Characterization of trehalose phosphorylase from *Schizophyllum commune*

Christian EIS and Bernd NIDETZKY[1]

Division of Biochemical Engineering, Institute of Food Technology, Universität für Bodenkultur (BOKU), Muthgasse 18, A-1190 Wien, Austria

During growth on D-glucose, the basidiomycete *Schizophyllum commune* produces an intracellular α,α-trehalose phosphorylase. Specific phosphorylase activity increases steadily during the exponential growth phase, up to a maximum of approx. 0.08 unit/mg of protein, and decreases after the available D-glucose in the medium has been fully depleted. The variation with time of the concentrations of intracellular α,α-trehalose and P_i is reciprocal to that of trehalose phosphorylase activity, indicating that the enzyme makes temporary use of the pool of α,α-trehalose (approx. 0.42 mmol/g dry cell) via phosphorolysis. The enzyme has been purified, 150-fold, to homogeneity in 55% yield and characterized. It is a monomeric 61 kDa protein, which seems to lack regulation at the level of enzyme activity. The enzyme catalyses the reversible phosphorolysis of α,α-trehalose into α-D-glucose 1-phosphate and α-D-glucose in the absence of cofactors, with a catalytic-centre activity at 30 °C of 14 s^{-1}. Double-reciprocal analysis of the initial velocities for trehalose phosphorolysis and synthesis yields intersecting patterns, and no exchange reaction occurs between α-D-glucose 1-phosphate and the phosphate analogue arsenate. Therefore trehalose phosphorylase operates by a ternary-complex, rather than a Ping-Pong, kinetic mechanism. The specificity constants (k_{cat}/K_m) of phosphate (6000 $M^{-1}\cdot s^{-1}$) and α-D-glucose 1-phosphate (3500 $M^{-1}\cdot s^{-1}$) compared with those of α,α-trehalose (161 $M^{-1}\cdot s^{-1}$) and D-glucose (260 $M^{-1}\cdot s^{-1}$), together with the inhibition by NaCl, which is competitive with respect to phosphate with a K_i of 67 mM, suggest an important role for ionic enzyme–phosphate interactions in the catalytic mechanism of trehalose phosphorylase. The isolated enzyme requires α,α-trehalose (0.1–0.3 M) for its conformational stability.

Key words: fungi, function, kinetic mechanism, properties, trehalose phosphorolysis.

INTRODUCTION

The non-reducing disaccharide α,α-trehalose occurs in fungi and yeasts, and also in different classes of prokaryotes, plants and animals [1]. Trehalose is a very common sugar in vegetative cells and spores of fungi, where it is found in concentrations that can exceed significantly those of other storage carbohydrates, such as glycogen or D-mannitol [1]. The enzyme known to be responsible for the breakdown of trehalose in fungi is α,α-trehalase (EC 3.2.1.28), which catalyses the hydrolysis of trehalose into glucose [2].

In the early 1970s, Belocopitow and Marechal isolated and characterized a trehalose phosphorylase (EC 2.4.1.64) from the alga *Euglena gracilis* [3,4]. This enzyme was found to catalyse the phosphorolysis of α,α-trehalose, according to eqn. (1):

$$\alpha,\alpha\text{-Trehalose} + P_i \leftrightarrow \beta\text{-D-glucose 1-phosphate} + \text{D-glucose} \quad (1)$$

The identification of a phosphoglucomutase in *E. gracilis* that was capable of converting β-D-glucose 1-phosphate into D-glucose 6-phosphate [5] supported a physiological role for trehalose phosphorylase in a catabolic pathway for trehalose. Low intracellular activities of trehalose phosphorylase, typically less than 0.01 unit/mg of protein, have subsequently been detected in a number of other organisms [6–13]. Bacterial trehalose phosphorylases from *Catellatospora ferruginea* [12] and *Micrococcus varians* [13] have been isolated and characterized. They are large oligomers of approx. 400–600 kDa composed of identical subunits of approx. 90 kDa, and their activities are not dependent on exogenous or enzyme-bound cofactors. Trehalose phosphorylase from *M. varians* catalyses the phosphorolysis of α,α-trehalose by using a sequential kinetic mechanism in which both substrates, i.e. phosphate followed by trehalose, must add to the enzyme to form a ternary enzyme–substrate complex before the first product (glucose) is released [13]. Like the enzyme from *E. gracilis* [3,4], the bacterial trehalose phosphorylases catalyse glucosyl transfer from α,α-trehalose to phosphate with net inversion of the configuration at the anomeric centre of the glucosyl donor, and hence β-glucose 1-phosphate is produced [12,13].

At present, little is known about properties and physiological function of trehalose phosphorylase from fungal sources [6,8,9,14,15]. An important feature, however, which seems to distinguish the fungal enzymes from their bacterial counterparts is a reaction mechanism in which the anomeric configuration of the glucosyl donor is retained in the resulting product, leading to the formation of α-D-glucose 1-phosphate (α-D-Glc 1-P) from α,α-trehalose [6,8,9,14,15], as shown in eqn. (2):

$$\alpha,\alpha\text{-Trehalose} + P_i \leftrightarrow \alpha\text{-D-Glc 1-P} + \alpha\text{-D-glucose} \quad (2)$$

Recently, the first primary structure of a fungal trehalose phosphorylase (termed trehalose synthase in the paper [14]), from the basidiomycete *Grifola frondosa*, was described. The gene encodes a polypeptide of 732 amino acids (approx. 84 kDa), which lacks entire-chain similarity to other glycosyl transferases [14]. The mature enzyme protomer appears to have a molecular size which is significantly smaller than expected from the corresponding gene, and the occurrence of enzymically active protein forms with molecular masses of 78 kDa and 61 kDa is described. A trehalose phosphorylase from *Agaricus bisporus* has recently been isolated and characterized [9]. The enzyme is a tetramer composed of 61 kDa subunits, and its activity was found to be inhibited by phosphate ions and regulated to some extent by the cellular energy charge [9].

The present paper describes the isolation and characterization of trehalose phosphorylase from the basidiomycete *Schizophyllum commune*. The enzyme differs clearly from known bacterial trehalose phosphorylases [12,13] in its subunit size (61 kDa), the quartenary structure (monomer) and kinetic

Abbreviations used: α-D-Glc 1-*P*, α-D-glucose 1-phosphate; MALDI-MS; matrix-assisted laser-desorption/ionization MS; PEG, poly(ethylene glycol).

[1] To whom correspondence should be addressed (e-mail nide@mail.boku.ac.at).

properties. It appears similar to the trehalose phosphorylase from *Agaricus bisporus* [9], although differences in substrate specificity [15] and subunit organization were found. In addition, the paper addresses the question of the physiological function of trehalose phosphorylase in *S. commune*, which has thus far been completely unknown.

