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Single Amino Acid Mutations Interchange the Reaction Specificities of Cyclodextrin Glycosyltransferase and the Acarbose-Modifying Enzyme Acarviosyl Transferase†

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ABSTRACT: Acarviosyl transferase (ATase) from *Actinoplanes* sp. SE50/110 is a bacterial enzyme that transfers the acarviosyl moiety of the diabetic drug acarbose to sugar acceptors. The enzyme exhibits 42% sequence identity with cyclodextrin glycosyltransferases (CGTase), and both enzymes are members of the α-amylase family, a large clan of enzymes acting on starch and related compounds. ATase is virtually inactive on starch, however. In contrast, ATase is the only known enzyme to efficiently use acarbose as substrate (2 μmol min^{-1} mg^{-1}); acarbose is a strong inhibitor of CGTase and of most other α-amylase family enzymes. This distinct reaction specificity makes ATase an interesting enzyme to investigate the variation in reaction specificity of α-amylase family enzymes. Here we show that a G140H mutation in ATase, introducing the typical His of the conserved sequence region I of the α-amylase family, changed ATase into an enzyme with 4-α-glucanotransferase activity (3.4 μmol min^{-1} mg^{-1}). Moreover, this mutation introduced cyclodextrin-forming activity into ATase, converting 2% of starch into cyclodextrins. The opposite experiment, removing this typical His side chain in CGTase (H140A), introduced acarviosyl transferase activity in CGTase (0.25 μmol min^{-1} mg^{-1}).

The α-amylase family, or glycoside hydrolase family 13 (*1*), is a large family of enzymes acting on α-glycosidic bonds in starch and related compounds (*2*). About 20 different reaction specificities have been identified in this family, including hydrolysis of α-(1,4)- and α-(1,6)-glycosidic linkages (e.g., α-amylase and isoamylase, respectively), as well as the formation of α-(1,4)- and α-(1,6)-glycosidic bonds (e.g., amylosucrase, acarviosyl transferase, cyclodextrin glycosyltransferase, and branching enzyme, respectively; Figure 1) (*2*, *3*). All members use an α-retaining double displacement mechanism (*4*) in which reactions proceed via a covalent glycosyl-enzyme intermediate (*5*). Glycosidic bond cleavage occurs between subsites −1 and +1 (Figure 2A), and after cleavage the glycosyl reaction intermediate remains bound at the donor subsites (−1, −2, −3, etc.) (*6*).

Cyclodextrin glycosyltransferase (CGTase,[1] EC 2.4.1.19) is also a member of the α-amylase family. It is a 75 kDa extracellular enzyme that produces circular α-(1,4)-glucans (cyclodextrins) from starch via an intramolecular transglycosylation reaction (Figure 1). The major products are α-, β-, and γ-cyclodextrin (with six, seven, or eight glucose residues) (*7*, *8*), which are subsequently imported into the bacteria excreting CGTase and are used as a carbon and energy source (*9*). CGTase also catalyzes a disproportionation reaction in which a segment of an α-(1,4)-glucan is transferred to the 4-hydroxyl group of a second sugar to yield a linear product (Figure 1). This reaction is also catalyzed by 4-α-glucanotransferase (also called amylomaltase and disproportionation enzyme), which is also a member of the α-amylase family. CGTases are organized in five domains (A−E) (*10*, *11*). The N-terminal part consists of the catalytic $(\beta/\alpha)_8$-barrel fold (domain A) with a loop of 60 residues protruding at the third β-strand (domain B). Domains A and B together form the substrate binding groove, which consists of at least seven donor substrate binding subsites (−1 to −7) and two acceptor subsites (+1 and +2) (*5*, *10*, *12*). Domains C and E are involved in starch binding (*13*), while the function of domain D remains to be elucidated.

Acarviosyl transferase (ATase,[1] EC 2.1.4.19) is a 76 kDa extracellular enzyme from *Actinoplanes* sp. SE50/110 that transfers the acarviosyl moiety of acarbose to the 4-hydroxyl group of various sugars (*14*) (Figure 1). This is the only enzyme known to catalyze this reaction. ATase has the highest sequence identity (42%) with CGTases (*14*), and the protein is most likely organized in five domains (A−E), similar to CGTases. The substrate of ATase, acarbose, is a pseudotetrasaccharide, composed of the C7-cyclitol valienamine, 4-amino-4,6-dideoxyglucose, and maltose (Figure 3),

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[1] Abbreviations: ATase, acarviosyl transferase; CGTase, cyclodextrin glycosyltransferase; HPAEC, high-performance anion-exchange chromatography; Tabium, *Thermoanaerobacterium thermosulfurigenes* strain EM1.

