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# Partial Purification and Properties of a Highly Specific Trehalose Phosphate Phosphatase from *Mycobacterium smegmatis*

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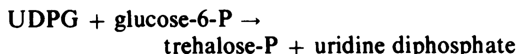
A specific trehalose phosphate phosphatase was purified approximately 50-fold from *Mycobacterium smegmatis*. The enzyme had a *p*H optimum of about 7.0 and was stimulated by $Mg^{2+}$. The optimum concentration of $Mg^{2+}$ was about $1.5 \times 10^{-3}$ M. Of other divalent cations tested, only $Co^{2+}$ showed some activity. The $K_m$ for trehalose phosphate was found to be about $1.5 \times 10^{-3}$ M. The enzyme showed slight activity toward mannose-6-P and fructose-6-P but was inactive on a large number of other phosphorylated compounds. Citrate was a competitive inhibitor of the enzyme both with respect to trehalose phosphate concentration and $Mg^{2+}$ concentration. This inhibition appears to be due to chelation of $Mg^{2+}$ by this compound. Ethylenediaminetetraacetic acid and NaF were also inhibitors of the enzyme, but these inhibitions were noncompetitive.

Trehalose is a naturally occurring disaccharide of D-glucose which is widespread throughout the plant and animal kingdoms. It is the major blood sugar in many insects and appears to serve as a reserve food material in a number of organisms (2). In addition, in mycobacteria trehalose also has a structural role and appears to be an important cell wall component (14).

The synthesis of trehalose from uridine diphosphate D-glucose (UDPG) was first described in brewer's yeast by Cabib and Leloir (3) according to the following reaction:



UDPG + glucose-6-P →
trehalose-P + uridine diphosphate

This reaction was also demonstrated in insects (4, 18), in *Mycobacterium tuberculosis* (11), in *Dictyostelium discoideum* (20), in baker's yeast (6), and in other fungi (8). Trehalose phosphate was also shown to be synthesized by a number of species of *Streptomyces*, but these organisms used guanosine diphosphate glucose (GDPG) rather than UDPG as the glucosyl donor (8). However, cell-free extracts prepared from a number of species of *Mycobacterium* were able to use either UDPG or GDPG as glucosyl donors for the synthesis of trehalose phosphate (15).

Although trehalose is synthesized by way of the phosphate derivative, it is stored as the neutral disaccharide, trehalose. Many workers have demonstrated a high intracellular concentration of trehalose in various organisms (7, 19, 23). The removal of the phosphate group of trehalose phosphate might serve to pull the reaction in the direction of synthesis and thereby augment trehalose accumulation. Such a mechanism would probably require a rather specific trehalose phosphate phosphatase. This report describes the partial purification and properties of such a highly specific phosphatase from *M. smegmatis*. A similar enzyme was previously isolated and purified from insects (10).

## MATERIALS AND METHODS

**Materials.** α,α-Trehalose-6-phosphate and trehalose-6,6′-diphosphate were synthesized by the method of MacDonald and Wong (16). Calcium phosphate gel was prepared as described by Keilen and Hartree (13). All other materials were obtained from commercial sources.

**Analytical methods.** Inorganic phosphate was determined either by the method of Chen et al. (5) or by the method of Fiske and Subbarow (9) as modified by Bartlett (1). Protein was measured as described by Sutherland et al. (21). Trehalose was assayed with a specific trehalase isolated from *Streptomyces hygroscopicus* (12).

**Growth of cells and preparation of crude extracts.** *M. smegmatis* was grown in Trypticase Soy Broth at 37 C for 2 to 4 days on a rotary shaker. Cells were harvested by centrifugation, washed with water, and stored at −20 C until used.

A 20-g amount of cell paste was suspended in 100 ml of 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.0) and subjected to sonic oscillation with a Sonifier Cell Disrupter, Model W185D (Heat Systems-

Ultrasonics, Inc.) for a total of 10 min at 0 C (2.5 min of sonic oscillation followed by a 5-min period of cooling). Cell debris was removed by centrifugation at 30,000 × *g* for 20 min. The supernatant fraction contained the enzymatic activity.

**Assay of trehalose phosphate phosphatase.** Incubation mixtures contained the following components (in micromoles) in a final volume of 0.25 ml: trehalose-phosphate, 1; $MgCl_2$, 1; Tris buffer (*p*H 7.0), 10; and an appropriate amount of enzyme. After incubation for 15 min at 37 C, the reaction was stopped by heating in a boiling-water bath for 5 min. The complete reaction mixture, or a sample thereof, was then tested for its content of inorganic phosphate as described. Control tubes were also run with trehalose-P or enzyme omitted during the incubation period. In these cases, the missing component was added just before placing the tubes in a boiling-water bath.

