Table II

Effect of Glucagon Concentration on Cell Homogenates

Glucagon Molarity	Phosphodiesterase Activity (pmol/min/mg protein)	
	Low Km	High Km
0	43	2660
2.7×10^{-12}	39	2560
2.7×10^{-11}	45	2550
2.7×10^{-10}	44	2590
2.7×10^{-9}	45	2620
2.7×10^{-8}	43	2590
2.7×10^{-7}	41	2610
2.7×10^{-6}	36	2600
2.7×10^{-5}	36	2490

The procedure used was essentially that described for the effect of glucagon concentration on phosphodiesterase activity with the exception that glucagon was added immediately after disruption of the cells. Results are the means of 2 experiments.

be observed when glucagon was added after the disruption of the cells (Table II).

Phosphodiesterase Activity as a Function of Time

A suspension containing approximately 3×10^6 cells/ml was incubated with 2.7μM glucagon and aliquots removed at 5 min intervals for phosphodiesterase assay. Activation was compared with a similar suspension incubated without glucagon. Fig. 3 shows that the maximal stimulation of the low Km form was attained within 5 min of the addition of glucagon. Once stimulated the activity remained unchanged for at least 35 min. These results differ from those observed in intact fat cells by Pawlson *et al* (15) and by Zinman and Hollenberg (16) where a sustained activation of low Km

was prepared as described previously (3). The protein fraction was obtained after centrifugation of the extract for 15 min at 1,200 x g over a Sephadex G 75 gel cake lacking void volume water (7). The beads had been preswollen in 50 mM phosphate buffer, pH 6.2. The low mol. fraction was obtained after ultrafiltration of the extract through Diaflo UM 10 membrane in an Amicon model 12 cell (The Amicon Corp., Lexington, Mass.) at a pressure of 57 p.s.i.

Purification of cryptic trehalase and activating factor protein. An X-press homogenate of 30 g of baker's yeast was prepared as described previously (3). To the thawed paste 20 ml of 50 mM phosphate buffer, pH 6.2, 0.2 mM EDTA (50 mM PE buffer) was added and thoroughly mixed. After centrifugation for 30 min at 38,000 x g the supernatant was diluted with 50 mM PE buffer to a protein conc. of 20 mg/ml. Under continuous stirring 10 % (vol./vol.) of a solution containing 15 mg/ml of protamine sulphate was added and the resulting precipitate was removed by centrifugation. The supernatant solution was then fractionated by addition of solid ammonium sulphate. The precipitate between 35 and 50 % saturation was collected by centrifugation for 10 min at 38,000 x g and subsequently dissolved in 20 mM PEM buffer (20 mM phosphate buffer, pH 6.2, 0.2 mM EDTA, 0.57 mM mercapto-ethanol). This solution was rapidly desalted by centrifugation over Sephadex G 50 coarse beads (7). Cryptic trehalase was purified by applying part of the desalted solution to a DEAE-cellulose column (20 x 1.2 cm) equilibrated with 100 mM PEM buffer, pH 6.2. Elution was carried out with a linear gradient of PEM buffer (100-400 mM). The remaining part of the ammonium sulphate fraction was used to purify activating factor protein (AF protein) by applying it to a CM-cellulose column (20 x 1.2 cm) equilibrated with 20 mM PEM buffer. After washing the unbound proteins thoroughly, the AF protein was eluted by changing the buffer to 200 mM PEM, pH 6.2. For both columns the flow rate was about 40 ml/hr and fractions of 6 ml were collected. Subsequent buffer changes were performed by centrifugation over Sephadex G 50.
Both ion-exchange celluloses (DE 52 and CM 52) were purchased from Whatman; Sephadex gels from Pharmacia, Uppsala; Salmon sperm protamine sulphate Grade I from Sigma.