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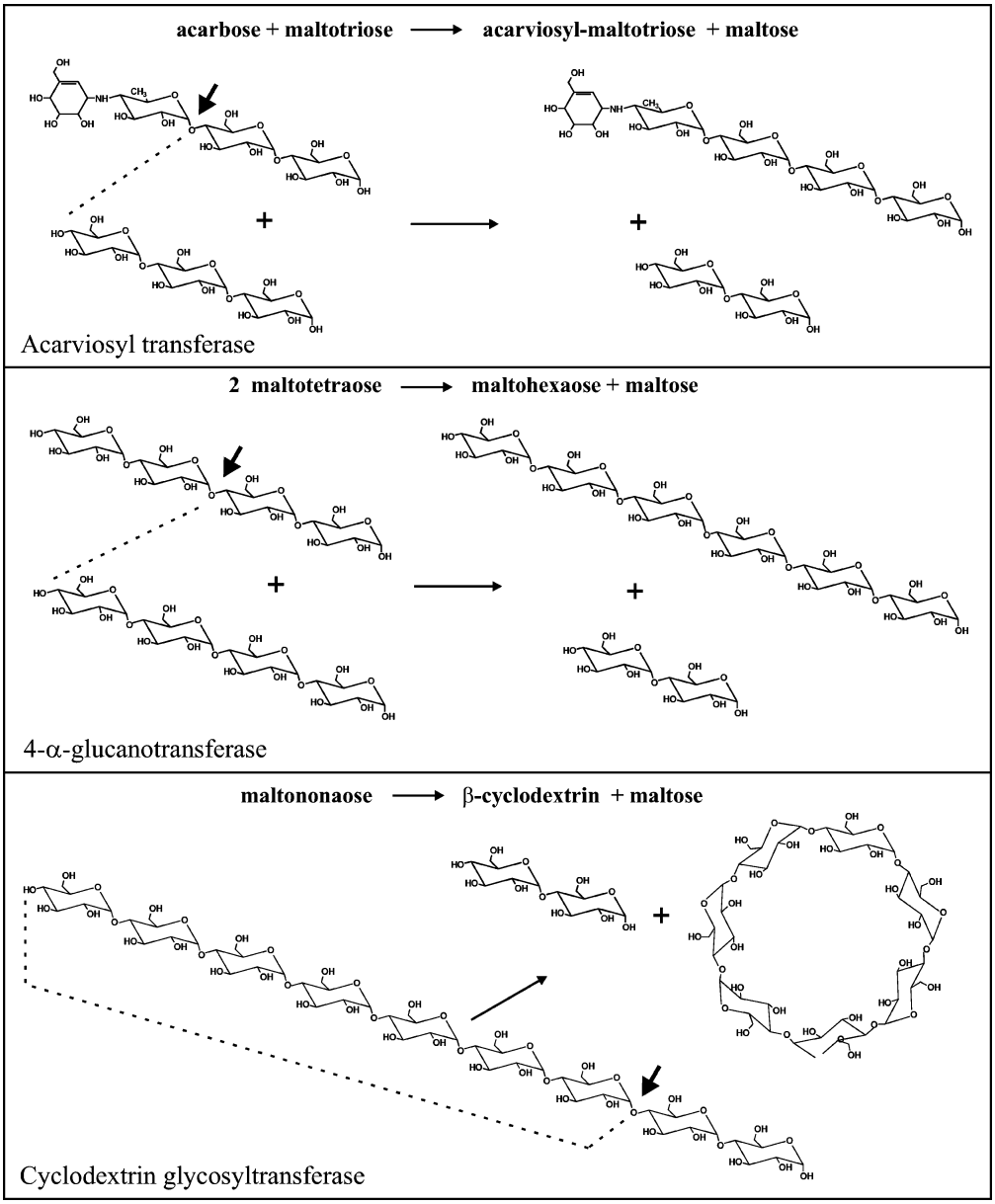

FIGURE 1: Reactions catalyzed by the enzymes acarviosyl transferase, 4-α-glucanotransferase, and cyclodextrin glycosyltransferase. The thick arrows indicate the scissile bonds, and the dashed lines indicate the bonds to be formed.

that is found in culture medium from *Actinoplanes* sp. SE50/110 when it is grown on starch or maltooligosaccharides (*15*). This secondary metabolite is produced industrially using engineered strains of *Actinoplanes* sp. SE50/110 (*16*), and it is used as a drug (Glucobay/Precose) in the treatment of diabetes patients to slow the intestinal release of glucose (*17*). Acarbose is a strong reversible inhibitor of many α-amylase family enzymes, including the cyclization reactions with starch catalyzed by the CGTases of *Bacillus circulans* strain 251 [K_i = 0.2 μM (*18*)] and *Thermoanaerobacterium thermosulfurigenes* strain EM1 [Tabium;[1] K_i = 0.1 μM (*19*)]. Protein crystallography studies have shown that acarbose inhibits α-amylase family enzymes by binding in the active site with the acarviosyl moiety at the −1 and +1 subsites (*20*−*25*). In ATase, in contrast, acarbose must bind at the −2 to +2 subsites to allow catalysis of the acarviosyl transferase reaction (Figure 1). The gene encoding ATase (*acbD*, part of accession number AJ293724) has been cloned, and the protein has been purified from culture medium (*14*). The *acbD* gene is located in a biosynthetic gene cluster for acarbose production from *Actinoplanes* sp. SE50/110 (*26*, *27*). Despite its high similarity to CGTases, it has been reported that ATase has no cyclodextrin-forming activity and is not inhibited by acarbose (*14*). Rather, acarbose is its main substrate, with a K_M value of 0.65 mM (*14*).

Here we describe single amino acid mutations that interchange the reaction specificities of ATase (from acarviosyl transferase into a cyclodextrin-producing 4-α-glucanotransferase) and CGTase (from a cyclodextrin-producing 4-α-glucanotransferase into an acarviosyl transferase).

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions. Escherichia coli DH5α (*28*) was used for cloning experiments and was grown at 37 °C in LB medium (*29*) supplemented with ampicillin (100 μg/mL). *Streptomyces lividans* 66 strain 1326 (*30*) was used as host for heterologous expression of ATase protein. *S. lividans* was cultured on

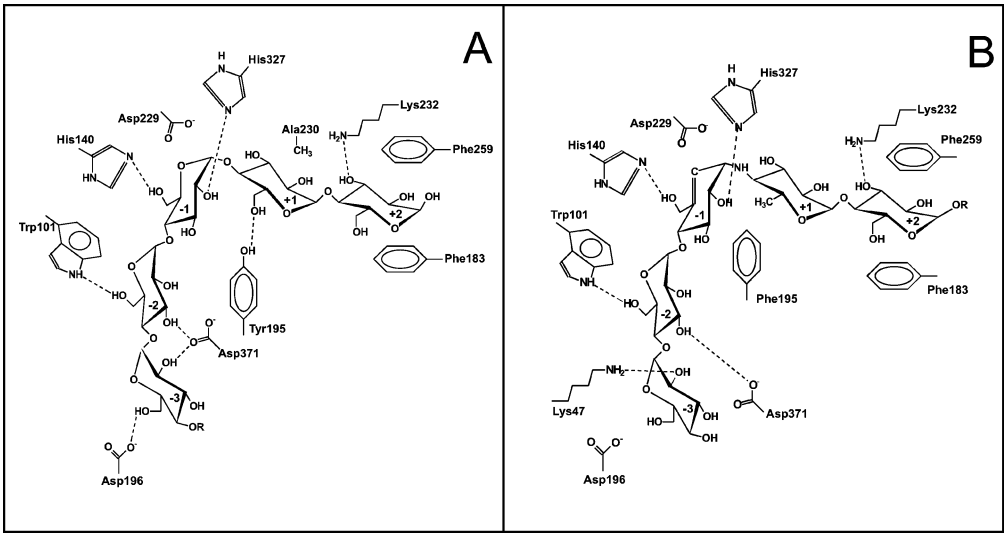

FIGURE 2: Schematic overview of the interactions between substrate binding subsites −3 to +2 of CGTase and maltooligosaccharides: (A) adapted from a *B. circulans* strain 251 CGTase−maltononaose substrate crystal structure (*5*); (B) adapted from a Tabium CGTase crystal structure with a maltohexaose inhibitor (extended acarbose) (*24*). For clarity, not all residues at the −2, −1, and +1 subsites are shown.

