A Protein Inhibitor of Mitochondrial Adenosine Triphosphatase (F₁) from Saccharomyces cerevisiae

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A heat-stable protein has been detected in Saccharomyces cerevisiae which inhibits mitochondrial ATPase activity. The protein inhibitor has been isolated from extracts prepared by brief heat treatment of unbroken cell suspensions. The isolated inhibitor is a small basic protein (molecular weight close to 7000, isoelectric point 9.05) devoid of tryptophan, tyrosine, and cysteine as well as proline. The NH₂-terminal amino acid is serine. The ultraviolet absorption spectrum shows the vibrational fine structure of the phenylalanine band. Like the ATPase inhibitor from bovine heart mitochondria the yeast inhibitor is rapidly destroyed by trypsin. It is also inactivated by the yeast proteinases A and B.

Radioimmunological analysis indicates that the inhibitor is synthesized on cytoplasmic ribosomes. Its accumulation seems to be connected to the formation of the mitochondrial ATPase complex, since its specific activity is greatly reduced both in extracts obtained from the F₁-ATPase-deficient nuclear mutant pet 936 and from the cytoplasmic petite mutant D 273-10B-1.

The ATPases of mitochondria, chloroplasts, Escherichia coli cell membranes, and myofibrils are known to be associated with endogenous protein inhibitors (1-4). Owing to their possible function in regulation of energy transformation the inhibitors have received much attention.

The protein inhibitor of mitochondrial ATPase has first been investigated in bovine heart (1, 5-11). It is a heat-stable protein with a molecular weight of approximately 10,000, is not destroyed by precipitation with trichloroacetic acid, but is very sensitive to trypsin (1, 7). It strongly suppresses the ATPase activity of soluble mitochondrial ATPase (F₁-ATPase) by binding to the F₁ molecule (1, 5, 6). In submitochondrial particles the inhibitor efficiently blocks ATP-driven energy transfer reactions and it is believed to control the back flow of energy from ATP to the mitochondrial electron and ion transport systems (8-11).

The recent identification of the F₁ inhibitor in various yeast species (12-15) facilitates new kinds of experimentation. Both cytoplasmic and nuclear mutations are known in yeast which affect the mitochondrial ATPase complex (16-22). These mutations offer the possibility to study the genetic control of the F₁ inhibitor. Furthermore, the biosynthesis of the mitochondrial ATPase system has been extensively investigated in Saccharomyces cerevisiae (23-25) and it would seem desirable to characterize the ATPase inhibitor in a similar fashion. Finally, the F₁ inhibitor can be obtained in radioactive form (13) and may serve as a specific probe for F₁ protein.

This communication describes the F₁ inhibitor of S. cerevisiae. Preliminary evidence will be presented concerning its biosynthesis. Its similarities and dissimilarities with the inhibitors from Candida utilis (13) and beef heart will be discussed.

A preliminary account of this publication has been presented at the Ninth Meeting of the Federation of European Biochemical Societies, Budapest (14).

MATERIALS AND METHODS

Yeast Strains and Cell Growth

Commercially grown bakers' yeast (Pleser Hefe, obtained from BÄKO Gesellschaft mbH, D-78 Freiburg) was used for purification of the ATPase inhibitor and for preparation of mitochondria.

In addition, the following laboratory strains were used for the immunological determination of F₁ inhibitor: the "grande" strain D 273-10B (αPET₁) (26) and two "petite" mutants derived from it, the cytoplasmic petite mutant α D 273-10B (αPET₂) (16) and the nuclear F₁-deficient mutant pet 936 (αPET₂) (21). The cells were grown aerobically at 28° to the early stationary phase in YPD medium (1% yeast extract (Difco), 2% Bacto-peptone (Difco), and 1% glucose). Approximately 19 g (wet weight) of wild type cells and 14 g, respectively, of mutant cells were obtained per liter of culture medium.