EXPERIMENTAL

Chemicals

α-D-Glc 1-*P*, calmodulin from hog brain, protein phosphatase 2B (calcineurin) from bovine brain and phosphoglucomutase from rabbit muscle were obtained from Boehringer (Mannheim, Germany). Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* and cAMP-dependent protein kinase from bovine heart were obtained from Sigma (Deisenhofen, Germany). $NAD(P)^+$-dependent D-glucose dehydrogenase (100 units/ml) was from Amano (Milton Keynes, Bucks., U.K.). Glucanex, which is a technical-grade β-1,3-glucanase preparation, was obtained from Novo Nordisk (Dittingen, Switzerland). Trehalose dihydrate, poly(ethylene glycol) (PEG) and glycerol (98 %) were purchased from Roth (Karlsruhe, Germany). Technical-grade trehalose dihydrate was from the British Sugar Technical Centre (Norwich, U.K.). Phenyl-Sepharose 4B-CL Fast Flow High Sub, DEAE-Sepharose Fast Flow, Superose 12 (preparative grade), Sephacryl S-300 and Superdex 75 (preparative grade) were from Amersham-Pharmacia (Uppsala, Sweden). Fractogel TSK-DEAE 650(S) was obtained from Merck (Darmstadt, Germany). All other chemicals were of reagent grade and were purchased from Sigma or Fluka (Basel, Switzerland).

Cultivation of fungi and enzyme production

All strains used in this study were obtained from culture collections maintained at the Institute of Applied Microbiology (MB), BOKU, Vienna, Austria, or the Institute of Biotechnology (BT), Technical University of Graz, Graz, Austria. Different strains of *Schizophyllum* and other fungi were cultivated at 30 °C in 1 litre baffled Erlenmeyer flasks containing 300 ml of medium with an initial pH of 6.0. A constant agitation rate of 100 rev./min was used in an Infors model Multitron HT rotary incubator (Infors, Bottmingen, Switzerland). The medium consisted of 20 g/l D-glucose as the carbon source, 5 g/l peptone from soy bean, 5 g/l yeast extract and 1 g/l malt extract as the nitrogen source, as well as 1 g/l K_2HPO_4. Unless noted otherwise, the cultivation time was 72 h. The inoculum was prepared in exactly the same way as described above, with the exception that 300 ml flasks containing 30 ml of medium were used. To reduce the formation of the extracellular glucan polymer schizophyllan during growth of *S. commune* on D-glucose, the nitrogen source was added in excess [16]. After 48 h of growth, 100 mg/l Glucanex was added to the main culture, to hydrolyse any schizophyllan that had formed. Aqueous solutions containing this polymer are very viscous, which leads to complications in the purification of trehalose phosphorylase.

Enzyme purification

The mycelial biomass of *S. commune* BT 2115 was washed twice with water, and harvested by a 15-min centrifugation at 8000 ***g*** and 4 °C in a Sorvall RC 26 PLUS centrifuge (DuPont, Stevenage, Herts., U.K.). To remove any remaining schizophyllan associated with the fungal mycelium, an ultracentrifugation step was employed using a Beckman L-70 ultracentrifuge (Beckman, Fullerton, CA, U.S.A.) operated at 4 °C and 80000 ***g*** for 15 min. The cell material thus obtained was resuspended (1:2, w/v) in 20 mM Mes buffer, pH 6.8, containing 4 mM EDTA, 2 mM mercaptoethanol and 40 % (v/v) glycerol. The fungal mycelium was then disrupted for 15 min at 4 °C, by using an Ultra Turrax T25 homogenizer (IKA Labortechnik, Staufen, Germany), with an instrument setting of 24000 rev/min. The crude cell extract was obtained by ultracentrifugation of the resulting slurry for 15 min at 80000 ***g*** and 4 °C.

Protein purification was carried out on Pharmacia GradiFrac and FPLC systems, at 6 °C. Unless noted otherwise, 20 mM Mes buffer, pH 6.8, containing 4 mM EDTA, 2 mM mercaptoethanol and 30 % (v/v) glycerol was used. The detection of proteins on elution from columns was at 280 nm.

Step 1

The initial purification of trehalose phosphorylase was by anion-exchange chromatography using DEAE-Sepharose Fast Flow. The crude cell extract was applied to the column (5 cm × 15 cm), with a maximum loading of approx. 15 mg of protein/ml of gel. Elution was carried out by using a step gradient, with steps of 0, 50 and 200 mM NaCl in buffer. Trehalose phosphorylase was eluted at 200 mM NaCl under these conditions.

Step 2

The enzyme preparation was diluted at 4 °C with constant stirring with 70 %-satd. $(NH_4)_2SO_4$ dissolved in buffer containing 30 % (v/v) glycerol to give a solution that was 25 %-satd. in $(NH_4)_2SO_4$. The protein precipitate was removed by a 15 min ultracentrifugation at 80000 ***g*** and 4 °C. The clear supernatant was loaded on to a 2.6 cm × 5 cm column of phenyl-Sepharose 4B-Cl Fast Flow High Sub. Trehalose phosphorylase was eluted by using a step gradient, with steps at 25 %, 12 % and 0 % $(NH_4)_2SO_4$ saturation in buffer containing 30 % (v/v) glycerol. Trehalose phosphorylase was eluted at 0 %-satd. $(NH_4)_2SO_4$.

Step 3

The enzyme preparation was concentrated by using aqueous two-phase partition in a system containing 7 % (w/v) PEG 4000 and $(NH_4)_2SO_4$ at 35 % saturation. The PEG-enriched top phase, which constitutes approx. 10 % of the total volume, contained more than 90 % of the enzyme activity. The top phase was carefully removed and dialysed against a glycerol-free buffer containing 50 % (w/v) saccharose. For dialysis, a cellulose membrane with 1 kDa cut-off (Spectrum, Houston, TX, U.S.A.) was used.

Step 4

The dialysed enzyme preparation was loaded on to a 1.6 cm × 6 cm column of the anion-exchange resin Fractogel TSK-DEAE, and eluted with NaCl using the step gradient described in step 1.

Step 5

A final gel filtration step was carried out using three columns in series. Each of these columns was 16 mm in diameter. The first column contained 140 ml of Sepharose 12, the second contained

180 ml of Sephacryl S-300, and the third contained 140 ml of Superdex 75. The columns were equilibrated with 20 mM Mes buffer, pH 6.8, containing 200 mM trehalose, 200 mM NaCl, 4 mM EDTA and 2 mM mercaptoethanol. The partially purified trehalose phosphorylase, typically 5 ml with a protein concentration of about 10 mg/ml, was loaded on to the first column. Protein was eluted using a flow rate of 0.5 ml/min.

Enzyme assays

Unless otherwise noted, the activity of trehalose phosphorylase in the direction of phosphorolysis was determined at 30 °C by using 250 mM trehalose and 40 mM phosphate as the substrates, in 20 mM Mes buffer, pH 6.6. Two methods of measurement were used. (1) The α-D-Glc 1-*P* liberated on enzyme action was measured by using a continuous coupled enzymic assay [17], which contained (in addition to substrates and trehalose phosphorylase) phosphoglucomutase (4 units/ml), NAD^+-dependent D-glucose-6-phosphate dehydrogenase (3 units/ml), 3 mM NAD^+, 10 mM $MgCl_2$ and 10 μM glucose 1,6-bisphosphate. The formation of NADH with time was monitored spectrophotometrically at 340 nm. (2) D-glucose, which is released on incubation of substrates with trehalose phosphorylase in a discontinuous phosphorolysis reaction, was measured by using glucose dehydrogenase. Samples (60 μl) taken after 5, 10 and 30 min of reaction time were heat-treated (5 min) and diluted into buffer containing 10 units of glucose dehydrogenase/ml and 3 mM NAD^+. After incubation at 30 °C for 45 min, the endpoint of the absorbance at 340 nm was monitored, which corresponds to the amount of NAD^+ reduced upon oxidation of β-D-glucose. The activity of trehalose phosphorylase in the direction of trehalose synthesis was determined in the presence of 200 mM D-glucose and 20 mM α-D-Glc 1-*P*, dissolved in 20 mM Mes buffer, pH 6.6. The release of P_i after a 10 min reaction time was measured colorimetrically at 850 nm [18]. Appropriate control reactions lacking the enzyme or the substrate were recorded in all cases, and enzyme activities were corrected for the control readings.

