role either as catabolic enzymes of starch/maltodextrin utilization or as enzymes of storage polysaccharide metabolism (BOOS & SCHUMAN, 1998; TAKAHA & SMITH, 1999; XAVIER et al., 1999). In plants, 4- α -glucanotransferase (D-enzyme) ap-

Table 1. Amylolytic enzymes from *Thermotoga maritima*.^a

| | | Size ^b | pH _{opt} | 'T _{opt} ' ^c | Reference |
|------|---|-------------------|-------------------|----------------------------------|----------------------------|
| AmyA | α -Amylase | 63 | 7.0 | 85–90 | LIEBL et al., 1997 |
| PulA | Pullulanase | 96 | 6.0 | 90 | KRIEGSHÄUSER & LIEBL, 2000 |
| MgtA | 4- α -Glucanotransferase (GTase) | 53 | 7.5 | 70 | LIEBL et al., 1992 |
| MmtA | Maltosyltransferase (MTase) | 2 × 74 | 6.5 | 85–90 | MEISSNER & LIEBL, 1998 |
| AgIA | α -Glucosidase | 52 | 7.0 | 80 | RAASCH et al., 2000 |
| AgpA | Maltodextrin phosphorylase | 96 | ? | ? | BIBEL et al., 1998 |
| MalE | Maltodextrin binding protein | 41 | n.a. | n.a. ^d | WASSENBERG et al., 2000 |

^a The enzymes listed have been expressed as recombinant proteins and have been (partially) characterized. The genes for further putative amylolytic enzymes and transport proteins have been cloned or were postulated from the genome sequence (NELSON et al., 1999; LIEBL et al., unpublished) but were not studied in detail.

^b Size in kDa.

^c 'T_{opt}' in °C measured in 10–20 min assays.

^d stable for > 2 h at 100 °C (far-UV-CD); n. a., not applicable.

Table 2. Comparison of 4- α -glucanotransferase (GTase) and maltosyltransferase (MTase) properties.

| Property | GTase | MTase |
|------------------------------------|---------------------|---------------------|
| Size | 1 \times 51.8 kDa | 2 \times 73.7 kDa |
| pH _{opt} | 7.5 | 6.5 |
| 'T _{opt} ' (10 min assay) | 70 °C | 85–90 °C |
| t ^{1/2} (80 °C) | 3 h | 36 h |
| Inhibition by EDTA | yes | no |
| DP ^a | ≥ 2 | strictly 2 |

^a Size of transferred dextrinyl segment.

parently plays a role in starch breakdown (CRITCHLEY et al., 2001). *T. maritima* GTase transfers maltodextrinyl segments as small as maltotri-syl or maltosyl units but also very large ones (see LIEBL et al., 1992; HUBER & LIEBL, 1994). MTase, on the other hand, has a strict transfer specificity, i. e. the enzyme can only transfer maltosyl units (MEISSNER & LIEBL, 1998). Malto-oligosaccharides with a given length DP X are disproportionated by MTase to a set of products with DP $X \pm 2n$ ($n = 0, 1, 2, 3, \dots$). Thus, the products of disproportionation of an odd-numbered substrate (e. g., $X = 5$) by MTase are exclusively odd-numbered malto-oligosaccharides (DP = 1, 3, 5, 7, 9, 11, \dots), while incubation of MTase with an even-numbered substrate (e. g., $X = 6$) yields exclusively even-numbered products (DP = 2, 4, 6, 8, 10, 12, \dots). A similar transfer specificity has not been described for any other MGTase before.

Therefore, MTase represents the first ex-acting MGTase known. To date, *T. maritima* is the only organism described to have an enzyme of this type. Interestingly, extensive incubation of the enzyme with amylose or amylopectin in the absence of any low-molecular mass acceptor gives rise principally to the production of a series of even-numbered small malto-oligosaccharides (Fig. 1). In this case MTase presumably releases some maltose from the polysaccharides via weak hydrolytic activity (i. e. water acts as an acceptor for maltosyl transfer to a limited extent), which subsequently serves as the acceptor for further transfer reactions. The role of MTase in the metabolism of *T. maritima* is still unclear.

