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PHYSIOLOGICAL AND GENETIC MODIFICATIONS OF THE EXPRESSION OF THE YEAST MITOCHONDRIAL ADENOSINE TRIPHOSPHATASE INHIBITOR*

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SUMMARY

1. The oligomycin-sensitive ATPase activity of submitochondrial particles of the glycerol-grown "petite-negative" yeast: *Schizosaccharomyces pombe* is markedly stimulated by incubation at 40 °C and by trypsin activations are treatment. Both increased in Triton-X 100 extracts of the submitochondrial particles.

2. A trypsin-sensitive inhibitory factor of mitochondrial ATPase with properties similar to that of beef heart has been extracted and purified from glycerol-grown and glucose-grown *S. pombe* wild type, from the nuclear pleiotropic respiratory-deficient mutant *S. pombe* M126 and from *Saccharomyces cerevisiae*.

3. ATPase activation by heat is more pronounced in submitochondrial particles isolated from glycerol-grown than from glucose-grown *S. pombe*. An activation of lower extent is observed in rat liver mitochondrial particles but is barely detectable in the "petite-positive" yeast: *S. cerevisiae*. No activation but inhibition by heat is observed in the pleitotropic respiratory-deficient nuclear mutant *S. pombe* M126.

4. The inhibition of *S. pombe* ATPase activity by low concentrations of dicyclohexylcarbodiimide dissapears at inhibitor concentrations above 25 μM. In Triton-extract of submitochondrial particles net stimulation of ATPase activity is observed at 100 μM dicyclohexylcarbodiimide. The pattern of stimulation of ATPase activity by dicyclohexylcarbodiimide in different genetic and physiological conditions parallels that produced by heat and trypsin. A similar mode of action is therefore proposed for the three agents: dissociation or inactivation of an ATPase inhibitory factor.

5. We conclude that "petite-positive" and "petite-negative" yeasts contain an ATPase inhibitor factor with properties similar to those of the bovine mitochondrial ATPase inhibitor. The expression of the ATPase inhibitor, measured by ATPase activation by heat, trypsin or high concentrations of dicyclohexylcarbodiimide, is sensitive to alterations of the hydrophobic membrane environment and dependent on both physiological state and genetic conditions of the yeast cells.

Abbreviation: DCCD, dicyclohexylcarbodiimide.

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INTRODUCTION

Treatment of beef-heart submitochondrial particles or of solubilized preparations of ATPase by moderate heat, trypsin or elution through Sephadex increase the ATPase activity [1–6]. These activation effects are usually interpreted as resulting from either an inactivation or a dissociation of a specific protein, the ATPase inhibitor first isolated by Pullman and Monroy [2]. Several suggestions have been made that this ATPase inhibitor displays some regulatory function either as a "respiratory-control factor" [2] or as a "directional regulator of the respiratory-chain-linked energy-transfer" [7]. Transition to an active form of the ATPase inhibitor is promoted in vitro by low pH and $Mg^{2+}ATP$ [6, 8] while ADP and coupled electron transport favor an inactive form [7, 8]. The physiological significance of these observations is still uncertain especially since the mitochondrial ATPase inhibitor has been demonstrated only in bovine heart and has not yet been reported in rat liver, yeast or any other mitochondria. The respiratory functions of yeast mitochondria can be easily modified by glucose repression and by genetic manipulations. It is therefore possible to study the activation of ATPase, as a possible index of the inhibitor activity, in yeast subjected to different physiological and genetical conditions. This paper compares activations of mitochondrial ATPase activity of rat liver mitochondria to that of two yeast species: *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* subjected or not to glucose repression and to hereditary respiratory deficiencies.

MATERIAL AND METHODS

Yeast strains and growth conditions

Schizosaccharomyces pombe 972h$^-$ is the wild strain from which the chromosomal pleiotropic respiratory-deficient mutants M126 [9, 10] and the chromosomal glucose-derepressed strain COB6, have been isolated [11]. *Saccharomyces cerevisiae* D261 is a diploid prototrophic strain.

The glycerol medium contains: 3.6 % (w/v) glycerol, 0.1 % (w/v) glucose, 2.0 % (w/v) yeast extract (Amber) and HCl to bring pH to 4.5. The glucose medium contains 5.8 % (w/v) glucose, 2.0 % (w/v) yeast extract and HCl to bring pH to 4.5. The ethanol medium contains: 3 % (v/v) ethanol, 0.1 % (w/v) glucose, 2.0 % (w/v) yeast extract (Difco), 0.2 % (w/v) ammonium sulfate, 0.2 % (w/v) KH_2PO_4, and HCl to bring pH to 5.8. Cultures were inoculated at 10^6 cells/ml, from an actively growing preculture, and grown aerobically for 40 h in glycerol medium, 20 h in glucose medium, or 16 h in ethanol medium at 30 °C.

Sonicated submitochondrial particles

Sonicated yeast submitochondrial particles were prepared as previously described [10]. The final pellet was suspended in 35 mM Tris/acetate, pH 8.3, 2 mM ATP, 1 mM EDTA at 10 mg protein per ml and frozen at −18 °C. Mitochondria isolated from rat liver (Sprague-Dawley) according to the procedure of Hogeboom et al. [12] were sonicated in 0.25 M sucrose, 10 mM Tris/acetate, pH 7.5, for 1 min at room temperature and centrifuged for 30 min at 100 000 × *g*. The pellet was washed and resuspended in 35 mM Tris/acetate, pH 8.3, 2 mM ATP, 1 mM EDTA at 8.3 mg protein per ml and frozen at −18 °C.