Trehalase activity was determined using 50 mM α,α-trehalose as the substrate, in the absence of phosphate, and by measuring the D-glucose in samples (10 μl) taken after 30 and 60 min incubation times, using the method described above. Phosphatase, phosphoglucomutase and D-glucose-6-phosphate dehydrogenase activities were measured by reported methods [17]. Protein was measured by a dye-binding assay, using BSA (fraction V; USB, Cleveland, OH, U.S.A.) as the standard.

Electrophoresis

SDS/PAGE and non-denaturing anionic PAGE were carried out on a Pharmacia PhastSystem, by using PhastGel Homo 20 and Gradient 8-25 respectively. Visualization of protein bands was by staining with Coomassie Blue. Staining for trehalose phosphorylase activity in non-denaturing PAGE was performed using an assay based on the enzyme activity in the direction of phosphorolysis. The gel was incubated in a solution, pH 6.6, containing 250 mM trehalose and 40 mM phosphate together with 5-methylphenazinium methylsulphate (1.0 mM) and Nitroblue Tetrazolium chloride (1.0 mM). The control reaction did not contain trehalose.

Quarternary structure, molecular mass and isoelectric point

The molecular mass of the trehalose phosphorylase subunit was determined by SDS/PAGE and matrix-assisted laser-desorption/ionization MS (MALDI-MS). The MALDI-MS measurements were carried out with a DYNAMO linear time-of-flight spectrometer (Thermo BioAnalysis, Hemel Hempstead, Herts., U.K.), operated with delayed extraction off and deflector on. Trehalose phosphorylase was mixed with an equal volume of matrix solution [20 g/l sinapinic acid in a 7:3 (v/v) mixture of acetonitrile and 1.0 g/l trifluoroacetic acid in water]. The sample (1 μl; containing approx. 0.1 pmol of protein) was dried on platen with wells and analysed. The mass axis was externally calibrated with BSA (66.441 kDa).

The quarternary organization of the protein was deduced from gel filtration analysis, carried out on Superose 12 HR 10/30 (Pharmacia). Elution during analytical gel filtration was at a constant flow rate of 0.3 ml/min, by using 50 mM potassium phosphate buffer, pH 6.8, containing 200 mM NaCl. The calibration standards comprised cytochrome *c* (12.4 kDa), myoglobulin (17.6 kDa), β-lactoglobulin (35 kDa), egg albumin (43 kDa), BSA (67 kDa), hexokinase (100 kDa) and aldolase (158 kDa). The molecular mass standards for SDS/PAGE consisted of marker proteins with masses of 15, 25, 35, 75, 100 and 150 kDa (Sigma).

The pI of trehalose phosphorylase was determined by isoelectric focusing, using an Ampholine PAGplate IEF, pH 4.0–6.5, and an isoelectric calibration kit, both obtained from Pharmacia.

Kinetics

Initial-velocity measurements in the phosphorolysis and synthesis directions were carried out at 30 °C in 20 mM Mes buffer, pH 6.6, containing 4 mM EDTA and 2 mM 2-mercaptoethanol. For determination of the apparent kinetic constants, one substrate was varied while the other substrate was kept constant at a saturating concentration, i.e. about 4–5 × its apparent K_m. For phosphorolysis, the release of α-D-Glc 1-*P* was routinely monitored; in the synthesis direction, the formation of phosphate was measured. Substrate depletion was usually less than 5% during the assays. Thus product formation was linear with time and could be used directly to derive the initial velocity. All kinetic constants were calculated by non-linear least-squares regression, fitting the observed data to a simple hyperbola, which was extended as required to account for substrate inhibition (eqn. 3):

$$V(S) = k_{cat}[E][S]/(K_m + [S] + [S]^2/K_{IS}) \quad (3)$$

where $V(S)$ is the observed reaction rate, k_{cat} is the catalytic constant, [E] is the total concentration of trehalose phosphorylase active sites, [S] is the concentration of the varied substrate, K_m is the apparent Michaelis constant for S and K_{IS} is the substrate inhibition constant for S. To distinguish between a sequential (ternary-complex) and a non-sequential (Ping-Pong) kinetic mechanism, initial-velocity measurements were carried out in the direction of phosphorolysis, with one substrate being varied at several constant concentrations of the second substrate. Reciprocal initial velocities were plotted against reciprocal substrate concentrations, and the kinetic pattern (intersecting or non-intersecting) was identified from these plots [19].

The equilibrium constant for the enzyme-catalysed interconversion of α,α-trehalose and phosphate, and α-D-Glc 1-*P* and D-glucose, was determined at 30 °C and different pH values by incubating approx. 1 unit/ml trehalose phosphorylase in the presence of substrates until constant concentrations of α-D-Glc 1-*P* and phosphate were reached. It was proved that enzyme inactivation with time did not interfere with the determination of the reaction equilibrium.

Spectroscopic analysis

Pyridoxal 5′-phosphate was determined according to Wada and Snell [20]. UV/visible spectra were recorded on a Hitachi (Tokyo, Japan) U-3000 spectrophotometer at 25 ± 0.2 °C. Fluorescence emission spectra were recorded on a Hitachi F2000 spectrofluorimeter at 25 ± 1 °C in cells of 1 cm optical path length. Band widths of 5 nm for the excitation and emission wavelengths were used.

Intracellular metabolites

For the determination of intracellular metabolites, thoroughly washed and centrifuged cells of *S. commune* were dried slowly at 105 °C to constant weight, by using an infrared moisture analyser (model MA 30; Sartorius, Göttingen, Germany). The dried cells were ground to fine powder, and metabolites were extracted with water (1:2, w/v) for 4 h at 4 °C. The slurry was centrifuged at 10000 ***g*** and 4 °C for 15 min. The composition of the supernatant was analysed by HPLC (trehalose, D-glucose, phosphate) as well as by colorimetric assays (see above; D-glucose, α-D-Glc 1-*P*, D-glucose 6-phosphate, phosphate). The HPLC system for carbohydrate analysis (trehalose, D-glucose) consisted of a Merck Model L 6200 A pump and a Model AS 2000 A autosampler. An HPX-87H (300 mm × 7.8 mm internal diam.) column from Bio-Rad, together with a high-sensitivity refractive index detector (Erma Optical Works, Tokyo, Japan), were used routinely. The column was kept at room temperature, and 5 mM H_2SO_4 was used as eluent at a flow rate of 0.3 ml/min. To confirm results obtained in colorimetric assays, phosphate was quantified by anion analysis on a Dionex HPLC system, model DX-120 (Dionex, Sunnyvale, CA, U.S.A.) with conductivity detection. An IonPac AS14 (250 mm × 4.0 mm internal diam.) with the same type of guard column (AG14; 50 mm × 4.0 mm internal diam.) was used, operated at room temperature. Elution was with 3.5 mM Na_2CO_3/1 mM $NaHCO_3$ at a flow rate of 1.8 ml/min. Both methods gave consistent results, which rules out the possibilty of overestimation of the free phosphate concentration by the colorimetric method because of the hydrolysis of other phosphorylated compounds, such as ATP, under the acidic conditions of the assay.

