Identification of key amino acid residues in *Neisseria polysaccharea* amylosucrase

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Abstract Amylosucrase from *Neisseria polysaccharea* catalyzes the synthesis of an amylose-like polymer from sucrose. Sequence alignment revealed that it belongs to the glycoside hydrolase family 13. Site-directed mutagenesis enabled the identification of functionally important amino acid residues located at the active center. Asp-294 is proposed to act as the catalytic nucleophile and Glu-336 as general acid base catalyst in amylosucrase. The conserved Asp-401, His-195 and His-400 residues are critical for the enzymatic activity. These results provide strong support for the predicted close structural and functional relationship between the sucrose-glucosyltransferases and enzymes of the α-amylase family.

Key words: Amylosucrase; Site-directed mutagenesis; Active site; Catalytic nucleophile; General acid catalyst

1. Introduction

Amylosucrase (AS) was first discovered in cultures of *Neisseria perflava* [1]. In 1974, *Neisseria polysaccharea* was isolated from the throats of healthy children in Europe and Africa [2]. This non-pathogenic strain was shown to produce an extracellular AS that uses sucrose to generate a linear polymer composed of α-(1,4)-glucopyranosyl residues having strong similarities with amylose [3]. The gene encoding AS from *N. polysaccharea* has been cloned, sequenced and expressed in *Escherichia coli* [4,5]. The recombinant protein was purified to homogeneity using a glutathione *S*-transferase (GST) fusion protein system [6].

Recently, investigations on the catalytic properties of the pure recombinant enzyme [7,8] confirmed that AS catalyzes the synthesis of an amylose-like polymer from sucrose only, without participation of α-D-glucosyl nucleoside-diphosphate, unlike other bacterial amylopolysaccharide synthases [9]. In addition to polymer synthesis, AS also catalyzes sucrose hydrolysis and oligosaccharide synthesis by transfer of the glucosyl residue of sucrose to either water or a sugar acceptor molecule [8].

Sequence alignment revealed that AS belongs to the glycoside hydrolase family 13 [10–14] and therefore most likely possesses a $(\beta/\alpha)_8$-barrel domain [6]. AS also shares common features with all other known glucansucrase sequences from *Leuconostoc* and *Streptococcus* species [6,11,15]. These sequences, however, were longer and contained additional N- and C-terminal domains; moreover, the $(\beta/\alpha)_8$-barrel was circularly permutated [11]. The structure prediction of AS revealed that it is the only glucansucrase for which the sequence is known that does not contain a circularly permutated $(\beta/\alpha)_8$-barrel [6,11]. Six of the eight highly conserved regions in amylolytic enzymes were identified in the AS sequence, and invariant amino acid residues [12,13] known as essential for catalysis in enzymes of glycoside hydrolase family 13 were shown to be conserved in AS [6].

On the basis of structure prediction, it was suggested that the catalytic mechanism of AS resembles that of α-amylases, especially for the first part of the reaction [16,17] consisting in the cleavage of the glycosidic linkage and the formation of the β-glucosyl-enzyme covalent intermediate. Indeed, AS which is an α-retaining enzyme probably uses a double-displacement mechanism. In the first step, sucrose is cleaved via the formation of a β-glucosyl ester of a carboxylic acid and release of fructose. In the second step, the glucosyl residue is displaced by reaction with the oxygen of the hydroxyl group either from a sugar molecule or from water. Several conserved residues have been shown to be involved in the α-retaining catalytic process of glycoside hydrolase of family 13 [18]. A glutamic acid (equivalent to Glu-230 of Taka-amylase from *Aspergillus oryzae*, TAA) acts as a general catalyst and protonates the oxygen of the glycosidic linkage to be cleaved and an aspartic acid (Asp-206 in TAA) exerts the nucleophile attack with formation of the β-glucosyl-enzyme intermediate. Three other conserved residues are important: a second aspartic acid (Asp-297 in TAA) is also essential for catalysis [18] while two histidines (His-122 and His-296 in TAA) are needed to maintain normal activity levels. The former residue plays a major role in substrate distortion and the histidines in the transition state stabilization at subsite −1 [18].

