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CATALYTIC VERSATILITY OF TREHALASE: SYNTHESIS OF α-D-GLUCOPYRANOSYL α-D-XYLOPYRANOSIDE FROM β-D-GLUCOSYL FLUORIDE AND α-D-XYLOSE*

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ABSTRACT

Trehalase was previously shown (see ref. 5) to hydrolyze α-D-glucosyl fluoride, forming β-D-glucose, and to synthesize α,α-trehalose from β-D-glucosyl fluoride plus α-D-glucose. Present observations further define the enzyme's separate cosubstrate requirements in utilizing these nonglycosidic substrates. α-D-Glucopyranose and α-D-xylopyranose were found to be uniquely effective in enabling *Trichoderma reesei* trehalase to catalyze reactions with β-D-glucosyl fluoride. As little as 0.2mM added α-D-glucose (0.4mM α-D-xylose) substantially increased the rate of enzymically catalyzed release of fluoride from 25mM β-D-glucosyl fluoride at 0°. Digests of β-D-glucosyl fluoride plus α-D-xylose yielded the α,α-trehalose analog, α-D-glucopyranosyl α-D-xylopyranoside, as a transient (i.e., subsequently hydrolyzed) transfer-product. The need for an aldopyranose acceptor having an axial 1-OH group when β-D-glucosyl fluoride is the donor, and for water when α-D-glucosyl fluoride is the substrate, indicates that the catalytic groups of trehalose have the flexibility to catalyze different stereochemical reactions.

INTRODUCTION

Recent studies of glycosylation reactions catalyzed without glycosidic-bond cleavage have shown the inadequacy of traditional notions of the capabilities and specificity of carbohydrases. Strong evidence has been found[1–7] for the functional flexibility of the catalytic groups of various glycosidases, including well known

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"inverting" enzymes long considered limited to catalyzing hydrolysis or its reversal. A recent examination of trehalase[5], for example, revealed the catalytic flexibility of this classic enzyme previously represented as strictly specific for hydrolyzing α,α-trehalose or close analogs of the same steric bulk, and as devoid of transglucosylative ability[8]. Trehalase preparations from rabbit kidney cortex and *Candida tropicalis* were shown to catalyze stereochemically complementary reactions with α- and β-D-glucosyl fluoride[5]. The enzymes hydrolyzed the α anomer (forming β-D-glucose and HF) faster than trehalose, which they likewise hydrolyzed with inversion as reported[9] for flesh fly trehalase. The conversion of β-D-glucosyl fluoride to glucose and HF by the rabbit kidney and *Candida* enzymes was extremely slow by comparison, and distinguished by being much enhanced by the addition of α-D-glucose. Digests of β-D-glucosyl fluoride plus α-D-[^{14}C]glucose yielded small amounts of α,α-trehalose with one ^{14}C-labelled residue[5], a transient product that subsequently undergoes hydrolysis. Trehalase thus catalyzes a glucosyl transfer-reaction that is sterically opposite to (though it is not a reversal of) the hydrolysis of trehalose or α-D-glucosyl fluoride. We now report findings which further delineate the range and the acceptor requirements of reactions catalyzed by trehalase with α- and β-D-glucosyl fluoride. The results were obtained with a purified *Trichoderma reesei* enzyme having high specific activity for α,α-trehalose[10] and known to hydrolyze the latter with configurational inversion[11].

RESULTS

Purified *T. reesei* trehalase was found to use both α- and β-D-glucosyl fluoride as substrates, as previously shown[5] for trehalase of rabbit kidney and of *Candida* yeast. The reaction with α-D-glucosyl fluoride gave glucose and HF as sole products, and showed a relationship between initial rate and substrate concentration consistent with direct hydrolysis. Rates of enzymically catalyzed fluoride release for digests containing 2.5–25mM α-D-glucosyl fluoride and 3 μg/mL trehalase (30°, 15 min, pH 5.4), plotted as (μmol F^-/min/mg)$^{-1}$ *vs* (μmol substrate/mL)$^{-1}$, gave a linear curve showing V_{max} 29 μmol/min/mg and K_m 5.4 mM. The *T. reesei* enzyme attacked α-D-glucosyl fluoride faster than α,α-trehalose; for instance, with substrates at 25mM, 23.4 μmol of α-D-glucosyl fluoride (11.9 μmol of trehalose) was hydrolyzed/min/mg at 30°.

