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ATPase INHIBITOR FROM YEAST MITOCHONDRIA. PURIFICATION AND PROPERTIES

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SUMMARY

1. Mitochondria from *Candida utilis* CBS 1516 and *Saccharomyces cerevisiae* JB 65 possess an ATPase-inhibitor activity. The inhibitor activity depends on the growth conditions of the yeast cells. It is markedly decreased when the cells are grown in the presence of a high concentration of glucose, which suggests that glucose represses the synthesis of the ATPase inhibitor or of a protein required for the insertion of the inhibitor into the inner mitochondrial membrane.

2. The ATPase inhibitor has been isolated from *C. utilis* mitochondria and purified to homogeneity. The minimal molecular weight calculated from amino acid composition is close to 7500. Determination of the molecular weight by sodium dodecylsulfate-polyacrylamide gel electrophoresis gives a value close to 6000.

3. The ATPase inhibitor of *C. utilis* mitochondria differs from the beef heart ATPase inhibitor by a number of properties. It has a lower molecular weight (6000–7500 vs 10500), a different amino acid composition, and a more acidic isoelectric point 5,6 vs 7,6). In spite of these differences, the *C. utilis* inhibitor cross-reacts with the ATPase of beef heart submitochondrial inhibitor-depleted particles.

4. The interaction of the *C. utilis* inhibitor with the ATPase of inhibitor-depleted particles requires the addition of Mg^{2+}-ATP or ATP in the incubation medium.

5. ^{14}C labelling of the *C. utilis* inhibitor has been achieved by growing *C. utilis* in a medium supplemented with [^{14}C]leucine. It has been found by titration experiments that the *C. utilis* ^{14}C-labelled inhibitor binds to the homologous submitochondrial inhibitor-depleted particles with a K_D of about 10^{-7} M. The number of binding sites is of the order of 0.1 nmol/mg protein.

INTRODUCTION

A low-molecular-weight protein able to inhibit noncompetitively the mitochondrial ATPase has been characterized in beef heart mitochondria [1–8]. This protein, referred to as natural ATPase inhibitor, is heat-stable and very sensitive to

Abbreviation: HEPES: hydroxyethylpiperazine ethane sulfonic acid.

trypsin digestion. The ATPase inhibitor inhibits not only the ATP hydrolysis, but also all the mitochondrial energy-linked reactions driven by ATP. Ernster et al. [3] and Van de Stadt et al. [4, 5] have studied the equilibrium between the mitochondrial ATPase and its natural inhibitor in various metabolic conditions and their results suggest that the ATPase inhibitor exerts a regulatory function during energy conservation. An ATPase inhibitor has also been isolated from chloroplasts [9] which inhibits specifically chloroplast ATPase.

The use of yeast offers specific advantages in the study of the mechanism of interaction of the ATPase inhibitor with the mitochondrial ATPase since phenotypic variations which may alter the ATPase inhibitor can be obtained easily by environmental changes or mutations. Also a radioactive ATPase inhibitor can be prepared by growing the cells in the presence of a labelled amino acid.

In this paper, we describe the isolation and some properties of an ATPase inhibitor form the mitochondria of the yeast *Candida utilis*. An abstract of this work has been previously published [10] and similar data on *Saccharomyces cerevisiae* have also been reported at the same time in an abstract form and independently [11].

EXPERIMENTAL PROCEDURE

Culture and preparation of C. utilis mitochondria

General conditions were described previously [12]. *C. utilis* (strain CBS 1516) was grown in a semi-synthetic liquid medium [13] containing "Difco" yeast extract (0.5 %), $(NH_4)_2SO_4$ (0.12 %), NaCl (0.05 %), $MgCl_2$ (0.07 %), KH_2PO_4 (0.1 %), $CaCl_2$ (0.01 %), $FeCl_3$ (0.005 %) and glycerol (3 %) as carbon source. The cells were harvested after 36 h of growth (late stationary phase). Labelling of the proteins was achieved by addition of [^{14}C]leucine to the above medium. After a 12-h period of growth, [^{14}C]leucine (400 μCi/l) dissolved in sterile water was added to the culture. At the end of the stationary phase (36 h) more than 80 % of the radioactivity was incorporated in the cells. Mitochondria were isolated after mechanical disruption of yeast cells according to the method of Balcavage and Mattoon [14] with minor modifications [12].

Materials

L-[^{14}C]Leucine (54 Ci/mol) was obtained from "Commissariat à l'Energie Atomique" (Saclay).

Beef heart mitochondria [15], submitochondrial inhibitor-depleted particles [16] and beef heart mitochondrial inhibitor [6] were prepared according to published procedures.

Standard assay of ATPase inhibitor activity

To assay the ATPase inhibitor activity of yeast mitochondrial extracts, mitochondrial particles depleted of ATPase inhibitor (inhibitor-depleted particles) were used. The assay was performed as described by Horstman and Racker [6] with the following modifications. Beef heart inhibitor-depleted particles (0.1 mg protein, specific ATPase activity 2–3 μmol P_i released/min per mg protein) were preincubated for 15 min at 30 °C with increasing amounts of ATPase inhibitor in the presence of 0.25 M sucrose, 2 mM morpholino-propane sulfonic acid, 0.5 mM ATP, 0.5 mM

$MgSO_4$. ATP was always added as the last component. The volume was adjusted to 0.5 ml and final pH was 6.8.

