*Biochimica et Biophysica Acta*, 391 (1975) 154—161


BBA 67483

# SOME PROPERTIES OF TREHALASE FROM *PHYCOMYCES BLAKESLEEANUS*

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(Received November 20th, 1974)

## Summary

Trehalase ($\alpha$, $\alpha$-trehalase glucohydrolase EC 3.2.1.28) from *Phycomyces* spores occurs in two different forms which are convertible in vivo: a form with low activity found in dormant spores and an active form after breaking the dormancy. Between the two forms no difference in molecular weight and electrophoretic mobility can be detected. The molecular weight is estimated by gel filtration at about 210 000. The relation between substrate concentration and trehalase activity follows the Michaelis-Menten equation ($K_m$ ± 55 mM) in activated spores whereas in dormant spores trehalase shows a different substrate binding, indicating a negative cooperative effect. They differ further in thermostability and in sensitivity to inhibition by ATP. Other nucleosidephosphates have no inhibiting effect. Heating the spores at different temperatures between 38 and 44°C results in a partial breaking of dormancy of the spore population and a corresponding partial activation of trehalase. This suggests a close connection between breaking dormancy and trehalase activation.

## Introduction

In a previous paper we showed that a heat treatment (3 min; 50°C) of *Phycomyces* spores, which breaks dormancy, causes a sudden 10—15-fold increase of trehalase activity, independently of protein synthesis. Within 1 h, this activity decays to the level found in dormant spores. We suggested that the heat treatment converts temporarily a less active form of trehalase in a more active one (Van Assche et al. [1]).

The experiments described in this paper were performed to find some difference between the less active form of trehalase in dormant spores and the active state after inducing germination. We studied furthermore the influence of several nucleosidephosphates on trehalase in vitro activity and th· influence

of different temperatures in vivo both on trehalase and germination induction of spores.

## Material and Methods

*Material*

Strain 1+ of *Phycomyces blakesleeanus* was grown for about 11 days on brown bread and spores were collected as previously described. In order to break dormancy the spores were suspended in water and shaked in a water bath at 50°C for 3 min.

*Preparation of a crude enzyme extract and trehalase assay*

We used the procedure as described in the previous paper (Van Assche et al. [1]) except where stated otherwise. The spores were homogenized in 0.01 M phosphate buffer (pH 6.0) by vibration with glass beads. The crude extract was centrifuged for 2 min at 11 000 × *g* and the supernatant dialyzed during at least 4 h against 5 mM potassium phosphate buffer (pH 7.5) at 1°C. The trehalase activity was determined by measuring the amount of glucose liberated with the glucose-oxidase-peroxidase method. The activity was expressed as nmol substrate hydrolyzed per minute per mg protein.

*Sephadex column chromatography*

The crude extract of 100—300 mg spores was centrifuged at 55 000 × *g* for 20 min and applied to an inversed Sephadex G-200 column (2.5 cm × 35 cm) at 1—2°C. The column was eluted with 5 mM potassium phosphate buffer (pH 7.5) and fractions of 15 drops were collected. For an estimation of the molecular weight, a mixture of reference proteins was applied five fractions behind the trehalase. As references the following enzymes were used: jack bean urease (mol. wt 483 000); radish catalase (mol. wt 244 000); rabbit aldolase (mol. wt 158 000) and *Escherichia coli* alkaline phosphatase (mol. wt 86 000). The protein content of the fractions was determined by measuring the absorbance at 280 nm. Recovery of trehalase and the reference proteins was based on enzymatic analysis.

*Electrophoresis*

Electrophoresis of protein extracts was carried out in the cold room on polyacrylamide gels (7% acrylamide; 0.18% bisacrylamide) in 0.06 M Tris · HCl buffer (pH 8.8). After the run the gels were stained with amidoblack and scanned; duplicate gels were sliced in 5-mm sections which were assayed for trehalase.

*Trehalase assay with labelled trehalose*

[U-$^{14}$C]Trehalose was purchased from The Radiochemical Centre (Amersham). The specific radioactivity was 540 Ci/mol. The labelled trehalose was previously chromatographed in order to remove traces of glucose. Unlabelled trehalose was added and the specific activity determined (approximately 30 000 cpm/μmol for trehalase from activated spores: approximately 85 000 cpm/μmol for the enzyme of dormant spores).

The reaction occurred in 0.05 M phosphate buffer (pH 7.5) at 30°C. At 10-min intervals aliquots (20 or 50 µl) were withdrawn and spotted on a Whatman 3 MM paper chromatogram together with 0.2 mg glucose. The chromatograms were run for 2.5 days with 1-butanol/pyridine/water (10/3/3, v/v/v). The chromatograms were sprayed with aniline-oxalic acid. The glucose spots were cut out and eluted in 5 ml ethanolamine/2-methoxy-ethanol (1/2, v/v). To this solution 10 ml toluene (containing 4 g PPO/l) was added for counting with a liquid scintillation spectrometer. Plotting the amount of freed radioactive glucose in function of time showed that the trehalase activity was constant during one hour. The activity was calculated from the slope of the regression line.