Table 1: Primers Used for PCR Amplification of the ATase Encoding Gene (*acbD*) and for Site-Directed Mutagenesis[a]

	primer (5′ to 3′ direction)
ATase	
F1	AAAAAACATATGCAACGTCACGCCAGGCA
R1	TTTGGATCCTCAATGATGATGATGATGATGGCGCTGCCAGGTGAGCGT
D229N for	GGCATCCGGGTCAACGCCGTCAAGCACAT
D229N rev	ATGTGCTTGACGGCGTTGACCCGGATGCC
E257Q for	GCCATCTTCGGCCAGTGGTACAT
E257Q rev	ATGTACCACTGGCCGAAGATGGC
D328N for	CTGGACAACCAGAACACCCGGCGCTT
D328N rev	AAGCGCCGGGTGTTCTGGTTGTCCAG
G140H for	TGGACGCCGAACCACACCAACCCG
G140H rev	CGGGTTGGTGTGGTTCGGCGTCCA
Q327H for	CTGGACAACCACGACACCCGGCGC
Q327H rev	GCGCCGGGTGTCGTGGTTGTCCAG
T329M for	AACCAGGACATGCGGCGCTTCGGG
T329M rev	CCCGAAGCGCCGCATGTCCTGGTT
Q327H/T329M for	CTGGACAACCACGACATGCGGCGCTTCG
Q327H/T329M rev	CGAAGCGCCGCATGTCGTGGTTGTCCAG
CGTase	
H140A	TGCACCAAATGCAACTTCACCTGC

[a] Restriction sites used are underlined.

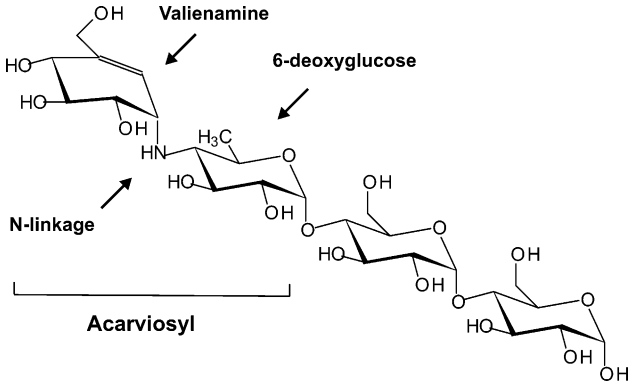

FIGURE 3: Structure of acarbose. The arrows indicate the three differences between acarbose and maltotetraose.

R2YE agar plates (*30*) or in liquid YEME medium with 34% (w/v) sucrose (*30*). *E. coli* vector pET3b (Stratagene) was used for cloning experiments, and the *E. coli*/actinomycete shuttle vector pUWL201PW was used for heterologous expression of ATase protein using streptomycetes host strains (*31*). This vector contains a constitutive *erm*EP promoter. *S. lividans* transformants were selected on R2YE agar plates with thiostrepton (20 μg/mL). CGTase (mutant) proteins of Tabium were produced using the pCScgt-tt vector (*19*) and *Bacillus subtilis* strain DB104A (*32*) as host.

General DNA Manipulations. DNA manipulations and calcium chloride transformation of *E. coli* were according to standard protocols (*29*). Preparation of protoplasts and subsequent transformation of *S. lividans* with plasmid were carried out as described (*30*). The *acbD* gene from *Actinoplanes* sp. SE50/110 (encoding ATase) was amplified by PCR from plasmid pAS5 (*26*) using forward primer F1 with a *Nde*I restriction site and reverse primer R1 that added six histidine codons to the 3′ end of the gene and with a *Bam*HI restriction site (Table 1). The PCR product (2.1 kb) was restricted with *Nde*I and *Bam*HI and cloned into pET3b to yield pET3b-AT. Site-directed mutations were introduced in pET3b-AT using the QuickChange site-directed mutagenesis

kit from Stratagene and the primers as listed in Table 1. For expression of ATase (mutant) proteins, the *Nde*I−*Bam*HI fragment of pET3b-AT was cloned in pUWL201PW to obtain pUWL201PW-AT, followed by transformation to *S. lividans* 66 strain 1326. Mutation H140A was introduced into Tabium CGTase as described (*33*).

Expression and Purification of ATase and CGTase. Liquid YEME medium (10−500 mL) was inoculated with mycelium from *S. lividans*/pUWL201PW-AT transformants. After 3−4 days of cultivation in Erlenmeyer flasks at 30 °C and shaking at 200 rpm, cultures were centrifuged (10000*g*, 40 min), and the supernatants were filtered by gravity through filtrate paper. Subsequently, imidazole (to 10 mM), sodium chloride (to 250 mM) and 1 mL of a Ni−agarose suspension (Qiagen) were added per 100 mL of supernatant, and the mixture was incubated at 4 °C for 4 h. After the Ni−agarose material was washed with 10 volumes of 20 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$, 250 mM NaCl, and 10 mM imidazole, bound ATase protein was eluted with the same buffer containing 200 mM imidazole. Tabium CGTase proteins were produced and purified as described (*8*). The purity of the proteins was checked with SDS−PAGE. Protein concentrations were determined with the Bradford reagent of Bio-Rad and bovine serum albumin as standard.

