Biochimica et Biophysica Acta, 1148 (1993) 303–307

BBAMEM 75991

The interaction of *Saccharomyces cerevisiae* trehalase with membranes

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(Received 15 December 1992)

Key words: Trehalase; Protein–lipid interaction; Enzyme regulation; Enzyme phosphorylation; Phosphorylation; (*S. cerevisiae*)

Plasma membranes isolated from cells of *Saccharomyces cerevisiae* previously submitted to a heat-shock showed a 10-fold increase in membrane-bound trehalase activity. Trehalase was purified to a high specific activity and was shown to be inhibited by glucose 6-phosphate and by the addition of a neutral phospholipid-like surfactant. Purified trehalase binds spontaneously to egg phosphatidylcholine small unilamellar vesicles, when in its active, phosphorylated form. When the enzyme was treated with alkaline phosphatase no binding was observed. The significance of this reversible binding for the control of trehalose metabolism in yeast cells is still unknown.

Introduction

The levels of trehalose in cells of *Saccharomyces cerevisiae* have been related to the ability of yeast to survive under stress (for a review, see Ref. 1). The enzyme trehalase, present in the cytosol of those cells, hydrolyzes trehalose to two molecules of glucose, and is strictly regulated by phosphorylation, the active form being the phosphorylated one [2,3].

This activation can be triggered by the addition of glucose to yeast cells grown on non-fermentable carbon sources [4] or by heat-shock [5], both conditions resulting in an increase in cyclic-AMP and phosphorylation of the cryptic form of trehalase.

Another form of trehalase which is confined to the vacuole has an acidic pH optimum and is permanently active, not regulated by phosphorylation [6,7]. Both enzymes have been characterized in various degrees of purification and correspond to different protein species.

In this paper we present some evidence that cytosolic trehalase, when active (phosphorylated) is able to bind to membranes, both plasma membranes or model membranes represented by PC vesicles. In order to show the binding to vesicles, active trehalase was purified and characterized.

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Abbreviations: C_{16}-PN, n-hexadecylphosphorylcholine; PC phosphatidylcholine; PMSF, phenylmethylsulfonyl fluoride.

Materials and Methods

Yeast cell plasma membrane preparations and the ATPase assay were performed as previously described [8]. Briefly, the procedure is as follows: *S. cerevisiae* cells were harvested by centrifugation for 10 min at $2000 \times g$, and washed twice in ice-cold water and once in homogenization buffer (50 mM Mops-KOH buffer (pH 7.5), containing 250 mM sucrose and 1 mM $MgCl_2$). Homogenization was carried out in a Bead-Beater (Biospec Products, USA) using the proportion of 120 g glass beads for 40 g of cells (wet weight) in 100 ml homogenization buffer for seven 1-min periods with 1-min intervals. After removing the glass beads the supernatant was adjusted to pH 7.5 with 1 M KOH and centrifuged for 10 min at $500 \times g$ two times. The supernatant obtained was centrifuged for 30 min at $20000 \times g$. This crude membrane fraction was resuspended in 10 mM Tris-acetate buffer (pH 5.2), containing 1 mM $MgCl_2$. Protein concentration was adjusted to 5 mg/ml and the pH of the suspension was carefully adjusted to 5.2 with 1:5 diluted acetic acid. This fraction was centrifuged for 10 min at $2000 \times g$ for the removal of mitochondria, and the supernatant was adjusted to pH 7.5 with 1 M KOH. After centrifugation at $50000 \times g$ the pellet containing the plasma membranes was vigorously vortexed for 10 min with 10 mM

Tris-acetate buffer (pH 7.5), containing 1 mM EDTA, and the protein concentration was adjusted to about 1 mg/ml.

Stable, freeze-dried, trehalase preparation and its assay were conducted according to published methods [9]. Inorganic phosphate was measured by the method of Geladopoulos [10]. n-Hexadecylphosphorylcholine was synthesized as previously described [11]. PC was purified from egg yolks by established procedures [12]. PC small unilamellar vesicles were prepared by sonication according to Schenkman et al. [13]. Phospholipid concentrations were measured after hydrolysis with perchloric acid [14]. Protein was determined by the method of Bradford [15] using bovine serum albumin as the standard. All special chemicals used were purchased from Sigma, except for alkaline phosphatase which was from Boehringer-Mannheim.