β-D-Glucosyl fluoride, in contrast, was converted only very slowly into glucose and HF by the mold enzyme. This difference provided the opportunity to investigate the acceptor specificity of trehalase. α-D-Glucose had been found to stimulate β-D-glucosyl fluoride utilization by rabbit kidney and *Candida* trehalase, and to serve as an acceptor cosubstrate in a reaction producing trehalose[5]. As α-D-glucose was the only compound previously examined in this respect, study was undertaken to learn which compounds can (or cannot) function as acceptors for trehalase in reactions catalyzed with β-D-glucosyl fluoride as donor.

Initial tests probed the effect of various carbohydrate additives (25mM) on

the rate of fluoride-ion release from 5mM β-D-glucosyl fluoride in digests incubated for 14 min at 30°, namely, under conditions where α-D-glucose had been found to be highly stimulatory, with the following results. The rate of enzymically catalyzed release of fluoride was found to be 0.48 μmol/min/mg for the substrate without additive; 2.97 or 2.32 μmol/min/mg with added α-D-glucose or α-D-xylose, respectively; and 0.71 or 0.65 μmol/min/mg with added β-D-glucose or D-galactose (86% α anomer), respectively. No enhanced utilization of β-D-glucosyl fluoride was detected on addition of the methyl glycosides of α-D-glucose, α-D-xylose, or α-D-galactose; or with D-mannose, 2-deoxy-D-glucose, D- or L-arabinose, or α-D-ribose. These results set glucose, xylose, and possibly galactose apart from various related structures, but they do not suffice to show (except in the case of α-D-glucose) which anomeric form(s) of these sugars have stimulatory activity. It is not clear, for example, that the modest enhancement found on addition of 25mM β-D-glucose can be attributed to the β anomer per se. The latter's mutarotation rate[12] at 30° is such that anomerization during the 14-min incubation would provide 4mM α-D-glucose, perhaps enough to account for the observed effect. Similarly, the finding of strong enhancement with added α-D-xylose does not exclude the possibility that β-D-xylose, which would reach a level of 13.5mM in 14 min at 30° from the 25mM added α-D-xylose ($k_1 + k_2$ 0.0532)[13], might be partly or wholly responsible for the observed stimulation.

Tests of the ability of various carbohydrates to promote the use of β-D-glucosyl fluoride by trehalase were therefore made in the cold to minimize the anomerization of added sugars as well as the nonenzymic hydrolysis of the substrate (~4.2% in 14 min at 30°; 0.4–0.8% at 0°). For each carbohydrate additive, rates of enzymically catalyzed release of fluoride were determined in a series of digests containing 25mM β-D-glucosyl fluoride, 0–3mM additive, and 90 μg/mL enzyme, kept in an ice–water bath for 14 min.

As illustrated in Fig. 1, digests with 0.2–2.8 mM added α-D-glucopyranose showed increasingly enhanced rates of fluoride release relative to the control digest without added glucose. Similar results were obtained with 0.4–3.0mM α-D-xylopyranose, although enhancement was less than with α-D-glucose at each concentration. In contrast, 0.4–3.0mM β-D-glucose, methyl α-D-glucopyranoside, or other carbohydrates (listed in Fig. 1 legend) caused no detectable change in the rates of the respective control digests of substrate without additive. The substantial stimulation produced by α-D-glucopyranose at levels far lower than would have arisen from the 25mM β-D-glucose in the 30° experiments, as well as the negative results with β-D-glucose in 0° digests where no significant anomerization could occur[12,14], indicate that β-D-glucose does not enhance utilization of β-D-glucosyl fluoride by trehalase. In contrast, the substantial stimulation produced by low concentrations of α-D-xylopyranose under conditions ($k_1 + k_2$ 0.00245)[14] where 5% anomerization would occur during the reaction period strongly suggests that α-D-xylopyranose, like α-D-glucopyranose, functions as an acceptor cosubstrate. Further evidence that this is the case is presented in the next section.