Primary structure characteristics of the *T. maritima* MGTases

MGTases (4- α -glucanotransferases, amylomalta-ses) or genes for these enzymes have been reported to occur in many phylogenetically diverse

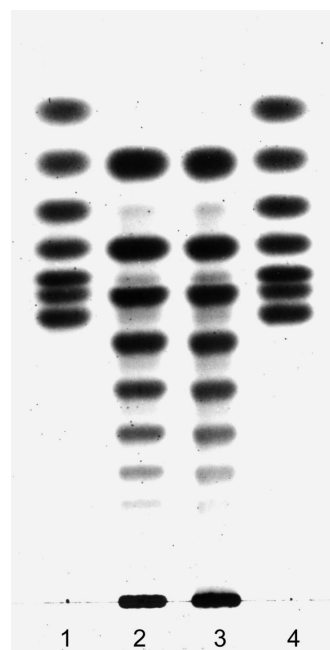


Fig. 1. Thin layer chromatography analysis of products formed by MTase after extended incubation with amylose and amylopectin. Reaction mixtures, containing 0.25% polysaccharide substrate, 8 μ g/mL MTase, and McIlvaine buffer pH 6.5 were incubated for 16 h at 75 °C. Lanes 1 and 4, 2 μ L malto-oligosaccharide standard solution containing glucose (G1) through maltoheptaose (G7) (0.1% [w/v] each); lane 2, 10 μ L products obtained with amylose as the substrate; lane 3, 10 μ L products obtained with amylopectin as the substrate.

organisms of the three domains *Archaea*, *Bacteria*, and *Eucarya*. Based on their primary structures, the MGTases have been classified into three glycoside hydrolase families (GHFs): GHF 13, GHF 57, and GHF 77 (see <http://afmb.cnrs-mrs.fr/CAZY/GH.html>). The majority of these enzymes are members of GHF 77. In contrast, interestingly, the MGTases of *Thermotoga* (i. e. both *T. maritima* enzymes MTase and GTase, and the *T. neapolitana* 4- α -glucanotransferase) belong to GHF 13 and represent the only MGTases in this enzyme family. Finally, two MGTases, the 4- α -glucanotransferases of *Thermococcus litoralis* and *Pyrococcus kodakaraensis*, have been assigned to GHF 57 (TACHIBANA et al., 1997; JEON et al., 1997). It should be noted that the enzymes of GHF 77 share limited sequence similarity with GHF 13 (HEINRICH et al., 1994). The members of GHF 57 are not obviously related to GHF 13 or GHF 77

