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Regulation of Trehalose Mobilization in Fungi

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INTRODUCTION

Trehalose (α-D-glucopyranosyl α-D-glucopyranoside) is an important storage compound in vegetative cells and spores of fungi. Biosynthesis of trehalose is mediated by trehalose-6-phosphate synthetase and trehalose-6-phosphate phosphatase. The only enzyme known to be involved in trehalose hydrolysis in fungi is trehalase (α,α-trehalose 1-D-glucohydrolase, EC 3.2.1.28). The regulation of trehalose synthesis in fungi is quite complex and has attracted a lot of attention, especially in relation to the sporulation process (e.g., references 85, 177). Trehalose mobilization, on the other hand, was long considered to be a relatively simple process. In many fungi, trehalase appeared to be a purely hydrolytic enzyme, and compartmentation between trehalose and trehalase was thought to be the only control mechanism for trehalose hydrolysis. Recently, however, evidence has been obtained for the involvement of cyclic adenosine 3′,5′-monophosphate (cAMP)-induced phosphorylation in the regulation of trehalase in certain fungi. This has raised a lot of new interest in the regulation of trehalose mobilization, especially because of its apparently close relationship with other cAMP-induced phosphorylation processes. In addition, trehalose mobilization is an important biochemical event in fungal spore germination and related developmental processes, such as the resumption of growth in resting cells. Hence, detailed elucidation of its regulation might also lead to an understanding of the complex interrelationships that exist in the regulation of metabolism during fungal development.

This review summarizes the available information on fungal trehalases and focuses on the two principal mechanisms proposed for the regulation of trehalose mobilization. From the data reviewed, it appears that two distinct types of trehalases can be discerned, a nonregulatory enzyme and a regulatory enzyme. In fungi with the first type of enzyme, compartmentation of trehalose and trehalase might be responsible for the control of trehalose breakdown. In fungi with the latter type of enzyme, trehalose mobilization appears to be regulated by cAMP-induced phosphorylation of trehalase.

TREHALOSE

Occurrence in Fungi

Trehalose is the most widely distributed disaccharide in fungi. It is very common in both vegetative and reproductive stages (47). In vegetative structures, it is usually found together with sugar alcohols and glycogen (14, 31, 93). This is also true of reproductive structures, but in this case trehalose is often present in much higher concentrations than the other storage carbohydrates (25, 76, 101, 154, 171, 175, 181).

Accumulation and Mobilization in the Life Cycle

The accumulation of trehalose in fungi appears to be associated in general with periods of reduced growth rate. In particular, trehalose synthesis is very intensive during differentiation processes and starvation periods. Synthesis of trehalose during sporulation and the resulting accumulation of trehalose in the spores have been observed in *Aspergillus niger* (119), *Dictyostelium* spp. (24, 25, 30, 64, 150, 155), *Phycomyces blakesleeanus* (154; J. M. Thevelein, unpublished results), *Schizosaccharomyces pombe* (77), and *Sac-*

charomyces cerevisiae (151), and during *Neurospora crassa* conidiation (65). In addition, trehalose has been found in spores of *Myrothecium verrucaria* (101), *Trichoderma reesei* (99), and *Pithomyces chartarum* (3); uredospores of *Puccinia* spp. (40, 147, 178, 218); ascospores of *Neurospora tetrasperma* (175); conidia of *Penicillium* spp. (7, 44); and spores of *Schizophyllum commune* (1) and *Helminthosporium* spp. (104). Trehalose is also an important storage carbohydrate of sclerotia. It has been found in large quantities in sclerotia of *Sclerotinia* spp. (32, 34, 86, 92) and *Claviceps* spp. (34, 136).

Starvation for glucose (60, 90, 94, 126, 129, 131, 137, 143, 169), nitrogen (90, 94), phosphate (94), and sulfur (94) induces trehalose accumulation in yeasts. The increase of the trehalose level starts just before the arrest of growth and continues for several hours in the resting cells. A typical example is the synthesis of trehalose at the end of the first exponential phase of growth on glucose (60, 94, 126, 129, 131, 137, 143, 169). When the resulting glucose-starved cells are given very low doses of glucose, e.g., by continuous feeding of glucose at a low concentration into the medium, the cells continue to accumulate trehalose for many hours (60). Several mutants with altered trehalose accumulation patterns have been isolated (35, 132, 133).

Correlations between growth rate and trehalose accumulation have been observed directly in continuous cultures of *Saccharomyces cerevisiae* (90), *Aspergillus niger* (119), and *Aureobasidium pullulans* (22). Slower growth rates were always associated with stronger trehalose accumulation. Using synchronized yeast populations, Küenzi and Fiechter (89) also found a positive correlation between budding activity and trehalose mobilization during the cell cycle itself. Trehalose mobilization took place within the period that immediately preceded the swelling of the buds, whereas trehalose accumulation took place during the maturation phase of the cell cycle. In *Aureobasidium pullulans*, the reduction of growth rate and the accumulation of trehalose were associated with a morphological transition from mycelial growth to yeast growth, i.e., multiplication by budding (22). In *Aspergillus niger*, reduced growth rate and trehalose accumulation were associated with increasing conidiation of the cultures (119). Trehalose accumulation in fungi is not solely confined to periods of differentiation or nonproliferation. Growth of yeast cells on maltose or galactose (131) and growth on glucose at high temperature (60, 61) also result in a high trehalose content. The growth rate in these cases, however, is also lower compared with growth on glucose at normal temperatures.

Whereas reduced growth rate is associated with trehalose accumulation, stimulation of growth and especially the induction of growth in resting stages is associated with rapid mobilization of trehalose. Breakdown of storage trehalose is usually one of the main biochemical events during early germination of fungal spores. This has been observed in *Dictyostelium discoideum* (25, 78), *Phycomyces blakesleeanus* (154, 186), *Schizosaccharomyces pombe* (76), *Schizophyllum commune* (1), *Neurospora* ascospores (46, 95, 175), *Saccharomyces cerevisiae* (153), *Pichia pastoris* (9, 181), *Aspergillus oryzae* (74), *Myrothecium verrucaria* (101), and uredospores of *Puccinia graminis tritici* (218). Germination of *Claviceps* sclerotia is also associated with mobilization of storage trehalose (34). A close correlation was found between conditions of germination and the extent of trehalose breakdown in ascospores of *Pichia pastoris* (181). The addition of both glucose and a nitrogen source is necessary for complete germination. Glucose alone causes partial trehalose mobilization followed by resynthesis of trehalose. Glucose combined with a nitrogen source causes much stronger trehalose breakdown and no resynthesis.

When growth is induced in stationary-phase cultures of *Saccharomyces cerevisiae* (17, 127, 169, 192, 193, 203) and *Candida albicans* (4), the previously accumulated trehalose is rapidly mobilized. In *Saccharomyces cerevisiae*, trehalose breakdown can be induced by the addition of glucose only. In this case, however, it is rapidly followed by resynthesis, often resulting in a final net increase of the trehalose content. The addition of nitrogen sources enhances trehalose breakdown and prevents resynthesis (17, 192, 193, 203). The situation in vegetative cells therefore, is analogous to the situation in yeast ascospores mentioned before (181). In general, one could state that resting yeast cells and yeast ascospores always accumulate trehalose when they are fed with glucose only. With high glucose concentrations, however, there is first a temporary breakdown of trehalose followed by resynthesis. In the presence of glucose, nitrogen sources stimulate trehalose breakdown and prevent resynthesis. Hence, glucose apparently serves as the trigger for the induction of trehalose mobilization, whereas nitrogen sources appear to control the continuation of trehalose mobilization. In wild-type *Saccharomyces cerevisiae*, nitrogen has no effect on trehalose accumulation in the absence of glucose. In certain yeast mutants (133) and in nitrogen-deficient *Torulopsis* yeasts (219), however, nitrogen alone is able to inhibit trehalose accumulation or to induce trehalose breakdown. An interesting correlation might also exist between the degree of catabolite repression exerted by a certain sugar and the capacity of nitrogen sources to induce trehalose breakdown in its presence (131, 133).

Resumption of growth on ethanol during the second logarithmic growth phase of vegetative yeast cells is also associated with trehalose mobilization (126, 137, 143, 166). Prolonged starvation (130 days) of trehalose-containing yeast cells also causes gradual mobilization of the trehalose, but in this case the disappearance of trehalose is extremely slow (94). Correlation between growth induction and trehalose hydrolysis was also observed in *Aureobasidium pullulans* by Catley and Kelly (22). During the temporary reduction of the growth rate associated with the budding of hyphae, trehalose accumulation occurred, whereas subsequent intensification of growth was associated with trehalose mobilization. Increases in the growth rate are also observed upon addition of glucose to yeast cells grown on maltose or galactose. In this case, the cellular trehalose content is also rapidly depleted (131).

In *Dictyostelium discoideum*, trehalase and trehalose are both present in the fruiting bodies. Trehalase activity is much higher in the stalk cells than in the spores. Besides, the activity increases in the stalk cells from the top of the fruiting body to the bottom. Trehalose shows a concentration gradient in the opposite direction. Lysing stalk cells contain high, transient glucose levels which are probably derived from trehalose (79, 214, 217).