Preparation of Submitochondrial Particles

The small scale procedure detailed by Mason et al. (27) was followed omitting the sonication procedure. Particles were stored frozen at -20° and thawed before use.

Enzyme and Inhibitor Measurements

ATPase was assayed titrimetrically at 30° and pH 7.4 with a pH stat assembly (Radiometer, Copenhagen). The assay mixture contained 80 µmol of MgATP and 400 µmol of KCl in an initial volume of 4 ml. NaOH (10 mM) was used for titration. Acid production was roughly linear with time until 10 µmol of alkali had been consumed.

ATPase inhibitor was determined by measuring its inhibitory effect on the ATPase activity of submitochondrial particles. The inhibitor was added directly to the ATPase reaction mixture and the reaction was allowed to proceed for 4 to 5 min until it reached an approximately constant inhibited rate. Only the linear part of the titration plot was used for the determination of activity. Care was...
taken that the total amount of NaOH consumed during the measurement did not exceed 10 µmol.

Published procedures were used to determine cytochrome c oxidase (27), succinic cytochrome c reductase (28), DNA-dependent RNA polymerase (29), and yeast proteinases A and B (30).

Definition of Unit

A unit of ATPase activity is defined as the amount of enzyme causing the formation of 1 µmol of titratable H⁺/min under the above described assay conditions. According to the calculation of Chance and Nishimura (31) this corresponds to 1.18 pmol of P₈ formed/min.

A unit of ATPase inhibitory activity is defined as that amount which results in a 50% inhibition of 2 units of mitochondrial ATPase under the specified assay conditions.

Electrophoresis and Electrotocusing

Cationic polyacrylamide gel electrophoresis (running pH 2.7) was carried out according to the instruction manual of Buchler Instruments, Inc., Fort Lee, N.J. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to Mason et al. (27) and analytical gel electrofocusing with a pH range of 3 to 10 according to Wrigley (32). Gels were stained with 1% Amido black in 10% acetic acid for up to 4 h at 28°C and destained by repeated washing in 10% acetic acid. Gels from which the protein had to be eluted were not stained but only fixed with 10% trichloroacetic acid prior to slicing in order to render the protein band visible. Gel slices were suspended in distilled water and washed in a 5% glass ionomer. The gel suspensions were neutralized afterwards.

To determine the isoelectric point of the ATPase inhibitor, isoelectric focusing was performed with an LKB model 8100 preparative apparatus and the 110-ml column. One percent ampholine, pH range 3 to 10, was used. The cathode was at the top of the column. The experiments were performed at 5-8°C in a Spinco model E ultracentrifuge. 

Current had dropped to a stable value of 0.5 mA. Fractions (1.5 ml each) were collected and used for assaying inhibitory activity and pH.

Other Analytical Methods

Meniscus depletion and conventional sedimentation equilibrium experiments were performed at 6-8°C in a Spinco model ultracentrifuge. Rotor speed was varied between 18,000 and 32,000 rpm. The initial inhibitor concentrations for the analytical runs ranged from 0.56 to 5.0 mg/ml. The samples were dissolved in 87 mM Na₃P₄, pH 7.6. The partial specific volume of the inhibitor was estimated from the amino acid composition (33).

Amino acid analysis was carried out in a Biocal amino acid analyzer, model BC-200. Protein samples (500 µg each) were hydrolyzed with 6 N HCl at 110°C for 24, 48, and 96 h in sealed, evacuated ampules. Hydrolysates were analyzed by the procedure of Eepmaan et al. (34). Cysteic acid and methionine sulfone were determined after performic acid oxidation according to Moore (35). The NH₂-terminus residue of the inhibitor was determined by the method of Hartley (36).

Protein concentration was measured according to Lowry et al. (37); 1 mg of inhibitor protein corresponded to 0.9 mg of inhibitor protein as determined by dry weight measurements.