Protein phosphorylation

Dephosphorylation

This was measured as described in [21]. Trehalose phosphorylase (0.5 mg/ml) was incubated with an unspecific, calmodulin-dependent protein (serine/threonine) phosphatase 2B (6 units/ml), and the change in phosphorylase activity and the concentration of free phosphate ion with time were measured (see above). The incubation was carried out at 20 °C in 50 mM Tris buffer, pH 7.0, containing 2 units/ml calmodulin, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 0.1 mM EGTA, 1 mM dithiothreitol and 1 mg of BSA. The control reaction did not contain phosphatase.

Phosphorylation

This was measured as described in [22]. Trehalose phosphorylase (0.2 mg/ml) was incubated with a broad-specificity protein (serine/threonine) kinase (1.4 mg/ml; equivalent to 1.4 m-units) in 50 mM Tris buffer, pH 7.0, containing 5 mM $MgCl_2$, 0.2 mM ATP and 20 μM cAMP. The change in phosphorylase activity with time was monitored. The control reaction did not contain cAMP.

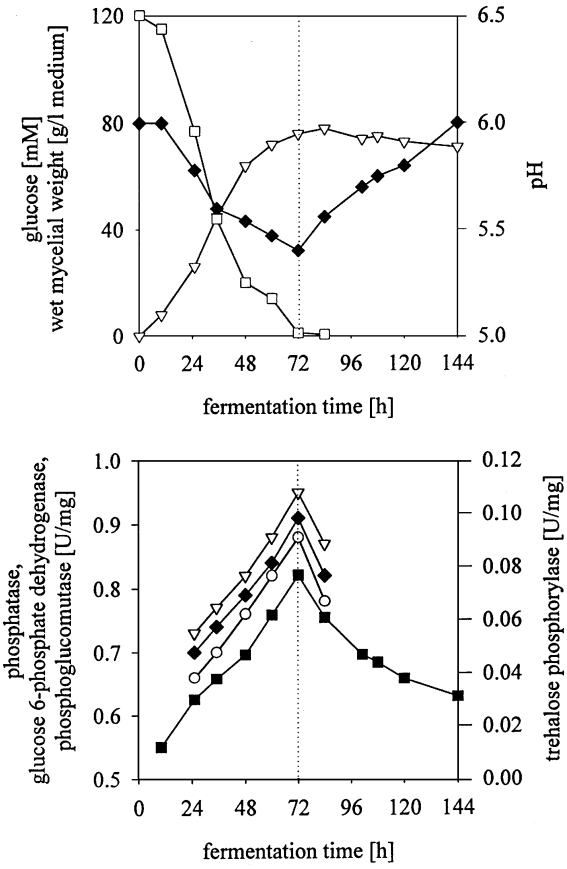

Figure 1 Formation of trehalose phosphorylase during growth of *S. commune* on D-glucose

Upper panel: production of mycelial biomass (▽), consumption of D-glucose (□) and acid formation (◆, pH) in relation to incubation time in shaken-flask culture at 30 °C. Lower panel: changes in specific enzyme activities with incubation time: ■, trehalose phosphorylase; ○, phosphatase; ◆, glucose-6-phosphate dehydrogenase; ▽, phosphoglucomutase. For other details, see the Experimental section.

RESULTS AND DISCUSSION

Occurrence of trehalose phosphorylase

Of the different strains of *Schizophyllum* studied, such as *S. commune* (BT 2115), *S. acer* (MB145, MB 146), *S. picea* (MB 143, MB 144) and *S. fagus* (MB 147, MB 148), all contained trehalose phosphorylase activity. The species-dependent variation in specific activity on exhaustive growth on D-glucose (72 h) was in the range 0.04–0.08 unit/mg. Furthermore, similar trehalose phosphorylase activities were found in other related fungi, such as *Trametes multicolor* (MB 49), *T. versicolor* (MB 53, MB 54), *T. suaveolea* (MB 51), *Sclerotium rolfsii* or *Pleurotus ostreatus*. Therefore trehalose phosphorylase seems to be a common enzyme in basidiomycetes. For further studies, the enzyme from *S. commune* BT 2115 was selected, because this organism produced the highest specific phosphorylase activities.

Enzyme production

During growth on D-glucose as the carbon source, the formation of wet mycelial biomass and the consumption of D-glucose varied with time, typically as shown in Figure 1 (upper panel). The metabolism of D-glucose by the organism was coupled with acid

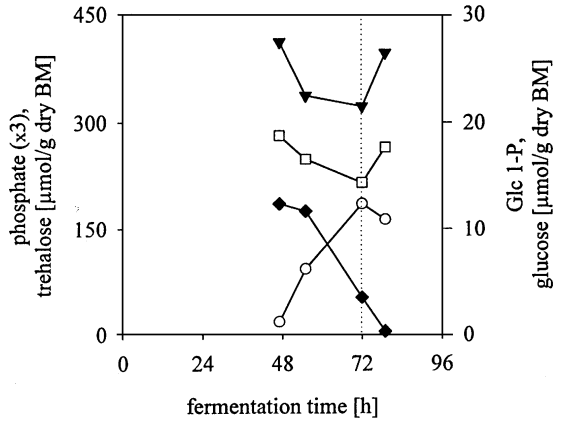

Figure 2 Changes in the concentrations of intracellular metabolites during growth of *S. commune* on D-glucose

Mycelial cell mass was harvested at the times indicated, and metabolite concentrations were determined as described in the Experimental section: ○, α-D-Glc 1-*P*; ◆, D-glucose; □, phosphate; ▼, trehalose. BM, biomass.

formation, and the pH decreased from an initial value of 6.0 to about 5.5. After the available D-glucose in the medium had been utilized completely, the pH increased to 6.0 again. Even in the presence of a large excess of nitrogen source over carbon source, the production of extracellular polymer was high. Therefore glucanase was added at 48 h. The consumption of D-glucose by *S. commune* was apparently slowed after the addition of the glucanase (Figure 1, upper panel), reflecting the release of D-glucose from the glucan polymer schizophyllan by enzymic hydrolysis. After approx. 72 h, the D-glucose in the medium was fully depleted, and growth stopped.

