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Trehalose synthase of *Mycobacterium smegmatis*

Purification, cloning, expression, and properties of the enzyme

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Trehalose synthase (TreS) catalyzes the reversible interconversion of trehalose (glucosyl-α,α-1,1-glucose) and maltose (glucosyl-α1-4-glucose). TreS was purified from the cytosol of *Mycobacterium smegmatis* to give a single protein band on SDS gels with a molecular mass of ≈ 68 kDa. However, active enzyme exhibited a molecular mass of ≈ 390 kDa by gel filtration suggesting that TreS is a hexamer of six identical subunits. Based on amino acid compositions of several peptides, the *treS* gene was identified in the *M. smegmatis* genome sequence, and was cloned and expressed in active form in *Escherichia coli*. The recombinant protein was synthesized with a $(His)_6$ tag at the amino terminus. The interconversion of trehalose and maltose by the purified TreS was studied at various concentrations of maltose or trehalose. At a maltose concentration of 0.5 mM, an equilibrium mixture containing equal amounts of trehalose and maltose (42–45% of each) was reached during an incubation of about 6 h, whereas at 2 mM maltose, it took about 22 h to reach the same equilibrium. However, when trehalose was the substrate at either 0.5 or 2 mM, only about 30% of the trehalose was converted to maltose in ≥ 12 h, indicating that maltose is the preferred substrate. These incubations also produced up to 8–10% free glucose. The K_m for maltose was ≈ 10 mM, whereas for trehalose it was ≈ 90 mM. While β,β-trehalose, isomaltose (α1,6-glucose disaccharide), kojibiose (α1,2) or cellobiose (β1,4) were not substrates for TreS, nigerose (α1,3-glucose disaccharide) and α,β-trehalose were utilized at 20 and 15%, respectively, as compared to maltose. The enzyme has a pH optimum of about 7 and is inhibited in a competitive manner by Tris buffer. [^{3}H]Trehalose is converted to [^{3}H]maltose even in the presence of a 100-fold or more excess of unlabeled maltose, and [^{14}C]maltose produces [^{14}C]trehalose in excess unlabeled trehalose, suggesting the possibility of separate binding sites for maltose and trehalose. The catalytic mechanism may involve scission of the incoming disaccharide and transfer of a glucose to an enzyme-bound glucose, as [^{3}H]glucose incubated with TreS and either unlabeled maltose or trehalose results in formation of [^{3}H]disaccharide. TreS also catalyzes production of a glucosamine disaccharide from maltose and glucosamine, suggesting that this enzyme may be valuable in carbohydrate synthetic chemistry.

Keywords: maltose; *Mycobacteria*; sugar interconversions; trehalose biosynthesis; trehalose metabolism.

Trehalose is a nonreducing disaccharide of glucose that is widespread in the biological world and may have a variety of functions in living organisms. Although there are three different anomers of trehalose (i.e. α,α-1,1-, α,β-1,1- and β,β-1,1-), the only known biologically active form of trehalose is α,α-1,1-glucosyl-glucose [1]. Trehalose has been isolated from a large number of prokaryotic and eukaryotic cells including mycobacteria, streptomycetes, enteric bacteria, yeast, fungi, insects, slime molds, nematodes, and plants [2,3]. Originally, it was believed to function solely as a reserve energy and carbon source in a manner similar to that of glycogen and starch [4]. However, trehalose is also a major component of a number of cell wall glycolipids in *Mycobacterium tuberculosis* and other mycobacteria, as well as in closely related organisms such as corynebacteria [5,6]. As a cell wall component, it adds to the impermeability and helps protect these organisms from antibiotics and toxic agents [7].

Trehalose functions as a protectant in yeast, fungi, brine shrimp and nematodes [8]. Thus, when yeast are subjected to heat stress, the amount of trehalose in these cells is greatly increased, and this trehalose protects proteins from denaturation, and membranes from damage and inactivation [9]. In addition, in yeast [10] and plants [11] trehalose may play a role as a signaling molecule to direct or control pathways related to energy metabolism [12], or even to affect cell growth [13].

Three distinct biosynthetic pathways can lead to the formation of trehalose [14]. The most widely distributed and best-known pathway involves two enzymes called trehalose-phosphate synthase (TPS here or OtsA in *Escherichia coli*) and trehalose-phosphate phosphatase (TPP here or OtsB

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Abbreviations: TPP, trehalose-phosphate phosphatase; TPS, trehalose-phosphate synthase; TreS, trehalose synthase.

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in *E. coli*). TPS catalyzes the transfer of glucose from UDP-glucose to glucose-6-phosphate to form trehalose-P and UDP [15]. TPP then removes the phosphate to give free trehalose [16]. A second pathway, involving the enzyme trehalose synthase (TreS), interconverts maltose and trehalose by catalyzing an intramolecular rearrangement of the α1,4-glycosidic bond of maltose to the α,α1,1-linkage of trehalose, or vice versa [17]. It is not known whether TreS functions to lower trehalose levels in cells by converting it to maltose, or whether its role is to synthesize trehalose. A third pathway involves two enzymes; the first, TreY, converts the reducing end of a glycogen or maltooligosaccharide chain from an α1,4-linkage to the α,α1,1-linkage of trehalose, while the second enzyme, TreZ, hydrolyzes the reducing-end disaccharide to produce one molecule of trehalose, and leave a glycogen that is two glucose residues shorter [18].

Because all three of these pathways appear to be present in *M. tuberculosis* [19], the question arises as to the function of each pathway, as well as how they are regulated. That is, does one pathway produce trehalose for cell wall function, while another synthesizes trehalose as a stress response? Or, are the pathways overlapping and/or coordinately controlled? In order to determine the potential role of TreS in the formation of cell wall and/or cytoplasmic trehalose, as compared to the other two biosynthetic pathways, we have cloned the *Mycobacterium smegmatis treS* gene and expressed it as active enzyme in *E. coli*. In this report, we describe the purification of TreS from *M. smegmatis*, as well as the isolation of active recombinant TreS, and its enzymatic properties. Experiments suggesting the possible mechanism of action of this enzyme are also presented.

Experimental procedures

Bacterial strains and culture conditions

M. smegmatis was obtained from the American Type Culture Collection (ATCC 14468). *M. smegmatis* mc^2155 was provided by W. R. Jacobs Jr., Albert Einstein College of Medicine, New York. The *E. coli* strains TOP10 and BL21Star (DE3) (Invitrogen) were used for cloning and expression studies, respectively. *E. coli* strains were cultured in Luria–Bertani (LB) broth and on LB agar supplemented with 100 $\mu g \cdot mL^{-1}$ ampicillin, 20 $\mu g \cdot mL^{-1}$ kanamycin or 10 $\mu g \cdot mL^{-1}$ tetracycline, individually or in combination where applicable. *M. smegmatis* was cultured in Middlebrook 7H9 broth and on Middlebrook 7H10 agar, supplemented in each case with the 10% (v/v) oleic acid–albumin–dextrose complex. All bacterial strains were cultured at 37 °C.

