NEUROSPORA TREHALASE AND ITS STRUCTURAL GENE

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ABSTRACT

We have isolated *Neurospora* trehalaseless mutants and mapped the trehalase structural gene to linkage group I. The structural gene mutations not only affect thermostability and other characteristics of the enzyme but also affect the production of an inhibitor of the wild-type trehalase. The inhibitor appears to be the mutant trehalase. We suggest that the mutant subunits act as inhibitors by entering into the multimeric forms of the enzyme and altering the ability of the normal wild-type subunits to catalyze the cleavage of trehalose.—Wild type trehalase has been purified to near homogeneity, and its characteristics have been studied. It was purified as a tetramer, with each subunit having a molecular weight of 88,000.—We have studied the regulation of trehalase and found the production of trehalase to be glucose repressible. Cells begin to produce trehalase 60 min after being transferred to glucose-free medium.

NEUROSPORA crassa has been shown to produce trehalase in response to glucose deprivation (HANKS and SUSSMAN 1969a, b). The enzyme is found in the periplasmic space where it catalyzes the hydrolysis of the disaccharide trehalose (HECKER and SUSSMAN 1973a, b). Mutations that affect glucose repression also affect the production of trehalase activity (METZENBERG 1962; GRATZNER and SHEEHAN 1969; GRATZNER 1972; MURAYAMA and ISHIKAWA 1973).

A purification of trehalase has been reported (HILL and SUSSMAN 1963; HECKER and SUSSMAN 1973b). The procedure involves a harsh alkaline treatment. We report a milder procedure for the purification of trehalase and have purified the enzyme to near homogeneity.

SUSSMAN *et al.* (1971) previously described the isolation of a class of *Neurospora* trehalaseless mutants that contained an inhibitor of trehalase. This inhibitor is a polypeptide that stoichiometrically binds trehalase (GARRETT, SUSSMAN and YU 1972).

We have isolated a number of trehalaseless mutants and characterized them. The trehalase mutations, like previously isolated trehalase mutations, cause an overproduction of a trehalase inhibitor (GARRETT, SUSSMAN and YU 1972; SUSSMAN *et al.* 1971). One of these mutations also renders the enzyme thermolabile. All of these mutations map in a tight cluster on the left arm of linkage group I. We present evidence that this locus is the trehalase structural gene.

MATERIALS AND METHODS

Strains: Strain R501 (a temperature-sensitive colonial strain by virtue of the *cot-1* mutation) was obtained from R. L. METZENBERG and served as the parental strain for the isolation of most of the mutants described. One of the mutants, SF189, was isolated from an *al-3 in1* (albino, inositol requirement) parent obtained from R. L. METZENBERG. The initial mapping of trehalase mutations was done using the *alcoy csp-2* strains (DAVIS and DE SERRES 1970; PERKINS and BJORKMAN 1979). The exact location of the trehalase mutations was determined using a multiply marked strain containing *nuc-1*, *ad-9*, *cyR*, *al-2P* and *nic-1* (defective in the production of repressible phosphatases, adenine requirement, cycloheximide resistance, albino-pink and nicotinamide requirement) obtained from R. L. METZENBERG. Forced heterokaryons were prepared as described by DAVIS and DE SERRES (1970).

Materials: Electrophoretic supplies were obtained from the BioRad Company, Richmond, California. Materials for isoelectric focusing gels were purchased from the LKB Company, Bromma, Sweden. All other supplies were obtained from the Sigma Chemical Company, St. Louis, Missouri.

Mutant isolation: A large scale screening procedure was used to isolate trehalaseless mutants. R501 conidia were mutagenized with UV radiation (1% survival) and plated out at 34° on Vogel's medium with 2% xylose as a carbon/energy source. After 5 days of growth, individual colonies were picked into small test tubes containing Vogel's N medium supplemented with 2% trehalose as a carbon/energy source. Those few tubes containing colonies that failed to grow were saved. To these tubes 1 ml of Vogel's N medium containing 2% glucose was added. Colonies able to grow on glucose as a carbon source, but unable to grow on trehalose, were then individually grown; cellular extracts were prepared, and these extracts were assayed for trehalase activity. Strains T7 and 72, which contain previously described *tre* mutations (SUSSMAN *et al.* 1971), were obtained from J. C. SCHMIDT, Department of Chemistry and Biochemistry, Southern Illinois University, Carbondale, Illinois.

