Trehalose-6-phosphate synthase/phosphatase complex from bakers' yeast: purification of a proteolytically activated form

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A protein of about 800 kDa with trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) activity was purified from bakers' yeast. This TPS/P complex contained 57, 86 and 93 kDa polypeptides. The 86 and 93 kDa polypeptides both appeared to be derived from a polypeptide of at least 115 kDa in the native enzyme. A TPS-activator (a dimer of 58 kDa subunits) was also purified. It decreased the Michaelis constants for both UDP-glucose (three-fold) and glucose 6-phosphate (G6P) (4.5-fold), and increased TPS activity at 5 mM-UDP-glucose/10 mM-G6P about three-fold. It did not affect TPP activity. The purification of TPS/P included an endogenous proteolytic step that increased TPS activity about three-fold and abolished its requirement for TPS-activator, but did not change TPP activity. This activation was accompanied by a decrease of some 20 kDa in the molecular mass of a cluster of SDS-PAGE bands at about 115 kDa recognized by antiserum to pure TPS/P, but by no change in the 57 kDa band. Phosphate inhibited TPS activity (K_i about 5 mM), but increased TPP activity about six-fold (K_a about 4 mM). Phosphate (6 mM) stimulated the synthesis of trehalose from G6P and UDP-glucose and decreased the accumulation of trehalose 6-phosphate.

Introduction

Large amounts of trehalose (α -D-glucopyranosyl- α -D-glucopyranose) occur in resting cells of bakers' yeast. Lillie & Pringle (1980) showed that during growth on glucose, accumulation of trehalose began distinctly later than that of glycogen, and that during subsequent starvation, trehalose consumption began only after depletion of the glycogen. Later work has established a strong correlation between the trehalose content of cells of *Saccharomyces cerevisiae* and their resistance to various stresses, such as temperature extremes and dehydration (Hottiger *et al.*, 1987a, b; Attfield, 1987; Gadd *et al.*, 1987). This correlation holds both for commercial bakers' yeast strains (Oda *et al.*, 1986) and for mutants with defective control of trehalose metabolism (Tenan *et al.*, 1985; Hottiger *et al.*, 1989). It appears that the primary function of trehalose in yeast is not as a C-reserve but as a protectant of cell membranes and proteins under conditions that deplete the activity of intracellular water, a role for which this non-reducing sugar is well suited (Crowe *et al.*, 1987).

Trehalose is degraded in yeast by a trehalase that is activated by phosphorylation (reviewed by Thevelein, 1988). Cabib & Leloir (1958) showed that trehalose is synthesized from uridine diphosphate glucose (UDPG) and glucose 6-phosphate (G6P) via trehalose 6-phosphate (T6P). Since then, rather few publications have dealt with the properties of the enzymes responsible, T6P synthase (TPS) and T6P phosphatase (TPP). In partial purifications, the two enzymes have co-purified (Cabib & Leloir, 1958; Vandercammen *et al.*, 1989). Inhibition of TPS by physiological concentrations of inorganic phosphate has been noted (Elander, 1968; Vandercammen *et al.*, 1989). Panek's group has claimed that some strains of *S. cerevisiae* contain distinct UDPG- and ADPG-linked TPS enzymes (Paschoalin *et al.*, 1989), and that the UDPG-linked enzyme is reversibly inactivated by phosphorylation (Panek *et al.*, 1987). The latter claim has been contested by Vandercammen *et al.* (1989), who could find no effect of exposure to phosphorylating conditions either *in vivo* or *in vitro* when a reliable TPS assay was used.

This paper reports the purification of a proteolytically modified complex containing both TPS and TPP activities. The purification of a protein that activates the TPS of this complex is also reported. Some catalytic properties of the complex, including its unexpected response to inorganic phosphate, are described.

Abbreviations: G6P, glucose 6-phosphate; T6P, trehalose 6-phosphate; TPP, trehalose-6-phosphate phosphatase; TPS, trehalose-6-phosphate synthase; TPS/P, trehalose-6-phosphate synthase/phosphatase complex; UDPG, uridine diphosphate glucose.

Methods

Materials. Anion exchange resin [AG1-X8 (formate: 200 to 400 mesh)] and hydroxyapatite (Biogel HT) were from BioRad, DEAE-cellulose (DE52) from Whatman, UDP-glucuronate-agarose from Sigma, and other chromatographic materials from Pharmacia. [^{14}C]Glucose 6-phosphate was from Amersham. Phenylmethylsulphonyl fluoride (PMSF), pepstatin A, glucose 6-phosphate, nucleotides, marker proteins, lactate dehydrogenase (pig heart), pyruvate kinase (rabbit muscle) and glucose-6-phosphate dehydrogenase (G6PDH) were from Sigma or Boehringer. Anti-rabbit IgG-alkaline phosphatase conjugate was from Promega.

