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Regulation of Trehalose Metabolism by *Streptomyces griseus* Spores

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Spores of *Streptomyces griseus* contain trehalose and trehalase, but trehalose is not readily hydrolyzed until spore germination is initiated. Trehalase in crude extracts of spores, germinated spores, and mycelia of *S. griseus* had a pH optimum of approximately 6.2, had a K_m value for trehalose of approximately 11 mM, and was most active in buffers having ionic strengths of 50 to 200 mM. Inhibitors or activators or trehalase activity were not detected in extracts of spores or mycelia. Several lines of evidence indicated that trehalose and trehalase are both located in the spore cytoplasm. Spores retained their trehalose and most of their trehalase activity following brief exposure to dilute acid. Protoplasts formed by enzymatic removal of the spore walls in buffer containing high concentrations of solutes also retained their trehalose and trehalase activity. Protoplasts formed in buffer containing lower levels of solutes contained low levels of trehalose. The mechanism by which trehalose metabolism is regulated in *S. griseus* spores is unresolved. A low level of hydration of the cytoplasm of the dormant spores and an increased level of hydration during germination may account for the apparent inactivity of trehalase in dormant spores and the rapid hydrolysis of trehalose upon initiation of germination.

Large amounts of the disaccharide trehalose are present in the spores and cysts of a variety of organisms, including actinomycetes, fungi, nematodes, and brine shrimp (2, 4, 13). In these organisms trehalose is degraded very slowly during periods of dormancy but is rapidly metabolized following the onset of vegetative growth (1, 9, 14, 20, 22).

Spores of *Streptomyces griseus* contain large amounts of trehalose (13). Extracts of the spores also contain a high specific activity of the enzyme trehalase (14). Nongerminating spores metabolize their endogenous trehalose reserves very slowly. Upon transfer to conditions allowing spore germination, trehalose is rapidly metabolized, while the level of trehalase activity remains constant (14).

In this paper we report the results of an investigation of the properties of trehalase and the localization of trehalase activity and trehalose in spores of *S. griseus*. The goal of this study was to determine the basis for the apparent coexistence of trehalase and trehalose in dormant spores and the rapid utilization of trehalose following the initiation of germination.

MATERIALS AND METHODS

Growth conditions. *S. griseus* NRRL B-2682 was maintained as described previously (13). Spores were harvested from solid DMC medium after 7 days of incubation at 30°C unless indicated otherwise. DMC medium was modified to obtain lysozyme-sensitive spores by adding glycine to a final concentration of 0.35% and by adjusting the pH to 6.6 prior to sterilization. Spores were germinated in the complex germination medium described previously (13). Mycelia were grown in liquid DMC medium containing 0.15 M KCl. Protoplast regeneration medium consisted of 25 mM morpholinopropanesulfonic acid (MOPS) (pH 7.0), 5 mM $(NH_4)_2SO_4$, 20 mM $CaCl_2$, 20 mM $MgCl_2$, 0.4 mM KPO_4 (pH 7.0), 50 mM glucose, 0.3% yeast extract (Difco Laboratories), 0.05% casein hydrolysate, 0.45 M sucrose, 2.0% (wt/vol) Bacto-Agar (Difco), and 2.5 ml of the trace salts solution described previously (13) per liter.

Preparation of cell extracts. Mycelia suspended in 10 mM potassium phosphate buffer (pH 7.0) were disrupted by sonication for 3 min with a model 350 Sonifer (Branson Sonic Power Co.) at a power setting of 7. Spores in 10 mM phosphate buffer were broken by agitation with 0.1-mm-diameter glass beads as described previously (14). Spores were also broken in buffer containing 10 mM phenylmethylsulfonyl fluoride and 10 mM ethylene-dinitrilotetraacetic acid as a precaution against protease activities. Spore and mycelial walls were removed by centrifugation for 3 min in a Microfuge (Beckman Instruments, Inc.). Cells and extracts were kept at 0 to 4°C throughout the procedures described above.

Spore permeabilization. Spores (2×10^9 spores per ml) were permeabilized by incubating them at 0°C for 2 min in 10 mM 2-morpholinoethanesulfonic acid (MES) buffer (pH 6.2) containing 0.4 mg of cetyltrimethyl ammonium bromide per ml. The spores were sedimented by centrifugation for 3 min in a Beckman Microfuge, washed once with cold 10 mM MES buffer (pH 6.2), and then resuspended in the same buffer.

Preparation of spore protoplasts. Protoplasts were obtained from spores grown on DMC medium (pH 6.6) containing 0.35% glycine. The spores were incubated in the osmotically stabilized buffers described below with lysozyme at 30°C until digestion was complete. Protoplasts were harvested by centrifugation and washed in the appropriate buffers at 0 to 4°C as described below.