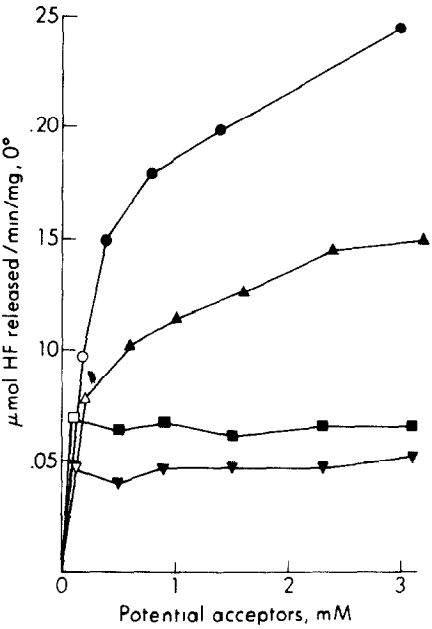


Fig. 1. Specificity and effectiveness of α-D-glucose and α-D-xylose in enabling *T. reesei* trehalase to attack β-D-glucosyl fluoride. Rates of enzymically catalyzed fluoride release (μmol/min/mg protein) from 25mM β-D-glucosyl fluoride (in mixtures kept at 0°, 14 min) *vs* concentrations (μmol/mL) of other carbohydrates present. ○, △, □, ▽, Digests prepared with β-D-glucosyl fluoride only, but containing 0.1–0.2mM endogenous glucose from nonenzymic hydrolysis of the substrate (taken as equal to the free fluoride ion concentration in the substrate–buffer control incubated with each test series) ●, Digests with 0.2–2.8mM α-D-glucopyranose added; ▲, with 0.4–3.0mM α-D-xylopyranose added, ▼, with 0.4–3.0mM β-D-glucopyranose added; ■, with 0.4–3.0mM methyl α-D-glucopyranoside added. Other digest series (not illustrated), containing 0.4–3.0mM of added methyl α-D-xylopyranoside, α(86%)-D-galactose, α(86%)-D-mannose, equilibrated 2-deoxy-D-*arabino*-hexose, α-D-glucosyl fluoride, or β-D-glucosyl fluoride, gave negative results comparable to those illustrated with β-D-glucopyranose and methyl α-D-glucopyranoside.

The finding (Fig. 1) that very low concentrations of α-D-glucose have stimulatory activity has special significance with respect to understanding the nature of the slow reaction catalyzed with β-D-glucosyl fluoride in the absence of any additive. β-D-Glucosyl fluoride undergoes nonenzymic hydrolysis in buffer and, in the several experiments of Fig. 1, the 25mM substrate–buffer control mixtures kept at 0° (14 min) showed free fluoride levels of 0.1–0.2mM. These values were taken as a measure of "endogenous" glucose concentrations in the several digests of β-D-glucosyl fluoride without additive (open data points at the left in Fig. 1). This level (0.1–0.2mM) is sufficient to account for the slow reactions observed for the digests without additive, and is consistent with the view that water does not serve as an acceptor when β-D-glucosyl fluoride is the donor.

Trehalase-catalyzed synthesis of α-D-*glucopyranosyl* α-D-*xylopyranoside.* — In view of the indication (Fig. 1) that α-D-xylose might function as an acceptor

cosubstrate, tests were made to learn whether trehalase acting on β-D-glucosyl fluoride in the presence of α-D-xylose might catalyze the formation of a transfer product analogous to α,α-trehalose from β-D-glucosyl fluoride and α-glucose. Chromatograms of incubated β-D-glucosyl fluoride–α-D-xylose–trehalase digests did indeed show, in addition to glucose and residual glucosyl fluoride and xylose, a product of R_{Glc} ~0.8 that stained extremely slowly with silver nitrate. A sample of this product (9.6 μmol, calculated as glucosyl xyloside) was recovered chromatographically from digests (7.5 mL) comprising 40mM β-D-glucosyl fluoride, 120mM α-D-xylose, and 0.12 mg/mL *T. reesei* trehalase (30°, 40 min). This yield corresponds to 7.1% of the amount of enzymically utilized β-D-glucosyl fluoride (135 μmol). The latter figure was determined from the amount of fluoride ion released in the digest, minus that released in an incubated substrate–buffer control-mixture (which control showed no sign of oligosaccharide formation).