Purification of TPS/P. Bakers' yeast from our Rajamäki factory was disintegrated by suspending 20 g lots in 20 ml 25 mM-HEPES/KOH (pH 7.0) containing 1 mM-benzamidine, 1 mM-MgCl₂, 0.1 mM-EDTA and 1 mM-dithiothreitol (HBMED), adding 0.4 ml methanol containing 0.1 M-PMSF and 1 mg pepstatin A ml⁻¹, and shaking with 50 ml 0.5 mm diameter glass beads in a Braun MK II homogenizer for two 1 min periods.

The homogenate (0.5 g yeast ml⁻¹) was centrifuged for 20 min at 28000 g. More PMSF (0.5 mM) and pepstatin A (5 µg ml⁻¹) were added to the supernatant. Then 10 ml protamine sulphate [15 mg (ml HBMED)⁻¹] was added to each 60 ml of supernatant. After 20 min the precipitate was discarded (20 min at 12000 g).

To every 50 ml of supernatant was slowly added 16.5 g powdered (NH₄)₂SO₄. After 40 min, precipitated proteins were collected (10 min at 28000 g) and dissolved in HBMED. PMSF (0.7 mM) and pepstatin A (7 µg ml⁻¹) were added, insoluble material was removed (5 min at 28000 g) and the solution was stored at -70 °C.

Material from 50 g yeast was thawed. PMSF (0.3 mM) and pepstatin A (3 µg ml⁻¹) were added and the solution centrifuged for 10 min at 8000 g. The supernatant (22 ml) was passed in two equal portions through a column (2.5 × 17 cm) of Sephadex G25 equilibrated with HBMED/0.1 M-KCl and then applied at 13 ml h⁻¹ to a column (2.5 × 13 cm) of Heparin-Sepharose equilibrated with HBMED/0.1 M-KCl. The column was washed with 200 ml HBMED/0.1 M-KCl and developed with a linear gradient of 0.1–1.5 M-KCl in 500 ml HBMED at 24 ml h⁻¹ (see Fig. 2).

TPS active fractions were kept at about 3 °C for 5 d without PMSF or pepstatin A. Their activity increased two- to three-fold. To the pooled fractions (15 ml) were added 0.15 ml 0.1 M-EDTA and 6 g (NH₄)₂SO₄. After 40 min the precipitate was collected (15 min at 8000 g), dried (5 min at 17000 g) and dissolved in 2 ml HBMED. PMSF (0.5 mM) and pepstatin A (5 µg ml⁻¹) were added and insoluble material was removed (10 min at 17000 g).

The supernatant was passed at 20 ml h⁻¹ through a column (2.8 × 32 cm) of Sepharose 6B equilibrated with HBMED. Fractions from the leading half of the enzyme peak were pooled and run at 9 ml h⁻¹ into a column (0.7 × 7.3 cm) of UDP-glucuronate-agarose equilibrated with HBMED. The column was washed with 10 ml HBMED. Bound proteins were eluted at 4.5 ml h⁻¹ with about 20 ml HBMED/0.2 M-NaCl (until the A₂₈₀ of the eluate indicated no more protein was being eluted) and then with HBMED/0.4 M-NaCl/10 mM-UDPG.

Purification of TPS activator. The Heparin-Sepharose fractions containing TPS-activator were pooled (see Fig. 2) and centrifuged for 10 min at 12000 g. To 20 ml of the supernatant was added 0.2 ml 0.1 M-EDTA and 7.0 g (NH₄)₂SO₄. The precipitate was discarded. A further 2.3 g (NH₄)₂SO₄ was added. The precipitate was collected (10 min at 28000 g) and dissolved in 2.0 ml HBMED. PMSF (1 mM) and pepstatin A (10 µg ml⁻¹) were added.

The solution was run at 37 ml h⁻¹ through a column (2.6 × 34 cm) of Sephadex G 100 equilibrated with 25 mM-HEPES/KOH (pH

7.0)/1 mM-MgCl₂/0.1 mM-EDTA (HME). TPS-activator emerged between the elution volumes of γ-globulin (55 ml) and bovine albumin (72 ml).