Function of Trehalose

From the above-mentioned results, trehalose appears to serve mainly as a storage carbohydrate during periods of nonproliferation. This applies to the life cycle, the cell cycle, and other conditions under which cessation of growth occurs, such as (for example) starvation. During the life cycle, trehalose is accumulated in the reproductive stages and mobilized during germination. The importance of trehalose for spore germination is most obvious for spores that are

able to germinate in distilled water, such as *Neurospora* ascospores (170) and *Dictyostelium* spores (37). The importance of trehalose for spores that are able to germinate only in sugar-containing media is less obvious, especially since the amount of glucose produced in trehalose breakdown can be relatively small compared with the glucose taken up from the medium, at least in rich germination media (9, 181). The internal trehalose reserve in these spores might be more important for germination under poor conditions. In the cell cycle trehalose is accumulated during the maturation phase and is mobilized before cell division. Storage and mobilization of energy reserves at specific times appear to be a general aspect of the cell cycle (8, 209). During starvation, trehalose is formed upon depletion of essential substrates in the medium. It is mobilized again during the resumption of growth in a more complete medium. Apparently, it serves as a storage carbohydrate for survival during the starvation period. The importance of trehalose for the viability of resting yeast cells is well known because of its industrial importance (138, 169). It was shown by Lillie and Pringle (94) that during prolonged starvation, the survival of the yeast cell ultimately depends on the trehalose level. Trehalose might also be important for the provision of energy and intermediates during another resting condition, i.e., spore dormancy. It was shown by Barton et al. (9) that the trehalose reserve in dormant yeast spores can be used for the supply of energy. The adenosine 5'-triphosphate (ATP) concentration was suggested to be the controlling factor for the mobilization of trehalose during dormancy (181). In other spores, such as the ascospores of *Neurospora* spp., lipids appear to serve as the endogenous substrate during dormancy whereas trehalose is only used during germination (95).

The high trehalose levels in fungal spores are also believed to enhance the resistance of the spores under extreme environmental conditions, such as high and low temperatures, desiccation, etc. Direct evidence on this matter, however, is scarce. Emyanitoff and Wright (48) were able to show that *Dictyostelium* spores are afforded more protection by higher trehalose levels. They succeeded in growing spores with different trehalose levels and showed that the higher trehalose levels enhanced the heat resistance of the spores considerably. Glycogen, on the other hand, did not seem to play a role. The possible importance of trehalose in enhancing spore resistance might explain why glycogen is rarely used as a storage carbohydrate in spores. Mannitol, on the other hand, can be expected to give protection similar to that given by trehalose. It is also a common constituent of spores and is sometimes present in even higher concentrations than trehalose (59, 93).

TREHALASE

The only enzyme known to be responsible for trehalose hydrolysis in fungi is trehalase (47). Trehalose phosphorylase, discovered in *Euglena gracilis* (10, 102), is also capable of mobilizing trehalose. Phosphotrehalase, discovered in *Bacillus popilliae* (12), is able to cleave trehalose-6-phosphate. There is no evidence, however, for the occurrence of these enzymes in fungi.

Nonregulatory and Regulatory Trehalases

A survey of the literature (Table 1) shows that fungal trehalases can be divided relatively easily into two groups. The first group contains trehalases with an acid pH optimum (between pH 3.5 and 5.5) and high heat stability (no significant inactivation when heated in vitro for a few minutes at 50°C). Rapid activity changes of trehalase (as measured in cell extracts) during periods of fast trehalose mobilization have never been observed in fungi containing such a trehalase. In addition, no evidence has ever been obtained for activation of these trehalases by protein phosphorylation. I propose calling these enzymes nonregulatory trehalases. The acid pH optimum and the high stability fit with the purely hydrolytic character of these enzymes. This first group of trehalases is found in species of slime molds, the zoosporic fungi, the ascomycetes, the basidiomycetes, and the deuteromycetes, but not the zygomycetes.

A second type of trehalase has been found up to now only in a few species: *Phycomyces blakesleeanus, Mucor rouxii, Pichia pastoris, Piptocephalis* spp. *Saccharomyces cerevisiae*, and a *Saccharomyces* hybrid (Table 1). The genera *Phycomyces, Mucor*, and *Piptocephalis* belong to the *Mucorales* (zygomycetes), whereas *Pichia* and *Saccharomyces* are closely related yeasts, belonging to the ascomycetes. The trehalase of these fungi has a neutral pH optimum (between 6 and 7.5) and a low heat stability (substantial inactivation when heated in vitro at 50°C for a few minutes). In *Phycomyces blakesleeanus, Mucor rouxii, Pichia pastoris*, and *Saccharomyces cerevisiae*, periods of fast trehalose mobilization are always associated with both a rapid, drastic activation of trehalase and a rapid increase of the cAMP level. In addition, evidence for the activation of trehalase in vitro by AMP-dependent protein phosphorylation was obtained for these fungi. I propose calling these enzymes regulatory trehalases. The neutral pH optimum and the low stability fit in with the regulatory, and therefore presumably more complex character of these enzymes.

The only exceptions to this scheme are two commercial strains of *Saccharomyces cerevisiae* studied by Panek and Souza (134) and Kelly and Catley (81). Trehalase from the first strain was reported to have an acid pH optimum and a high heat stability; trehalase from the second strain had an acid pH optimum. These *Saccharomyces* strains are not, however, true exceptions since no data are available on the course of their trehalase activity during periods of trehalose mobilization. The possibility of in vitro activation of trehalase by phosphorylation has also not been investigated in these strains. It cannot be excluded that both the nonregulatory and regulatory trehalases occur in *Saccharomyces cerevisiae*. The distribution of the two forms might depend on the strain and perhaps also on the growth conditions. In *Coprinus lagopus*, two pH optima were observed for trehalose hydrolysis: an acid pH optimum (around 3.5) and an alkaline pH optimum (around 8). Some variation was observed in these optima with extracts from different stages of the life cycle. The heat stability of the acid trehalase was higher than that of the alkaline trehalase. According to the authors, the alkaline trehalase might have been a general α-glucosidase with broad substrate specificity (145).

Another characteristic of the regulatory type of trehalase might be a higher K_m than that for the nonregulatory trehalases. The K_m values of the nonregulatory trehalases fall in the range of about 0.5 to 3 mM (70, 74, 81, 82, 108, 109, 128, 134, 139, 208, 211). K_m values reported for regulatory trehalases are 4.5 (88), 10 (6), and 55 (198) mM. More data will be needed, however, before any firm conclusion can be drawn on the existence of a correlation between the K_m and the type of trehalase.

When the fungal trehalases are divided in this way into two groups, a clear correlation emerges with the different mechanisms that have been proposed in the literature for the regulation of trehalose breakdown (see below). This classifi-

TABLE 1. Properties of fungal trehalases

Species	Rapid activation of trehalase during trehalose mobilization[a]	pH optimum[b]	Heat stability[c]	In vitro evidence for activation by phosphorylation
Nonregulatory trehalases				
Aspergillus oryzae	No (74)	Acid (74)	High (74)	
Aureobasidium pullulans (*Pullularia pullulans*)		Acid (109); pH 5.4 (22)[d]		
Candida albicans	No (4)	Acid (4)		
Candida tropicalis		Acid (97)	High (97)	
Coprinus lagopus		Acid and alkaline (145)	High (145) (acid > alkaline)	
Dictyostelium discoideum	No (78)	Acid (23, 82)	High (23, 82)	
Lagenidium sp.		Acid (108)	High (108)	
Myrothecium verrucaria		Acid (101)[e]		
Neurospora crassa and *N. tetrasperma*	No (172, 221)	Acid (68, 70, 141, 176)	High (68, 70, 141, 222)	
Saccharomyces cerevisiae		Acid (81, 134); pH 5.5 (80)[d]	High (134)	
Schizophyllum commune		Acid (213)	High (213)	
Schizosaccharomyces pombe	No (76)	pH 5.6 (76)[d]		
Sclerotinia sclerotiorum		Acid (211)	High (211)	
Thermomyces lanuginosus		Acid (139)	High (139, 141)	
Trichoderma reesei		Acid (208)	High (208)	
Regulatory trehalases				
Mucor rouxii	Yes[f]	Neutral[f]		Yes[f]
Phycomyces blakesleeanus	Yes (42, 186, 199)[g]	Neutral (199)	Low (199)	Yes (204)
Pichia pastoris	Yes (181)[h]	Neutral[i]	Low[j]	Yes[i]
Piptocephalis spp.		Neutral (49)		
Saccharomyces carlsbergensis		Neutral (88)	Low[k] (88)	
Saccharomyces cerevisiae	Yes (203)	pH 6 (122); pH 6.2 (212); pH 7 (89, 96, 203, 207)[d]		Yes (96, 122, 196, 203, 207, 212)
Saccharomyces spp. hybrid		Neutral (6)	Low (6)	

[a] For regulatory trehalases, "yes" indicates rapid increase in cAMP level in addition to rapid activation of trehalase during trehalose mobilization.
[b] Acid, pH optimum between pH 3.5 and 5.5; neutral, pH optimum between pH 6 and 7.5.
[c] High, No significant inactivation when heated for a few minutes at 50°C in vitro; low, substantial inactivation when heated for a few minutes at 50°C in vitro.
[d] pH optimum not shown or mentioned, but trehalase assay carried out at the indicated pH.
[e] Measured with permeabilized spores.
[f] M. Dewerchin and J. M. Thevelein, unpublished results.
[g] Increase in cAMP level: R. Van Mulders and A. Van Laere, unpublished results.
[h] Increase in cAMP level: J. M. Thevelein, Arch. Microbiol., in press.
[i] J. M. Thevelein, unpublished results.
[j] J. M. Thevelein, Curr. Microbiol., in press.
[k] Inferred from the optimum temperature which was 30°C.

cation also provides, in my opinion, a good basis for future research. The regulatory type of trehalase appears to be present in the zygomycetes and in certain yeast genera which might be closely related to the zygomycetes. The nonregulatory type of trehalase appears to be present in all other fungi. Although it is still too early to derive any taxonomic or evolutionary conclusions from this distribution, future work along these lines might turn out to be very interesting.