Immunological Procedures

Rabbit antisera to the F₁ inhibitor were prepared according to Mason et al. (27). For immunoprecipitation of F₁ inhibitor, we followed essentially the procedure published earlier (38) except that we washed the immunoprecipitates with 10 mM Tris/sulfate, pH 7.4, and 0.14 M NaCl. The inhibitor content of crude extracts was determined with an excess of antiserum. A calibration curve obtained with purified inhibitor was used to determine the inhibitor content of the immunoprecipitate.

Extracts from boiled yeast suspension were prepared as described below.

Isolation of ATPase Inhibitor from Yeast

All operations subsequent to the boiling procedure in Step 1 were conducted at 4°C in order to retard bacterial contamination. Prior to chromatography, the adsorbents were equilibrated with the buffers used as starting eluents. A Beckman model 131 gradient pump was used for gradient chution.

Step 1: Preparation of Extract from Boiled Yeast Suspension—

Twelve kilograms of pressed bakers' yeast were suspended in 6 liters of distilled water. The smooth suspension was pumped at 3 liters/h through a glass coil (1.5 m long, inner diameter 1 cm) immersed in boiling water (98°C). The effluent was collected in a glass beaker immersed in a cooling water bath (15°C) and slowly stirred to allow rapid cooling to approximately 30°C. Slow stirring of the boiled suspension was continued overnight in the cold room (4°C). The slurry was centrifuged in a Sorvall G-3 rotor (Sorvall, Inc., Norwalk, Connecticut) for 5 min at 6000 rpm (5000 x g). The supernatant was filtered through 10 layers of cheesecloth (A. H. Thomas Co., Philadelphia, Pa.) to remove floating material. Approximately 8 liters of a yellowish, slightly turbid extract were obtained.

The crude extract of CM-cellulose (Serva) (CM23, Serva, Heidelberg, Germany, 5 x 80 cm) was equilibrated with 10 mM sodium acetate, pH 5. The CM-cellulose was then removed from the column, mixed with the extract, stirred for 30 min, and allowed to settle. The CM-cellulose to which the inhibitor had been adsorbed was subsequently washed three times by decantation with 8 liters of 10 mM sodium acetate, pH 5, put back into the column, and packed. A linear sodium chloride gradient was applied to the column. The gradient volume was 2 liters. The starting buffer was 10 mM sodium acetate, pH 5; the limiting buffer contained in addition 1.5 M NaCl. The flow rate was 100 ml/h. Fractions of 20 ml were collected and assayed for the inhibitor which emerged at approximately 1 M NaCl. To the pooled fractions containing inhibitor (approximately 0.5 liter), 50 ml of 100% (w/v) trichloroacetic acid were added.

The precipitated protein was immediately collected by centrifugation at 10,000 x g for 10 min and suspended in 0.9 M sodium acetate. The suspension was homogenized and the pH was adjusted to 7 with 1 M Tris base. The slightly turbid solution was centrifuged at 27,000 x g for 10 min and the precipitate discarded.

Step 2: Chromatography on Hydroxyapatite—
The clear supernatant solution obtained in the preceding step was pumped at a flow rate of 20 ml/h through a column of hydroxyapatite (2.5 x 15 cm) equilibrated with 10 mM NaH₂PO₄, pH 7.0. The column was eluted at the same flow rate with 250 ml of a linear gradient varying from 10 mM to 500 mM NaH₂PO₄, pH 7. Fractions of 4 ml each were collected and their absorbances at 220 nm were measured. The peak eluting at approximately 0.3 M P₂₄ was found to contain the inhibitory activity. The peak fractions were pooled and the protein was precipitated with trichloroacetic acid as described above. The precipitate was suspended in 1 ml of distilled water and neutralized with 1 M Tris base. In order to desalt the protein solution, the sample was filtered through a column (2.5 x 80 cm) of Sphadex G-50 medium equilibrated with 0.1 M ammonium acetate, pH 5.5. The flow rate was 20 ml/h. The protein-containing fraction of the column eluate was lyophilized.

