Maltooligosaccharide disproportionation reaction: an intrinsic property of amylosucrase from *Neisseria polysaccharea*

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Received 24 July 2002; accepted 26 July 2002

First published online 12 August 2002

Edited by Barry Halliwell

Abstract Amylosucrase from *Neisseria polysaccharea* (AS) is a remarkable transglycosidase of family 13 of the glycoside hydrolases that catalyses the synthesis of an amylose-like polymer from sucrose and is always described as a sucrose-specific enzyme. Here, we demonstrate for the first time the ability of pure AS to catalyse the disproportionation of maltooligosaccharides by cleaving the α-1,4 linkage at the non-reducing end of a maltooligosaccharide donor and transferring the glucosyl unit to the non-reducing end of another maltooligosaccharide acceptor. Surprisingly, maltose, maltotriose and maltotetraose are very poor glucosyl donors whereas longer maltooligosaccharides are even more efficient glucosyl donors than sucrose. At least five glucose units are required for efficient transglucosylation, suggesting the existence of strong binding subsites, far from the sucrose binding site, at position +4 and above. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Amylosucrase; Sucrose; Maltooligosaccharides; Disproportionation; Transglycosylation

1. Introduction

Amylosucrases (EC 2.4.1.4) are enzymes known to be specific for the cleavage of the α1–β2 linkage of sucrose and for the formation of an insoluble α-1,4-linked glucan [1]. Among the glucansucrases, which catalyse polymer synthesis from sucrose, amylosucrases are the only ones that synthesise mainly α-1,4 linked glucan.

In 1988, Tao et al. observed a transglycosidase activity on maltooligosaccharide substrates in a crude preparation of amylosucrase from *Neisseria perflava* [2]. This activity was not attributed to amylosucrase but to a separate transglycosidase present in the enzyme mixture. Later, reactions carried out with recombinant AS in the presence of maltooligosaccharides as unique substrates also revealed the presence of a transglycosidase activity in the preparation [3]. Here again, the origin of the activity was uncertain and was attributed to a contaminant amylomaltase also expressed in *Escherichia coli*.

Amylosucrase from *Neisseria polysaccharea* (AS) has been purified to homogeneity [4] and was shown to catalyse sucrose hydrolysis and oligosaccharide formation in addition to polymer synthesis [5]. Besides, glycogen can be used as an acceptor and has a very efficient activator effect on the sucrose conversion rate [6].

Unlike the glucansucrases from lactic bacteria, all classified in family 70 of the glycoside hydrolases [7–8], AS belongs to family 13 (α-amylase family) [4]. The three-dimensional structure of amylosucrase [9–11] consists of three domains common for the enzymes of family 13: a $(\beta/\alpha)_8$-barrel domain containing all the conserved catalytic residues involved in the cleavage of the osidic bond through an α-retaining mechanism [12–14], a protuberant B-domain and a β-sandwich C-domain. Structural comparison of AS to amylolytic enzymes also revealed major differences [10]. AS contains two additional and specific domains: a helical N-terminal domain and a long loop between barrel strand β-7 and helix α-7, which forms the B′ domain. The active site is located at the bottom of a narrow pocket, contrary to most of the α-amylases which possess a large cleft on their surface [15]. This architecture is mainly due to the B′ domain, which partially covers the active site entrance. The glucosyl and the fructosyl rings of sucrose occupy the −1 and +1 positions respectively (referring to the subsite numbering used for α-amylases [16]). In addition, four specific residues are believed to be the primary determinants for the sucrose specificity: Asp144 and Arg509 which form a salt bridge at the bottom of the pocket and Asp394 and Arg446 which interact with the fructosyl ring in subsite +1 [11].

Here, we have addressed the question of the ability of AS to catalyse transglycosylation reactions from maltooligosaccharide substrates. The investigation was conducted using AS purified to homogeneity and maltooligosaccharides of degree of polymerisation ranging from 2 to 7. The minimum size required for efficient substrate transglycosylation and a proposed mechanism are discussed with regard to structural data.