The recovered enzymic product, rechromatographed to remove traces of impurities, had R_{Glc} 0.84 in 6:4:3 1-butanol–pyridine–water, as reported[15] for chemically synthesized α-D-glucopyranosyl α-D-xylopyranoside. Incubation of the product with *Candida* trehalase (2 h, 30°) yielded glucose and xylose in a molar ratio of 1.02:1.00. A Fourier transform ^{1}H-n.m.r. spectrum of a 9mM solution in deuterium oxide, recorded at 200 MHz, showed a resonance doublet of two-proton intensity at 5.13 p.p.m. ($J_{1,2}$ 3 Hz) assignable to the equatorial C-1 protons of α-D-glucopyranose and α-D-xylopyranose (no signal referable to the axial C-1 proton of β-D-glucose or β-D-xylose was present). A nearly identical H-1 and H-1′ resonance value was reported by Belcopitow *et al.*[16] for α-D-glucopyranosyl α-D-xylopyranoside synthesized by trehalose phosphorylase acting on β-D-glucosyl phosphate plus D-xylose; Bar-Guilloux *et al.*[15] reported 5.62 p.p.m. ($J_{1,2}$ 3 Hz) for H-1 and H-1′ of the chemically synthesized compound (the reference standard used in ref. 15 was external Me_4Si as compared with sodium 4,4-dimethyl-4-silapentanesulfonate in the present case).

A ^{13}C-n.m.r. spectrum of the same 9mM solution of the trehalase product in deuterium oxide, recorded at 125.7 MHz, showed nine resonance peaks of one-carbon intensity and one of two-carbon intensity (Table I). The pattern of resonances in this spectrum is indistinguishable from the pattern reported[15] for chemically synthesized α-D-glucopyranosyl α-D-xylopyranoside (resonances listed in Table I for comparison). Each resonance in the spectrum of the enzymically synthesized product appears 1.2–1.3 p.p.m. upfield of the corresponding peak in the spectrum of the chemically synthesized compound. This uniform difference in chemical shifts probably reflects the use of different reference standards in the two studies; in particular, the lowest-field resonances at 93.39 and 93.25 p.p.m. (our results) correspond, respectively, to the anomeric carbon atoms of α-D-xylopyranose and α-D-glucopyranose; the larger peak at 70.99 p.p.m. corresponds to C-5 of α-D-glucopyranose and C-2 of α-D-xylopyranose[15]. These ^{13}C-n.m.r. data provide direct evidence that the product of trehalase action on β-D-glucosyl fluoride plus α-D-xylose is the α,α-trehalose analog, α-D-glucopyranosyl α-D-xylopyranoside.

TABLE I

^{13}C-N.M.R. RESONANCES OF PRODUCT SYNTHESIZED FROM β-D-GLUCOSYL FLUORIDE PLUS α-D-XYLOSE BY *T. reesei* TREHALASE, COMPARED WITH RESONANCES REPORTED[15] FOR CHEMICALLY SYNTHESIZED α-D-GLUCOPYRANOSYL α-D-XYLOPYRANOSIDE

Assignment			*Resonance (p.p.m.)*	
α-D-Glucopyranosyl residue		*α-D-Xylopyranosyl residue*	*Enzymically synthesized product*[a]	*Chemically synthesized product*[b]
		C-1	93.39	94.6
C-1			93.25	94.5
		C-3	72.58	73.9
C-3			72.44	73.7
C-2			72.07	73.3
C-5	plus	C-2	70.99	72.2
C-4			69.59	70.9
		C-4	69.41	70.7
		C-5	61.46	62.8
C-6			60.40	61.8

[a]72,000 scans were collected at 293K with a 9mM solution of sample in deuterium oxide. Chemical shifts are in p.p.m. relative to 1,4-dioxane in deuterium oxide at 67.4 p.p.m. [b]Ref. 15. Spectra recorded in deuterium oxide. Chemical shifts are in p.p.m. relative to external tetramethylsilane; temperature not specified.