Comparison of amino acid sequences enabled unequivocal localization of the two aspartic acids and the two histidine residues in the sequence of AS. However, three glutamic acids were candidates for the general catalyst. A site-directed mutagenesis approach was used to investigate the role in activity of the two aspartic acid, two histidine and three glutamic acid target residues in AS and to identify the putative general acid catalyst.

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Abbreviations: AS, amylosucrase; GST, glutathione *S*-transferase; TAA, Taka-amylase from *Aspergillus oryzae*; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis

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2. Materials and methods

2.1. Bacteria and plasmid

E. coli JM109 was used as a host for site-directed mutagenesis and for purification of wild-type and mutated AS. The strain was maintained and grown on Luria–Bertani (LB) agar plates containing ampicillin (100 μg ml^{-1}) [19]. The pGST-AS plasmid [6] encoding the GST gene fused to that of AS, was used for site-directed mutagenesis and for expression of the fusion gene. Plasmid DNA was prepared using a Qiaprep Spin miniprep kit (Qiagen).

2.2. Site-directed mutagenesis

Site-directed mutagenesis of the AS gene was carried out with the QuikChange™ site-directed mutagenesis kit (Stratagene). The procedure utilizes the pGST-AS double-stranded DNA vector and two synthetic oligonucleotide primers containing the desired mutation (Table 1). The oligonucleotide primers (synthesized by Isoprim, Toulouse, France), each complementary to opposite strands of the vector, were extended during temperature cycling by *pfu* Turbo DNA polymerase (Stratagene). On incorporation of the oligonucleotide primers, a mutated plasmid containing staggered nicks is generated. Following temperature cycling (95°C, 30 s; 55°C, 1 min; 68°C, 15 min for 12 cycles), the product is treated with *Dpn*I. The *Dpn*I endonuclease (target sequence: 5′-G^{m}ATC-3′) is specific for methylated and hemimethylated DNA and is used to digest the methylated parental DNA template and to select for mutation-containing synthesized DNA. The nicked vector DNA incorporating the desired mutation is then transformed into *E. coli* JM109 competent cells, as previously described [19]. Mutagenic oligonucleotides were designed to create a restriction site which was used to screen the correct mutation. The mutations were confirmed by DNA sequencing which was carried out using the dideoxy chain-termination procedure [20], by Genome Express (Grenoble, France).

2.3. Purification of wild-type and mutated AS

E. coli carrying the recombinant plasmid encoding wild-type or mutated AS gene was grown on LB containing ampicillin (100 μg ml^{-1}) and isopropyl-β-thiogalactopyranoside (IPTG) (2 mM) for 10 h. The cells were harvested by centrifugation, resuspended and concentrated to an OD_{600} of 80 in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3). The intracellular enzyme was extracted by sonication and the lysate supernatant was used as the source for enzyme purification. Overexpression was verified by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Purification of wild-type and mutated enzyme was performed as previously described [6] by affinity chromatography of the GST/AS fusion protein on glutathione–Sepharose 4B (Amersham Pharmacia Biotech). The fusion protein solution was subjected to proteolysis to remove the GST-tag, using the PreScission protease (Amersham Pharmacia Biotech). The purified AS was finally eluted in PreScission buffer (50 mM Tris–HCl pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol). The enzyme was concentrated using Microsep™ microconcentrators 10K (Filtron Technology Corporation, Northborough, MA, USA).

2.4. Electrophoresis of proteins

Electrophoresis was carried out with the PHAST system (Amersham Pharmacia Biotech) as recommended by the manufacturer, using PhastGel™ gradient 8–25 (Pharmacia Biotech) ready made gels under denaturing conditions.

