Characterization of two trehalases in baker's yeast

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Trehalase activities at pH 5 (not inhibited by EDTA) and pH 7 (inhibited by EDTA) were present in the soluble fraction of disintegrated commercial baker's yeast. The pH 5 activity binds strongly to concanavalin A, is only partially salted out by saturated (NH₄)₂SO₄, has an apparent M₉ of 215000 (by gel filtration) and is an acidic protein. It has a Kₘ of 1.4 mM, a broad pH optimum (at 40 mM-trehalose) between pH 4 and 5, and is activated by about 30% by 20–300 mM neutral salts such as KCl, NaNO₃ and MnCl₂. The enzyme is strongly inhibited by acetic acid/acetate buffers, with a Kᵢ of about 15 mM-acetic acid. The pH 7 activity does not bind to concanavalin A, is salted out at 20–32% (w/v) (NH₄)₂SO₄ and has an M₉ of 170000 (by gel filtration). It is absolutely dependent on Ca²⁺ or Mn²⁺ ions (Mg²⁺ is ineffective) and strongly inhibited by neutral salts in the 20–100 mM range. It can be activated by treatment with MgATP in the presence of cyclic AMP. Activation decreases, but does not abolish, the Ca²⁺ requirement, and does not change the Kₘ for trehalose (5.7 mM) or shift the sharp pH optimum at pH 6.7 (at 40 mM-trehalose).

Trehalose (α-D-glucopyranosyl-α-D-glucopyranose), a major reserve carbohydrate in yeast, is mobilized by hydrolysis to glucose catalysed by trehalases (EC 3.2.1.28). Yeast trehalase has generally been found to be a soluble enzyme with an acid pH optimum. Such an enzyme was purified by Kelly & Catley (1976), and was specific for trehalose, as are the trehalases from other sources (Labat-Robert, 1980). van der Plaat (1974) found that a rapid degradation of trehalose was preceded by a sudden 7-fold increase in trehalase activity at pH 7. This increase could be mimicked in vitro, and putative cyclic AMP-dependent protein kinase-and 'cryptic' trehalase-containing fractions were separated (Van Solingen & van der Plaat, 1975).

The relationship between the trehalase studied at pH about 5 and the cyclic AMP/ATP-activatable trehalase was unclear. Wiemken & Schellenberg (1982) reported that the trehalase activity at pH 5 was confined to the vacuoles, but that the cytosol contained a 'trehalase zymogen' that became active after treatment with cyclic AMP/ATP. We now show that trehalase activities at pH 5 and 7 can also be separated by protein fractionation, and differ in their physical and catalytic properties. At least two active interconvertible forms of the neutral trehalase occur.

Methods

Fractionation of yeast

Commercial baker's yeast from Alko was suspended in an equal volume of fractionation buffer [20 mM-potassium phosphate (pH 7.0)/0.2 mM-EDTA/1.4 mM-mercaptoethanol] containing 1 mM-phenylmethanesulphonyl fluoride, broken in a Braun MSK cell homogenizer and centrifuged for 30 min at 40000 g. Supernatant from 60 g of yeast was diluted to 150 ml, 750 μl of methanol containing 0.1 M-phenylmethanesulphonyl fluoride and 15 ml of water containing 225 mg of protamine were added to it, and the mixture was centrifuged for 10 min at 40000 g. The supernatant (protamine supernatant in Tables 1 and 2) was then fractionated with (NH₄)₂SO₄. (NH₄)₂SO₄ concentrations are reported as g of (NH₄)₂SO₄ added per 100 ml original volume of protamine supernatant. Precipitates formed on increasing the (NH₄)₂SO₄ concentration in steps to 20, 32, 43 and 63% (w/v) were collected by
centrifugation for 10 min at 40000g, and the pellets were packed dry by a further 10 min centrifugation at 40000g and then dissolved in fractionation buffer.