Protoplasts were formed with sucrose as the osmotic stabilizer by incubating spores for 2 to 2.5 h in P buffer (10 mM MOPS buffer [pH 7.0], 2 mM $MgCl_2$, 2 mM $CaCl_2$) containing 0.45 M sucrose and 0.5 mg of lysozyme per ml. Following incubation, intact spores and spore wall debris were removed by centrifugation for 5 min at $100 \times g$. The supernatant containing the protoplasts was diluted with 2 volumes of P2 buffer (10 mM MOPS [pH 7.0], 10 mM $MgCl_2$, 25 mM $CaCl_2$) containing 0.45 M sucrose, and the protoplasts were sedimented by centrifugation for 15 min at $1,500 \times g$. The pellet consisted of two layers. The top layer containing brown-pigmented wall debris was carefully washed away with P2 buffer containing 0.45 M sucrose, leaving the bottom layer of protoplasts behind. The proto-

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plasts were suspended in P2 buffer containing 0.45 M sucrose and washed three times by centrifugation. Protoplasts were formed in P buffer containing 1.25 M sucrose as described above, except that the lysozyme digestion time was extended to 3.5 to 4 h. Wall debris was removed by centrifugation at 600 × *g* for 5 min. The protoplasts suspended in 1.25 M sucrose did not sediment readily when they were centrifuged, so it was necessary to dilute the suspension with 5 volumes of P2 buffer containing 1.0 to 1.25 M KCl. The protoplasts were then sedimented by centrifugation for 15 min at 2,000 × *g*.

Protoplasts were formed with KCl as the osmotic stabilizer by incubating spores for 3 h in P buffer containing 0.3 M KCl and 20 mg of lysozyme per ml. The protoplasts were diluted with 2 volumes of P2 buffer containing 0.3 M KCl and sedimented by centrifugation for 10 min at 1,000 × *g*.

Protoplasts were formed with sorbitol as the osmotic stabilizer by one of three procedures. Spores were incubated for 3 h in P2 buffer containing 1.25 M sorbitol and 2.5 mg of lysozyme per ml, for 5 h in P2 buffer containing 2.5 M sorbitol and 10 mg of lysozyme per ml, or for 12 h in P2 buffer containing 4 M sorbitol and 20 mg of lysozyme per ml. Debris was removed by centrifugation at 150 to 250 × *g* for 5 min. The supernatants containing the protoplasts were diluted with 5 volumes of P2 buffer containing 1.0, 2.0, or 3.2 M KCl, respectively, and the protoplasts were collected by centrifugation for 15 min at 1,000, 2,500, or 3,000 × *g*, respectively.

The efficiency of conversion of spores to stable protoplasts was determined by diluting the protoplasts in protoplasting buffer or distilled water and counting the number of intact protoplasts with a Petroff-Hauser cell-counting chamber and a phase-contrast microscope.

Electron microscopy. Spores were washed and suspended in P2 buffer containing 0.4 M KCl. Protoplasts formed in P buffer containing 0.4 and 1.25 M sucrose were washed and suspended in P2 buffer containing 0.4 and 1.25 M KCl, respectively. Cells were fixed with 2% glutaraldehyde for 1 h at 25°C, washed with fresh buffer of the same composition, postfixed with 1% OsO_4 for 8 h at 25°C, dehydrated, and embedded in vinyl cyclohexene dioxide (19). Thin sections were stained with uranyl acetate and lead citrate and viewed with a Hitachi electron microscope.

Measurement of trehalase activity. Trehalase activity was measured in 40 mM MES–50 mM KCl buffer (pH 6.2). In some experiments MES was replaced by MOPS (pH 7.0). Trehalase activity was routinely assayed with approximately 400 μg of crude extract protein per ml. Trehalose was added to a final concentration of 100 mM to start the reaction. After incubation at 33°C for 15 min, the reaction was stopped by immersing the reaction tubes in a boiling water bath for 3 min. Precipitated material was removed by centrifugation when necessary. In experiments in which intact or permeabilized spores were used, the reaction was stopped by adding Tris (pH 7.0) to a final concentration of 300 mM, and cells were removed by centrifugation. Tris is a competitive inhibitor of trehalase (8, 15); we verified this for *S. griseus* trehalase (data not shown). The glucose formed by trehalose hydrolysis was measured enzymatically by the glucose oxidase-peroxidase method (Sigma Chemical Co.). One unit of trehalase activity was defined as the amount of enzyme that hydrolyzed 1.0 nmol of trehalose per min at 33°C.

The effect of pH on trehalase activity was determined by using 20 mM sodium citrate buffer (pH 4.5 to 7.5), 40 mM MES buffer (pH 5.2 to 6.2), 40 mM potassium phosphate buffer (pH 5.8 to 7.5), 40 mM MOPS buffer (pH 6.2 to 7.6), and 20 mM glycine buffer (pH 7.6 to 8.0).

Trehalase activity associated with the spore surface was measured by incubating spores in 40 mM MES buffer (pH 6.2) containing 100 mM trehalose and 20 mM NaF at 33°C for 30 min. The reaction was stopped by adding Tris (pH 7.0) to a final concentration of 300 mM, and the spores were sedimented by centrifugation for 3 min in a Beckman Microfuge. The glucose in the supernatant was measured enzymatically. The purpose of the NaF in the assay buffer was to block the uptake of glucose by the spores; more than 98% of the glucose uptake was inhibited by 200 mM NaF (data not shown). Spores were incubated with NaF in the absence of added trehalose to correct for any glucose released from spores.