Reagents and materials

Trehalose, maltose, trehalase, α-glucosidase, DEAE–cellulose, ω-aminohexyl-agarose, phenyl-Sepharose, CL-4B, glucose oxidase/peroxidase assay kit, various chromatographic resins and materials, molecular mass markers for gel filtration, and buffers, were all from Sigma Chemical Co. Bio-Rad protein reagent, hydroxyapatite, DE-52, and all electrophoresis materials were from Bio-Rad. Trypticase soy broth was from Becton Dickenson, and LB broth was from Fisher Scientific Co. Sephacryl S-300 and Sephacryl S-200, and [^{14}C]maltose and [^{3}H]glucose, were from Amersham Pharmacia Biotech Inc. [^{3}H]Trehalose was prepared by incubating UDP-[^{3}H]glucose plus glucose-6-phosphate with the purified mycobacterial trehalose-P synthase as described previously [20]. The radioactive trehalose-P was isolated by ion-exchange chromatography and treated with the trehalose-P phosphatase [16] to obtain free trehalose. Ni–nitrilotriacetic acid His-binding resin was from Novagen. Except where otherwise specified, all DNA manipulation enzymes, including restriction endonucleases, polymerases and ligase, were from New England Biolabs and were used according to the manufacturer's instructions. Custom oligonucleotide primers were commercially synthesized by Integrated DNA Technologies (Coralville, IA). PCR reagents were from Applied Biosystems. All other reagents were from reliable chemical companies and were of the best grade available.

Assay of trehalose synthase activity

The enzymatic activity of TreS was routinely measured by determining the formation of reducing sugar when enzyme was incubated with trehalose. Assays were carried out in a final volume of 100 μL, containing 40 mM potassium phosphate buffer pH 6.8, various amounts of trehalose (usually 50–100 mM), and an appropriate amount of enzyme. After incubation at 37 °C for 10 min, the mixture was heated in boiling water for 5 min to stop the reaction. The amount of maltose produced was measured by the Nelson reducing sugar method [21]. A unit of enzyme is defined as that amount of enzyme that causes the conversion of 1 nmole of trehalose to maltose in 1 min. TreS could also be assayed by determining the formation of trehalose from maltose. In this case, an aliquot of the incubation mixture was subjected to HPLC on the Dionex carbohydrate analyzer to separate and quantify maltose and trehalose. Trehalose formation could also be measured using a specific trehalase to convert trehalose to glucose, and then determining the amount of glucose with the glucose oxidase reagent.

Purification of the TreS

Growth and harvesting of bacteria. M. smegmatis was grown in 2-L flasks containing 1 L trypticase soy broth. Cells were harvested by centrifugation, washed with phosphate-buffered saline, and stored as a paste in aluminum foil at −20 °C until used.

Preparation of crude extract (Step 1). All purification steps were carried out at 4 °C unless otherwise specified. One hundred grams of cell paste were suspended in 500 mL of ice-cold 10 mM potassium phosphate buffer, pH 6.8 (Buffer A), and cells were disrupted by sonic oscillation. Cell walls and membranes were removed by centrifugation and the supernatant liquid was designated 'crude extract'.

Ammoniun sulfate fractionation (Step 2). Solid $(NH_4)_2SO_4$ was added to 30% saturation, and the precipitate was removed by centrifugation and discarded. The supernatant liquid was brought to 60% saturation by the

addition of solid $(NH_4)_2SO_4$, and the precipitated protein was isolated by centrifugation and suspended in a minimal volume of Buffer A.

Gel filtration on Sephracryl S-300 and Sephracryl S-200 (Step 3). The ammonium sulfate fraction was applied to a column of Sephracryl S-300 that had been equilibrated with 10 mM potassium phosphate buffer, pH 6.8, containing 1 M KCl (Buffer B). Fractions (3 mL) were collected and an aliquot of each fraction was removed and assayed for TreS activity. Active fractions were pooled, concentrated on the Amicon apparatus, and applied to a column of Sephracryl S-200 equilibrated with Buffer B. The column was eluted with Buffer B and fractions (3 mL) were collected and assayed for TreS activity. Active fractions were pooled and concentrated on the Amicon apparatus.

DEAE–cellulose chromatography (Step 4). A column of DE-52 was prepared and equilibrated with Buffer A. The concentrated enzyme fraction from Step 3 was applied to the column, which was first washed with Buffer A, and the TreS was then eluted from the column with a 0–0.5 M linear gradient of NaCl in Buffer A. Fractions containing active enzyme were pooled and concentrated on the Amicon apparatus to a small volume.

Chromatography on hydroxyapatite columns (Step 5). The concentrated enzyme fraction from the DE-52 column was applied to a column of hydroxyapatite that had been equilibrated with Buffer A. The column was washed with buffer, and enzyme was eluted with a linear gradient of 10–250 mM potassium phosphate buffer, pH 6.8. Fractions containing TreS were pooled and concentrated on the Amicon filtration apparatus.

ω-Aminohexyl-agarose chromatography (Step 6). A column of aminohexyl-agarose was equilibrated with Buffer A. The enzyme preparation from Step 5 was applied to the column which was washed with Buffer A containing 250 mM NaCl. TreS was eluted from the column with a 250–400 mM linear gradient of NaCl in Buffer A. Those fractions containing active enzyme were pooled and concentrated on the Amicon filtration apparatus.

Phenyl-Sepharose CL-4B chromatography (Step 7). A column of phenyl-Sepharose was equilibrated with Buffer B. The enzyme fraction from Step 6 was applied to the column which was washed with Buffer A and then TreS was eluted with a linear gradient of 0–75% (v/v) ethylene glycol in Buffer A. Fractions containing active TreS were pooled and concentrated on the Amicon filtration apparatus. The ethylene glycol was removed by the repeated addition and removal of Buffer A using the Amicon filtration apparatus.