Table 1
Oligonucleotides used for site-directed mutagenesis

Mutant	Sequence													Restriction site
D401E		Val	Arg	Ser	His	Asp	Asp	Ile	Gly	Trp	Thr	Phe	Ala	
	C	GTC	CGC	AGC	CAC	GAC	GAC	ATC	GGC	TGG	ACG	TTT	GC	
	C	GTC	CGC	AGC	CAC	**GAA**	GAT	ATC	GGC	TGG	ACG	TTT	GC	*Eco*RV
						Glu								
D401N		Val	Arg	Ser	His	Asp	Asp	Ile	Gly	Trp	Thr	Phe	Ala	
	C	GTC	CGC	AGC	CAC	GAC	GAC	ATC	GGC	TGG	ACG	TTT	GC	
	C	GTC	CGC	AGC	CAC	**AAC**	GAT	ATC	GGC	TGG	ACG	TTT	GC	*Eco*RV
						Asn								
D294N	Gly	Val	Asp	Ile	Leu	Arg	Met	Asp	Ala	Val	Ala			
	GGC	GTT	GAC	ATC	CTG	CGT	ATG	GAT	GCG	GTT	GCC			
	GGC	GTT	GAT	ATC	CTG	CGT	ATG	**AAT**	GCG	GTT	GCC			*Eco*RV
								Asn						
D294E	Gly	Val	Asp	Ile	Leu	Arg	Met	Asp	Ala	Val	Ala			
	GGC	GTT	GAC	ATC	CTG	CGT	ATG	GAT	GCG	GTT	GCC			
	GGC	GTT	GAT	ATC	CTG	CGT	ATG	**GAA**	GCG	GTT	GCC			*Eco*RV
								Glu						
E352Q		Gln	Tyr	Ile	Gly	Gln	Asp	Glu	Cys	Gln	Ile	Gly		
	C	CAA	TAC	ATC	GGG	CAG	GAC	GAA	TGC	CAA	ATC	GG		
	C	CAA	TAC	ATC	GGG	CAG	GAC	**CAA**	TGC	CAA	ATC	GG		*Ava*II
								Gln						
E336Q		Ala	Val	Phe	Phe	Lys	Ser	Glu	Ala	Ile	Val	His		
	CC	GCC	GTG	TTC	TTC	AAA	TCC	GAA	GCC	ATC	GTC	CAC	C	
	CC	GGC	GTG	TTC	TTC	AAA	TCC	**CAA**	GCC	ATC	GTC	CAC	C	*Sac*II
								Gln						
E308Q		Lys	Gln	Met	Gly	Thr	Ser	Cys	Glu	Asn	Leu	Pro		
	GG	AAA	CAA	ATG	GGG	ACA	AGC	TGC	GAA	AAC	CTG	CCG		
	GG	AAA	CAA	ATG	GGG	ACT	AGT	TGC	**CAA**	AAC	CTG	CCG		*Spe*I
										Gln				
H195Q		Asp	Phe	Ile	Phe	Asn	His	Thr	Ser	Asn	Glu	His		
	C	GAT	TTT	ATC	TTC	AAC	CAC	ACC	TCC	AAC	GAA	CAC	G	
	C	GAT	TTT	ATC	TTC	AAC	**CAG**	ACG	TCC	AAC	GAA	CAC	G	*Aat*II
							Gln							
H400N		Val	Arg	Ser	His	Asp	Asp	Ile	Gly	Trp	Thr	Phe	Ala	
	C	GTC	CGC	AGC	CAC	GAC	GAC	ATC	GGC	TGG	ACG	TTT	GC	
	C	GTC	CGC	AGC	**AAC**	GAC	GAT	ATC	GGC	TGG	ACG	TTT	GC	*Eco*RV
					Asn									

Gene (upper) and direct mutagenic oligonucleotide (lower) sequences. Underlining of the lower sequence indicates the site introduced by the mutagenesis.