Triton-extracts

Triton-extracts were obtained by centrifugation at 115 000 × *g* for 20 min at 4 °C of yeast or rat liver submitochondrial particles suspended at 2 mg protein per ml in 35 mM Tris/acetate, pH 8.3, 2 mM ATP, 1 mM EDTA and 0.2 % (w/v) Triton-X 100. The supernatant is called the "Triton-extract". The remaining pellet, resuspended at 2 mg protein per ml in 35 mM Tris/acetate, pH 8.3, 2 mM ATP and 1 mM EDTA, is the "Triton-extracted pellet". When indicated, 1 ml of Triton-extract was put on top of an 11 ml linear sucrose gradient (10 to 25 %) containing 35 mM Tris-acetate, pH 8.3, 2 mM ATP, 1 mM EDTA, and centrifuged 3 h at 150 000 × *g* at 4 °C. The ATPase activity was measured in each fraction of 1.2 ml.

Heat treatments

Heat treatments were performed by incubations at 40 °C of either submitochondrial particles suspended at 2 mg protein per ml in 35 mM Tris/acetate, pH 8.3, 2 mM ATP, 1 mM EDTA or their Triton-extract obtained as described above. At the indicated times of incubation, aliquots were used immediately for ATPase assay and compared to the activity of the same sample measured before incubation at 40 °C.

ATPase

Mitochondrial ATPase (F1) was purified according to Goffeau et al. [10] from glycerol-grown *Schizosaccharomyces pombe*.

ATPase assays were performed at 30 °C for 8 min in a total volume of 1.0 ml containing: 0.3 M mannitol, 3 mM ATP, 20 mM Tris, 6 mM $MgCl_2$, 5 mM phosphoenolpyruvate, 40 μg pyruvate kinase (Sigma type II), 10 μl ethanol and when indicated, 80 μg oligomycin. The pH of the reaction mixture was brought to 8.6 with NaOH. Phosphate liberated was measured by the Fiske and SubbaRow method as described by Leloir and Cardini [13]. The ATPase specific activities are expressed in μmol phosphate liberated/min/mg protein. The "total ATPase" is measured in the absence of oligomycin. The "oligomycin-sensitive ATPase" is the fraction of "total ATPase" activity specifically inhibited by oligomycin. When indicated, oligomycin and dicyclohexyldiimide were added directly to the ATPase reaction mixture. Protein contents were measured according to Lowry et al. [14].

ATPase inhibitor

The following procedure modified from that of Horstman and Racker [6] for beef heart was carried out at 4 °C. A pH electrode was inserted in 30 ml of sonicated submitochondrial particles suspended in 4 mM Tris/acetate, pH 7.5, 1 mM ATP, 2 mM EDTA at 9 mg proteins per ml with magnetic stirring. The pH was brought to 11.5 with 1 M KOH. After 1 min, the pH was brought to 5.4 with 1 M acetic acid and quickly neutralized to pH 7.4 with 1 M KOH. The suspension was centrifuged for 10 min at 16 000 × *g*. 8.7 g of ammonium sulfate were added to the supernatant and further stirred for 20 min. After centrifugation for 10 min at 16 000 × *g*, the pellet was suspended in 30 ml of a solution of 500 mg ammonium sulfate per ml and centrifuged again for 10 min at 16 000 × *g*. The floating solid layer was collected and solubilized in 20 ml of 0.25 M sucrose. After addition of 10 ml of 50 % trichloroacetic acid and centrifugation for 10 min at 16 000 × *g*, the pellet was suspended in 6.0 ml of 10 mM

2-(N-morpholino) ethane sulfonic acid buffer pH 6.0, 0.5 mM ATP, 1 mM $MgCl_2$. The pH was brought to 5.0 with 1 M KOH and the particulate material was removed by centrifugation at 16 000 × *g* for 10 min. The supernatant was brought to pH 6.0 with 1 M KOH and frozen at −18 °C until use.

The ATPase inhibitor assay was carried in two steps. 5 µl of the peak fractions of the glycerol gradient, last step for purification of the mitochondrial ATPase (F1) of glycerol-grown *Schizosaccharomyces pombe* 972h⁻ [10], were first preincubated for 20 min at room temperature with 20 µl of the final inhibitor fraction at pH 6.0. This preincubation mixture contained about 2.5 to 4 µg of F1 (specific activity of 50 to 100) and 3 to 4 µg inhibitor. In a second step, the ATPase assay was carried out at pH 8.6 as previously described using 5 µl of the preincubated F1-inhibitor mixture as starting reagent.