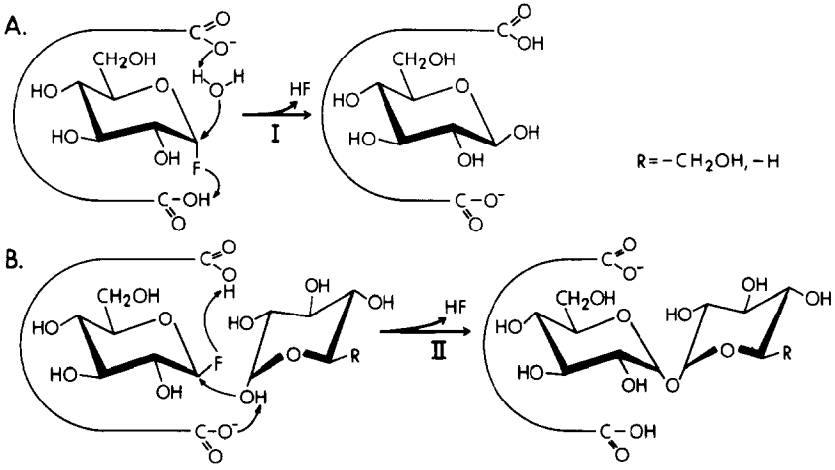
DISCUSSION

The cosubstrate requirements of trehalase in catalyzing reactions with α- and β-D-glucosyl fluoride, respectively, have been further defined. α-D-Glucopyranose and α-D-xylopyranose were found to be highly efficient and specific in promoting utilization of β-D-glucosyl fluoride by *T. reesei* trehalase. As little as 0.2mM of added α-D-glucose substantially increased the low rate of enzymically catalyzed fluoride release from 25mM β-D-glucosyl fluoride in digests at 0°. Because of this efficiency, the very slow reaction catalyzed without added glucose can be accounted for as one of transfer to the traces of endogenous glucose formed from β-D-glucosyl fluoride in buffer alone. It is thus highly probable that *T. reesei* trehalase does not directly hydrolyze β-D-glucosyl fluoride. On the other hand, the enzyme's ability to catalyze glucosyl transfer from the latter was shown by the isolation of α-D-glucopyranosyl α-D-xylopyranoside from digests of β-D-glucosyl fluoride plus α-D-xylose. The transfer product does not greatly accumulate as it undergoes subsequent enzymic hydrolysis. Its recovered yield was ~7% of the enzymically utilized substrate (measured as enzymically catalyzed release of fluoride). In sum, the cosubstrate requirement of *T. reesei* trehalase in utilizing β-D-glucosyl fluoride appears to be for an aldopyranose having an axial 1-OH group and equatorial 2-, 3-, and 4-hydroxyl groups. It is possible that some compounds which differ slightly from this structure (e.g., α-D-galactopyranose) may prove to be low-affinity acceptors with β-D-glucosyl fluoride.

Kinetic findings for the reactions catalyzed by *T. reesei* trehalase with α-D-glucosyl fluoride were consistent with a direct hydrolysis, as reported for rabbit kidney and *Candida* trehalase on steric grounds[5]. Preliminary experiments have further shown that the additions of 3mM α-D-glucose or α-D-xylose to enzymic digests of 4mM α-D-glucosyl fluoride caused 35–40% inhibition of fluoride release from this substrate (at 0°), whereas 3mM β-D-glucose, methyl α-D-xyloside or other carbohydrates had no appreciable effect. That the sugars which specifically promote β-D-glucosyl fluoride utilization also suppress the utilization of α-D-glucosyl fluoride, possibly by blocking seating of its F atom, supports the conclusion that trehalase requires water as cosubstrate when α-D-glucosyl fluoride is the substrate.

The demonstration that *T. reesei* trehalase (like rabbit kidney and *Candida* trehalase) effects the hydrolysis of α-D-glucosyl fluoride, but catalyzes glucosyl-transfer reactions with β-D-glucosyl fluoride, has parallels in findings[1,2] of similar dual modes of action for beta amylase, glucoamylase, and glucodextranase. That each of these "inverting hydrolases" catalyzes nonhydrolytic reactions contradicts the long-accepted characterization of each as being strictly limited to promoting only hydrolysis or its reversal. A significant new point shown by the present and earlier[1,2,5] studies is that trehalase and the exo-α-glucanases catalyze glycosyl-transfer reactions in a way other than by partitioning a common glycosyl–enzyme intermediate between water and an organic acceptor molecule, previously the only way known to account for glycosyl-transfer reactions catalyzed by a glycosidase.