The incubation was initiated by addition of 0.5 ml of an ATPase assay mixture containing 0.1 M Tris · SO_4, 20 mM ATP, 10 mM $MgSO_4$, 20 mM phosphoenolpyruvate, 30 μg pyruvate kinase (Boehringer Mannheim), final pH 7.8. It was carried out for 10 min at 30 °C and terminated with 0.25 ml of 2.5 M $HClO_4$. After another 10-min period at 0 °C, denatured proteins were centrifuged for 3 min at $8000 \times g$ and inorganic phosphate was determined on 0.2-ml aliquots by the method of Fiske and Subbarow [17] except when the ATPase inhibitor activity was assayed on fractions obtained by isoelectric focusing; in this case the ampholine material was found to interfere with the Fiske and Subbarow assay and therefore phosphate was determined by the method of Nielsen and Lehninger [18]. The yellow phosphomolybdate complex was extracted in isobutanol-benzene and its absorbance read at 420 nm.

One unit of ATPase inhibitor activity is defined [6] as the amount of protein required to produce a 50 % inhibition of the ATPase activity in beef heart mitochondrial particles, under the specified assay conditions.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis

Electrophoresis in the presence of sodium dodecylsulfate for determination of molecular weights was carried out on 10 % polyacrylamide gel in a 0.1 M sodium phosphate buffer, pH 7.6, according to Weber and Osborn [19]. The amount of protein layered on the gel was of the order of 10 μg. Marker proteins were treated in parallel. The following reference values were used: soybean inhibitor, M_r 20 100, cytochrome *c*, M_r 12 400, CNBr-cleavage products of cytochrome *c*, Peptides I and II M_r 7760 and 2780, insulin chains, M_r 2900, bacitracin, M_r 1450. The gels were stained with Coomassie Blue and destaining was accomplished by diffusion with repeated changes of destaining solution. In experiments where the ATPase inhibitor was eluted from the gels for assaying the activity, phosphate buffer was replaced by 0.1 M NaCl, 50 mM Tris · HCl, pH 7.8.

Isolelectric focusing

A discontinuous 0–45 % sucrose gradient containing 1 % (w/v) ampholine, pH 3–10, was used in a LKB 110-ml column at 4 °C [20]. The cathode was at the bottom of the column and the protein solution was applied in the middle of the ampholine column. Electrophoresis was performed at 400 V for the first 15 h and 800 V for the further 30 h. Upon completion of electrophoresis, 2-ml fractions were collected and assayed for pH, protein content and ATPase-inhibitor activity.

Protein estimation

The biuret method of Gornall et al. [21] or the method of Zack and Cohen [22] with the Folin reagent were used with bovine serum albumin as a standard.

RESULTS

Unmasking of mitochondrial ATPase in submitochondrial particles from C. utilis

In submitochondrial particles from beef heart, the ATPase activity is masked, due to an interaction with its specific inhibitor. It is unmasked either by selective

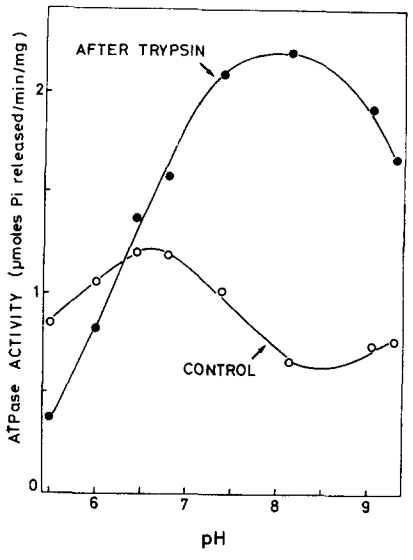

Fig. 1. Effect of trypsin on the ATPase activity of *C. utilis* submitochondrial particles. *C. utilis* mitochondria (10 mg/ml) suspended in 0.63 M mannitol, 2 mM morpholino-propane sulfonic acid pH 6.8, were sonicated with a Branson sonifier at 0 °C for four 30-s periods separated by 1-min intervals. The suspension was incubated with trypsin (1 μg/mg protein) for 45 min at 30 °C and the reaction was stopped by addition of soybean trypsin inhibitor (3 μg/μg trypsin). In the control, trypsin inhibitor was added before trypsin. ATPase activity was measured with 0.1 mg protein in 0.5 ml of the following medium: 10 mM ATP, 5 mM $MgSO_4$, 10 mM phosphoenolpyruvate, 30 μg pyruvate kinase, 0.1 M Tris/morpholino-ethane sulfonic acid buffer at the indicated pH. After 10 min at 30 °C, incubation was stopped by 0.1 ml of 2.5 M $HClO_4$ and inorganic phosphate was measured as described under Experimental Procedure.

degradation of the bound ATPase inhibitor by trypsin [23] or by removal of the inhibitor by Sephadex treatment (inhibitor-depleted particles) [16].

