The trehalose-phosphate phosphatase (TPP) was purified from the cytosol of Mycobacterium smegmatis to near homogeneity using a variety of conventional steps to achieve a purification of about 1600-fold with a yield of active enzyme of about 1%. Based on gel filtration, the active enzyme had a molecular weight of about 27,000, and the most purified fraction also gave a major band on SDS-PAGE corresponding to a molecular weight of about 27,000. A number of peptides from the 27-kDa protein were sequenced and these sequences showed considerable homology to the trehalose-P phosphatase of Escherichia coli. Based on these peptides, the M. smegmatis gene for TPP was cloned and expressed in E. coli. The recombinant protein was synthesized with a (His)_6 tag at the amino terminus. Most of the TPP activity in the crude E. coli sonicate was initially found in the membrane fraction, but it became solubilized in the presence of 0.2% Sarkosyl. The solubilized protein was purified to apparent homogeneity on a metal ion column and this fraction had high phosphatase activity that was completely specific for trehalose-P. The purified enzyme, either isolated from M. smegmatis, or expressed in E. coli, rapidly dephosphorylated trehalose-6-P, but had essentially no activity on any other sugar phosphates, or on p-nitrophenyl phosphate. The K_m for trehalose-6-P was about 1.6 mM, and the pH optimum was about 7.5. The native enzyme showed an almost absolute requirement for Mg^{2+} and was not very active with Mn^{2+}, whereas both of these cations were equally effective with the recombinant TPP. The enzyme activity was inhibited by the antibiotics, diumycin and moenomycin, but not by a number of other antibiotics or trehalose analogs. TPP activity was strongly inhibited by the detergents, Sarkosyl and deoxycholate, even at 0.025%, but it was not inhibited by Nonidet P-40, Triton X-100, or octyl glucoside, even at concentrations up to 0.3%. The purified enzyme was stable to heating at 60 °C for up to 6 min, but was slowly inactivated at 70 °C. Circular dichroism studies on recombinant TPP indicate that the secondary structure of this protein has considerable β-pleated sheet and is very compact. TPP may play a key role in the biosynthesis of trehalose compounds, such as trehalose mycolates, and therefore may represent an excellent target site for chemotherapy against Mycobacterium tuberculosis, and other mycobacterial pathogens. Thus, the mechanisms of action and the properties of the enzymes involved in the biosynthesis of trehalose represent important information for the design and development of new antimycobacterial drugs.

Trehalose is an α,α-1,1-linked disaccharide of D-glucose and is found in various plants, insects, and microorganisms. In these organisms, it is believed to serve diverse roles, one of which is as a storehouse of glucose for energy and/or for the synthesis of cell components, another is for protection of cells against environmental pressures such as desiccation and freezing, and for stabilization of proteins against denaturation (1). In addition to these several functions for trehalose that have been demonstrated in yeast, fungi, and insects, some other organisms such as mycobacteria and corynebacteria also utilize trehalose as a structural component in their cell walls (2). Thus, in these organisms, trehalose serves as the backbone of several different glycolipids, such as trehalose mycolate, various sulfolipids, and other acylated-trehalose compounds (3–5). Because trehalose is neither synthesized by, nor utilized in, mammalian cells, but is probably an essential molecule for the structural integrity of the mycobacterial cell wall, the reactions involved in the biosynthesis of trehalose and trehalose glycolipids should represent excellent potential target sites for chemotherapy against Mycobacterium tuberculosis, and other mycobacterial pathogens. Thus, the mechanisms of action and the properties of the enzymes involved in the biosynthesis of trehalose represent important information for the design and development of new antimycobacterial drugs.