Activity During the Life Cycle

Nonregulatory trehalases. The most detailed studies of the course of trehalase activity during the fungal life cycle were carried out with species of *Dictyostelium* and *Neurospora*. In *Dictyostelium* spp., trehalase activity, as measured in cell extracts, increases during spore germination and reaches a maximum during the emergence of the myxamoebae from the spore cases (24, 26, 38). The trehalase activity remains approximately constant in free-living myxamoebae (24). Aggregation of the vegetative myxamoebae upon starvation is associated with secretion of trehalase into the medium, which results in a drastic decrease in the intracellular activity (24, 83, 84, 152). Commitment to aggregation is enough to induce trehalase secretion since aggregation itself can be inhibited without preventing the secretion of trehalase (24). During formation of the fruiting bodies, trehalase activity increases again and reaches a value of between 50 and 200% of the value found in free-living myxamoebae (83, 84, 152). The increase in the activity is restricted completely to the stalk cells of the fruiting body. In addition, trehalase activity increases from the top of the fruiting body to the bottom; the concentration of trehalose increases in the opposite direction (79, 214). Two major isozymes of *Dictyostelium* trehalase were discovered by Killick (83). Isozyme I is present in vegetative myxamoebae and is secreted into the medium by aggregating cells. Isozyme II is the major isozyme during fruiting body formation and is at that stage also the major isozyme secreted into the medium. According to Hames and Ashworth (64), cells grown axenically possess at all stages of the life cycle a 10-fold higher trehalase activity than cells grown on bacteria, although the overall pattern of activity is the same. Preliminary evidence has been present-

ed that the increase of the trehalase activity during spore germination is due to new synthesis (36, 38). For the other stages of the life cycle, no information is available concerning the molecular basis of the modulation of trehalase activity (85).

The specific activity of trehalase in *Neurospora* is highest in conidia and lowest in ascospores (71). The activity in conidia declines for some time during germination, whereas the activity in ascospores increases during germination. Development of the mycelium and subsequent conidiation are associated with a continuous increase in trehalase activity, resulting in maximal activity in the conidia. The small amount of trehalase activity located in the cell wall does not follow the pattern of the bulk of activity in the soluble fraction (71). Heavily conidiating strains of *Neurospora* develop higher trehalase activity than poorly conidiating strains, whereas aconidial strains show virtually none. Suppression of conidiation in certain media also leads to lower levels of trehalase activity (65). Subsequent experiments, however, showed that the increase in the trehalase activity was not responsible for the onset of conidiation. High trehalase activity could also be obtained in the absence of any conidiation (65). According to Urey (197), conidia can be formed without any increase in trehalase activity. This was confirmed by the isolation of trehalaseless mutants of *Neurospora crassa* which were apparently still able to sporulate (156, 174). Whether the observed changes of the trehalase activity during the life cycle of *Neurospora* are entirely due to de novo synthesis of the enzyme is not known. The trehalaseless mutants isolated by Sussman and colleagues (57, 174) were found to possess a protein inhibitor of trehalase. Indications were obtained that the protein might have been a precursor of trehalase. Since the inhibitor appeared to be present also in wild-type ascospores and wild-type mycelium containing low trehalase activity (57), the actual trehalase activity present in *Neurospora* might be determined (in part) by the degree of post-translational processing of the enzyme.

Sporulation in *Aspergillus niger* (119) and *Schizosaccharomyces pombe* (77) and hyphal budding in *Aureobasidium pullulans* (22) are also associated with a sharp increase in trehalase activity. In these cases, however, it is not known whether the increase is necessary for the initiation or progress of conidiation. It should be kept in mind also that these increases in trehalase activity all occur simultaneously with a strong accumulation of trehalose. In *Thermomyces lanuginosus*, on the other hand, trehalase activity was found to be higher in nonsporulating cultures than in sporulating cultures (140).

In vivo activation of regulatory trehalases. The interconversion of trehalase from a form with low activity to a form with a 10-fold higher activity was discovered originally by Van Assche et al. (199) as an in vivo heat activation of the enzyme in *Phycomyces blakesleeanus* sporangiospores. The discovery was made because these spores need a heat shock for the induction of germination, and it had been found before that early germination was associated with rapid trehalose mobilization (154). *Phycomyces* spores can also be activated by an acetate pretreatment, and during this treatment the trehalase activity also increases 10-fold (42).

Subsequently, glucose-induced activation of trehalase was discovered in stationary-phase cells of yeast by van der Plaat (203). In this case, the omission of nitrogen led to faster decay of the trehalase activity afterwards. Indirect evidence for the activation of trehalase by glucose in yeasts was obtained before by Souza and Panek (166). They observed stimulation of fermentation on exogenous trehalose after preincubation of yeast cells with glucose. Later on, it was shown that induction of germination by glucose in ascospores of the yeast *Pichia pastoris* was also associated with activation of trehalase (181). Although the presence of nitrogen is not necessary for trehalase activation in the spores, it is necessary for the continuation of germination, and it keeps the trehalase activity high for a much longer time. The addition of glucose to acetate-grown yeast cells also results in trehalase activation (Thevelein, Arch. Microbiol., in press). In all these cases, activation of trehalase is very rapid: it occurs within 5 to 10 min. When the cells or spores are left in the glucose-containing medium, the trehalase activity decreases slowly back to approximately its initial value (181, 186, 203). When the glucose is removed, however, the activity diminishes in about the same time as needed for trehalase activation, i.e., 5 to 10 min (182).

In the meantime, several activation treatments of *Phycomyces* spores had been found during which no trehalase activation was observed (200, 201, 205). It was also found that activation of trehalase during spore heat activation could be inhibited completely by the application of high pressure, addition of long-chain alcohols, or addition of more apolar phenols during the heat shock without affecting the heat activation of the spores (184, 185). Later, however, it was shown that in all these cases, trehalase also becomes activated, but the activation occurs only upon suspension of the spores in the (glucose-containing) germination medium (186). It has been discovered recently that activation of trehalase by heat shock is also possible with yeast ascospores, despite the fact that these spores do not need a heat shock for the induction of germination in a glucose-containing medium (Thevelein, Curr. Microbiol., in press). Unpublished results from our laboratory (M. Dewerchin and J. M. Thevelein) indicate that the induction of germination by glucose in sporangiospores of *Mucor rouxii* is also associated with activation of trehalase. Nitrogen sources which are necessary for complete germination markedly stimulate the activation. Relatively rapid changes of the trehalase activity also appear to take place during the budding cycle of yeasts (89). It is not clear, however, whether this constitutes de novo protein synthesis or post-translational modification.

So far, no other examples of in vivo activation of trehalase have been reported. In all other cases in which the course of trehalase activity was investigated during trehalose mobilization, no changes of the trehalase activity were found (Table 1). Other examples of fungi showing rapid in vivo activation of trehalase will most probably be found among the zygomycetes. Yeasts closely related to *Saccharomyces* and *Pichia* might also turn out to show in vivo activation of trehalase. It is certainly intriguing that the yeast genera *Candida* and *Schizosaccharomyces*, and apparently even certain strains of *Saccharomyces cerevisiae*, have a nonregulatory trehalase, whereas other strains of *Saccharomyces cerevisiae* and the genus *Pichia* have a regulatory trehalase.

The activation in vivo of trehalase by glucose correlates very well with the changes observed in the trehalose level. This is true for both vegetative cells (192, 193, 203) and ascospores of yeasts (181). The addition of glucose causes in both cases a transient increase of the trehalase activity. The addition of nitrogen sources or cycloheximide stimulates trehalose breakdown and also prolongs the activation of trehalase. In yeast ascospores, trehalose breakdown and trehalase activation by glucose in the absence of phosphate are limited. The addition of phosphate markedly stimulates both processes (181). Recently, Uno et al. (196) also report-

ed a strong correlation between the basal activity of trehalase and the content of trehalose in a series of yeast mutants. Avigad (5) found that in vegetative yeast cells the turnover of trehalose was very rapid during the first hour of glucose feeding. After 1 h, however, resynthesis of trehalose was already very vigorous. Measurement of the turnover of trehalose during actual trehalose breakdown might reveal whether trehalose synthesis is also affected by glucose.

SUBCELLULAR LOCALIZATION OF TREHALOSE AND TREHALASE

The simultaneous occurrence of large quantities of trehalose and relatively high trehalase activity in yeast cells was considered already a long time ago as an indication of a spatial separation of substrate and enzyme (115, 117). Procedures which affect membrane integrity, such as autolysis, heating, drying, and treatment with toluene and other organic solvents (13, 17, 116, 117, 144, 166), were found to cause rapid breakdown of trehalose. These results strengthened belief in compartmentation between trehalose and trehalase.