Other Preparations

Yeast proteinases A, B, and C were a gift of Dr. A. Hasilik (Biochemisches Institut Freiburg) and purified yeast F₁-ATPase (specific activity 80 units x mg⁻¹) was provided by Dr. E. Agsteribbe (Biozentrum Basel). Hydroxypapitite was obtained according to O. Levin (39) from H. Hinze (Biochemisches Institut Freiburg). Kallikrein trypsin inhibitor from Bayer, Werk Elberfeld was a gift of Dr. Schmidt-Kastner (Beverkken).

Sodium dodecyl sulfate (Serva, Heidelberg) was recrystallized from ethanol (40).

Dialysis tubing with molecular weight cut-off of 3500 was purchased from Spectrum Medical Industries, Inc., Los Angeles, Calif.

RESULTS

Isolation of Inhibitor—The yeast extract prepared as outlined under "Materials and Methods" contains only approximately 1% of the total yeast protein. It is capable of inhibiting mitochondrial ATPase activity. As can be seen from Fig. 1, the ATPase activity affected is oligomycin-sensitive and, therefore, seems to represent F₁ activity. Duration of boiling did not seem to be overly important, since small samples could be boiled for as short a period as 1 min without significantly reducing the amount of inhibitory activity liberated. Stirring of the yeast suspension subsequent to boiling somewhat increased the yield of inhibitory activity, especially under alka-
behind kallikrein (molecular weight 6200). Its apparent molecular weight is approximately 7000. On the basis of the amino acid analysis (cf. Table II) the minimal molecular weight was calculated to be 7400, which is in good agreement with the electrophoretically determined value.

**Fig. 1 (left).** Inhibition of mitochondrial ATPase activity by an extract prepared from boiled yeast suspension and by oligomycin. ATPase activity was determined in the pH-stat as described under "Materials and Methods" except that the initial volume of the reaction mixture was 2.5 ml. The reaction was started by addition of 1.1 mg of mitochondria. ,--X, yeast extract; O--O, oligomycin.

**Fig. 2 (right).** Electrophoresis and electrofocusing of purified F, inhibitor from yeast in polyacrylamide gels. *a*, cationic disc electrophoresis, pH 2.7 (7.5% acrylamide, 20 μg of inhibitor); *b*, cationic disc electrophoresis, pH 2.7 (15% acrylamide, 10 μg of inhibitor); *c*, electrophoresis in the presence of 0.2% sodium dodecyl sulfate, pH 9 (15% acrylamide, 6 μg of inhibitor); *d*, electrofocusing in 7.5% acrylamide, 1% ampholine, pH range 3 to 10 (30 μg of inhibitor). Wires denote the position of the tracking dye: *a* and *b*, methylene blue; *c*, bromphenol blue.

The purified material from Step 2 could not be further enriched by any of the following procedures: chromatography on Sephadex G-50, isoelectric focusing, or fractionation with acetone. It gave a single protein band upon electrophoresis or disc electrophoresis, pH 2.7 (15% acrylamide, 20 μg of inhibitor); disc electrophoresis, pH 2.7 (15% acrylamide, 10 μg of inhibitor); electrofocusing in the presence of 0.2% sodium dodecyl sulfate, pH 9 (15% acrylamide, 6 μg of inhibitor); electrofocusing in 7.5% acrylamide, 1% ampholine, pH range 3 to 10 (30 μg of inhibitor). Wires denote the position of the tracking dye: *a* and *b*, methylene blue; *c*, bromphenol blue.

**Molecular Weight—**Upon electrophoresis in 15% or 20% polyacrylamide gels containing sodium dodecyl sulfate, the inhibitor migrates as a single protein band (cf. Fig. 2) slightly behind kallikrein (molecular weight 6200). Its apparent molecular weight is approximately 7000. On the basis of the amino acid analysis (cf. Table II) the minimal molecular weight was calculated to be 7400, which is in good agreement with the electrophoretically determined value.