Trehalase was purified using a FPLC from Pharmacia LKB Biotechnology. Freeze-dried crude trehalase [9] was dissolved in a small volume of water and filtered through a Sephacryl S-300 HR column (2.0 × 65 cm) equilibrated with 20 mM imidazole-HCl buffer (pH 6.8) containing 0.5 mM PMSF, 1 mM benzamidine, 1.5 mM β-mercaptoethanol and 2.5 mM $CaCl_2$ (column buffer), operated at a flow rate of 0.7 ml/min. Fractions of 1.5 ml were collected. The peak fractions were pooled and applied to a DEAE-cellulose (DE-52, Whatman, 1.6 × 40 cm) equilibrated with the same buffer. After an initial washing with one column volume of buffer, trehalase was eluted from the column with a 50–300 mM KCl linear gradient in the equilibration buffer with a flow rate of 0.5 ml/min. Fractions of 3 ml were collected. The active fractions were pooled and dialyzed overnight against column buffer at 4°C. The dialyzate was applied to a ω-amino-pentyl-agarose column (1.0 × 6.0 cm) equilibrated with column buffer. The column was washed with 20 ml of column buffer and the enzyme activity was eluted with a 0–500 mM KCl gradient in column buffer with a flow rate of 0.2 ml/min. Fractions of 3 ml were collected. The active fractions were pooled and desalted in a FPLC fast desalting column equilibrated with column buffer. Trehalase thus obtained was maintained at 4°C and could be used for a few days without appreciable loss of activity.

Results and Discussion

During the course of our studies on the production of a stable trehalase preparation with high activity we employed a heat-shock treatment to achieve a five-fold activation of the enzyme [9]. In order to obtain more information on this activation process, and considering that all the activity recovered in the crude extract was found in the soluble fraction (cf., Table III in Ref. 9) we decided to investigate the presence of trehalase in the plasma membrane of yeast cells. The involvement of membrane proteins in this type of modulation of enzymatic activity is well-documented [1,5,16].

The results shown in Table I clearly indicate that in the purified plasma membrane fraction of cells of *S. cerevisiae* that have been submitted to a heat-shock treatment there was a 10-fold increase in trehalase activity linked to the membranes, when compared to those of control cells. This membrane-linked trehalase activity could not be removed by repeated washings in the final buffer used in the membrane preparation. However, when treated with *Escherichia coli* alkaline phosphatase (10 μg/ml), which is not specific for phosphorylated groups in proteins, a decrease in trehalase activity was observed (results not shown).

The ATPase activity of these plasma membranes assayed in the presence of 50 mM NaN_3 (to inhibit any remaining mitochondrial ATPase activity) and 0.2 mM ammonium molybdate (to inhibit phosphatase activity) [8] was 1780 ± 89 ($n = 3$) mU/mg of protein, which corresponds to more than 10-fold the activity observed in the homogenate (153 ± 10 mU/mg protein). These results correlate well with those published for purified membrane preparations [17–20]. These membranes exhibited a negligible succinate dehydrogenase activity, measured as described by King [21], indicating a minimum mitochondrial contamination.

A ratio of 4 was found between trehalase activity in the homogenates of heat-shock-treated cells and control cells, while the ratio between trehalase activity in the plasma membrane fraction of heat-shock-treated cells and control cells was found to be 14 (Table I). This 3-fold increase in the ratio suggests a preferential binding of trehalase to the plasma membranes.

Although this interaction of active trehalase with plasma membranes was unexpected, it is not unique. Several examples of soluble enzymes that interact with membranes are known. CTP : phosphocholine cytidyltransferase, the regulating enzyme in phosphatidylcholine synthesis [22] is translocated from the cytosol to membranes in response to various stimuli [23], in-

TABLE I

Trehalase activity in Saccharomyces cerevisiae subcellular fractions, strain D273-10B

Results are shown as the average ± S.E. of three separate experiments. Trehalase activity is expressed as specific activity of the enzyme; where one unit corresponds to the amount of the enzyme that produces 1 μmol of glucose per minute at 30°C under the assay conditions [9]. Protein (mg) in the fractions was assayed according to Bradford [15].