Trehalase preparations

The properties of the two trehalases (V- and C-trehalase; see the Results section) reported here were studied by using partially purified preparations of each enzyme, which appeared to be free from contamination with the other enzyme.

The following preparations of V-trehalase were used. (1) Dialysed 63 P (sp. activity 20 munits/mg); the precipitate collected between 43 and 63% (w/v) (NH₄)₂SO₄ was dialysed overnight against two changes of 50 mM-Mes (4-morpholine-ethanesulphonic acid)/KOH (pH 5.0)/0.1 mM-EDTA/1.4 mM-mercaptoethanol. (2) Gel-filtered 63 P (sp. activity 200 munits/mg); the preparation was run through a column (1.6 cm x 35 cm) of Sephadex G-150 in 50 mM-Mes/KOH (pH 5.5)/0.1 mM-EDTA/1.4 mM-mercaptoethanol and the peak fraction was used. (3) Concentrated 63 S (sp. activity 300 munits/mg); material soluble in 63% (w/v) (NH₄)₂SO₄ was run into a column (0.9 cm x 11 cm) of DEAE-cellulose equilibrated with fractionation buffer, and the column was washed with fractionation buffer containing 63 g of (NH₄)₂SO₄/100 ml and then with ordinary fractionation buffer: the trehalase first bound to the column, presumably by hydrophobic forces, and was eluted sharply in (NH₄)₂SO₄-free buffer. (4) Highly purified V-trehalase (sp. activity 5 units/mg); this is material purified by a method being developed in our laboratory (K. Varimo, unpublished work). Briefly, yeast was broken with a Manton–Gaulin homogenizer, the 100 000 g supernatant adjusted to 45% (w/v) (NH₄)₂SO₄ and centrifuged, and the supernatant fractionated by chromatography on octyl-Sepharose, DEAE-Sepharose and Sephacyr S200, with a purification of about 100-fold in 15% yield. The final product still contained about 15 times more invertase than trehalase, but only small amounts of other proteins.

For C-trehalase, the precipitate (32 P) collected between 20 and 32% (w/v) (NH₄)₂SO₄ was gel-filtered: 32 P was made by passing 0.8 ml of 32 P through a column (0.9 cm x 14 cm) of Sephadex G-25 equilibrated with either 20 mM-potassium phosphate (pH 7.0)/50 μM-EDTA/1.4 mM-mercaptoethanol or 20 mM-Pipes (1,4-piperazinediethanesulphonic acid)/KOH (pH 7.0)/1 mM-CaCl₂/0.1 mM-EDTA/1.4 mM-mercaptoethanol, as stated in the text, and the protein-containing fraction was collected in 1.5 ml.

Activation of C-trehalase

Preparation 32 P diluted to 22 mg of protein/ml of fractionation buffer was incubated with 3.6 mM-MgATP and 180 μM-cyclic AMP for 1.0 min at 30°C and then rapidly cooled in ice and passed through Sephadex G-25 as described above, to remove nucleotides and metal ions.

Enzyme assays

A unit of trehalase catalyses the hydrolysis of 1 μmol of trehalose/min at 30°C and 10 mM-trehalose in either (for C-trehalase) 50 mM-Pipes/ KOH (pH 7.0) containing 1 mM-MnCl₂ or 2.5 mM-CaCl₂, or (for V-trehalase) 50 mM-Mes/KOH (pH 5.0) containing 1 mM-EDTA. Initial rates were estimated with three 1.0 ml reaction mixtures, started by addition of enzyme and stopped by heating in boiling water at once or after two incubation times, usually 15 and 30 min. The glucose liberated was determined enzymically (see van der Plaat, 1974). The pH of spent reaction mixtures was determined with a glass electrode at 30°C. Bivalent-metal-ion-free reaction mixtures were made by passing 50 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/KOH (pH 7.0)/10 mM-trehalose through a column (1.5 cm x 27 cm) of Chelex 100 at 50 ml/h.