RESULTS

Heat-activation of ATPase activity of glycerol-grown Schizosaccharomyces pombe

Schizosaccharomyces pombe is a "petite-negative" yeast from which stable chromosomal respiratory-deficient mutants are easily obtained [9–11, 15–18]. Fig 1A shows that treatment at 40 °C of submitochondrial particles of glycerol-grown *S. pombe*, stimulates markedly their ATPase activity. During the first 10 min of heat treatment, the increase of oligomycin-sensitive ATPase activity parallels that of the total ATPase activity and is thus not caused or accompanied by inactivation or dissociation of any of the several proteins and lipids necessary for oligomycin sensitivity. Treatment of the submitochondrial particles with 0.2% Triton X-100 extracts 59% of the proteins and 67 % of the ATPase activity of the glycerol-grown submitochondrial particles. Fig. 1B shows that the Triton-extracted ATPase activity is more stimulated by heat treatment than the corresponding membrane-bound activity. However in-

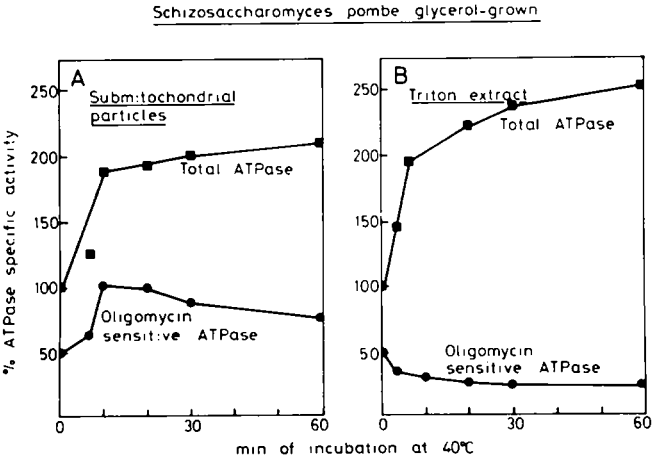

Fig. 1. Activation by heat of ATPase activity in submitochondrial particles and Triton-extracts of glycerol-grown *Schizosaccharomyces pombe*. The specific activities of the total ATPase of the untreated (100 %) submitochondrial particles (A) and Triton-extract (B) were 5.0 and 6.0, respectively.

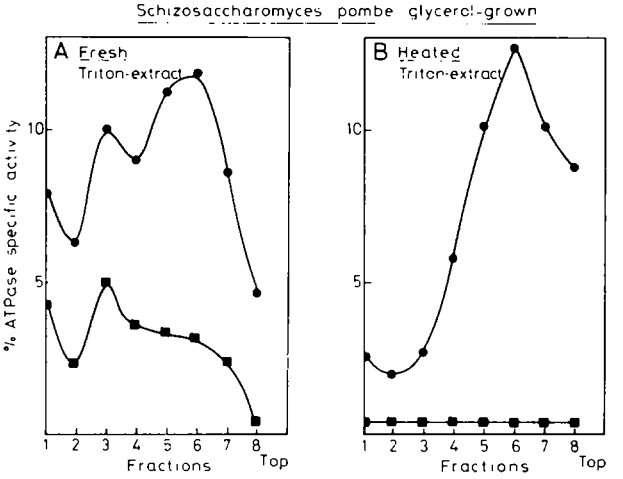

Fig. 2. Dissociation by heat treatment of the ATPase complex. The Triton-extracts from glycerol-grown *Schizosaccharomyces pombe*, freshly prepared (A) or heated 30 min at 40 °C (B) were centrifuged in sucrose density gradient as described in Methods. (● – ●): total ATPase specific activity; (■ – ■): oligomycin-sensitive ATPase specific activity.

hibition of oligomycin-sensitive ATPase is observed following the heat treatment. Under these conditions, dissociation by heat of the ATPase complex is demonstrated by centrifugation of the heated Triton-extract in a sucrose density gradient (Fig. 2). Two peaks of ATPase activities are distinguished in unheated Triton-extract (Fig. 2A). The heavier peak (fraction 3) is 50 % oligomycin-sensitive whereas only 26 % of the ATPase of the peak of lower density (fraction 6) is sensitive to oligomycin. The position of the latter peak coincides with that produced under similar conditions by highly purified F1. After treatment of the extract at 40 °C for 35 min and centrifugation for 3 h at 4 °C in the sucrose density gradient, the oligomycin-sensitivity is completely lost, the heavier peak is markedly decreased while the ATPase specific activity of the light peak is increased (Fig. 2B), indicating dissociation of the Triton-extracted oligomycin-sensitive ATPase complex.

Activations by trypsin

In order to determine whether the heat treatment of glycerol-grown *S. pombe* Triton-extract was modifying an ATPase inhibitor protein similar to that of beef-heart mitochondria, the effects of trypsin, another agent known to inactivate the bovine ATPase inhibitor was investigated [2, 3, 6]. Trypsin digestion of the Triton-extract of glycerol-grown *S. pombe* submitochondrial particles produces two effects (Fig. 3A). Low concentrations of trypsin inhibit 27 % of the ATPase activity in 10 min. This effect is not due to inhibition of the ATPase molecule itself, since F1 is not inhibited, but is slightly stimulated by trypsin. Loss of oligomycin-sensitivity parallels the decrease of total ATPase activity, indicating functional or physical dissociation of the ATPase complex. Increasing trypsin concentrations up to 60 μg/ml stimulates the total ATPase activity which in Fig. 3A passes from 73 % to 128 % of the untreated value. In the experiment of Table I, the stimulation of 62 %