2.5. AS activity assays

AS catalyzed the synthesis of an α-1,4 glucan from sucrose without any oligosaccharide or polysaccharide primer. Studies on the purified recombinant AS showed, however, that the sucrose consumption rate greatly increased when oyster glycogen was added to the reaction mixture [7,8]. In order to facilitate the characterization of mutants that exhibited a highly reduced activity, all the initial velocities therefore were determined in the presence of glycogen. AS standard assay was carried out at 30°C in PBS 1× or PreScission buffer supplemented with sucrose (50 g l^{-1}) and glycogen (0.1 g l^{-1}). One unit of AS activity corresponds to the amount of enzyme that catalyzes the production of 1 μmol of fructose per min under these assay conditions. The concentration of fructose was measured by the dinitrosalicylic acid method, using fructose as a standard [21]. Protein content was determined by the Bradford and micro-Bradford methods, using bovine serum albumin as the standard [22].

2.6. Sugar analysis

Sucrose, glucose and fructose concentrations were measured by high performance liquid chromatography (HPLC) at 25°C, with an Aminex HP87H column (Bio-Rad Chemical Division, Richmond, CA, USA), with 8.5 mM H_2SO_4 as the eluant, at a flow rate of 0.5 ml min^{-1}.

3. Results and discussion

3.1. Identification of amino acid residues that may be involved in the active site of AS

Identification of potential critical or essential residues was approached by AS sequence comparison to related enzymes belonging to the α-amylase superfamily (Fig. 1). AS Asp-294 and Asp-401 corresponding to TAA Asp-206 and Asp-297 respectively were first selected as targets for site-directed mutagenesis. Both were replaced by Asn, to minimize the structural changes (D294N, D401N), and Glu to maintain the charge (D294E, D401E). Multiple alignment did not unequivocally point out the general acid catalyst in AS equivalent to TAA Glu-230. Three glutamic acid residues Glu-308, Glu-336 and Glu-352 were potential candidates and each of these was replaced by Gln (E308Q, E336Q, E352Q) to measure the effect on activity and in this manner identify the putative general catalyst.

The AS His-195 and His-400, equivalent to the conserved His-122 and His-296 at the active site of TAA, were also chosen as targets for substitution (Fig. 1). In the other glucansucrases, the first histidine residue is substituted by a glutamine, while the second is conserved [11]. AS His-195 and His-400 were replaced by Gln (H195Q) and Asn (H400N) respectively. In both cases, the N of the side chain carboxyamide group was able to superimpose with one of the nitrogens, NE2 or ND1 respectively of the imidazole ring in histidine.

3.2. Amino acid substitution by site-directed mutagenesis and purification of wild-type and mutant AS

The pGST-AS plasmid was used as a template for site-directed mutagenesis. Mutation was confirmed by sequencing part of the mutated AS gene. Recombinant *E. coli* JM109 strains carrying the plasmid (pGST-AS or pGST-AS*) with the wild-type or mutated AS gene were grown. The expression levels in *E. coli* of genes encoding GST-AS or GST-AS mutants were shown to be very similar. Both wild-type and mu-

