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Aromatic interactions at the catalytic subsite of sucrose phosphorylase: Their roles in enzymatic glucosyl transfer probed with $Phe^{52} \rightarrow Ala$ and $Phe^{52} \rightarrow Asn$ mutants

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Mutants of *Leuconostoc mesenteroides* sucrose phosphorylase having active-site Phe^{52} replaced by Ala (F52A) or Asn (F52N) were characterized by free energy profile analysis for catalytic glucosyl transfer from sucrose to phosphate. Despite large destabilization (⩾3.5 kcal/mol) of the transition states for enzyme glucosylation and deglucosylation in both mutants as compared to wild-type, the relative stability of the glucosyl enzyme intermediate was weakly affected by substitution of Phe^{52}. In reverse reaction where fructose becomes glucocylated, "error hydrolysis" was the preponderant path of breakdown of the covalent intermediate of F52A and F52N. It is proposed, therefore, that Phe^{52} facilitates reaction of the phosphorylase through (1) positioning of the transferred glucosyl moiety at the catalytic subsite and (2) strong cation-π stabilization of the oxocarbenium ion-like transition states flanking the covalent enzyme intermediate.
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1. Introduction

Recognition of carbohydrates by proteins often involves π-interactions from aromatic side chains of Phe, Tyr, and Trp. Aromatic rings undergo stacking with pyranoses and furanoses whereby their π-electron cloud interacts with aliphatic protons of the sugar ring [1–4]. The resulting interaction energies were reported to vary between −0.5 and −0.8 kcal/mol, depending on the nature of the aromatic ring and the carbohydrate [5]. Not surprisingly, therefore, carbohydrate-π interactions are widely utilized by glycoside hydrolases (e.g. [6,7]), glycosyltransferases (e.g. [8–10]) and carbohydrate-binding modules [11,12] to achieve recognition and specificity for their natural substrates. A striking commonality among the diverse families of glycoside hydrolases is the occurrence of a family-specific hydrophobic motif in their catalytic subsites. This motif usually consists of one or more aromatic residues and is found irrespective of whether the glycoside hydrolase utilizes an α or β-configured pyranosyl substrate and hydrolyzes it with retention or inversion of anomeric configuration [13]. Its proposed role in catalysis is to aid in the conformational itinerary of the glycopyranoside upon moving from the ground state (e.g. $^4C_1$ chair) to the oxocarbenium ion-like transition state (e.g. $^4H_3$ half-chair). Non-polar contacts with the hydrophobic C_4–C_5-hydroxymethyl moiety of the glycopyranoside that become tightened in the transition state are believed to be the source of the catalytic facilitation provided. In addition to this *conformational* stabilization derived from carbohydrate-π interactions, there is the interesting question if aromatic side chains contributed to the catalytic subsite might also be important for *electrostatic* stabilization of the transition state of the glycoside hydrolase reaction.

Family GH-13 constitutes a large group of retaining hydrolases and transglycosidases that act on α-*O*-glycosidic substrates such as starch and sucrose [14]. Its members contain a special aromatic motif (Fig. 1; [15–21]) where a highly conserved Phe/Tyr is positioned at the B-face of the glucopyranosyl ring bound in the catalytic subsite. Sucrose phosphorylase (from *Leuconostoc mesenteroides*; LmSPase) was chosen for examining with site-directed mutagenesis the function of the relevant aromatic residue (Phe^{52}) in the catalytic mechanism. Note: the aromatic motif in Fig. 1 must be distinguished from the likewise present hydrophobic

Abbreviations: Glc1P, α-D-glucopyranosyl phosphate; Glc1F, α-D-glucopyranosyl fluoride; SPase, sucrose phosphorylase (EC 2.4.1.7); BaSPase, *Bifidobacterium adolescentis* SPase; LmSPase, *Leuconostoc mesenteroides* SPase

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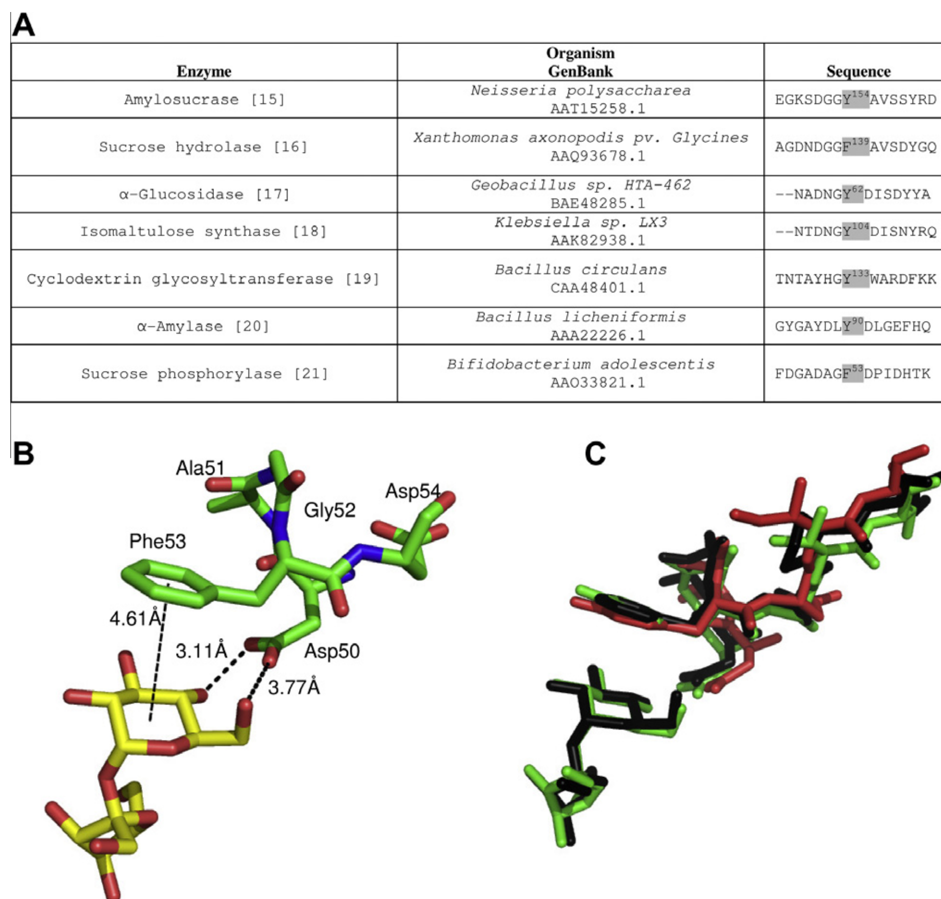