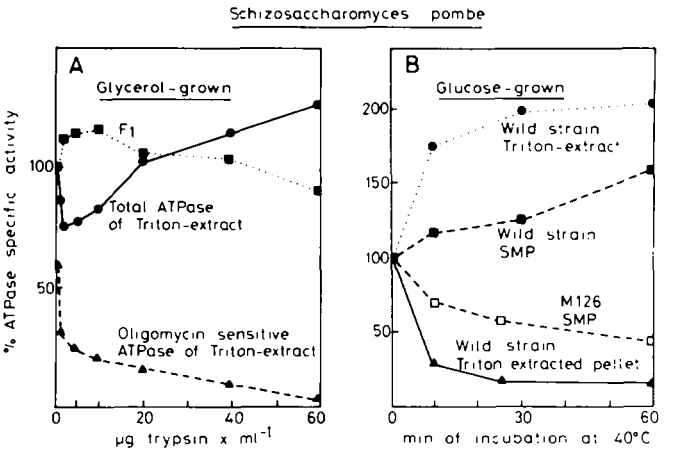

Fig. 3. (A) Trypsin digestion of purified ATPase (F1) and Triton-extract from glycerol-grown *Schizosaccharomyces pombe*. The total ATPase activities of the untreated samples (100 %) were: 82.0 (purified F1 ATPase) and 5.5 (Triton-extract). The digestion by the indicated trypsin concentrations were carried out as in Table I at the following mitochondrial protein concentrations: 0.2 mg per ml (F1), 1,2 mg per ml (Triton-extract). (B) Activation by heat of ATPase activities of the submitochondrial particles (SMP) and Triton-extract of glucose-grown *Schizosaccharomyces pombe*, wild strain and mutant M126. The total ATPase specific activities of the untreated fractions (100 %) were 3.8 (SMP, wild strain), 1.3 (SMP, M126), 2.8 (Triton-extract, wild strain) and 3.9 (Triton-extracted pellet, wild strain).

TABLE I

EFFECTS OF HEAT AND COLD TREATMENTS ON THE TRYPSIN STIMULATED ATPase ACTIVITY OF TRITON-EXTRACT OF GLYCEROL-GROWN *SCHIZOSACCHAROMYCES POMBE*

60 µg of trypsin are added to 1 ml of freshly prepared Triton-extract containing 2 mg protein, 2 mM ATP, 1 mM EDTA, 35 mM Tris/acetate, pH 8.3, and 0.2 % Triton X-100. After 10 min of incubation at 22 °C, the reaction is stopped by addition of 80 µg trypsin inhibitor and the ATPase activity is measured immediately or after incubation at 2 °C or 40 °C for 30 min.

Treatment	Total ATPase activity	
	Specific activity	%
None	5.8	100
60 µg trypsin	9.4	162
30 min at 2 °C	5.5	95
60 µg trypsin – 30 min at 2 °C	5.2	89
30 min at 40 °C	11.2	192
60 µg trypsin + 30 min at 40 °C	9.4	162

obtained by trypsin digestion of the Triton-extract was totally abolished by a subsequent cold treatment which in the absence of trypsin is ineffective. Since the beef heart ATPase inhibitor is trypsin sensitive, and since ATPase is protected against cold inactivation by the ATPase inhibitor [2, 6], our data suggest that in this yeast also, trypsin inactivates a similar ATPase inhibitor, In addition, Table I shows that

TABLE II

EFFECTS OF PURIFIED YEAST ATPase INHIBITORS ON MITOCHONDRIAL F1

F1 was purified from glycerol-grown *S. pombe* [10]. In Expt 1, 40 μg of inhibitor was incubated with 200 μg trypsin in 0.5 ml of 0.25 M sucrose, 20 mM Tris/sulfate pH 8.0, 2 mM EDTA, 4 mM ATP for 20 min at 20 °C. The reaction was stopped with 100 μg of soja trypsin inhibitor. The proteins were precipitated with 12.5% trichloroacetic acid, resuspended in 260 μl 10 mM 2-(*N*-morpholino)ethane sulfonic acid, pH 6.0, 0.5 mM ATP, 1 mM $MgCl_2$ and assayed as described in methods. Controls were carried out with no addition of trypsin and trypsin inhibitor or with the addition of trypsin inhibitor at the beginning of the preincubation. In Expt 2, the assays for inhibition were carried out as described in methods except that in one case the pH of the inhibitor solution was brought to 8.0 with HCl. In Expt 3, the inhibitors were purified from glycerol grown (Gly) or glucose-grown (Glu) *S. pombe* wild type or nuclear respiratory-deficient mutant M126 (10). When indicated, commercial baker's yeast (*S. cerevisiae*) was used.

Experiment	Preincubation conditions	ATPase specific activities (μmol $P_i \cdot min^{-1} \cdot mg^{-1}$)
1	1.5 μg F1	71
	+ 3.2 μg inhibitor from *S. pombe* Gly	12
	+ 3.2 μg trypsinized inhibitor	75
	+ 3.2 μg inhibitor + trypsin + trypsin inhibitor	9
2	2.6 μg F1 at pH 6.0	96
	+ 4.8 μg inhibitor from *S. pombe* Gly	15
	2.6 μg F1 at pH 8.0	81
	+ 4.8 μg inhibitor from *S. pombe* Gly	68
3	2.6 μg F1	93
	+ 4.8 μg inh from *S. pombe* Gly	14
	2.6 μg F1	99
	+ 4.0 μg inh from *S. pombe* Glu	3
	2.6 μg F1	127
	+ 4.0 μg inh from *S. pombe* M126	54
	2.6 μg F1	99
	+ 3.6 μg inh from *S. cerevisiae*	18

after trypsin digestion, further stimulation by heat is not obtained in yeast Triton-extract, as expected if indeed both heat and trypsin treatments were inactivating the same ATPase inhibitor.