The first direct investigation of the subcellular localization of trehalose and trehalase was carried out by Souza and Panek (166) with yeast protoplasts. They found that trehalase was located in the cytoplasm, whereas trehalose was associated with a particulate membrane fraction. The methods used in their paper have been criticized, however, by Keller et al. (80), who also pointed out that it was very unlikely that the cellular membranes would be able to bind large quantities of a neutral sugar like trehalose. They carried out a more careful study, using a gentle polybase method for lysis of the yeast protoplasts. Trehalase was found to be located in the vacuoles and trehalose in the cytosol. In a subsequent paper, the authors found that only the activated form of yeast trehalase was located in the vacuoles, whereas the nonactivated form (trehalase-zymogen) was located in the cytosol (212). Other detailed investigations of the subcellular localization of trehalase and trehalose were carried out by Sussman and colleagues with *Neurospora*, in particular with *Neurospora* ascospores. Immunofluorescent labeling of trehalase showed that the enzyme was associated with the innermost layer of the ascospore wall. Trehalose, on the other hand, was believed to reside in the cytosol (69). Homogenization of the ascospores apparently caused nearly complete release of trehalase into the soluble fraction (71, 173). Isolated cell walls of conidia and mycelium, on the other hand, contained appreciable trehalase activity which could be released only by enzymatic hydrolysis of the walls (27, 71). In ascospore extracts of *Schizosaccharomyces pombe*, trehalase activity was only detected in the cell wall fraction. Trehalose, on the other hand, was only found in the soluble fraction (76).

In spores of *Phycomyces blakesleeanus* and *Myrothecium verrucaria*, trehalase is found only in the soluble fraction and not in isolated cell walls. In both cases, however, indirect evidence has been presented for a peripheral localization of trehalase in intact spores (100, 101, 201). As for *Myrothecium* spores, Mandels et al. (101) found that heat and toluene treatment resulted in rapid degradation of the trehalose reserve. These data were taken as additional evidence for the existence of compartmentation between trehalose and trehalase. Other fungi in which trehalase was found only in the soluble fraction of cell extracts include *Dictyostelium discoideum* (82), *Pullularia pullulans* (109), and ascospores of *Pichia pastoris* (Thevelein, unpublished results). It was suggested that in spores of *Dictyostelium discoideum*, trehalose was localized in vesicles (62, 114), as opposed to the localization of trehalase in the cytosol. In *Candida albicans* (4), *Lagenidium* sp. (108), and conidia of *Aspergillus oryzae* (74), trehalase was found in both soluble and particulate fractions.

In addition to compartmentation between trehalose and trehalase, separation of trehalose into distinct pools has also been observed. Budd et al. (20) found that after absorption of labeled glucose by *Neurospora* ascospores, the labeled trehalose existed in two pools. One pool was highly radioactive and extractable without breaking the spores; the other pool was low in radioactivity and only extractable after spore breakage. Mandels et al. (101) obtained similar results with *Myrothecium* spores. Thirty percent of the trehalose could be released from the spores with a mild, nonlethal procedure, whereas drastic, lethal treatments were necessary to extract the remainder of the trehalose.

REGULATION OF TREHALOSE MOBILIZATION

Regulation of Trehalase at the Transcriptional Level

The regulation of fungal trehalases at the transcriptional level has never received much attention. This is most probably due to the fact that in most cases trehalose mobilization in fungi is a rapid process which never appears to be induced by de novo synthesis of trehalase. Synthesis of trehalase during sporulation in, for example, *Neurospora* spp. (65, 156, 174, 197), *Aspergillus niger* (119), and *Schizosaccharomyces pombe* (77) is presumably not relevant for the sporulation process itself but constitutes a preparatory step for the subsequent developmental process of spore germination. In *Dictyostelium discoideum*, however, this is not the case. The increase of the trehalase activity during fruiting body construction is completely restricted to the stalk cells (79). It might function, therefore, in sustaining fruiting body construction by mobilizing endogenous trehalose reserves.

New synthesis of trehalase during spore germination and subsequent mycelial development is probably directed towards maximal utilization of potential substrates. This is corroborated by the evidence that has been obtained for the control of trehalase synthesis by catabolite repression. Hanks and Sussman (65) discovered that high sugar concentrations repressed trehalase expression in *Neurospora* mycelium. Subsequent and detailed investigation of this effect revealed that carbon sources allowing abundant mycelial growth, such as glucose, sucrose, maltose, etc., caused trehalase activity to be low (66). Poor carbon sources, on the other hand, produced mycelia with high trehalase activity. De novo synthesis of the enzyme was demonstrated by the incorporation of radioactive leucine into purified trehalase (66). Indications for catabolite control of trehalase were also obtained in *Aspergillus niger* (119) and *Thermomyces lanuginosus* (140). In *Candida tropicalis*, synthesis of trehalase could be induced by the addition of trehalose to the medium (15, 97).

It was suggested by Metzenberg (110) that invertase and trehalase were coordinately controlled in *Neurospora crassa*. A series of carbon sources exerted a similar degree of repression on both enzymes, a single-locus mutant was isolated which produced much higher amounts of the two enzymes, and aging of the cultures caused similar fluctuations in the activities of both enzymes. The hypothesis was later criticized by Sussman and colleagues. They made detailed investigations of the activity of both invertase and trehalase during the life cycle of *Neurospora* spp. and found several discrepancies between the levels of activity of the

two enzymes (71). They also claimed that the existence of isozymes of both invertase (45, 111) and trehalase (70, 220) made the hypothesis unlikely. Another argument was that the difference in trehalase activity between wild-type strains and aconidial mutants was much larger than the difference in invertase activity (65). It was also discovered by North (121) that trehalase activity can be induced in *Neurospora crassa* by incubation at low temperature, whereas the activity of invertase does not increase under these conditions. It is difficult to assess the validity of these criticisms since nothing is known for *Neurospora* spp. about the possible regulation of trehalase and invertase by post-translational modification. The discovery in *Neurospora crassa* of a protein inhibitor of trehalase which might represent a precursor of the enzyme (57, 174) certainly emphasizes the tentative character of these conclusions and criticisms.

Compartmentation of Trehalose and Nonregulatory Trehalases

In fungi with a nonregulatory trehalase, rapid changes in trehalase activity during trehalose mobilization have never been observed (Table 1). Several authors have suggested that compartmentation between trehalose and trehalase was responsible for prevention of the trehalose breakdown. Breakdown of the permeability barrier was believed to induce trehalose mobilization. The most clear-cut example in this respect is the ascospore of *Schizosaccharomyces pombe* (76). In extracts of these spores, trehalase is found only in the isolated cell walls and not in the soluble fraction. No changes in the subcellular localization of trehalase or in its activity were observed during spore germination, which is associated with rapid trehalose mobilization. Hence, it was suggested that the spatial separation between trehalose and trehalase was responsible for the lack of trehalose degradation in the dormant spores. Increased availability of trehalose to trehalase was believed to cause trehalose breakdown during early germination on glucose (76).

The compartmentation hypothesis was proposed originally by Hecker and Sussman to explain the rapid degradation of trehalose upon heat or chemical activation of *Neurospora* ascospores (69). The dormant ascospores have large quantities of trehalose together with relatively high trehalase activity. During dormancy, no trehalose is consumed. The induction of germination is associated with rapid mobilization of a large part of the trehalose without a significant change in the trehalase activity (71, 95, 171, 172, 175). The situation is not as clear, however, as it is for *Schizosaccharomyces* ascospores since nearly all the trehalase activity in *Neurospora* ascospores is found in the soluble fraction (71, 173). By using immunofluorescent labeling of trehalase and other, more indirect, evidence, Hecker and Sussman (69) were able to show that trehalase was located in the innermost layer of the cell wall of intact ascospores. Heat and chemical activation were then postulated to cause a permeability change in the plasma membrane, allowing trehalose to diffuse towards the trehalase located in the cell wall. The increased availability of energy and carbon from trehalose breakdown (*Neurospora* ascospores are able to germinate in distilled water; 170) was believed to break spore dormancy and to sustain further germination of the spore (69). The permeability change was later suggested to be caused by a phase transition of plasma membrane phospholipids (173). Membranes are often thought to be involved in heat-mediated events (2, 173). Proteins, nucleic acids, and polysaccharides, however, are also able to undergo heat-induced transitions (173, 179, 180). *Neurospora* conidia also contain high levels of trehalose (up to 10% of the dry weight), and they have very high levels of trehalase activity (the specific activity of trehalase is 20 times higher in conidia than in ascospores) (65, 71). Conflicting evidence was obtained for the precise location of trehalase in conidia (176). The mobilization of trehalose during conidial germination has never been investigated in detail, and no hypothesis has ever been proposed to explain the coexistence of trehalose and the very high trehalase activity in conidia. Mutant strains of *Neurospora crassa* which lack trehalase activity have been isolated by Sussman and colleagues (174). However, detailed investigations of these strains with respect to the germination capacity of their ascospores and conidia were never made or at least were never reported. Apparently, however, the conidia are still able to germinate, at least in a glucose-containing medium (156, 174) (wild-type conidia are able to start germination in distilled water [156]). A protein inhibitor of trehalase was discovered in extracts of the trehalaseless mutants (57, 174). Although it was reported that the inhibitor was also present in mycelia and ascospores of wild-type *Neurospora* spp., no further studies appear to have been done on its possible role in the regulation of trehalase. It is evident that as long as the precise role of this protein inhibitor in the regulation of *Neurospora* trehalase is not clarified, it will remain difficult to make a definite judgment about the compartmentation model proposed by Sussman for the regulation of trehalose breakdown during dormancy and germination of the ascospores.