Enzyme Assays. All assays were carried out in Tris−maleic acid buffer (20 mM, pH 6.5, total volume 0.3−1.0 mL) supplemented with 1 mM $CaCl_2$ at 30 °C (ATase) or 60 °C (CGTase). Reactions were started by adding enzyme and stopped by boiling samples for 5 min. Acarviosyl transferase activity was determined by measuring the transfer rate of the acarviosyl moiety from acarbose (donor substrate) to maltotriose (acceptor substrate), resulting in maltose release and the formation of acarviosylmaltotriose (Figure 1). Activity is defined as the amount of acarviosylmaltotriose produced per minute. Reaction mixtures contained 1−20 mM acarbose, 20 mM maltotriose, and 6−50 μg of (mutant) ATase enzyme/mL. 4-α-Glucanotransferase activity was determined by incubating 10 μg of (mutant) ATase enzyme/mL with 20 mM maltotetraose and measuring maltose transfer, resulting in the formation of maltose and maltohexaose. Activity is defined as the amount of maltohexaose produced per minute. Products formed, in both reactions, were analyzed by HPLC using an econosphere NH2 5U column (250 by 4.6 mm; Alltech, Breda, The Netherlands) linked to a refractive index detector. A mobile phase of 60−75% acetonitrile and 40−25% water at a flow rate of 1 mL/min was used.

Starch Degradation. Starch-degrading activities were measured by incubating soluble potato starch (1% w/v; Sigma) with 10 μg of (mutant) ATase enzyme/mL at 30 °C. Formation of β-cyclodextrin was measured with phenolphthalein (*8*). Formation of α-, β-, and γ-cyclodextrin was determined with HPAEC[1] analysis as described below. Hydrolysis of starch was assayed by measuring the amount of reducing sugars with dinitrosalicylic acid as described (*13*). CGTase activities were determined with 0.01−1.0 μg of enzyme/mL. Acarbose inhibition of (mutant) CGTases was determined with 1 μg of enzyme/mL and acarbose concentrations between 0 and 2 mM.

HPAEC Analysis. The amounts of cyclodextrins formed were quantified using a HPAEC system. Before analysis, the reaction mixtures containing residual starch were incubated with 1500 units of amyloglucosidase (Megazyme, County Wicklow, Ireland) per gram of starch substrate at 50 °C overnight to degrade all starch into glucose. Subsequently, the samples were diluted in 80% dimethyl sulfoxide. α-, β-, and γ-cyclodextrins from Fluka were used as standards. Separation was achieved with a CarboPac PA1 anion-exchange column (250 × 4 mm; Dionex, Sunnyvale, CA) with a CarboPac1 guard column (50 × 4 mm; Dionex) and a mobile phase of 0.1 M NaOH (solution A) and 0.6 M sodium acetate in 0.1 M NaOH (solution B) at a flow rate of 1 mL/min. The following gradient program was used: 0−10 min 5−35% solution B, 10−20 min 35−45% solution B, 20−50 min 45−65% solution B, 50−54 min 65−100% solution B, 54−60 min 100% solution B, 60−61 min 100−5% solution B, and 61−65 min 5% solution B. Detection was performed with an ED40 electrochemical detector (Dionex). The pulse program used was +1.0 V (0−0.40 s), +0.7 V (0.41−0.60 s), and −0.1 V (0.61−1.00 s).

Mass Spectrometry. Mass spectra of samples containing cyclodextrins and acarviosylmaltotriose were recorded on a Perkin-Elmer Sciex Instruments API3000 triple quadrupole LC/MS/MS mass spectrometer. In each case samples were diluted in MeOH/H_2O (50/50) and directly injected into the mass spectrometer. For detection of cyclodextrins 1 mM NaCl was added to the samples. Cyclodextrins were detected as Na^+, $Tris^+$, and $Tris/Na^+$ complexes; these complexes were also formed with commercially available cyclodextrins.

RESULTS AND DISCUSSION

Heterologous Expression and Purification of ATase. Heterologous expression of the ATase protein has been described previously (*14*). However, these *S. lividans* transformants appeared to be unstable, as the results were not reproducible or the expression failed completely. Therefore, new expression clones were constructed. Attempts to express ATase in *E. coli* BL21(DE3) under control of a T7 promoter and in *B. subtilis* under control of an erythromycin promoter failed (data not shown). In contrast, ATase overproduction was readily achieved in *S. lividans* when the *E. coli*/actinomycete shuttle vector pUWL201PW-AT was used. Acarviosyl transferase activity was found in the culture medium, demonstrating that the signal sequence for ATase secretion is also recognized by *S. lividans.* The ATase protein was purified from the culture medium using a standard His-tag purification protocol, yielding about 4 mg of ATase protein/L of culture.

The acarviosyl transferase activity of this recombinant ATase is 2.0 μmol min^{-1} mg^{-1} (Table 2), which is very similar to the value measured for partially purified ATase from *Actinoplanes* sp. SE50/110 (data not shown). In addition, the activity of the recombinant ATase is in the same order of magnitude as the 4.6 μmol min^{-1} mg^{-1} measured by Hemker et al. (*14*) for the ATase enzyme purified from *Actinoplanes* sp. SE50/110. Note that we measured the formation of acarviosylmaltotriose (Figure 1), whereas Hemker et al. (*14*) determined the exchange rate of the maltose moiety of acarbose for ^{14}C-labeled maltose. Our activities were determined at an acarbose concentration of 20 mM far above the K_M value of wild-type ATase, about 1 mM (*14*). K_M values for acarbose were not determined as our detection method was not sensitive enough at the very

	Region I	Region II	Region III	Region IV
	132-140	224-233	253-260	322-329
	|	*	*	|*|
ATase	VIM***D***WTPNG	DGI***R***V***D***AVKH	AIFG***E***WYM	TFLDNQ***D***T
CGTase	VII***D***FAPN***H***	DGI***R***M***D***AVKH	FTFG***E***WFL	TFIDN***HD***M
4-α-Glucanotransferase	VVL***D***LPIH***H***	DGF***R***F***D***AAKH	IFLA***E***IWA	NFTSN***HD***M
α-Amylase	LMV***D***VVAN***H***	DGL***R***I***D***TVKH	YCIG***E***VLD	TFVEN***HD***N
Cyclomaltodextrinase	VLL***D***AVFN***H***	DGW***R***L***D***VANE	AYIL***E***VWH	NLLDS***HD***T
Oligo-1,6-glucosidase	LMM***D***LVVN***H***	DGF***R***M***D***VINF	MTVG***E***MPG	LYWNN***HD***Q
Amylosucrase	AVV***D***FIFN***H***	DIL***R***M***D***AVAF	FFKS***E***AIV	NYVRS***HD***D
Branching enzyme	VIL***D***WVPG***H***	DAL***R***V***D***AVAS	VTMA***E***EST	VLPLS***HD***V