Extraction of yeast ATPase inhibitor

The presence of an ATPase inhibitor in glycerol-grown *S. pombe* submitochondrial particles was directly demonstrated by its extraction and partial purification. Table II shows that ATPase inhibitor activity is present in a fraction obtained by alkaline extraction of *S. pombe* submitochondrial particles followed by differential ammonium sulfate and acid precipitations. The yeast inhibitor is similar to the one obtained from beef heart in its sensitivity to trypsin digestion and its requirement for acid pH during preincubation [2, 6]. The yeast inhibitor preparation exhibits a major component migrating slightly ahead of cytochrome *c* during sodium dodecyl-sulfate polyacrylamide gel electrophoresis.

The same extraction and purification procedures yield significant inhibitory activity not only from glycerol-grown *S. pombe* but also from glucose-grown *S. pombe* wild type, as well as from the pleiotropic nuclear respiratory-deficient mutant *S. pombe* M126 and the commercial baker's yeast, *Saccharomyces cerevisiae*. These data show the existence of a mitochondrial ATPase inhibitor in all tested yeasts. However the amount of ATPase inhibitor extracted and purified from different membranes can hardly be considered as a quantitative estimate of the inhibitor activity present in situ because of the uncertainties concerning the extraction and purification yields as well as the existence of the inhibitor in active or inactive states [2–8]. We have therefore attempted to compare the activation of the ATPase activities in different sonicated membranes as a possible global index of the ATPase inhibitor activity.

In order to allow comparison between the different membrane preparations, all data are expressed in per cent of the ATPase activity of untreated preparations.

Physiological and genetic modifications of ATPase activations

Treatment for 60 min at 40 °C is needed for 50 % stimulation of the total ATPase activity of submitochondrial particles isolated from glucose-grown *S. pombe* (Fig. 3B), whereas only a 10 min heat treatment causes an 85 % stimulation in glycerol-grown particles (Fig. 1A). However, when the ATPase complex is extracted by Triton, the relative activations by heat become similar in the two cases (Figs 1B and 3B). These data show that the ATPase inhibitory activities present in mitochondria of both glucose and glycerol-grown membranes are expressed differently. The ATPase inhibitor of glucose-grown submitochondrial particles is present in a form that requires pretreatment by Triton to be activated by heat. Although 0.2 % Triton extracts 72 % of the proteins and 35 % of the total ATPase activity of glucose-repressed submitochondrial particles, the remaining Triton-extracted particulate pellet still possesses oligomycin-sensitive ATPase of about the same specific activity as the unextracted membranes. This activity is inhibited by heat treatment (Fig. 3B). Such inhibition is not observed in the Triton-extracted pellet prepared from glycerol-grown cells where under identical conditions heat treatment stimulates the ATPase activity even after several successive Triton-extractions.

S. pombe M126 is a pleiotropic, respiratory-deficient, single-gene mutant of Mendelian heredity lacking the cytochrome aa_3 absorption peak and having oligomycin-insensitive ATPase activity [10]. Fig. 3B shows that the ATPase of glucose-grown M126 submitochondrial particles is not stimulated but inhibited by heat treatment like that of the Triton-extracted pellet of the glucose-grown *S. pombe* wild type.

Fig. 4 shows that the response to heat given by submitochondrial ATPase preparations from ethanol-grown *Saccharomyces cerevisiae* is very different of that of glucose-or glycerol-grown *Schizosaccharomyces pombe*. Stimulation by heat cannot be observed in submitochondrial particles (Fig. 4A) and a weak stimulation of only 23 % is observed in Triton-extracted ATPase (Fig. 4B). In addition, no stimulation by trypsin could be observed (Fig. 4C). In spite of previous unsuccessful attempts [19], Table II demonstrates that ATPase inhibitor can be extracted from *S. cerevisiae*. This inhibitor is thus either fully activated in untreated membranes or requires more drastic treatments than Triton and heat to be activated.

It is also of interest that the conditions which permit observation of ATPase

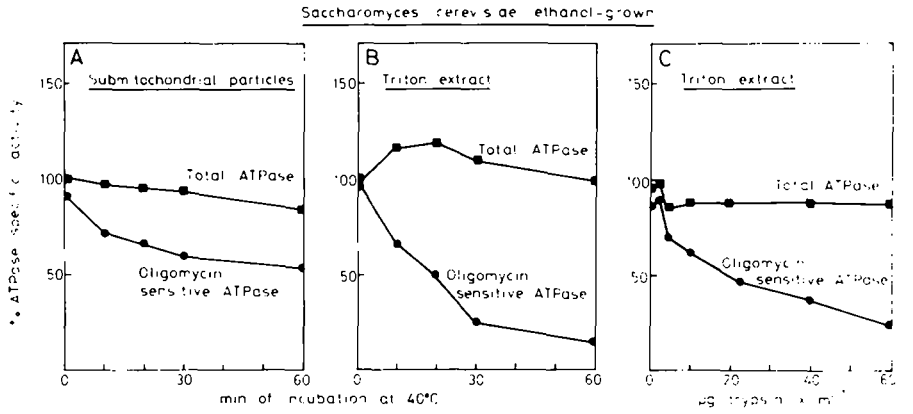

Fig. 4. Activation by heat and trypsin of ATPase activity in submitochondrial particles and Triton-extracts from ethanol-grown *Saccharomyces cerevisiae*. The total ATPase specific activities of the untreated samples (100 %), were: 2.5 (A SMP) and 2.8 (B and C Triton-extract). The trypsin treatment was carried out as in Fig. 3A except that 1.9 mg mitochondrial protein per ml was used.

activation in *S. pombe* are also effective in rat liver mitochondria, where no activation has been reported so far (Fig. 5). The stimulation is, however, less pronounced that that obtained in *S. pombe* and is barely increased by Triton-extraction (Fig. 5B compared to Fig. 5A).