Trehalase assay: Cellular extracts were prepared by grinding mycelium (approximately 0.5 g wet weight) in a mechanical dounce homogenizer in 3 ml of ice-cold 50-mM sodium acetate, pH 5.0. These cellular extracts were dialyzed overnight against 50 mM sodium acetate, pH 5.0, to remove glucose and trehalose from the cellular extracts. Trehalase activity was assayed by incubating 0.2 ml of cellular extract with 0.8 ml of 15 mM trehalose (in 50 mM sodium acetate, pH 5.0) for 1 hr at 37°. The reaction was stopped by placing the assay tubes in boiling water for 5 min, and the glucose released was determined as described by GASCON and LAMPEN (1968).

The thermostability of trehalase was determined by incubating dialyzed cellular extracts at 62° for varying lengths of time prior to carrying out the assay for enzymatic activity. Extract-mixing experiments were carried out as described by SUSSMAN *et al.* (1971).

Purification procedure: Wild-type cells (strain 74-OR23-1A) were grown in 12 liters of Vogel's N medium supplemented with 2% sucrose and grown with constant shaking (150 rpm) at 30° for 10 days. The cells were harvested on a Buchner funnel and frozen. We normally obtained between 400 and 500 g of wet weight mycelium from 12 liters of medium. The frozen mycelium (400–500 g) was ground to a fine powder in a Waring blender using dry ice as the abrasive. The enzyme was extracted from the ground mycelium by incubating the mycelium with 500 ml of 50 mM sodium acetate buffer, pH 5.0, overnight. The extract was heated to 60° for a 15-min incubation. Following the heat treatment the extract was cooled on ice, and the particulate material and denatured protein were removed by centrifugation at 8000 × *g* for 20 min.

Cold ethanol (95%, –70°) was slowly added to a final concentration of 60%, and the trehalase was allowed to precipitate overnight at –20°. The precipitate was collected by centrifugation at –20° (8000 × *g* for 20 min), resuspended in 100 ml of 50 mM sodium acetate, pH 5.6, and dialyzed overnight against the same. The enzyme was applied to a diethylaminoethyl (DEAE)-cellulose column (1.5 × 25 cm) that had been equilibrated with 50 mM sodium acetate, pH 5.6, and eluted with a linear salt gradient of NaCl (0 to 500 mM in the acetate buffer). The fractions containing the bulk of the trehalase activity were pooled, and the enzyme was concentrated by an ethanol precipitation as described above. This material was applied to a column (1.5 × 50 cm) of Sephadex G-200 (40–120 µm particle size) and eluted with 50 mM sodium acetate, pH 5.6.

Analytical gel electrophoresis: Isoelectric focusing was carried out in 5.0% polyacrylamide gels as suggested by LKB company. pH gradients of ampholines from pH 2.5 to 4.5 and from 3.5 to 9.5

TABLE 1

The purification of Neurospora trehalase

Step	Volume (ml)	Total protein (mg)	Total activity	Specific activity	Fold purified
Crude extract	577	9463	507	0.054	
After heating	577	8655	646	0.075	1.4
Ethanol precipitation	93	1507	699	0.46	8.5
Dialysis	125	900	549	0.61	11.3
DEAE pooled peak fractions	39	78	677	8.66	160
Ethanol precipitation	1.5	19.8	486	24.5	454
G-200 peak fractions	4.0	3.0	238 ^a	79.0	1463

^a Recovery from pooled peak fractions. Total recovery from the G-200 column was 405 units. One unit of activity equals 1 μ mol of trehalase cleaved/min at 37°.

were employed in various experiments. Gels were stained for protein using Coomassie blue and for trehalase activity using the procedure of GABRIEL and WANG (1969) to stain for aldo sugars after first incubating the gel with trehalose. Electrophoresis in sodium dodecyl sulfate (SDS)-polyacrylamide gels was carried out according to the procedure of LAEMMLI (1970).