The major pathway for the synthesis of trehalose and trehalose derivatives in most organisms involves the transfer of glucose from UDP-glucose (or GDP-glucose) to glucose-6-P to produce trehalose 6-phosphate plus UDP (or GDP) (6). This reaction is catalyzed by trehalose-P synthase (TPS), but this enzyme apparently differs from one organism to another in terms of the nucleoside diphosphate glucose that is able to serve as the glycosyl donor (7–9). For example, the TPS of Mycobacterium smegmatis and M. tuberculosis can utilize either GDP-glucose or UDP-glucose (or even other glucose nucleotides) as glycosyl donors for trehalose synthesis (10). On the other hand, the TPS from Saccharomyces cerevisiae is apparently specific for UDP-glucose (11), although there is one report of an ADP-glucose-dependent trehalose synthase in these yeast (12).

Organisms that utilize TPS for trehalose synthesis also contain a phosphatase that converts trehalose-P to free trehalose (6). Although the gene (otsB) for this enzyme has been identified in Escherichia coli (13, 14) and other organisms, the specificity and properties of the phosphatase have not been described in any detail. We previously reported on the presence of TPS in M. smegmatis (15), and this phosphatase appeared to be relatively specific for trehalose-P, with fairly low activity to-
ward glucose-6-P or other sugar phosphates. Here we describe the biochemical properties of TPP.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions

*M. smegmatis* was obtained from the American Type Culture Collection (ATCC 14465). *M. smegmatis* mc^2^155 was provided by Dr. W. R. Jacobs Jr., Albert Einstein College of Medicine, New York. The *E. coli* strains DH5α and HMS-F (18) were used for cloning and expression studies, respectively. HMS-F is a derivative of the expression strain HMS174(DE-3) (Novagen). HMS174(DE3) contains a chromosomal IPTG-inducible T7 RNA pol gene. HMS-F contains an additional copy of the lac repressor lacI^B^ on an F episome, which was transferred from *E. coli* cloning strain XL-1 (Strategene). This addition effectively represses expression from the T7 promoter on the *E. coli* expression vector pET15b (Novagen) in the absence of IPTG. HMS-F was routinely cultured in the presence of 10 μg/ml ampicillin, 20 μg/ml kanamycin, or 10 μg/ml 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 phosphate, buffers, DEAE-cellulose, and other chromatographic resins and materials were from Sigma. Sephadex G-75 and all electrophoresis materials were from Bio-Rad. Except where otherwise specified, all DNA manipulation enzymes, including restriction endonucleases, polymerases, and ligase, were supplied by New England Biolabs (Beverly, MA), and used according to the manufacturer’s instructions. Custom oligonucleotide primers were commercially synthesized by Integrated DNA Technologies (Coralville, IA). PCR reagents were supplied by Applied Biosystems (Foster City, CA). All other reagents were from reliable chemical companies, and were of the best grade available. Moenomycin was a generous gift from Aventis Pharma Deutschland GmbH and diumycin was kindly supplied by Bristol Myers Squibb Pharmaceutical Research Institute.

Assay of the Trehalose-P Phosphatase Activity

The enzymatic activity of TPP was measured by determining the release of inorganic phosphate from trehalose-6-P, or other sugar phosphates. Briefly, the assay was done in a final volume of 100 μl, containing the following components: 1 mm trehalose-6-P, 2 mm MgCl₂, 50 mm Tris-HCl buffer, pH 7.5, and an appropriate amount of enzyme. After an incubation at 37 °C for 1 to 30 min, two volumes of a filtered solution containing 0.15% m/m ammonium molybdate, and 12.5% (v/v) concentrated HCl were added to the incubation, and the mixture was allowed to incubate for an additional 2 min to allow for development of color. The absorbance of the mixture was read at 630 nm and compared with a standard solution of inorganic phosphate treated in the same manner (15).

Purification of the Trehalose-P Phosphatase

Growth and Harvesting of Bacteria—*M. smegmatis* was grown in 2-liter flasks containing 1 liter of trypticase soy broth. One ml of an overnight culture containing 10^5 unbroken cells and cellular debris, and the resulting supernatant liquid was allowed to stand on ice for 15 min, and the precipitate was isolated by centrifugation and resuspended in 40 ml of 50 mm Tris-HCl buffer, pH 7.5, containing 5 mm 2-mercaptoethanol.