Trehalose phosphorylase activity increased linearly during the exponential growth phase, from about 0.01 unit/mg to a maximal value of 0.08 unit/mg, which was observed at approx. 72 h (Figure 1, lower panel). With regard to the isolation of trehalose phosphorylase, the time of cell harvesting was critical, because the specific enzyme activity decreased rapidly after D-glucose had been consumed fully. The specific activity of trehalose phosphorylase at the time of D-glucose depletion was independent of the initial D-glucose concentration (10–40 g/l). Osmotic shock, achieved by adding 300 mM NaCl in a pulse at 48 h (for comparison, see Figure 1, upper panel), or temperature shock, achieved by heating the submerged fungal culture to 50 °C for 10 min, did not have a significant effect on enzyme activity, either immediately after the shock treatment or after growth to depletion of the carbon source. It seems, therefore, that trehalose phosphorylase is not a stress-inducible enzyme. The variation with cultivation time of key enzyme activities that are involved in the metabolism of α-D-Glc 1-*P* are shown in Figure 1 (lower panel). The formation of phosphoglucomutase, glucose-6-phosphate dehydrogenase and phosphatase paralleled that of trehalose phosphorylase. However, the specific activities of the selected enzymes were approx. 10 times that of trehalose phosphorylase.

Intracellular metabolites

The intracellular concentrations of α-D-Glc 1-*P*, D-glucose, phosphate, trehalose and D-glucose 6-phosphate were measured, and changes in these concentrations with cultivation time were monitored, as shown in Figure 2. Notably, the concentration of trehalose was as high as 0.42 mmol/g dry cells (equivalent to approx. 14% of the dry cell material). Trehalose and phosphate decreased from 48 h to minimal levels at 72 h, followed by an increase back to the concentrations seen at 48 h. The change in α-D-Glc 1-*P* with time was reciprocal to those of phosphate or trehalose. The molar ratio of phosphate to α-D-Glc 1-*P* decreased from a high value of approx. 78 to a low value of 6 at the time when trehalose phosphorylase activity was a maximum. In contrast, the α-D-Glc 1-*P*/D-glucose 6-phosphate molar ratio increased from a value of less than 0.1 at 48 h to a maximum value of approx. 1 at 72 h (results not shown). The intracellular D-glucose concentration decreased from 48 h onwards, and this metabolite was not detectable after approx. 84 h.

Table 1 Purification of trehalose phosphorylase from *S. commune*

The processed material was 700 g wet weight of mycelial biomass. All steps were carried out at 6 °C.

Step	Total volume (ml)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
Crude cell extract	2000	156	0.06 ± 0.01	100	1
Anion-exchange (DEAE-Sepharose)	260	154	0.41	98	6.3
Hydrophobic interaction (phenyl-Sepharose)	163	104	0.90	67	14
Aqueous two-phase partition	44	94	1.40	60	22
Anion-exchange (Fractogel TSK-DEAE)	24	92	1.60	56	25
Gel filtration (Sepharose 12, Sephacryl S-300 and Sepharose 75, in series)	33	85	9.85	55	152

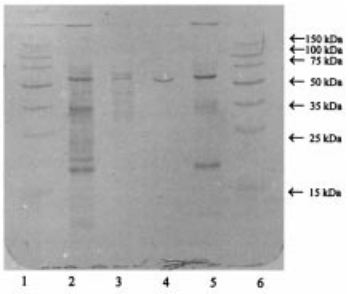

Figure 3 Purification of trehalose phosphorylase from *S. commune* documented by SDS/PAGE

Lanes 1 and 6, molecular-mass standards; lane 2, crude cell extract; lane 3, protein fraction eluting before trehalose phosphorylase during gel filtration; lane 4, pure trehalose phosphorylase; lane 5, enzyme preparation prior to gel filtration.

Enzyme purification

Trehalose phosphorylase was purified to apparent homogeneity (approx. 95%) by using a five-step procedure, which is summarized in Table 1. The purity of the enzyme was assessed by SDS/PAGE (Figure 3), non-denaturing anionic PAGE (Figure 4) and isoelectric focusing (results not shown). In all cases, trehalose phosphorylase migrated as a single protein band. Staining for enzyme activity in non-denaturing PAGE (see the Experimental section) allowed unequivocal identification of the protein as trehalose phosphorylase (Figure 4). The isolated

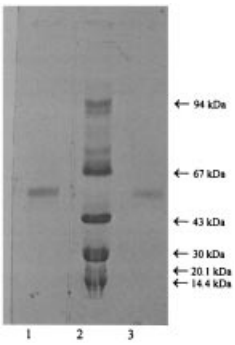

Figure 4 Non-denaturing anionic PAGE of purified trehalose phosphorylase and identification of the enzyme by staining for activity

Lane 1, trehalose phosphorylase, stained for enzyme activity; lane 2, molecular-mass standards; lane 3, trehalose phosphorylase stained with Coomassie Blue.

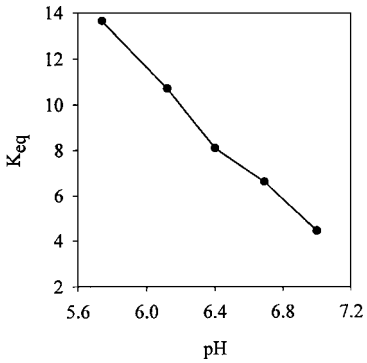

Figure 5 K_{eq} for the reaction catalysed by trehalose phosphorylase at 30 °C and its dependence on pH

Trehalose phosphorylase was incubated with 20 mM D-glucose and 20 mM α-D-Glc 1-*P* for up to 24 h. The equilibrium concentrations of α-D-Glc 1-*P*, phosphate, D-glucose and trehalose were measured, and the equilibrium constant was calculated using the formula K_{eq} = [trehalose][phosphate]/([α-D-Glc 1-*P*][D-glucose]).

Table 2 Apparent kinetic parameters for trehalose phosphorylase from *S. commune*

Reactions in the directions of phosphorolysis and synthesis of α,α-trehalose were carried out at 30 °C in 20 mM Mes buffer, pH 6.6. Results are means ± S.D.

Varied substrate	Constant substrate (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	K_{IS} (M)
α,α-Trehalose	Phosphate (50)	15 ± 1	91 ± 10	165	4.50 ± 2.20
Phosphate	α,α-Trehalose (300)	14 ± 1	2.4 ± 0.2	5833	0.61 ± 0.10
D-Glucose	α-D-Glc 1-*P* (25)	10 ± 1	39 ± 4	256	0.48 ± 0.05
α-D-Glc 1-*P*	D-Glucose (200)	10 ± 1	2.8 ± 0.2	3571	0.33 ± 0.08

enzyme had a specific activity of 9.8 units/mg and was recovered in an overall yield of 55%.

Anion-exchange chromatography on DEAE-Sepharose (step 1) gave an efficient initial purification of the crude cell extract and removed any remaining polysaccharide. Hydrophobic-interaction chromatography was required to remove contaminating phosphatase activity. Since ultrafiltration could not be used in the presence of 30% (v/v) glycerol as a means of concentrating the enzyme, an aqueous two-phase partition was employed. The major advantage of the $(NH_4)_2SO_4$/PEG 4000 system was that PEG 4000 stabilized the enzyme activity in a similar way as glycerol. As a final purification step, high-resolution gel filtration was employed. This procedure (see the Experimental section) allowed us to separate active trehalose phosphorylase with an apparent molecular mass of 61 kDa from several contaminant proteins (Figure 3).

Physicochemical properties

The results obtained by SDS/PAGE suggested that trehalose phosphorylase has a molecular mass of 61 kDa per protomer (see Figure 3). By using MALDI-MS, in which trehalose phosphorylase appeared as a single peak, a value of 61.081 kDa was obtained. The molecular mass of functional trehalose phosphorylase was determined by analytical gel filtration to be 60 kDa, indicating that the enzyme is a monomer. The isoelectric point of trehalose phosphorylase is 4.8.

