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 $(\text{NH}_4)_2\text{SO}_4$, has an apparent M_r of 215 000 (by gel filtration) and is an acidic protein. It has a K_m 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, NaNO_3 and MnCl_2 . The enzyme is strongly inhibited by acetic acid/acetate buffers, with a K_i of about 15 mM-acetic acid. The pH 7 activity does not bind to concanavalin A, is salted out at 20–32% (w/v) $(\text{NH}_4)_2\text{SO}_4$ and has an M_r of 170 000 (by gel filtration). It is absolutely dependent on Ca^{2+} or Mn^{2+} ions (Mg^{2+} 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 Ca^{2+} requirement, and does not change the K_m 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 Plaats (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 Plaats, 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 40 000g. Supernatant from 60 g of yeast was diluted to 150 ml, 750 μ l 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 40 000g. The supernatant (protamine supernatant in Tables 1 and 2) was then fractionated with $(\text{NH}_4)_2\text{SO}_4$. $(\text{NH}_4)_2\text{SO}_4$ concentrations are reported as g of $(\text{NH}_4)_2\text{SO}_4$ added per 100 ml original volume of protamine supernatant. Precipitates formed on increasing the $(\text{NH}_4)_2\text{SO}_4$ 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) $(\text{NH}_4)_2\text{SO}_4$ 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 above preparation was run through a column (1.6 cm $\times$ 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) $(\text{NH}_4)_2\text{SO}_4$ was run into a column (0.9 cm $\times$ 11 cm) of DEAE-cellulose equilibrated with fractionation buffer, and the column was washed with fractionation buffer containing 63 g of $(\text{NH}_4)_2\text{SO}_4$ /100 ml and then with ordinary fractionation buffer: the trehalase first bound to the column, presumably by hydrophobic forces, and was eluted sharply in $(\text{NH}_4)_2\text{SO}_4$ -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 100000g supernatant adjusted to 45% (w/v) $(\text{NH}_4)_2\text{SO}_4$ and centrifuged, and the supernatant fractionated by chromatography on octyl-Sepharose, DEAE-Sepharose and Sephacryl 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) $(\text{NH}_4)_2\text{SO}_4$ was used: gel-filtered 32 P was made by passing 0.8 ml of 32 P through a column (0.9 cm $\times$ 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- CaCl_2 /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- MnCl_2 or 2.5 mM- CaCl_2 , 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 Plaats, 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 $\times$ 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) $(\text{NH}_4)_2\text{SO}_4$ (Table 1), whereas an activity at pH 5 only began to precipitate above 43% (w/v) $(\text{NH}_4)_2\text{SO}_4$ and was appreciably soluble in 63% $(\text{NH}_4)_2\text{SO}_4$, though not so soluble as invertase. The recovery of the pH 7 activity depended on the type (Mg^{2+} , Mn^{2+} or Ca^{2+}) of bivalent metal ion used in the assay, and was highest with Ca^{2+} or Mn^{2+} , lower with Mg^{2+} 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

Table 1. $(\text{NH}_4)_2\text{SO}_4$ fractionation of the protamine supernatant

The protamine supernatant was prepared and fractionated as described in the Methods section. Trehalase was assayed in 50mM-Pipes/KOH (pH 7.0)/2.5mM- CaCl_2 or 50mM-Mes/KOH (pH 5.0)/100mM-KCl/1mM-EDTA. Enzyme and protein amounts per g of fresh yeast are shown.

	Protein (mg)	Invertase (units)	Trehalase (munits)	
			pH 7/Ca	pH 5/EDTA
Protamine supernatant	52	71.4	1970	395
$(\text{NH}_4)_2\text{SO}_4$ fractions:				
20% precipitate	5.5	0.8	120	14
32% precipitate	20.5	2.9	1100	8
43% precipitate	15.4	5.2	34	48
63% precipitate	6.6	3.9	31	143
63% supernatant	2	54	26	112
Recovery	96%	94%	67%	82%

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

Protamine supernatant was dialysed against fractionation buffer and 4ml was then applied to a column (0.9cm $\times$ 4cm) of concanavalin A-Sepharose equilibrated with the same buffer at 5°C. The column was washed with this buffer without and with 0.2M-KCl and then with 0.1M-Mes/KOH (pH 6.0)/0.2mM-EDTA/1.4mM-mercaptoethanol containing 0.2M-, 0.5M- and 1.0M- 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 0.5M- and 1.0M- 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 50mM-Pipes/KOH (pH 7.0)/1mM- MnCl_2 or in 50mM-Mes/KOH (pH 5.0)/1mM-EDTA. Abbreviation: ND, not determined.