Activation of trehalase and its assay. Trehalase-c was activated immediately before assay. The reaction mixture, unless otherwise indicated, contained 50 mM phosphate buffer, pH 8.2, 4 mM ATP, 4 mM $MgSO_4$, 50 mM cAMP, trehalase-c plus an excess of AF protein. Total protein concentration was usually 1 - 20 mg/ml. Preincubation was at 30° C for 10 min, unless otherwise stated. Blanks were performed with omission of Mg^{2+}, ATP and cAMP. Preincubation was terminated by suitable dilution with ice-cold 50 mM phosphate buffer, pH 7.0, 0.2 mM EDTA. The subsequent assay of trehalase-a has been described in an earlier publication (3). Protein was estimated by the method of Lowry et al. (8). Bovine serum albumin, dibutyryl cAMP and ATPase (Apyrase Grade II) were from Sigma. The source of the other chemicals was reported previously (3).

Assay of protein kinase activity. The protein kinase activity of AF protein was assayed by measuring the radioactivity of γ-^{32}P ATP incorporated into an acid-precipitable material as described by Ljungström and Engström (9). The standard reaction mixture had the same composition as for trehalase-c activation except that ATP was replaced by 10 nmoles per ml of γ-^{32}P ATP (11.5 Ci/mmol), which was obtained from The Radiochemical Centre, Amersham. Radioactivity was determined by liquid scintillation counting. cAMP-binding activity of AF protein was assayed under the conditions described by Brown et al. (10).

RESULTS

Our previous results (3) showed that the cryptic form of trehalase

Table I. Effect of low mol. weight compounds on trehalase activation.

Sample	Protein conc. (mg/ml)	Addition	Trehalase activity (mU/mg protein)
Crude supernatant	96.1	-	10.8
		dibutyryl cAMP	11.3
		cAMP	74.3
		cAMP + ATPase	5.5
Sephadex G 75 filtrate	26.2	-	9.9
		cAMP	10.2
	13.7	UM 10 filtrate + cAMP	25.9
	26.2	Mg^{2+} + ATP + cAMP	75.4
		Mn^{2+} + ATP + cAMP	75.2
		Co^{2+} + ATP + cAMP	79.2

Activation conditions were as earlier described (3), concentrations used were Mg^{2+}, Mn^{2+}, Co^{2+}, and ATP 4mM, 50 µM cAMP.
ATPase (16 mg/ml) treatment was for 5 min at 30°C followed by cAMP addition.

Table II. Summary of purification of trehalase-c from baker's yeast.

Fraction	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg)
Crude supernatant	38	2.250	141	0.062[*]
Ammonium sulphate	3	256	127	0.495
DEAE-cellulose	27	6.5	122	18.76

[*] Activated under standard conditions for 3 min.

(trehalase-c) could only be completely activated at protein concentrations higher than 60 mg/ml. We therefore decided to investigate whether this was due to the protein itself or to the concentration of other substances in the extract. The results presented in table I show that addition of ATPase destroys the activating ability. It is also evident that the dibutyryl analogue cannot replace cAMP as an activator. The requirement for low mol. weight compounds was confirmed by showing that after filtration over Sephadex G 75 the protein mixture cannot be activated. Recombination of Sephadex G 75 filtrate and UM 10 membrane filtrate partially restores the activating ability

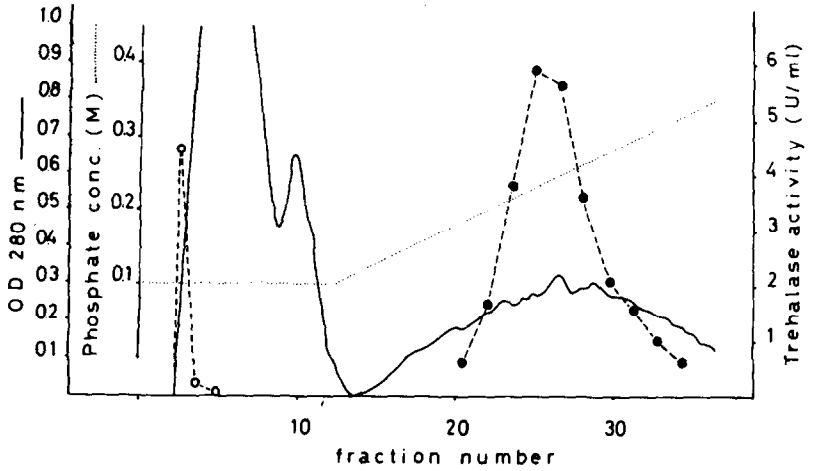

Fig. 1. DEAE-cellulose chromatography of trehalase-c and AF protein from baker's yeast.