Paper chromatographic separation of disaccharides

In several experiments, the conversion of radioactivity from maltose to trehalose (or vice versa) was measured in the presence of large amounts of unlabeled trehalose in order to gain evidence for two separate substrate binding sites. In these cases, it was necessary to separate the large amount of product (trehalose) from the radioactive starting substrate (maltose), to be able to determine whether radioactive trehalose had been produced. While the Dionex analyzer separates maltose and trehalose very well, it cannot be used to separate large amounts (i.e. milligram quantities) of sugars. On the other hand, paper chromatography is useful for separating large amounts of material, although the separation is not as good. Thus, a number of individual papers can be streaked with the sugar solution and all run at the same time in the same solvent. Standards of trehalose and maltose are applied to the sides of the paper to determine the locations of these sugars, and those areas of the papers can be eluted to isolate the individual sugars which can then be re-chromatographed for additional purification, if necessary. The solvent used for chromatography was ethyl acetate/pyridine/water (12 : 5 : 4, v/v/v).

Other methods

Protein was measured with the Bio-Rad protein reagent using BSA as the standard. The molecular mass of the native TreS was estimated by gel filtration on Sephracryl S-300. Molecular mass standards included thyroglobulin (669 kDa), apoferritin (443 kDa), α-amylase (200 kDa) and carbonic anhydrase (29 kDa). SDS/PAGE was performed according to Laemmli in 10% polyacrylamide gel [22]. The gels were stained with 0.5% Coomassie blue in 10% acetic acid.

Equilibrium analysis

Equilibrium analysis studies were conducted using high performance anion-exchange chromatography. Eluents were distilled water (E1) and 400 mM NaOH (E2).

Appropriate aliquots (0–3 nmol) from each time point were injected into a CarboPac PA-1 column equilibrated with a mixture of E1 and E2 (E1/E2 = 98/2). The elution and resolution of the carbohydrate mixtures was performed as follows: T_0 = 2% E2 (v/v); T_{15min} = 100% E2 (v/v); T_{25min} = 100% E2 (v/v). Each constituent was detected by pulse amperometry as recommended by the manufacturer (Dionex, technical note, March 20, 1989) at a range setting of 300 K.

Sequence analysis

ORFs were identified by BLASTP alignment with predicted amino acid sequences on GenBank™. Multiple amino acid alignments were performed using the online CLUSTALW alignment program at a web site maintained by the European Bioinformatics Institute (EMBL-EBI; http://www.ebi.ac.uk/clustalw/). Basic sequence analysis, including identification of restriction sites, translations, and DNA sequence alignment, were performed using the GENEJOCKEY program (Biosoft, Cambridge, UK).

Results

Purification of *M. smegmatis* TreS

TreS was purified about 3800-fold from the cytosolic extract of *M. smegmatis* as outlined in Table 1. The steps in the purification procedure included gel filtration on Sephracryl

Table 1. Purification of TreS. Steps in the purification are described in the Experimental procedures. The protein profiles at each step in the purification are shown in Fig. 1. One unit of enzyme is that amount that causes the converion of 1 nmole trehalose to maltose in 1 min.

Step	Total protein (mg)	Total activity (units)	Specific activity (units·mg^{-1} protein)	Purification (fold)	Yield (%)
Crude	11448	65 250	5.7	0	100
$(NH_4)_2SO_4$	4040	31 416	7.9	1.4	49
Gel filtration	1720	18 748	10.9	2.0	29
DE-52	120	9168	76.4	13	14
Hydroxy-apatite	42	7804	185	33	12
Aminohexyl-agarose	1.2	5250	4375	768	8
Phenyl-sepharose	0.15	3269	21791	3825	5

S-200 and S-300, ion exchange chromatography on DEAE–cellulose, chromatography on hydroxyapatite columns, and hydrophobic chromatography on columns of aminohexyl-agarose and phenyl-sepharose. Figure 1 shows the protein profiles obtained at each of these steps, as demonstrated by SDS/PAGE. It can be seen in lane 8 that the final elution from the phenyl-sepharose column gave one major protein band with a molecular mass of ≈ 68 kDa. The recombinant TreS purified from *E. coli* extracts (see below) also showed a single protein band (Fig. 1, lane 9) with the same migration

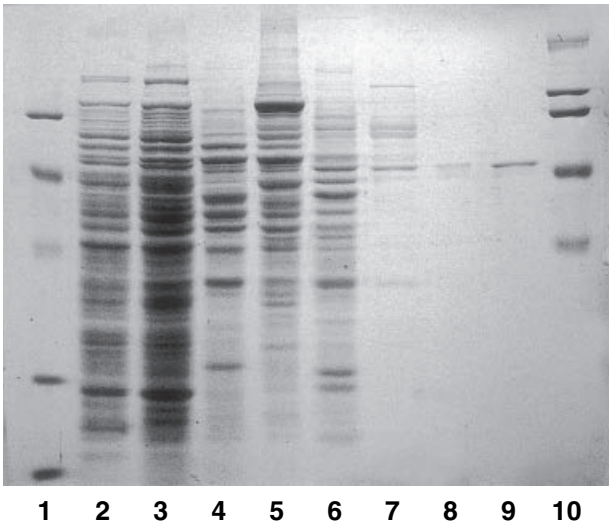

Fig. 1. Purification of *M. smegmatis* TreS. At each step in the purification an aliquot of the sample was subjected to SDS/PAGE and the proteins were visualized by staining with Coomassie blue. Lanes 1 and 10 are protein standards (from the top: left, 97, 66, 45, 31, 21 kDa; right, 200, 116, 97, 66, 45 kDa). Lanes 2–8 are various steps in the purification: 2, crude extract; 3, ammonium sulfate precipitate; 4, gel filtration; 5, DE-52 elution; 6, hydroxylapatite elution; 7, aminohexyl-agarose fraction; 8, phenyl-sepharose elution; 9, recombinant enzyme purified on nickel column.