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Enzyme	Organism GenBank	Sequence
Amylosucrase [15]	*Neisseria polysaccharea* AAT15258.1	EGKSDGGY^{154}AVSSYRD
Sucrose hydrolase [16]	*Xanthomonas axonopodis pv. Glycines* AAQ93678.1	AGDNDGGF^{139}AVSDYGQ
α-Glucosidase [17]	*Geobacillus sp. HTA-462* BAE48285.1	--NADNGY^{62}DISDYYA
Isomaltulose synthase [18]	*Klebsiella sp. LX3* AAK82938.1	--NTDNGY^{104}DISNYRQ
Cyclodextrin glycosyltransferase [19]	*Bacillus circulans* CAA48401.1	TNTAYHGY^{133}WARDFKK
α-Amylase [20]	*Bacillus licheniformis* AAA22226.1	GYGAYDLY^{90}DLGEFHQ
Sucrose phosphorylase [21]	*Bifidobacterium adolescentis* AAO33821.1	FDGADAGF^{53}DPIDHTK

Fig. 1. The aromatic motif at the catalytic subsite of selected glucoside hydrolases and transglucosidases from family GH-13, revealed by Phe/Tyr residue conservation in both sequence (A) and three-dimensional structure (B and C). Panel B shows relevant interactions with the glucopyranosyl moiety of sucrose in the catalytic subsite of *B. adolescentis* sucrose phosphorylase (BaSPase). Note: Phe^{52} of LmSPase is homologous to the shown Phe^{53} of BaSPase. Panel C is a structural overlay of the corresponding catalytic subsite parts of BaSPase (2GDU; black), *N. polysaccharea* amylosucrase (1JGI; green) and *Klebsiella sp.* isomaltulose synthase (1M53; red).

motif (Phe^{160} in LmSPase) of conformational stabilization [13] that is not considered herein. LmSPase belongs to a particular group of transglycosidases within family GH-13 that promote reversible glucosyl transfer from sucrose to phosphate (P_i), yielding α-D-glucopyranosyl phosphate (Glc1P) and D-fructose as products [22]. Like in other GH-13 enzymes [23–26], catalytic reaction of LmSPase involves formation (*glucosylation*) and breakdown (*deglucosylation*) of a covalent β-glucosyl enzyme intermediate [27]. The proposed role of Phe^{52} involves stabilization, by strong cation-π interactions, of the oxocarbenium ion-like transition states leading to and from the glucosylated enzyme.

2. Materials and methods

Materials used have been described elsewhere [22]. α-D-Glucopyranosyl fluoride (Glc1F) was prepared by Zemplen deprotection from 2,3,4,6-tetra-*O*-α-D-glucopyranosyl fluoride that was obtained from TCI Europe.

2.1. Site-directed mutagenesis and enzyme preparation

Mutations resulting in substitution of Phe^{52} by Ala (F52A) or Asn (F52N) were introduced using a reported two-stage PCR protocol [28] in which a pQE30 expression vector encoding wild-type LmSPase was used as template [22]. Oligonucleotide primers and details of conditions used for PCR are summarized in Supplementary data. For expression, we subcloned native and mutated genes, via *Bam*HI and *Pst*I restriction sites, from pQE30 into pASK-IBA7+ (IBA GmbH). The pASK-IBA7+ constructs introduce *Strep*-tag II at the N-terminus of each enzyme. The new tag (8 amino acids) is comparable in length to the previously used His-tag (11 amino acid), which however did not facilitate protein isolation [22]. All inserts were confirmed by sequencing in sense and antisense directions. *Escherichia coli* Top10 cells harboring pASK-IBA7+ construct were cultivated under standard conditions (Supplementary data) using induction with 200 μg/l anhydrotetracycline (6 h, 25 °C). Crude cell extract was prepared with a French press and protein was purified using chromatography on a Strep-Tactin Superflow column (bed volume 5 ml) (for details, see the Supplementary data). Purification was monitored by SDS–PAGE.

2.2. Assays

Enzyme activity in direction of phosphorolysis of sucrose was determined at 30 °C using a continuous coupled enzymatic assay with phosphoglucomutase and glucose-6-phosphate dehydrogenase [22]. Protein concentration was measured with the BioRad dye-binding method referenced against BSA. Glucose was determined using an assay with hexokinase and glucose-6-phosphate dehydrogenase [22]. Glucose in the presence of fructose was determined using an assay with glucose oxidase and peroxidase [29]. Glc1P was assayed using phosphoglucomutase and glucose-6-phosphate dehydrogenase [22]. Phosphate was determined colorimetrically at 850 nm [22]. Other analytical methods (NMR, HPLC)

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