The trypsin-induced activation of the mitochondrial ATPase activity has been used to test the presence of an ATPase inhibitor in *C. utilis* mitochondria. The ATPase of yeast submitochondrial particles was measured as a function of pH, before and after the addition of trypsin. As shown in Fig. 1 treatment by trypsin stimulated 2- to 3-fold the ATPase activity at alkaline pH. This result was indicative of the presence of an ATPase inhibitor in *C. utilis* mitochondria.

The ATPase activity of *C. utilis* submitochondrial particles can also be increased 2- to 4-fold by a passage of the particles on a Sephadex column (inhibitor-depleted particles). Tzagoloff [24] reported a similar observation for *S. cerevisiae* mitochondria, but he did not relate this effect to the removal of an ATPase inhibitor.

Effect of growth conditions on the content of C. utilis mitochondrial in ATPase

The presence of an ATPase-inhibitor activity in extracts of *C. utilis* mitochondria is shown in Fig. 2. The ATPase inhibitor was extracted from mitochondria by heat treatment at 90 °C for 5 min followed by centrifugation at $100\,000 \times g$ for 15 min and the activity of the inhibitor was measured in the extract. In order to verify whether glucose repression could affect the synthesis of the mitochondrial ATPase inhibitor, a 3 % glycerol medium and a 5 % glucose medium were used for growing the

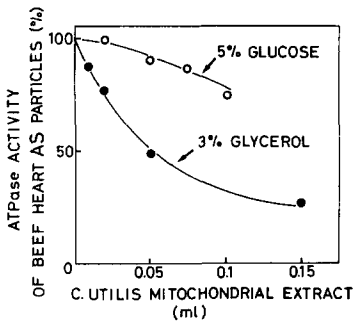

Fig. 2. Effect of growth conditions on the activity of the ATPase inhibitor in *C. utilis* mitochondria. *C. utilis* mitochondria (30 mg protein) isolated from cells grown in the presence of 3 % glycerol or 5 % glucose suspended in 0.63 M mannitol, 2 mM morpholino-propane sulfonic acid, pH 6.8, in a final volume of 1.5 ml. After heating for 5 min at 90 °C, the suspension was cooled at 0 °C and centrifuged at 25 000 × *g* for 15 min. Increasing amounts of the resulting supernatant were assayed for ATPase inhibitor activity, using beef heart inhibitor-depleted particles as described under Experimental Procedure.

cells. A constant amount of beef heart inhibitor-depleted particles was incubated with increasing amounts of an extract of *C. utilis* mitochondria and the inhibitory effect of the mitochondrial extract on the ATPase activity of the inhibitor-depleted particles was measured.

It was observed that *C. utilis* mitochondria contain a measurable ATPase inhibitor activity which was approximately 4-fold lower when the cells were grown in the presence of glucose than in the presence of glycerol (Fig. 2). This suggests that growth of *C. utilis* under conditions of glucose repression results in a decrease of the synthesis of the ATPase inhibitor or of the insertion of the inhibitor into the inner mitochondrial membrane. As shown in Fig. 2 and in Table I approximately 1 unit of ATPase inhibitor can be extracted from 1 mg of mitochondrial protein when *C. utilis* is grown with glycerol as carbon source. *S. cerevisiae* mitochondria also contain an

TABLE I

For details cf. Results.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield %	Specific radioactivity (dpm/μg)
Mitochondria	4940	—	—	—	—
1. Alkaline extract	1180	5040	4.3	100	120
2. $(NH_4)_2SO_4$ precipitate	238	6240	26	124	96
3. Trichloroacetic acid precipitate	64	3245	51	65	40
4. DEAE-cellulose	3.9	1940	500	39	135
5. Isoelectric focusing	1.6	974	630	19	114

ATPase inhibitor. Using *S. cerevisiae* JB 65, we found that the inhibitor activity was decreased by glucose repression like in *C. utilis*.

We have also measured the amount of ATPase inhibitor in the cytosol of *C. utilis* cells. Post-mitochondrial supernatant was first centrifuged for 2 h at 100 000 $\times g$ to sediment the mitochondrial fragments. Proteins were then precipitated with 10 % trichloroacetic acid and the pellet was extracted at pH 5 exactly as described in the purification procedure of the ATPase inhibitor from *C. utilis* mitochondria, Step 3 (see the following section). We found that about one unit of ATPase inhibitor can be extracted from 2 mg of the original cytosol protein. The presence of a significant ATPase-inhibitor activity in the post-mitochondrial supernatant is likely due to the release of loosely bound ATPase inhibitor from mitochondria.

Purification of ATPase inhibitor

Previously published procedures for the purification of ATPase inhibitor from beef heart mitochondria [1, 6] proved ineffective when used as such with *C. utilis* mitochondria. The conditions of Horstman and Racker [6] were followed up to Step 3 with slight modifications. For convenience the whole procedure is detailed thereafter and results of a typical experiment are reported in Table I. All the purification steps were performed at 0–4 °C.