***M.smegmatis* Trehalose synthase aa sequence .**

MEEHTQGSHV EAGIVEHPNA EDFGHARTLP TDTNWFK**HAE**
VLVRAFYDSN ADGIGDLRGL TEKLDYI KWL GVDCLWLPPF
YDSPLR**DGYD IRDFYK**VLPE FGTVDDFVTL LDAAHRRGIR
IITDLVMNHT SDQHEWFQES RHNPDGPYGD FYVWSDTSDR
YPDARIIFVD TEESNW TFDP VR**RQFY WHR**F FSHQPDLNYD
NPAVQEAMLD VLRFWLDLGI DGFR**LDAVPY LFEREGTNCE**
NLPETHAFLK RCRKAIDDEY PGRVLLAEAN QWPADVVAYF
GDPDTGGDEC HMAFHFPLMP R**IFMAVR**RES RFPISEILAQ
TPPIPDTAQW GIFLR**NHDEL TLEMVTDEER DYMYAEYAKD**
PRMKANVGIR RRLAPLLEND RNQIELFTAL LLSLPGSPVL
YYGDEIGMGD IIWLGDRDSV RTPMQWTPDR NAGFSKATPG
RLYLPPNQDA VYGYHSVNVE AQLDSSSSLL NWTRNMLAVR
SRHDAFAVGT FRELGGSNPS VLAYIREVTR QQGDGGAKTD
AVLCVNNLSR FPQPIELNLQ QWAGYIPVEM TGYVEF PSIG
QLPYLLTLPG HGFYWFQLRE PDPEPGAQQ

Fig. 2. Predicted amino acid sequence of *M. smegmatis* TreS based on gene sequence. A number of peptides isolated from purified TreS were identified by Q-TOF MS, and identified in the *M. smegmatis* genome (shown in bold type and underlined). These peptides allowed the gene for TreS to be identified in the genome and its cloning and expression in *E. coli*.

properties as the purified 68-kDa protein from *M. smegmatis*. On the other hand, active TreS, subjected to gel filtration on a column of Sephracryl S-300 eluted at a position indicating a molecular mass of about 390 000 (data not shown), suggesting that the native enzyme is a hexamer of six identical 68-kDa subunits. The purified enzyme was stable to storage at −20 °C for at least several weeks, but was inactivated by repeated freezing and thawing. It could be stored on ice for several months with no apparent loss of activity.

The 68-kDa protein from lane 8 of the SDS gels was excised from the gels and subjected to trypsin digestion and amino acid analysis using Q-TOF MS to determine amino acid compositions of the various peptides. The data from these peptides (Fig. 2) was used to locate the ORF coding for TreS in the *M. smegmatis* genome.

Cloning and sequencing of *M. smegmatis* TreS cDNA

The TIGR unfinished *M. smegmatis* genome sequence was screened using the TBLASTN program for DNA sequences corresponding to the amino acid sequences obtained from purified *M. smegmatis* TreS. All of the primary amino acid sequences aligned with a region of contig 3426. The possible ORF in this region (1781 bp) is located at nucleotides 4158182–4156401 (−2 frame) of the *M. smegmatis* mc^{2}155 genome sequence. This ORF potentially encodes a 593-residue polypeptide with a predicted molecular mass of 71 kDa. Figure 2 presents the amino acid sequence of this ORF and the underlined areas correspond to the predicted matches based on the amino acid compositions that we obtained from MS.

BLASTP analysis of this ORF amino acid sequence indicated homology with hypothetical proteins Rv O126 from *M. tuberculosis* (85% identity) and putative TreS from *Streptomyces avermitilis* (72% identity), from *Corynebacterium glutamicum* (69% identity) and from *Pseudomonas* sp. (61% identity).

This ORF was amplified by PCR using the oligonucleotide primers TSFP 5′-CACC**ATG**GAGGAGC ACACGCAGGGCAGC-3′ (4 158 182–4 158 159) and TSRP 5′-CGACAC**TCA**TTGCTGCGCTCCCGGTTC-3′ (4 156 393–4 156 419). The bold '**ATG**' in the forward primer represents the start codon, and bold 'TCA' in TSRP represents the stop codon of the recombinant ORF. PCR products were directionally cloned into precutpET100D-TOPO (Invitrogen) generating the plasmid pTS-TOPO. The overhang into the cloning vector (GTGG) invaded the 5′ end of the PCR product, annealed to the four bases (CACC; underlined) and stabilized the PCR product into the correct orientation. The entire cloned $(His)_6$–*treS* gene fusion was sequenced to confirm the fidelity of the amplification. The pTSTOPO was transformed into *E. coli* expression strain BL21 star (DE3). pTSTOPO in BL21 star (DE3) was used for further expression studies.

The *E. coli* expression strain BL21 was grown and induced by addition of 1 mM isopropyl thio-β-D-galactoside for 4 h. The crude sonicate of these cells was subjected to high-speed centrifugation and TreS activity was located both in the supernatant fraction and in the pellet. However, the majority of the activity in the pellet could be released into the soluble fraction upon repeated sonication. The solubilized protein was applied to a nickel ion column and after thorough washing in 10 mm imidazole, the column was eluted batchwise with various concentrations of imidazole. Most of the activity was eluted in 100 mM imidazole, and as shown in Fig. 1, lane 9, this fraction contained a single protein band on SDS gels that migrated with the TreS purified from *M. smegmatis* extracts. The enzymatic properties of recombinant TreS were identical to those of enzyme purified from the mycobacterial extract.

Properties of the TreS purified from *M. smegmatis*

Effect of time and protein concentration on formation and characterization of the products. The conversion of trehalose to maltose was measured by determining the amount of reducing sugar resulting from the production of maltose. The amount of maltose increased with increasing incubation times up to 10 h, and then slowly leveled off with longer incubation times (data not shown). The formation of maltose was also proportional to the amount of enzyme added to the incubation mixtures (data not shown). The formation of trehalose from maltose was also linear with time of incubation and enzyme concentration, but the rate of this conversion was much slower than that of maltose to trehalose. This data showed that all measurements were made in the linear range.

The product produced from maltose was characterized as α,α1,1-trehalose on the basis of the following criteria: (a) identical rates of migration to that of standard trehalose on paper chromatograms in several different solvent systems; (b) identical elution position on the Dionex carbohydrate analyzer to that of standard trehalose; (c) hydrolysis to glucose by a specific trehalase as also shown by authentic trehalose; (d) similar resistance as authentic trehalose to hydrolysis by α-glucosidase. Likewise, the product produced from trehalose showed identical mobilities on paper chromatograms and by HPLC to those of authentic maltose, as well as identical susceptibility to α-glucosidase but resistance to trehalase.