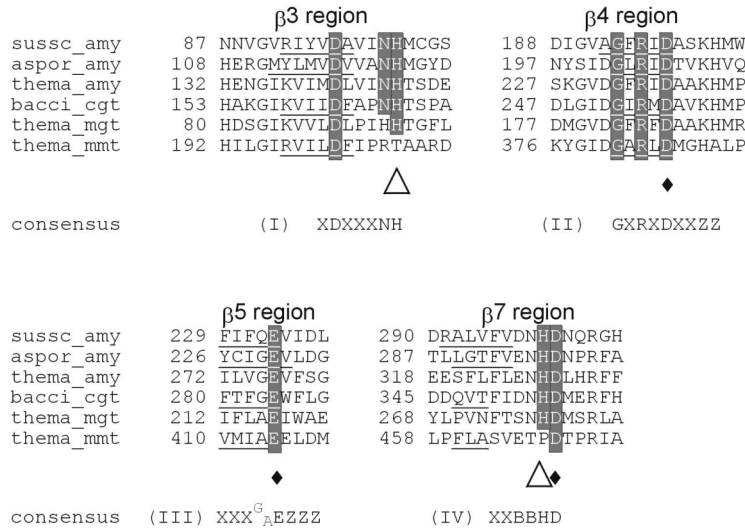


Fig. 2. Amino acid sequence alignment of conserved regions in representative hydrolases and transferases of the α -amylase enzyme family. Some highly conserved or invariant residues are emphasized by dark boxes. The numbering of the regions corresponds to the β -strands of the $(\beta/\alpha)_8$ -barrel of the α -amylase family. For enzymes with known crystal structure, the sequences comprising the β -strands of the $(\beta/\alpha)_8$ -barrel are underlined. The three catalytic acidic residues are marked with diamond symbols. Two highly conserved His residues involved in substrate binding in most GHF 13 enzymes are marked with open triangle symbols. The ‘consensus’ line shows the conserved motifs as described by MACGREGOR et al. (2001), with X meaning usually hydrophobic, B meaning usually hydrophilic, and Z standing for residues important for specificity. Abbreviations (accession numbers in brackets): sussc_amy, pig pancreatic α -amylase (SwissProt: P00690); aspor_amy, *Aspergillus oryzae* amylase Taka-amylase A (SwissProt: P10529); them_a_amy, *Thermotoga maritima* α -amylase AmyA (TrEMBL: P96107); bacci_cgt, *Bacillus circulans* cyclodextrin glycosyltransferase (SwissProt: P43379); them_a_mgt, *T. maritima* 4- α -glucanotransferase (GTase) (SwissProt: P80099); them_a_mmt, *T. maritima* maltosyltransferase (MTase) (EMBL: AJ001090).

and do not share the highly conserved sequence motifs of GHF 13 shown in Figure 2, although a possible distant relationship between families 57 and 13 has been discussed (JANECEK, 1998).

All enzymes of the ‘ α -amylase family’ (comprising GHFs 13, 70 and 77; for an overview see JANECEK, 1997; MACGREGOR et al., 2001) possess a central super-secondary structure motif which represents a $(\beta/\alpha)_8$ -barrel. This $(\beta/\alpha)_8$ -barrel (domain A) is interrupted between β -strand 3 and α -helix 3 by an inserted domain (domain B) with variable size and low crosswise similarity. Generally, one or more C-terminal domains are present, but only a limited number of GHF 13 enzymes carry additional N-terminal domains.

The domain structure of the *T. maritima* GHF 13 enzymes α -amylase (AmyA), GTase and MTase can be predicted from their primary structures, and in the case of MTase the predicted domain architecture (MEISSNER & LIEBL, 1998) was recently verified and defined more precisely by solving the enzyme’s three-dimensional structure

(ROUJEINIKOVA et al., 2001a). Figure 3 shows a comparison of the domain organisation of MTase with several starch hydrolases and related enzymes. Remarkably, the size of domain B of MTase (about 155 residues) is larger than most other B-domains (typically about 40 to 130 residues; JESPERSEN et al., 1991). The B domains of the other GHF 13 enzymes of *T. maritima*, GTase and AmyA, both have predicted sizes of about 70 residues. The core (domains A1-B-A2) of MTase is flanked by two additional domains. The sequences of the N-terminal and C-terminal domains of MTase lack significant amino acid sequence similarity with known protein sequences.

The conserved sequence regions typical for the α -amylase family can be found in the GHF 13 enzymes of *T. maritima* (Fig. 2). Strikingly, MTase lacks two histidine residues that are highly conserved throughout the family and, in the other GHF 13 enzymes, are important for substrate binding. In addition, the MTase sequence regions C-terminal to β -strands 4 and 5 of the $(\beta/\alpha)_8$ -

barrel of domain A (residues 207-209 and 231-234 in Taka-amylase A numbering), which are thought to be of relevance for the reaction specificity of the α -amylase family enzymes (MACGREGOR et al., 2001), are not similar to the signatures of typical α -amylases or 4- α -glucanotransferases.