Step 1. Alkaline extraction of mitochondria. 5 g of *C. utilis* mitochondria were suspended in 250 ml of 0.25 M sucrose containing 5 mM Tris · SO_4 and 2 mM EDTA, pH 8.0. While rapid stirring was maintained, 1 M KOH was added dropwise to bring the pH up to 11.8–12.0. After 1 min of contact at alkaline pH, 10 M acetic acid was added to lower the pH to 8 and after a further 2-min period, 1 M acetic acid was added to bring the pH to 5.4. After 1 min at pH 5.4 the pH was finally brought to 7.4 with 1 M KOH and the suspension was centrifuged at 100 000 $\times g$ for 15 min. The clear yellow supernatant was saved and the pellet was discarded.

Step 2. $(NH_4)_2SO_4$ precipitation. $(NH_4)_2SO_4$ (0.29 g/ml) was added to the supernatant from Step 1 and the solution stirred for 30 min. The precipitate was removed by centrifugation at 20 000 $\times g$ for 10 min. More $(NH_4)_2SO_4$ (0.194 g/ml) was then added to the corresponding supernatant. After 1 h the suspension was centrifuged for 10 min at 20 000 $\times g$ and the pellet was dissolved in 0.25 M sucrose, 2 mM Tris · HCl, pH 7.4, to a concentration of about 10 mg protein/ml.

Step 3. Trichloroacetic acid precipitation. Concentrated trichloroacetic acid was added dropwise to the solution from Step 2 to a final concentration of 10 % and the suspension was immediately centrifuged at 20 000 $\times g$ for 5 min. The supernatant was discarded; the pellet was homogenized in distilled water and adjusted to pH 5.0 with 1 m KOH. The mixture was centrifuged at 20 000 $\times g$ for 10 min. The pellet was discarded and the supernatant was dialyzed overnight against 100 vol. of 30 mM hydroxyethylpiperazine ethane sulfonic acid (HEPES), 75 mM sucrose, 2 mM EDTA, pH 7.0 (Buffer A).

Step 4. DEAE-cellulose chromatography. Dithioerythritol was added to a final concentration of 1 mM to the dialyzed fraction from the preceding step, and the fraction was applied to a column of DEAE-cellulose (Whatman DE 52, 10 cm $\times$ 2 cm) equilibrated with Buffer A. The column was eluted with 200 ml of a linear NaCl gradient (0 to 0.2 M) in Buffer A containing 1 mM dithioerythritol and the salt concentration was followed by conductivity. The ATPase inhibitor was eluted for a

salt concentration corresponding to 50 mM NaCl (Fig. 3). Trichloroacetic acid was added to the pooled fractions to a final concentration of 10 %. The precipitate was immediately centrifuged for 5 min at 140 000 × *g*. The pellet was dissolved in 0.25 M sucrose, 2 mM Tris · HCl and the pH was adjusted to 7.4 with 1 M KOH.

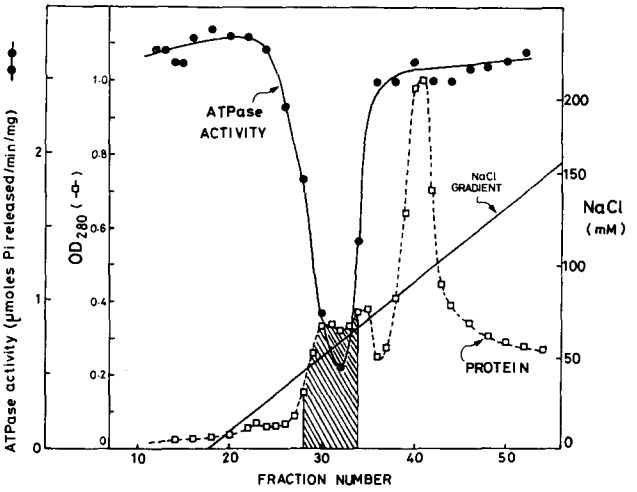

Fig. 3. Purification of *C. utilis* ATPase inhibitor. DEAE-cellulose chromatography. ATPase inhibitor (Step 3) was applied to a DEAE-cellulose column and eluted at a rate of 1 ml/min as described in the text. Fractions (2.7 ml) were analyzed for ATPase inhibitor activity (●—●), absorbance at 280 nm (□- - -□) and NaCl concentration (—) as deduced from conductivity measurements. The hatched area indicates fractions pooled for further purification.