	Volume (ml)	Protein (ml $\cdot$ A_{280} units)	Invertase (units)	Trehalase (munits)	
				pH 7/Mn	pH 5/EDTA
Dialysed supernatant	4	63.7	91.2	1840	396
Eluate:					
Wash-through	12	60.2	18.5	1750	66
0.2M-KCl	13	1.3	0	ND	0
0.2M- α -Me-Man	18	1.4	5.0	ND	13
0.5M- α -Me-Man	20	1.1	29.9	ND	45
1.0M- α -Me-Man	9	ND	19.7	ND	30
Still bound to matrix		ND	ND	ND	79
Recovery		100%	80%	95%	59%

amount (66munits, 17%) of the pH 5 activity. By washing the column with α -methyl D-mannoside solutions, 88munits (22%) of the pH 5 activity was eluted, and a further 79munits (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 (54.6units) 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 carefully 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 50mM-glycine/HCl and eluted in 70% yield at 0.14M-NaCl (results not shown). During gel-filtration on Sephadex G-150 in 20mM-potassium phosphate (pH 7.0) or 10mM-Mes/KOH (pH 5.5)/0.1M-KCl it was eluted immediately after invertase, with an activity recovery of over 80% and apparent M_r of 215000 (results not shown).

The activity of V-trehalase was not altered by replacing the 1mM-EDTA in assay mixtures by 1mM- MgCl_2 , - MnCl_2 or - CaCl_2 or by 0.1mM- ZnCl_2 . Higher concentrations of MnCl_2 (20 and 50mM) caused 30% activation, but similar activa-

tions (23–37%) were caused by comparable concentrations (50–300mM) of NaCl, NaNO₃ and KCl. The activity decreased by less than 10% when the concentration of Mes was lowered to 10mM, and tetramethylammonium (about 1mM) was used as counterion, suggesting that there was no absolute dependency on metal ions.

The K_m for trehalose was 1.4 ± 0.1 mM at pH 5.0, 5.8 (Fig. 1, inset) and 6.5 in 50mM-Mes/KOH/100mM-KCl. At 40mM-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 10mM-trehalose and a constant K⁺ concentration of 100mM, plots of $1/v$ against [acetate + acetic acid] were linear up to 75mM, and at pH 5.0 the K_m was 1.1mM at 100mM-potassium acetate and 1.4mM at 100mM-KCl), with K_i values of 33mM- and 17mM- (acetate + acetic acid) at pH 5.0 and 4.0 respectively. For a pK_a of acetic acid of 4.75, these K_i values correspond to 12mM- and 15mM-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 Plaats, 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 2.5mM-CaCl₂) or 4-fold (measured at 1mM-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 32P) 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 50mM-Pipes/KOH (pH 7.0)/10mM-trehalose by using fraction 32P gel-filtered in 20mM-potassium phosphate (pH 7.0)/50μM-EDTA/1.4mM-mercaptoethanol as described in the Methods section. EDTA (1mM) and ZnCl₂ (0.1mM) each completely inhibited the enzyme, and MgCl₂ caused about 50% inhibition at 1mM and complete inhibition at 50mM, whereas MnCl₂ and CaCl₂ (1mM) caused 2- and 4-fold activations respectively, the optimum concentration for CaCl₂ being about 2.5mM (results not shown). Higher concentrations of MnCl₂ and CaCl₂ were strongly inhibitory, so that at 50mM 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 1mM-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

Fig. 1. pH- and substrate-dependence of V-trehalase. The activity (munits/ml of gel-filtered 63P) was measured in reaction mixtures containing 40mM-trehalose, 2mg of albumin/ml, 100mM-KCl and 50mM-Hepes/KOH (□), -Pipes/KOH (●), -Mes/KOH (○) or -glycine/HCl (Δ) or 25mM-sodium citrate (■). The theoretical curve for a pH-independent velocity of 104 and pK of 6.1 is shown (continuous line). Inset shows a Hofstee (1959) plot for the same enzyme sample in 50mM-Mes/KOH (pH 5.8) containing 100mM-KCl, 1mg of albumin/ml and 1.5–40mM-trehalose.