FIGURE 4: The four conserved amino acid sequence regions in enzymes of the α-amylase family. The numbering is according to *B. circulans* strain 251 CGTase. The seven most conserved residues are shown in bold and italic, catalytic residues are marked with an asterisk (*), and the residues that are different in ATase are marked with a vertical line (|). The following sequences were used in the alignment: ATase of *Actinoplanes* sp. SE50/110 (Q9K5L5); CGTase of *B. circulans* strain 251 (P43379); 4-α-glucanotransferase of *T. maritima* (P80099); α-amylase of *Aspergillus oryzae* (P10529); cyclomaltodextrinase of alkalophilic *Bacillus* sp. I-5 (Q59226); oligo-1,6-glucosidase of *Bacillus cereus* (P21332); amylosucrase of *Neisseria polysaccharea* (Q9ZEU2); branching enzyme of *Escherichia coli* (P07762).

Table 2: Acarviosyl Transferase and 4-α-Glucanotransferase Activities of (Mutant) ATase Proteins[a]

enzyme	acarviosyl transferase activity (acarviosylmaltotriose formation) (μmol min^{-1} mg^{-1})	4-α-glucano-transferase activity (maltohexaose formation) (μmol min^{-1} mg^{-1})
wild type	2.0 ± 0.2	0.05 ± 0.01
D229N	<0.001	<0.001
E257Q	<0.001	<0.001
D328N	<0.001	<0.001
G140H	0.23 ± 0.04	1.1 ± 0.2
Q327H	1.2 ± 0.2	0.02 ± 0.01
T329M	1.7 ± 0.2	0.6 ± 0.1
Q327H/T329M	1.2 ± 0.2	0.6 ± 0.1
G140H/Q327H	0.23 ± 0.03	1.0 ± 0.1
G140H/Q327H/T329M	0.24 ± 0.05	3.4 ± 0.3

[a] Acarbose and maltotriose were used as substrates in the acarviosyl transferase reaction and maltotetraose in the 4-α-glucanotransferase reaction, as shown in Figure 1.

Table 3: Typical CGTase Residues that Are Conserved in the ATase Sequence and Their Functional Roles Previously Evaluated in CGTases.

residue in CGTase[a]	residue in ATase	function in CGTase
subsite +2		
Phe183	Phe185	cyclization (*46*, *47*)
Lys232	Lys234	general activity (*47*)
Phe/Tyr259	Tyr261	essential for transglycosylation specificity (*33*)
subsite +1		
Leu194	Leu196	cyclization (*48*)
Ala230	Ala232	essential for transglycosylation specificity (*49*)
His233	His235	general activity (*39*)
subsite −1		
Asp229	Asp231	catalytic nucleophile (*5*, *50*)
Glu257	Glu259	acid/base catalyst (*5*)
Asp328	Asp331	transition state stabilization (*5*)
Tyr100	Tyr106	general activity (*51*)
Asp135	Asp141	essential for conformation of Glu257 and Arg227 (*52*)
His140	Gly146	general activity (*39*)
Arg227	Arg229	general activity (*52*)
His327	Gln330	general activity (*39*)
subsite −2		
His98	His104	general activity (*53*)
Trp101	Trp107	unknown function
Arg375	Arg386	unknown function
subsite −3		
Arg/Lys/His47	Arg55	cyclization (*54*)
Asp196	Asp198	cyclization (*24*)
Asp371	Asp382	cyclization (*24*)
other		
Tyr/Phe195	Ala197	cyclization (*39*), central aromatic residue
Met329	Thr332	unknown function, close to the acceptor subsites +1/+2
Phe283	Phe287	cyclization, close to Glu257 (*24*)
maltose binding sites I, II and III (*11*)		
Trp616/Trp662	Trp627/Trp674	binding to starch, E-domain (*13*)
Tyr633	Glu644	binding to starch, E-domain (*13*)
Trp413	Trp424	binding to starch, C-domain (*13*)

[a] These are all typical CGTase residues; numbering is according to *B. circulans* strain 251 CGTase (accession number P43379).

low acarbose concentrations needed to determine the K_M values for this compound.

Also the maltogenic amylases from *Bacillus stearothermophilus* ET1 and *Thermus* strain IM6501 are able to use acarbose as substrate (*34*−*35*). However, these enzymes are obviously different from ATase as they cleave the α-(1,4)-glycosidic bond between the two glucose residues of acarbose (Figure 3) to release glucose. The remaining pseudotrisaccharide is subsequently transferred to water (hydrolysis) or the 4- or 6-hydroxyl group of a sugar acceptor (transglycosylation). Thus, in maltogenic amylases a native glucose residue is bound at the catalytic subsite −1 during bond cleavage, whereas ATase is the only enzyme of the α-amylase family to bind a modified glucose moiety at subsite −1 during catalysis. Unfortunately, reaction rates for acarbose degradation by maltogenic amylases have not been reported, although they must be rather efficient as acarbose is completely degraded upon incubation with large amounts of maltogenic amylase (*35*).

Catalytic Residues. Sequence alignments of ATase with various α-amylase family enzymes revealed that the four short sequence regions (I−IV) characteristic of the α-amylase family (*2*, *37*) are present in ATase (Figure 4). Of the seven residues most stringently conserved in the α-amylase family, two were different in ATase (Figure 4; see below). Asp229, Glu257, and Asp328 were identified as the putative catalytic nucleophile, acid/base catalyst, and transition state stabilizer, respectively (Table 3); throughout the paper all amino acids are numbered according to *B. circulans* strain 251 CGTase. Mutating these residues into the corresponding amide

residues resulted in loss of catalytic activity (Table 2), demonstrating that Asp229, Glu257, and Asp328 are essential for catalysis. Therefore, we assume that the catalytic site architecture of ATase is similar to those of other α-amylase family enzymes and that the preference of ATase for acarbose is probably the result of relatively small differences (e.g., point mutations) in or close to its active site.