**Sedimentation equilibrium analysis** in the analytical ultracentrifuge under nondissociating conditions gave a molecular weight of 22,000. A similar value was obtained from gel filtration experiments in Sephadex G-50 in the absence of denaturing agents. We take these data as an indication that the protein aggregates in the absence of dissociating agents.

**Amino Acid Analysis and NH₂-terminal Group—**The amino acid composition of the yeast inhibitor is shown in Table II. The data obtained for the bovine heart inhibitor by Brooks and Senior (7) and for the inhibitor from Candida utilis by Sarre et al. (13) are given for comparison. Both yeast inhibitors lack cysteine. The bovine heart inhibitor and the inhibitor from Saccharomyces cerevisiae do not contain proline. In agreement with the CF₂ inhibitor (2) tyrosine is missing in the S. cerevisiae protein. The absence of tryptophan in the inhibitor from S. cerevisiae was established by spectral analysis (cf. below). The large number of lysine and arginine residues in the inhibitor from S. cerevisiae is in agreement with its alkaline isoelectric point (cf. below). The polarity index according to Capaldi and Vanderkooi (41) was 70% which explains the extreme solubility of the inhibitor in water.

The authors' report that most soluble proteins have polarities within the narrow range of 47 ± 6%. The F₂ inhibitor, therefore, seems to be considerably more hydrophilic than an "average" protein.

**Protein determination according** to Lowry et al. (37) with bovine serum albumin as standard was in good agreement with the dry weight determination and the value expected from amino acid analysis. Thus, there is no indication that the F₂ inhibitor from yeast contains compounds other than amino acids. The determination of the NH₂-terminal amino acid was performed by dansylation. After hydrolysis of the dansylated inhibitor the amino acid mixture was separated on polyamide thin layers according to Hartley (36). The chromatogram showed the spots corresponding to dansyl acid, dansylamide, dansyl serine, and ε-dansyl lysine. No other dansylated amino acid could be detected using another solvent system such as 1 M ammonia/ethanol (1/1, v/v). It is concluded that serine is the NH₂-terminal amino acid.

**Ultraviolet Absorption Spectrum—**Fig. 3 shows the ultraviolet spectrum of a 0.2 mM inhibitor solution in water. Very little absorption in the wavelength range between 270 and 280
Yeast $F_{i}$ Inhibitor

**Table II**

Amino acid composition of $F_{i}$ inhibitors from yeast and beef heart

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Saccharomyces cerevisiae ATPase inhibitor</th>
<th>Beef heart ATPase inhibitor</th>
<th>Candida utilis ATPase inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Residues</td>
<td>Extrap.</td>
<td>Protein</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>48</td>
<td>mol</td>
</tr>
<tr>
<td>Lysine</td>
<td>11.9</td>
<td>12.7</td>
<td>8</td>
</tr>
<tr>
<td>Histidine</td>
<td>5.6</td>
<td>7.4</td>
<td>4</td>
</tr>
<tr>
<td>Arginine</td>
<td>17.6</td>
<td>21.1</td>
<td>11</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>8.1</td>
<td>8.5</td>
<td>5</td>
</tr>
<tr>
<td>Threonine</td>
<td>6.2</td>
<td>5.6</td>
<td>4</td>
</tr>
<tr>
<td>Serine</td>
<td>9.7</td>
<td>9.0</td>
<td>6</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>21.7</td>
<td>20.0</td>
<td>14</td>
</tr>
<tr>
<td>Proline</td>
<td>6.2</td>
<td>6.5</td>
<td>4</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.7</td>
<td>1.6</td>
<td>1</td>
</tr>
<tr>
<td>Cysteine*</td>
<td>3.6</td>
<td>3.2</td>
<td>2</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.4</td>
<td>1.4</td>
<td>1</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2.9</td>
<td>2.9</td>
<td>2</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.3</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>8.2</td>
<td>8.0</td>
<td>5</td>
</tr>
<tr>
<td>Phenytoalanine</td>
<td>8.2</td>
<td>7.4</td>
<td>4</td>
</tr>
<tr>
<td>Tryptophan†</td>
<td></td>
<td></td>
<td>NDc</td>
</tr>
<tr>
<td>Total number of amino acids</td>
<td>64</td>
<td>91</td>
<td>66-67</td>
</tr>
<tr>
<td>Minimal molecular weight</td>
<td>7,400</td>
<td>10,500</td>
<td>7,500</td>
</tr>
</tbody>
</table>