## Results

Since trehalase seems to occur in two different forms with different activity, gel filtration was used to detect a possible difference in molecular weight. In all experiments trehalase from activated spores as well as that from dormant spores was eluted in one single peak behind the proteins in the void volume. In comparison with the reference proteins the molecular weight of both forms is estimated at about 210 000 (Fig. 1).

These results are confirmed by acrylamide gel electrophoresis of both enzyme extracts (Fig. 2). Trehalase is found at the same position on the gel. It may be noted in passing that no significant difference is seen between the protein pattern of dormant and activated spores.

In order to study the influence of substrate concentration on trehalase activity, a more sensitive enzyme assay with labelled trehalose was used. The obtained results are plotted according to Hanes [2] ($s$ versus $s/v$) for estimating the parameters of the Michaelis-Menten equation (Fig. 3).

Trehalase from activated spores obviously obeys the Michaelis-Menten equation, the $K_m$ being about 55 mM and V 39 nmol · $min^{-1}$ · mg $protein^{-1}$. Two experiments with partially purified trehalase (through Sephadex G-200 chromatography) gave the same $K_m$ value. Trehalase from dormant spores, on

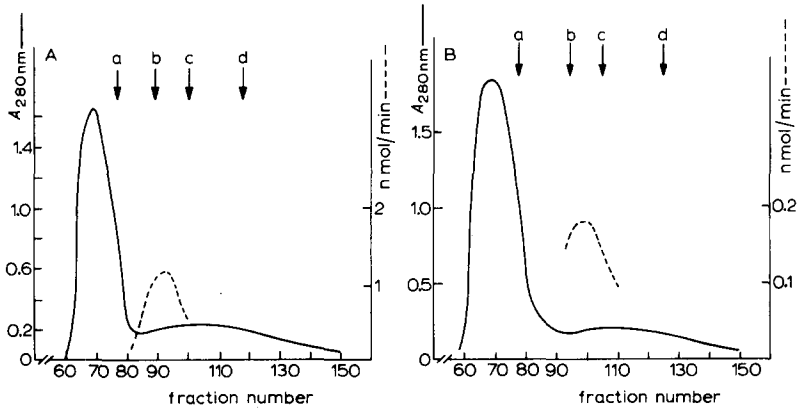


Fig. 1. Elution profile of trehalase and position of reference proteins from a Sephadex G-200 column. A, from activated spores; B, from dormant spores. a: urease; b: catalase; c: aldolase; d: alkaline phosphatase. ———, absorbance; - - - - - -, trehalase activity.

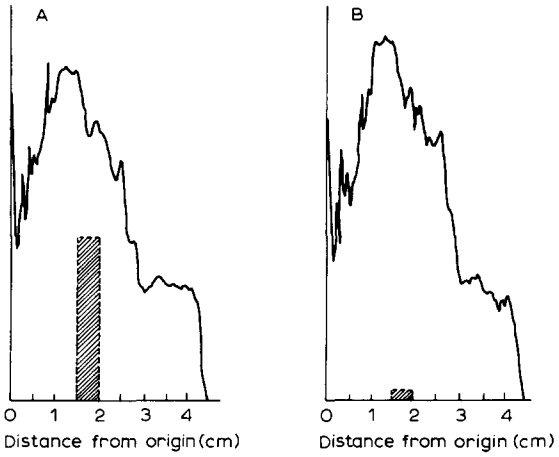


**Fig. 2. Localization of trehalase after acrylamide gel electrophoresis of soluble proteins. A, from activated spores; B, from dormant spores. ———, densitometric tracing; the column indicates the place and relative activity of trehalase.**

the contrary, shows a deviation from the Michaelis-Menten equation. This deviation is even more clearly illustrated by an Eadie-Hofstee [3] plot ($v$ versus $v/s$) from another experiment where lower substrate concentrations (0.1—40 mM) are used (Fig. 4). The substrate saturation of the enzyme from dormant spores and the shape of the Eadie-plot are consistent with the kinetic behaviour described as negative cooperativity.

If trehalase is heated in the absence of its substrate for various times at 36°C, trehalase from activated spores is more rapidly inactivated than from dormant spores (Table I).