Invertase was assayed at 30°C in 50 mM-Mes/KOH (pH 5.0) containing 10 mM-sucrose in the same way as trehalase.

Materials

Chelex 100 from Bio-Rad (Richmond, CA, U.S.A.) was washed with 1 M-HCl and 1 M-KOH and then equilibrated with 50 mM-Hepes/KOH (pH 7.0) before use. Concanavalin A–Sepharose and Sephadex G-25 and G-150 were from Pharmacia Fine Chemicals AB, Uppsala, Sweden. D- (+)-Trehalose dihydrate was from Fluka A.G., Buchs, Switzerland, and phenylmethanesulphonyl fluoride from Sigma Chemical Co., St. Louis, MO, U.S.A.

Results

Fractionation of trehalase activity

The distribution of trehalase observed during fractionation of the protamine supernatant depended on the pH and metal content of the assay mixtures. An activity at pH 7 was salted-out between 20 and 32% (w/v) (NH₄)₂SO₄ (Table 1), whereas an activity at pH 5 only began to precipitate above 43% (w/v) (NH₄)₂SO₄ and was appreciably soluble in 63% (NH₄)₂SO₄, though not so soluble as invertase. The recovery of the pH 7 activity depended on the type (Mg²⁺, Mn²⁺ or Ca²⁺) of bivalent metal ion used in the assay, and was highest with Ca²⁺ or Mn²⁺, lower with Mg²⁺ and very low if no bivalent metal ion was used (results not shown). The pH 7 activity ran through a concanavalin A–Sepharose column (Table 2) together with most of the protein and a small
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Table 1. (NH₄)₂SO₄ fractionation of the protamine supernatant

The protamine supernatant was prepared and fractionated as described in the Methods section. Trehalase was assayed in 50 mM-Pipes/KOH (pH 7.0)/2.5 mM-CaCl₂ or 50 mM-Mes/KOH (pH 5.0)/100 mM-KCl/1 mM-EDTA. Enzyme and protein amounts per g of fresh yeast are shown.

<table>
<thead>
<tr>
<th>Protamine supernatant</th>
<th>Protein (mg)</th>
<th>Invertase (units)</th>
<th>Trehalase (munits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH₄)₂SO₄ fractions:</td>
<td></td>
<td></td>
<td>pH7/Ca pH5/EDTA</td>
</tr>
<tr>
<td>20% precipitate</td>
<td>52</td>
<td>71.4</td>
<td>1970 395</td>
</tr>
<tr>
<td>32% precipitate</td>
<td>5.5</td>
<td>0.8</td>
<td>120 14</td>
</tr>
<tr>
<td>43% precipitate</td>
<td>20.5</td>
<td>2.9</td>
<td>1100 8</td>
</tr>
<tr>
<td>63% precipitate</td>
<td>15.4</td>
<td>5.2</td>
<td>34 48</td>
</tr>
<tr>
<td>63% supernatant</td>
<td>6.6</td>
<td>3.9</td>
<td>31 143</td>
</tr>
<tr>
<td>Recovery</td>
<td>2</td>
<td>54</td>
<td>26 112</td>
</tr>
<tr>
<td></td>
<td>96%</td>
<td>94%</td>
<td>67% 82%</td>
</tr>
</tbody>
</table>

Table 2. Concanavalin A–Sepharose fractionation of the protamine supernatant

Protamine supernatant was dialysed against fractionation buffer and 4 ml was then applied to a column (0.9 cm x 4 cm) of concanavalin A–Sepharose equilibrated with the same buffer at 5°C. The column was washed with this buffer without and with 0.2 mM-KCl and then with 0.1 M-Mes/KOH (pH 6.0)/0.2 mM-EDTA/1.4 mM-mercaptoethanol containing 0.2 M-, 0.5 M- and 1.0 M-α-methyl D-mannoside (α-Me-Man). For the washes with α-Me-Man, the column was filled with buffer and then turned off for 1 h before continuing the elution, and at 0.5 M- and 1.0 M-α-Me-Man the temperature was raised to about 22°C. Finally, the concanavalin A–Sepharose was forced out of the column, made into a slurry and assayed directly for trehalase. Trehalase assays were in 50 mM-Pipes/KOH (pH 7.0)/1 mM-MnCl₂ or in 50 mM-Mes/KOH (pH 5.0)/1 mM-EDTA. Abbreviation: ND, not determined.