---

**Mechanism and Specificity of Action** — When the inhibitor is mixed with submitochondrial particles and the mixture subsequently subjected to differential centrifugation, a substantial part of the inhibitor is removed from the supernatant, as is shown in Table III. Concomitantly the ATPase activity of the sedimanted particles is inhibited. Washing of the particles with a large excess of water does not relieve the inhibition. This suggests that a stable complex between inhibitor and submitochondrial particles is formed. Addition of ATP or Mg$^{2+}$, or both to the incubation mixture did not affect absorption of inhibitor by the particles. However, it cannot be ruled out that the mitochondria themselves contained traces of these compounds which seem to be required for the inhibitory action of the bovine heart inhibitor (6) and the inhibitor of C. utilis (13).

The activities of cytochrome c oxidase and succinate cytochrome c reductase are not significantly affected by the yeast inhibitor (cf. Table III). These preliminary data suggest that the action of the inhibitor on the mitochondrial inner membrane is restricted to $F_{i}$-ATPase activity. This idea is further supported by the fact that highly purified soluble $F_{i}$-ATPase is inhibited with similar efficiency as the ATPase activity of submitochondrial particles. This demonstrates that the inhibitor binds to the $F_{i}$ molecule itself.

The action of the $F_{i}$ inhibitor is not mimicked by proteinase B inhibitor I from yeast (46), various trypsin inhibitors such as ovomucoid, and the inhibitors from soybean and pancreas or a variety of small basic proteins such as horse heart cytochrome c, pancreatic RNase, lysozyme, salmine protamine, calf thymus histone, or the polyamines, spermine and spermidine. Conversely, the yeast $F_{i}$ inhibitor did not inhibit yeast proteinases A, B, or C, but, as already mentioned, is rapidly destroyed by either yeast proteinases A or B. Furthermore, the inhibitor does not display histone-like activity, since it does not inhibit the DNA-dependent RNA polymerase from Escherichia coli.2

**Bioisynthesis** — Rabbit antiserum to yeast $F_{i}$ inhibitor is able to precipitate the inhibitor from crude extracts prepared by heating. As was shown with extracts from cells labeled with $[^{14}C]$leucine, the precipitated antigen co-migrates with the purified inhibitor protein upon sodium dodecyl sulfate-acrylamide gel electrophoresis (cf. Fig. 4). When cells are pulse-labeled in the presence of cycloheximide, an inhibitor of cytoplasmic protein synthesis, incorporation of radioactivity into the inhib-
Interaction between submitochondrial particles and inhibi-

tor protein is completely suppressed (cf. Table IV). In the presence of acriflavine, a potent inhibitor of mitochondrial protein synthesis (47), incorporation of [3H]leucine into the inhibitor protein still proceeds, although at a lower rate than in uninhibited control cells. We feel that the data shown in Table IV are good evidence that the F, inhibitor (like F,-ATPase itself (25)) is synthesized by cytoplasmic ribosomes.