Preparation of antibody: Purified enzyme (800 μ g) was injected intramuscularly and subcutaneously into each of two male New Zealand white rabbits. Three weeks later a second injection of 800 μ g of purified enzyme was given subcutaneously. The rabbits were bled 8 days after the second injection and antisera prepared and used as previously described (FREE and METZENBERG 1982).

RESULTS

The purification of trehalase: HILL and SUSSMAN (1963) previously reported purifying trehalase. Since their purification involved treatment with 1.0 M NaOH, which might adversely affect the enzyme, we sought a gentler procedure. Table 1 shows that the procedure we have devised resulted in a 1460-fold purification of the enzyme from strain 74-OR23-1A. The specific activity of the purified enzyme was 79 μ mol of substrate cleaved/min/mg of protein as compared to a value of 13.6 μ mol of substrate cleaved/min/mg for the previous procedure (HECKER and SUSSMAN 1973a). Upon electrophoresis in SDS-polyacrylamide the purified trehalase migrated as a single band having a molecular weight of 88,000 (Figure 1).

When subjected to isoelectric focusing, purified trehalase focused as a single polypeptide having an isoelectric point (pI) of 3.7. Figure 2 shows an isoelectric focusing gel in which one lane was stained for trehalase activity and a second lane was stained for protein with Coomassie blue. On the basis of isoelectric focusing and SDS-polyacrylamide gels we found trehalase to have been purified to near homogeneity. Only on heavily overloaded gels did we find other bands of protein.

Two lines of evidence show that trehalase exists as a multimer. One is that during the purification the enzyme was subjected to chromatography on a G-200 Sephadex column. The approximate molecular weight of the enzyme on the G-200 column was 360,000, some four times larger than the 88,000 molecular weight seen in SDS-gel electrophoresis. A second line of evidence is

FIGURE 1.—SDS-polyacrylamide gel electrophoresis of purified trehalase. Purified trehalase (lane 1) was subjected to electrophoresis as described by LAEMMLI (1970). Lane 2 contains the molecular weight markers phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000) and carbonic anhydrase (31,000). The gel was stained with Coomassie blue.

FIGURE 2.—Isoelectric gel focusing of purified trehalase. Purified trehalase was loaded into two adjacent lanes of a slab gel and subjected to isoelectric gel focusing as described in MATERIALS AND METHODS. The gel was cut and one lane stained with Coomassie blue (lane A) and one lane stained for trehalase activity (lane B).

that, when purified enzyme was subjected to gel electrophoresis in the absence of SDS, three bands of polypeptide were seen, all three bands staining positively for trehalase activity (data not shown). Since the same preparation showed the presence of only the 88,000 molecular weight polypeptide on SDS-

FIGURE 3.—Time course of trehalase production following transfer to carbon-free medium. Wild-type conidia were germinated and grown for 24 hr in Vogel's glucose medium. The cells were then harvested, washed and placed in Vogel's medium devoid of a carbon source. Cells were harvested at various times after the transfer, extracts prepared, and the specific activity of trehalase present determined by assay.

gel electrophoresis we ascribe the multiple bands to multimeric states. The three bands might possibly represent monomers, dimers and tetramers. One observation consistently made was that in gels in which the three bands of activity were evident the larger molecular weight species contained much less trehalase activity than we would have expected for the amount of Coomassie blue-staining material. This suggests that the enzymatic activity is reduced whenever trehalase exists in larger multimeric states.

The purified enzyme was characterized with regard to its enzymatic properties. The enzyme is active over a broad pH range with optimum activity at pH 5.0. The K_m of the purified enzyme was determined to be 0.36 mM. The purified enzyme has a half-life of 13 min when heated to 65° and shows the inactivation kinetics expected for a single-enzyme species.