		I	II	III	IV	V	VI	VII	VIII
		β2	β3	L3	L3	β4	β5	β7	β8
A	**AS**	134-**GLTYLHLM-P**-142	190-**DFIFNH**-195		262-**QWDLN**-266	290-**ILRMDAVAF**-298		396-**YVR--SHD**-401	488-**GLPLIYLGD**-496
	AMY	36-**GFGGVQVS-P**-44	96-**DAVINH**-101	150-SYND-153	165-**LLDLA**-169	193-**GFRIDASKH**-201	233-**EVID**-236	295-**FVD--NHD**-300	334-**GFTRVMSSY**-342
	OGL	44-**GIDVIWLS-P**-52	98-**DLVVNH**-103	150-QYDE-153	167-**QPDLN**-171	195-**GFRMDVINF**-203	255-**EMPG**-258	324-**YWN--NHD**-329	360-**GTPYIYQGE**-368
	AGL	52-**GVDAIWVC-P**-60	106-**DLVINH**-111	164-TFDE-167	181-**QVDLN**-185	210-**GFRIDTAGL**-218	276-**EVAH**-279	344-**YIE--NHD**-349	381-**GTLYVYQGQ**-389
	PUL	210-**GVTHVELL-P**-218	281-**DVVYNH**-286	308-AYGN-311	319-**GNDIA**-323	348-**GFRFDLMGI**-356	381-**EGWD**-384	464-**YVE--SHD**-469	505-**GIPFLHSGQ**-513
	APU	435-**GISVIYLN-P**-443	488-**DGVFNH**-493	527-PYGD-530	565-**WADFI**-569	593-**GWRLDVANE**-601	626-**ELWG**-629	698-**LLG--SHD**-703	745-**GMPSIYYGD**-753
	CMD	187-**GVNALYFN-P**-195	240-**DAVFNH**-245	284-TYDT-287	294-**MPKLN**-298	323-**GWRLDVANE**-331	356-**EIMH**-359	418-**LLG--SHD**-423	450-**GTPCIYYGD**-458
	MTH	50-**GFSAIWMPVP**-59	112-**DVVPNH**-117	145-NYPN-148	160-**ESDLN**-164	189-**GFRFDFVRG**-197	219-**ELWK**-222	289-**FVD--NHD**-294	327-**GTPVVYWSH**-335
	ISA	217-**GVTAVEFL-P**-225	291-**DVVYNH**-296	328-TSGN-331	341-**GANFN**-345	370-**GFRFDLASV**-378	416-**EFTV**-419	502-**FID--VHD**-507	570-**GTPLMQGGD**-578
	DGL	44-**GVMAIWLS-P**-52	98-**DLVVNH**-103	145-QYDD-148	162-**QPDLN**-166	190-**GFRMDVIDM**-198	236-**ETWG**-239	308-**FWN--NHD**-313	344-**GTPYIYQGE**-352
	MHH	38-**GITAVWIP-P**-46	102-**DVVMNH**-107	166-DWDQ-169	203-**YADID**-207	232-**GFRIDAVKH**-240	266-**EFWK**-269	328-**FVD--NHD**-333	362-**GYPSVFYGD**-370
	NPU	188-**GINGIYLT-P**-196	241-**DAVFNH**-246	284-NYDT-287	294-**MPKLN**-298	323-**GWRLDVANE**-331	356-**EVWH**-359	418-**LLG--SHD**-423	450-**GTPCIYYGD**-458
	BRE	280-**GFTHLELL-P**-288	335-**DWVPGH**-340	356-LYEH-359	367-**HQDWN**-371	401-**ALRVDAVAS**-409	458-**EEST**-461	519-**FVLPLSHD**-526	555-**GWMWAFPGK**-563
	CGT	70-**GVTALWISQP**-79	135-**DFAPNH**-140	185-SLEN-188	197-**LADFN**-201	225-**GIRVDAVKH**-233	257-**EWFL**-260	323-**FID--NHD**-328	354-**GVPAIYYGT**-362
	GDE	137-**GYNMIHFT-P**-145	198-**DVVYNH**-203	248-KYKE-251	451-**LRNFA**-455	505-**GVRLDNCHS**-513	538-**ELFT**-541	604-**FMD-ITHD**-610	642-**GYDELVPHQ**-650
	TAA	56-**GFTAIWIT-P**-64	117-**DVVANH**-122	155-YEDQ-158	173-**LPDLD**-177	202-**GLRIDTVKH**-210	230-**EVLD**-233	292-**FVE--NHD**-297	323-**GIPIIYAGQ**-331
			★			★	★	★★	