Trehalase activity can be inhibited specifically by ATP (Fig. 5 and Table II). The less active form from dormant spores is clearly more hindered by ATP. All other tested nucleosidephosphates (at a concentration of 2 mM) show no significant inhibitory effect. It is unlikely that ATP would inhibit trehalase

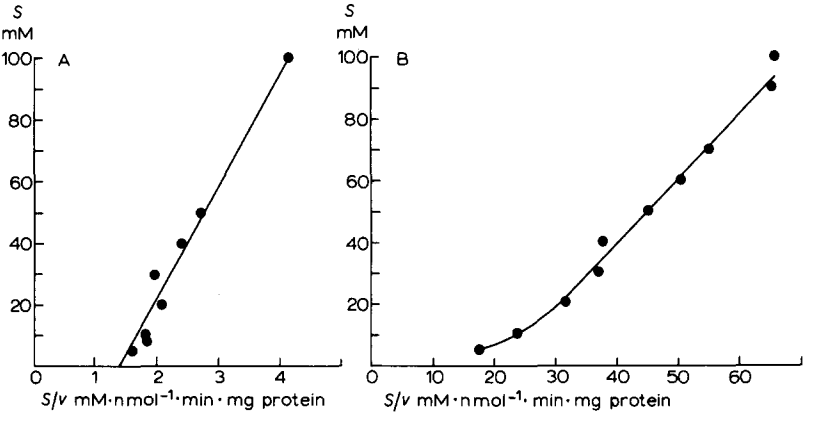


**Fig. 3. Hanes-plot ($s$ versus $s/v$) showing the relation between substrate concentration and trehalase activity. A, from activated spores; B, from dormant spores.**

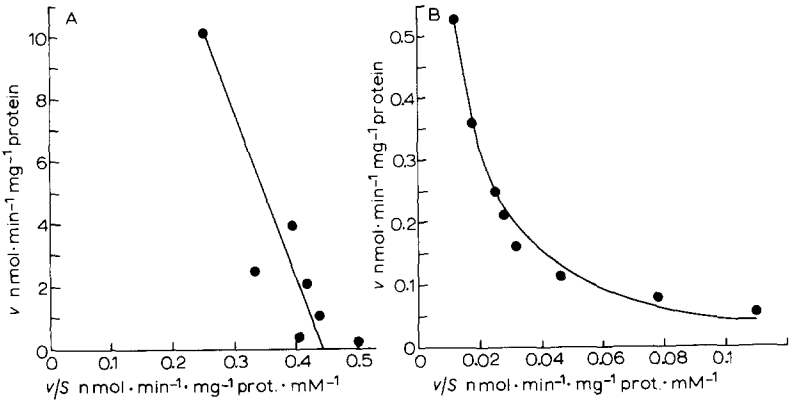


Fig. 4. Eadie-Hofstee-plot ($v$ versus $v/s$) of an experiment where lower substrate concentrations are used (0.5 to 40 mM). A, from activated spores; B, from dormant spores.

TABLE I

TREHALASE ACTIVITY AFTER DIFFERENT PERIODS OF HEATING AT 36°C

| Heating period (min) | From activated spores | From dormant spores |
|---|---|---|
| 0 | 17.68 | 1.09 |
| 15 | 8.19 | 0.47 |
| 30 | 4.22 | 0.47 |
| 60 | 1.44 | 0.51 |
| 90 | 1.19 | 0.36 |
| 120 | 0.72 | 0.21 |
| 150 | 0.52 | 0.27 |
| 180 | 0.45 | 0.27 |

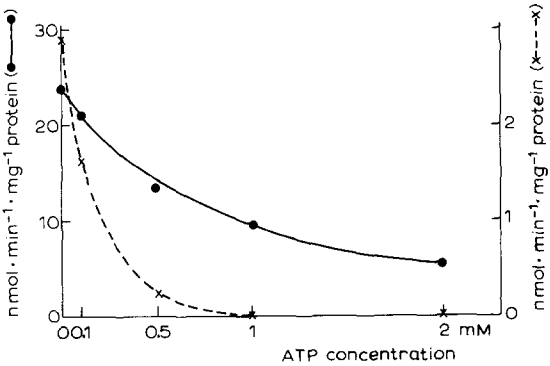


Fig. 5. Influence of different concentrations of ATP on trehalase activity. ———, from activated spores; - - - - - -, from dormant spores.

TABLE II

INFLUENCE OF NUCLEOSIDEPHOSPHATES ON TREHALASE ACTIVITY

| Additions | Activated spores | Dormant spores |
|---|---|---|
| Control | 27.1 | 2.9 |
| ATP $2 \cdot 10^{-3}$ M | 3.9 | 0 |
| GTP $2 \cdot 10^{-3}$ M | 24.9 | 2.9 |
| CTP $2 \cdot 10^{-3}$ M | 25.7 | 2.6 |
| UTP $2 \cdot 10^{-3}$ M | 24.8 | 2.9 |
| ADP $2 \cdot 10^{-3}$ M | 20.6 | 1.8 |
| AMP $2 \cdot 10^{-3}$ M | 24.4 | 2.9 |
| cyclic AMP $2 \cdot 10^{-3}$ M | 27.5 | 2.4 |

activity by forming complexes with divalent ions which are necessary for the activity (Van Assche et al. [1]) as the same could be expected with other nucleosidephosphates. Moreover we found the same inhibitory effects by using the magnesium salt of ATP.