Trehalase production following transfer to carbon-free medium: We have characterized the production of trehalase as wild-type cells (strain 74-OR23-1A) enter carbon deficiency. Mycelium from conidia that had been germinated and allowed to grow for 24 hr in Vogel's glucose medium were harvested, extensively washed, and resuspended in Vogel's medium devoid of a carbon/energy source. At various times thereafter cells were harvested, and the level of trehalase was determined. Starting 60 min after the transfer we found a dramatic increase in trehalase activity (Figure 3). The specific activity of trehalase was found to increase more than 400-fold during the first 5 hr of carbon starvation (from a value of less than 0.0001 at 0 time to 0.044 at 5 hr).

Mutant isolation: Selection procedures have proven unsuccessful for the isolation of mutants affected in enzymes used to acquire carbon (FASS 1969). The problem can be attributed to (1) large endogenous energy reserves within fungal spores which allow the germinating spores to grow without the need for exogenous energy sources and (2) the problem of cross-feeding that occurs

TABLE 2

Fine mapping of the trehalaseless mutations relative to each other

Cross	No. of progeny tested	No. of wild-type recombinants	Map distance (cM)
1. SF 189 × CW 210	4,000	1	0.05
2. SF 189 × CW 230	4,000	3	0.15
3. SF 189 × CW 412	4,000	0	>0.05
4. SF 189 × CW 415	4,000	2	0.10
5. SF 189 × CW 315	1,167	1	0.17
6. SF 189 × T7	7,200	4	0.11
7. SF 189 × 72	13,000	6	0.09

Matings were made between the indicated strains, and ascospores were tested for the ability to utilize trehalose as a carbon source. For all crosses except cross 5 (SF 189 × CW 315) ascospores were plated out on Petri dishes containing Vogel's medium with trehalose as a carbon source. For cross 5, 1167 germinating ascospores were individually picked and placed in small test tubes containing 1 ml of Vogel's trehalose liquid medium.

when selecting for extracellular activities. Therefore, the screening procedure outlined in MATERIALS AND METHODS was used to isolate trehalaseless mutants.

Mutants SF 189, CW 210, CW 230, CW 315, CW 412 and CW 415 were isolated as colonies that were unable to utilize trehalose. In preliminary mapping experiments using the *alcoy csp-2* strain developed by PERKINS and BJORKMAN (1979) all six mutations were found to be located on linkage group I. We crossed SF 189 to the *nuc-1*, *ad-9*, *cyR*, *al-2P*, *nic-1* tester strain to locate the position of the trehalase mutation more precisely. It maps 10 cM centromeric to the *ad-9* loci on linkage group I. The other trehalase mutations all map in the same region on linkage group I.

To test for allelism, crosses were performed between SF 189 and the other mutants. Ascospores from these crosses were tested for the ability to grow on trehalose as a carbon source (Table 2). Only a very limited number of progeny able to utilize trehalose as a carbon source were found. The mutations in CW 210, CW 230, CW 315, CW 412 and CW 415 all map at a distance of less than 0.2 cM from that in SF 189. Alterations within a structural gene usually map within 0.1 cM from each other (DAVIS and DE SERRES 1970). These data suggest that all the trehalase mutations could be allelic.

To demonstrate that these various mutations reside in a single gene two forced heterokaryons (having nuclei that contained the mutations from SF 189 and CW 315 and from SF 189 and CW 230) were prepared. In both cases the nuclei were unable to complement each other as demonstrated by an inability of the heterokaryon to utilize trehalose as a carbon source. We conclude that the trehalase mutations reside in a single gene.