		I	II	IV	V	VI	VII	VIII
B	**AS**	134-**GLTYLHLM-P**-142	190-**DFIFNH**-195	262-**QWDLN**-266	290-**ILRMDAVAF**-298		396-**YVR--SHD**-401	488-**GLPLIYLGD**-496
	DSRB	939-**GITSFQLA-P**-947	1004-**DWVPDQ**-1009	491-**ANDVD**-495	529-**GIRVDAVDN**-537	571-**EDWS**-574	639-**FVR--AHD**-644	709-**TVPRVYYGD**-717
	DSRA	699-**GITSFEMA-P**-707	764-**DWVPDQ**-769	244-**ANDVD**-248	282-**GYRVDAVDN**-290	324-**QFWK**-327	392-**FIR--AHD**-397	464-**TIPRVYYGD**-472
	GTFD	894-**GVTSFEMA-P**-902	959-**DWVPDQ**-964	423-**ANDID**-427	461-**GVRVDAVDN**-469	503-**EAWS**-506	579-**FIR--AHD**-584	649-**SITRLYYGD**-657
	GTFK	893-**GITSFEIA-P**-901	958-**DWVPDQ**-963	417-**SNDID**-421	457-**GIRVDAVDN**-465	499-**EAWS**-502	577-**FVR--AHD**-582	647-**AATRVYYGD**-655
	GTFS	849-**GITQFEMA-P**-857	914-**DLVPNQ**-919	495-**ANDVD**-499	433-**GVRVDAVDN**-441	475-**EAWS**-478	542-**FIR--AHD**-547	614-**TVTRVYYGD**-622
	GTFI	860-**GITDFEMA-P**-868	926-**DWVPDQ**-931	405-**ANDVD**-409	443-**SIRVDAVDN**-451	485-**EAWS**-488	553-**FAR--AHD**-558	623-**SIPRVYYGD**-631
	GTFC	889-**GVTDFEMA-P**-897	954-**DWVPDQ**-959	435-**ANDVD**-439	473-**SIRVDAVDN**-481	515-**EAWS**-518	583-**FIR--AHD**-588	653-**SVPRVYYGD**-661
	GTFB	862-**GVTDFEMA-P**-870	927-**DWVPDQ**-932	409-**ANDVD**-413	447-**SIRVDAVDN**-455	489-**EAWS**-492	557-**FIR--AHD**-562	627-**SVPRVYYGD**-635
	DSRS	958-**GVTSFQLA-P**-966	1023-**DWVPDQ**-1028	509-**ANDVD**-513	547-**GIRVDAVDN**-555	589-**EDWS**-592	657-**FVR--AHD**-662	727-**TVPRVYYGD**-735
			★		★	★	★★	

Fig. 1. Conserved sequence stretches (roman numbers) in AS, in the α-amylase superfamily (A) and in glucosyltransferases (B). The second line denotes the elements of secondary structure, as determined for pig pancreatic α-amylase. The enzymes are numbered from the N-terminal end. The invariant residues are in bold. Enzyme sources (A): AS, AS (*N. polysaccharea*); AMY, α-amylase (pig pancreatic); OGL, oligo-1,6-glucosidase (*Bacillus cereus*); AGL, α-glucosidase (*Saccharomyces cerevisiae*); PUL, pullulanase (*Bacillus stearothermophilus*); APU, amylopullulanase (*Clostridium thermohydrosulfuricum*); CMD, cyclomaltodextrinase (*Bacillus sphaericus*); MTH, maltotetraohydrolase (*Pseudomonas saccharophila*); ISA, isoamylase (*Pseudomonas amyloderamosa*); DGL, dextran-glucosidase (*Streptococcus mutans*); MHH, maltohexaohydrolase (*Bacillus sp.* strain 707); NPU, neopullulanase (*B. stearothermophilus*); BRE, branching enzyme (*E. coli*); CGT, cyclodextrin-glycosyltransferase (*Bacillus circulans*); GDE, glycogen debranching enzyme (Human muscle); TAA, α-amylase (*A. oryzae*). Enzyme sources (B): AS, (*N. polysaccharea*); DSRB (*Leuconostoc mesenteroides* NRRL B-1299); DSRA (*L. mesenteroides* NRRL B-1299); GTFD (*S. mutans* GS5); GTFK (*Streptococcus salivarius* ATCC 25975); GTFS (*Streptococcus downei* Mfe 28); GFTI (*Streptococcus sobrinus* OMZ176 Serotype D); GTFC (*S. mutans* GS5); GTFB (*S. mutans* GS5); DSRS (*L. mesenteroides* NRRL B-512F). All the glucansucrases except AS shown in block B are circularly permutated. Stars correspond to functionally important amino acids that have been replaced by site-directed mutagenesis.