Kinetics and thermodynamics of the reaction catalysed by trehalose phosphorylase

The apparent kinetic constants, determined at 30 °C in the presence of a saturating, constant concentration of the second substrate, were obtained from fits of eqn. (3) to the kinetic data, and are shown in Table 2. The k_{cat} values are similar in both directions of the reaction, as are the K_m values for α-D-Glc 1-*P* and phosphate, which reflect the apparent binding of the phosphoryl substrates to the enzyme. The K_m of trehalose is 2.3 times that of D-glucose, suggesting weaker apparent binding of the disaccharide compared with the monosaccharide. A comparison of the specificity constants (k_{cat}/K_m) in Table 2 reveals a large difference between reactants that contain the phosphoryl group (α-D-Glc 1-*P*, phosphate) and those that do not (D-glucose, trehalose). This could indicate that enzyme–phosphate interactions are important for binding and catalysis by trehalose phosphorylase. Sucrose phosphorylase, in contrast, displays similar catalytic efficiencies with sucrose, phosphate, D-fructose and α-D-Glc 1-*P* [23,24]. Notably, bacterial (inverting) phosphorylases show very similar k_{cat}/K_m values for phosphate, trehalose, β-D-Glc 1-*P* and D-glucose [12,13]. To determine whether the interaction of the enzyme with the substrate phosphoryl group is predominantly ionic or hydrogen-bonding in nature, the inhibition of trehalose phosphorylase by NaCl was studied; phosphate was the varied substrate and trehalose was kept constant at 400 mM. NaCl acted as a competitive inhibitor, with a K_i of 67 ± 4 mM, suggesting that ionic interactions are important for binding of phosphate to the enzyme.

The pH-dependencies for the forward and reverse reactions (eqn. 2) at apparent saturation with substrates are both symmetrical, decreasing above and below pH optima of 6.6 and 6.2 respectively. The temperature-dependence of the trehalose-phosphorylase-catalysed reaction follows Arrhenius' law, and the optimum temperature (determined for phosphorolysis) is 40 °C. The resulting activation energy is 24 ± 2 kJ/mol.

The equilibrium constant (K_{eq}) for the enzymic interconversion of trehalose + phosphate and α-D-Glc 1-*P* + D-glucose was determined at 30 °C in the pH range 5.5–7.0 as [trehalose][phosphate]/([α-D-Glc 1-*P*][D-glucose]). The formation and consumption of reactants by the action of trehalose phosphorylase matched exactly with the reaction shown in eqn. (2).

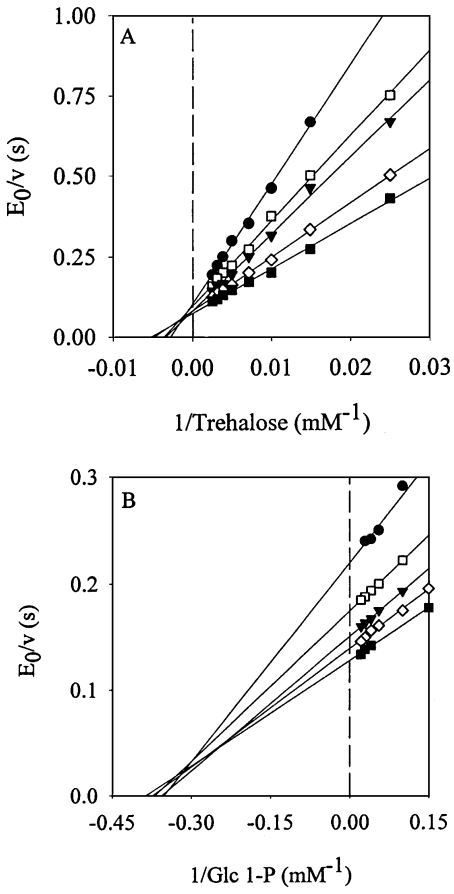

Figure 6 Evidence of a ternary-complex kinetic mechanism for trehalose phosphorylase

(**A**) Double-reciprocal plot from initial-velocity measurements with the concentration of α,α-trehalose varied and the concentration of phosphate held constant at 4.3 (●), 6.3 (□), 8.3 (▼), 12.5 (◇) and 18.8 (■) mM. (**B**) Double-reciprocal plot from initial-velocity measurements with the concentration of α-D-Glc 1-*P* varied and the concentration of D-glucose held constant at 30 (●), 50 (□), 75 (▼), 110 (◇) and 150 (■) mM. Reactions were carried out at 30 °C in 50 mM Mes, pH 6.6, containing 2 mM EDTA and 2 mM β-mercaptoethanol. v is the initial velocity (μM/s), and E_0 is the concentration of trehalose phosphorylase (μM).

K_{eq} increased with decreasing pH, as shown in Figure 5, which indicates that the reaction equilibrium for eqn. (2) is far on the educt side.

Ternary-complex kinetic mechanism of trehalose phosphorylase

Three pieces of evidence rule out a Ping-Pong kinetic mechanism for trehalose phosphorylase from *S. commune*. They suggest that, unusually for a retentive glucosyl transferase [25], this phosphorylase utilizes a ternary-complex kinetic mechanism in which both substrates must bind to the enzyme before a product is released.

Initial-velocity patterns

Both the initial velocities obtained with trehalose as the varied substrate when phosphate was constant, and initial velocities obtained with phosphate as the varied substrate when trehalose was constant, yielded straight converging lines that intersected to the left of the ordinate for the double-reciprocal plot of initial velocity against varied reactant concentration (Figure 6A). The secondary plots of the resulting slopes and intercepts against the reciprocals of the second fixed substrate or coenzyme concentration were linear (results not shown). In the direction of trehalose synthesis, both when α-D-Glc 1-*P* was varied and D-glucose was constant and when D-glucose was varied and α-D-Glc 1-*P* was constant, linear intersecting primary Lineweaver–Burk plots were obtained (Figure 6B), and the re-plots of slopes and intercepts against the reciprocal of the second fixed substrate concentration were linear (results not shown). These results indicate that the kinetic mechanism is sequential and not Ping-Pong, which would require that lines in double-reciprocal plots be parallel [19].

Absence of exchange reactions

A characteristic feature of the Ping-Pong kinetic mechanism is the occurrence of exchange reactions [19]. The arsenolysis of α-D-Glc 1-*P* (eqn. 4) is quite useful for the study of exchange reactions with phosphorylases, and takes place with sucrose phosphorylase, whose kinetics have been shown unequivocally to be Ping-Pong [23]. Since α-D-glucose 1-arsenate is an unstable intermediate, the net reaction observed on arsenolysis of α-D-Glc 1-*P* is the formation of D-glucose and phosphate:

$$\alpha\text{-D-Glc 1-}P + \text{arsenate} \leftrightarrow \alpha\text{-D-glucose 1-arsenate} + \text{phosphate} \rightarrow \alpha\text{-D-glucose} + \text{arsenate} + \text{phosphate} \quad (4)$$

Trehalose phosphorylase utilizes arsenate in trehalose phosphorolysis with a catalytic efficiency that is approx. 20 % of that observed with phosphate [15]. However, even with incubation times of 24 h in the presence of a high enzyme concentration (50–100 μg/ml), there was no detectable arsenolysis of α-D-Glc 1-*P* (40 mM) catalysed by trehalose phosphorylase in the presence of 10 mM arsenate (30 °C, 50 mM Mes, pH 6.6). In this experiment, the appearance of both phosphate and D-glucose with incubation time was measured. In addition, possible *de novo* production of trehalose from D-glucose and α-D-Glc 1-*P* (see eqn. 4) was monitored by TLC and high-performance anion-exchange chromatography [15]. No reaction was observed.