Determination of equilibrium. The enzyme purified from *M. smegmatis* catalyzed the reversible interconversion of the α1,4-linked glucose disaccharide, maltose, to the nonreducing α,α1,1-linked disaccharide, trehalose, or vice versa. Figure 3 presents the results of several experiments in

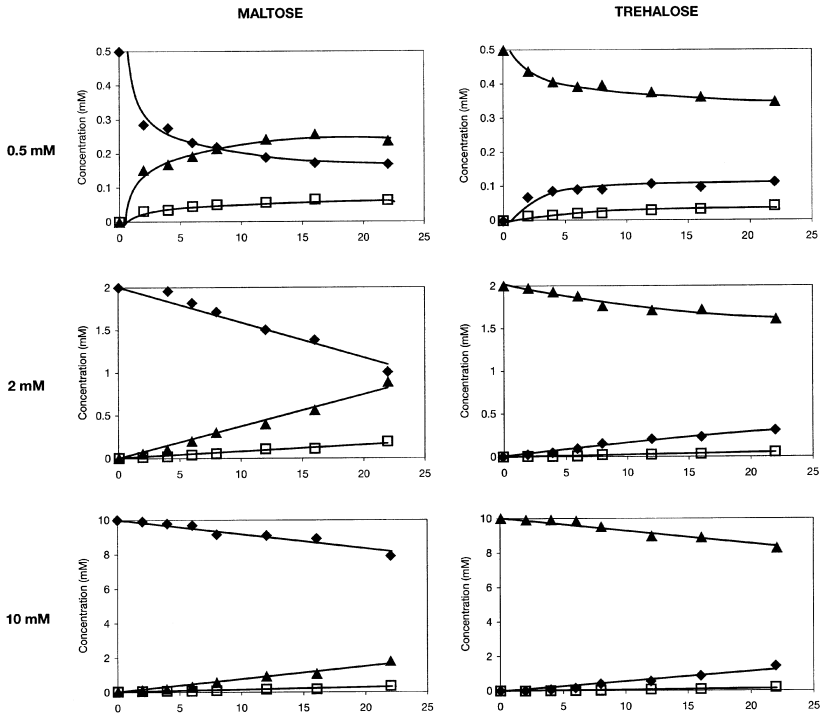

Fig. 3. Time-course studies to reach equilibrium of disaccharides with purified TreS. Enzyme was incubated with various concentrations of maltose (left profiles) or trehalose (right profiles) and aliquots of the incubation mixtures were removed at the times indicated in the graphs and subjected to Dionex HPLC to determine the ratios of maltose (■) and trehalose (▲). Glucose (□) was also produced in these incubations and its concentration was also determined. These were carried out at 0.5, 2 and 10 mM initial concentrations of maltose (left side) or trehalose (right side). Samples were removed at times up to 22 h.

which TreS was incubated with various concentrations of either maltose or trehalose, and the amounts of the two sugars were measured at increasing times of incubation following their separation by HPLC. Profile A (left) shows that when the substrate was maltose at an initial concentration of 0.5 mM an equilibrium mixture was reached in about 6 h; this contained equal amounts of both trehalose and maltose (42–45% of each) as well as around 8–10% glucose. The other figure in Profile A (right) shows the conversion of 0.5 mM trehalose to maltose. In this case, the rate of conversion of trehalose to maltose was much slower and equilibrium was not reached, even after an incubation of 22 h. In this reaction also, small amounts of glucose were produced.

Similar experiments were carried out at 2 and 10 mM maltose or trehalose and the results are shown in Fig. 3B,C. With 2 mM maltose, it took about 22 h to reach equilibrium, but again the ratio of trehalose to maltose was approximately 1 : 1 (40–45% of each disaccharide). However, when TreS was incubated with 2 mM trehalose, the conversion to maltose was again much slower, and after 22 h only 30% of the trehalose had been utilized with the formation of about 22% maltose. Figure 3C shows that at 10 mM maltose or 10 mM trehalose, the attainment of equilibrium was even slower than with 0.5 or 2 mM concentrations. These data indicate that the time necessary for reaching equilibrium depends on the concentration of the starting substrate, and that TreS prefers maltose over trehalose as the substrate. These results are in agreement with experiments presented below that also demonstrate that TreS has a greater affinity for maltose than for trehalose.

Determination of substrate affinities

Because TreS catalyzes the interconversion of maltose and trehalose, but converts maltose to trehalose more rapidly than trehalose to maltose, it was of interest to determine the affinity (K_m) of TreS for these two substrates. The amount of the product, trehalose, increased with increasing concentrations of maltose in the incubation up to about 5 mM, and then leveled off with further increases in substrate concentration. When this data was plotted by the method of Lineweaver and Burk, the K_m for maltose was estimated to be $\approx$ 10 mM and the V_{max} for maltose was determined as 16 nmol·min^{-1}. A similar experiment using trehalose as the substrate showed that the formation of maltose increased with increasing concentrations of trehalose to give a K_m of $\approx$ 90 mM and a V_{max} of 25 nmol·min^{-1}. These data support the equilibrium experiments indicating that TreS has a greater affinity for maltose than it does for trehalose.

Substrate specificity of TreS

The substrate specificity of TreS in the trehalose to maltose direction was examined by determining whether maltose could also be produced from either α,β-trehalose or β,β-trehalose. The results of this experiment are presented in Table 2. The naturally occurring, or α,α-anomer of trehalose was by far the best substrate, but TreS could also convert the α,β-trehalose to maltose, although only about 15% as well as with the natural trehalose. However, the β,β-anomer of trehalose was inactive as a substrate.

Table 2. Substrate specificity of TreS. Various trehalose anomers and other glucose disaccharides were added to incubation mixtures instead of trehalose and incubated with purified (or recombinant) TreS as described in Experimental procedures. The amount of reducing sugar was determined and the product was identified as maltose by paper chromatography.

	Activity (nmol·min^{-1})
Linkage of trehalose activity	
α,α	11.1
α,β	1.6
β,β	0.03
Glucose disaccharides as substrates	
Maltose (α1,4)	10.0
Isomaltose (α1,6)	0
Cellobiose (β1,4)	0
Nigerose (α1,3)	2.0
Kojibiose (α1,2)	0
Maltitol	0

A number of glucose disaccharides were also tested as substrates to replace maltose in the synthesis of trehalose. Table 2 shows that isomaltose (α1,6-glucosyl-glucose), kojibiose (α1,2-glucosyl-glucose) and cellobiose (β1,4-glucosyl-glucose) were not utilized as substrates for TreS, but nigerose (α1,3-glucosyl-glucose) was converted to trehalose, although only about 20% as well as maltose.