<table>
<thead>
<tr>
<th>Eluate:</th>
<th>Volume (ml)</th>
<th>Protein (ml-Å₂₈₀ units)</th>
<th>Invertase (units)</th>
<th>Trehalase (munits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialysate supernatant</td>
<td>4</td>
<td>63.7</td>
<td>91.2</td>
<td>1840 396</td>
</tr>
<tr>
<td>Eluate:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wash-through</td>
<td>12</td>
<td>60.2</td>
<td>18.5</td>
<td>1750 66</td>
</tr>
<tr>
<td>0.2 M-KCl</td>
<td>13</td>
<td>1.3</td>
<td>0</td>
<td>ND 0</td>
</tr>
<tr>
<td>0.2 M-α-Me-Man</td>
<td>18</td>
<td>1.4</td>
<td>5.0</td>
<td>ND 13</td>
</tr>
<tr>
<td>0.5 M-α-Me-Man</td>
<td>20</td>
<td>1.1</td>
<td>29.9</td>
<td>ND 45</td>
</tr>
<tr>
<td>1.0 M-α-Me-Man</td>
<td>9</td>
<td>ND</td>
<td>19.7</td>
<td>ND 30</td>
</tr>
<tr>
<td>Still bound to matrix</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND 79</td>
</tr>
<tr>
<td>Recovery</td>
<td></td>
<td>100%</td>
<td>80%</td>
<td>95% 59%</td>
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</table>

amount (66 munits, 17%) of the pH 5 activity. By washing the column with α-methyl D-mannoside solutions, 88 munits (22%) of the pH 5 activity was eluted, and a further 79 munits (20%) could be detected still bound to the matrix at the end of the experiment. Evidently, the pH 5 trehalase was more strongly bound by concanavalin A–Sepharose than was invertase, which was eluted in 60% yield (54.6 units) by α-methyl D-mannoside.

All the trehalase activity at pH 7 and pH 5 found in the unfractionated disintegrated-yeast preparation was recovered in the protamine supernatant, so that both activities seem to be soluble. Unbroken cells exhibited negligible trehalase activity.

Properties of V-trehalase

We call the EDTA-insensitive trehalase activity at pH 5 ‘V-trehalase’, because it probably corresponds to the trehalase found in the vacuoles of careftully lysed yeast spheroplasts (Keller et al., 1982). We have investigated its properties by using both crude and partially purified preparations (dialysed 63P, gel-filtered 63P, concentrated 63S and highly purified V-trehalase, as described in the Methods section). No differences were noted between these preparations. V-trehalase is an acidic protein that can be adsorbed by DEAE-Sephadex at pH 3.0 in 50 mM-glycine/HCl and eluted in 70% yield at 0.14 M-NaCl (results not shown). During gel-filtration on Sephadex G-150 in 20 mM-potassium phosphate (pH 7.0) or 10 mM-Mes/KOH (pH 5.5)/0.1 mM-KCl it was eluted immediately after invertase, with an activity recovery of over 80% and apparent Mr, of 215000 (results not shown).

The activity of V-trehalase was not altered by replacing the 1 mM-EDTA in assay mixtures by 1 mM-MgCl₂, -MnCl₂ or -CaCl₂ or by 0.1 mM-ZnCl₂. Higher concentrations of MnCl₂ (20 and 50 mM) caused 30% activation, but similar activa-
tions (23–37%) were caused by comparable concentrations (50–300 mM) of NaCl, NaNO₃ and KCl. The activity decreased by less than 10% when the concentration of Mes was lowered to 10 mM, and tetramethylammonium (about 1 mM) was used as counterion, suggesting that there was no absolute dependency on metal ions.