The most active fractions (57 to 72 ml) were applied to a column (0.7 × 4.7 cm) of DE52-cellulose equilibrated with HME. The column was developed with a linear gradient of 0.0 to 0.5 M-NaCl in 100 ml HME at 7 ml h⁻¹ and 2.2 ml fractions were collected. The activator eluted sharply at about 50 mM-NaCl.

The purest fraction (2.2 ml) was applied to a column (0.7 × 4.5 cm) hydroxyapatite equilibrated with HME. The column was developed at 2 ml h⁻¹ with a linear gradient of 0 to 10% (w/v) (NH₄)₂SO₄ in 60 ml HME. The activator emerged between 3 and 4% (w/v) (NH₄)₂SO₄.

Enzyme assays. Enzymes were assayed at 30 °C. The amount of enzyme producing 1 µmol product min⁻¹ in the respective standard assay is 1 U. Samples were diluted, when necessary, with 50 mM-HEPES/KOH (pH 7.0)/0.1 mM-EDTA/5 mg bovine albumin ml⁻¹, because the TPS activity was unstable at low protein concentrations.

TPS was assayed by a modification of the method of Cabib & Leloir (1958). Standard assay mixtures (100 µl final volume) contained 40 mM-HEPES/KOH (pH 6.8), 10 mM-MgCl₂, 10 mM-G6P, 5 mM-UDPG and 1 mg bovine albumin ml⁻¹. Reactions were started with enzyme and stopped after appropriate times (a zero and two times between 1 and 10 min) by placing the tubes in boiling water for 2 min. UDP in the cooled reaction mixtures was determined by adding 0.9 ml 40 mM-HEPES/KOH (pH 6.8) containing 10 mM-MgCl₂, 2.5 mM-phosphoenolpyruvate and 0.24 mM-NADH, centrifuging to remove precipitated protein, and measuring spectrophotometrically at 340 nm the disappearance of NADH on addition of pyruvate kinase and lactate dehydrogenase. When substrate concentrations were varied in TPS assays, rates at each concentration were measured over two different time intervals. They generally agreed within 10%, and the mean was used. However, when a significant amount of substrate was consumed (≥15% at initial concentrations below K_m), the rate from each time interval was handled separately and plotted against the average substrate concentration in the time interval, as recommended by Glick *et al.* (1979).

TPP was assayed by measuring the production of [^{14}C]trehalose from [^{14}C]trehalose 6-phosphate ([^{14}C]T6P). Standard assay mixtures (110 µl final volume) contained 25 mM-potassium phosphate (pH 6.8), 5 mM-MgCl₂, 0.5 mM-[^{14}C]T6P (924 c.p.m. nmol⁻¹) and 1 mg bovine albumin ml⁻¹. Reactions were started with enzyme and stopped after appropriate intervals (a zero and two times between 1 min and 20 min) by immersion in boiling water. AG1-X8 (formate) anion-exchange resin (1.0 ml of a slurry made by mixing 300 g resin with 730 ml H₂O to give 1 litre; 1.0 ml of slurry contained 0.86 ml of liquid because the moisture content of the slurry was 44%) was added to each tube. After 20 min equilibration, the tubes were centrifuged. The radioactivity of supernatant samples (400 µl) was measured by liquid scintillation counting. With fresh reaction mixtures less than 2% of the total radioactivity remained in the supernatant when enzyme was omitted (much higher blanks were obtained with the chloride form of the resin). When reactions were allowed to go to completion, 100 ± 5% of the total radioactivity was recovered in the supernatant.

The overall reaction from UDPG and G6P to trehalose was measured as follows. Reaction mixtures (1 ml) contained 50 mM-HEPES/KOH (pH 6.8), 5 mM-MgCl₂, 5 mM-UDPG, 4.25 mM-[^{14}C]G6P (870 c.p.m. nmol⁻¹), 1 mg bovine albumin ml⁻¹ and zero or 6 mM-potassium phosphate (pH 6.8). UDP and [^{14}C]trehalose were determined in duplicate 100 µl samples as described for the TPS and TPP assays. T6P was calculated as the difference between the UDP and trehalose. Because the T6P formed binds less tightly to the AG1-X8 resin than does G6P, the blank increases during the reaction. Apparent trehalose concentrations were corrected accordingly (this negative correction decreased with time from ≤14% at 5 min to ≤6% at 20 min).

Table 1. Purification of TPS/P complex

The data are from the particular preparation described in Methods. Several other preparations have been performed with similar results. Protein was determined according to Warburg & Christian (1941) (W) or by assuming an $A_{280}^{1\%}$ of 10. Values in parentheses for the TPS/TPP ratio were determined in another preparation. ND, Not determined.