Sequence Comparison of ATase and CGTase. To explain why ATase uses acarbose as substrate and lacks the ability to form cyclodextrins from starch (see Results), its amino acid sequence was compared with those of CGTases. Although the total identity between ATase and CGTase (*B. circulans* strain 251, accession number P43379) is only 42% (not shown), the sequence alignment revealed that most of the typical CGTase residues are conserved in ATase. This is especially significant for active site residues, e.g., the residues of the −3 to +2 subsites (Figure 2). At the acceptor subsites +1 and +2, all residues providing substrate interactions in CGTase are conserved in ATase (Table 3), which is in agreement with the identical acceptor substrate preference of both enzymes; both prefer the OH4 group of glucose or longer oligosaccharides as acceptor substrate.

At donor subsite −1, in contrast, there are striking differences between CGTase and ATase. His140 and His327 of CGTase (Figure 2) are replaced by Gly and Gln in ATase, respectively (Table 3). Both histidines are conserved in roughly 95% of the α-amylase family enzymes (*38*), and they are found in all CGTases known to date. Structural and mutagenesis studies on α-amylases and CGTases have demonstrated that these His residues have a function in distortion of the substrate toward transition state planarity (His140) and in transition state stabilization (His327) (*5*, *39*, *40*). In the absence of these two His residues acarbose most likely obtains a different binding mode in the active site of ATase (acarviosyl moiety at the −2 and −1 subsites) compared to α-amylases and CGTases (acarviosyl moiety at the −1 and +1 subsites; Figure 2B), thereby explaining why ATase is not inhibited by acarbose.

Opposite to the variation at subsite −1, the residues forming the −2 and −3 subsites in CGTase are all conserved in ATase (Table 3). This was surprising as ATase binds the valienamine moiety of acarbose at subsite −2, whereas CGTase binds a glucose ring at this subsite. Although this indicates that subsite −2 is conserved between ATase and CGTases, this is no proof that ligand binding at subsite −2 in ATase is identical to that observed in CGTases. The presence of a putative −3 subsite in ATase is not needed for the acarviosyl transferase reaction as shown in Figure 1. However, a −3 donor subsite may be beneficial in the processing of acarbose variants with one or more glucose residues attached to the nonreducing end, compounds that are found in the culture medium of *Actinoplanes* sp. SE50/110 (*15*). In contrast, the residues forming the remote donor subsite −6, which are characteristic for CGTases (*41*), are not conserved in ATase.

Other interesting differences between ATase and CGTase are the replacement of the invariant CGTase residues Tyr/Phe195 by Ala and Met329 by Thr (Table 3). Tyr/Phe195 is important for cyclization and cyclodextrin size specificity (*39*, *42*), whereas the function of Met329 for CGTase is unknown. However, the fact that Met329 follows the transition state stabilizer Asp328 (*5*) and its proximity to the acceptor subsites suggest that it is important for catalysis and/or reaction specificity.

Mutations in Gly140, Gln327, and Thr329 Decrease Acarviosyl Transfer Activity. To investigate whether the distinct reaction specificities of ATase and CGTase are the result of point mutations in their active sites, G140H, Q327H, and T329M mutations were introduced in ATase to increase its similarity to CGTase. All three mutations decreased the acarviosyl transferase activities (Table 2), most pronounced for the G140H mutation. Combinations of the mutants did not further reduce the acarviosyl transferase activity (Table 2). The reduced activities were not due to acarbose inhibition (not detectable; data not shown), even though the mutations increased the similarity to CGTase, which is strongly inhibited by acarbose. This indicated that acarbose binds in the −2 to +2 subsites in these mutant ATases, as in the wild-type enzyme, which is the binding mode needed to transfer the acarviosyl moiety of acarbose. In this binding mode acarbose cannot benefit from hydrogen bonding with G140H since the substrate ring at subsite −1 has no OH group. In contrast, inhibition would indicate binding at the −1 to +3 subsites, the acarbose binding mode observed in crystal structures of CGTases (*24*, *25*) (Figure 2B).

The precise reason for the strongly decreased acarviosyl transferase activity of mutant G140H is not known, but possible explanations are the larger size and the basic character of the His side chain. Nevertheless, it is concluded that the acarviosyl transferase activity of ATase is strongly reduced by introducing a His side chain at the position of Gly140, whereas mutagenesis studies have shown that the His side chain at the equivalent position in CGTases and α-amylases is important for the catalytic efficiency of these enzymes with starch substrates (*39*, *40*).

G140H and T329M Mutations Give ATase 4-α-Glucanotransferase Activity. Subsequently, the (mutant) ATase enzymes were tested for their ability to catalyze the 4-α-glucanotransferase reaction. Maltotetraose was chosen as substrate, because this oligosaccharide is similar to acarbose. Wild-type ATase has a very low activity on this substrate (Table 2), forming maltose and maltohexaose, by transferring a maltose moiety of one maltotetraose to a second maltotetraose molecule (see Figure 1, 4-α-glucanotransferase). The ATase catalytic residue mutants lacked this activity (Table 2), indicating that the 4-α-glucanotransferase activity was not due to, for instance, a contamination in the ATase protein preparation. The low 4-α-glucanotransferase activity therefore must be a property of ATase.

The G140H and T329M mutations strongly enhanced (12−22-fold) the 4-α-glucanotransferase activity, whereas it was reduced in mutant Q327H (Table 2). Triple mutant G140H/Q327H/T329M increased the 4-α-glucanotransferase activity 68-fold, reaching a value even higher than the acarviosyl transferase activity of the wild-type enzyme. The mutant ATases formed maltose and maltohexaose as initial products and, somewhat later, also maltooctaose. After prolonged incubation, also oligosaccharides of uneven lengths appeared (Figure 5A). In contrast to maltotetraose, the shorter maltotriose was only very slowly used as donor substrate by the wild-type and mutant ATases (not shown). Thus, the wild-type and mutant ATases have a preference for maltose transfer with oligosaccharide substrates, although the specificity for maltose is not absolute.