Fraction	Control cells (mU/mg)	Heat-shock-treated cells (mU/mg)
Homogenate	21.62 ± 2.19	87.6 ± 6.02
Plasma membranes	1.8 ± 0.21	24.7 ± 3.6

cluding reversible phosphorylation. For this enzyme the active form, bound to membranes is the dephosphorylated one [24]. Protein kinase C is translocated from the cytosol upon cell activation [25], and it was shown in model systems that it binds to negatively-charged monolayers [26] and vesicles, in a Ca^{2+}-dependent manner [27]. Also, in cell-free extracts of rat liver Kupfer cells, phospholipase A_2 reversibly associates with cellular membranes in the presence of Ca^{2+} concentrations in the range of 0.1–1.0 μM. It is released from the membranes in the presence of EGTA [28].

To corroborate these results obtained with the isolated plasma membranes it was necessary to purify trehalase and demonstrate that the active trehalase could bind to lipid bilayers while the dephosphorylated enzyme would not bind to vesicles.

Table II summarizes the data of a typical purification of active trehalase. After ω-amino pentyl agarose chromatography the specific activity of trehalase was 14.2 U/mg protein and a purification of 47.3-fold was obtained. This purification refers to the crude, stable trehalase preparation used as the starting material. If the purification is calculated starting with the cell-free extract [9] then its value is 163-fold. Non-denaturating polyacrylamide gel electrophoresis of the purified trehalase performed by the method of Davis [29] in 7.5% gels showed a major protein band that coincided with the band stained for trehalase activity [30], however, four minor contaminants were still present (results not shown). Since the amount of protein obtained at the last stage of purification was very small (total of 60 μg protein), we could not achieve homogeneity by preparative electrophoresis as App and Holzer [31] did in their procedure. Furthermore, at the last stage we could not stock the enzyme, because it became very unstable losing activity in a few days.

Mg^{2+} and Ca^{2+} up to 10 mM had no effect on the activity of the purified trehalase. An activation of 50% was achieved in the presence of 50% (v/v) glycerol, or 1 mM AMP. However, ATP or ADP at the same concentration had no effect. High salt concentration

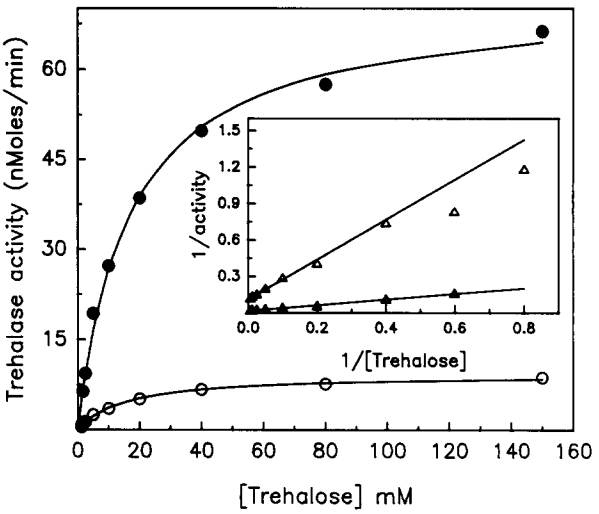

Fig. 1. Trehalase activity at various substrate concentrations. Open symbols in the presence of 10 mM glucose 6-phosphate; closed symbols in the absence. The inset shows the double-reciprocal plots to visualize that the effect of the inhibitor is only on k_{cat}.

such as 1 M KCl had a 30% inhibitory effect, which could explain the apparent activation of trehalase after Sephacryl S-300 chromatography by the removal of the salts present in the freeze-dried crude trehalase (Table II). The inhibitory effect of glucose 6-phosphate is shown in Fig. 1. The inhibition affects only k_{cat} which showed an 8-fold decrease in the presence of 10 mM glucose 6-phosphate. This result is consistent with our former suggestion that trehalase is susceptible to inhibition by glucose-like structures [9].

