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The stereochemical course of the reaction mechanism of trehalose phosphorylase from *Schizophyllum commune*

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Abstract Phosphorolysis of α,α-trehalose catalyzed by trehalose phosphorylase from the basidiomycete *Schizophyllum commune* proceeds via net retention of anomeric configuration and yields α-D-glucose 1-phosphate and α-D-glucose as the products. In reverse reaction, only the α-anomers of D-glucose 1-phosphate and D-glucose are utilized as glucosyl donor and acceptor, respectively, and give exclusively the α,α-product. Trehalose phosphorylase converts α-D-glucose 1-fluoride and phosphate into α-D-glucose 1-phosphate, a reaction requiring the stereospecific protonation of the glucosyl fluoride by a Brønsted acid. The results are discussed with regard to a plausible reaction mechanism of fungal trehalose phosphorylase.

Key words: Trehalose phosphorylase; Stereochemistry; Retaining mechanism

1. Introduction

The common concept concerning the catalytic mechanisms of glycosyl transferases (EC 2.4) suggests a separation into two distinct mechanistic classes (for review, see [1]). One class comprises enzymes acting via net inversion of anomeric configuration, that is the glycosyl donor and the product have different anomeric forms. Enzymes belonging to the other class catalyze the reaction with net retention. Inverting glycosyl transferases operate via a direct displacement of the substrate leaving group by a carbohydrate acceptor. In the case of oligo- or polysaccharide phosphorylases which catalyze the interconversion of a glycosidic and glycoside-phosphate linkage, the acceptor is inorganic phosphate when the direction of enzymic action is phosphorolysis. The reaction pathway leading to net retention is based on an even number of successive displacements, commonly two, each occurring with inversion [1]. The double-displacement mechanism involves either an inverted covalent glycosyl-enzyme intermediate, as unequivocally demonstrated for sucrose phosphorylase [2], or an ion pair intermediate in which the glycosyl carbonium ion is stabilized by electrostatic forces in the active site [3,4]. In classifying glycosyl transferases including the phosphorylases as retaining or inverting, direct stereochemical evidence serves the most important function [1] and thereby provides valuable, albeit indirect mechanistic information on enzymes that have not been characterized.

Trehalose phosphorylase (EC 2.4.1.64) has only recently been found to be more widely distributed in nature than originally thought [5], and its occurrence was reported in soil bacteria [6,7], fungi [8] and yeast [9]. The physiological function of trehalose phosphorylase in these organisms is unknown as yet. Trehalose phosphorylase catalyzes the phosphorolysis of α,α-trehalose into an equimolar mixture of D-glucose and D-glucose 1-phosphate (D-Glc 1-P) as the products, and this reaction is readily reversible. Two bacterial trehalose phosphorylases were shown to produce the β-anomer of D-Glc 1-P on phosphorolysis of α,α-trehalose [6,7], whereas partially purified enzymes from fungal and yeast source seem to yield the α-anomer of D-Glc 1-P [8,9]. However, direct stereochemical evidence for the trehalose phosphorylase-catalyzed reaction has not been presented so far. Here, we report the complete stereochemical course of the reaction mechanism of purified trehalose phosphorylase from the basidiomycete *Schizophyllum commune*.

2. Materials and methods

2.1. Materials

α,α-Trehalose was from Merck, α,β-trehalose and β,β-trehalose were from Chemprosa (Lannach, Austria). α-D-Glc 1-P, β-D-Glc 1-P and β-D-Glc were obtained from Sigma. α-D-Glucopyranosyl 1-fluoride (α-D-Glc 1-F) was synthesized by reported methods [10].

2.2. Trehalose phosphorylase

Trehalose phosphorylase was produced in *S. commune* BT 2115 and isolated by procedures to be published elsewhere. Judging from denaturing and non-denaturing PAGE, isoelectric focusing and matrix-assisted laser desorption-ionization mass spectrometry, the enzyme was pure (>95%). It is a monomeric protein of M_r about 60 000 (Eis and Nidetzky, to be published) and has a specific activity of 9.8 units/mg, determined in phosphorolysis at 30°C and pH 6.6, using 250 mM α,α-trehalose and 40 mM orthophosphate as substrates. The D-Glc released after 15 min reaction time was measured using the glucose oxidase/peroxidase assay. One unit of enzyme activity refers to 1 μmol D-Glc produced per minute. The purified enzyme was free of phosphatase activity, determined by using 30 mM D-Glc 1-P as substrate and measuring the release of inorganic phosphate in 60 min, and trehalase activity, determined by using 50 mM α,α-trehalose as substrate in the absence of phosphate and measuring the release of D-Glc in 30–60 min. Trehalose phosphorylase was stored in 40% glycerol (by volume), 20 mM MES, 300 mM NaCl, 5 mM EDTA, 2 mM 2-mercaptoethanol, pH 6.8.