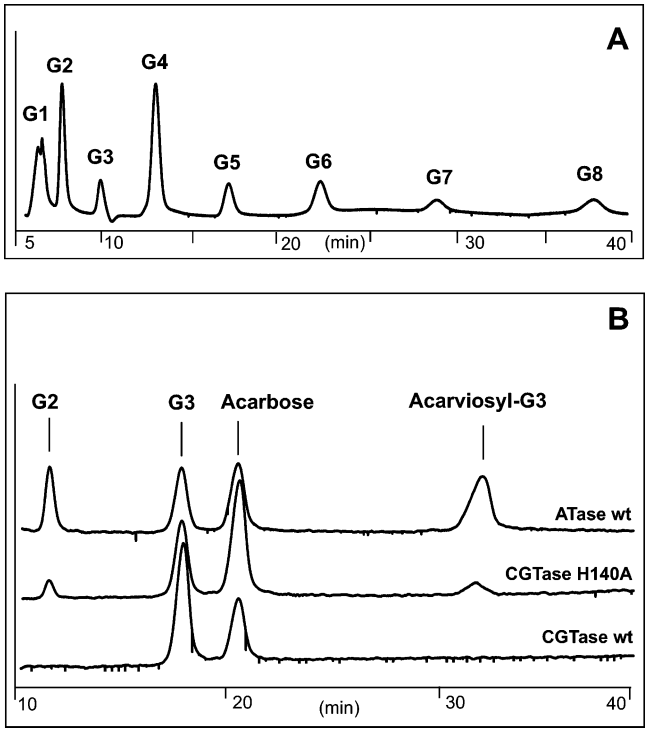

FIGURE 5: HPLC elution profiles of substrates and reaction products of ATase and (mutant) CGTase enzymes. (A) ATase mutant G140H/Q327H/T329M (10 μg/mL) incubated with 20 mM maltotetraose for 18 h. (B) Wild-type ATase (10 μg/mL), wild-type CGTase (2 μg/mL), and CGTase mutant H140A (2 μg/mL) incubated with 20 mM acarbose and 20 mM maltotriose (40 mM for wild-type CGTase) for 24 h. G1−G8, glucose to maltooctaose. The G1 peak in panel A overlaps with the buffer used in the assay.

The stimulatory effect of mutation T329M on the 4-α-glucanotransferase activity of ATase is not understood; this Met residue (following the invariant Asp of conserved sequence region IV; Figure 4) has no interactions with substrates in CGTase. However, in another member of the α-amylase family (amylosucrase), a mutation in the equivalent residue decreased the glucan-forming reaction specificity of the enzyme (*43*), also showing the importance of the side chain at this position for reaction specificity within the α-amylase family. A likely explanation for the increased 4-α-glucanotransferase activity of mutant G140H is that the introduced His side chain enables ATase to form a hydrogen bond with the OH6 group of the glucose residue in subsite −1, similar to the interactions observed in a CGTase−substrate structure (Figure 2A). Moreover, the increased 4-α-glucanotransferase activity (22-fold; Table 2) of mutant G140H corresponds well with the decreased cyclization activities [10−25-fold (*39*) and below] of CGTase mutants where this His side chain was substituted. This suggests that the His140 side chain introduced in ATase plays a similar role as in CGTase. Thus, both G140H and T329M mutations, in conserved sequence regions I and IV of the α-amylase family, strongly increase the 4-α-glucanotransferase activity of ATase.

In comparison with other 4-α-glucanotransferase enzymes (e.g., amylomaltase and maltosyltransferase), the 4-α-glucanotransferase activity of the most active ATase mutant is relative low, with an activity of 3.4 μmol min^{-1} mg^{-1} (Table 2), which is equivalent to a turnover rate of 4.3 s^{-1}. *Aquifex aeolicus* 4-α-glucanotransferase is four times more active with maltotetraose as substrate, with a k_{cat} of 17 s^{-1} (*44*), while the amylomaltases of *Pyrobaculum aerophilum* and *Thermus thermophilus* have k_{cat} values of 115 and 317 s^{-1}, respectively, using maltotriose as substrate (T. Kaper, personal communication). A major difference between the (mutant) ATases and other 4-α-glucanotransferase is that ATase is virtually inactive with maltotriose, whereas amylomaltases and maltosyltransferase have high activities with maltotriose as well as longer maltooligosacchraides (*44*, *45*). This difference may reflect the similarity between ATase and CGTases, as *B. circulans* strain 251 and Tabium CGTase are inactive on maltotriose (*8*, *19*). Interestingly, the ATase variants have a strong preference for maltose transfer, which is also observed for the maltosyltransferase from *Thermotoga maritima*, although for the later enzyme this specificity appears to be absolute (*45*).

Table 4: Production of Cyclodextrins by (Mutant) ATase Proteins after Incubation with 1% (w/v) Starch at 30 °C for 2 Weeks

enzyme	conversion of starch into CDs[a] (%)	α-CD[a] (mg/L)	β- and γ-CD[a,b] (mg/L)
CGTase[c]	25	700	1800
wild type	0	nd[d]	nd
G140H	1.9	80	110
Q327H	0	nd	nd
T329M	0	nd	nd
Q327H/T329M	0.1	6	5
G140H/Q327H	1.2	38	80
G140H/Q327H/T329M	7.4	460	280

[a] CD = cyclodextrin. [b] β- and γ-cyclodextrin eluted at the same retention time. [c] Tabium CGTase and data from ref *41*. [d] nd, not detectable.

Activity on Starch. Previously it has been reported that ATase has no activity with starch as substrate (*14*). However, upon prolonged incubation with 10 μg of enzyme/mL, a very low starch hydrolysis activity was detectable, with about 1 mM reducing sugars formed after 168 h of incubation. This ATase hydrolytic activity with starch of 0.01 μmol min^{-1} mg^{-1} is much lower than the hydrolytic activity of Tabium CGTase, 54 μmol min^{-1} mg^{-1} (*33*), which is, in turn, much lower than the hydrolytic activity of α-amylases (*2*). The different data for starch degradation by ATase in Hemker et al. (*14*) and in the present study may be explained either by the longer incubation times and larger amounts of enzyme used by us or by the different starch substrates used in both studies. No starch hydrolysis was observed with the ATase catalytic residue mutants (data not shown), indicating that this activity is not due to a contamination but a true ATase property. The hydrolytic activity of the mutant ATase was not significantly different from the wild-type (recombinant) ATase. Thus, ATase has a detectable but very low starch degrading activity.