The $K_m$ for trehalose was $1.4 \pm 0.1$ mM at pH 5.0, 5.8 (Fig. 1, inset) and 6.5 in 50 mM Mes/KOH/100 mM-KCl. At 40 mM-trehalose, a pH optimum between pH 4 and 5 and a $pK$ of 6.1 were found (Fig. 1). We have not estimated the $pK$ below pH 4 because of poor enzyme stability: in reaction mixtures containing no trehalose, the loss of enzyme activity during 30 min was less than 4% at or above pH 4, but 50% at pH 3. With Good buffers (Good et al., 1966) no specific buffer effects were observed, but acetate buffers inhibited strongly. The inhibition was essentially non-competitive (results not shown: at 10 mM-trehalose and a constant $K^+$ concentration of 100 mM, plots of 1/v against [acetate + acetic acid] were linear up to 75 mM, and at pH 5.0 the $K_m$ was 1.1 mM at 100 mM-potassium acetate and 1.4 mM at 100 mM-KCl), with $K_v$ values of 33 mM- and 17 mM- (acetate + acetic acid) at pH 5.0 and 4.0 respectively. For a $pK_v$ of acetic acid of 4.75, these $K_v$ values correspond to 12 mM- and 15 mM-acetic acid, suggesting that the inhibition is mainly caused by acetic acid. At pH 5.0, propionic acid behaved quantitatively similarly to acetic acid, whereas formic acid had no significant effect.

**Properties of C-trehalase**

We call the bivalent-metal-ion-stimulated trehalase activity at pH 7 ‘C-trehalase’, because it probably corresponds to the cytosolic ‘trehalase zymogen’ reported by Wiemken & Schellenberg (1982). As previously reported (Van Solingen & van der Plaat, 1974; Londesborough, 1982; Wiemken & Schellenberg, 1982), this C-trehalase can be activated by treatment with cyclic AMP and MgATP. Such treatment of the protamine supernatant increased its activity at pH 7.0 2-fold (measured at 2.5 mM-CaCl₂ or 4-fold (measured at 1 mM-MgCl₂), but increased the activity at pH 5.0 by less than 10% (results not shown).

During gel filtration in Pipes/CaCl₂, activated C-trehalase showed an apparent $M_r$ of 170000 [Fig. 2, (○)]. In phosphate/MgCl₂ the main peak was eluted at the same volume, but recovery of activity fell to between 14 and 29% [17% in Fig. 2, (●)], and a minor high-$M_r$ (> 250000) peak was observed. When non-activated C-trehalase (fraction 32 P) was passed through Sephadex G-150 in the Pipes/CaCl₂ buffer, the main peak also had an apparent $M_r$ of 170000, but recovery was again low (35%) and a minor high-$M_r$ peak was again observed (results not shown).

The effects of metal salts on C-trehalase were investigated further in reaction mixtures containing 50 mM-Pipes/KOH (pH 7.0)/10 mM-trehalose by using fraction 32 P gel-filtered in 20 mM-potassium phosphate (pH 7.0)/50 μM-EDTA/1.4 mM-mercaptoethanol as described in the Methods section. EDTA (1 mM) and ZnCl₂ (0.1 mM) each completely inhibited the enzyme, and MgCl₂ caused about 50% inhibition at 1 mM and complete inhibition at 50 mM, whereas MnCl₂ and CaCl₂ (1 mM) caused 2- and 4-fold activations respectively, the optimum concentration for CaCl₂ being about 2.5 mM (results not shown). Higher concentrations of MnCl₂ and CaCl₂ were strongly inhibitory, so that at 50 mM of either salt the activity was less than 25% of that at the respective optimum. KCl and MgCl₂ caused similar inhibitions when added together with 1 mM-CaCl₂.