Crystal structure of MTase and implications for its catalytic mechanism

X-ray analysis of the structure of MTase provides the basis for coming to an understanding of its reaction mechanism and of features at the molecular level contributing to the resistance of MTase against thermoinactivation. Crystals of recombinant MTase have been obtained by the hanging-drop vapor-diffusion method using ammonium phosphate as a precipitating agent at pH 4.8 (BURKE et al., 2000), and a MTase-maltose crystal complex was obtained by co-crystallization with maltose. The structures of MTase and its maltose complex were solved at 2.4 and 2.1 Å resolution, respectively (ROUJEINIKOVA et al., 2001a). Each subunit of the homo-dimer consists of four domains: domain N (about 70 residues), the $(\beta/\alpha)_8$ -barrel-domain A (about 350 residues), which carries domain B (about 155 residues) inserted between its 3rd β -strand and 3rd α -helix, and domain C (about 65 residues).

Domain N of MTase contains two α -helices connected by a three-stranded antiparallel β -sheet, and its α -helices are located at the outside of, and interact with, the first two α -helices of the $(\beta/\alpha)_8$ -barrel structure of domain A (helices $\alpha 5$ and $\alpha 6$ in MTase numbering) via hydrophobic interactions. It is not known if domain N is important for MTase activity or specificity, but this domain is not situated in the vicinity of the active site. However, the N-terminal domain does contribute to the inter-subunit interaction of the MTase dimer (ROUJEINIKOVA et al., 2001a). For other GHF 13 enzymes, like isoamylase and the dimeric *Thermus* maltogenic amylase, a role in active site formation and an indirect role for enzyme specificity (through interaction with the active site region of the other monomer), respectively, have been discussed (KATSUYA et al., 1998; KIM et al., 1999). The C-terminal domain of MTase, like the C-terminal domains of most other enzymes of the α -amylase family, is made up of β -strands. This domain is comprised of eight antiparallel β -strands which form an open-sided "jelly roll" Greek key β -barrel. A remote similarity between the folds of this domain of MTase and family III cellulose-binding domains has been noted (ROUJEINIKOVA

et al., 2001a), but there is no experimental evidence in support of carbohydrate-binding by the C-terminal part of MTase.

Generally, in GHF 13 enzymes, substrate binding takes place in a cleft formed by domains A and B. Residues of both these domains participate in substrate binding. The three catalytically-essential acidic residues are located at the C-terminal ends of three β -strands of the $(\beta/\alpha)_8$ -barrel of domain A. In MTase these catalytic residues, which are conserved in the primary structures of all enzymes of the α -amylase family (see Fig. 2), are Glu414 (proton donor, protonates the glycosidic oxygen), Asp385 (nucleophile, for nucleophilic attack at C1), and Asp468 (catalytic base, thought to assist in attack of incoming acceptor) (MEISSNER & LIEBL, 1998). In the crystal structure of MTase the arrangement of the catalytic residues and sugar binding at the -1 subsite were found to be similar to those in other GHF 13 enzymes of known crystal structure, but the side chain of Asp385 takes a position that is not in accordance with its proposed function as the catalytic nucleophile. However, this is presumably the result of an experimental artifact caused by the low pH of crystallization (ROUJEINIKOVA et al., 2001a). Strikingly, MTase lacks two highly conserved histidine residues present in most other GHF 13 enzymes (see above, and Figure 2; His122 and His296 in Taka-amylase A numbering) where they play a role at the -1 substrate-binding subsite. His122 of Taka-amylase A and the equivalent histidine of other GHF 13 enzymes forms a hydrogen bond with the O6 atom of the sugar at the -1 subsite (QIAN et al., 1994; BRZOZOWSKI & DAVIES, 1997; UITDEHAAG et al., 1999). The substitute for this histidine in MTase, Thr206, is not directly involved in maltose binding in the MTase-maltose crystal structure. However, Thr206 is hydrogen bonded to a water molecule (Wat63) which itself forms a hydrogen bond to the O6 atom of the glucose unit bound at the -1 subsite. The other conserved histidine (at position 296 of Taka-amylase A, equivalent to Pro467 of MTase) has been implicated in hydrogen bonding to the substrate at the -1 subsite in some GHF 13-acarbose complexes (QIAN et al., 1994; STROKOPYTOV et al., 1995). In the Taka-amylase A-acarbose complex, on the other hand, His296 is thought to hydrogen bond to Tyr82, thereby stabilizing the aromatic stacking of Tyr82 over the -1 subsite (BRZOZOVSKI & DAVIES, 1997). Pro467 in MTase can not fulfill a similar role.