Step 3: Heat Inactivation—Small aliquots of the above ammonium sulfate fraction were heated at 60 °C for 45 s. The tubes were cooled in an ice bath for 10 min, and the precipitated protein was removed by centrifugation at 100,000 × g for 1 h. The supernatant liquids were pooled and concentrated with an Amicon Filtration Apparatus using a Millipore YM-10 membrane.

Step 4: Gel Filtration on Sephadex G-75 and Sephacryl S-300—A 3-ml aliquot of the supernatant liquid from Step 3 was applied to a 2.6 × 95-cm column of Sephadex G-75 that had been equilibrated with 50 mm Tris-HCl buffer, pH 7.5, containing 5 mm 2-mercaptoethanol and 1 mm KCl (Buffer A). Five-ml fractions of the eluate were collected and examined for TPP activity, as described above. Active fractions were pooled and concentrated on the Amicon apparatus. This fraction was then applied to a Sephacryl S-300 (1.6 × 90 cm) column equilibrated with 50 mm Tris-HCl buffer, pH 7.5, containing 5 mm 2-mercaptoethanol and 10% glycerol (Buffer B). The column was eluted with the same buffer and 5-ml fractions were collected and assayed for TPP activity. Active fractions were pooled and used in subsequent steps.

Step 5: DE-52 Cellulose Chromatography—The enzyme from the Sephacryl S-300 column was applied to a 1.4 × 8-cm column of DE-52 that had been equilibrated with Buffer B. After application of the column, the sample was washed with the same buffer, containing 50 mm NaCl. The enzyme was then eluted with 90 mm NaCl in the same buffer. Fractions were collected and those containing active enzyme were pooled and concentrated to 3 ml on the Amicon concentrator. The concentrated enzyme was desalted on an Econo-Pac 10 DG chromatography column that had been equilibrated with 25 mm piperizine-HCl buffer, pH 5.5, containing 5 mm 2-mercaptoethanol and 10% glycerol (Buffer C). This buffer was also used to elute the enzyme.

Step 6: Chromatofocusing—The enzyme from the above step was applied to a 0.9 × 14-cm column of Polybuffer exchange (PBE 94) that had been equilibrated with Buffer C. The enzyme was eluted from the column with Polybuffer 74-HCl, pH 4.0, containing 5 mm 2-mercaptoethanol and 10% glycerol. Fractions of 2 ml were collected and each fraction was analyzed for protein, enzymatic activity, and pH. The enzyme emerged from the column at pH 4.3 to 4.4. Active fractions were pooled, and concentrated on the Amicon apparatus.

Step 7: Native Polyacrylamide Gel Electrophoresis—Preparative polyacrylamide gel electrophoresis was done at 4 °C in tubes containing 7% acrylamide (17). During electrophoresis, the voltage was maintained at 300. One gel was stained with Coomassie Blue stain to detect protein, whereas other gels were cut into 2-mm sections and protein was eluted by overnight diffusion at 4 °C into Buffer B. Elutions were assayed for TPP activity. Table I presents a summary of the purification procedure, including changes in specific activity, amount of purification, and yield of enzyme at each step of the procedure.

Other Methods

Protein was measured with the Bio-Rad protein reagent (Bio-Rad) using bovine serum albumin as the standard. The molecular weight of TPP was estimated by gel filtration on Sephacryl S-300, as well as by gel electrophoresis. Molecular weight standards included β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), cyanocobalamin (29 kDa), and cytochrome c (12 kDa).

Sequence Analysis

Open reading frames (ORFs) were identified by BLASTP alignment with predicted amino acid sequences on GenBank. Multiple amino acid alignments were performed using on line CLUSTALW alignment program at a web site maintained by the European Bioinformatics Institute (EMBL-EBI; www.ebi.ac.uk/clustalw). Basic sequence analysis, including identification of restriction sites, translations, and DNA sequence alignments, were performed using the “Genejockey” program (Biosoft, Cambridge, United Kingdom).