Purification of trehalase. For some experiments the trehalase activity was partially purified from spore extracts. Membranes and nucleic acids were precipitated by treatment with 50 mM $MnCl_2$ followed by centrifugation at 140,000 × *g* for 90 min. The supernatant containing the trehalase activity was brought to 30% saturation with $(NH_4)_2SO_4$, and the precipitate was pelleted by centrifugation for 20 min at 20,000 × *g* and discarded. The supernatant was brought to 60% saturation with $(NH_4)_2SO_4$, and the precipitate containing the trehalase activity was pelleted by centrifugation, dissolved in 10 mM MES buffer (pH 6.5), and dialyzed three times against 4 liters of 10 mM MES buffer. The extracts were kept at 0 to 4°C throughout the procedures described above. This procedure resulted in a fourfold purification of trehalase activity, with approximately 85% recovery.

Determination of K_m of trehalase. The K_m values of trehalase were measured by using extracts of spores, spores that were germinated for 2 h, and 16-h mycelia. Spores and mycelia were suspended in 10 mM potassium phosphate buffer (pH 6.5) containing 60 mM KCl and disrupted as described above. The cell extracts were passed through 10-ml Sephadex G-25 desalting columns to remove any residual trehalose. Trehalase activity was measured with the desalted extracts in 40 mM MES buffer (pH 6.2) containing 50 mM KCl as described above, except that trehalose was added to final concentrations of 0, 0.5, 1.0, 2.5, 5.0, 10.0, 20.0, 50.0, and 100.0 mM. The K_m values were determined from double-reciprocal plots of velocity versus trehalose concentration. Standard deviations were calculated from three determinations.

Analytical methods. Malic acid dehydrogenase was measured spectrophotometrically. The reaction mixture contained 62.5 μmol of MOPS buffer (pH 7.0), 0.175 μmol of NADH, 2.5 μmol of oxaloacetic acid, and cell extract in a final volume of 1.25 ml. Oxidation of NADH was measured by the change in A_{338}. One unit of activity was defined as the amount of enzyme needed to oxidize 1 μmol of NADH per min at 37°C.

Trehalose and protein were extracted from cells and measured as previously described (13). The trehalose assay involved hydrolysis to glucose by a Tris-insensitive trehalase preparation from a gram-negative soil bacterium.

RESULTS

Properties of trehalase. We reported previously that dormant spores of *S. griseus* metabolize their endogenous trehalose slowly despite the presence of high levels of trehalase activity in spore extracts (14). We examined the trehalase activity of spores in more detail in order to understand this apparent paradox. The level of trehalase

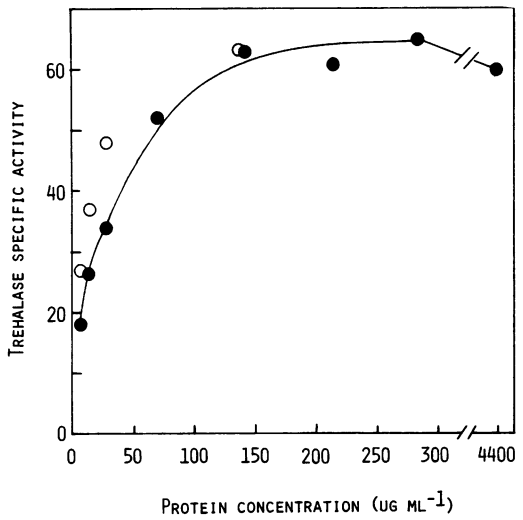

FIG. 1. Effect of concentration of cell extract protein on trehalase specific activity. Spores were disrupted, and trehalase was measured in the presence (○) and in the absence (●) of bovine serum albumin (200 μg/ml).

activity in spores was determined by three different procedures. Spores that were permeabilized with detergent and extracts that were prepared by agitating spores with glass beads had 36 to 38 U of trehalase activity per mg of total spore protein. Spore extracts that were prepared by digesting lysozyme-sensitive spores with lysozyme had 44 U of trehalase activity per mg of protein. Disruption of spores in the presence of 10 mM phenylmethylsulfonyl fluoride or ethylenedinitrilotetraacetic acid and 1.5 mM mercaptoethanol did not alter the level of trehalase activity (data not shown). Permeabilized spores or spore extracts hydrolyzed trehalose at a linear rate immediately following spore disruption or permeabilization.

The properties of trehalase in crude extracts of dormant spores, germinating spores, and mycelia were examined. The trehalase activities in each of the extracts had broad pH profiles with optimal activities at approximately pH 6.2 and half-maximal activities at approximately pH 5.0 and 7.7. The K_m values for trehalose measured in extracts of dormant spores, germinated spores, and vegetative mycelia were 10.6 ± 1.9, 10.7 ± 0.5, and 12.0 ± 1.0 mM, respectively.