Some positions in the conserved regions of enzymes of the α -amylase family following β -

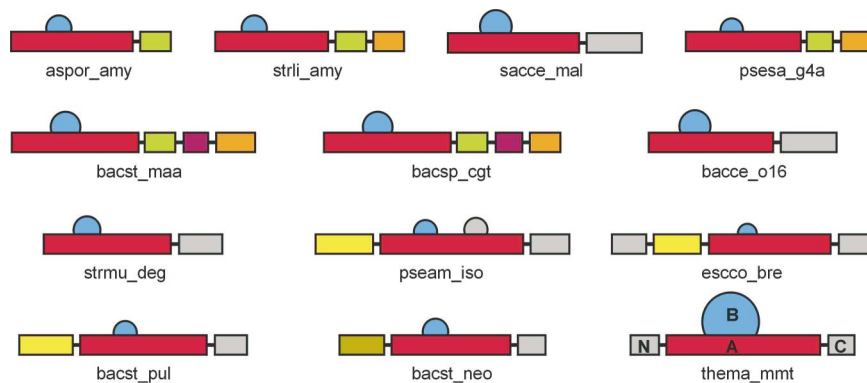


Fig. 3. Schematic comparison of the domain organisation of *T. maritima* MTase with other amylytic enzymes. The architecture of the other enzymes was drawn in a manner similar to that of JESPERSEN et al. (1991), but with modifications. The $(\beta/\alpha)_8$ -barrel core domain (domain A) is drawn as a red box. Circles mark loop domains inserted in the core domain. Domain B (blue circle) represents an insertion between β -strand 3 and α -helix 3, characteristic for the α -amylase-class of $(\beta/\alpha)_8$ -barrel proteins. The empty circle in *Pseudomonas amyloclavata* isoamylase indicates an extra loop between β -strand 7 and α -helix 7. Additional boxes drawn in grey indicate additional domains without significant homology to domains of the other enzymes, while coloured boxes represent domains with significant sequence similarity to other domains drawn in the same colour. Abbreviations: asp_or_amy, *Aspergillus oryzae* Taka-amylase A; strli_amy, *Streptomyces limosus* α -amylase; sacce_mal, *Saccharomyces cerevisiae* maltase; psesa_g4a, *Pseudomonas saccharophila* maltotetraohydrolase; bacst_maa, *B. stearothermophilus* maltogenic α -amylase; bacsp_cgt, *Bacillus* sp. cyclodextrin glycosyltransferase; bacce_o16, *B. cereus* oligo-1,6-glucosidase; strmu_deg, *Streptococcus mutans* dextran glucosidase; pseam_iso, *Pseudomonas amyloclavata* isoamylase; escce_bre, *E. coli* branching enzyme; bacst_pul, *B. stearothermophilus* pullulanase; bacst_nep, *B. stearothermophilus* neopullulanase; thema_mmt, *T. maritima* maltosyltransferase.

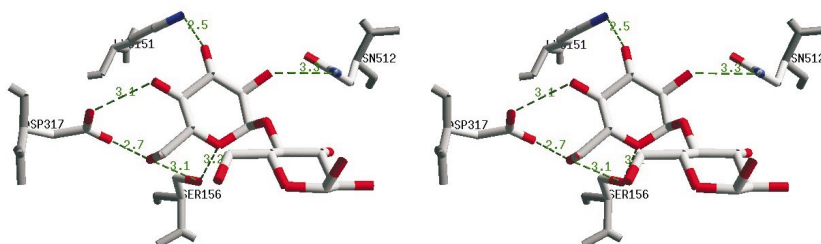


Fig. 4. Stereoview of the hydrogen-bonding environment of the non-reducing-end glucosyl residue of maltose at the -2 subsite of MTase. An additional water-mediated (Wat293) hydrogen bond between Asn512 OD1 and the C2 hydroxyl of the non-reducing-end glucosyl residue is not shown.