Circular Dichroism (CD) Measurements

To investigate the conformation of the recombinant TPP, near-UV and far-UV were recorded at 25 °C with a Jasco Model 715 spectrophotometer. Protein concentrations of 1 and 0.1 mg/ml, respectively, in 50 mm phosphate buffer, pH 7.4, were used for recording near-UV and far-UV CD spectra. The reported CD spectra are the average of five scans, which were smoothed and corrected for buffer blanks. CD data were expressed as mean residue molar ellipticity with 115 as the mean residue molecular weight. Secondary structure parameters were estimated by the computer program PROSECF derived by Yang et al. (17).
A Highly Specific Trehalose-phosphate Phosphatase from Mycobacteria

TABLE I

Purification of trehalose-phosphate phosphatase

| Purification step   | Protein (mg) | Total activity (units) | Specific activity (units/mg) | Purification (fold) | Yield (%) |
|---------------------|--------------|------------------------|------------------------------|---------------------|-----------|
| Crude extract       | 1,430        | 69,184                 | 48                           | 0                   | 100       |
| (NH₄)₂SO₄ fraction  | 550          | 54,488                 | 99                           | 2                   | 79        |
| Gel filtration      | 107          | 31,772                 | 259                          | 5.4                 | 45        |
| DE-52               | 28           | 17,042                 | 604                          | 12.5                | 27        |
| Chromatofocusing    | 2.6          | 7,227                  | 2,780                        | 55                  | 10        |
| Native gel          | 0.01         | 856                    | 81,571                       | 1,688               | 1.2       |

* Units are expressed as nanomole of Pi released from trehalose-P in 1 min.

RESULTS

Purification of the M. smegmatis TPP—TPP was purified about 1600-fold to near homogeneity using the procedure described under “Experimental Procedures,” and outlined in Table I. Two of the best steps in the purification are shown in Fig. 1. In Fig. 1A, the ammonium sulfate fraction (40 to 75% saturation) was applied to a column of Sephadex G-75. Because the TPP is a small protein (27 kDa), it was readily separated from much of the protein in the extract by gel filtration, and the activity eluted in a symmetrical peak after the major protein peak. Fig. 1B shows the chromatofocusing step that also gave a sharp peak of TPP activity that was separated from much of the protein in the DE-52 eluate. At the final stage of purification, the enzyme fraction showed a major protein band of 27 kDa, and several minor bands on SDS gels. This purification procedure gave a yield of enzyme of about 1%, but that provided a sufficient amount of protein to isolate peptides and obtain amino acid sequence data to use for cloning the gene, and expressing recombinant TPP.

The major 27-kDa protein band was cut from the SDS gels and sent to Harvard Microchemical Systems Inc. for protein sequencing by mass spectrometry. Based on the amino acid sequence information, several primers were prepared and used in PCR as described below.

Cloning and Sequencing of M. smegmatis TPP—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 TPP. All of the primary amino acid sequences aligned with a region of Contig 3312. However, because this region of the genome sequence has not yet been corrected or edited, we were unable to identify the boundaries of the ORF corresponding to the amino acid sequence. We used oligonucleotide primers DC172 (TCATCGCGGAGTGCGCGACCGTA) and DC173 (CGAGCCGATCTTCCGCGACCCGAACG), corresponding to nucleotides 1108256–1108281 of M. smegmatis contig 3312) to PCR amplify a 2.8-kb region of the M. smegmatis genome that contained the putative TPP coding region. The PCR product was ligated into the vector pCR4 TOPO (Invitrogen), generating the plasmid p996A481. Sequence analysis of the p996A481 insert demonstrated the presence of 2 complete ORFs and 1 partial ORF, ORF1 extended from 384 to 1121. The predicted 245-residue product was highly homologous (90% amino acid identity plus 6% similarity) with the M. tuberculosis ORF Rv3574, which is annotated as a possible transcriptional regulator of the Tet/AcrR family.