We tested the effect on the trehalase activity in spore extracts of various concentrations of various salts in the assay mixture. The activity more than doubled when the concentration of KCl, NaCl, or KPO_4 was increased from 0 to 25 mM, remained nearly constant at concentrations ranging from 25 to 100 mM, and then declined as the concentrations were increased further (data not shown). The effects of the salt concentrations on trehalase activity in extracts of mycelia were nearly identical.

Trehalase-specific activity was dependent on the concentration of crude spore extract protein in the assay mixture (Fig. 1). The specific activity of trehalase remained constant at protein concentrations between 120 and 4,300 μg/ml. However, when the concentration of the crude spore extract in the reaction mixture was decreased below 120 μg of protein per ml, the specific activity of trehalase decreased dramatically. The addition of bovine serum albumin to reaction mixtures containing low levels of spore extract protein increased the level of trehalase activity slightly. Possible explanations for the decreased activity in dilute spore extracts are absorption of the enzyme to glass tube walls or a low level of inhibitory molecules in the assay reagents (e.g., contaminating heavy metals). The increase in activity obtained with added serum albumin is consistent with either possibility.

It is possible that the concentration of some chemical regulates the activity of trehalase in spores. Crude spore extracts were mixed with mycelial extracts or boiled spore extracts in 1:1 ratios. The trehalase activities in mixtures of spore and mycelial extracts were equivalent to the sum of the activities in the individual extracts. The addition of boiled extracts did not inhibit the trehalase activity in spore extracts. A variety of chemicals that might be found in the spores were examined for their effects on trehalase activity. Glutamate, which is present in high concentrations in spores (13), was tested along with isoleucine, leucine, valine, and adenosine, which are required for optimal spore germination in defined media. Addition to the assay mixture of 5 mM $CaCl_2$, 5 mM $MgCl_2$, 50 mM glutamate, 10 mM alanine, 10 mM isoleucine, 10 mM leucine, 10 mM valine, 6 mM adenosine, 6 mM ATP, 1 mM AMP, 1 mM cAMP, 10 mM GTP, 10 mM GMP, 10 mM cGMP, 10 mM glucose, 500 mM trehalose, 50 mM sucrose, 50 mM fructose, 50 mM mannose, 50 mM maltose, 50 mM mannitol, or 50 mM sorbitol, had no significant effect on the trehalase activity of spore extracts. The trehalases of some fungal spores are activated by a cAMP-mediated phosphorylation (20). Incubation of crude extracts of *S. griseus* spores with cAMP and ATP or with alkaline phosphatase did not alter the trehalase activity (data not shown). Likewise, disruption of spores in 10 mM MES buffer (pH 6.2) containing 100 mM NaF to inhibit phosphatase activity did not affect the level of activity measured.

Hydrolysis of endogenous trehalose by trehalase. Crude spore extracts contain both trehalose and trehalase activity. The ability of trehalase in the crude spore extracts to hydrolyze this endogenous trehalose was tested. Dilute spore extracts containing 200 to 1,000 μg of protein per ml contained about 0.5 to 3.0 mM trehalose. This is considerably lower than the K_m of trehalase for trehalose. Trehalose was hydrolyzed slowly in these extracts. In an attempt to mimic the trehalose content of the spore cytoplasm, extracts were concentrated by lyophilization. Samples of spore extracts containing approximately 1.0 mg of spore protein and 900 μg of endogenous trehalose were lyophilized to dryness in test tubes. The amounts of trehalase activity in the extracts and the effect of lyophilization on trehalase activity were determined by diluting the extracts and adding 100 mM trehalose as described in Materials and Methods. Trehalase activity was nearly completely stable to lyophilization followed by rehydration. To measure hydrolysis of endogenous trehalose, duplicate samples of lyophilized extract were hydrated with 10 to 50 μl of distilled water, stoppered to prevent evaporation, and incubated at 33°C. At various times the samples were diluted to a volume of 1.0 ml with hot (100°C) distilled water and boiled for 3 min. The amounts of glucose and trehalose in the samples were measured to determine the rate of hydrolysis of endogenous trehalose. Reaction mixtures containing 21 mg of protein per ml of water hydrolyzed endogenous trehalose at an initial rate of approximately 10.9 nmol/mg of protein per min (Fig. 2). The rate of trehalose hydrolysis decreased during the incubation. This may have been due to the lack of mixing in the gellike hydrated reaction mixture. The decrease in trehalose concentration from about 50 mM to about 20 mM as a result of hydrolysis may also have contributed to the decreased rate of trehalose hydrolysis. Reaction mixtures containing 42 and