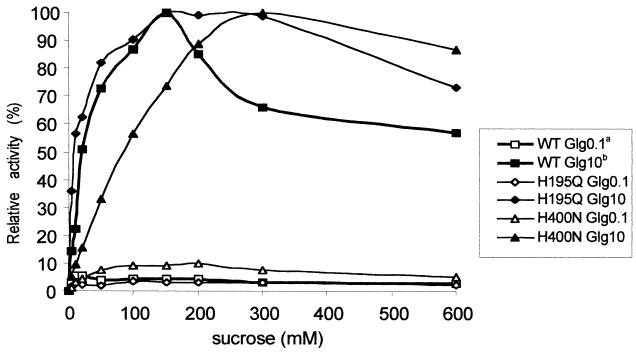
tant ASs were purified to homogeneity by affinity chromatography and the tag GST was removed by proteolysis. The AS protein size after proteolysis was verified by SDS–PAGE. Each mutant enzyme produced a single band of correct size.

3.3. Activity of wild-type and mutant ASs

AS mutant activities were compared to that of the wild-type enzyme (Table 2). Replacement of the putative nucleophile Asp-294 by Asn or Glu (D294N and D294E mutants) led to a complete loss of AS activity. Concerning the localization of the glutamic acid that could act as a general catalyst, the three AS mutants harboring either E308Q, E336Q or E352Q replacement were produced, purified and tested. The mutant carrying E308Q substitution retained 100% of the wild-type AS activity, whereas the specific activity of the E352Q mutant was reduced by 30%. In contrast, the mutation of Glu-336 (E336Q) most drastically affected the activity. Neither sucrose consumption nor polymer production were detectable, even in the presence of 30 g l^{-1} of glycogen, known to be a very efficient activator at this high concentration [8]. These data provide evidence for the essential catalytic role of Asp-294 and Glu-336 in AS. By analogy to α-amylases, Asp-294 is thus proposed as the catalytic nucleophile and Glu-336 as the general acid-base catalyst.

Similarly, the residue Asp-401 equivalent to Asp-297 of TAA was replaced by Asn or Glu (D401N and D401E mutants). No consumption of the substrate sucrose was detected in the presence of 0.1 g l^{-1} of glycogen. However, the D401E mutant, in the presence of 30 g l^{-1} glycogen that most efficiently activates native AS, exhibited 0.15% of the wild-type AS activity. Under these conditions, insoluble glucan synthesis was visually detected after 2 days of incubation. For several enzymes of glycoside hydrolase family 13, residues equivalent to Asp-401 are shown to participate in the distortion of substrate. Moreover, these residues are considered to stabilize the geometry of the catalytic groups and presumably contribute to maintain the general acid catalyst in the protonated form at the pH range for optimum activity [23]. These residues indeed are also critical for AS activity. However, when the charge was maintained as in the D401E mutant, the mutant still had detectable catalytic activity when large amounts of glycogen were added.

Finally, H195Q and H400N mutant enzymes were shown to retain respectively 2 and 3% of the wild-type enzyme activity (Table 2). In the presence of glycogen, a low level of polymer production was observed for both of these mutant enzymes, but their activity was too low for a kinetic characterization in the reaction with sucrose as sole substrate. Instead, the activator effect at two different glycogen concentrations (0.1 g l^{-1} and 10 g l^{-1}) on mutant and wild-type enzyme activity was compared (Fig. 2). Glycogen still exerts an activating effect on sucrose consumption for the two mutants. This effect is dependent on glycogen concentration and is more pronounced for the wild-type enzyme and the mutant H195Q than for the mutant H400N. At 100 mM sucrose, the initial activity increased 25 and 18 times for H195Q and wild-type enzyme respectively, when the glycogen concentration was increased from 0.1 to 10 g l^{-1}, whereas for the H400N mutant, only a six-fold increase was observed. In addition, at 10 g l^{-1} of glycogen, the profiles of activation of the two mutants differ from the profile of the wild-type enzyme (Fig. 2).