2. Materials and methods

2.1. Bacterial strains

Cloning of the AS gene ATCC 43768 was carried out as previously

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Abbreviations: AS, amylosucrase from *Neisseria polysaccharea*; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose; G7, maltoheptaose; pNP-G_n, paraNitroPhenyl-maltooligosaccharide with a degree of polymerisation of *n*; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography

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PII: S0014-5793(02)03168-X

described [4]. *E. coli* strain JM109 was used as the host of pGEX-6P-3 for glutathione *S*-transferase (GST) fusion protein expression.

2.2. Enzyme extraction methods

E. coli carrying the recombinant plasmid encoding the AS gene was grown on Luria–Bertani medium containing ampicillin (100 μg ml^{-1}) and isopropyl-β-thiogalactopyranoside (1 mM) for 10 h. The cells were harvested by centrifugation (8000×*g*, 10 min, 4°C), resuspended and concentrated to an $OD_{600\,nm}$ 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 1% (v/v) Triton X-100 was added to the extract and mixed for 30 min at 4°C. After centrifugation (10 000×*g*, 10 min, 4°C), the supernatant obtained was used as the source for enzyme purification.

2.3. Purification of AS

Purification of amylosucrase was performed by affinity chromatography of the GST/AS fusion protein on glutathione–Sepharose 4B (Amersham Pharmacia Biotech) as previously described [6]. The enzyme was obtained in PreScission buffer (50 mM Tris–HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol). The protein content was determined by the micro-Bradford method, using bovine serum albumin as standard [17].

Electrophoresis of pure enzyme was carried out with the PHAST system (Amersham Pharmacia Biotech), using PhastGel™ gradient 8–25 (Pharmacia Biotech) ready-made gels under denaturing conditions. Staining with 0.5% (w/v) $AgNO_3$ led to a single band profile.

2.4. Disproportionation assay

The disproportionation assay, using 100 mg l^{-1} purified AS, was carried out at 30°C in the PreScission buffer supplemented with maltooligosaccharides with a degree of polymerisation ranging from 2 to 7. Maltooligosaccharides (G2 to G7; Sigma) and maltooligosaccharides labelled with paraNitroPhenyl tag at the reducing end (pNP-G2 to pNP-G7; Fluka), were successively tested at a concentration of 10 mM. Reactions with maltohexaose (G6) were analysed in more detail using concentrations of G6 from 5 to 450 mM (maximum solubility).

Initial rates are expressed in μmol of maltooligosaccharide substrates converted per minute and per gram of pure AS in the assay conditions.

2.5. Carbohydrate analysis

Maltooligosaccharide substrates and products were separated by reverse-phase chromatography using a 250×4 mm column (C18) (Bischoff Chromatography), eluted with water at 0.5 ml min^{-1}. Retention times were compared to that of commercial maltooligosaccharides used as standards (G2 to G7).

pNP-labelled-maltooligosaccharide substrates and products were separated by thin-layer chromatography (TLC) on silica-gel plates (silica gel 60, 5×10 cm, thickness = 0.20 mm, Alugram® Macherey-Nagel) in 4:1 (v/v) acetonitrile–water. Detection was achieved both by quenching of $UV_{254\,nm}$ fluorescence (for direct detection of pNP-tagged sugars) and by spraying the plate with 2:1 (v/v) methanol–sulphuric acid followed by heating for 10 min at 110°C.