Hypotheses similar to the compartmentation model of Sussman have been proposed for other fungal spores which also contain high trehalase activity together with a high trehalose content. In *Myrothecium verrucaria* spores, trehalase activity is found only in the soluble fraction of spore extracts. A peripheral location of trehalase is indicated, however, by the fact that all trehalase activity can be destroyed by treatment with 0.1 N HCl without affecting the viability of the spores. Germination is associated with rapid utilization of the trehalose reserve. Heat and toluene treatment also result in rapid trehalose degradation, and this was taken as additional evidence for the existence of compartmentation between trehalose and trehalase (101). Later on, it was suggested that only the endogenous reserves of *Myrothecium* spores, and not the enzymatic systems responsible for their utilization, were sequestered (99). No evidence, however, was given to support such a distinction.

In *Dictyostelium discoideum*, spore germination is associated with trehalose mobilization and increase in trehalase activity (24–26, 38). The latter might be caused by new synthesis of the enzyme (36, 38). A similar increase in trehalase activity has also been observed during germination of other resting stages of the *Dictyosteliaceae* (187). The increase in trehalase activity was generally believed to be responsible for trehalose utilization during spore germination (79, 85). However, closer examination of the time course of these two processes and selective inhibition of the increase in the trehalase activity indicated that no causal relationship existed in *Dictyostelium discoideum* spores between trehalose mobilization and new synthesis of trehalase (78). Therefore, the basal level of trehalase activity found in dormant spores had to be responsible for trehalose breakdown during early germination. Since no trehalose consumption was observed in dormant spores, it was suggested that the breakdown of a permeability barrier elicited trehalose mobilization during early germination. It has been suggested that trehalose might be located in vesicles of *Dictyostelium* spores, as opposed to location of trehalase in the cytosol (62,

114). No direct evidence, however, for such a compartmentation has been presented. An increase of the trehalase activity during late spore germination, as opposed to the mobilization of trehalose during early germination, has also been observed in *Neurospora* ascospores (71, 172) and *Aspergillus* conidia (74).

Compartmentation of trehalose and trehalase was long believed to exist also in yeasts. Arguments in favor of this hypothesis included the simultaneous occurrence of large quantities of trehalose and relatively high trehalase activity and the initiation of trehalose hydrolysis by procedures affecting membrane integrity, such as treatment with organic solvents (13, 115, 117, 144, 166). When trehalose was rapidly utilized, as during the induction of growth in trehalose-containing cells, an alteration in the membrane structure was supposed to be the initiating mechanism (166). The discovery of in vivo trehalase activation during periods of trehalose mobilization in *Saccharomyces cerevisiae* (203; Thevelein, Arch. Microbiol., in press) and *Pichia pastoris* (181) has rapidly overshadowed these previously held concepts. They might, however, still be valid for other yeast genera containing a nonregulatory trehalase, such as *Candida* and *Schizosaccharomyces*. It has been found recently that permeabilization of yeast cells initiates a time- and temperature-dependent activation of trehalase (Thevelein, manuscript in preparation). This finding offers an alternative explanation for the old observations on the initiation of trehalose hydrolysis by membrane-disrupting agents in *Saccharomyces cerevisiae*. It also indicates that care has to be taken in interpreting measurements of enzyme activity in permeabilized cells. Usually, such in situ measurements are considered to reflect more accurately the true activity of an enzyme existing in vivo than measurements made in cell extracts, i.e., in vitro (146, 160, 163, 164). Sols et al. (163) cite a personal communication by Reyes and Delafuente that the trehalase activity in yeast cells is much higher when measured in situ than in vitro. The results mentioned above indicate that this is most likely an artifact caused by the permeabilization procedure.

Although the compartmentation model provides an obvious explanation for the coexistence of high trehalase activity and a high trehalose level, the problem arises when the stimulation of trehalose breakdown has to be explained. Most authors deal with this aspect in very hazy terms, and the proposal by Sussman (173) that a phase transition of membrane phospholipids would be involved still lacks direct evidence. In fungi with a regulatory trehalase, the progress of trehalose mobilization appears to be strictly controlled by the growth conditions (181, 192, 193, 203). Similar data exist for fungi with nonregulatory trehalases (78), but they are relatively scarce, perhaps because they do not fit very well with the relatively simple mechanism proposed for the induction of trehalose breakdown, i.e., sudden, uncontrolled collapse of a permeability barrier. On the other hand, it must also be emphasized that the data presented for ascospores of *Schizosaccharomyces pombe* strongly suggest involvement of compartmentation (76). This type of spore is perhaps best suited for further studies into the molecular mechanism of glucose-induced decompartmentation of trehalose and trehalase.

cAMP-Induced Phosphorylation of Regulatory Trehalases

The glucose-induced, cAMP-dependent phosphorylation cascade. van der Plaat and van Solingen were the first to provide evidence for the involvement of cAMP-induced phosphorylation in the activation of trehalase (203, 207). Their results showed that it was possible to activate trehalase from sixfold to eightfold in vitro by the addition of cAMP, ATP, and $MgSO_4$ to phosphate-buffered extracts of stationary-phase yeast cells containing the low-activity form of trehalase. They also succeeded in partial purification of the cryptic (low-activity) form of trehalase and the activating factor protein (presumably a protein kinase) (207). Upon purification of the activating factor protein, however, its cAMP dependence was partially lost. van der Plaat was also the first to show that the addition of glucose to stationary-phase yeast cells is associated with a transient eightfold increase of the cAMP level (203). From the results of van der Plaat, the following sequence could be constructed: the addition of glucose to yeast cells activates in some way adenylate cyclase, and this results in a rapid increase of the cAMP level. The rise of the cAMP concentration activates one or more protein kinases, which phosphorylate and thereby activate trehalase.

It was not until 1982 that the evidence of van der Plaat for in vitro phosphorylation of trehalase was confirmed. Londesborough (96) and Wiemken and Schellenberg (212) showed that trehalase could be activated in vitro by the addition to yeast cell extracts of all ingredients necessary for protein phosphorylation. Similar activation of trehalase was achieved later with extracts of *Phycomyces* spores (204), yeast ascospores (Thevelein, unpublished results), and *Mucor* spores (Dewerchin, unpublished results).

Recently, compelling evidence for the involvement of cAMP-dependent protein phosphorylation in the activation of yeast trehalase was presented by Uno et al. (196). They were able to activate partially purified inactive trehalase by the addition of cAMP-dependent protein kinase from yeasts. Fifty percent activation was obtained with a cAMP concentration of only 20 nM. The addition of protein kinase from mutant cells in which the enzyme had lost its cAMP dependence resulted in trehalase activation in the absence of cAMP. The in vitro activation of inactive trehalase was correlated with increased phosphorylation of a protein which most probably, but not certainly, was a subunit of trehalase. Since in these experiments trehalase was not purified to homogeneity, unequivocal evidence that trehalase itself is phosphorylated is not yet available. It cannot be excluded that another protein is phosphorylated which activates trehalase upon phosphorylation. At present, however, this merely appears to be a theoretical possibility. The results of Uno et al. (196) have made it very probable that trehalase itself is phosphorylated. From the results of van der Plaat (203), Wiemken and Schellenberg (212), and Uno et al. (196), it appears that the nonactivated form of trehalase has virtually no activity and that therefore this zymogen of trehalase can only be detected after in vitro activation by cAMP-induced phosphorylation.

The increase of the cAMP level upon the addition of glucose to stationary-phase yeast cells has been confirmed by several authors (106, 142, 189). It has also been observed with acetate-grown yeast cells, with ascospores of yeasts (Thevelein, Arch. Microbiol., in press), with sporangiospores of *Phycomyces blakesleeanus* (R. Van Mulders and A. J. Van Laere, unpublished results), and with *Mucor rouxii* (Dewerchin, unpublished results) and *Neurospora crassa* (124). Membrane depolarizing agents, such as 2,4-dinitrophenol, CCCP (carbonyl cyanide-*m*-chlorophenyl hydrazone), and nystatin, which are known to cause a rapid increase of the cAMP level in yeasts (106, 194), also cause activation of trehalase, both in vegetative cells and ascospores (Thevelein, J. Bacteriol., in press). The induction of trehalose breakdown in yeasts by the addition of 2,4-dinitro-

phenol was observed a long time ago by Berke and Rothstein (11). They also found a high rate of ^{14}C incorporation from glucose-1-^{14}C into trehalose during its depletion in the presence of 2,4-dinitrophenol. Based on this result, they suggested that 2,4-dinitrophenol acted by increasing the rate of trehalose dissimilation rather than by inhibiting assimilation. This has been confirmed by the recent discovery of trehalase activation in the presence of 2,4-dinitrophenol (Thevelein, J. Bacteriol., in press). This finding might also provide an explanation for other reports (16, 168) showing pronounced stimulation of endogenous fermentation in yeasts in the presence of dinitrophenol.

Other important evidence for the involvement of cAMP-induced phosphorylation in trehalase activation was provided by the group of Panek and Mattoon. They isolated yeast mutants deficient in glycogen synthesis with a simple iodine screening procedure based on the absence of color formation in glycogen-deficient mutants. These glc1 mutants were also deficient in trehalose accumulation. The deficiency was due to abnormally high trehalase activity. The authors were able to show that the high trehalase activity was caused by the presence of an abnormally active protein kinase which had lost its normal dependence on cAMP. This protein kinase also acted on glycogen synthase, maintaining it in the phosphorylated low-activity form (29, 122, 123).