ORF2 was located immediately downstream of ORF1, extending from 1122 to 1874. This potentially encoded a 250-residue 27-kDa polypeptide. BLASTP analysis of the ORF2 amino acid sequence indicated homology with trehalose-6-P phosphatases from Corynebacterium glutamicum (31% identity, 13% similarity, E = 7e⁻¹⁷), and from E. coli (33% identity, 11% similarity, E = 1e⁻¹⁵). Lesser degrees of homology were detected between ORF2 and the putative M. tuberculosis putative trehalose-6-P phosphatases Rv2006 (otsB, 28% identity, 12% similarity, E = 6e⁻⁵⁵) and Rv3372 (otsB2, 28% identity, 13% similarity, E = e⁻⁶⁰). ORF3 was oriented in opposition to ORF1/ORF2 extended from 2838 to 1875. This encoded a 35.2 kDa 328-residue with a high degree of homology (73% identity plus 10% similarity) with M. tuberculosis ORF Rv3575c, a possible transcriptional regulator related to the E. coli raffinose repressor.

FIG. 1. A. gel filtration of TPP on Sephadex G-75. The supernatant fraction from the heat inactivation step was applied to a 2.6 × 95-cm column of Sephadex G-75 and eluted with 50 mM Tris-HCl buffer, pH 7.5, containing 5 mM 2-mercaptoethanol and 1 mM KCl. Five-ml fractions were collected and assayed for TPP activity (solid squares) and protein (solid diamonds). B, purification of TPP by chromatofocusing. The active fraction from DE-52 was applied to a 0.9 × 14-cm column of Polybuffer exchange as described under “Experimental Procedures.” Enzyme was eluted with Polybuffer 74-HCl, pH 4.0, containing 5 mM 2-mercaptoethanol and 10% glycerol. Two-ml fractions were collected and assayed for TPP activity (squares) and protein content (diamonds).
ORF2 (otsB) was PCR amplified using the primers DC186 (GGGGCATAATGCGCAGCGC, corresponding to nucleotides 1136–1153 of the p996A481 insert sequence) and DC187 (GGGGGATCCAGCTTCTGGAGC, complementary to nucleotides 1915–1936 of the p996A481 insert sequence). These contained the requisite base mismatches (indicated underlines) to generate an upstream CATATG NdeI site and a downstream BamHI site. The central “ATG” in DC186 represents the start codon of the recombinant ORF. The 0.8-kb DC186/DC187 PCR product was amplified, digested with NdeI and BamHI, and ligated with the NdeI- and BamHI-digested expression plasmid pET15b (Invitrogen), generating the plasmid p986A504. The identity of the PCR product and the fidelity of the amplification were confirmed by DNA sequencing. p986A504 was transformed into the E. coli expression strain HMS-F. The predicted amino acid sequence of _M. smegmatis_ TPS is presented in Fig. 2 and is compared with similar enzymes in other organisms.

**Preparation of Recombinant TPS—** The _E. coli_ expression strain HMS-F transformed with p986A504 was grown and induced as described under “Experimental Procedures.” The crude sonicate, as well as the supernatant (cytosolic) and pellet (i.e. membrane) fractions resulting from high speed centrifugation were subjected to SDS-PAGE, and the proteins were visualized with Coomassie Blue. Fig. 3 presents the results of these studies. In the absence of IPTG induction, only a small amount of 27-kDa band was detected (lane 2) and this band was apparently removed by centrifugation (lane 3). Incubation of the cells in IPTG resulted in production of substantial amounts of the 27-kDa band (lane 4), but again much of this protein was pelleted by high-speed centrifugation (lanes 5 and 6). The protein could be mostly solubilized by addition of 0.2% Sarkosyl to the crude homogenates before the centrifugation as seen in lanes 8 and 9. The solubilized fraction had strong trehalose-P phosphatase activity and could be purified to a single band as shown below.