Because the degree of activation of 32P by cyclic AMP/ATP depended on the type of bivalent metal ion used in the trehalase assays (see above), it seemed likely that two active forms of C-trehalase, with different responses to bivalent metal ions, were involved. When the Pipes buffer in the assay mixtures was replaced by Hepes/KOH, pH 7.0, and the assay mixtures were treated with Chelex 100, a clear difference between the behaviour of the activated and non-activated enzymes became apparent (Fig. 3). With no additions to the assay...
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Fig. 2. Gel filtration of activated C-trehalase

Fraction 32P was activated with cyclic AMP/ATP as described in the Methods section and passed through a Sephadex G-25 column equilibrated with 20 mM-Pipes/KOH (pH 7.0)/1 mM-CaCl₂/0.1 mM-EDTA/1.4 mM-mercaptoethanol. A portion of the eluate (970 units in 1.0 ml) was then passed through a column (1.6 cm × 34 cm) of Sephadex G-150 in the same Pipes/CaCl₂ buffer at 12 ml/h. Trehalase (○, assayed at pH 7.0/2.5 mM-CaCl₂) and A₂₈₀ (----) of the eluate were measured. The recovery of trehalase was 63% through the G-150 column. The column was calibrated (■) with γ-globulin (205000), lactate dehydrogenase (135000), bovine serum albumin (71000) and ovalbumin (41000), by using $M_r$ values recommended by Andrews (1965). Also shown is the trehalase profile observed (●) in an otherwise identical experiment but with both Sephadex columns equilibrated with 20 mM-potassium phosphate (pH 7.0)/1 mM-MgCl₂/0.1 mM-EDTA/1.4 mM-mercaptoethanol. The recovery of trehalase through the G-150 column in this experiment was only 17%.

Fig. 3. Effect of bivalent metal ions and EDTA in Chelex-treated reaction mixtures on C-trehalase activity before and after activation by cyclic AMP/ATP

The Chelex-treated reaction mixtures (see the Methods section) all contained 2 µM-EDTA derived from the enzyme sample and 50 mM-Hepes/KOH (pH 7.0)/10 mM-trehalose. Activity is plotted against the total concentration of EDTA (○, ●; leftwards from zero on the abscissa), or the net concentration (i.e. added concentration of metal chloride minus 2 µM-EDTA) of Ca²⁺ (○, ●; rightwards from zero on the abscissa), Mn²⁺ (△, ▲) or Mg²⁺ (□, ■). The enzyme used was fraction 32P that had not been activated (○, △, □) or had been activated with cyclic AMP/ATP as described in the Methods section (●, ▲, ■), and then passed through Sephadex G-25 equilibrated with 20 mM-potassium phosphate (pH 7.0)/50 µM-EDTA/1.4 mM-mercaptoethanol.
mixtures except the 2 μM-EDTA introduced with the enzyme sample, activated enzyme hydrolysed 23 nmol of trehalose/min per mg of protein, which was 22% of its maximum activity (at 2.5 mm-CaCl₂), whereas non-activated enzyme was essentially inactive (<2% of its activity at 2.5 mm-CaCl₂). Between 3 and 100 μM free Ca²⁺, activated enzyme exhibited a plateau at about 40% of the maximum activity, but the activity of non-activated enzyme slowly increased to 20% of that at 2.5 mm-CaCl₂ (progress curves with non-activated enzyme below 50 μM-Ca²⁺ accelerated, so that, e.g., at 48 μM- and 3 μM-Ca²⁺ respectively the rates in the second 15 min of reaction were 60 and 180% greater than in the first 15 min: this effect, probably a slow binding of Ca²⁺ by apoenzyme, does not exceed the size of the experimental points in Fig. 3). Addition of boiled activated enzyme to non-activated enzyme did not increase the latter’s activity at 4 μM-EDTA, and passing activated enzyme slowly through a Chelex 100 column did not change its activity at 2 μM-EDTA relative to that at 2.5 mm-CaCl₂. Nevertheless, activated enzyme was inhibited by EDTA (Fig. 3). Non-activated enzyme was completely inactive in the presence of 10 μM–1 mM-MgCl₂, whereas activated enzyme exhibited somewhat less activity than with 3 μM free Ca²⁺ alone.