When acid-labile phosphorylated compounds were tested, phosphate was assayed by the method of Bartlett (1), since this method does not involve the use of strong acid. In other cases, such as with sugar 6-phosphates, the method of Chen et al. (5) was used.

## RESULTS

**Purification of the trehalose phosphate phosphatase: manganese precipitation and calcium phosphate gel treatment.** A solution of 1 M $MnCl_2$ was added to the crude extract to a final concentration of 0.05 M. The mixture was allowed to stand for 5 min in an ice bucket, and the precipitate was removed by centrifugation and discarded. To 100 ml of supernatant liquid, 40 ml of calcium phosphate gel (14.5 mg/ml) was added slowly with stirring. The mixture was allowed to stand in an ice bucket for several minutes and the precipitate was removed by centrifugation and discarded.

**Ammonium sulfate precipitation.** To 100 ml of ice-cold calcium-phosphate gel supernatant fraction, 27 g of solid ammonium sulfate (0 to 45% saturation) was added slowly with stirring. The precipitate was removed by centrifugation and discarded; to the supernatant fluid, 26 g of solid ammonium sulfate (45 to 80% saturation) was added with stirring. The precipitate was collected by centrifugation, dissolved in 10 ml of distilled water, and dialyzed overnight against several liters of 0.01 M Tris buffer, *p*H 7.0.

**DEAE-cellulose column chromatography.** Diethylaminoethyl (DEAE)-cellulose was treated with 1 N NaOH followed by 1 N HCl and was then stored in 1 M KCl until used. Columns were washed well with 0.01 M Tris buffer (*p*H 7.0) before applying the enzyme. A 10-ml amount of ammonium sulfate fraction was applied to a column (1.5 by 10 cm) of DEAE-cellulose which was then washed with Tris buffer. Columns were eluted in two different ways: either batchwise with increasing concentrations of KCl or with a linear gradient of KCl from 0 to 1 M. In both cases, the enzyme activity eluted at about 0.2 M KCl. Active fractions were pooled, concentrated by precipitation with ammonium sulfate, and dialyzed against 0.01 M Tris buffer, *p*H 7.0. This enzyme fraction was used in the following experiments.

By the use of these procedures, the enzyme was purified about 50-fold with a recovery of approximately 40% (Table 1). This fraction had negligible phosphatase activity for other phosphorylated compounds and did not contain any trehalase activity. At this stage of purification, the enzyme slowly lost activity in the freezer over a period of several weeks. However, the enzyme could be kept in ice as a suspension of the enzyme in an 80% ammonium sulfate solution without any apparent loss of activity over a period of several weeks.

**Effect of time and protein concentration.** The liberation of inorganic phosphate from trehalose phosphate was linear with respect to time for at least 15 min and was also proportional to protein concentration over a fourfold range.

**Effect of $Mg^{2+}$ concentration and other divalent cations.** As shown in Fig. 1, phosphatase activity was markedly stimulated by the addition of $Mg^{2+}$. The optimum concentration of $Mg^{2+}$ was about $1.5 \times 10^{-3}$ M. The enzyme was relatively specific for $Mg^{2+}$ and was much less active with other divalent cations. Most divalent cations were not able to replace $Mg^{2+}$ or were only slightly effective in this regard (Table 2). However, $Co^{2+}$ was the most effective of those tested.

**Effect of trehalose phosphate concentration and other phosphate compounds.** Figure 2 shows the effect of increasing concentration of trehalose phosphate on phosphatase activity. The initial reaction rate was proportional to trehalose phosphate concentration to about $2 \times 10^{-3}$ M, and the $K_m$ for trehalose phosphate was estimated to be about $1.5 \times 10^{-3}$ M.

A large number of other phosphorylated compounds were also tested as substrates for the enzyme (Table 3). The phosphatase was fairly specific for trehalose phosphate but did show slight activity with mannose-6-P and fructose-6-P. All other compounds, including glucose-6-P and glucose-1-P, were essentially inactive under the conditions of assay.

**Effect of *p*H.** The *p*H optimum for the enzyme was about 7.0 in Tris buffer (Fig. 3). Tris-citrate buffer was inhibitory, and this was shown to be due to competitive inhibition by citrate as indicated below.