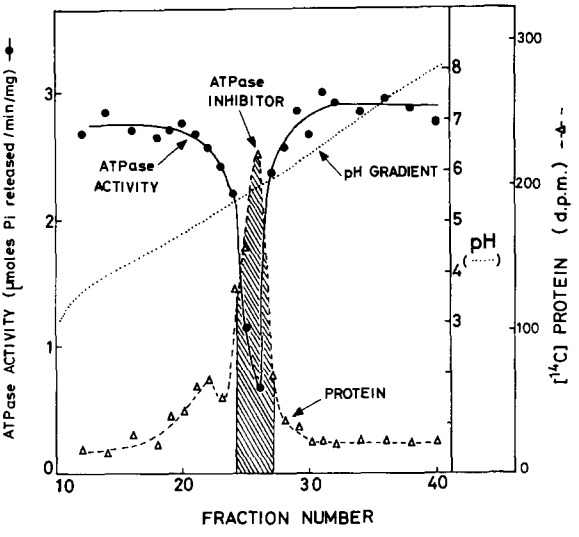

Fig. 4. Purification of *C. utilis* ATPase inhibitor. Isoelectric focusing. Isoelectric focusing was carried out as described in the text. Upon completion, 2-ml fractions were eluted. The pH of each fraction was recorded with a glass electrode (. . .), radioactivity of the protein was measured by liquid scintillation counting (△- - -△) and ATPase-inhibitor activity was measured on aliquots after addition of an equal volume of 1 M morpholino-propane sulfonic acid buffer, pH 6.8 (●—●).

Step 5. Isoelectric focusing. Electrofocusing was the final purification step. An electrophoretic profile is presented in Fig. 4. The isoelectric point of *C. utilis* ATPase inhibitor was pH 5.6. Contaminant proteins with isoelectric points around and below pH 5 could be eliminated during this step. The recovery of the ATPase inhibitor together with the removal of ampholine material was obtained by trichloroacetic acid precipitation. Trichloroacetic acid was added to a final concentration of 10 % to the pooled active fractions and the suspension was immediately centrifuged at 140 000 × *g* for 5 min. The pellet was dissolved in a minimal volume of water and neutralized to pH 7.0 with 1 M KOH. To ensure complete removal of ampholine, precipitation by trichloroacetic acid was repeated once again.

Comments on the purification procedure

The purification factor relative to the alkaline extract of *C. utilis* was in the range of 150–200 and the yield about 20 %. The specific radioactivity of proteins was not constant during the purification procedure, which indicates a variation in the mean leucine content of the mitochondrial proteins.

Electrofocusing was found necessary to remove low-molecular-weight contaminants. The purified inhibitor obtained after electrofocusing yielded only one band in sodium dodecylsulfate-polyacrylamide gel electrophoresis. At this stage, if a duplicate unstained acrylamide gel was sliced and eluted in the presence of bovine serum albumin to trap sodium dodecylsulfate, the eluted ATPase-inhibitor activity was found entirely at the level of the stained band (Fig. 5). When dithioerythritol and

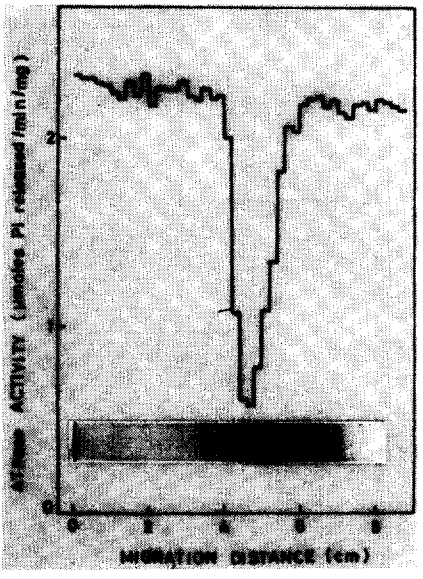

Fig. 5. Sodium-dodecylsulfate-polyacrylamide gel electrophoresis of ATPase inhibitor. Electrophoresis on 10 % polyacrylamide gels was run in duplicate, as described under Experimental Procedure. One gel was stained with Coomassie Blue and the other was cut in 2-mm slices which were immersed into 0.25 ml of a medium made of 0.25 M sucrose, 2 mM morpholino-propane sulfonic acid, 5 mM dithioerythritol, pH 6.8 and 2 mg bovine serum albumin. Slices were soaked overnight at 4 °C and the ATPase-inhibitor activity was measured as described in the standard assay.

mercaptoethanol were omitted during the purification procedure and prior to gel electrophoresis, respectively, a minor supplementary band with a molecular weight approximately double of that found in the presence of dithioerythritol was detected in the acrylamide gel. This band may correspond to a dimer of the ATPase inhibitor.

Estimation of molecular weight

An estimate of the molecular weight of purified *C. utilis* mitochondrial ATPase inhibitor was made by electrophoresis on 10 % polyacrylamide gels containing 0.1 % sodium dodecylsulfate according to the method of Weber and Osborn [19]. The protein and peptides were located by staining with Coomassie Blue. ATPase inhibitor was coelectrophoresed with various proteins and peptides standards and by comparison with migration distances of standards, its molecular weight was found in the neighbourhood of 6000 (Fig. 6).