strands 4 and 5 of the $(\beta/\alpha)_8$ -barrel (positions 209, 210, 231, 232 and possibly 233 and 234 in Taka-amylase A numbering) have been linked with the glycosidic-bond specificity of the enzymes (see Figure 2). For example, the position equivalent to His210 in Taka-amylase A, which was found to be a histidine or a glycine in 97% of the α -1,4-specific GHF 13 and GHF 77 enzymes, may be important for cleavage specificity (MACGREGOR et al., 2001). Interestingly, *T. maritima* MTase, an enzyme that cleaves and subsequently re-forms α -1,4-glucosidic bonds, does not closely resemble the

other α -1,4-specific enzymes in this region (MACGREGOR et al., 2001).

Structural features that putatively contribute to the exo-mode of action and to the strict transfer specificity of MTase are: (i) domains A and B form a deep substrate-binding cleft; loop 314-317 of domain B and particularly residue Lys151 of domain A apparently form a 'barrier' in the substrate-binding cleft; these structures are involved in binding the non-reducing end of the bound substrate (Fig. 4) and thus may contribute to the failure of MTase to cleave off longer segments than malto-

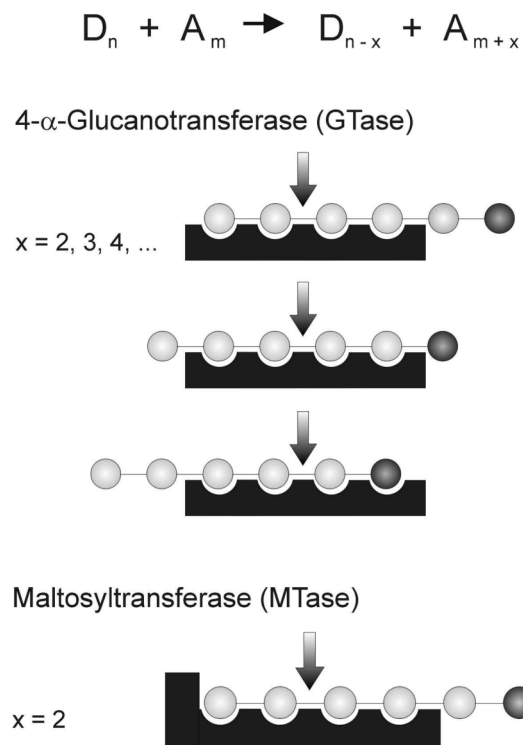


Fig. 5. Model for the binding of maltopentaose at the substrate binding regions of GTase (top) and MTase (bottom). MTase contains two subsites for binding the glucose units at the non-reducing end of maltodextrin substrates. The number of subsites on the other side of the catalytic center has not been determined. Also, the number of subsites in the substrate-binding region of GTase is unknown, but biochemical data (unpublished) indicate that there may be more than the four depicted here. The vertical box drawn in the MTase model indicates a proposed barrier for non-reducing-end substrate binding within the substrate binding cleft (see text).

syl units from donor malto-oligo- or polysaccharides; (ii) the dimeric nature of MTase may play a role because the dimer interface is in the immediate vicinity of the substrate-binding region of each subunit; however, the substrate-binding region does not cross the subunit border, i. e. each substrate-binding region is comprised of residues from one subunit only. Preliminary data indicate that the dimeric nature of MTase may also be of importance for the extraordinarily high thermostolerance of the enzyme (unpublished results).

Based on the results of the crystal structure of MTase, a model of the substrate-binding region of this enzyme can be proposed and compared with

the hypothetical situation in GTase as shown in Figure 5. Recombinant GTase of *T. maritima* has been crystallized recently (ROUJEINIKOVA et al., 2001b). Analysis of the three-dimensional structure of this enzyme and comparison with the crystal structures of MTase and other GHF 13 enzymes is expected to yield further insight into substrate binding and the reaction mechanism of starch- and matodextrin-converting transglycosidases.

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