The elution profile of trehalase in the Sephacryl S-300 column showed a single activity peak (Fig. 2) which after calibration of the column with molecular mass marker proteins, corresponded to 180 kDa, a result very close to previously published ones [31,32].

The effect of 10 mM C_{16}-PN, which associates in zwitterionic micelles, with a polar head-group forming an interface with similar properties as phosphatidylcholine (for a review, see Ref. 33) showed a strong inhibitory effect (over 80%) at various substrate con-

TABLE II

Purification of S. cerevisiae trehalase

The purification was performed as described in Materials and Methods. One unit of trehalase is defined as the amount of enzyme that catalyzes the hydrolysis of trehalose under the assay conditions giving rise to 1 μmol of glucose per min at 30°C and pH 6.0 [9]. Specific activities are expressed as U/mg protein.

	Volume (ml)	Total activity (U)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Crude trehalase	2.5	6.0	0.30	1	100
Sephacryl S-300 chromatography	28	9.2	0.44	1.5	150
DEAE-cellulose chromatography	30	4.4	2.64	8.8	73
Dialysate	32	3.9	3.85	12.8	65
ω-Amino-pentyl-agarose chromatography	6	0.1	14.2	47.3	2

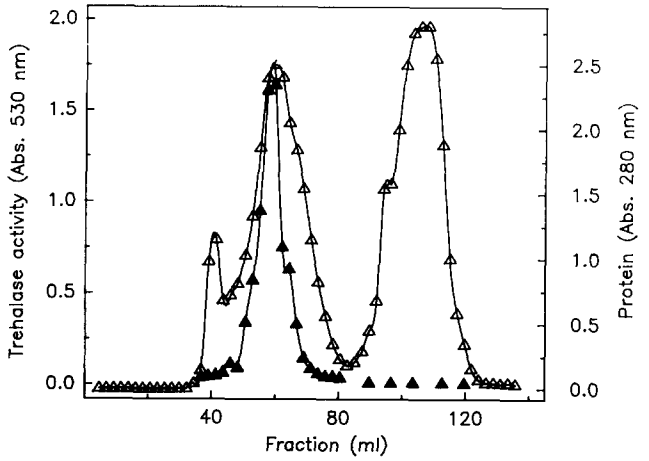

Fig. 2. Elution profile of crude trehalase in Sephacryl S-300 HR 2.5 U trehalase in 2.0 ml was applied to the column. (▲) Trehalase activity; (△) absorbance at 280 nm. Fractions of 3 ml were collected. For experimental details, see Materials and Methods.

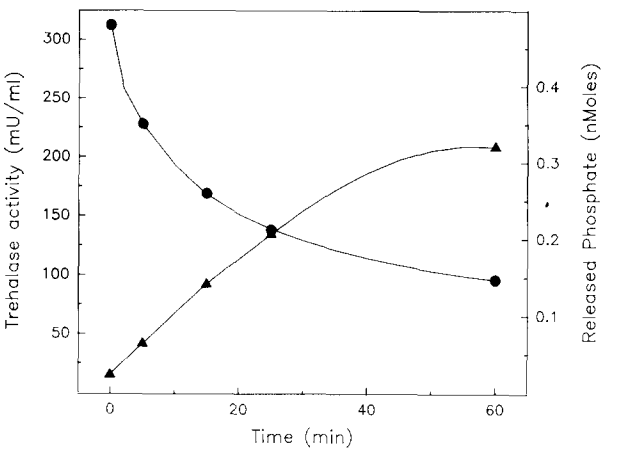

Fig. 3. Dephosphorylation of partially-purified trehalase. Trehalase (320 mU/ml) in 20 mM imidazole buffer (pH 6.8) containing 1 mM $MgCl_2$ was incubated with *E. coli* alkaline phosphatase (10 μg/ml) at 30°C. At the indicated times samples were taken for activity determination (●) and for inorganic phosphate released (▲) as described in Materials and Methods.

centrations. This suggests that trehalase is separated in its monomers in the presence of micelles, even when the interface is similar to that formed by phospholipids. When a sample of partially purified trehalase which contained 10 mM C_{16}-PN was filtered through the Sephacryl S-300 column, in the same buffer as in Fig. 2 containing 1 mM of the same surfactant, no activity could be detected in the fractions eluted. However, the elution profile of the single protein peak obtained corresponded to approx. 70 kDa. Similar values for the molecular mass of the monomer were found by other authors [6,31,32]. Therefore, it appears that active trehalase corresponds to the dimeric species, even when in its phosphorylated form.