3. Results

3.1. Activity of amylosucrase onto maltooligosaccharide substrates

Maltooligosaccharides with a degree of polymerisation ranging from 2 to 7 (G2 to G7) were successively used as substrates. Fig. 1 shows the variation with time of the C18 high-performance liquid chromatography (HPLC) profile of the reaction products obtained from G6 substrate. Comparison of retention times with retention times of standard maltooligosaccharides indicates that the action of AS only results in the formation of linear maltooligosaccharides. The evolution of the concentrations of substrate and products released from 10 mM G6 substrate is reported in Fig. 2. The production profiles show that G5 and G7 are synthesised simultaneously at the beginning of the reaction whereas G4 and G8

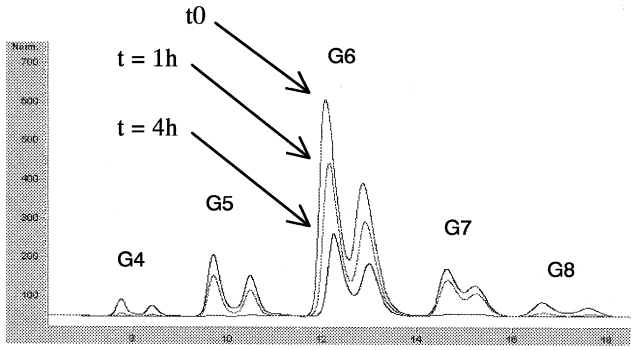

Fig. 1. C18 HPLC profile of the reaction medium in the presence of 10 mM G6 substrate at *t*0, *t* = 1 h and *t* = 4 h.

formation is delayed. No other oligosaccharides were detected in any amount in the medium, at any time, in the conditions of the assay. Substrate and product concentrations varied linearly during the first hour of the reaction. A decrease of 1 mol of G6 is correlated to the production of 0.5 mol of G5 and 0.5 mol of G7. G4 and G8 appear in equivalent amounts after a certain delay required for their formation.

This indicates that AS cleaves a terminal α-1,4 linkage of G6, releases a G5 and transfers G1 to an accepting G6 to form G7. This emphasises the functional relatedness of AS and amylolytic enzymes.

3.2. Mode of action

Products of the reaction carried out with maltooligosaccharide substrates labelled with pNP at the reducing end were separated by TLC. The direct UV detection of all the products testifies that they all carried the pNP tag. Indeed, classical treatment of the plates for sugar detection does not reveal additional saccharides, indicating that no glucose or any non-tagged saccharides are released. This shows that AS does not catalyse the hydrolysis of maltooligosaccharides.

After 20 min of reaction in the presence of pNP-G_n (paraNitroPhenyl-maltooligosaccharide with a degree of polymerisation of *n*) substrate, we observed the formation of pNP-G_{n-1} and pNP-G_{n+1} (data not shown). pNP-G_{n-2} and pNP-G_{n+2} appear later in the medium. This is in accordance with the HPLC results described above.

It is clearly demonstrated therefore that AS operates as a glucosyltransferase, transferring the non-reducing glucose residue from a donor oligosaccharide to the non-reducing end of an acceptor oligosaccharide. This is correlated with the geom-

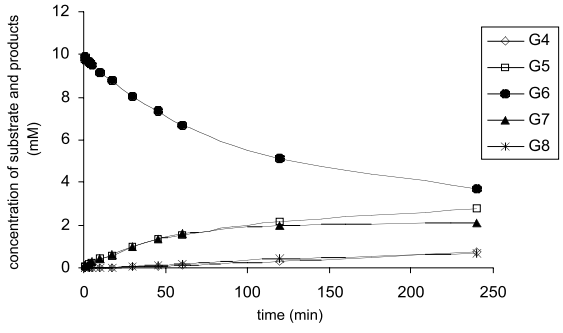

Fig. 2. Concentrations of substrate (initially 10 mM G6) and products released versus time.

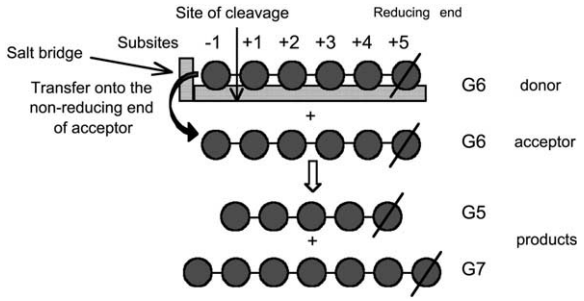

Fig. 3. Schematic representation of the disproportionation mechanism of G6 substrate.