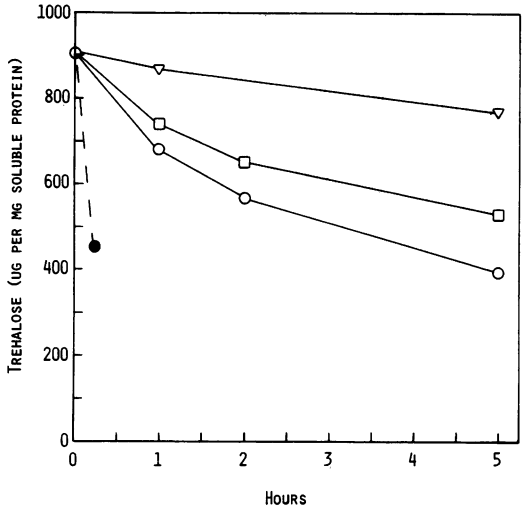

FIG. 2. Hydrolysis of endogenous trehalose by extracts of *S. griseus* spores. Samples of spore extracts containing approximately 1.0 mg of protein and 900 μg of endogenous trehalose were lyophilized to dryness and then hydrated with small volumes of water and incubated at 33°C. The amounts of trehalose and glucose present in the samples were determined at various times. Symbols: ○, 21 mg of protein per ml of water; □, 42 mg of protein per ml of water; ▽, 104 mg of protein per ml of water. The dashed line indicates the rate of trehalose hydrolysis in an assay mixture containing 200 μg of extract protein per ml and 100 mM added trehalose.

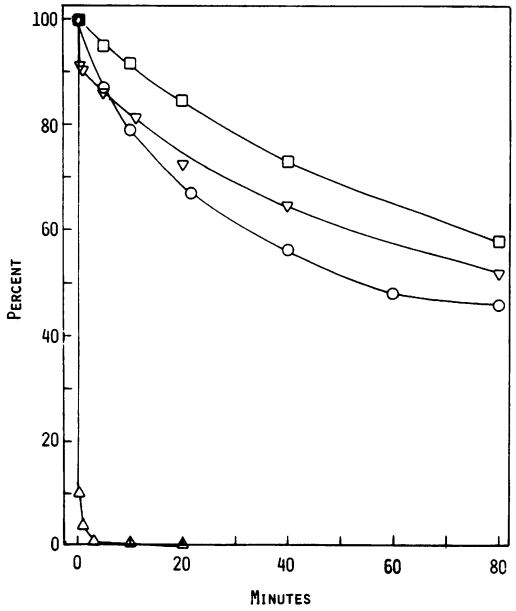

FIG. 3. Effect of incubation in dilute acid on spore viability, trehalose content, and trehalase activity. Spores were incubated in 0.01 N HCl at 0°C. At various times samples were neutralized by dilution with 9 volumes of 100 mM MES buffer (pH 6.3) and dispersed by gentle sonic treatment in a sonic water bath for 2 min. The effects of incubation in acid on viability (○), total trehalase activity (measured by permeabilizing spores with cetyltrimethyl ammonium bromide) (▽), surface-associated trehalase (△), and trehalose content of spores (□) were determined.

104 mg of protein per ml of water hydrolyzed 8.2 and 1.6 nmol of endogenous trehalose per mg of protein per min, respectively. All of these values were significantly lower than the maximal level of trehalase activity in the fully hydrated crude spore extracts measured with added trehalose, which was 80 nmol/mg of protein per min. These data suggest that the specific activity of trehalase was dependent on the hydration state of the reaction mixture, but we do not know how our conditions compare with the hydration state of spore cytoplasm.

Localization of spore trehalase. A possible explanation for the failure of trehalase to hydrolyze trehalose in dormant spores is a physical separation by compartmentalization. The possibility that trehalase is located exterior to the spore membrane was tested by incubating spores with trehalose in the presence of 200 mM NaF to inhibit glucose uptake by spores. Following incubation the spores were sedimented by centrifugation, and the amount of glucose in the supernatant was measured as an index of surface-associated trehalase. Trehalose was hydrolyzed by the spores at a rate of 3.6 nmol/mg of total spore protein per min. This is approximately 10% of the activity found in permeabilized spores or in spore extracts. Washing the spores with 1 M KCl did not remove the surface-associated trehalase activity.

Trehalase in spore extracts is sensitive to exposure to acid (data not shown), whereas spores are relatively resistant to acid (14). We tested the effect of incubating spores in 0.01 N HCl on surface-associated and total trehalase activities. Surface-associated trehalase was nearly completely inactivated by 1.0 min of exposure to acid (Fig. 3). Spores remained viable and germinated normally following inactivation of surface trehalase by this short acid treatment. Total trehalase activity decreased slowly during the 80-min incubation in acid. This decrease paralleled the decreases in spore viability and trehalose content. These data suggest that trehalase and trehalose are located predominately in the cytoplasm of the spores, but a small amount of trehalase activity appears to be located at the spore surface.

The location of spore trehalase was further investigated by using spore protoplasts. Lysozyme-sensitive spores for these studies were obtained from media containing 0.35% glycine. These spores appeared to be identical to spores grown on DMC medium when they were observed by phase-contrast or transmission electron microscopy and germinated normally to produce vegetative mycelia when they were incubated in the complex germination medium. Protoplasts obtained by digestion with lysozyme as described in Materials and Methods were osmotically sensitive but viable. Protoplasts formed in the presence of 0.45 M sucrose regenerated to form vegetative colonies on regeneration medium with efficiencies of up to 60%.