When active trehalase was incubated with alkaline phosphatase, the loss of activity was accompanied by an increase in released inorganic phosphate (Fig. 3). In spite of the large excess of alkaline phosphatase, a plateau was reached after 30 min of incubation and no further loss of activity was observed.

Although zwitterionic micelles disrupted the activity of the enzyme, no inhibition of trehalase activity was observed upon the addition of small unilamellar vesicles of egg PC. In fact, a small activation in the order of 15% was observed. When partially purified trehalase was incubated for a minimum of 2 h with lipid vesicles, at room temperature or for up to 12 h at 4°C, and then the mixture was filtered through a small Sepharose 4B column, most of the trehalase activity eluted in a peak coincident with the elution peak of the vesicles alone (Fig. 4). However, when trehalase that was previously treated with alkaline phosphatase (it was submitted to the same conditions as those used in Fig. 3) the elution peak of enzymatic activity shifted toward higher elution volumes, and was no longer coincident with the elution of PC vesicles. These results strongly indicate that trehalase, when phosphorylated is able to spontaneously bind to small unilamellar PC vesicles, albeit not when dephosphorylated, confirming the results obtained in vivo as shown in Table I.

Since the surface of these PC vesicles is uncharged the interaction must be of hydrophobic nature. When active trehalase was incubated with PC vesicles in the presence of 500 mM KCl, the same results as those shown in Fig. 4 were obtained. This high salt concentration drastically reduces electrostatic interactions, thus corroborating the hydrophobic nature of the trehalase interaction with membranes.

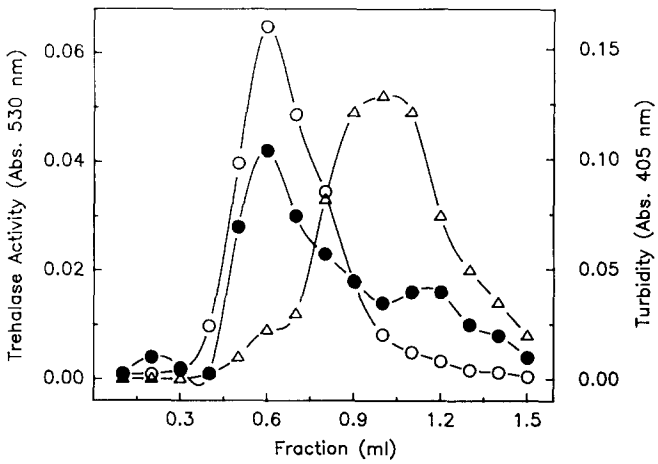

Fig. 4. Elution profiles of partially purified trehalase incubated with PC vesicles. Trehalase (100 μl) was incubated for 12 h at 4°C with 200 μl of PC small unilamellar vesicles (phospholipid concentration: 25 mg/ml) and 100 μl of the reaction mixture was applied to a 2 ml Sepharose 4B column (0.8×4 cm, void volume 0.6 ml). Fractions of 100 μl were collected and used for activity determination or turbidity measurements to follow the elution of vesicles. Active trehalase (7.3 mU)+vesicles (●); trehalase (28 mU) treated with alkaline phosphatase (same conditions as in Fig. 3)+vesicles (△) and vesicles alone (○).

The mechanism of this reversible binding and its significance in the regulation of trehalose metabolism in yeast cells are still obscure and will be the object of further studies.

Acknowledgements

We are very grateful for helpful discussions with Dr. Johan M. Thevelein (Leuven, Belgium), during the course of this work. We thank Marcia Aparecida da Silva for her skilled technical assistance. This work was supported by grants from CNPq and FAPESP (Brazil) and Third World Academy of Sciences (Trieste, Italy).

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