2.3. Phosphorolysis of trehalose and α-D-Glc 1-F

The phosphorolysis reaction was carried out in a total volume of 0.3 ml at 20°C in 20 mM MES, pH 6.6, using an enzyme activity of 1 U/ml. Incubation at 20°C rather than 30°C (see Section 2.2) was chosen to minimize enzyme inactivation. Mixing in the reaction tubes was accomplished using an Eppendorf Thermomixer with agitation at 500 rpm. The substrate concentrations were 25 mM α,α-trehalose (or the α,β- and β,β-anomer) and 5 mM orthophosphate. The reaction with α-D-Glc 1-F (1–30 mM) was carried out in the presence of 10 mM inorganic phosphate. A control reaction which lacked the enzyme was used to monitor the spontaneous hydrolysis of α-D-Glc 1-F. At

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Abbreviations: D-Glc 1-P, D-glucose 1-phosphate; α-D-Glc 1-F, α-D-glucose 1-fluoride

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appropriate time intervals (0.1–3 h) samples (20 μl) were taken, cooled to 4°C and analyzed immediately (see below).

2.4. Trehalose synthesis

This was carried out as otherwise described for phosphorolysis, but in the absence of inorganic phosphate, using 50 mM glucosyl acceptor (such as D-Glc) and 5–20 mM glucosyl donor: α-Glc 1-P, β-Glc 1-P or α-D-Glc 1-F. To prevent mutarotation of β-D-Glc or α-D-Glc, the reaction was started by adding the solid acceptor substrate. Reactions with α-D-Glc 1-F were carried out with 0–10 mM added orthophosphate.

2.5. Identification of products of trehalose phosphorolysis

2.5.1. High performance anion exchange chromatography (HPAEC). The HPAEC was conducted with a Dionex model AI 450 system and a model PAD 2 pulsed amperometric detector. The column was a Dionex CarboPac PA-1 (250×4 mm) equipped with the same type of guard column (50×4 mm). The eluents were (A) 20 mM NaOH and (B) 20 mM NaOH and 200 mM sodium acetate, prepared daily on diluting a carbonate-free sodium hydroxide solution (50% NANO-pure II reagent grade; Baker, The Netherlands) in deionized water with a maximum conductivity of 18 MΩ/cm. Eluents were filtered (0.2 μm), degassed and kept under a stream of helium. Each sample was centrifuged (19 000×*g*; 10 min) and diluted to a sugar concentration of about 20–80 μM. Twenty μl of the sample was injected, and elution was carried out at room temperature with a flow rate of 1 ml/min, and the following gradient was used: 0–10 min (100% A), 10–20 min (100% B), 20–40 min (100% A).

2.5.2. High performance liquid chromatography (HPLC). Separation of α- and β-anomers of D-Glc was achieved by standard HPLC using a Bio-Rad HPX-87C column (300×7.8 mm) and refractive index detection. The column was cooled on ice to prevent mutarotation of D-Glc during analysis. Deionized water (4°C) was used as eluent at a constant flow rate of 0.25 ml/min. Samples were diluted to a concentration of about 20 mM, and 20 μl was injected.

2.5.3. Measurement of release of phosphate, α-D-Glc 1-P, β-D-Glc and other analyses. Inorganic phosphate was measured colorimetrically [11]. α-D-Glc 1-P was determined by a coupled NAD-dependent enzymatic assay [12]. β-D-Glc was measured using NAD-dependent glucose dehydrogenase (Amano, Milton Keynes, UK). Thin layer chromatography was carried out using high performance-TLC silica plates (Kieselgel 60 F254, Merck). An appropriately diluted sample was applied to the plate (1.2 μl), and development was with *n*-butanol/pyridine/water (6:4:3). Thymol reagent was used for detection of carbohydrate components.