Mechanisms consistent with the present and previously reported[5] hydrolytic and glucosyl-transfer reactions catalyzed by trehalase with α- and β-D-glucosyl fluoride, respectively, are envisioned as concerted displacements of opposed types. The functionally active groups are assumed, by analogy with those of cockchafer trehalase[8] and of hen egg lysozyme, to comprise a pair of cooperatively interacting catalytic groups (possibly carboxyl groups) located above and below the glycosidic bond of the bound substrate. The proposed mechanism (A) for hydrolysis of α-D-



glucosyl fluoride is as reported[5] for trehalase preparations shown to hydrolyze this substrate to form β-D-glucose*. In this mechanism, the protonated carboxyl group acts as a general acid to aid cleavage of the axial glycosylic 1-F bond while the other carboxyl group, as an anion, acts as a general base to aid the specifically directed attack of solvent water. The postulated mechanism (B) for the transfer reactions catalyzed with β-D-glucosyl fluoride assumes the presence of a suitably bound, specific aldopyranose cosubstrate whose axial 1-OH group is positioned at the active center, that is, oriented oppositely to the water in (A). In the (Type II) transfer-reaction, the roles of the enzyme's two functional groups are reversed in promoting a concerted displacement reaction between a specific donor and an organic acceptor-molecule bound simultaneously at the active site. Thus, the present as well as earlier[5] results show that the catalytic groups of trehalase, like those of other glycosylases[1-4,6,7], are functionally flexible beyond requirements of the principle of microscopic reversibility. The results also show that trehalase has two different carbohydrate binding-sites — one specific for an α- or β-D-glucopyranosyl residue, the other for α-D-aldopyranose. This is of special interest with respect to the recent finding[17] of different binding modes for phloridzin (a β-D-glycoside) and phloretin (its aglycon) in inhibiting the trehalase-catalyzed hydrolysis of α,α-trehalose.

Further evidence of the catalytic versatility of trehalase, and of the functional flexibility of its catalytic groups, has recently been obtained from the observation that *T. reesei* trehalase catalyzes the hydration of (*Z*)-3,7-anhydro-1,2-dideoxy-D-*gluco*-oct-2-enitol[18], which can be expected to proceed by a still different mechanism from the (A) and (B) displacements involved in the reactions with α- and β-D-glucosyl fluoride. Again, parallels exist in that beta amylase catalyzes the hydration of maltal[3], and glucodextranase the hydration of 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol[6], in addition to reactions with the α and β anomers of suitable glycosyl fluorides. The findings with trehalase thus add to the growing evidence that a glycosylase may act on different types of substrates by different mechanisms, contrary to widely found references to "the mechanism" of an enzyme.

EXPERIMENTAL

Materials and methods. — Trehalase from *Trichoderma reesei* QM 9414, prepared as described by Vijayakumar *et al.*[10], was further subjected to precipitation with one volume of acetone to decrease the hydrolytic activity for *p*-nitrophenyl β-D-glucoside to an insignificant level. Specific activity for α,α-trehalose, assayed at 5.3mM final concentration, 50° and pH 4.4 (ref. 10) was 104 μmol trehalose hydrolyzed/min/mg protein. A stock of enzyme (0.6 mg/mL) in 0.4 saturated ammonium sulfate was kept at 4°. For use, known volumes were dialyzed

*The steric course of the hydrolysis by *T. reesei* trehalase has not been examined, but the enzyme is known[11] to hydrolyze α,α-trehalose with inversion of the mobilized glucosyl residue

against 0.05M acetate buffer of pH 5.4 for 1 h; or, for reactions involving less than 8 μg/mL enzyme, the stock was directly diluted with the appropriate buffer.

Highly purified α- and β-D-glycosyl fluoride were prepared from the pure crystalline tetraacetates and structurally characterized as previously reported[2]. Stock solutions of known concentration in dry methanol were kept at −20°, protected from moisture. Immediately before use, desired amounts of each anomeric glucosyl fluoride were obtained by drying aliquots of stock solution in 10 × 75 mm plastic test-tubes in a rotary vacuum evaporator (30°, 0.6 mmHg). The free fluoride content was <0.1% of total fluoride in the case of the α anomer; 0.4–0.8% in the case of the more labile β anomer.

α- And β-D-glucopyranose were chromatographically pure and essentially anomerically pure compounds; the α anomer, $[\alpha]_D^{25}$ +112° (*c* 1, 90% dimethyl sulfoxide), was prepared according to Hudson and Dale[12], the β anomer, $[\alpha]_D^{25}$ +17.6° (*c* 3, 90% methyl sulfoxide), by Behrend's method[19]. Crystalline (86% α)-D-galactopyranose was made according to Hudson and Yanovsky[13]; crystalline (86% α)-D-mannopyranose according to Isbell and Frush[20]. Pure methyl α-D-glucopyranoside was prepared from a reagent-grade sample by removing accompanying impurities[2]. α,α-Trehalose dihydrate, α-D-xylopyranose $[\alpha]_D^{23}$ +92.3° (*c* 2, 90% dimethyl sulfoxide), and methyl α-D-xylopyranoside were of reagent grade (Sigma). Other carbohydrates were chemically and chromatographically pure.