Absence of nucleophilic competition for reaction with a glucosyl-enzyme intermediate

The reaction catalysed by sucrose phosphorylase follows a retentive double-displacement mechanism that involves the formation of a glucosyl-enzyme intermediate, in which the configuration at C-1 of the glucosyl moiety is opposite to that in the substrates, sucrose or α-D-Glc 1-*P* [23]. The glucosyl intermediate can react with phosphate or D-fructose, or with other acceptors such as alcohols or water [23], to give products that have retained the anomeric configuration of the substrate. To determine whether trehalose phosphorylase catalyses glucosyl transfer to acceptors other than phosphate or arsenate, nucleophilic competition experiments (e.g. [23,25–27]) were carried out in the direction of trehalose phosphorolysis. Glycerol was used as alternate glucosyl acceptor. In the case when no glucosyl transfer occurs, the formation of D-glucose and α-D-Glc 1-*P* on trehalose phosphorolysis will be stoichiometric, and thus the velocity ratio, $V_{Glc1\text{-}P}/V_{Glc}$, will be 1.0 (where $V_{Glc1\text{-}P}$ and V_{Glc} are the velocities of the release of α-D-Glc 1-*P* and D-glucose respectively). When a glucosyl-phosphorylase intermediate is trapped with high concentrations of glycerol, $V_{Glc1\text{-}P}/V_{Glc}$ is expected to decrease. On using glycerol at concentrations between 10 and 50 % (w/v), we found no change in the $V_{Glc1\text{-}P}/V_{Glc}$ ratio, which was equal to unity within experimental error. However, concentrations of

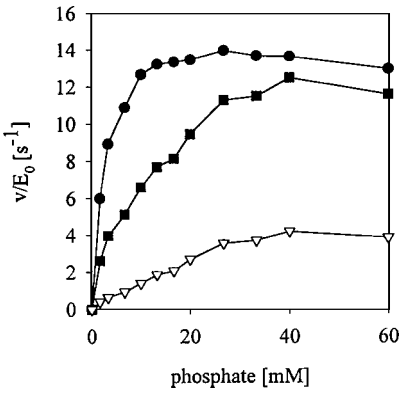

Figure 7 Glycerol inhibits trehalose phosphorylase

The effect of increasing concentrations of glycerol was determined by assaying trehalose phosphorylase activity with phosphate as the varied substrate. Trehalose was held constant at 400 mM. Symbols: ●, no glycerol; ■, 20% (w/v) glycerol; ▽, 50% (w/v) glycerol. v is the initial velocity (μM/s), and E_0 is the concentration of trehalose phosphorylase (μM).

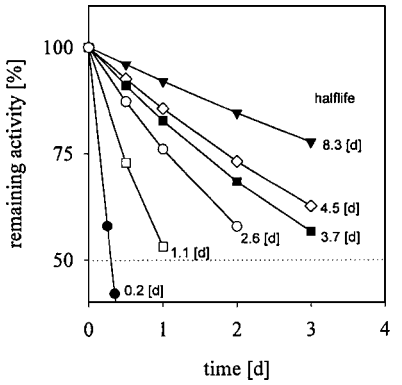

Figure 8 Stability and stabilization of trehalose phosphorylase

The enzyme (75 μg/ml) was incubated at 30 °C in 50 mM Mes buffer, pH 6.6, in the absence or presence of added stabilizers. ●, None added; □, 20% (w/v) glycerol; ○, 200 mM trehalose; ■, 400 mM trehalose; ◇, 20% (w/v) PEG 4000; ▼, 50% (w/v) glycerol. The half-lives of trehalose phosphorylase (days; d) under the respective conditions of incubation were calculated with the assumption of apparent first-order decay of activity.

glycerol greater than 30% (w/v) reversibly inhibited enzyme activity, and at 50% (w/v) glycerol the residual activity in phosphorolysis, at saturation with substrate, was reduced to less than 20% of the original activity (Figure 7). In contrast with glycerol, PEG 4000 (up to 40%, w/v) did not inhibit enzyme activity.

Spectral characterization

Pyridoxal 5′-phosphate is an essential cofactor in α-1,4-D-glucan phosphorylases [28], but has so far not been found in disaccharide phosphorylases [24]. Trehalose phosphorylase is no exception to this rule. No evidence of pyridoxal 5′-phosphate was obtained, on using either a specific assay for the cofactor [20] or spectrophotometric and spectrofluorimetric analysis of the protein. The absorption spectrum of trehalose phosphorylase (results not shown) indicates the absence of other protein-bound chromophores. A molar absorption coefficient of the enzyme at 280 nm of 72600 $M^{-1} \cdot cm^{-1}$ was calculated from this spectrum. The maximum fluorescence emission on excitation at 295 nm was at 340 nm, which is indicative of tryptophan residues located in a rather polar, probably solvent-exposed, environment [29].

Stability and regulation of enzyme activity

Isolated trehalose phosphorylase is a rather unstable protein, which loses activity within a few hours when stored or incubated at 4 °C or 20 °C in diluted buffered solution. On addition of glycerol (20–50%, w/v), PEG 4000 (20%, w/v) or trehalose (0.1–0.4 M) to the buffer, marked stabilization is observed (Figure 8). Note that the optimum concentration of trehalose for stabilizing the enzyme activity, i.e. approx. 0.3 M, corresponds roughly to the concentration of trehalose found in *S. commune*. Interestingly, PEG compounds with a molecular mass greater than 4000 Da were less efficient than PEG 4000 in the stabilization of trehalose phosphorylase. In contrast with trehalose phosphorylases from *C. ferruginea* [12] and *E. gracilis* [4], whose thermostabilities are critically dependent on phosphate ions, the fungal enzyme is not stabilized against thermal denaturation by phosphate, sulphate or other ions at concentrations up to 0.2 M.

Trehalase from zygomycetes and yeasts of the genera *Pichia* and *Saccharomyces* are regulated by cAMP-dependent covalent phosphorylation [2]. We investigated whether the activity of trehalose phosphorylase is under the control of phosphorylation/dephosphorylation processes, but found no evidence in support of this hypothesis. Incubation with a non-specific protein phosphatase did not lead to the release of phosphate from the enzyme, and had no effect whatsoever on enzyme activity. In addition, treatment of trehalose phosphorylase with a non-specific protein kinase in the presence of cAMP and ATP did not affect enzyme activity.

A number of metabolic intermediates were tested for their ability to activate or inhibit trehalose phosphorylase activity in the direction of phosphorolysis. None of ATP, AMP, D-glucose 6-phosphate, α-D-glucose 1,6-bisphosphate and D-fructose 2,6-bisphosphate had any effect when used in the concentration range 10–50 μM. Therefore the enzyme seems not to show control of activity by allosteric effectors or feedback inhibition.