Effect of pH and various inhibitors on TreS activity

The pH optimum of TreS was determined using two different buffers as shown in Fig. 4. The pH optimum of this enzyme was 7–7.2 using phosphate buffer. Tris buffer was inhibitory, and this inhibition was of a competitive nature, with 50% inhibition occurring at a concentration of

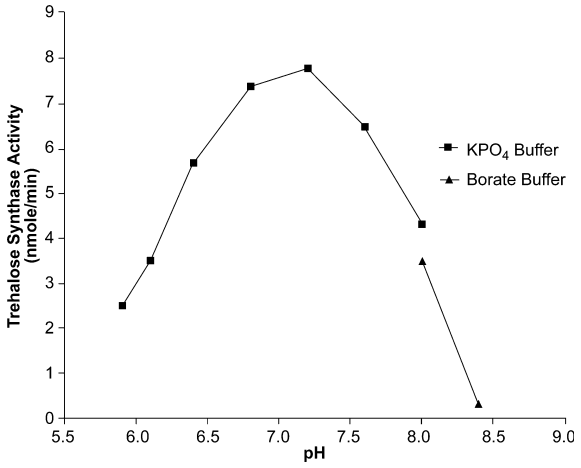

Fig. 4. Effect of pH of the incubation mixture on the activity of TreS. Incubations were as described in the text using trehalose as substrate, but contained phosphate buffer or borate buffer at various pH values. Enzyme activity was measured by determining the reducing sugar value as maltose was formed from trehalose. In these experiments incubations were for 10 min.

about 2.5 mM Tris. On the other hand, phosphate was somewhat stimulatory and caused a 25–30% increase in activity at about 20 mM concentration (data not shown).

A number of other compounds were tested as possible inhibitors of this reaction. The glucosidase inhibitor, castanospermine, was examined and found to inhibit the conversion of maltose to trehalose and trehalose to maltose with 50% inhibition of either reaction occurring at about 50 μg of castanospermine per incubation mixture. On the other hand, trehalase inhibitors such as trehazolin did not affect the reaction. In addition, vancomycin, moenomycin and diumycin, antibiotics that have been found to inhibit other enzymes in trehalose metabolism (23,24), did not inhibit TreS.

Mechanism of action of TreS

Evidence compatible with two substrate binding sites. In order to determine the catalytic mechanism of TreS, each of the radioactive substrates was incubated with the enzyme in the presence of high concentrations of the unlabeled product, and the formation of radioactive product was determined. Thus, [^{14}C]maltose, at micromolar concentrations, was incubated with TreS in the presence of 50 mM unlabeled trehalose. The incubation mixture was subjected to paper chromatography on a number of papers, in order to separate the large amount of trehalose from [^{14}C]maltose. The radioactivity in each area of the paper chromatograms was then determined. Figure 5 shows that in the control incubations with heat-inactivated enzyme (open bars), the radioactivity was present only in the maltose area of the papers, whereas when radioactive maltose was incubated with active TreS, even in the presence of a very large excess of trehalose, radioactive trehalose was still produced (filled bars). Similar results were observed when TreS was incubated with radioactive trehalose in the presence of a large excess of unlabeled maltose (data not shown).

The above experiment was repeated at various incubation times to compare maltose and trehalose as substrates in the presence of excess product. In this experiment, aliquots of each incubation were removed at the times indicated in Table 3 and treated either with trehalase (when [^{3}H]trehalose was the substrate) or with α-glucosidase (when [^{14}C]maltose was the substrate) to convert any remaining substrate to free glucose. After this incubation, the mixture was passed through a column of Biogel P-2 to separate the disaccharide product from the radioactive glucose, and the disaccharide product was isolated by paper chromatography and its radioactive content was determined. Table 3 shows that radioactive maltose was readily converted to trehalose even in the presence of a 100-fold excess of unlabeled trehalose and the amount of radioactivity converted to trehalose continued to increase in an almost linear manner for about 6 h. Radioactive maltose was also formed from [^{3}H]trehalose in the presence of a large excess of unlabeled trehalose, but in this case the reaction was not linear beyond 1 h and was much slower. However, the fact that maltose is still converted to trehalose in excess unlabeled trehalose suggests that TreS may have two separate binding sites, one for trehalose and another for maltose.

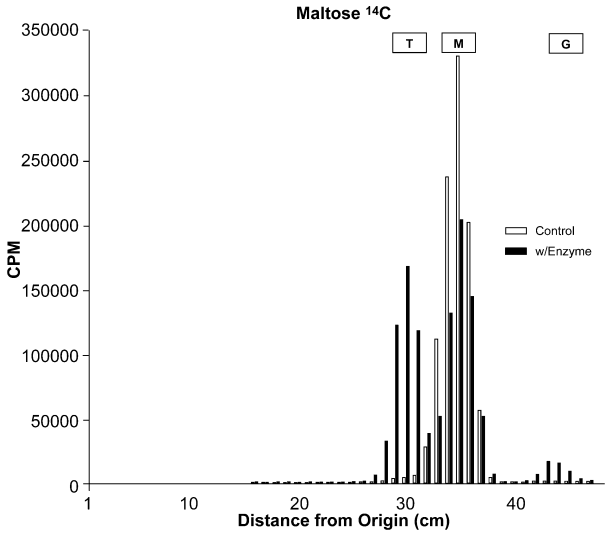

Fig. 5. Production of radioactive trehalose from [^{14}C]maltose in the presence of unlabeled trehalose. Radioactive maltose (10 μCi, 10 μM) was incubated with purified TreS in the presence of 50 mM unlabeled trehalose and after an incubation of 2 h, the reaction was stopped by heating. Control incubations contained all the reaction components but were incubated with 'heat-inactivated' enzyme and processed in the same way as with active enzyme. The supernatant liquid was deionized with mixed-bed ion-exchange resin and subjected to paper chromatography in ethyl acetate/pyridine/H_2O (12 : 5 : 4). Radioactive areas of the paper were detected by cutting papers into 1 cm strips, from the origin to the solvent front. Each strip was placed in a scintillation vial and its radioactive content was determined. Standard sugars, i.e. glucose (G), trehalose (T) and maltose (M), were run on the sides and detected by the silver nitrate dip. Their locations on the paper are shown at the top of the figure.

Table 3. Evidence for two separate binding sites in TreS. Incubations contained radioactive disaccharide (either [^{3}H]trehalose or [^{14}C]maltose) at μM concentration and 20 mM concentration of the unlabeled other disaccharide (cold mM maltose with radioactive trehalose and vice versa). The amount of radioactive maltose produced from trehalose, or vice versa, was determined.