In addition to trehalase, other enzymes appear to be involved in the glucose-induced cAMP-dependent phosphorylation cascade in yeasts. The addition of glucose to stationary-phase or acetate-grown yeast cells causes rapid activity changes of whole series of enzymes. The plasma membrane adenosine triphosphatase is rapidly activated (159), whereas the following enzymes are rapidly inactivated: fructose-1,6-bisphosphatase (54; W. Harris and J. J. Ferguson, Jr., Fed. Proc. **26**:678, 1967), cytoplasmic malate dehydrogenase (43, 50, 118, 206, 215, 216), phosphoenolpyruvate carboxykinase (55, 63), isocitrate lyase (28), α-isopropylmalate synthase (19), aminopeptidase I (51), maltose permease (58, 133, 149, 206), and the galactose uptake system (103). Most of these enzymes are gluconeogenic or are for other reasons not needed for growth on glucose. Inactivation of the gluconeogenic enzymes by glucose and activation of trehalase by glucose have in common the fact that the (final) product of the reaction is the trigger for the change in enzyme activity. Glucose-induced inactivation was first called inactivation repression (50) and later on catabolite inactivation (72). By analogy, one could call glucose-induced activation of trehalase catabolite activation (J. M. Thevelein, K.-A. Jones, J. A. den Hollander, and R. G. Shulman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, K215, p. 212). At present, however, it seems better to abandon these terms since the glucose catabolites are most probably not involved in the inactivation process (see below). Glucose-induced (in)activation or in general substrate-induced (in)activation are in my view more accurate terms. They are valid in all cases in which the addition of a new substrate to an organism causes rapid and selective activation or inactivation of specific enzymes, allowing the organism in some way to grow or to survive better with the new substrate. The use of the terms repression and induction, on the other hand, should be restricted completely to new synthesis of enzymes.

Glucose-induced inactivation of fructose-1,6-bisphosphatase has been studied most intensively. Conclusive evidence for the involvement of protein phosphorylation in inactivation has been obtained by several groups (105, 107, 113). Involvement of cAMP was indicated by the absence of inactivation in cAMP-deficient yeast strains and also by other, more indirect, evidence (189, 190). The inactivation of fructose-1,6-bisphosphatase occurs in two steps. In the first step, the enzyme is phosphorylated, and this results in rapid inactivation by about 60%. This inactivation is completely reversible (56, 91, 96, 106, 142, 188). In the second, irreversible, step, the enzyme is slowly degraded by proteolysis (52). It has been suggested that phosphorylation of the enzyme serves as a signal for the initiation of proteolysis (142, 188).

The glucose-induced increase of the cAMP level is thought to be mediated by depolarization of the plasma membrane (H. Holzer *in* P. Cohen, ed., *Molecular Aspects of Cellular Regulation*, vol. 3, in press). Cotransport of the sugar with protons is known to cause a temporary collapse of the proton gradient across the membrane (41, 67, 87, 112, 157, 158, 162). This depolarization is assumed to activate adenyl cyclase. Membrane-depolarizing agents such as 2,4-dinitrophenol, CCCP, and nystatin are known to cause a rapid increase of the cAMP level in yeast cells and in cells of other fungi (106, 124, 194, 195). Whether membrane depolarization itself directly activates adenyl cyclase or whether it causes the formation or release of specific activators is not known. The activation of adenyl cyclase by depolarizing agents in brain cells is thought to be mediated by adenosine (75, 98). Depolarizing agents usually cause a permanent depolarization of the plasma membrane and a permanent increase of the cAMP level in yeasts, whereas the increase caused by glucose is only temporary. In the latter case, the recently discovered glucose-induced activation of plasma membrane-bound adenosine triphosphatase (159) might play a role in the restoration of the proton gradient across the membrane. The presumed activation of adenyl cyclase by depolarization of the plasma membrane is in sharp contrast with the situation in bacteria, in which both sugar-induced depolarization and artificial depolarization by, e.g., CCCP cause strong inhibition of adenyl cyclase (135). Since activation of adenyl cyclase by membrane depolarization has also been found in other eucaryotic cells (39), this might point to an important difference in metabolic regulation between procaryotes and eucaryotes.

In summary, the glucose-induced, cAMP-dependent phosphorylation cascade in yeasts is thought to comprise the following sequence of events (Holzer, in press): glucose causes depolarization of the plasma membrane because of cotransport with protons (41, 67, 87, 112, 157, 158, 162); this depolarization is assumed to cause activation of adenyl cyclase, which results in the observed increase in the cAMP concentration (106, 142, 189, 203); one or more cAMP-dependent protein kinases become activated and phosphorylate a series of enzymes; and this causes either inactivation (e.g., fructose-1,6-bisphosphatase) or activation (e.g., trehalase) of the enzymes involved. In addition, phosphorylation of the enzymes might serve as a signal for the initiation of their proteolytic breakdown (142, 188).

Phosphorylation-triggered initiation of proteolysis was also suggested by Wiemken and Schellenberg (212) to be involved in the inactivation of trehalase after its activation in the presence of glucose. Previous investigations of the subcellular localization of trehalose and trehalase by Keller et al. (80) indicated that trehalase was located in the vacuoles. In a subsequent and more detailed study, it was found that only the activated form of trehalase was located in the vacuoles, whereas the nonactivated form (trehalase-zymogen) was located in the cytosol (212). The authors therefore suggested that phosphorylation of trehalase might serve as a signal for the transfer of the activated trehalase into the vacuoles and that as a consequence trehalase inactivation

after its initial activation in the presence of glucose was due to proteolysis. Recently, however, it has been found that immediate and complete reactivation of trehalase during or after its inactivation in the presence of glucose is possible by the addition of nitrogen sources (182; Thevelein, Arch. Microbiol., in press). Therefore, it is very unlikely that the inactivation of trehalase after its activation by glucose is caused by proteolysis. It remains possible, on the other hand, that transfer of the activated form of trehalase into the vacuoles occurs only many hours after glucose-induced trehalase activation or only in the presence of an adequate amount of nitrogen sources. It was observed before that nitrogen source-induced reversibility of trehalase activation decreases with increasing concentration of the nitrogen source (182). Further investigation of these possibilities might help to solve this intriguing problem.

Evidence that the regulation of trehalase by phosphorylation-dephosphorylation is linked to the regulation of other enzymes is relatively scarce. Ortiz et al. (122) showed that in the glc1 mutants of yeasts, trehalase and glycogen synthase were simultaneously phosphorylated, maintaining the former in the activated state and the latter in the inactivated state. Simultaneous phosphorylation and dephosphorylation of glycogen synthase and trehalase is also indicated by the fact that glycogen synthesis and trehalose resynthesis start at the same moment after the addition of glucose to yeasts. Both processes are also inhibited in the same way by nitrogen sources, azide, and high phosphate levels (193). The addition of plasma membrane-depolarizing agents to yeast cells causes both activation of trehalase (Thevelein, J. Bacteriol., in press) and inactivation of fructose-1,6-bisphosphatase (106) by artificially increasing the cAMP level and thereby activating one or more cAMP-dependent protein kinases. In this case, however, there is no evidence that the same kinase acts on both enzymes. Indirect evidence includes the fact that approximately the same group of sugars (glucose, sucrose, maltose, and mannose) is active in trehalase activation (Thevelein, unpublished results) and in glucose-induced inactivation (72). As for the latter, a regulatory mutant that was isolated by Van de Poll et al. (202) and whose fructose-1,6-bisphosphatase was not inactivated by addition of glucose also lacked inactivation of phosphoenolpyruvate carboxykinase and malate dehydrogenase (55). In addition, cAMP-deficient yeast mutants, studied by Tortora et al. (190), lacked inactivation of fructose-1,6-bisphosphatase and phosphoenolpyruvate carboxykinase as well as of cytoplasmic malate dehydrogenase. Investigation of trehalase activation in these mutants could provide more evidence for a common regulatory mechanism with the process of glucose-induced inactivation.

Cellular cAMP levels and the basal activity of regulatory trehalase. In the past, most attention has been paid to rapid changes of the trehalase activity and the trehalose level induced by the addition of glucose. Recent data, however, show that the basal level of trehalase activity in yeast cells is also under cAMP control. Uno et al. (196) reported a strong correlation among the cAMP level, the level of trehalase activity, and the trehalose content in a series of yeast mutants altered in cAMP metabolism. In addition, the amount of ^{32}P incorporated in vivo in a protein which was presumed to be a subunit of trehalase also showed a strong correlation with the previous parameters. Other evidence for cAMP control of the basal trehalase activity in yeast cells includes the elevated trehalase activity and low trehalose level found in mutants whose protein kinase has lost its cAMP dependence (122).