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 20mM-Pipes/KOH (pH 7.0)/1mM-CaCl₂/0.1mM-EDTA/1.4mM-mercaptoethanol. A portion of the eluate (970 munits in 1.0ml) was then passed through a column (1.6cm × 34cm) of Sephadex G-150 in the same Pipes/CaCl₂ buffer at 12ml/h. Trehalase (○, assayed at pH 7.0/2.5mM-CaCl₂) and *A*₂₈₀ (—) 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 20mM-potassium phosphate (pH 7.0)/1mM-MgCl₂/0.1mM-EDTA/1.4mM-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 μ M-EDTA derived from the enzyme sample and 50mM-Hepes/KOH (pH 7.0)/10mM-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 μ M-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 20mM-potassium phosphate (pH 7.0)/50 μ M-EDTA/1.4mM-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- CaCl_2), whereas non-activated enzyme was essentially inactive (<2% of its activity at 2.5 mM- CaCl_2). Between 3 and 100 μM free Ca^{2+} , 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- CaCl_2 (progress curves with non-activated enzyme below 50 μM - Ca^{2+} accelerated, so that, e.g., at 48 μM - and 3 μM - Ca^{2+} 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 Ca^{2+} 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- CaCl_2 . Nevertheless, activated enzyme was inhibited by EDTA (Fig. 3). Non-activated enzyme was completely inactive in the presence of 10 μM –1 mM- MgCl_2 , whereas activated enzyme exhibited somewhat less activity than with 3 μM free Ca^{2+} 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 Ca^{2+} concentrations. No significant effects were observed.

In the presence of 2.5 mM- CaCl_2 , C-trehalase had a K_m 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 $V_{\max}$, did not change the K_m 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 - CaCl_2 the pH profile of activated enzyme was essentially the same as at 2.5 mM- CaCl_2 (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

Fig. 4. pH- and substrate-dependence of C-trehalase

Untreated (open symbols) and cyclic AMP/ATP-treated (closed symbols) fraction 32P was used after gel filtration through Sephadex G-25 as described in Fig. 3. For panel (a), reaction mixtures contained 50 mM-Pipes/KOH (pH 7.0), 2.5 mM- CaCl_2 and 1.0–100 mM-trehalose, and rates are expressed as percentages of that at 40 mM-trehalose. For panel (b), reaction mixtures contained 2.5 mM- CaCl_2 , 40 mM-trehalose, 50 mM-KCl, 4 mg of albumin/ml and 10 mM-glycylglycine (∇ , $\blacktriangledown$), -Hepes ($\circ$, $\bullet$), -Pipes ($\triangle$, $\blacktriangle$) or -Mes ($\square$, $\blacksquare$) buffer adjusted to the appropriate pH with KOH. Below pH 6.5, the continuous and broken lines refer to non-activated and activated enzyme respectively. The bars at low and high pH with non-activated enzyme indicate corrections for enzyme instability observed in the absence of trehalose (activated enzyme was stable throughout the pH range).

Table 3. *Properties of V- and C-trehalases*

	V-trehalase	C-trehalase
Behaviour to $(\text{NH}_4)_2\text{SO}_4$	Soluble at 63% (w/v)	Precipitates before 32% (w/v)
Concanavalin A	Binds strongly	No binding
$10^{-3} \times M_r$	215	170
pH optimum	4–5	6.7
K_m (mM)	1.4	5.7
Bivalent metal needed	None	Ca^{2+} or Mn^{2+} (affinity increases on activation)
Effect of 100 mM-KCl	30% activation	65% inhibition
Effect of 0.1 mM-ZnCl ₂	None	Complete inhibition

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 Ca^{2+} - or Mn^{2+} -dependent trehalase with an activity and affinity for Ca^{2+} that are both increased by treatment with cyclic AMP/ATP. Ca^{2+} 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 (M_r 320 000) partially purified from yeast was accompanied by phosphorylation of a polypeptide of M_r 80 000, and confirmed the proposal (Van Solingen & van der Plaats, 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 $M_r > 250 000$ (Fig. 2). At least two active forms of C-trehalase occur, because treatment with cyclic AMP/ATP changed the enzyme's response to Ca^{2+} (Fig. 3). As a corollary, the degree of activation by treatment with cyclic AMP/ATP will depend on the concentrations of Ca^{2+} and Mn^{2+} 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 trehalose concentrations are not known, but V-trehalase has a relatively small K_m). The high activities of C-trehalase shown in Tables 1 and 2 result from the unphysiologically high concentrations of Ca^{2+} and Mn^{2+} used (both ions are below 10 μM in the cytosol; Nieuwenhuis *et al.*, 1981; Martonosi, 1980). In the absence of added Ca^{2+} or Mn^{2+} , considerable activity at pH 7.0 is found in crude cell disintegrates (about 0.6 unit/g, results not shown; see also van der Plaats, 1974), but this activity is presumably supported by Ca^{2+} and Mn^{2+} derived from the cell wall and vacuole (which accumulates Mn^{2+} ; Okorokov *et al.*, 1977),

and disappears during $(\text{NH}_4)_2\text{SO}_4$ 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 K_m 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 K_m 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 M-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 M-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 K_m 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 50% inhibited by 50 mM-EDTA, although its K_m (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

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 (Schekman, 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 20 h 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 *et al.*, 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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