3. Results and discussion

3.1. Stereochemical requirements of the glucosyl donor

In direction of phosphorolysis (5 mM inorganic phosphate), trehalose phosphorylase utilizes α,α-trehalose as the glucosyl donor, but not β,β-trehalose or α,β-trehalose. Under the conditions used for phosphorolysis, at least 0.2% of the α,α-trehalose-linked enzyme activity would have been detectable with the other anomeric forms of trehalose. On exhaustive phosphorolysis of α,α-trehalose, D-Glc 1-P and D-Glc were formed in exactly equimolar amounts. At 30°C and pH 6.6, the equilibrium constant, [α,α-trehalose][inorganic phosphate]/([D-Glc 1-P][D-Glc]), was 6.7. The time course of production of D-Glc 1-P from α,α-trehalose was monitored by using HPAEC analysis. That method [13] separates the anomeric forms of D-Glc 1-P and trehalose, and the results in Fig. 1A show that α-D-Glc 1-P but not β-D-Glc 1-P is produced from α,α-trehalose. The results of the analysis by HPAEC were fully consistent with those obtained by an indirect enzymatic method for the measurement of α-D-Glc 1-P [12], which uses rabbit muscle phosphoglucomutase whose activity is dependent on the α-anomer of D-Glc 1-P. In direction of trehalose synthesis, only the α-anomer of D-Glc 1-P is utilized as glucosyl donor, and α,α-trehalose is the exclusive product made by trehalose phosphorylase (Fig. 1B). Release of phosphate on incubation with trehalose phosphorylase and D-Glc was detectable only when α-D-Glc 1-P was the substrate, thus proving indirectly that the enzyme activity is dependent on the α-anomeric form of D-Glc 1-P.

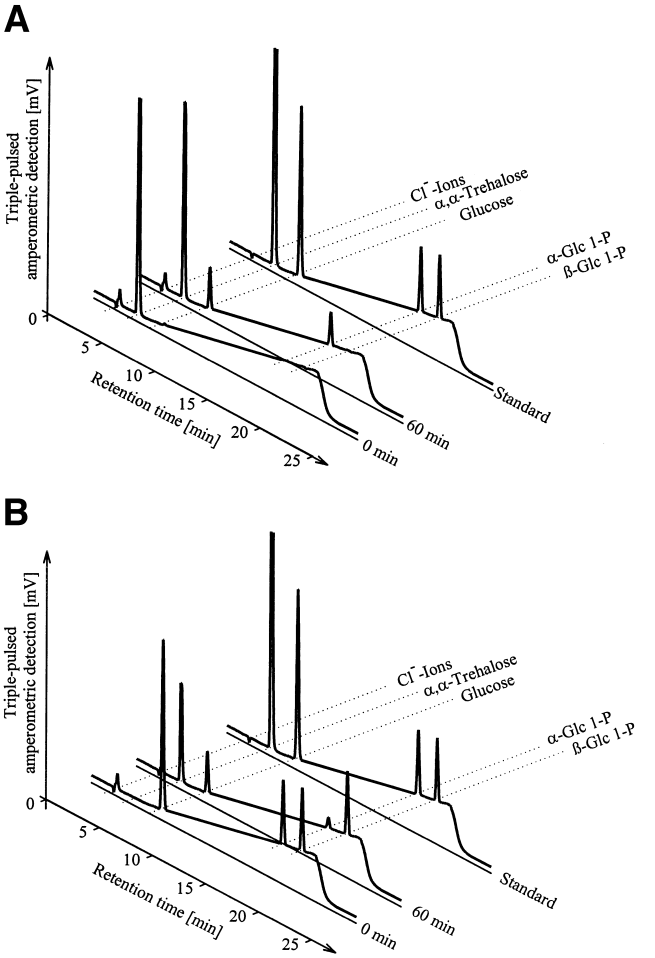

Fig. 1. Configuration of the products from the action of trehalose phosphorylase from *S. commune*, analyzed by HPAEC. Samples were taken at zero time and after 60 min. A: Phosphorolysis of α,α-trehalose (25 mM); and B: synthesis of α,α-trehalose in the presence of an equimolar mixture of α-D-Glc 1-P and β-D-Glc 1-P (5 mM each). In synthesis direction, an anomeric mixture of 50 mM D-Glc (in mutarotation equilibrium) was used as glucosyl acceptor.