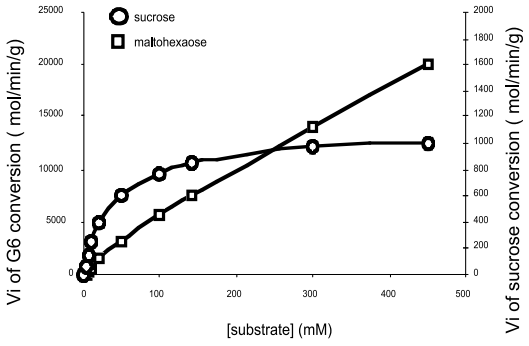

Fig. 4. Initial velocity of sucrose and G6 substrate conversion versus substrate concentration.

etry of the active site which is situated in a narrow pocket, the bottom of which is formed by a salt bridge between Asp144 and Arg509 [11]. Consequently, only one glucosyl ring can bind in the glycon site ($-n$) (subsites corresponding to -2 and -3 subsites in α-amylases being disrupted by the salt bridge). The non-reducing end of G_n maltooligosaccharide substrate binds the active site in the -1 subsite, the other glucose units occupying the aglycon site ($+n$). A mechanism including the following stages is suggested (Fig. 3):

1. exo-cleavage of the G_n maltooligosaccharide at the non-reducing end
2. formation of a β-glucosyl-enzyme covalent intermediate and release of G_{n-1} product
3. transfer of the glucosyl residue onto the non-reducing end of a G_n acceptor molecule leading to the formation of product G_{n+1}

This disproportionation reaction leads, in a first step, to the formation of G_{n-1} and G_{n+1} products from G_n substrate. In a second step, multiple reactions can occur involving all the products when they are accumulated in sufficient concentrations in the medium. New maltooligosaccharides such as G_{n-2} and G_{n+2} are therefore synthesised.

3.3. Kinetic studies

3.3.1. Size selectivity. The initial rates of G_n substrate conversion and G_{n+1} and G_{n-1} product formation from maltooligosaccharide substrates ranging from G2 to G7 are presented in Table 1. The rates increase with the size of the maltooligosaccharide substrates. Maltooligosaccharides with a degree of polymerisation lower than five are converted very slowly indicating that at least four glucose units are required in the aglycon sites ($+n$) for efficient transglucosylation. The different subsites are expected to make an additional contribution to binding. In particular, the +4 appears to be crucial for the binding of maltooligosaccharides. Besides, as suggested above, the relationship $V_{iGn-1} = V_{iGn+1} = 1/2\,V_{iGn}$ is verified, where V_{iGn} is the initial rate of G_n substrate conversion and V_{iGn-1} and V_{iGn+1} that of G_{n-1} and G_{n+1} product formation.

3.3.2. Effect of sucrose and maltooligosaccharide concentration on AS activity. Reactions in the presence of various G6 concentrations (ranging from 5 to 450 mM) were carried out. The initial rate of G6 conversion versus substrate concentration is shown in Fig. 4, which also presents the previously established curve of the initial rate of sucrose conversion versus sucrose concentration [5]. The initial rate of G6 conversion is directly proportional to substrate concentration and follows first-order kinetics. At 100 mM G6, the initial rate of G6 conversion is 5600 μmol/min/g and is 7 times higher than the initial rate of sucrose conversion at 100 mM. But whereas the V_{max} is almost reached at 100 mM in the case of sucrose substrate, it can never be reached with G6 substrate. Indeed, it was not possible to use higher concentration of G6 because of its limited solubility. However, the Lineweaver–Burk representation of the data enabled the determination of apparent kinetic constants of the G6 disproportionation reaction: the catalytic constant (k_{cat}) and Michaelis constant (K_m) are estimated to be 58.3 s^{-1} and 790 mM, respectively. These kinetic data are very different from those previously established in the presence of sucrose [5]. Indeed, it was shown that AS does not present a classic Michaelis-Menten behaviour for sucrose conversion. The values of apparent k_{cat} and K_m for initial sucrose concentrations higher than 20 mM were estimated to be 1.3 s^{-1} and 50.2 mM, respectively. Then, the values of apparent k_{cat} and K_m are 45-fold and 16-fold higher for G6 than for sucrose, respectively. Consequently, considering the ratio k_{cat}/K_m, the AS is three-fold more efficient in the presence of G6 than in the presence of sucrose.