Activation of regulatory trehalase by nitrogen sources in the presence of glucose. Brandt discovered in 1941 (17) that trehalose was accumulated in *Saccharomyces* cells in the presence of sugar, whereas the addition of both sugar and nitrogen induced trehalose breakdown. More detailed studies of this effect were carried out by Trevelyan and Harrison in 1956 (192, 193). They found that trehalose breakdown in *Saccharomyces cerevisiae* was stimulated, and trehalose resynthesis was prevented, by the addition of nitrogen sources in the presence of glucose. The addition of NH_4Cl some time after resynthesis of trehalose also caused rapid depletion of the trehalose. No effect was observed when the nitrogen sources were added in the absence of glucose. Later on, the stimulating effect of nitrogen sources on trehalose mobilization was confirmed for both vegetative yeast cells (203) and yeast ascospores (181). Whether the presence of glucose is necessary might depend on the physiological status of the yeast or might simply be different in other yeast species, since Yemm and Folkes (219) found that the addition of nitrogen alone was sufficient to induce trehalose mobilization in nitrogen-deficient *Torulopsis* yeast. Similar results were obtained by Panek et al. (132, 133) with several mutants (e.g., EB7-3B) of *Saccharomyces cerevisiae*. These mutants are not able to accumulate trehalose after glucose exhaustion during diauxic growth. Apparently, the nitrogen left in the medium is able to inhibit trehalose accumulation in the absence of glucose. This was not due to deficiency in trehalose synthesis since the mutants accumulated large amounts of trehalose in the presence of glucose only. With maltose as a nitrogen source, on the other hand, the presence of nitrogen did not prevent trehalose accumulation.

Before the discovery of trehalase activation, changes observed in the trehalose level in yeasts were thought to arise from changes in the rate of trehalose synthesis rather than from changes in the rate of trehalose hydrolysis (131, 192, 193). Trevelyan and Harrison, for example, tried to explain the nitrogen effect by assuming competition for common coenzymes between the reactions of protein and trehalose synthesis (192, 193). No evidence could be found, however, for this hypothesis. An explanation for the nitrogen effect came when van der Plaat (203) found that glucose-induced activation of trehalase in stationary-phase yeast cells was prolonged by the addition of nitrogen. Similar results were obtained with yeast ascospores (181). Besides, it was shown that in both vegetative cells and ascospores of yeasts, the addition of a nitrogen source some time after trehalase inactivation caused a new increase of the trehalase activity up to the same level found after the addition of glucose (182; Thevelein, Arch. Microbiol., in press). In the absence of glucose, nitrogen sources had no effect (182). These results provide an explanation for the observations of Trevelyan and Harrison (192, 193). They indicate that the effect of nitrogen sources on the trehalose level in yeasts can be explained by changes in the trehalase activity rather than by changes in the capacity for the synthesis of trehalose.

Some attempts have been made to find out how the nitrogen sources could influence the cAMP-dependent phosphorylation cascade. It was shown by van der Plaat (203) that the glucose-induced increase of the cAMP level in yeasts was not significantly affected by the presence of nitrogen sources. The possible existence of a correlation between nitrogen source-induced trehalase reactivation and the cAMP level was recently investigated in vegetative cells and ascospores of yeasts (Thevelein, Arch. Microbiol., in press). Both glucose-induced trehalase activation and subsequent nitrogen source-induced reactivation of trehalase were

associated with an increase of the cAMP level in ascospores. In vegetative cells (both stationary phase and acetate grown), only glucose-induced trehalase activation was associated with an increase of the cAMP level. These data, together with the data obtained by van der Plaat, indicate that the effect of nitrogen sources on trehalase activity is not exerted through a change of the total cellular cAMP content. A large part of the cAMP in yeast cells, however, could be bound to protein (125). Therefore, it cannot be excluded that changes in the level of free cAMP are involved in the nitrogen effect.

In this connection, it also has to be mentioned that the addition of cycloheximide produces the same inhibition of trehalase inactivation as the nitrogen sources (181, 182). In addition, the presence of either cycloheximide or a nitrogen source causes glucose-induced trehalase activation to be completely reversible. In their absence, glucose-induced trehalase activation is only partially reversible, and the extent of the activation decreases with each activation-deactivation step (182). Cycloheximide is known to block protein synthesis; the addition of amino acids to cells under nitrogen starvation would normally stimulate protein synthesis. In this respect, the two effects are seemingly in contradiction. Both treatments, however, might also have common consequences, such as raising the intracellular concentration of amino acids. According to van der Plaat (203), the glucose-induced increase of the cAMP level is smaller in the presence of cycloheximide than in its absence. From the effect of cycloheximide on the activity of trehalase, one would normally expect the opposite. Azide was also reported to stimulate trehalose breakdown (191–193) and to inhibit protein synthesis (167) in yeasts. The effect of azide on trehalase activation, however, has not yet been investigated.

The stimulation of trehalase inactivation by nitrogen starvation resembles somewhat the stringent response in bacteria. In this case, nitrogen starvation leads to the rapid shutdown of a whole series of metabolic processes, and much has been learned already about the molecular mechanisms involved (18, 21, 53). In eucaryotes, however, virtually nothing is known about this (161). Whether similar mechanisms are involved in the effect of nitrogen starvation on trehalase inactivation in yeasts remains to be investigated. Elucidation of the molecular mechanism involved in this process might certainly produce interesting information concerning the integration of metabolism in yeasts and possibly in other eucaryotes.

Activation of regulatory trehalase in fungal spores by heat shock. Activation of trehalase by heat shock has been observed in *Phycomyces blakesleeanus* sporangiospores (199) and in yeast ascospores (Thevelein, Curr. Microbiol., in press). In both cases, the optimal temperature is around 50°C, and at this temperature a heat shock of only 1 min is required for full activation. In the temperature range from 40 to 50°C, there is a gradual increase in the degree of activation.

The heat activation of regulatory trehalase in vivo in fungal spores forms a marked contrast with the in vitro heat stability of the enzyme. A short heat treatment at 50°C causes nearly complete denaturation of both *Phycomyces* (198) and yeast trehalase in spore extracts (Thevelein, Curr. Microbiol., in press). Presumably, the enzyme is protected by the high trehalose concentration which is known to exist inside *Phycomyces* (154) and yeast (181) spores. The absence of such a high sugar concentration in vegetative yeast cells might be the reason why in vivo heat activation of trehalase is not possible with these cells (Thevelein, Curr. Microbiol., in press).

The molecular mechanism of trehalase heat activation has long remained obscure. The effect of high pressure on the heat activation of trehalase in *Phycomyces* spores was investigated to distinguish between a protein conformational change and a phospholipid phase transition as the triggering mechanism (184). The same technique had been used before to identify the molecular nature of the triggering process in the breaking of dormancy by heat shock in *Phycomyces* spores. In that case, the data pointed unambiguously to a protein conformational change (183). In the case of trehalase heat activation, however, the results did not fit very well with either mechanism (184). Indications for a thermal transition in a polysaccharide matrix were also not very convincing (180, 185). Recently, however, important progress has been made in the understanding of the molecular mechanism of trehalase heat activation. First of all, it has been found that in yeast ascospores, trehalase is not activated during the heat treatment itself, but only during cooling of the spores at the end of the heat treatment (Thevelein, Curr. Microbiol., in press). This was shown by experiments in which the spores were rapidly frozen in liquid nitrogen instead of cooled in the usual way, i.e., by addition of an excess of ice-cold water. Upon freezing in liquid nitrogen, no activation of trehalase was observed, and this was not due to denaturation of trehalase by the freezing procedure. When the cAMP level was measured in yeast spores during the heat treatment, no increase was found when the spores were frozen in liquid nitrogen. However, when the spores were cooled with an excess of ice-cold water, as was done for trehalase activation, an increase of the cAMP level was found which was about twofold to threefold. The increase of the cAMP level roughly paralleled the increase in trehalase activity when measured as a function of temperature (Thevelein, Curr. Microbiol., in press). These results might finally shed some light on the molecular mechanism of trehalase heat activation. They indicate that cAMP-induced phosphorylation could also be involved in this trehalase activation process, with the increase of the cAMP level being caused by the rapid cooling of the spores. Presumably, heating of the spores causes melting of the plasma membrane lipids, whereas subsequent rapid cooling causes the membrane to solidify in a disorganized way. This might result in temporary leakiness of the membrane. Leakage of cations into the spores might depolarize the plasma membrane and thereby activate adenylate cyclase. It is well known that depolarization of the plasma membrane causes rapid increases of the cAMP level in fungi (106, 124, 194). The addition of depolarizing agents to yeast spores was shown to result in immediate activation of trehalase (Thevelein, J. Bacteriol., in press).

Activation of regulatory trehalase and the induction of fungal spore germination. As mentioned before, trehalase activation was discovered because of its close association with spore activation in *Phycomyces blakesleeanus*. In general, the interest in the regulation of trehalose mobilization stems mainly from its possible involvement in the induction of fungal spore germination. Rapid activation of trehalase during the induction of germination has been observed in *Phycomyces* (42, 186, 199) and *Mucor* sporangiospores (Dewerchin and Thevelein, unpublished results) and in yeast ascospores (181). It represents one of the earliest and most dramatic metabolic changes observed in fungal spore germination.