Parallel activations by dicyclohexylcarbodiimide and by heat

That the variations of ATPase activation by heat in different membranes are not fortuitous, is indicated by parallel responses of ATPase activities of different membranes to two very different agents: high dicyclohexylcarbodiimide concentrations and heat.

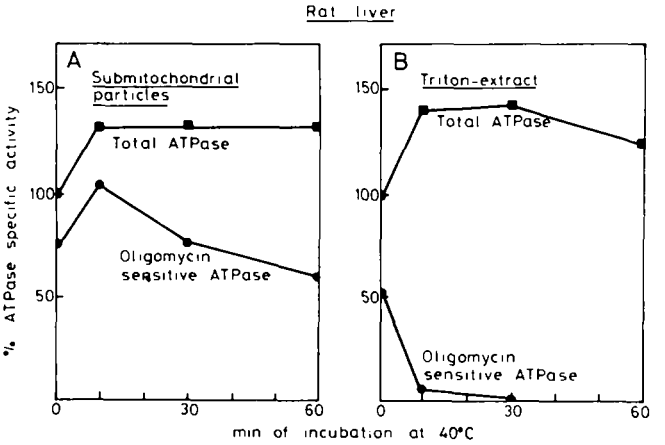

Fig. 5. Activation by heat of ATPase activity in rat liver submitochondrial particles and Triton-extract. The total ATPase specific activities of the untreated samples (100 %), were: 2.8 (A SMP) and 1.3 (B Triton-extract).

Low concentration of 1 to 5 nmol DCCD per mg protein inhibits the oligomycin-sensitive ATPase activities of beef-heart and rat liver mitochondria [4, 20–29] by binding covalently to a lipoprotein of 10 000 to 13 000 molecular weight. As expected, the ATPase activity of sonicated submitochondrial particles isolated from glycerol-grown *S. pombe* is about 50 % inhibited by 25 μM DCCD (1.25 μmol DCCD/mg protein) (Fig. 6A). We have, however, discovered that at higher DCCD concentrations, a gradual relief of inhibition is observed, and between 100 μM to 1000 μM DCCD the ATPase specific activity approaches the value observed in the absence of inhibitor. The effects of high DCCD concentration are more pronounced in the Triton-extract of the same particles where a net stimulation is produced, since at 250 μM DCCD, the ATPase specific activity is 25 to 50 % higher than that obtained in the absence of DCCD.

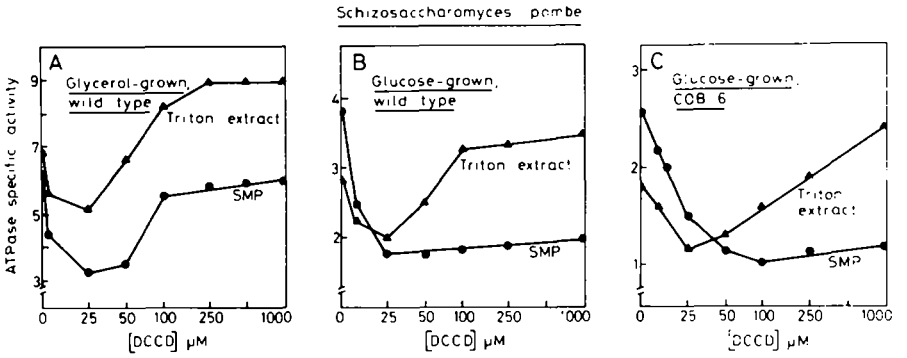

Fig. 6. Effects of DCCD on the total ATPase activity of submitochondrial particles and Triton-extract of *Schizosaccharomyces pombe*. The glycerol-grown (A) or glucose-grown (B) wild type was used as described in Methods. The glucose-derepressed strain *S. pombe* COB 6 (C) was grown for 30 h in the glucose medium.

The effects of high DCCD concentrations were also analyzed in submitochondrial particles and Triton-extracts prepared from glucose-grown wild type (Fig. 6B) and derepressed mutant COB6 (Fig. 6C) of *S. pombe*, the ethanol grown *Saccharomyces cerevisiae* (Fig. 7A) and from rat liver (Fig. 7B). The responses of ATPase activities to heat or high DCCD concentrations were parallel. Three types of responses could be distinguished.

1. *High sensitivity.* In this case, heat activation and relief of inhibition by high DCCD concentration are observed in both membranes and Triton-extracts (as in glycerol-grown *S. pombe*).

2. *Medium sensitivity.* Little or no effects of heat or high DCCD are obtained in submitochondrial particles but both agents stimulate in Triton-extracts (as in glucose-grown *S. pombe* wild type and COB6 and rat liver).

3. *Insensitivity.* No or little stimulation either in membranes or in Triton-extracts (ethanol-grown *S. cerevisiae*).