**Effect of inhibitors.** Since Tris-citrate buffer was found to be inhibitory, the effect of citrate was examined to investigate the possibility that

TABLE 1. *Purification of trehalose phosphate phosphatase*

| Fraction | Specific activity[a] | Total units |
|---|---|---|
| Crude[b] | 0.0185 | 92.5 |
| Ammonium sulfate | 0.266 | 53.2 |
| DEAE | 0.87 | 37.7 |

[a] Expressed as units per milligram of protein. One unit of activity is that amount of enzyme that will catalyze the release of 1 μmole of inorganic phosphate from trehalose phosphate in 1 min at 37 C.

[b] Actual purification by manganese precipitation and calcium phosphate gel treatment could not be determined, since the assay could not be run after these two steps.

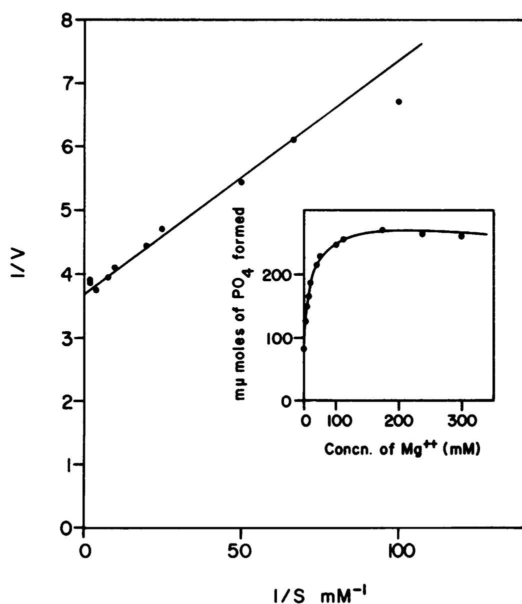



FIG. 1. *Effect of $Mg^{2+}$ concentration on the reaction velocity. Incubation mixtures were as described and contained 0.02 unit of enzyme. Various concentrations of $MgCl_2$ were used as indicated.*

TABLE 2. *Requirement for divalent cations by trehalose phosphate phosphatase*[a]

| Cation tested | Activity[b] | Activity relative to $Mg^{2+}$ [c] |
|---|---|---|
| None | 64 | 18 |
| $Ba^{2+}$ | 96 | 28 |
| $Ca^{2+}$ | 80 | 23 |
| $Cd^{2+}$ | 24 | 7 |
| $Co^{2+}$ | 200 | 59 |
| $Fe^{2+}$ | 72 | 21 |
| $Mg^{2+}$ | 336 | 100 |
| $Mn^{2+}$ | 84 | 24 |
| $Pb^{2+}$ | 52 | 15 |

[a] Assay mixtures were as described in the text and contained 0.03 unit of enzyme. $Mg^{2+}$ was replaced as indicated by 1 μmole of the indicated cations (added as the chlorides).

[b] Expressed as nanomoles of Pi released per 15 min.

[c] Activity with $Mg^{2+}$ taken as 100.

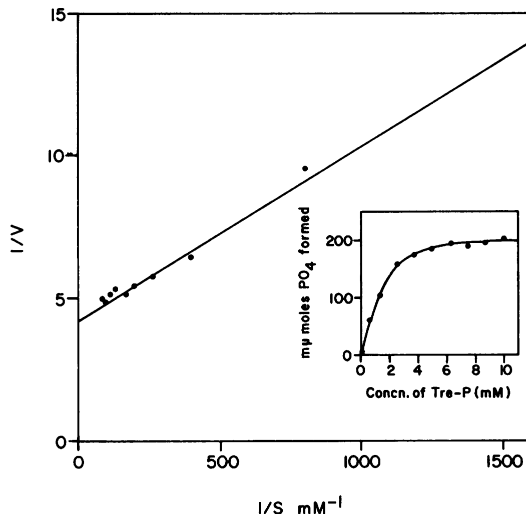



FIG. 2. *Effect of trehalose phosphate concentration on the reaction velocity. Incubation mixtures were as described and contained 0.02 unit of enzyme. Various concentrations of trehalose phosphate were used as indicated.*

this inhibition functions as a control. Figure 4 shows the effect of citrate on the reaction velocity as a function of trehalose phosphate concentration. Citrate acted as a competitive inhibitor of the phosphatase under these conditions. Citrate was also a competitive inhibitor of the phosphatase with respect to $Mg^{2+}$ concentration. Although this inhibition may have some physiological significance and could be a control, at least part of the effect of citrate appears to be due to its chelation of $Mg^{2+}$. This could be shown by the reversal of inhibition by citrate at high $Mg^{2+}$ concentration.