A Single G140H Mutation Gives ATase Cyclodextrin-Forming Activity. Despite the 42% sequence identity with CGTases, ATase did not form cyclodextrins from starch, not even upon 2 weeks of incubation with 10 μg/mL enzyme (detection limit of ∼5 μM, using the color reagent phenolphthalein). In contrast, ATase variants containing the G140H mutation clearly formed cyclodextrins (Table 4). Mass spectrometry confirmed that the G140H mutants produced α-, β-, and γ-cyclodextrins from starch. Compounds with these masses were not detectable following incubation of

starch with ATase wild type or derived Q327H, T329M, and Q327H/T329M mutant proteins for 2 weeks. HPAEC analysis revealed that the G140H mutants converted 1.2−7.4% of the starch into cyclodextrins (Table 4). By comparison, CGTases can reach up to 40% conversion of starch into cyclodextrins, depending on the particular CGTase (25% in the case of Tabium CGTase; Table 4). HPAEC analysis also showed a very low amount of cyclodextrins in the sample incubated with mutant Q327H/T329M (Table 4). A cyclization activity of 0.002 μmol min^{-1} mg^{-1} was calculated, equivalent to 0.15 turnovers per minute, for ATase mutant G140H/Q327H/T329M, which is 10^5-fold lower than that of Tabium CGTase [250 μmol min^{-1} mg^{-1} (*19*)]. Nevertheless, the ATase mutants converted a significant amount of the starch into cyclodextrins after prolonged incubation (Table 4), reflecting the similarity between ATase mutants and CGTases.

CGTase Gains Acarviosyl Transferase Activity upon a H140A Mutation. Since mutation G140H introduced cyclodextrin-forming activity into ATase, the question arose whether the opposite mutation would give CGTase acarviosyl transferase activity. Since a Tabium CGTase H140A mutant was already available in our laboratory, this mutant was tested for its ability to catalyze the acarviosyl transferase reaction (Figure 1). Mutation H140A reduced the cyclization activity of Tabium CGTase from 250 to 10 μmol min^{-1} mg^{-1}. CGTase mutant H140A displayed indeed considerable acarviosyl transferase activity (Figure 5B), only 8 times less than that of ATase (0.25 μmol min^{-1} mg^{-1}; equivalent to a turnover rate of about 0.3 s^{-1}). In contrast, wild-type CGTase did not form acarviosylmaltotriose (Figure 5B), not even during much longer incubation periods (data not shown). Mass spectrometry confirmed the formation of a compound with the mass of acarviosylmaltotriose − H^+ (808 Da) by CGTase-H140A but not by wild-type CGTase. Interestingly, to catalyze this reaction, acarbose must bind at the −2 to +2 subsites of CGTase mutant H140A, whereas it is bound in the −1 to +3 subsites of wild-type CGTase proteins according to crystal structural information (*24*, *25*), with the C−N−C linkage at the cleavage site (Figure 2B). The CGTase structures also showed a hydrogen bond between the His140 side chain and the OH6 group of the valienamine moiety of acarbose at subsite −1, similar to the hydrogen bond formed with a natural substrate (Figure 2A), indicating the importance of this His residue for acarbose inhibition. Indeed, CGTase mutant H140A required a 400-fold higher acarbose concentration for 50% inhibition of cyclization activity (40 versus 0.1 μM for wild-type Tabium CGTase; data not shown). This is in agreement with the much higher K_i values for acarbose measured for His to Asn mutations at the same positions in barley α-amylase I and alkalophilic *Bacillus* sp. 1011 CGTase (*39*, *40*). Thus, our data demonstrate that acarbose can also bind in the −2 to +2 subsites of CGTase mutant H140A and that a single H140A mutation is sufficient to give CGTase acarviosyl transferase activity.

CONCLUSIONS

By comparing the ATase and CGTase sequences and using site-directed mutagenesis, we have identified two residues (Gly140 and Thr329) that are essential for the acarviosyl transferase versus 4-α-glucanotransferase specificity of ATase (Table 2). A single G140H mutation introduced cyclodextrin-forming activity into ATase, whereas Tabium CGTase acquired acarviosyl transferase activity with a single H140A mutation in its active site. The data thus indicate that the active sites of the ATase and CGTase proteins are very similar. The different reaction specificities of the two enzymes can be interchanged by single mutations at their active sites (Tables 2 and 4).

These observations suggest that *Actinoplanes* has recruited a CGTase enzyme to perform a new and special task in its acarbose/starch metabolism. The acarbose (*acb*) gene cluster of *Actinoplanes* sp. SE50/110 encodes a mixture of proteins related to enzymes of the primary and secondary metabolism (*27*), supporting a new type of starch metabolism based on acarbose as a central structure. Acarbose serves as an inhibitor for starch-degrading enzymes used by competitors in the natural environment and also serves as an acceptor molecule for glucose and longer oligosaccharides (*16*, *27*). Acarbose is structurally related to starch molecules (Figure 3), and acarbose metabolism involves several enzymes closely related to starch-modifying enzymes (*16*). Since our data show that a single amino acid change in the active site of a CGTase is sufficient to make it an acarbose-modifying enzyme (and vice versa), the α-amylase encoded by the acarbose (*acb*) gene of the *Actinoplanes* sp. SE50/110 cluster may have become adapted in a similar manner. Also, this α-amylase (AcbE) is resistant to acarbose inhibition (*27*). The molecular basis for this resistance is not understood so far, since the two histidine residues studied in this paper are both present, and this α-amylase is highly related to other long-chain α-amylases. The detailed characterization of this α-amylase in future work may reveal an alternative adaptation mechanism.

The detailed knowledge and understanding at the molecular level of the effects of the modifications described in this paper allow the design of enzymes with new reaction specificities which may find application in the enzymatic synthesis of a new (or modified) carbohydrate type of primary and secondary metabolites.

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