Phycomyces spores have to receive a short heat shock or some other activation treatment; otherwise, they will not germinate in a glucose-containing medium (148, 165). Two

activation treatments, heat shock (199) and acetate pretreatment (42), are associated with activation of trehalase during the activation treatment itself. Several other activation treatments are not associated with trehalase activation (200, 201, 205). In this case, however, trehalase is activated as soon as the spores are suspended in the glucose-containing germination medium (186). Other results also indicate that trehalase activation is not the trigger of spore activation: high pressure, long-chain alcohols, and more apolar phenols all exert opposite effects on the temperature dependence of trehalase heat activation and spore heat activation (184, 185). Elucidation of the molecular mechanism of trehalase activation, however, might also lead to the mechanism of spore activation. Since in *Phycomyces blakesleeanus* also trehalase activation appears to be caused by cAMP-dependent phosphorylation (204), one of the earlier steps in this cascade might be the trigger of spore activation. Increase of the cAMP level was observed a few minutes after the breaking of spore dormancy, and by using different activation methods, a good correlation was observed between the cAMP increase and the activation of trehalase (R. Van Mulders and A. Van Laere, unpublished results). When dormant spores are suspended in a glucose-containing medium, there is no activation of trehalase. The addition of glucose to activated spores, however, causes immediate activation of trehalase (see above). Hence, it is clear that if a cAMP-dependent phosphorylation cascade is indeed involved in trehalase activation, one of the components of the chain must have been changed by the spore activation treatment. If the cAMP-dependent phosphorylation cascade constitutes the trigger for the induction of spore germination, it still has to be determined whether in that case trehalase activation is a necessary event in the sequence of metabolic changes leading to germination.

Similar reservations have to be made in the case of yeast ascospores. Here, the addition of glucose together with a nitrogen source immediately leads to germination without any other activation treatment being necessary (181). Glucose alone induces activation of trehalase, but the activity drops quickly back to the original level. A small amount of trehalose is broken down and is slowly replenished in the continuous presence of glucose. In this case, there is no germination. The addition of nitrogen sources keeps the trehalase activity high, resulting in extensive trehalose breakdown and allowing germination to occur. Glucose was recently shown to trigger a transient increase of the cAMP level when added to yeast ascospores (Thevelein, Arch. Microbiol., in press). These data point to the cAMP-dependent phosphorylation cascade being operative also during the induction of yeast ascospore germination by glucose. It remains to be established in this case too, however, whether the regulatory cascade coincides only fortuitously with the breaking of dormancy or whether it constitutes in itself the trigger for the induction of germination. Recently, Panek and Bernardes (130) succeeded in isolating trehalose-deficient mutants of *Saccharomyces cerevisiae* that were still able to sporulate. Germination of the resulting spores, however, was extremely poor. These data might confirm the importance of trehalose for the induction of germination. However, they might also point to the importance of trehalase in maintaining viability during dormancy. Lack of germination capacity and lost spore viability could both have been responsible for the lack of germination in the trehalose-deficient spores.

There has been speculation that the low-activity form of trehalase plays a crucial role in maintaining yeast ascospores in the dormant state (181). It was found that partially purified trehalase from dormant spores was inhibited by ATP in the concentration range existing in these spores. The partially purified, activated form of trehalase was not inhibited by ATP. It was suggested that the low activity and the ATP feedback inhibition of trehalase were responsible for the very slow mobilization of the large amount of trehalose in dormant spores (181). Indirect evidence for this hypothesis was provided by Barton et al. (9). They showed that the addition of 2-deoxyglucose to yeast ascospores resulted in both ATP depletion (because of the formation of nonmetabolizable 2-deoxyglucose-6-phosphate) and slow trehalose mobilization. Isolation of trehalase-deficient mutants might provide conclusive evidence for this hypothesis.

The results obtained recently for trehalose metabolism in sporangiospores of *Mucor rouxii* appear to be very similar to those obtained for yeast ascospores. Glucose alone causes activation of trehalase, but in the presence of nitrogen sources the activation is much more pronounced (Dewerchin and Thevelein, unpublished results). The cAMP content increases rapidly during early germination, and in vitro activation of trehalase is also possible with conditions suitable for cAMP-dependent protein phosphorylation (Dewerchin, unpublished results).

Whether the mobilization of trehalose is crucial for the induction of fungal spore germination is not yet known. The studies concerning the regulation of this process, however, have already produced new insights into the regulation of fungal spore germination. From the effects of glucose and nitrogen sources on trehalose mobilization in yeast ascospores (181), it appears that glucose is actually the inducer of spore germination, whereas the presence of nitrogen sources causes germination to continue. Control mechanisms exist which quickly shut down germination in the absence of nitrogen sources and also turn the clock back by restoring the original metabolic status of the spores (e.g., replenishment of storage trehalose). For a long time, studies of dormancy and the induction of germination in fungal spores have mainly been descriptive, without many attempts to identify directly the regulatory mechanisms involved. Besides, these data were obtained independently with different fungal species without serious efforts to verify whether the proposed models and hypotheses were also valid for spores of other species. The results obtained on the molecular mechanisms involved in the regulation of trehalose mobilization in *Phycomyces* and *Mucor* sporangiospores (zygomycetes) and in ascospores of yeasts (ascomycetes) present a striking similarity between both different spore types and different fungal groups. In addition, a clear correlation exists between the progress of germination and the course of trehalose metabolism. *Phycomyces* spores have a more stringent type of dormancy (constitutive dormancy) than *Mucor* and yeast spores. In my view, complete elucidation of the molecular mechanisms involved in the induction of trehalose mobilization might also lead to a better understanding of the differences in the stringency of dormancy in these spores.

Other Mechanisms

A completely different mechanism for the regulation of trehalose mobilization was suggested to be operative during the germination of *Aspergillus* conidia (74). In addition to trehalose, the conidia also contain a large amount of D-mannitol. The molar ratio between D-mannitol and trehalose is about 10:1. It was found by Horikoshi and Ikeda (74) that *Aspergillus* trehalase is competitively inhibited by D-manni-

tol. At the concentration of D-mannitol existing in the spores, trehalase should be strongly inhibited. Since both the concentration of D-mannitol and trehalose were found to decrease during germination, it was suggested that trehalose mobilization was controlled by the decrease of the D-mannitol concentration (73, 74). A detailed comparison of the time course of the two processes, however, has never been made.

Other fungal spores containing both D-mannitol and trehalose include *Penicillium* conidia (7, 44), *Puccinia* uredospores (40, 147, 178, 218), *Schizophyllum commune* basidiospores (1), and *Myrothecium* spores (101). In *Puccinia graminis* (40) and *Schizophyllum commune* (1, 120), both sugar alcohols and trehalose are utilized during early germination. Sugar alcohols, however, do not have an inhibitory effect on trehalase from *Schizophyllum commune* (mycelium). Therefore, the mechanism proposed for the regulation of trehalose utilization in *Aspergillus* conidia (74) was not believed to be operative in *Schizophyllum* basidiospores (213). Most probably, this mechanism is also not operative during germination of *Myrothecium* spores since the mannitol concentration in these spores increases during trehalose mobilization. Indications were even obtained that part of the trehalose was converted into mannitol (101). Trehalase from the oomycete *Lagenidium* sp. was reported not to be inhibited by mannitol (108), whereas the enzyme from *Sclerotinia sclerotiorum* was inhibited by mannitol (211). Sclerotia and mycelia of the latter fungus contain both mannitol and trehalose (32, 33, 92, 210). Hence, it was suggested that mannitol might regulate trehalase activity during vegetative growth and germination of sclerotia (211). According to unpublished results mentioned in the same paper, however, trehalose disappeared more rapidly than mannitol during germination of the sclerotia. The fate of trehalose and mannitol during germination of *Claviceps* sclerotia also contradicted regulation by this mechanism (34). The data on the proposed regulation of trehalase by the mannitol concentration in *Aspergillus* conidia are certainly too preliminary to allow any definite judgment. Although the results of Horikoshi and Ikeda (74) have never been questioned, no further evidence in favor of their hypothesis has been presented.

CONCLUSIONS

The disaccharide trehalose is an important storage carbohydrate in fungi, especially during periods of nonproliferation. This applies to the life cycle, the cell cycle, and periods of starvation. Resumption of growth is associated in all these cases with rapid mobilization of trehalose.

Fungal trehalases can be divided into two groups. The first group contains so-called nonregulatory trehalases. The inherent activity of these enzymes does not change during periods of trehalose mobilization. These nonregulatory trehalases have an acid pH optimum and a high heat stability. Evidence for in vitro activation of these enzymes by cAMP-dependent protein phosphorylation has never been obtained. The second group contains so-called regulatory trehalases. In fungi containing such a trehalase, periods of trehalose mobilization are always associated with both a drastic, rapid activation of trehalase and a rapid increase in the cAMP level. The activation can be performed in vitro by providing all conditions necessary for cAMP-dependent protein phosphorylation. The regulatory trehalases have a neutral pH optimum and a low heat stability. The nonregulatory type of trehalase appears to be present in all fungal groups except the zygomycetes. The regulatory type of trehalase has been found only in the zygomycetes and in the closely related yeast genera *Saccharomyces* and *Pichia*.

The principal mechanisms that have been proposed for the regulation of trehalose breakdown in fungi fit with the subdivision of the trehalases. Compartmentation of trehalose and trehalase was proposed for nonregulatory trehalases, and regulation by cAMP-dependent protein phosphorylation was proposed for the regulatory trehalases. Although very suggestive evidence has been presented for involvement of compartmentation in the regulation of nonregulatory trehalases, conclusive evidence is still lacking. In particular, the mechanism responsible for breaking down the permeability barrier remains a mystery. Compelling evidence for involvement of cAMP-dependent protein phosphorylation in the regulation of regulatory trehalases has been obtained in several species. Conclusive evidence that the enzyme itself is phosphorylated, however, is not yet available.

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