The isolated spore protoplasts were concentrated by centrifugation and lysed by dilution in distilled water. The specific activities of trehalase and of the cytoplasmic marker enzyme malic acid dehydrogenase were nearly the same in extracts prepared by disrupting spores with glass beads and by osmotically lysing protoplasts (Table 1). The levels of recovery of total trehalase and malic acid dehydrogenase activities from osmotically lysed protoplasts were approximately 20% of the levels of recovery of the activities found in extracts of spores. The loss of activity can be explained by the level of efficiency of recovery of protoplasts from spores, which was 20% in this experiment. These data indicate that nearly all of the spore trehalase activity was in the cytoplasm.

Localization of spore trehalose. The possibility that trehalose is located external to the spore membrane was tested by measuring the trehalose content of spore proto-

TABLE 1. Enzyme activities in extracts prepared from spores and spore protoplasts[a]

Extract	Sp act		Total activity (%)	
	Trehalase[b]	Malate dehydrogenase[c]	Trehalase	Malate dehydrogenase
Spore extract	76	5.0	100	100
Protoplast lysate	80	5.6	22	17

[a] Spores were extracted by agitation with glass beads as described in Materials and Methods. Protoplasts were prepared in P2 buffer containing 1.25 M sorbitol, washed in P2 buffer containing 1.0 M KCl, and lysed by suspension in distilled water at 0 to 4°C.

[b] Expressed as nanomoles of trehalase hydrolyzed per milligram of protein per minute at 33°C.

[c] Expressed as micromoles of NADH oxidized per milligram of protein per minute at 37°C.

plasts (Table 2). Protoplasts formed in buffer containing relatively low levels of osmotic stabilizers (0.3 M KCl, 1.25 M sorbitol, or 0.45 M sucrose) retained only 4 to 21% of the trehalose content of intact spores. Protoplasts formed in buffer containing higher concentrations of sorbitol or sucrose retained virtually all of their trehalose content. It was not possible to obtain protoplasts in buffer containing higher concentrations of KCl, presumably because of inhibition of lysozyme activity. The specific activities of trehalase were nearly identical in lysates from protoplasts formed in buffer containing 0.3 M KCl and in buffers containing the various concentrations of sucrose or sorbitol (data not shown).

There are several possibilities for the observed decreased trehalose contents of protoplasts formed in buffers containing low concentrations of osmotic stabilizers. These protoplasts might have been more permeable to trehalose than the protoplasts stabilized by the higher concentrations of osmotic solutes, and thus trehalose would have been released. It is also possible that the level of hydration of the protoplast cytoplasm was increased during protoplasting in the low concentrations of osmotic stabilizers, resulting in increased trehalose hydrolysis within the protoplasts. To distinguish between these two possibilities, protoplasts were formed in P buffer containing 0.3 M KCl and 20 mM Tris buffer (pH 7.0), and the total amount of trehalose present was compared with the amount present in spores prior to protoplasting. Any trehalose released by protoplast lysis or leakage would not have been hydrolyzed because of the presence of Tris, a trehalase inhibitor. The protoplasts and residual spore debris were sedimented. The supernatant fraction was heated to 100°C for 5 min and saved for trehalose analysis. The pellet was suspended in cold distilled water and incubated for 5 min at 0°C to osmotically lyse the protoplasts. Intact spores and debris were sedimented by centrifugation and suspended in distilled water. The pellet and protoplast lysate fractions were heated at 100°C for 30 and 5 min, respectively. The amounts of trehalose in the supernatant remaining after removal of protoplasts by sedimentation, in the protoplast lysate fraction, and in the pellet obtained after protoplast lysis and centrifugation were determined. The protoplast lysate fraction contained approximately 5% of the trehalose present in the original untreated spores. The protoplasting buffer and the final pellet (intact spores and wall debris) contained approximately 59 and 11%, respectively, of the amount of trehalose found in untreated spores. Only 75% of the original amount of trehalose was accounted for in these fractions. The remainder was apparently metabolized during protoplast formation. The apparent increase in trehalose metabolism during protoplasting may have been the result of changes in the state of the cytoplasm following wall removal. Intact spores incubated at 30°C for 3 h in this buffer without lysozyme retained more than 95% of their trehalose. The large amount of trehalose found in the buffer after protoplast formation may have originated from lysis of protoplasts during wall digestion since only approximately 23% of the spores were converted to stable protoplasts. Any trehalose released by lysis of protoplasts would not have been degraded because of the Tris buffer, which inhibited trehalase.

TABLE 2. Trehalose contents of protoplasts of spores prepared in various stabilizing media

Extract	Trehalose content (μg/mg of protein)
Spore extract[a]	292
Protoplast lysates[b]	
0.3 M KCl	10
1.25 M sorbitol	44
0.45 M sucrose	61
2.5 M sorbitol	120
1.25 M sucrose	272
4.0 M sorbitol	318

[a] Spores were disrupted by agitation with glass beads as described in Materials and Methods.

[b] Protoplasts were prepared with the osmotic stabilizers as described in Materials and Methods and were lysed by dilution in distilled water.