Purification Step	Volume (ml)	Protein (mg ml ⁻¹)	TPS		TPP (U)	TPP/TPS (%)
			(U mg ⁻¹)	(U)		
Homogenate	106	ND	ND	780	ND	(34)
After protamine sulphate	79	20 (W)	0.38	590	ND	(36)
1st (NH ₄) ₂ SO ₄ precipitate	19	52 (W)	0.60	570	ND	ND
Sephadex G25 eluate	42	30	0.31	394	122	31
Heparin-Sepharose eluate:						
Fresh	17	4	0.80	54	22	41
5 d old	17	4	2.4	159	22	14
2nd (NH ₄) ₂ SO ₄ precipitate	1.4	41 (W)	1.9	106	ND	ND
Sepharose 6B eluate	20	0.11	15	33*	ND	ND
UDP-glucuronate-agarose eluate:						
0.2 M-NaCl	7.5	0.09	1.1	0.8	ND	ND
0.4 M-NaCl/10 mM-UDPG	7.2	ND	ND†	13	2.6	20

* A further 36 U (at 3.8 U mg⁻¹) was discarded.

† Coomassie blue staining indicated about 25 U mg⁻¹.

Preparation of [¹⁴C]T6P. [¹⁴C]T6P was made by a modification of the method of Vandercammen *et al.* (1989) by using TPS/P in the presence of excess EDTA, which inhibits the TPP activity but not the TPS. [U-¹⁴C]G6P (assayed with G6PDH: 87.0 µmol; 924 c.p.m. nmol⁻¹) and UDPG (100 µmol) were incubated for 4 h at 30 °C with 0.7 U pure TPS/P in 40 mM-HEPES/KOH (pH 7.0)/0.9 mM-EDTA/0.9 mg bovine albumin ml⁻¹. The reaction mixture was then heated at 100 °C for 5 min, cooled, mixed with 2 vols ethanol, and centrifuged. The supernatant was dried at 60 °C under a stream of N₂. The residue was dissolved in 4 ml 0.1 M-NaOH, heated at 80 °C for 15 min, adjusted to pH 5.7 with 1 M-H₃PO₄ and applied to a column (2.5 × 17 cm) of AG1-X8 (formate form) anion exchanger in H₂O. The column was developed at 50 ml h⁻¹ with 0.1 M-NH₄H₂PO₄ (pH 4.5). [¹⁴C]T6P (43 µmol) was eluted between 460 and 560 ml, before the G6P peak at 600 ml. The [¹⁴C]T6P fractions were lyophilized. The residues were maintained under vacuum for 24 h to remove ammonium formate (which inhibits TPP), dissolved in H₂O to about 20 mM and stored at -18 °C.

Electrophoresis. SDS-PAGE was performed essentially as described by Laemmli (1970). For Western blotting, samples run on 70 × 100 × 0.75 mm gels were blotted onto nitrocellulose, probed with antiserum as described in Fig. 5 and detected with goat anti-rabbit IgG-alkaline phosphatase conjugate (1/7500) from Promega according to the manufacturer's instructions.

Preparation of antisera. Antisera were prepared for us at the National Public Health Institute (Helsinki). Rabbits were injected with 0.2 ml of a 1:1 mixture of Freund's complete adjuvant and antigen (about 20 µg protein) into both popliteal lymph nodes. Booster injections were given after 2 and 6 weeks, and the animals bled out 1 week after the last injection. The antigens used were pure TPS/P (the preparation shown in Fig. 1), and 93 and 57 kDa polypeptides isolated by SDS-PAGE, located by immersing the gel in cold 0.5 M-KCl (Hager & Burgess, 1980), cut out and eluted.

Peptide mapping. The 57, 86, and 93 kDa polypeptides were separated by SDS-PAGE in an 8% (w/v) T acrylamide gel (aged for 48 h), blotted overnight and digested on the nitrocellulose with trypsin

essentially as described by Aebersold *et al.* (1987). The peptides were separated by reverse-phase chromatography at 1 ml min⁻¹ on a Vydac 218 TP B5 column (0.46 × 25 cm; The Sep/ara/tions Group) with a gradient from 0.1% trifluoroacetic acid to 60% (v/v) acetonitrile/0.04% trifluoroacetic acid in 90 min. The eluate was monitored at 218 nm.