Characterization of the trehalase activity in SF189, CW 210, CW 230, CW 315, CW 412 and CW 415: Each of the mutants contains some trehalase activity (Table 3). The residual trehalase activity in each of the six mutants was compared with the trehalase activity from the appropriate wild-type parent (74-

TABLE 3

Characteristics of the trehalase found in wild-type and mutant strains

Strain	Ability to retain activity upon heating	Ability to retain activity during ethanol precipitation	Presence of an inhibitor	% wild-type activity remaining
74-OR8-1a	+	+	—	100
SF 189	+	+	+	5
R501	+	+	—	100
CW 210	+	—	+	4
CW 230	+	—	+	3
CW 315	—	+	+	9
CW 412	+	+	+	3
CW 415	+	+	+	1

74-OR8-1a and R501 are wild-type strains and were used as controls. The percentage of wild-type trehalase activity remaining was determined in extracts prepared from cells that had been placed in carbon-free medium for 4 hr. The ability to retain activity upon heating and during ethanol precipitation were determined by comparing the mutant and wild-type enzymes. —, statistically significant difference noted.

FIGURE 4.—Thermostability of trehalase from CW 315 and from its *cot-1* parent, R501. Extracts from CW 315 (●) and R501 (○) were incubated at 62° for various lengths of time, and the activity remaining was assayed.

OR8-1a or R501) with regard to its heat lability, K_m value and behavior when subjected to the purification procedure described in MATERIALS AND METHODS. Table 3 shows that the trehalase present in each mutant differs in some important respect from the enzyme present in the parental strain. In particular, one of the mutants, CW 315, makes a thermolabile enzyme. Figure 4 shows the inactivation of CW 315 trehalase and the trehalase from its parent as a

function of time at 62°. The CW 210 and CW 230 trehalases were found to be unstable to ethanol precipitation, whereas the parental trehalase was stable (Table 3). The data show that the trehalase mutations alter the properties of the enzyme molecule and that different mutations affect it differently. We conclude that these mutations lie within the trehalase structural gene.

The relationship of mutants to the previously described tre mutants: SUSSMAN *et al.* (1971) isolated and characterized a number of trehalaseless mutants that produced an inhibitor of trehalase activity. The trehalaseless mutation was termed *tre* and mapped at a position close to the trehalase structural gene (see above). Since the different mutations all map to the same position, each of our mutants was tested for the presence of inhibitor. A simple way to test for an inhibitor is to mix an extract that might contain an inhibitor with an extract from the wild-type cell and look for loss of activity (SUSSMAN *et al.* 1971). Extracts from all six mutants inhibited wild-type trehalase activity in extract-mixing experiments (Table 3).

SUSSMAN *et al.* (1971) reported that the *tre* mutation is semidominant in heterokaryons, *i.e.*, *tre*/wild-type heterokaryons grow slower than the wild-type cell on trehalose and have reduced levels of trehalase activity. Heterokaryons between SF 189 and wild-type strains were constructed and found to grow slowly on trehalose as a carbon/energy source.

SF 189 was crossed with two of the previously described trehalaseless mutants, T7 and 72. Only a very limited number of progeny from these crosses were able to grow on trehalose (Table 2). The genetics and biochemistry of the SF 189, CW 210, CW 230, CW 315, CW 412 and CW 415 mutants suggest that these trehalase mutations are allelic with the previously isolated *tre* mutations (SUSSMAN *et al.* 1971).

Mutant extracts contain large amounts of antigen: The data show that both the presence of trehalase inhibitor and alterations of trehalase inhibitor are due to single mutations within the trehalase structural gene (Table 3). GARRETT, SUSSMAN and VU (1972) suggested that the inhibitor might be an altered trehalase molecule. If the altered trehalase is the inhibitor, mutant extracts should contain much more trehalase antigen than would be needed to account for the trehalase activity. Antibody directed against the wild-type enzyme was used to test this prediction. Figure 5 shows the amount of antibody needed to immunoprecipitate 0.009 units of trehalase activity from CW 230 and its parent. The results clearly show that it requires much more antibody to precipitate the enzymatic activity from the mutant extract. Although the mutant extract has only a fraction of the wild-type level of activity, it contains a larger than expected level of trehalase antigen.