[a] in the presence of 0.1 g.l^{-1} glycogen
[b] in the presence of 10 g.l^{-1} glycogen

Fig. 2. Relative activity of wild-type (WT), H195Q and H400N mutant ASs in presence of glycogen versus sucrose concentration.

Glycogen is known to play the role of acceptor and its external chains have been shown to be elongated by AS [6,7]. The effect of glycogen on the initial activity was speculated to stem from a change of the rate limiting step of the reaction. In the presence of glycogen, its external chains would efficiently displace the glucosyl residue from the covalent complex with the active site nucleophile resulting in elongation of the chain. Thus, the limiting step may be the formation of the glucosyl-enzyme, whereas in the absence of glycogen, it may be the transfer onto the growing linear α-glucan chain. This effect supports that an acceptor site

Table 2
Specific activity of wild-type and mutant ASs

Mutation	Site and type of mutation (corresponding to amino acids of TAA)	Activity[a] (U mg^{-1})	Relative activity[a] (%)	Activity[b] (U mg^{-1})	Relative activity[b] (%)
D294N	Asp-294 (Asp-206) → Asn	ND[c]	–	ND	–
D294E	Asp-294 (Asp-206) → Glu	ND	–	ND	–
D401N	Asp-401 (Asp-297) → Asn	ND	–	ND	–
D401E	Asp-401 (Asp-297) → Glu	ND	–	0.12	0.15
E308Q	Glu-308 → Gln	10	100		
E336Q	Glu-336 (Glu-230) → Gln	ND	–	ND	–
E352Q	Glu-352 → Gln	7.5	75		
H195Q	His-195 (His-122) → Gln	0.2	2	3.4	4
H400N	His-400 (His-296) → Asn	0.3	3	2.5	3
Wild-type		10	100	85	100

Corresponding amino acid residues of TAA are indicated in brackets.
[a]Activity in the presence of 50 g l^{-1} sucrose and 0.1 g l^{-1} glycogen.
[b]Activity in the presence of 50 g l^{-1} sucrose and 30 g l^{-1} glycogen.
[c]ND: not detectable.

exists which can bind the involved glycogen. At high sucrose concentration, sucrose may compete with glycogen for binding to this site and consequently the initial rate of the reaction would decrease [7,8]. The two different profiles of activation obtained with the two mutants suggest that the two histidine may interfere in a different way with the acceptor glycogen or with sucrose at the acceptor binding site.

The equivalents of His-195 in complexes of related α-amylases with inhibitors mimicking transition state such as the pseudotetrasaccharide acarbose [12,18,23,24] have been shown to form a hydrogen bond with OH-6 of the ring bound at subsite −1. Similarly, the equivalents of His-400 have been found to bind to OH-2 and OH-3 also in subsite −1. The stabilization of the transition state structure has a strong impact on the efficiency of the subsequent reaction steps in the mechanism. Apparently, the His195Gln mutant at 0–150 mM sucrose is most sensitive to glycogen activation suggesting either a superior replacement by glutamine compared to asparagine or that His-400 is more critical for this activation. In the known crystal structures [17,22,23], the NE2 of imidazole participates in the corresponding histidines in hydrogen bonding to the ligand. This supports that glutamine and not asparagine makes a preferred replacement.

The results presented here allowed us to pinpoint functionally important amino acid residues located at the active center of AS. By analogy with enzymes of glycoside hydrolase family 13, Asp-294 is proposed as the catalytic nucleophile and Glu-336 as the general acid base catalyst. The family 13 consensus residues moreover were confirmed by the mutation of Asp-401, His-195 and His-400. In addition, the results of these mutageneses strongly support the predictions of a close structural and functional relationship between the sucrose-glucosyltransferases of *Streptococcus* and *Leuconostoc* sp. and the enzymes of the α-amylase family [11]. The three-dimensional structure of AS is necessary to further improve insight in the roles of these amino acids in the formation of the glucosyl-enzyme intermediate and in the subsequent transfer onto sugar acceptor molecules.

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