Since yeast contains a calmodulin-like polypeptide (Hubbard et al., 1982), we tested whether ox heart calmodulin might activate either form of C-trehalase in the presence of suboptimal Ca²⁺ concentrations. No significant effects were observed.

In the presence of 2.5 mm-CaCl₂, C-trehalase had a Kₘ for trehalose of 5.7 mM both before and after treatment with cyclic AMP/ATP (Fig. 4). Addition of 100 mM-KCl, which caused a 3-fold decrease in Vₘₐₓ, did not change the Kₘ of non-activated enzyme (results not shown). At 40 mM-trehalose, both forms of C-trehalase had an unusually sharp pH optimum at pH 6.7. Activation appeared to broaden the pH optimum slightly (Fig. 4). At 50 μM-CaCl₂ the pH profile of activated enzyme was essentially the same as at 2.5 mm-CaCl₂ (results not shown).

Discussion

Our results show that baker’s yeast contains two distinct trehalases with very different properties, which are summarized in Table 3. Wiemken and colleagues showed that a trehalase active at pH 5.5 is confined to the vacuoles of baker’s yeast (Keller et al., 1982), whereas the cytosol contains a ‘trehalase zymogen’, which can be measured at
pH 6.2 in the presence of 2 mM-MgCl₂ after activation with cyclic AMP/ATP (Wiemken & Schellenberg, 1982). Presumably our V-trehalase corresponds to their vacuolar enzyme, and we find that the so-called ‘trehalase zymogen’ is in fact a Ca²⁺- or Mn²⁺-dependent trehalase with an activity and affinity for Ca²⁺ that are both increased by treatment with cyclic AMP/ATP. Ca²⁺ also stabilized activated C-trehalase during gel filtration (Fig. 2). The activation of C-trehalase by cyclic AMP/ATP is clearly a covalent modification, because we could separate activated enzyme from small molecules by gel filtration. Uno et al. (1983) have shown that activation of a trehalase (Mr 320000) partially purified from yeast was accompanied by phosphorylation of a polypeptide of Mr 80000, and confirmed the proposal (Van Solingen & van der Plaat, 1975) that the activation is caused by a protein kinase. The enzyme of Uno et al. (1983) is presumably a tetramer (in 50 mM-Tris/HCl, pH 7.4). Our C-trehalase may be the corresponding dimer, and we sometimes observed a minor component with Mr > 250000 (Fig. 2). At least two active forms of C-trehalase occur, because treatment with cyclic AMP/ATP changed the enzyme’s response to Ca²⁺ (Fig. 3). As a corollary, the degree of activation by treatment with cyclic AMP/ATP will depend on the concentrations of Ca²⁺ and Mn²⁺ during assay.

Since V-trehalase is in the vacuole (pH about 5.5; Slavik, 1982), it operates in vivo close to its optimal pH, and is probably the major trehalase activity in vivo when C-trehalase is not activated (vacuolar trehalase concentrations are not known, but V-trehalase has a relatively small Kₘ). The high activities of C-trehalase shown in Tables 1 and 2 result from the unphysiologically high concentrations of Ca²⁺ and Mn²⁺ used (both ions are below 10 μM in the cytosol; Nieuwenhuis et al., 1981; Martonosi, 1980). In the absence of added Ca²⁺ or Mn²⁺, considerable activity at pH 7.0 is found in crude cell disintegrates (about 0.6 unit/g, results not shown; see also van der Plaat, 1974), but this activity is presumably supported by Ca²⁺ and Mn²⁺ derived from the cell wall and vacuole (which accumulates Mn²⁺; Okorokov et al., 1977), and disappears during (NH₄)₂SO₄ fractionation. The abrupt pH-dependence of C-trehalase (Fig. 4) will also affect its activity in vivo, because the intracellular pH of yeast (7.5–6.4; Kotyk, 1963) varies from well above to somewhat below the enzyme’s optimal pH.