DISCUSSION

Trehalose has been shown to be an important form of glucose storage in a number of organisms (SUSSMAN and LINGAPPA 1959). It is ubiquitous in the fungi and often used as an energy source during the germination of fungal spores. *Neurospora* produces an extracellular trehalase which is found in the periplasmic space.

FIGURE 5.—Titration of mutant and wild-type enzyme with antitrehalase antisera. Trehalase activity (0.009 units) from CW 230 (●) and from its wild-type parent (○) were incubated with increasing amounts of antisera, and the immunoprecipitated protein was removed by centrifugation. The amount of trehalase removed by the antisera is graphed *vs.* the amount of antisera added.

Neurospora trehalase from the wild-type strain 74-OR23-1A has been purified to near homogeneity. The purified enzyme preparation shows a single band of protein both in SDS-polyacrylamide gels and in isoelectric focusing gels (Figures 1 and 2). It has a *pI* of 3.7, a *pH* optimum of 5.0, and a *K_m* value of 0.36 mM. We have found that trehalase from other wild-type strains shows differences in some of these characteristics (data not shown). The subunit molecular weight of trehalase is 88,000 as judged by SDS-gel electrophoresis in the absence of 2-mercaptoethanol. When subjected to electrophoresis in the presence of 2-mercaptoethanol the purified enzyme is found as a series of bands from 75,000 downward. Different preparations show differences in the number and intensity of these bands (data not shown). We ascribe these smaller molecular weight bands to the presence of nicks caused by proteases. A second extracellular enzyme, glucamylase, is also nicked when isolated from *Neurospora*. Evidence suggests that some of these glucamylase nicks are occurring *in vivo* and not during isolation (SIGMUND *et al.* 1985). Presumably, trehalase contains a number of disulfide bridges which hold the various fragments together and which are broken by the 2-mercaptoethanol. The 88,000 molecular weight could possibly be an underestimate of the size because we would be unable to see small fragments that were not held to the rest of the molecule by disulfide bonds. The enzyme exists as a multimer of identical subunits. The size of the multimer is 360,000 as estimated by chromatography on a G-200 Sephadex column. This would suggest that the molecule might exist as a tetramer.

Neurospora responds to glucose starvation by producing trehalase and other extracellular hydrolases. The production of trehalase begins 60 min following glucose deprivation (Figure 3). Mutants that have lost glucose repression constitutively synthesize extracellular hydrolases (METZENBERG 1962; GRATZNER and SHEEHAN 1969; MURAYAMA and ISHIKAWA 1973). The production of tre-

halase is not coordinate with the production of other extracellular hydrolases controlled by glucose repression. Invertase and glucamylase are both synthesized within 30 min after a transfer to glucose-free medium (LEE and FREE 1984; SIGMUND *et al.* 1985).

A number of trehalaseless mutants have been isolated and shown to be affected in the trehalase structural gene. The residual trehalase activity in these mutants differs from the parental trehalase and from each other in thermostability and in stability in the presence of ethanol (Table 1). These structural gene mutations not only alter the characteristics of the trehalase molecule but also cause the production of an inhibitor; this suggests that the inhibitor is the altered trehalase molecule.

Similar situations, in which structural gene mutations result in the production of defective subunits capable of inhibiting the native wild-type enzyme, have been reported. DAVILA *et al.* (1983) reported that *Neurospora* glutamine synthetase structural gene mutants produce glutamine synthetase subunits capable of inhibiting the native enzyme. MACPHEE, NELSON and SCHUSTER (1983) showed that mutations in the *Neurospora* asparagine synthetase gene result in the production of a subunit inhibitor of the enzymatic activity. A well-documented case of subunit interaction between a mutant subunit and the wild-type subunit is known from *E. coli* in which a nonfunctional multimer results from I^-d mutations in the lac repressor (WEBER and GEISLER 1978). In all of these cases the mutations are dominant or codominant to the wild type and the presence of inhibitor can be demonstrated *in vitro*. The *tre* mutations share these characteristics.

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