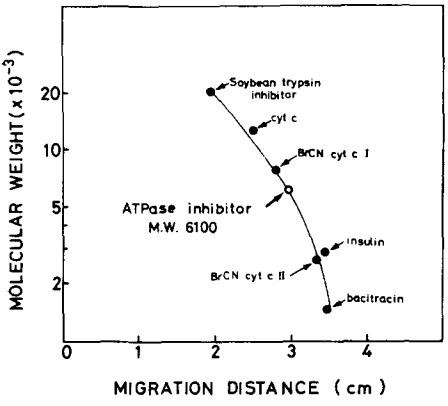

Fig. 6. Molecular-weight determination of *C. utilis* ATPase inhibitor by sodium dodecylsulfate-polyacrylamide gel electrophoresis. *C. utilis* ATPase inhibitor (10 μg) was submitted to co-electrophoresis with indicated standards (5 μg) as described in Experimental procedure. Samples were treated prior to electrophoresis by 1 % sodium dodecylsulfate and 1 % 2-mercaptoethanol for 20 min at 25 °C.

Amino acid analysis

Amino acid analysis was performed on *C. utilis* mitochondrial ATPase inhibitor and compared to the amino acid analysis made on the beef heart ATPase inhibitor. The amino acid composition shown in Table II is given in terms of nearest integral number of amino acid residues per mol of protein. Similarly to beef heart ATPase inhibitor, *C. utilis* ATPase inhibitor lacks methionine. In contrast to beef heart ATPase inhibitor, it contains threonine and proline. Its high leucine content (about 7 residues per mol) is favourable for the biosynthetic labelling obtained by growing the cells in the presence of [^{14}C]leucine. The isoelectric point of pH 5.6 (Fig. 4) is in agreement with the large number of glutamic and aspartic acid residues. The minimal molecular weight is close to 7500. Both data from amino acid analysis and sodium dodecylsulfate-acrylamide gel electrophoresis (Fig. 6) indicate that the ATPase inhibitor of *C. utilis* has a molecular weight lower than that of beef heart.

TABLE II

AMINO ACID COMPOSITION OF ATPase INHIBITORS

Amino acids	*C. utilis* ATPase inhibitor*			Beef heart ATPase inhibitor**
	Residues (mol)		Extrapolated integer	
	18 h	48 h		
Asp	7.25	7.90	8	8
Thr	4.80	4.40	5	—
Ser	3.10	3.00	3	6
Glu	12.95	13.00	13	20
Pro	0.70	0.85	1	—
Gly	3.20	3.30	3	6
Ala	5.90	6.00	6	11
Val	0.80	0.85	1	2
Met	—	—	—	—
Ile	1.70	2.10	2	4
Leu	6.80	6.80	7	5
Tyr	1.30	1.30	1–2	1
Phe	1.80	1.90	2	2
Lys	9.10	10.10	10	11
His	1.05	1.00	1	6
Arg	2.50	3.00	3	9
Cys***	—	—	—	—
Total number of amino acids			66–67	91
Minimal molecular weight			7500	10 500

* Analysis kindly performed by Dr J. Jollès on samples hydrolyzed for the indicated number of hours. Tryptophan not estimated.

** Values calculated from the data of Brooks and Senior [8].

*** Determined as cysteic acid after performic acid oxidation.

ATP requirement for the action of ATPase inhibitor

Horstman and Racker [6] have first demonstrated that Mg^{2+}-ATP was required to obtain a maximal inhibitory effect of the ATPase inhibitor on mitochondrial ATPase. *C. utilis* inhibitor-depleted particles were preincubated with the homologous ATPase inhibitor in 0.25 M sucrose, 2 mM morpholinopropane sulfonic acid, pH 6.5 (control medium) and in the same medium supplemented either with 0.05 mM Mg^{2+} (Mg^{2+} medium) or with 0.05 mM ATP (ATP medium) or with 0.05 mM Mg^{2+}-ATP (Mg^{2+}-ATP medium). After preincubation, the ATPase activity was measured at pH 7.8. Little inhibition of ATPase activity was obtained after preincubation in the control medium or in the Mg^{2+} medium; this contrasts with the marked inhibition obtained after preincubation with Mg^{2+}-ATP or with ATP (Fig. 7).

Cross-reactions of beef heart and C. utilis ATPase inhibitors with submitochondrial particles

The specificity and the potency of *C. utilis* and beef heart mitochondrial ATPase inhibitors was measured towards submitochondrial inhibitor-depleted parti-

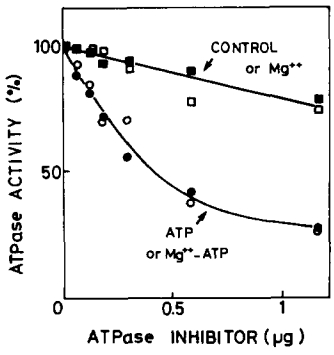

Fig. 7. ATP requirement for the action of ATPase inhibitor. *C. utilis* inhibitor-depleted particles (0.16 mg protein, specific ATPase activity 2.3 mol P_i released/min per mg protein) were incubated with increasing amounts of purified *C. utilis* ATPase inhibitor in the following mediums: control as medium (□—□), Mg^{2+} medium (■—■), ATP medium (●—●) or Mg^{2+}-ATP medium (○—○) detailed in the text. The volume was 0.5 ml. After 15 min at 30 °C, ATPase activity was assayed by addition of 0.5 ml of the ATPase-assay mixture as described under Experimental Procedure.