Physiological role of trehalose phosphorylase

The synthesis of trehalose is the thermodynamically favoured direction of the reaction catalysed by fungal trehalose phosphorylase. However, the prevailing P_i/α-D-Glc 1-*P* ratio in *S. commune* was shown to vary between upper and lower limits of 78 and 6 respectively. Accordingly, P_i is present in excess at all times, and, taking into account the large amount of stockpiled trehalose, phosphorolysis is clearly the strongly preferred direction of enzymic action *in vivo*. This conclusion is corroborated by the observed changes in the intracellular levels of trehalose, phosphate and α-D-Glc 1-*P* in an apparently direct response to a change in the specific activity of trehalose phosphorylase. Furthermore, the enzymic synthesis of trehalose is strongly inhibited by P_i *in vitro*, with inhibition being competitive against α-D-Glc 1-*P* with a K_i of approx. 2 mM (C. Eis and B. Nidetzky, unpublished work). This value is similar to that found for trehalose phosphorylase from *A. bisporus* [9]. Therefore this implies that trehalose phosphorylase would make an extremely poor 'trehalose synthase' under physiological reaction conditions, even at the minimal P_i/α-D-Glc 1-*P* ratio of 6.

Trehalose phosphorylase apparently does not operate close to 'at equilibrium' *in vivo*. This means that the observed steady-state concentrations of trehalose and P_i, and of α-D-Glc 1-*P* and D-glucose, do not correspond to the thermodynamic K_{eq}. By

assuming a value of 10 for K_{eq} and using the values for the intracellular metabolite levels at 72 h in Figure 2, the expression

$$\{[\alpha\text{-D-Glc 1-}P][\text{D-glucose}]/([\text{trehalose}][\text{phosphate}])\}K_{eq}$$

[30] gives a value of approx. 0.02. Note that, at equilibrium, this expression would give a value of 1.0. Therefore trehalose phosphorylase clearly fails to equilibrate its substrates and products, obviously because the enzyme catalyses the phosphorolysis of trehalose more slowly than downstream enzymes consume the resulting products via anabolic or catabolic reactions. Furthermore, the analysis for trehalose phosphorylase implies that, because of the rapid removal of α-D-Glc 1-*P* and D-glucose, there will always be a net flow of carbon metabolites through trehalose phosphorolysis (see above). Overall, the phosphorolysis of trehalose would seem to be an energetically favourable way of utilizing the disaccharide for catabolism, because much of the stored energy is conserved in the sugar/phosphate linkage of α-D-Glc 1-*P*.

However, the exact physiological role of trehalose phosphorylase remains puzzling, and the conversion of stockpiled disaccharide into energy for growth or maintenance energy is probably not the the primary function of this enzyme. For example, the production of D-glucose on trehalose phosphorolysis, when D-glucose is abundant in the medium anyway (cf. Figure 1, lower panel, and Figure 2), is completely contrary to what one would expect of trehalose phosphorylase if the function of the enzyme was the mobilization of reserve carbohydrate.

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REFERENCES

1 Elbein, A. D. (1974) Adv. Carbohydr. Chem. Biochem. **30**, 227–257
2 Thevelein, J. M. (1984) Mirobiol. Rev. **48**, 42–59
3 Belocopitow, E. and Marechal, L. R. (1970) Biochim. Biophys. Acta **198**, 151–154
4 Marechal, L. R. and Belocopitow, E. (1972) J. Biol. Chem. **247**, 3223–3228
5 Belocopitow, E. and Marechal, L. R. (1974) Eur. J. Biochem. **46**, 631–637
6 Kitamoto, Y., Akashi, H., Tanaka, H. and Mori, N. (1988) FEMS Microbiol. Lett. **55**, 147–150
7 Salminen, S. O. and Streeter, J. G. (1986) Plant Physiol. **81**, 538–541
8 Schick, I., Haltrich, D. and Kulbe, K. D. (1995) Appl. Microbiol. Biotechnol. **43**, 1088–1095
9 Wannet, W. J. B., Op den Camp, H. J. M., Wisselink, H. W., van der Drift, C., Van Griensven, L. J. L. D. and Vogels, G. D. (1998) Biochim. Biophys. Acta **1425**, 177–188
10 Aisaka, K. and Masuda, T. (1995) FEMS Microbiol. Lett. **131**, 47–51
11 Kizawa, H., Miyazaki, Y., Yokota, A., Kanegae, Y., Miyagawa, K. and Sugiyama, Y. (1995) Biosci. Biotechnol. Biochem. **59**, 1522–1527
12 Aisaka, K., Masuda, T., Chikamune, T. and Kamitori, K. (1998) Biosci. Biotechnol. Biochem. **62**, 782–787
13 Kizawa, H., Miyagawa, K. and Sugiyama, Y. (1995) Biosci. Biotechnol. Biochem. **59**, 1908–1912
14 Saito, K., Yamazaki, H., Ohnishi, Y., Fujimoto, S., Takahashi, E. and Horninouchi, S. (1998) Appl. Microbiol. Biotechnol. **50**, 193–198
15 Eis, C., Albert, M., Dax, K. and Nidetzky, B. (1998) FEBS Lett. **440**, 440–443
16 Rau, U. and Brandt, C. (1994) Bioprocess Eng. **11**, 161–165
17 Bergmeyer, H. U. (ed.) (1988) Methods in Enzymatic Analysis, 3rd edn., vols. 3 and 4, VCH, Weinheim
18 Saheki, S., Takeda, A. and Shimazu, T. (1985) Anal. Biochem. **148**, 277–281
19 Cleland, W. W. (1971) Adv. Enzymol. **45**, 273–387
20 Wada, H. and Snell, E. E. (1961) J. Biol. Chem. **236**, 2089–2095
21 Wang, Y. and Roach, P. J. (1993) in Protein Phosphorylation: A Practical Approach (Hardie, D. G., ed.), pp. 121–144, IRL Press, Oxford
22 MacIntosh, C. (1993) in Protein Phosphorylation: A Practical Approach (Hardie, D. G., ed.), pp. 197–230, IRL Press, Oxford
23 Mieyal, J. J. and Abeles, R. H. (1972) in The Enzymes (Boyer, P. D., ed.), pp. 515–532, Academic Press, New York
24 Vandamme, E. J., van Loo, J., Machtelinckx, L. and de Laporte, A. (1987) Adv. Appl. Microbiol. **32**, 163–201
25 Sinnott, M. L. (1990) Chem. Rev. **90**, 1171–1202
26 Viratelle, O. M. and Yon, J. M. (1973) Eur. J. Biochem. **33**, 110–116
27 Richard, J. P., Westerfeld, J. G., Lin, S. and Beard, J. (1995) Biochemistry **34**, 11713–11724
28 Palm, D., Klein, H. W., Schinzel, R., Buehner, M. and Helmreich, E. J. M. (1990) Biochemistry **29**, 1099–1107
29 Schmid, F. X. (1990) in Protein Structure: A Practical Approach (Creighton, T. E., ed.), pp. 251–285, IRL Press, Oxford
30 Burbaum, J. J., Raines, R. T., Albery, W. J. and Knowles, J. R. (1989) Biochemistry **28**, 9293–9305

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