3.2. Reaction with α-D-glucopyranosyl 1-fluoride

The glycosylic substrate analogue α-D-Glc 1-F is known to be reactive with sucrose phosphorylase [2] and glycogen phosphorylase [14]. The α-D-Glc 1-F is a substrate of trehalose phosphorylase, but it is poorly utilized: in the presence of inorganic phosphate (10 mM), α-D-Glc 1-F is converted slowly into α-D-Glc 1-P (Fig. 2), with only about 0.8% of the specific activity and 10% of the catalytic efficiency of the enzyme with α,α-trehalose, which is 190 s^{-1} M^{-1} in the presence of saturating concentrations of orthophosphate (50 mM). The nature of the leaving group obviously has a large effect on the catalytic competence of trehalose phosphorylase. Notably, sucrose phosphorylase converts α-D-Glc 1-F into α-D-Glc 1-P, with catalytic efficiencies that are even higher than those of phosphorolysis of sucrose [2]. In the direction of trehalose synthesis, that is in the absence of inorganic phos-

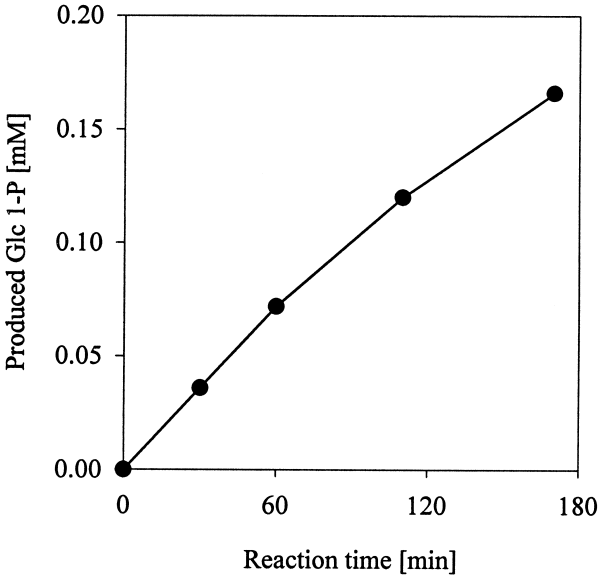

Fig. 2. The time course of the production of α-D-Glc 1-P by trehalose phosphorylase using α-D-Glc 1-F (10 mM) and inorganic phosphate (10 mM) as the substrates.

phate but with D-Glc (20 mM) as the acceptor, α-D-Glc 1-F (1–15 mM) does not serve as the donor of the glucosyl moiety at appreciable reaction rates. It seems therefore that α-D-Glc 1-F is recognized by trehalose phosphorylase as a substrate analogue of α,α-trehalose but not of α-D-Glc 1-P, at least under the conditions used here. In agreement with this notion, α-D-Glc 1-F did not inhibit the enzyme activity significantly ($K_i \gg 20$ mM), when α-D-Glc 1-P was the varied substrate and D-Glc was constant and saturating (50 mM). Like trehalose phosphorylase, glycogen phosphorylase utilizes α-D-Glc 1-F slowly [14], and the glucosyl fluoride is a substrate for polysaccharide synthesis, given the presence of orthophosphate and an oligo- or polysaccharide primer [14]. With glycogen phosphorylase, phosphate does not participate directly in the reaction, but serves a catalytic function as it is the proton donor activating the α-D-Glc 1-F [3,14]. In contrast, trehalose phosphorylase does not catalyze the transfer of a glucosyl residue from α-D-Glc 1-F to α-D-Glc, when 'catalytic' inorganic phosphate (0.1–10 mM) had been added to the reaction mixture. Instead, no reaction is observed under these conditions. If α-D-Glc 1-F is recognized by trehalose phosphorylase only as a substrate analogue of α,α-trehalose in phosphorolysis direction, D-Glc will act as an inhibitor of α-D-Glc 1-P formation from α-D-Glc 1-F, and not serve as glucosyl acceptor in the reaction with the glucosyl fluoride. This is entirely consistent with the experimental observations.