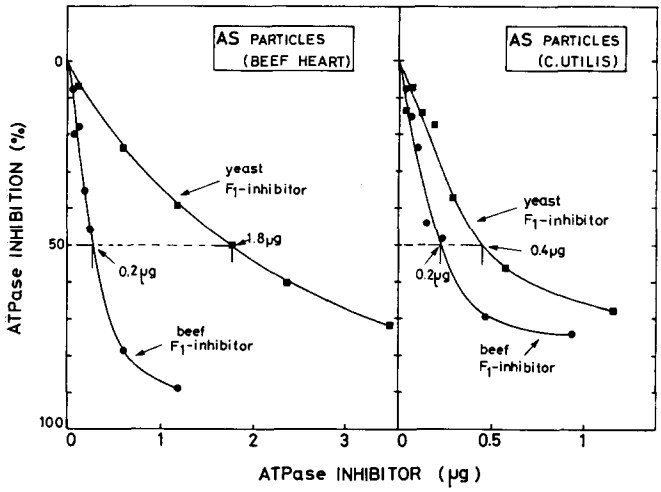

Fig. 8. Cross-reactions of beef heart and *C. utilis* ATPase inhibitors with submitochondrial inhibitor-depleted particles. Beef heart inhibitor-depleted particles (0.11 mg protein, specific ATPase activity 3.7 μmol P_i released/min per mg protein) and *C. utilis* inhibitor-depleted particles (0.16 mg protein, specific ATPase activity 2.7 μmol P_i released/min per mg protein) were incubated with increasing amounts of *C. utilis* and beef heart ATPase inhibitors as described under Experimental Procedure.

cles from beef heart and *C. utilis* (Fig. 8). A constant amount of inhibitor-depleted particles was titrated by increasing amounts of the purified inhibitors. *C. utilis* ATPase inhibitor was about 5 times more active towards homologous yeast particles than towards beef heart particles (50 % inhibition was obtained with 0.4 μg of inhibitor using *C. utilis* particles as compared to 1.8 μg when using beef heart mitochondrial particles).

Beef heart ATPase inhibitor, however, was equally active towards beef heart and yeast particles. Furthermore, the beef heart ATPase inhibitor was twice as active,

on weight basis, than *C. utilis* ATPase inhibitor towards *C. utilis* submitochondrial particles.

Effect of pH on interaction between ATPase and ATPase inhibitor

To test the effect of the pH on the interaction between ATPase and ATPase inhibitor, purified inhibitor from yeast or heart was incubated with homologous or heterologous inhibitor-depleted particles at a given pH for 15 min at 30 °C. Then the pH was brought to 7.8 and the incubation was started by addition of ATP. In all assays, with homologous or heterologous systems the maximal inhibition was obtained at acidic pH between 5.5 and 6.6 in agreement with data of Horstman and Racker [6] for the interaction between beef heart ATPase and the beef heart ATPase inhibitor.

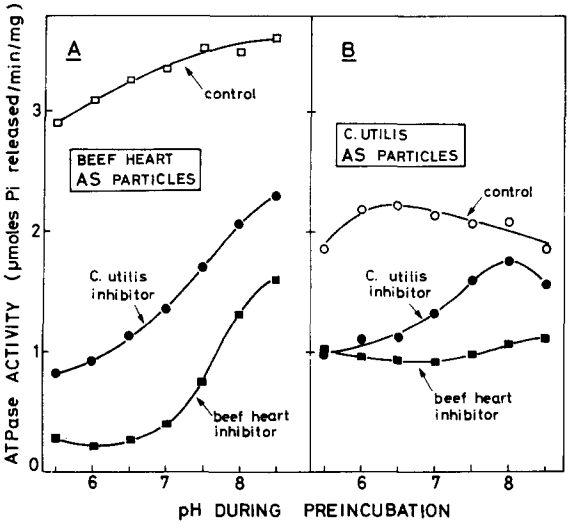

Fig. 9. pH dependence of the inhibition of ATPase activity of submitochondrial inhibitor-depleted particles by ATPase -inhibitors. (A) Beef heart inhibitor-depleted particles (0.1 mg protein) were preincubated for 15 min at 30 °C with purified beef heart ATPase inhibitor (1.2 μg, ■—■) or *C. utilis* ATPase inhibitor (concentration 3.0 μg, ●—●) in 0.25 M sucrose, 0.5 mM ATP, 0.5 mM $Mg \cdot SO_4$ and 10 mM Tris/morpholino-ethane sulfonic acid buffer at the indicated pH. The volume was 0.5 ml. ATPase activity was measured after addition of 0.5 ml of an ATPase-assay mixture as described under Experimental Procedure. The control curve (□—□) refers to the ATPase activity of the inhibitor-depleted particles without added ATPase inhibitor. (B) An experiment similar to that described above was performed with *C. utilis* inhibitor-depleted particles (0.18 mg protein). Control curve without added inhibitor (○—○); preincubation with *C. utilis* ATPase inhibitor (0.5 μg, ●—●); or with beef heart ATPase inhibitor (0.2 μg, ■—■).