Results

Purification of TPS/S complex

A typical purification is summarized in Table 1. SDS-PAGE analysis of purified TPS/P is shown in Fig. 1. The ratio of TPP and TPS activities was between 14 and 41% throughout the purification, suggesting the final product is a complex with both activities. The overall yield of TPS was low (1.7% in Table 1; 4% in our best preparation). Much TPS activity was lost during chromatography on Heparin-Sepharose because of resolution of an activator of TPS from TPS/P (see below and Fig. 2). However, the TPS activity of Heparin-Sepharose eluates increased by 2.5 to 4-fold during storage (see below). TPS/P was eluted from Sepharose 6B before the elution volume of ferritin, with an apparent molecular mass of about 800 kDa (not shown), and the more contaminated tailing half of the enzyme peak was discarded. Often (but not in the example shown in Table 1) 20 to 50% of the enzyme eluted from the UDP-glucuronate-agarose column in the 0.2 M-NaCl wash.

The three major polypeptides in the purified enzyme had molecular masses of 57 kDa, about 86 kDa and 93 kDa (Fig. 1). The 86 kDa band was often a doublet.

Fig. 1. SDS-PAGE of purified TPS/P and TPS-activator. Gels were stained with Coomassie blue. (a) Lanes 1, 2 and 3 contained 25, 106 and 24 mU of TPS, respectively, from successive fractions eluted from UDP-glucuronate-agarose with 0.4 M-NaCl/10 mM-UDPG. The positions of molecular mass standards are shown on the right. (b) Lane 4, standard proteins; lanes 5 and 6, 1.3 and 1.9 μ g (assuming an $A_{280}^{1\%}$ of 10) of TPS-activator from successive fractions eluted from hydroxyapatite.

Peptide maps of tryptic digests of blots of the 86 and 93 kDa polypeptides were essentially identical (not shown). This suggests that the 86 kDa polypeptide is a degradation product of the 93 kDa polypeptide. In contrast, the peptide map of the 57 kDa polypeptide was clearly distinct (not shown), and the 57 kDa polypeptide did not cross-react with antiserum against the 93 kDa polypeptide (see Fig. 5). When the bands were cut out of stained (but undried) gels and Coomassie blue eluted with 4% (w/v) SDS and determined at 560 nm, relative colour yields of 1.00 (57 kDa), 0.34 ± 0.20 (86 kDa) and 0.79 ± 0.07 (93 kDa) were found in four analyses of three different samples. Gels stained with Fast Green gave similar results. If the bands have equivalent affinities for the dyes, then these data suggest a molar ratio of about 1.4 for the 57 kDa polypeptide to the sum of the 93 and 86 kDa polypeptides.

TPS activator

Fig. 2 shows the chromatographic resolution of an activator of TPS from the TPS/P enzyme. The inset shows that TPP activity was unaffected by amounts of this TPS-activator that had a nearly saturating effect on the TPS activity. The maximum activation in Fig. 2 was 2.5-fold. Activations of up to four-fold have been obtained with some TPS/P samples. The inset of Fig. 2 shows that 3 μ l of the fraction with most TPS-activator caused a 90% of maximal activation of 7.1 mU of TPS.

Fig. 2. Separation of TPS-activator from the enzyme. Partially purified TPS/P (394 U of TPS) was chromatographed on Heparin-Sepharose as described in Methods. The A_{280} (---), KCl gradient (—), TPS activity ($\circ$) and TPS-activator ($\square$) are shown. The bar marked A shows the fractions pooled for purification of TPS-activator. TPS-activator was assayed by measuring the TPS activity of 1.7 μ l (7.1 mU) of the fraction at 360 ml in the presence and absence of 5 μ l of fractions collected between 20 and 120 ml. The inset shows the effect of various amounts of the TPS-activator-containing fraction eluted at 65 ml on the TPS ($\circ$) and TPP ($\bullet$) activities of 1.7 μ l of the fraction eluted at 360 ml. Variations in the size of the response of TPS to TPS-activator (from 2- to 4-fold) are described in the text.

Fig. 3. Effect of TPS-activator on the Michaelis constants of 'native' TPS/P. TPS/P eluted from Heparin-Sepharose and stored at -70°C in the presence of PMSF was used at $53\text{ mU (ml reaction mixture)}^{-1}$ in the presence (●) and absence (O) of $2.5\text{ }\mu\text{g ml}^{-1}$ of pure TPS-activator. Rates [v : $\mu\text{mol min}^{-1} (\text{ml enzyme})^{-1}$] were measured in standard assay mixtures but with variable substrate concentrations (S) of (a) 1.6 to 41 mM-G6P at 5 mM-UDPG and (b) 0.18 to 12.5 mM-UDPG at 25 mM-G6P. Also shown (with v in arbitrary units) are results (Δ) with pure TPS/P in the absence of TPS-activator. Assays were run for at least two time intervals at each initial substrate concentration and handled as described in Methods.