These data suggest that the target of high DCCD concentrations is similar to that of heat, most likely inactivation or dissociation of the ATPase inhibitor. This possibility is in agreement with the observation that the stimulation by high DCCD concentrations does not result from stimulation of F1 since the purified

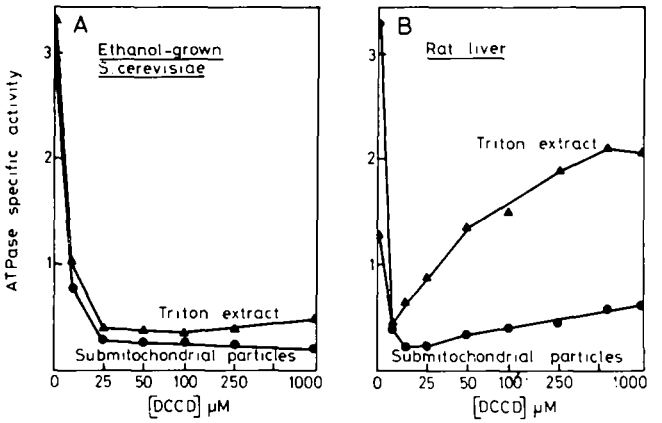

Fig. 7. Effects of DCCD on the total ATPase activity of submitochondrial particles and Triton-extract of ethanol-grown *Saccharomyces cerevisiae* (A) and rat liver (B).

mitochondrial enzyme is not sensitive to low or high DCCD concentrations. In addition, loss of the response to high DCCD concentrations can be obtained by previous ATPase activation by heat treatment at 40 °C for 20 min of sonicated submitochondrial particles of glycerol-grown *S. pombe* (Fig. 8A). This data indicates the existence of two distinct types of action for DCCD, since under these conditions the inhibition by low DCCD concentration is not modified and persists up to 1 mM DCCD.

Furthermore, high DCCD concentrations relieve the inhibition exerted by oligomycin in Triton-extracts of glycerol-grown *S. pombe* submitochondrial particles (Fig. 8B). This data suggests that high DCCD concentrations dissociate the catalytic

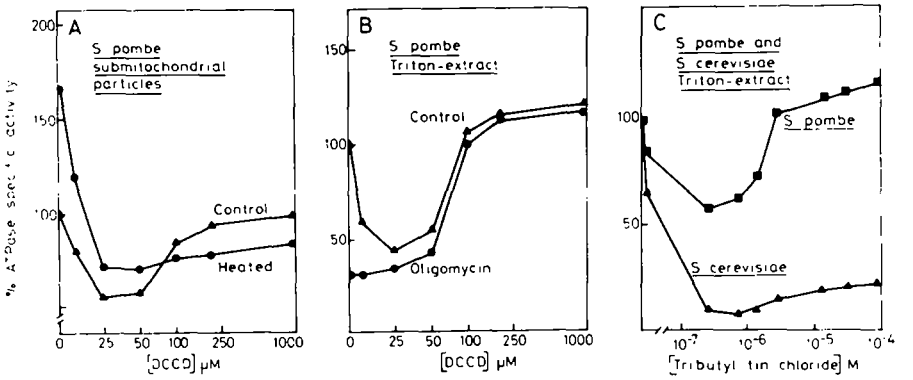

Fig. 8. Effects of DCCD and tributyl-tin chloride on the total ATPase activity of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. In A, the "heated" sample was pretreated at 40 °C for 20 min. 100 % represent a specific activity of 6.2. In B, 80 μg of oligomycin per ml were added in the reaction mixture of the "oligomycin" sample. 100 % represents a specific activity of 4.5. In C, the indicated concentrations of tributyltin chloride were added to the reaction mixture. 100 % represents specific activities of 6.0 for *S. pombe* and 3.0 for *S. cerevisiae*. All other conditions are indicated on the figure or described in Methods.

ATPase activity from the oligomycin-reactive sites of the Triton-extracted ATPase. Physical dissociation has been observed by slower rate of sedimentation through sucrose gradient of the ATPase activity of DCCD-treated Triton-extract. It is likely, however, that in submitochondrial particles, the functional uncoupling by DCCD of the catalytic and oligomycin-sensitive sites of ATPase is not accompanied by complete physical dissociation since after preincubation of sonicated particles from glycerol-grown cells with high DCCD concentration, no significant release of proteins could be detected in the supernatant fluid obtained after centrifugation for 30 min at $100\,000 \times g$.

The effects of high DCCD concentrations are reminiscent of the relief of inhibition of ATPase activity by high tributyltin chloride concentrations which has been observed in beef heart submitochondrial particles [30]. A similar response has been observed in Triton-extract of submitochondrial particles of glycerol-grown *S. pombe*. Concentrations up to $5 \cdot 10^{-7}$ M are inhibitory, but the inhibition is relieved at $5 \cdot 10^{-6}$ M tributyltin chloride (Fig. 8C). At still higher concentrations, F1 activity becomes inhibited. The effects of increasing tributyltin chloride concentrations parallel those of DCCD. Indeed, low inhibitor concentrations are more inhibitory in *S. cerevisiae* than in *S. pombe* preparations, but the inhibition is not relieved at high concentrations in *S. cerevisiae*.