Another chelating agent, ethylenediaminetetraacetic acid (EDTA) was also tested as an inhibitor. At concentrations of EDTA in the order of $2.5 \times 10^{-3}$ M, the reaction was inhibited at lower $Mg^{2+}$ concentrations (up to $5 \times 10^{-3}$ M). However, the kinetics of inhibition in this case were not competitive and appeared quite complex. Higher concentrations of EDTA (above 2.5 $\times 10^{-3}$ M) were completely inhibitory regardless of the $Mg^{2+}$ concentrations.

As might be expected, NaF was also an inhibitor of the enzyme. The effect of this inhibitor on the reaction is shown in Fig. 5. It can be seen

TABLE 3. *Substrate specificity of trehalose phosphate phosphatase*[a]

| Substrate tested | Pi released (nmoles/ 15 min) | Activity[b] |
|---|---|---|
| Expt I | | |
| Trehalose phosphate | 421 | 100 |
| Fructose-6-P | 69 | 16 |
| Mannose-6-P | 69 | 16 |
| Glucose-6-P | 0 | 0 |
| Mannose-1-P | 0 | 0 |
| Glucose-1-P | 19 | 4 |
| Galactose-1-P | 25 | 6 |
| Glucosamine-6-P | 0 | 0 |
| Ribose-5-P | 19 | 4 |
| Expt II[c] | | |
| Trehalose phosphate | 373 | 100 |
| Trehalose diphosphate | 21 | 5 |
| Phosphoenolpyruvate | 17 | 4 |
| 3-Phosphoglyceric acid | 21 | 5 |
| UTP | 11 | 3 |
| ATP | 8 | 2 |
| GTP | 0 | 0 |
| NADP | 0 | 0 |

[a] Incubation mixtures were as described in the test and contained 0.04 unit of the enzyme. Trehalose-P was replaced by 1 $\mu$mole of the indicated substrate.

[b] Relative to trehalose-P. Trehalose-P activity taken as 100.

[c] UTP, uridine triphosphate; ATP, adenosine triphosphate; GTP, guanosine triphosphate; NADP, nicotinamide adenine dinucleotide phosphate.

from these data that NaF acts as a noncompetitive inhibitor of the phosphatase.

**Characterization of the products.** One of the products of the action of the enzyme on trehalose phosphate was inorganic phosphate as shown by the method of Chen et al. (5) or Bartlett (1). The other product was identified as trehalose, since

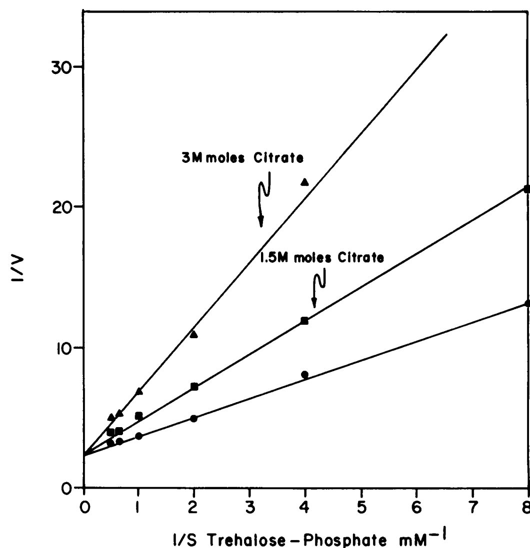



FIG. 4. *Lineweaver-Burk plots showing the effect of citrate on the velocity as a function of trehalose phosphate concentration. Incubation mixtures were as described, except that citrate was added as indicated. Incubations contained 0.02 unit of enzyme. Curves are as follows:* ●, *no citrate;* ■, *1.5 $\mu$mole of citrate;* ▲, *3 $\mu$moles of citrate.*