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. Fig. 4 shows that 250 mM imidazole eluted a single protein that migrated slightly faster than the 31-kDa protein standard and this fraction had strong TPP activity. This protein fraction was used in all subsequent studies to compare its properties with those of the native TPP isolated from mycobacteria.

The expressed protein containing a (His)_6_ tag was purified to near homogeneity on a metal ion column. As shown in Fig. 4 (lane 6), the elution from this column showed a single protein on SDS gels with a molecular weight of about 27,000. As demonstrated below, this protein had strong phosphatase activity, and this activity was very specific for trehalose-P.

**Results.** Lanes 1 and 10, protein standards; lane 2, crude extract of cells without induction by IPTG; lane 3, supernatant fraction of extract in lane 2; lanes 4–6, cells induced with IPTG, whole extract, supernatant fraction after high speed centrifugation, pellet from high speed centrifugation; lanes 7–9, whole extract, from cells induced with IPTG, mixed with 0.2% Sarkosyl, supernatant fraction after high speed centrifugation, and pellet from centrifugation.

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**Fig. 2.** CLUSTALW alignment of _M. smegmatis_ OtsB predicted amino acid sequence with those of homologous ORFs from _Mycobacterium leprae_ and _M. tuberculosis_. The _M. leprae_ homolog was identified by searching the Wellcome Trust Sanger Institute _M. leprae_ genome sequence (www.sanger.ac.uk/Projects/M_leprae/) with TBLASTN. The _M. tuberculosis_ ORFs Rv2006 (otsB; accession number Z74025) and Rv3372 (otsB2) were identified by searching the Wellcome Trust Sanger Institute _M. tuberculosis_ genome sequence (www.sanger.ac.uk/Projects/M_tuberculosis/) with TBLASTN. The _M. smegmatis_ homolog was identified by searching the Wellcome Trust Sanger Institute _M. smegmatis_ genome sequence (www.sanger.ac.uk/Projects/M_smegmatis/) with TBLASTN. The well-conserved and semi-conserved substitutions are indicated by dots (.), respectively. Gaps introduced by CLUSTALW to generate an upstream CATATG NdeI site and a downstream BamHI site. The central “ATG” in DC186 represents the start codon of the recombinant ORF. The 0.8-kb DC186/DC187 PCR product was amplified, digested with NdeI and BamHI, and ligated with the NdeI- and BamHI-digested expression plasmid pET15b (Invitrogen), generating the plasmid p986A504. The identity of the PCR product and the fidelity of the amplification were confirmed by DNA sequencing. p986A504 was transformed into the _E. coli_ expression strain HMS-F. The predicted amino acid sequence of _M. smegmatis_ TPS is presented in Fig. 2 and is compared with similar enzymes in other organisms.
Properties of the Trehalose-P Phosphatase—The enzyme purified from *E. coli* was characterized in terms of pH optima, substrate specificity, and metal ion requirements, and other properties as discussed below. The TPP purified from *M. smegmatis* and the recombinant TPP had almost identical properties except for their requirement for divalent cations (see below).

The pH optimum of TPP was determined using two different buffers as shown in Fig. 5. The pH optimum of this activity was 7.0–7.5 using either Tris-HCl or Tris maleate buffers. The enzyme had almost no activity at either pH 5 or 9, in contrast to many nonspecific acid or alkaline phosphatases that have maximum activity at around pH 5.0 or 9.0.

TPP was incubated with a variety of sugar phosphates, tested at 2 and 5 mM concentrations, to determine whether any of them could serve as substrates for this enzyme. Fig. 6 demonstrates that this enzyme had very strong phosphatase activity toward trehalose-P, but essentially no activity with any of the other sugar phosphates. The slight activity seen with glucose-6-P or glucose-1-P is probably because of small amounts of inorganic phosphate present in these substrates as a result of some degradation (i.e. loss of phosphate) during storage, because the substrate itself gives some background color in the phosphate determination. The enzyme also had no activity on the general phosphatase substrate, *para*-nitrophenyl phosphate. This data indicates that TPP is an unusual and very specific phosphatase, because these enzymes usually have a rather broad substrate specificity and can hydrolyze many different phosphate esters.