Relationship between ATPase activations and decreased oligomycin sensitivity

As a general rule the extent of inhibition by low DCCD concentration is identical to that produced by oligomycin. An inverse relationship between inhibition by oligomycin or low DCCD concentrations and stimulation by heat or high DCCD concentrations appears throughout our data. When inhibition by oligomycin or low DCCD concentrations is very pronounced (more than 80 % of the total ATPase activity) heat stimulation and relief by high DCCD concentrations are usually not observed. Also, the increased ATPase activations after Triton extraction

TABLE III

SUPPRESSION BY AMMONIUM SULFATE TREATMENT OF THE STIMULATION BY HEAT OF THE ATPase ACTIVITY OF TRITON-EXTRACT OF GLYCEROL-GROWN *SCHIZOSACCHAROMYCES POMBE*

The ammonium sulfate treatment was carried out by addition of 1.12 g ammonium sulfate to 1 ml of fresh Triton-extract. The suspension was centrifuged for 15 min at $100\,000 \times g$, the solid, floating, upper layer was suspended in 0.5 ml of 35 mM Tris/acetate pH 8.3, 2 mM ATP, 1 mM EDTA and either used immediately for ATPase assay or further incubated at 40 °C for 30 min.

Treatments	Specific activities	
	Total ATPase	Oligomycin-sensitive ATPase
None	5.5	3.7
30 min at 40 °C	10.3	0.5
Ammonium sulfate	7.5	5.1
Ammonium sulfate +30 min at 40 °C	5.7	2.8

are generally accompanied by some decrease of inhibition by oligomycin or low DCCD concentrations (see, for instance, Figs 1B, 5B, 6 and 7B). However, the data of Fig. 1A exclude the possibility that a heat-activable form of the ATPase inhibitor is specifically required for the integrity of the oligomycin-sensitive complex. In fact, increased oligomycin-sensitive ATPase can be observed after short term heat activation of submitochondrial particles of glycerol-grown *S. pombe*. The data of Table III also demonstrate that ATPase activation by heat and loss of oligomycin-sensitivity are not necessarily related, since treatment of glycerol-grown *S. pombe* Triton-extract by high concentrations of ammonium sulfate (80 % saturation) abolishes the stimulation by heat without causing dissociation of the oligomycin-sensitive ATPase complex. Actually, ammonium sulfate treatment not only increases the oligomycin-sensitive ATPase specific activity but partly prevents the loss of oligomycin-sensitivity induced by heat.

DISCUSSION

Our data demonstrate that under appropriate conditions, activations of the ATPase activity by heat, trypsin or high DCCD concentrations are observed in *S. pombe* mitochondrial fractions. These activations are most probably the result of dissociation or inactivation of the ATPase inhibitory factor that we have extracted and greatly purified from yeast mitochondrial membranes. This inhibitor is of a protein nature, since it is precipitated by ammonium sulfate, trichloroacetic acid and inactivated by trypsin. It is active at pH 6.0 but not at pH 8.0. It protects the ATPase activity against cold-inactivation. Its molecular weight is slightly less than that of cytochrome *c*. Similar properties have previously been reported for the bovine heart mitochondrial ATPase inhibitor [2, 6].

The ATPase inhibitory factor has been extracted not only in glycerol-grown *S. pombe* wild type but also in glucose-grown *S. pombe* wild type, the pleiotropic nuclear respiratory-deficient mutant *S. pombe* M126 and ethanol-grown *S. cerevisiae*. The activity of the ATPase inhibitor seems also to be reflected by the activations of the ATPase activity. Parallel ATPase activations are obtained by trypsin, heat treatment or high DCCD concentrations in different membranes. All three stimulations are increased by Triton-treatment and none is observed with the purified F1. They are manipulated similarly by physiological or genetic conditions. They are barely observed in ethanol-grown *S. cerevisiae* and glucose-grown *S. pombe* M126. They are obtained in Triton-extracts, but are low or absent in submitochondrial particles of glucose-grown *S. pombe* wild type and derepressed mutant COB6. They are prominent in glycerol-grown *S. pombe*. Furthermore, the stimulations are not additive; heat-activation prevents further stimulation by DCCD. Therefore it may be suggested that trypsin, heat or DCCD activations result all from a physical or functional uncoupling of the ATPase catalytic site from the ATPase inhibitor. The extent of this uncoupling seems to be dependent partly on the hydrophobicity of the association of the ATPase inhibitor in the ATPase complex since it is generally favored by previous treatment with Triton-X 100. It becomes apparent therefore that different activations do not necessarily reflect different inhibitor content but depend also on constraints exerted by the membrane and/or of the functional state of the inhibitor itself.

The use of yeast mutants has permitted us to draw two additional conclusions. In membranes obtained from glucose-grown *S. pombe*, the ATPase inhibitor is no longer inactivated unless pretreated by detergent. However, since the glucose-grown strain *S. pombe* COB6 which is not submitted to glucose repression [11] reacts like the glucose-grown wild type, one can exclude the possibility that the lower stimulations observed by DCCD, heat or trypsin, in glucose-grown compared to glycerol-grown mitochondria are specifically produced by glucose repression of mitochondrial development. In addition it must be noted that the extraction of the ATPase inhibitor, in spite of the absence of ATPase activation, in the nuclear pleiotropic respiratory-deficient mutant *S. pombe* M126 indicates that the membrane-bound Dio-9 sensitive ATPase which is detected in this mutant [10] contains the ATPase inhibitor in a form which is not activated by heat, trypsin or DCCD even after Triton-treatment. The loss of oligomycin-sensitivity in this mutant can thus not be related to an absence of ATPase inhibitor.

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