Paper chromatography (descending) was carried out with Whatman no. 1 paper and 6:4:3 1-butanol–pyridine–water. Staining was by a silver nitrate dipping method with papers hung in air for 12 min following application of the alkaline reagent. Reducing sugars were determined by a micro method employing the Somogyi and Nelson reagents, and total sugars by the phenol–sulfuric acid method[20]. Appropriate sugar standards were run concurrently in each case.

Fluoride ion concentrations, in the presence of absence of α- or β-D-glucosyl fluoride, were determined in the presence of TISAB buffer [M sodium acetate buffer, pH 5.2, M sodium chloride, 0.4% 1,4-cyclohexane bis(dinitrilotetraacetic acid) monohydrate] with an Orion specific ion meter Model 407A and combination fluoride electrode Model 96-09, as previously described[1,5].

The effect of carbohydrate additives on the rate of β-D-glucosyl fluoride utilization by trehalase at 0° was determined as follows. Five weighed samples of each additive were kept in test tubes in an ice–water bath. One sample at a time was dissolved, 1.5 min before use, in ice-cold water to give a solution of desired concentration (0.4–5.6mM). Meanwhile, seven 4.0-μmol samples of β-D-glucosyl fluoride from the stock in methanol were dried in small plastic test-tubes in a rotary vacuum evaporator (30°). At 2-min intervals, one dried sample was transferred from the evaporator to the 0° bath, chilled for 30 s, and then treated with 80 μL of one of the solutions of additive (or with ice-cold water). Without delay, 80 μL of ice-cold trehalase (0.18 mg/mL in pH 5.4 buffer) or buffer alone, was added and all components were mixed. Each digest was kept for 14.0 min at 0°, and then treated with 0.60 mL of TISAB buffer (25°) and immediately analyzed for free fluoride-ion concentration with the specific ion electrode.

Isolation and characterization of α-D-glucopyranosyl α-D-xylopyranoside transfer product. — Replicate 1.0-mL digests comprising 40 μmol of β-D-glucosyl fluoride (freshly dried from solution in methanol), 120 μmol of α-D-xylose, and 0.12 mg of *T. reesei* trehalase in 0.05M acetate buffer, pH 5.4, were incubated for 40 min at 30°. Each 0.5-mL digest was chromatographed as a 20-cm band on a sheet of Whatman no. 1 paper. End strips were stained with silver nitrate to locate the nonreducing R_{Glc} 0.85 product, which was then eluted from the unstained center panel. The combined eluted material from panels corresponding to 7.5-mL digest were concentrated *in vacuo* to a small volume and rechromatographed to remove trace impurities. Product yield by the phenol–sulfuric acid method, standardized with equimolar glucose and xylose, was 9.6 μmol (calculated as glycosyl xyloside).

^{1}H-N.m.r. spectra were recorded for a 9mM solution of the transfer product in deuterium oxide (99.8 atom % ^{2}H, Stoehler) at 20°, using a Varian 200 MHz spectrometer; 64 free-induction decays with 2-s repetition times were accumulated and Fourier transformed. Proton chemical-shift measurements were made with respect to sodium 4,4-dimethyl-4-silapentane-1-sulfonate as the external standard. ^{13}C-N.m.r. spectra of the same solution in deuterium oxide were recorded in the Fourier-transform mode at 125.7 MHz with broad-band proton decoupling in a Bruker WM-500 spectrometer. The solvent deuterium signal was used as the field–frequency lock. All chemical shifts are quoted from 1,4-dioxane in deuterium oxide as the internal standard at 67.4 p.p.m. at 20°. Sample tubes were 5 mm in diameter, and the probe temperature was controlled to ±0.5°.

A digest (0.2 mL) containing 1.4 μmol of the transfer product and 80 μg of *Candida tropicalis* trehalase[5] was incubated for 2 h at 30°, and then chromatographed as a band on paper, together with standards of glucose and xylose. The standards were stained and used to guide elution of the glucose and xylose in the digest chromatogram. The yields, measured with the Somogyi–Nelson reagents against the respective sugars as standards, were 1.10 μmol of glucose and 1.08 μmol of xylose.

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