To determine whether protoplasts containing a large amount of trehalose would release or hydrolyze trehalose when they were diluted with buffer having a lower osmotic strength, protoplasts suspended in P2 buffer containing 1.25 M KCl were slowly diluted with P2 buffers containing 0.85 and 0.45 M KCl. The fragile protoplasts invariably lysed during these dilutions, making the analysis impossible to perform.

Stability of trehalose in spore protoplasts. Trehalose in dormant spores incubated in buffer is degraded only slowly (14). We were interested in the stability of trehalose in spore protoplasts. Protoplasts (4×10^8 protoplasts per ml) in P2 buffer–1.0 M KCl containing 272 μg of trehalose per mg of protein were incubated at 33°C for 4 h while they were being shaken at 50 rpm. The protoplasts were then collected by centrifugation and lysed in distilled water. The protoplast lysate contained 242 μg of trehalose per mg of protein. The trehalase activity in the protoplasts should have been sufficient to hydrolyze all of the trehalose within 30 min under optimal conditions. The solute concentration in the cytoplasm may partially account for the apparent inhibition of trehalase within dormant spores or spore protoplasts. Trehalase activity in spore extracts or protoplast lysates was inhibited by 84% by P2 buffer containing 1.0 M KCl, but it is obvious that this buffer does not closely mimic the solute composition of the cytoplasm.

Fractionation of spore protoplasts. We designed a series of experiments to determine whether trehalose and trehalase are localized in different subcellular compartments of protoplasts. Protoplasts prepared in P buffer containing 1.25 M sucrose were osmotically lysed in distilled water and centrifuged at 100,000 × *g* for 90 min. All of the original trehalose and trehalase activity were found in the supernatant fraction. The distribution of trehalose and trehalase activity were also determined following disruption of protoplasts in osmotically stabilizing buffer. Protoplasts suspended in P2 buffer containing 1.25 M KCl were disrupted by sonication for 10 min at 0°C and centrifuged for 5 min at 10,000 × *g* and then

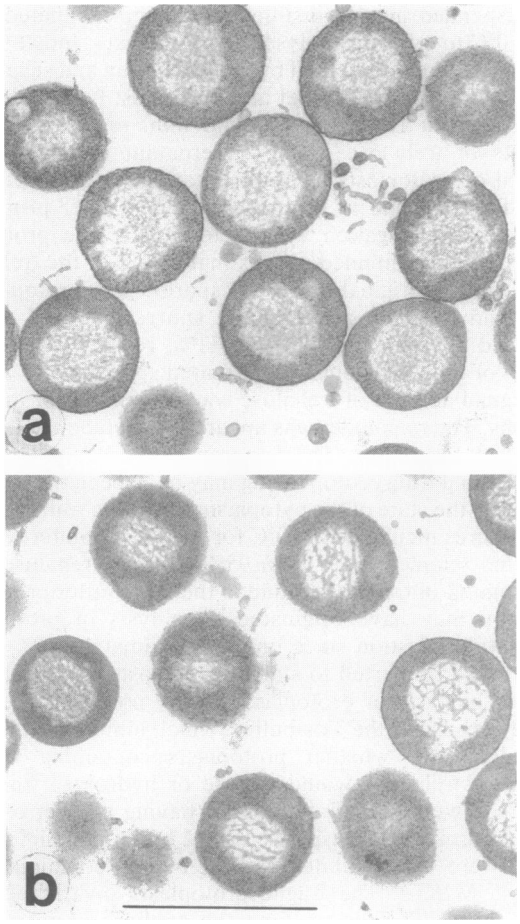

FIG. 4. Transmission electron micrographs of thin sections of spore protoplasts. (a) Protoplasts formed in 0.4 M sucrose. (b) Protoplasts formed in 1.25 M sucrose. Bar = 1 μm.

for 90 min at 140,000 × *g*. Virtually all of the trehalose and trehalase activity were found in the supernatant.

Ultrastructure of spore protoplasts. Electron micrographs of sectioned spore protoplasts revealed normal cytoplasmic contents surrounded by a cytoplasmic membrane (Fig. 4). We detected no obvious morphological differences between protoplasts formed in buffers containing high and low concentrations of sucrose or sorbitol and thus having large and small amounts of trehalose. Likewise, no obvious differences between spores containing high and low levels of trehalose were observed (data not shown).

DISCUSSION

Spores of *S. griseus* contain large amounts of the disaccharide trehalose (13). Trehalose is degraded very slowly during periods of dormancy but is rapidly hydrolyzed during germination (14). Extracts of spores also contain a high specific activity of the enzyme trehalase (14). The apparent K_m of *S. griseus* trehalase for trehalose of approximately 11 mM and the pH optimum of about 6.2 are similar to the values for the 80-fold-purified mycelial trehalase of *Streptomyces hygroscopicus* (8).