Based on this result, the total amount of TPS-activator recovered from the Heparin-Sepharose column was nearly enough to maximally activate all the TPS (68 U) recovered from the column. In agreement with this, TPS activity in crude yeast extracts and the material applied to Heparin-Sepharose was increased by less than 7% by addition of partially purified TPS-activator.

TPS-activator was completely destroyed by 10 min incubation at 70°C , but maintained about 50% activity through 10 min at 55°C . Gel-filtration indicated a native molecular mass of about 110 kDa (see Methods). SDS-PAGE analysis of pure TPS-activator showed a single Coomassie blue band, with a molecular mass of 58 kDa (Fig. 1). These results suggest that TPS-activator is a dimeric protein. TPS-activator migrated more slowly than the 57 kDa polypeptide of TPS/P during co-electrophoresis on SDS-PAGE, and was not recognized by antiserum prepared against pure TPS/P (not shown).

Pure TPS-activator activated enzyme eluted from Heparin-Sepharose with a saturation curve similar in form to that in the inset of Fig. 2, and 500 ng of activator

Fig. 4. Spontaneous activation of Heparin-Sepharose eluates. The TPS (open symbols) and TPP (filled symbols) activities were measured in the absence of TPS-activator during storage at 3°C of enzyme eluted from Heparin-Sepharose. The samples contained no protease inhibitor (O, ●), pepstatin A (□, ■), PMSF (Δ , ▲) or both pepstatin A and PMSF (∇ , ▼). Also shown are the TPS activities measured in the presence of a saturating amount of TPS-activator for a sample stored for 0 and 7 d without protease inhibitors (x) and for a sample stored for 7 d with both pepstatin A and PMSF (+). Two independent experiments are shown. In one, the original TPS and TPP activities were 1.86 and 0.77 U ml^{-1} , respectively, and assays were made at 0, 2 and 5 d. In the other, the original TPS was 2.57 U ml^{-1} and assays were made at 0 and 7 d.

was enough to completely activate (2.45-fold) 18 mU of the enzyme (not shown). This amount of 'native' enzyme is equivalent to about 50 mU or 2000 ng of purified TPS/P. These results are consistent with the notion that TPS-activator binds stoichiometrically to TPS/P, but are not accurate enough to define the molar ratio.

TPS-activator decreased the apparent Michaelis constants of 'native' enzyme (from 15 to 3.3 mM for G6P and from 1.0 to 0.34 mM for UDPG) towards the values (2.5 mM G6P and 0.16 mM UDPG) observed for pure TPS/S in the absence of TPS-activator (Fig. 3).

Proteolytic activation of TPS after Heparin-Sepharose chromatography

When enzyme eluted from Heparin-Sepharose was stored at 0 to 7°C for a few days, the TPS activity increased 2.5 to 4-fold (there was some variation between fractions and different preparations) but the TPP activity remained nearly constant (Table 1, Fig. 4). This spontaneous activation was not prevented by pepstatin A ($10\text{ }\mu\text{g ml}^{-1}$) but was prevented by the serine protease inhibitor PMSF (1 mM): with PMSF the activity decreased by about 40% (Fig. 4). The spontaneous

Fig. 5. Molecular mass changes during proteolytic activation. In (a) pure TPS/P (4.4 mU per lane) was electrophoresed and blotted as described in Methods. The blot was cut into four pieces, which were probed with (lanes 1 to 3) antiserum against pure TPS/P (1:30000 dilution), (lanes 4 and 5) antiserum against isolated 57 kDa polypeptide (1:40000), (lanes 6 and 7) antiserum against isolated 93 kDa polypeptide (1:20000), or (lanes 8 and 9) pre-immune serum (1:30000). In (b) enzyme freshly eluted from Heparin-Sepharose or enzyme stored for 5 d was boiled in 2% SDS, electrophoresed and blotted. The blot was probed with antiserum (1:10000) to pure TPS/P. Lanes 1 to 4 contained 4.3 mU (0.54 U mg⁻¹) original activity of enzyme stored with (lane 1) no addition, (lane 2) PMSF, (lane 3) pepstatin A and (lane 4) PMSF and pepstatin A. Lanes 5 and 6 contained 10 mU (1.0 U mg⁻¹) original activity of (lane 5) enzyme stored with no addition and (lane 6) freshly eluted enzyme. Numbers below the lanes show the activity at the time of boiling in 2% SDS, as percentages of the original activity. Molecular mass scales are on the left.

activation increased the TPS activity measured in the absence of TPS-activator to about that of freshly eluted enzyme measured with saturating amounts of TPS-activator. Furthermore, after spontaneous activation the enzyme became insensitive to TPS-activator (only 7% activation after 7 d storage) whereas enzyme stored in the presence of PMSF and pepstatin A could still be activated 2.3-fold even though it had lost 65% of its fresh activity (Fig. 4).