4. Discussion

This study clearly demonstrates that AS catalyses the cleavage of the α-1,4 glucosidic linkage at the non-reducing end of

Table 1
Initial rates of G_n substrate conversion and G_{n+1} and G_{n-1} product formation from maltooligosaccharide substrates ranging from G2 to G7

Substrate (10 mM)	Initial G_n substrate conversion rate (μmol/min/g)	Initial G_{n+1} and G_{n-1} product formation rate (μmol/min/g)
G2	4.0	1.9
G3	5.3	2.5
G4	8.1	4.2
G5	260.3	125.0
G6	555.5	263.2
G7	851.8	412.9

maltooligosaccharides. The demonstration of this new intrinsic property testifies that AS is not only specific for the cleavage of the α1-β2 linkage of sucrose and emphasises the polyspecific character of this enzyme. The transglycosidase activities previously observed in two preparations of amylosucrases from *N. polysaccharea* and *N. perflava*, that were attributed to contaminating enzymes, are probably the result of amylosucrase activity itself.

The disproportionation reaction proceeds via the formation of a glucosyl–enzyme intermediate and the transfer of a glucosyl residue to acceptor. This is in accordance with the geometry of the active site where the subsites corresponding to subsites −2 and −3 in α-amylases are disrupted by a salt bridge [10–11]. Assuming that K_m reflects the affinity of AS for its substrate, the high apparent K_m value of the disproportionation reaction suggests that AS has a lower affinity for maltooligosaccharides than for sucrose. But, the high apparent k_{cat} value and the first-order kinetics of this reaction show that, once the intermediate is formed, the transfer to maltooligosaccharides is very fast so the enzyme is never saturated by the substrate. We can assume that the efficiency of the reaction is due to the fact that maltooligosaccharides can act as both donor and acceptor, which is not the case of sucrose.

Surprisingly, in the presence of maltooligosaccharides, we never observed glucose release. The oligosaccharides occupy the acceptor binding site, preventing the access of water to the active site and consequently the hydrolysis reaction. With sucrose as substrate, the access of water to the active site is facilitated, especially in the first steps of the reaction, because of the lack of acceptor. Disproportionation reaction is comparable to the reaction in the presence of sucrose and glycogen acceptor [6]. In this former case, the transfer of the glucosyl residue onto glycogen branches is also favoured to the detriment of the hydrolysis reaction. In addition, the increase of initial rate is perfectly comparable to the initial rate observed in the presence of G6.

From the structural analysis of the AS active site and the comparison to amylolytic enzyme active sites, G2, G3 and G4 could have been expected to be good substrates. However, AS hardly catalyses their disproportionation showing that subsites +1, +2 and +3 are too weak to accommodate these substrates. To be efficient donor, maltooligosaccharides must be composed of at least five glucose units. More generally, this indicates that an efficient binding in subsite +4 controls the positioning of maltooligosaccharides in the active site, preventing the reactivity of oligosaccharides having a degree of polymerisation lower than 5. In addition, above +4, subsites are expected to make an additional contribution to binding which could be responsible for the increase of the initial rate with maltooligosaccharide size. Structural analysis of AS in complex with long maltooligosaccharides should allow mapping of the various subsites from −1 to +*n* and determination of their individual contributions to binding.

As maltooligosaccharides longer than G5 are more efficient glucosyl donors than sucrose, they can be used as sucrose substitute in transglycosylation reactions. This will be undoubtedly of interest for testing starch hydrolysates as glucosyl donors for oligosaccharide and glucoconjugate synthesis.

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