For V-trehalase, the constant Kₘ between pH 5.0 and 6.5, where the velocity at near-saturating substrate concentration has a pK of 6.1, argues that, as for invertase (Haldane, 1930), the Kₘ is a thermodynamic dissociation constant. The strong inhibition of V-trehalase by acetic acid explains the sharp pH optimum at 5.5 found by Panek & Souza (1964), because these authors used 0.1 mM-acetate buffers. It may also account for the prolonged disappearance of ATP and several days’ lag phase (Chu et al., 1981) when yeast grown on glucose is transferred to 0.1 mM-potassium acetate (pH 5.5) as sole carbon source. Much shorter lag phases occur when the cells are transferred to ethanol or pyruvate, or to lower concentrations (10 mM) of acetate: possibly the cells can then mobilize trehalose to maintain energy supplies, but their V-trehalase is inhibited at the higher acetate concentration.

The different properties of C- and V-trehalase explain some, but not all, of the discrepant reports concerning yeast trehalases. Our Kₘ values for V- and C-trehalase agree, respectively, with the values of 1.5 mM for a partially purified preparation (Panek, 1969) and 5.6 mM for unfractionated yeast extracts (Ortiz et al., 1983). Both lower (e.g. 0.5 mM at pH 5.6; Panek & Souza, 1964; Kelly & Catley, 1976) and higher (10 mM at pH 6.9; Avigad et al., 1965) values have been reported. The preparation of Avigad et al. (1965) resembled C-trehalase in its sharp pH optimum at pH 6.9, but was not inhibited by EDTA and only 20% inhibited by 1 mM-ZnCl₂. Panek’s (1969) trehalase preparation was 30% inhibited by 50 mM-EDTA, although its Kₘ (1.4 mM) and strong binding to DEAE-cellulose suggest that it was mainly V-trehalase. Possibly strain differences or proteolytic artifacts explain some of these discrepancies.

Wiemken & Schellenberg (1982) have proposed that phosphorylation of cytoplasmic trehalase may

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<thead>
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<th>Table 3. Properties of V- and C-trehalases</th>
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</thead>
<tbody>
<tr>
<td><strong>V-trehalase</strong></td>
</tr>
<tr>
<td>Behaviour to (NH₄)₂SO₄</td>
</tr>
<tr>
<td>Concanavalin A</td>
</tr>
<tr>
<td>10⁻¹ × Mr</td>
</tr>
<tr>
<td>pH optimum</td>
</tr>
<tr>
<td>Kₘ (mM)</td>
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<tr>
<td>Bivalent metal needed</td>
</tr>
<tr>
<td>Effect of 100 mM-KCl</td>
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<tr>
<td>Effect of 0.1 mM-ZnCl₂</td>
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</tbody>
</table>

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initiate its transport into vacuoles. Since V-trehalase probably contains more carbohydrate than does C-trehalase, this suggestion implies a novel mechanism for the glycosylation and translocation of vacuolar enzymes. These processes are usually concurrent with translation (Scheekman, 1982). If C-trehalase is indeed a precursor of V-trehalase, then the interconversion must involve drastic changes in catalytic properties, including loss of the dependence on free bivalent metal ions. Possibly the binding of Ca\(^{2+}\), which is already much tighter for activated C-trehalase than for the non-activated enzyme, becomes essentially irreversible. However, highly purified V-trehalase was not inactivated by 20h incubations with 2.5 mm-o-phenanthroline or 8-hydroxyquinoline at 30°C (J. Londesborough, unpublished work). On the present evidence, and by analogy with invertase (Carlson, 1983), we consider it more likely that V- and C-trehalase are synthesized from different mRNA species, and possibly even different genes.

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References