Western blots (Fig. 5) showed that during storage of the Heparin-Sepharose eluates there was a decrease in the molecular mass of the polypeptides recognized by

Fig. 6. Activation of TPP by potassium phosphate. TPP activity was measured in reaction mixtures containing 50 mM-HEPES/KOH (pH 6.8), 5 mM-MgCl₂, 0.24 mM-[¹⁴C]T6P, 1 mg ovalbumin ml⁻¹ and the indicated concentrations of potassium phosphate (pH 6.8). The points are means of duplicates that were within $\pm 12\%$ of the mean, except at 0.45 mM-phosphate ($\pm 26\%$). The inset shows the reciprocal of the increase in reaction rate above that without phosphate [$1/\Delta v$, (ml enzyme) min nmol⁻¹] as a function of the reciprocal of the phosphate concentration ($1/P_i$, mM⁻¹).

antiserum prepared against purified TPS/P complex. The antiserum recognised a band in the Heparin-Sepharose eluates at about 57 kDa which did not change during storage and presumably corresponds to the 57 kDa Coomassie-blue-stained band in pure TPS/P. It also recognized a cluster of bands between about 90 and 115 kDa. Within this cluster, the strongest bands in fresh preparations of enzyme or enzyme stored in the presence of PMSF were above 110 kDa, whereas after 5 d storage without PMSF these bands had almost disappeared and bands at about 90 to 100 kDa became much stronger.

These results suggest that the 'native' TPS/P in fresh Heparin-Sepharose eluates became proteolytically modified in such a way that full TPS activity no longer required TPS-activator. Varying extents of proteolytic modification, either *in vivo* or during early purification steps, might explain why the sensitivity to TPS-activator and the amount of activation during storage varied from preparation to preparation.

Catalytic activity

As previously reported for crude enzyme preparations (Cabib & Leloir, 1958; Elander, 1968), free divalent metal ions were not essential for the TPS activity of pure TPS/P but Mg²⁺ ions promoted the reaction. TPS rates at 1 mM- and 10 mM-MgCl₂ were 4% and 40% greater,

Table 2. Effect of phosphate on the overall reaction

The reaction from 5 mM-UDPG and 4.25 mM-[¹⁴C]G6P to trehalose was followed at pH 6.8 in the presence and absence of 6 mM-potassium phosphate as described in Methods. The data are from a single experiment.

Time (min)	Phosphate (mM) . . .	UDP (μM)		T6P (μM)		Trehalose (μM)	
		0	6	0	6	0	6
5		315	162	299	132	16	30
10		661	402	582	293	79	109
20		1280	810	971	437	309	373

respectively, than that with 5 mM-EDTA/75 μM-MgCl₂. In contrast, no TPP activity was observed at 4 mM-EDTA, and between 16 μM- and 1 mM-free Mg²⁺ a linear double-reciprocal plot was obtained with $K_{1/2}$ of 80 μM-Mg²⁺ (not shown). The optimal concentration of MgCl₂ was between 1 and 2 mM.

With pure TPS/P, no UDP or ADP were formed when UDPG in the standard TPS reaction mixture was replaced by UDP-glucuronate or by ADP-glucose, respectively. Although they were not substrates, both these compounds caused 20% inhibition when added at 5 mM to standard reaction mixtures.

Potassium phosphate (pH 6.8) inhibited TPS activity, as has been reported previously (Cabib & Leloir, 1958; Elander, 1968; Vandercammen *et al.*, 1989), with a K_i of 5 mM (not shown). This is somewhat higher than that (2 mM) reported by Vandercammen *et al.* (1989), but lower than that reported by the earlier workers. In contrast, phosphate strongly activated TPP activity (Fig. 6). From the double-reciprocal plot in the inset of Fig. 6 a maximum activation of 5.7-fold and a K_a of 3 mM-phosphate were calculated. Table 2 shows that the overall reaction from UDPG and G6P to trehalose was slightly stimulated by 6 mM-potassium phosphate. Furthermore, the higher rate of trehalose production occurred with markedly less accumulation of T6P. Native TPS/P, like the highly purified TPS/P, was similarly stimulated by phosphate: when the phosphate in the standard TPP assay was replaced by 50 mM-HEPES/KOH (pH 6.8), the activity of crude homogenate fell by 85%.