The effect of trehalose-P concentration on the reaction rate

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**Fig. 4.** Purification of recombinant TPP on the nickel-chelate column. Lanes 1 and 7, protein standards; lane 3, crude *E. coli* supernatant fraction before application to column; lane 3, fraction passing through nickel column; lane 4, fraction eluting with 10 mM imidazole; lane 5, fraction eluting with 25 mM imidazole; lane 6, fraction eluting with 250 mM imidazole.

**Fig. 5.** Effect of pH of incubation mixture on activity of native and recombinant TPP. Incubations were prepared as described in the text but contained buffers at various pH values as shown in the figure. Enzyme activity was determined as described under “Experimental Procedures.” Diamonds are results with TPP purified from *Mycobacterium smegmatis*. Squares are results with recombinant enzyme made in *E. coli*.
was determined. The rate of the reaction was proportional to the amount of trehalose-P in the incubation up to about 5 mM, and the $K_{m}$ for trehalose-P was calculated to be about 1.6 mM (data not shown).

Native TPP showed an almost absolute requirement for the divalent cation Mg$^{2+}$, as shown in Fig. 7. The data in this figure also show that Mn$^{2+}$ worked to some extent, but was much less effective than Mg$^{2+}$. The optimum concentration of Mg$^{2+}$ for the native TPP was about 1–2 mM. On the other hand, the recombinant TPP was somewhat different in regard to its reactivity with Mn$^{2+}$ as also shown in Fig. 7, because this TPP showed almost equal activity with both Mn$^{2+}$ and Mg$^{2+}$. In either case, the optimum concentration of cation was about 3–4 mM. The significance of this difference in cation requirement between the native and recombinant TPPs is not clear at this time, but it could be related to the presence of the His tag on the recombinant protein.

TPP was subjected to far-UV and near-UV circular dichroism measurements as described under “Experimental Procedures.” A summation of the data is presented in Table II and indicates that the protein has about 50% $\alpha$-pleated sheet, and is quite compact.

**Inhibition of Trehalose-P Phosphatase Activity**—The enzymatic activity was not inhibited by 10 mM sodium fluoride or 10 mM sodium orthovanadate. It was also not inhibited by a variety of sugars such as trehalose, maltose, glucose-6-P, glucose-1-P, GlcNAc-1-P, at up to 10 mM concentrations, nor by 10 mM $\beta$-glycerophosphate, or acetyl-coenzyme A. The enzyme also did not show any phosphatase activity toward any of these phosphorylated compounds.

Both the natural TPP and the recombinant enzyme were inhibited by two antibiotics, diumycin and moenomycin as shown in Fig. 8. It can be seen that the activity was progressively inhibited by adding increasing amounts of either of these antibiotics to the incubation mixtures, and 50% inhibition by either antibiotic occurred at about 100 $\mu$g/ml. These two antibiotics were previously shown to inhibit the growth of *M. smegmatis* (18).

**Stability of the Trehalose-P Phosphatase**—The purified enzyme (both native and recombinant) was stable to freezing and maintained its activity for at least several weeks when stored at $-20^\circ$C. The enzyme was also quite stable to heating as shown in Fig. 9. Thus, when the enzyme was placed in a hot water bath at various temperatures from 40 to 60 $^\circ$C, there was little loss of activity for at least 6 min of heating. In fact, even
at 70 °C, the enzyme retained significant activity for several minutes and then slowly lost activity. During the purification, we found that the crude extracts could be heated at 95 °C for 1 or 2 min, and in many cases most of the TPP activity was retained. However, this procedure was not completely reproducible from one extract to another, and therefore the heat step was used at 60 °C.