Binding of ^{14}C-labelled C. utilis ATPase inhibitor to submitochondrial particles

The preparation of radioactively labelled *C. utilis* ATPase inhibitor was mainly intended to investigate by direct means the properties of the interaction between the ATPase inhibitor and the mitochondrial ATPase. Along this line a titration experiment is shown in Fig. 10. ^{14}C-labelled ATPase inhibitor was incubated with *C. utilis* submitochondrial inhibitor-depleted particles. Bound and free ATPase inhibitor were measured directly after high-speed centrifugation as the amount of radioactivity

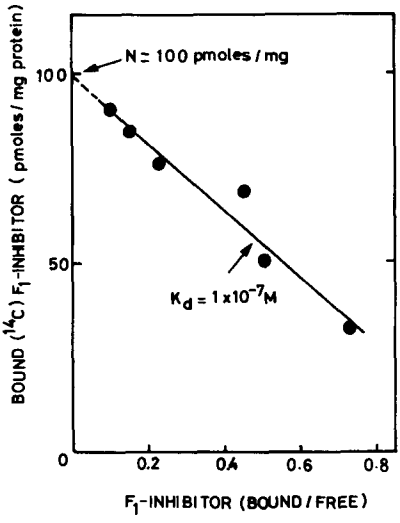

Fig. 10. Binding of ^{14}C-labelled *C. utilis* ATPase inhibitor to *C. utilis* inhibitor-depleted particles. *C. utilis* inhibitor-depleted particles (2.0 mg) were incubated for 10 min at 30 °C with increasing concentrations of *C. utilis* ^{14}C-labelled inhibitor (from 0.92 to 13.8 μg protein), specific radioactivity, 120 dpm/μg, in series of tubes containing 2 ml of 10 mM KCl, 50 mM Tris · SO_4 , 10 mM morpholino-propane sulfonic acid, 5 mM Mg^{2+}-ATP, 10 mM phosphoenolpyruvate, 60 μg pyruvate kinase, final pH 6.8.

associated with the particle pellet and the supernatant fluid, respectively. The data plotted according to Scatchard [2] show a limited amount of homogenous binding sites of the order of 0.1 nmol/mg protein. The dissociation constant, deduced from the slope, was about 10^{-7} M. The number of ATPase inhibitor sites is of the same order as the number of aurovertin sites in beef heart submitochondrial particles [5]. Although this similarity may be fortuitous, it strongly suggests that similarly to aurovertin [19] there is one mol of inhibitor-binding site per mol of ATPase.

DISCUSSION

1. Comparison of the properties of ATPase inhibitors from beef heart and C. utilis mitochondria

The present report demonstrates the presence of an ATPase inhibitor in mitochondria from the yeasts *C. utilis* and *S. cerevisiae*. The ATPase inhibitor from *C. utilis* has been purified to homogeneity and its properties compared to those of the ATPase inhibitor extracted from beef heart mitochondria. Like the beef heart ATPase inhibitor, the *C. utilis* inhibitor is stable to heat treatment and to concentrated trichloroacetic acid. Both proteins lack methionine. However, the *C. utilis* inhibitor differs from the heart inhibitor by a number of properties: (1) the molecular weight of the yeast inhibitor is lower than that of the beef heart inhibitor (6000–7500 vs 10 500); (2) the two enzymes exhibit significant differences in their amino acid composition; (3) the isoelectric points of the two enzymes differ by two pH units (5,6 for the yeast enzyme vs 7.6 for the heart enzyme). In spite of these differences, a cross-reactivity between the beef heart and *C. utilis* systems has been observed.

2. Inhibitors of enzymes involved in ATP-dependent systems

There are so far only a few reports in the literature on low-molecular-weight proteins which specifically inhibit enzymatic activities involving adenine nucleotides. The inhibitor of the cyclic AMP-dependent protein kinase, isolated from skeletal muscle [26, 27], is one of them; its low molecular weight (26 000), heat-stability, resistance to trichloroacetic acid and the noncompetitive character of its inhibition recall properties of the mitochondrial ATPase inhibitor.

A low-molecular-weight inhibitor (M_r 23 000) isolated from troponin which inhibits the actomyosin Mg^{2+}-ATPase is another example [28, 29]; interestingly, it also inhibits the mitochondrial ATPase [30]. This result is in agreement with cross-reactivity of the yeast mitochondrial ATPase inhibitor and the heat mitochondrial ATPase inhibitor (see Results), and it suggests that the ATPase inhibitors belong to a special class of enzymes with broad specificity.

3. Growth conditions and synthesis of the ATPase inhibitor in C. utilis

As shown in this paper, *C. utilis* and *S. cerevisiae* grown in the presence of a non-fermentable substrate contains much more ATPase inhibitor than the same yeast grown in a glucose medium. The repressing effect of glucose on the synthesis of the mitochondrial ATPase inhibitor is another example of glucose repression on mitochondrial development in yeast, which is known to affect the synthesis of mitochondrial membrane components belonging to the respiratory chain [31, 32] and to the oligomycin-sensitive ATPase [33, 34].

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