3.3. Stereochemical requirements of the glucosyl acceptor

HPLC analysis under conditions that minimize mutarotation of D-Glc [15] was used to determine the time course of production of D-Glc from α,α-trehalose by trehalose phosphorylase. The results in Fig. 3 show that the α-anomer of D-Glc is formed on phosphorolysis of α,α-trehalose. With increasing reaction times formation of β-D-Glc is observed because of mutarotation, the ratio of the α- and β-anomer finally approaching mutarotation equilibrium. In the direction of trehalose synthesis, α-D-Glc is the substrate for trehalose phosphorylase. When mixture of α- and β-D-Glc is used as the glucosyl acceptor, only α-D-Glc is converted into α,α-trehalose. The β-anomer is not utilized by the enzyme, even with high concentrations of 300 mM. Compared with sucrose phosphorylase, trehalose phosphorylase seems to be very specific for the natural acceptor, D-Glc, concerning the glucosyl transfer from α-D-Glc 1-P: of D-Gal, D-Man, L-Glc, D-Rib, D-Xyl, L-Xyl, L-Lyx, D-Lyx, L-Ara, D-Ara, D-Fruc, L-Sorb (each with 50 mM), only D-Man served as acceptor, albeit with only 5% catalytic efficiency of that observed with D-Glc, which is approximately 270 s^{-1} M^{-1} in the presence of saturating concentrations of α-D-Glc 1-P (30 mM). The putative α-D-mannopyranosyl-1,1′-α-D-glucopyranoside product was detected on TLC analysis (not shown), and is clearly distinguished from α,α-trehalose which could have been formed in the case of slight contamination of D-Man with D-Glc. Unlike sucrose phosphorylase [2], trehalose phosphorylase does not seem to transfer the glucosyl moiety from α,α-trehalose to a number of acceptors other than phosphate. For example, on phosphorolysis of α,α-trehalose (5 mM phosphate), no by-products were formed when competing nucleophiles such as glycerol (10–20%, by volume) or D-Glc (50–200 mM) had been added. One notable exception, however, is arsenate, which can replace inorganic phosphate as glucosyl acceptor in the direction of degradation of α,α-trehalose. The catalytic efficiency of arsenolysis is approximately 20% that of phosphorolysis (∼10 000 s^{-1} M^{-1}). As α-D-Glc 1-arsenate is unstable [2], the net reaction is the production of two moles of D-Glc on enzymatic arsenolysis of α,α-trehalose.

4. Conclusions

Fungal trehalose phosphorylase, from *S. commune*, is a glucosyl-transferring enzyme that proceeds with overall retention of the anomeric configuration at the scissile glycosidic or glycoside-phosphate bond. According to the designation of Sinnott [1], which distinguishes whether, in the preferred conformation, the orientation of the leaving group is axial (a) or equatorial (e), trehalose phosphorylase belongs to the class of a→a glucosyl transferases. Precedent mechanistic studies with a number of retaining glycosyl transferases were all in support of a double displacement reaction mechanism, and on grounds of the stereochemical evidence this reaction mecha-

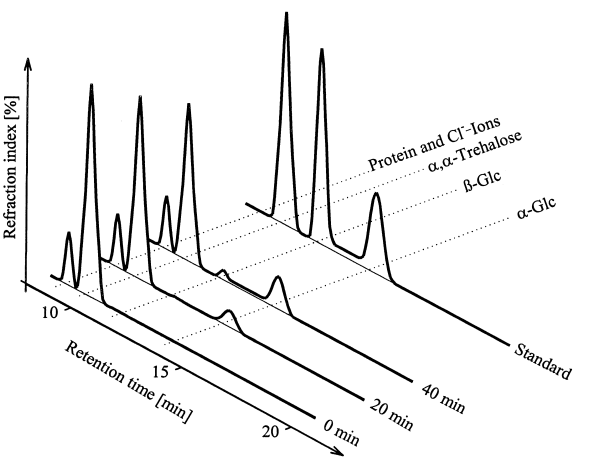

Fig. 3. The release of α-D-Glc on phosphorolysis of α,α-trehalose (25 mM) catalyzed by trehalose phosphorylase from *S. commune*. Samples were taken at zero time and after 20 and 40 min.

nism seems likely for trehalose phosphorylase. Utilization of α-D-Glc 1-F by trehalose phosphorylase in the presence of inorganic phosphate yields α-D-Glc 1-P and arguably requires stereospecific protonation of the glucosyl fluoride before the glucosyl residue is transferred, which is in agreement with the mechanism. In conclusion, fungal trehalose phosphorylase is clearly distinguished from the bacterial counterpart enzyme [6,7] by the stereochemical course of its reaction mechanism. With regard to the utilization of the substrate analogue and mechanistic probe α-D-Glc 1-F, it differs significantly from the well-characterized sucrose phosphorylase [2], which like trehalose phosphorylase is a retaining disaccharide phosphorylase.

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