After sample application the column was washed with 100 mM PEM buffer. The gradient was started when the OD at 280 nm had dropped below 0.1.

——, OD at 280 nm; o---o, activity of AF protein; ●---●, activity of trehalase-c; ....., molarity of PEM buffer.

at low protein concentration. The UM 10 filtrate can be replaced by ATP plus a divalent cation, such as Mg^{2+}, Mn^{2+} or Co^{2+}.

Once these conditions had been ascertained, attempts were made to purify the proteins. The results of a partial purification are presented in table II. The cell-free extract has to be prepared with the X-press, as French pressure cell extracts give a poor yield of total trehalase units after ammonium sulphate fractionation. The X-press extract is clarified by centrifugation (38,000 x g). The nucleic acids are precipitated from this cell-free extract with protamine sulphate. After centrifugation to remove the nucleic-acid precipitate the supernatant is fractionated with ammonium sulphate to separate the activatable protein mixture from the bulk of the active form of the trehalase (trehalase-a). The subsequent chromatographic separation on DEAE-cellulose is shown in fig. 1. As none of the fractions collected could be activated, it became apparent that a second activating factor-protein (AF protein), is involved in the activation of trehalase-c. The former can be recovered from the front peak in 100 mM phosphate, while trehalase-c elutes at about 200 mM phosphate. Recombination of these two protein fractions yields trehalase-a after preincubation with the necessary co-factors. Residual trehalase-a, present in the sample could not be recovered from the column, even after elution with 2.0 M NaCl, a concentration higher than the one mentioned by Panek (11). The presence of trehalase-a at the top of the column

Table. III. Isolation of AF protein from baker's yeast.

Fraction	Volume (ml)	Protein (mg)	Activity* (units) +cAMP	Activity* (units) -cAMP	[^{3}H] cAMP binding (c.p.m./mg protein)
Crude supernatant	70	1,772	0.039	0	---
Ammonium sulphate	25	240	0.155	0.076	3760
CM-cellulose	10	14	0.440	0.230	4954

*Activity of trehalase-a produced under standard conditions of trehalase-c preincubation. Although excess trehalase-c had been added these values cannot be considered as specific activity of AF protein.

Table IV. Protein kinase activity of the AF protein fraction.

Proteins	Incubation time (min)	cAMP	Protein phosphorylation* (^{32}P /mg protein) Trehalase-c	AF protein
Trehalase-c	60	+	20	
Trahalase-c + AF protein	10	-	327	
	10	+	615	
	60	+	750	
AF protein	10	-		81
	10	+		117

* Corrected for non- enzymic ^{32}P
Concentrations used were: Trehalase-c 62.5 µg/ml, AF protein 878 µg/ml.

is demonstrable by incubating the cellulose with trehalose and subsequently assaying for glucose.

The AF protein present in the 35 - 50 % ammonium sulphate fraction can also be bound to CM-cellulose. In this case trehalase-c elutes in the front peak. The results of partial purification of AF protein are presented in table III. It is obvious that during the fractionation with ammonium sulphate the dependence on cAMP is partially lost. However, the AF protein-containing fraction still shows cAMP-binding activity, whereas trehalase-c eluted from DEAE-cellulose does not contain, within the limits of experimental error, any significant cAMP-binding activity.