The spores of many species of fungi also contain large pools of trehalose that are utilized slowly by dormant spores but are rapidly mobilized during germination (20). Different groups of fungi exhibit different mechanisms for the regulation of trehalose mobilization. In some fungi the specific activity of trehalase increases dramatically prior to trehalose degradation. A cAMP-dependent protein kinase apparently generates the active form of trehalase from the inactive form by phosphorylation (3, 20). The spores of other fungi contain both trehalose and active trehalase (20). In some cases localization of trehalose and trehalase in separate compartments accounts for the apparent coexistence. Trehalase is localized in the spore walls of *Neurospora* and *Schizosaccharomyces* spores (7, 11) and in membrane-enclosed vesicles or vacuoles of *Saccharomyces* cells and *Dictyostelium* spores (12, 17), whereas trehalose is free in the cytoplasm of *Saccharomyces* cells (12). The barriers separating trehalose from trehalase in these organisms seem to be removed or altered during germination. In some other fungi the mechanism of regulation remains uncertain (20). Dormant fungal spores have a low water content (21). The water content of *Phycomyces blakesleeanus* spores increases during germination at about the same time that trehalose mobilization occurs. Increased hydration may contribute to the increased metabolism of trehalose by germinating spores.

Trehalose degradation in *S. griseus* spores does not seem to be regulated by activation of trehalase since the levels of trehalase activity are the same in extracts of dormant and germinating spores. We found no evidence of inhibitors or activators of trehalase activity in cell extracts.

Several lines of evidence indicate that trehalose and most of the spore trehalase activity are located in the cytoplasm. The high K_m for trehalose suggests that trehalase functions in the cytoplasm, in which we estimate the trehalose concentration in dormant spores to be at least several hundred millimolar. Spores retain their trehalose and most of their trehalase activity during brief incubation in dilute acid. Protoplasts formed from spores contain both trehalose and trehalase activity. Maintenance of trehalose and trehalase in separate subcellular compartments seems unlikely since no obvious organelles were observed in electron micrographs of thin sections of spores or spore protoplasts. Furthermore, neither trehalose nor trehalase activity was sedimented by ultracentrifugation following sonic disruption of protoplasts in osmotically stabilized buffer.

If trehalose and trehalase coexist in the cytoplasm, the conditions of the dormant spore cytoplasm must prevent the potential trehalase activity from being realized. Dormant spores appear bright when they are observed by phase-contrast microscopy. This brightness may be an indication of low water content (16). Low water content in the spore cytoplasm might inhibit trehalase activity. Spores of *S. griseus* are sufficiently hydrated to allow some enzymatic reactions to proceed. Spores maintain the ability to metabolize glucose and synthesize trehalose (13, 14). Trehalase activity might be more sensitive to the hydration level of the cytoplasm than are the enzymes involved in glucose catabolism or trehalose synthesis.

Several lines of evidence suggest that the water content of streptomyces spores increases during germination. As spores germinate, their spore walls decrease in thickness (6, 18). Weakening of the wall and its stabilizing pressure on the spore protoplast would allow an influx of water, spore swelling, and an efflux of solutes as the osmotic balance between the cytoplasm and the surrounding fluid is reestablished. Spores rapidly lose their refractility, as shown by decreased phase brightness and by a decrease in the optical density of spore suspensions during germination (6, 10). The decreased refractility probably results from increased hydration of the spores. The phase-dark spores increase in volume

before germ tubes are formed. The dry weight of *Streptomyces antibioticus* spores remains constant during phase darkening and swelling, suggesting that swelling is mainly due to hydration of the cytoplasm (6). The cytoplasm of germinated spores appears to be less condensed than that of dormant spores (6, 18), suggesting increased hydration during germination. The increased hydration could be partially responsible for the increased metabolic rate of germinated spores (5, 6) and the increased trehalose hydrolysis observed.

Spore protoplasts formed in buffer containing large amounts of solute (1.25 M sucrose) contained large amounts of trehalose. Protoplasts formed in buffer containing lower concentrations of solutes (0.3 M KCl or 0.45 M sucrose) retained much lower levels of their trehalose. Similar results were obtained for protoplasts formed in buffer containing sorbitol as the osmotic stabilizer, although higher levels of sorbitol were necessary to stabilize protoplasts and to allow retention of trehalose. The low content of trehalose in protoplasts in buffer containing low levels of solute may have been due to leakage from the protoplasts or to increased hydrolysis within the protoplasts. Protoplasts apparently did metabolize a significant portion of their trehalose during formation in buffer having a low solute concentration. A low level of hydration of the cytoplasm of protoplasts formed in buffers containing high concentrations of solutes might have inhibited trehalase activity, whereas increased hydration of the cytoplasm of protoplasts in buffers containing low levels of solutes could have allowed trehalose hydrolysis.

The mechanism of regulation of trehalose metabolism by *S. griseus* spores remains uncertain. Trehalose is hydrolyzed slowly by nongerminating spores but rapidly by germinating spores (14). In this paper we show that it is unlikely that compartmentalization in dormant spores separates trehalase and trehalose. No evidence for inhibitors or activators of trehalase was found. The most logical explanation is that increased hydration of the spore cytoplasm during germination facilitates trehalose hydrolysis. Rapid hydration would also occur during spore permeabilization or disruption and could account for the large amount of trehalase activity observed in extracts of dormant spores.

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