Discussion

By purifying the TPS activity we obtained a high molecular mass protein preparation that also had high TPP activity and contained three major polypeptides, with molecular masses of 57, about 86 and 93 kDa. The methods we have investigated so far (including gel-

filtration in the presence of various concentrations of urea or in phosphate buffers) do not separate these polypeptides without destroying both activities. The ratio of the standard activities of TPP and TPS was $36 \pm 5\%$ through the Heparin-Sepharose step and then decreased to $17 \pm 3\%$ after the spontaneous proteolytic activation of TPS (Table 1). The almost identical peptide maps of the two larger polypeptides suggest that the 86 kDa polypeptide is derived from the 93 kDa polypeptide. This is supported by preliminary data showing that corresponding pairs of peptides from the two polypeptides have identical amino acid sequences (N. Kalkkinen, R. Fagerström, J. Londesborough & O. Vuorio, unpublished work). The staining intensities of the bands indicated a molar ratio of the 57 kDa polypeptide to the sum of the 86 and 93 kDa polypeptides of 1.4. We conclude we have purified a complex that exhibits both TPS and TPP activities and contains roughly equal molar amounts of a short (57 kDa) polypeptide and a long (93/86 kDa) polypeptide that has been partially degraded. The Western blot (Fig. 5) suggests that the 'native' molecular mass of the long polypeptide is at least 115 kDa.

We do not yet know which polypeptide contains which active site. Possibly one of the polypeptides has a purely regulatory role. Changes in the molecular mass of the long polypeptide lead to increases in TPS activity with no change in TPP (Figs 4 and 5). This suggests that one end of the long polypeptide interacts with the TPS active site, but does not define the location of this active site.

TPS-activator was a relatively heat-stable protein (50% of its activity survived 10 min at 55 °C) and surprisingly large (a dimer of 58 kDa subunits; Fig. 1). It had no effect on TPP activity, in either the usual assay system (Fig. 2) or when HEPES buffer was used instead of phosphate (not shown). The TPS activity of 'native' TPS/P did not absolutely require TPS-activator. Instead, the Michaelis constants for UDPG and G6P were decreased by TPS-activator towards the values exhibited by the pure (proteolysed) TPS/P complex in the absence of TPS-activator (Fig. 3). In the absence of TPS-activator, the apparent maximum velocities of 'native' TPS/P at infinite G6P and 5 mM-UDPG and at infinite UDPG and 25 mM-G6P were, respectively, 80% and 67% of the corresponding values at a saturating level of TPS-activator (Fig. 3). Thus, at saturating levels of both substrates, TPS-activator would have a negligible effect. These results suggested, as a working hypothesis, that the TPS active site is partially occluded by a flap of protein formed from one end of the long polypeptide of TPS/P. Access to the active site would be facilitated either by interaction with TPS-activator or by limited proteolysis of the long polypeptide, forming the 93 and 86 kDa polypeptides observed in purified TPS/P. Panek & Panek

(1990) in a recent review state they have isolated a protein activator of TPS, but give no information about it.

Variation in the level of TPS-activator and limited proteolysis *in vivo* provide two new mechanisms by which trehalose synthesis might be regulated. The cytosolic concentration of phosphate may be another important factor, because as well as the previously reported (Cabib & Leloir, 1958; Elander, 1968; Vandercammen *et al.*, 1989) inhibition of TPS by phosphate, we found a strong activation of TPP at similar phosphate concentrations (Fig. 6). Lagunas & Gancedo (1983) consider that free phosphate in yeast cytosol probably varies by a factor of 2 within the range 1 to 5 mM. Table 2 shows that 6 mM-phosphate modestly accelerated the overall reaction and markedly decreased accumulation of the intermediate, T6P. Even if variations in phosphate do not have a regulatory role, it seems likely that about 5 mM-phosphate is required for the proper functioning of TPS/P, to produce trehalose at a moderate steady-state concentration of T6P. In this connection, it is interesting that Piper & Lockheart (1988) have isolated a temperature-sensitive mutant of *S. cerevisiae* that accumulates T6P above the restriction temperature.

We have not attempted to characterize the catalytic properties of the purified TPS/P in further detail, because it is not yet clear whether this form occurs *in vivo*. Further work is needed to investigate this possibility and to purify the native form of TPS/P.

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