[^{3}H]Trehalose → Maltose		[^{14}C]Maltose → Trehalose	
Time (h)	Maltose (c.p.m.)	Time (h)	Trealose (c.p.m.)
0	692	0	1087
1	5009	1	31365
3	5535	3	113086
6	5116	6	296145
22	7096	22	326974

Evidence for glucose as an intermediate in the conversion

Radioactive glucose was consistently produced when either purified or recombinant TreS was incubated with radioactive maltose or radioactive trehalose (Fig. 3). This observation suggested that glucose might be an intermediate in the

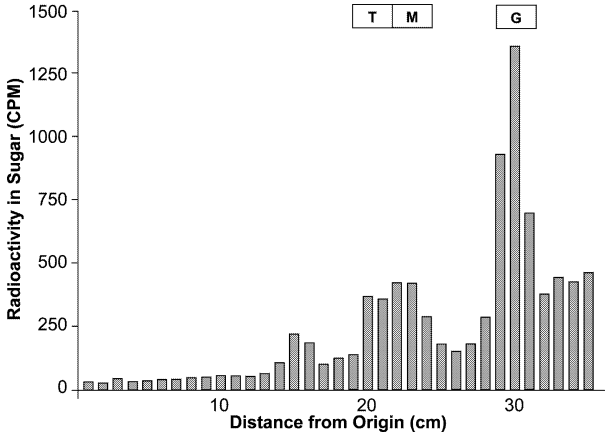

Fig. 6. Conversion of radioactive glucose to radioactive trehalose or radioactive maltose by purified TreS. Enzyme was incubated with [^{3}H]glucose (10 μCi, 10 μmoles) in the presence of either unlabeled maltose (50 mM) or unlabeled trehalose (50 mM). After an incubation of 20 h, the mixtures were deproteinized and deionized, and the supernatant liquid was subjected to paper chromatography as described in Fig. 5. Radioactive areas of the paper were detected by scintillation counting as in Fig. 5.

reaction, either as the free sugar or in an enzyme-bound form. A number of experiments were carried out in an attempt to isolate radioactive enzyme (TreS). These experiments included incubating TreS with [^{3}H]glucose, in the absence or presence of the unlabeled disaccharides, and then precipitating the protein with methanol and examining the precipitate for its radioactive content. Attempts were also made to reduce the enzyme with $NaBH_4$ in the event that the radioactive glucose was bound to the protein via a Schiff base intermediate. No evidence for a radioactive enzyme was obtained in any of these experiments.

However, when [^{3}H]glucose was incubated with active TreS, radioactive disaccharides were produced. This conversion of radioactive glucose to radioactive disaccharide was examined in more detail as indicated by the experiments reported below. In the chromatogram presented in Fig. 6, TreS was incubated with radioactive glucose for 2 h in the presence of unlabeled trehalose, and the reaction was subjected to paper chromatography to separate the disaccharide area from free glucose. A small peak of radioactivity, identified as maltose, was observed that did separate from the radioactive glucose peak. TreS was also incubated with radioactive glucose and unlabeled maltose. In this case, most of the radioactivity in the disaccharide area was in trehalose with a small peak in the maltose area and as expected a large peak of radioactivity in glucose (data not shown). As a control for these experiments, radioactive glucose was incubated with heat-inactivated enzyme in the presence of unlabeled trehalose, or unlabeled maltose. No radioactivity was found in the disaccharide areas of the paper in those experiments.

Exogenous glucose was also found to inhibit the conversion of maltose to trehalose, or trehalose to maltose, as shown in Fig. 7. In this experiment, TreS was incubated with either 50 mM trehalose or 50 mM maltose in the absence or in the presence of 10 or 50 mM glucose. Fig. 7 shows that 10 mM glucose inhibited both the conversion of maltose to trehalose and trehalose to maltose by 30–50% at 1 and 3 h of incubation, and this inhibition increased to > 75% at 50 mM glucose. These experiments strongly implicate glucose as an intermediate in the reaction, but its exact role remains to be established.

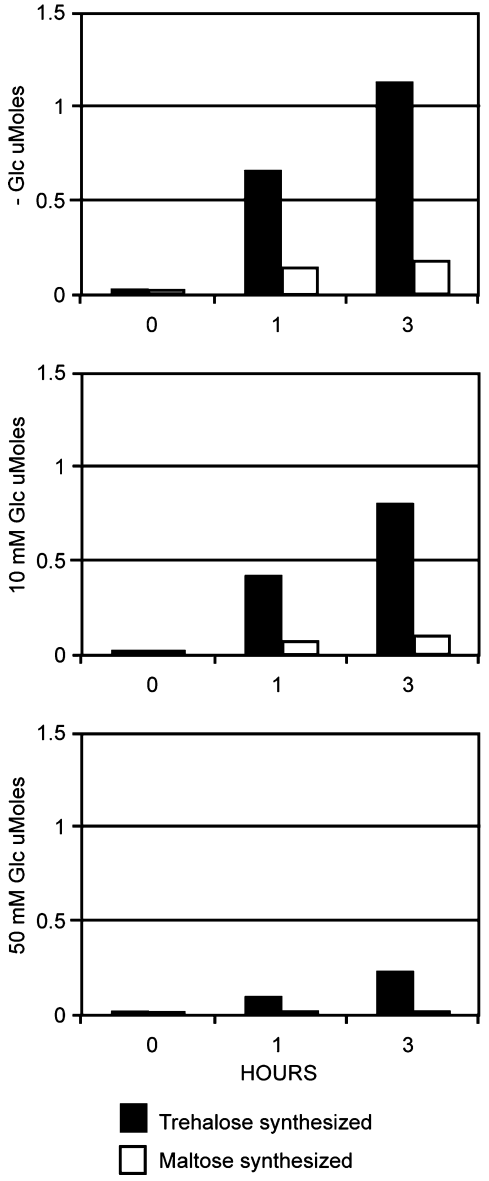

Fig. 7. Inhibition of TreS activity by free glucose. Incubations were as described in other figures and contained either 50 mM trehalose (filled bars) or 50 mM maltose (open bars), buffer and purified TreS. Either no glucose (upper graph), 10 mM glucose (middle graph), or 50 mM glucose (lower graph) were added to each incubation, and samples were removed and assayed for the presence of maltose (in the incubations where trehalose was substrate) or trehalose (in the maltose incubations) at 0 time, 1 h of incubation and after a 3 h incubation. Incubations were stopped by heating, deionized with mixed-bed ion-exchange resin and lyophilized. Sugars were detected and quantitated on the Dionex Carbohydrate Analyzer.

Formation of an amino-sugar disaccharide

[^{14}C]Maltose was incubated with TreS in the presence of unlabeled glucosamine and after an incubation of 3 h, the reaction was stopped by heating. The incubation mixture was passed through a column of Dowex-50-H$^+$, and after thorough washing with water, the column was eluted with HCl. A sharp symmetrical peak of [^{14}]C emerged in the acid elution. The eluted radioactive peak was pooled, concentrated, and separated by chromatography on a Biogel P-2 column (2 × 200 cm). The radioactive material eluted from the column at the same position as where disaccharides emerge. This radioactive material was N-acetylated in the presence of acetic anhydride and sodium bicarbonate, and following this treatment, the radioactive material no longer bound to the Dowex-50 column. These data suggests that the enzymatic product is a disaccharide of glucose and glucosamine, which becomes acetylated to give a disaccharide of [^{14}C]glucose and GlcNAc. Unfortunately, the amount of product is currently too small for NMR analysis, and thus far it is not known whether it is a reducing or nonreducing disaccharide.