Because the recombinant TPP was found mostly in the membrane fraction and required treatment with Sarkosyl for solubilization, we tested the effects of various detergents on en-
zyme activity. Fig. 10 presents the results obtained with a variety of detergents at increasing concentrations. It can be seen that the enzyme activity was rapidly lost in the presence of increasing concentrations of Sarkosyl or deoxycholate, and at 0.05% of these detergents there was almost complete loss of activity. On the other hand, detergents such as Nonidet P-40, Triton X-100, and octyl glucoside were relatively innocuous and even at 0.2–0.3% there was no loss of activity. We did use 0.2% Sarkosyl to solubilize the TPP in crude sonicates of the transformected E. coli cells, but the Sarkosyl was not in the buffer used to elute the TPP from the metal ion columns. This indicates that removal of that detergent results in restoration of activity.

DISCUSSION

The gene for trehalose-P phosphatase from E. coli (otsB) has been mapped and located on the E. coli chromosome (13), and that gene was also identified in the Arabidopsis thaliana genome by functional complementation of the yeast trehalose-P phosphatase (tps2) gene (19). In E. coli, the gene for trehalose-P phosphatase (otsB) and the gene for trehalose-P synthase (otsA) constitute an operon in which otsB are proximal to the promoter, and otsA is distal (20). In Saccharomyces cerevisiae, the synthase and the phosphatase copurify as a complex and the individual enzymes have not been separated (21). This complex also contains several regulatory proteins that affect the activities of these two enzymes. In E. coli, TPS and TPP apparently do not exist as a complex because the two proteins could be expressed separately in an otsAB deletion mutant (14).

Despite the considerable amount of information on the genetics of the trehalose pathway in these organisms, very little is known about the structure of the protein that removes the phosphate from trehalose-P or the protein that synthesizes trehalose-P (TPS) in these organisms, nor is there much information on the specificity or other properties of these two enzymes.

In this report, we describe the purification of the trehalose-P phosphatase (TPP) from M. smegmatis to near homogeneity and the identification of the complete amino acid sequence of this interesting protein. The translation start of the M. smegmatis TPP is undiscovered. The purified polypeptide located closest to the predicted amino terminus starts at residue 11 (1ALTAVAATPHLLVTSDFDGT
)
, suggesting that the actual translation start was located between residues 1 and 11.

CLUSTALW alignment of the M. smegmatis TPP predicted amino acid sequence with those of C. glutamicum and E. coli suggests that the most likely start codon was located at residue 8 (see Fig. 3). Almost 50% of the amino acids in the 27-kDa TPP are hydrophobic (i.e., 45 alanines, 7 Phe, 18 Gly, 7 Ile, 28 Leu, and 23 Val), but these amino acids are distributed throughout the protein sequence rather than occurring in specific domains. The hydrophobic nature of the protein may account for some of its unusual properties. For example, when E. coli cells transformected with the TPP gene were broken by sonication and the crude homogenate subjected to high speed centrifugation, most of the TPP activity was found in the pellet, i.e. membrane fraction. However, when 0.2% Sarkosyl was added to the crude homogenate, almost all of the TPP activity remained in the supernatant fraction. This protein is also quite resistant to heat denaturation. Thus, the protein could be heated at temperatures of up to 60 °C for 5 or 6 min with only a small loss in activity. This property suggests that the protein is tightly folded and fairly resistant to denaturation. Examination of the protein by circular dichroism also supports this hypothesis and indicates that the protein structure is largely in the β-pleated sheet and is very compact.

Both TPP and TPS are inhibited by the glycoside antibiotics, moenomycin and diumycin. These antibiotics also inhibit the growth of M. smegmatis (18). However, because these compounds also inhibit peptidoglycan synthesis in some organisms, we cannot be certain that the inhibition of growth of mycobacteria is because of inhibition in the trehalose biosynthetic pathway. We are currently designing experiments to determine whether growth inhibition and inhibition of trehalose synthesis are directly related. We are also designing experiments to determine whether this trehalose biosynthetic pathway is essential to the viability of these organisms.

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