Both the partially purified trehalase-c and the AF protein fractions could be stored at -20° C for a few days, with subsequent loss of activity of the latter. Some further investigations were performed with a mixture of these

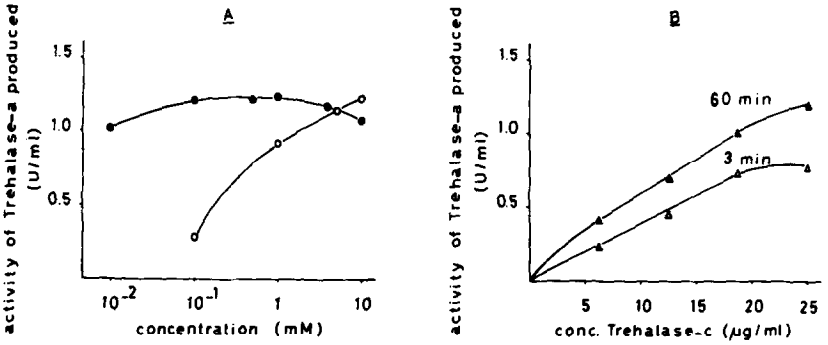

Fig. 2. Dependence of the activation on concentration of some reactants. Incubations under standard conditions, AF protein 0.46 mg/ml. Fig. 2A, trehalase-c 125 g/ml; o, Mg^{2+}, •, ATP.

two fractions. We were not able to demonstrate any dependence on pH, during the activation, in the range 6.0 - 7.5. As shown in fig. 2, the activation process leading to the production of trehalase-a is, however, dependent on protein concentration and duration of the incubation, and confirms the necessity for the presence of Mg^{2+} and ATP.

The question of how trehalase-c is converted into trehalase-a was further explored by testing for protein kinase activity. The results are presented in table IV, and show that trehalase-c does not have any significant kinase activity. The AF protein fraction, isolated from CM-cellulose, possesses a fairly high endogenous phosphorylating ability. Nevertheless it is clear from the data in table IV that trehalase-c is a good substrate for the kinase activity of the AF protein. The partial loss of cAMP dependence for trehalase-c activation agrees with the observed levels of incorporation of ^{32}P into the trehalase-c protein fraction in the presence and absence of cAMP respectively.

DISCUSSION

The results presented show that extracts of cells of baker's yeast can be separated in two protein fractions, neither of which possess the ability to split trehalose. When these fractions are recombined and incubated under the appropriate conditions - in the presence of Mg^{2+}, ATP and cAMP - an active trehalase is formed in a time- and concentration-dependent process. It could further be shown that one of the fractions (AF protein) contained cAMP-binding activity and was able to phosphorylate the other (trehalase-c) fraction. The latter fraction was devoid of both cAMP-binding and protein-kinase activities.

Due to the fact that the AF protein fraction appeared to be contaminated with other basic proteins, which can act as a substrate for phosphorylation (6) we could not demonstrate unequivocally that trehalase-a is

the phosphorylated product of trehalase-c. There are, however, some indications that this may be the case. Phosphorylation of trehalase-c as well as production of trehalase-a are roughly half as active when cAMP is omitted during preincubation. This partial loss of cAMP dependence may be due to the loss of a regulatory subunit during the fractionation procedure, thereby creating a type II protein kinase (12).

Each of the divalent cations Mg^{2+}, Mn^{2+} and Co^{2+} can act as the co-factor needed for activation of the protein kinase described by Takai et al.(6). The same cations function equally well in our system. However, further purification of the AP protein and extensive kinetic investigations of the activation process are necessary to be able to demonstrate conclusively that trehalase-c is (one of) the natural substrate(s) of this protein kinase.

The observation that trehalase-a is more strongly bound to DEAE-cellulose than is trehalase-c does not contradict phosphorylation as the activation mechanism. The higher affinity of trehalase-a for this cellulose as compared to the trehalase described by Panek (11) may be due to the isolation procedure of the latter, which allows some proteolytic degradation to occur.

The possible existence of cAMP-mediated protein phosphorylation reactions leading to activation of a metabolic process in yeast suggests that the action of cAMP is similar in both this organism and higher eukaryotes.

The observation that the trehalase activity in partially synchronised yeast populations, rises rapidly prior to the onset of budding (13), taken together with our findings, tempts us to suggest that cAMP may activate certain metabolic processes in a particular stage of the yeast cell cycle. Investigations into possible periodic changes of the cAMP level in relation to the mitotic cycle, as shown to exist in sea urchin eggs (14), certainly deserve further attention.

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