In the previous paper evidence was presented that trehalase is activated as a direct result of heating the spores. Since the standard heat treatment (50°C, 3 min) breaks dormancy of the spores and activates trehalase we followed the effect of different temperatures on both phenomena. Immediately after the heat treatment the spores are homogenized and trehalase assayed. Another sample of the heated spores is brought in nutrient medium and the percentage of germinated spores is determined after 8 h. From the results shown in Fig. 6 there seems to be a close correlation between activation of trehalase and induction of germination as a consequence of the heat treatment.

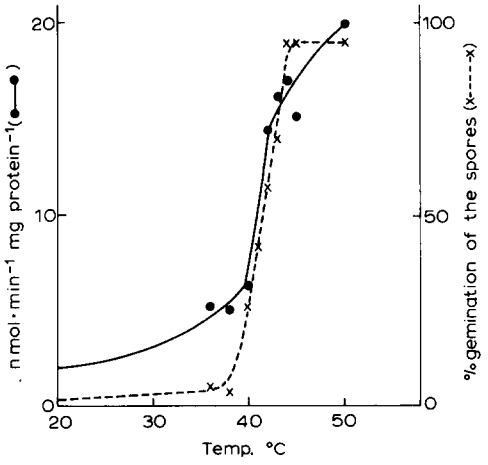


Fig. 6. Influence of treatment of the spores at different temperatures on trehalase activity and breaking of dormancy. ●——●, trehalase activity; X- - - - - X, percentage of germination.

## Discussion

Although both forms of trehalase have nearly the same molecular weight and electrophoretic mobility, there are several data indicating a different structure. The difference in substrate saturation, sensitivity to ATP-inhibition and thermostability indicate that two different forms of trehalase occur, one with high activity and another which is much less active. The interconversion of both forms does not involve an important change in molecular weight so that the mechanism of activation and inactivation is not based on association or dissociation of subunits.

For trehalase from mycelium of *Neurospora*, Hecker and Sussman [4] described different forms varying in molecular weight from 105 000 to more than 400 000 as a result of changes in ionic strength, however without major alteration of enzymatic activity.

Since both forms of *Phycomyces* trehalase are easily convertible in vivo the most plausible hypothesis would be that trehalase can be chemically modified, or that an effector can be tightly bound to the enzyme. Although ATP inhibits trehalase activity it is improbable that reversible adenylation or phosphorylation are involved in modification of trehalase. After removal of ATP by dialysis the activity of trehalase is restored. Furthermore, if the activity was diminished through adenylation or phosphorylation one should expect a higher inhibitory effect on the active enzyme form. Finally, after addition of ATP 0.5 mM and 2 mM, (which inhibits partially) to trehalase of activated spores, trehalose is broken down at a reduced but constant rate. In the case of inhibition by adenylation or phosphorylation of the enzyme, one should expect a decrease of activity in function of time. For all these reasons, ATP presumably inhibits by an allosteric effect, rather than by modifying chemically the enzyme.

Trehalose is a major reserve substance of *Phycomyces*. The inhibition of the enzyme by ATP could be an efficient feedback control of trehalase activity supplying glucose according as energy is required. The increased hydrolysis of trehalose in vivo after breaking of dormancy (Rudolph and Ochsen [5]) is not due to a decrease in ATP concentration. In preliminary and unpublished experiments in our laboratory we found that the ATP concentration is practically unchanged after heat treatment, while Furch [6] found even an increase by 50%. The amount of ATP in spores is higher than 200 nmol/100 mg spores according to Furch [6]; the overall concentration of ATP (without regard to intracellular compartmentation) in the spores is then higher than 2 mM. If the results of Fig. 5 are a real reflection of the in vivo properties, the functioning of trehalase would be almost completely suppressed in dormant spores. After the heat treatment trehalase functioning would not only increase by an activation but also by a decreased sensitivity to ATP inhibition.

The hydrolysis of trehalose is seen as a major trigger mechanism for the induction of germination of *Neurospora* ascospores. Trehalase is located in the spore wall so that in dormant spores trehalase and its substrate are separated. After a heat shock trehalose probably comes in contact with its hydrolase (Hecker and Sussman [7]).

Concerning *Phycomyces* spores we know that a heat treatment causes the

activation of trehalase and that trehalase activity and induction of germination seem to be closely correlated. If dormancy is broken by treatment with ammonium acetate instead of heating, trehalase is also activated (Delvaux [8]). It remains however unknown to what extent induction of germination in *Phycomyces* is dependent on the breakdown of trehalose since dormant spores do not germinate when supplied with external glucose (Rudolph and Ochsen [5]).

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