Discussion

M. tuberculosis and other mycobacteria utilize the glucose disaccharide α,α-trehalose in several different roles. It is a component of a number of cell wall lipids, such as trehalose-dimycolate, and other glycolipids [23], and is also present as the free disaccharide in the cytosol of mycobacteria as well as most bacteria, yeast and fungi [24]. In the cytosol, it serves as a storehouse of energy and carbon, and may also serve to protect cellular membranes and proteins from various stresses such as heat and pressure [25] or oxidation [8]. Any one of these functions could be essential to the organism's ability to survive within the host, and/or to cause an active infection. It is likely that some roles for trehalose may be more critical to survival of the pathogen than others.

Therefore, the biosynthesis of trehalose should be an excellent target for inhibiting mycobacterial growth, or for causing these organisms to become much more susceptible to various antibiotics, or to phagocytosis. Furthermore, as trehalose is not synthesized or required by mammalian cells, nor is it present in any mammalian cell structures, inhibitors of trehalose formation or utilization should not be toxic to humans. Isolation of a *M. smegmatis* strain defective in the synthesis of mycolic acid [7] provides evidence for the essential role of the trehalose glycolipids in cell wall function. As a result of this lesion, this mutant is unable to synthesize glycolipids such as trehalose-mono- and dimycolate. Although the mutant still grows well in artificial media such as trypticase soy broth, it is much more sensitive to various antibiotics, detergents, and other toxic agents. Presumably, the cell wall lacks the hydrophobic trehalose-glycolipids, and therefore has a permeability defect that allows toxic compounds to enter and kill the cells. Of course, in this case the sensitivity could be due entirely to loss of mycolic acid and not to the absence of trehalose-glycolipids.

While trehalose biosynthesis should be a useful target site for intervention in mycobacterial diseases, it is now clear that the metabolism of this sugar is more complicated than previously hypothesized. Thus, examination of the *M. tuberculosis* gene sequence has shown a number of ORFs with considerable homology to genes in other bacteria that code for various pathways that could potentially lead to the production of trehalose [14]. Those studies suggest three potential pathways of synthesis of trehalose as outlined in the Introduction, but they do not show whether these pathways are actually active and functioning in mycobacteria, nor do they indicate whether one pathway produces the trehalose that is incorporated into cell wall glycolipids while another pathway produces trehalose as a stress protectant, and so on. Therefore, it is essential to isolate and characterize the mycobacterial enzymes involved in each pathway, and then determine the role of each pathway in the production of trehalose in the intact organism, as well as to understand how the pathways interact with each other.

We recently cloned and expressed the two enzymes in the most widely known pathway, i.e. the trehalose phosphate synthase that transfers glucose from UDP-glucose to glucose-6-phosphate to form trehalose-6-phosphate and UDP [26], and the trehalose-phosphate phosphatase that cleaves trehalose-phosphate to form free trehalose and inorganic phosphate [27]. The recombinant proteins have been characterized and several antibiotics that inhibit these activities have been identified [28]. We are currently making mutant strains that are defective in these enzymatic activities in order to determine the role of that pathway in formation of cytoplasmic and/or cell wall trehalose.

The enzyme described in this report, trehalose synthase (TreS), may represent another pathway to synthesize trehalose from maltose, and it could also represent a link between glycogen and trehalose. Alternatively, TreS could be a mechanism to lower trehalose levels in cells such as after stress, or another pathway to convert trehalose to glucose. In *C. glutamicum*, the same three pathways have been identified and a number of deletion mutations have been made to determine the significance of each of these pathways [29]. When any one of the three pathways was inactivated by chromosomal deletion, there was relatively little effect on *C. glutamicum* growth. However, when all three pathways were deleted together, or the TPS/TPP and the TreYZ pathways were deleted together, the resulting mutants failed to produce trehalose, and failed to grow efficiently on various sugar substrates in minimal medium. However, addition of trehalose to the medium reversed the growth defect. In minimal medium and in the absence of trehalose, the double and triple mutants showed an altered cell wall lipid composition and lacked both trehalose mono- and trehalose di-corynomycolate.

Another study with *C. glutamicum* examined the role of the various pathways in the function of trehalose as an osmoprotectant [30]. Again strains defective in one or more of the trehalose biosynthetic pathways were used. These workers concluded that osmoregulated trehalose synthesis is mediated by the TreYZ, and not by the OtsAB (TPS/TPP) pathway. They also concluded that TreS is likely to be important for trehalose degradation rather than synthesis, as the ratio of trehalose to maltose in the cell is about 1000 : 1, whereas the conversion of trehalose to maltose is near equilibrium. We have also found that the levels of maltose in the cytoplasm of *M. smegmatis* are substantially lower than the amounts of trehalose, but we find ratios of

about 8–10 : 1 of trehalose : maltose. However, as the K_m for trehalose is about 10-fold higher than the K_m for maltose, TreS should function equally well in either direction. However, it is not clear what function maltose serves in mycobacteria: is it an energy source, or is it a means to reduce the concentration of trehalose? That is, TreS could be involved in controlling the levels of intracellular trehalose and this disaccharide, or its metabolites, could affect other energy-producing pathways, or it could act as a signaling molecule in mycobacteria as it apparently does in yeast.

The studies described here suggest that TreS may have a binding site for maltose that is distinct from the binding site for trehalose. This hypothesis is based on the observations that high concentrations of trehalose do not prevent the conversion of maltose to trehalose, or vice versa. In addition, it seems likely that TreS must have both maltase and trehalase activities, and these two different hydrolytic activities would likely be distinct from each other. In fact, as free glucose appears to be one of the products of the purified enzyme activity, and radioactive glucose, in the presence of maltose or trehalose can be converted by the enzyme into radioactive disaccharides, a likely mechanism would be cleavage of the maltose by an α-glucosidase activity (maltase) and transfer of one of the glucoses to an enzyme-bound glucose to give trehalose, or cleavage of the trehalose by a trehalase and transfer of glucose to another enzyme-bound glucose to give maltose. Unfortunately, we were not able to provide any evidence for an enzyme-bound glucose, but this may be due to the fact that the glucose is only transiently bound to the protein, and cycles on and off of the protein.

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