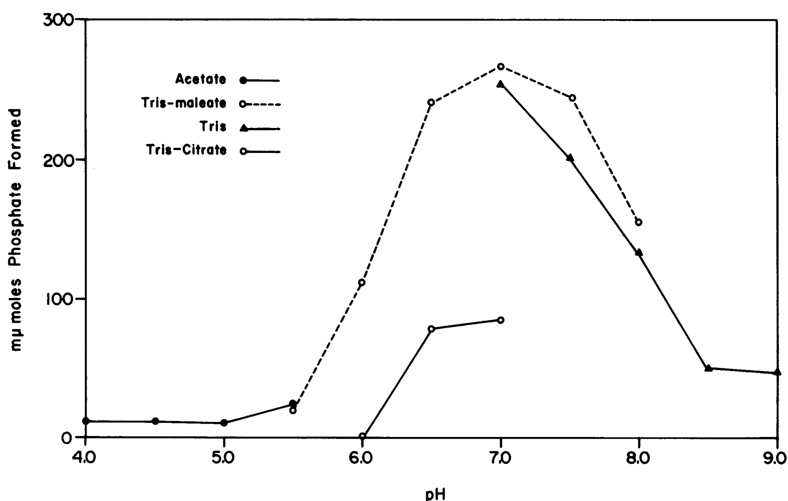



FIG. 3. *Effect of pH on the reaction velocity. Incubation mixtures were as described and contained 0.02 unit of enzyme. Various buffers were used as indicated:* ●, *acetate buffer;* ○ *(dashed line), Tris-maleate buffer;* ▲, *Tris buffer;* ○ *(solid line), Tris-citrate buffer.*

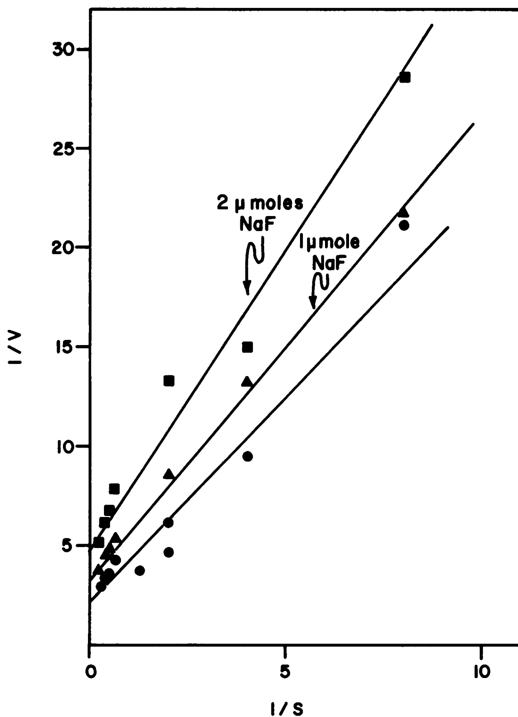



FIG. 5. *Lineweaver-Burk plots showing the effect of NaF on the velocity of the reaction as a function of trehalose phosphate concentration. Incubation mixtures were as described and contained 0.02 unit of enzyme except that NaF was added as indicated. Curves are as follows:* O, *no NaF;* ▲, *1 μmole of NaF;* ■, *2 μmoles of NaF.*

reaction of this sugar with a purified trehalase resulted in the liberation of glucose. The ratio of glucose released in the presence of trehalase to inorganic phosphate was approximately 2:1, (1.81:1).

## DISCUSSION

The isolation of a highly specific phosphatase from *Mycobacterium smegmatis* which cleaves trehalose phosphate to trehalose and inorganic phosphate is not surprising in view of the role of trehalose in these and other organisms. A specific trehalose phosphate phosphatase was first described in yeast by Cabib and Leloir (3). Friedman later isolated and purified a similar enzyme from adult blow flies (10) and demonstrated its specificity for trehalose phosphate. The phosphatase from insects is similar in many respects to the enzyme described here. Both have a *p*H optimum of about 7.0 in Tris-maleate buffer, both require $Mg^{2+}$ and are fairly specific for trehalose-P, and both have a $K_m$ for trehalose-P of about $1.5 \times 10^{-3}$ M.

In each of these organisms and many others which have been examined, trehalose appears to serve as a reserve food material, possibly in a manner analogous to sucrose and sucrose phosphate in plants (17). Thus, although organisms synthesize trehalose as the trehalose phosphate derivative, very little of the phosphorylated compound is found in cells, whereas high concentrations of trehalose may accumulate. It seems probable that this mechanism of synthesis coupled to the phosphatase activity leads to the accumulation of trehalose.

In mycobacteria, trehalose also serves as a reserve food material. Thus, the levels of trehalose vary according to age of cells and supply of nutrients (*unpublished data*). In addition, certain growth inhibitors such as isoniazid have been shown to cause an increase in the amount of trehalose in the cells (22). Since trehalose is also found in cell wall material (14), the synthesis and dephosphorylation of trehalose phosphate may also play an important role in growth of these organisms.

ACKNOWLEDGMENT

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