In order to investigate whether the inhibitor of F,-ATPase is synthesized in a coordinate fashion with F,, we have measured the F, inhibitor content of the S. cerevisiae normal strain D273-10B, the cytoplasmic petite mutant D273-10B-1 (16), and the nuclear "petite" mutant pet 936 (21). These two mutants are derived from D273-10B but contain less F,-ATPase. As can be seen from Table V, determinations of inhibitory activity agreed well with the results obtained by quantitative immunoprecipitation of the inhibitor protein. Both commercial baker's yeast and the normal haploid laboratory strain D273-10B contain comparable amounts of inhibitor. On the other hand, the nuclear petite mutant pet 936 contains less inhibitor and the cytoplasmic petite mutants appear to lack it completely, at least as far as the present assay methods are concerned. The lowered concentration of F,-ATPase in the mutants is thus accompanied by a lowered content of F, inhibitor.

DISCUSSION

In the present communication we have demonstrated that a protein inhibitor to mitochondrial ATPase can be isolated from S. cerevisiae. Since the isolation procedure did not start from mitochondria and involved drastic treatments like boiling and precipitation with trichloroacetic acid it may be argued that the protein described here is an artifactual product that acciden-
tally inhibits F,-ATPase. Several observations argue against this view. (a) Many physical properties of the S. cerevisiae inhibitor resemble those of inhibitors isolated from mitochondria of bovine heart and C. utilis (1, 13). All inhibitors are extremely heat-stable, are not destroyed by precipitation with trichloroacetic acid, are sensitive to proteinases, and are of similar size. Furthermore, all inhibitors seem to exist as polymers in the absence of dissociating agents (see "Results") and Refs. 9, 13, and 48). The similarities in amino acid composition already have been discussed (cf. "Results"). (b) The purified inhibitors from C. utilis and S. cerevisiae have approximately the same specific inhibitory efficiency, 1 mg of protein being able to inhibit 60 to 80 units of F,-ATPase. (c) The pH of the mixture was 6.7. After standing for 5 min at 25°, the mixture was centrifuged for 2 min at 8000 x g in a model 3200 Eppendorf centrifuge. F,-ATPase inhibitory activity of the supernatant and enzyme activities of the sedimented particles were assayed as described under "Materials and Methods.

<table>
<thead>
<tr>
<th>Inhibitor added</th>
<th>Inhibitor content of supernatant</th>
<th>Enzymic activities of sedimented particles</th>
<th>Total inhibitory activity recovered after centrifugation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units*</td>
<td>units*</td>
<td>%</td>
</tr>
<tr>
<td>0.9</td>
<td>0.1</td>
<td>1.6</td>
<td>0.75 0.37 89</td>
</tr>
<tr>
<td>9.0</td>
<td>1.3</td>
<td>0.4</td>
<td>0.9 0.37 87</td>
</tr>
</tbody>
</table>

* For definition of units, see "Materials and Methods.

FIG. 4. Electrophoretic analysis of immunoprecipitates of radioactive F, inhibitor from crude extracts of the normal laboratory strain D273-10B. Extraction of the labeled cells by brief heat treat-

ment, immunoprecipitation of the F, inhibitor, and analysis of the immunoprecipitates in 20% acrylamide gels containing sodium dode-
cyl sulfate were performed as outlined under "Materials and Meth-

ods." Prior to electrophoresis 35 &igrave;g of purified inhibitor were added to each gel. A, cells grown in the presence of radioac-
tive leucine, as described earlier (38). B, cells pulse-labeled with radioactive leucine according to Groot et al. (47): 9 g of washed cells (wet weight) were suspended in 40 ml of 40 mM phosphate buffer, pH 7.4, containing 1% galactose and incubated in a rotary shaker at 28° for 39 min in the presence of 0.5 &igrave;Ci/ml of [4,5-3H]leucine. Labeling was stopped by the addition of 40 ml of 0.1 M cold leucine. The bar indicates the position of the purified inhibitor after staining with Coomassie blue.

TABLE IV

Labeling of yeast F, inhibitor in presence and absence of antibiotics

Yeast cells were pulse-labeled with [4,5-3H]leucine as detailed in the legend of Fig. 4. The concentration of cycloheximide was 100 &igrave;g/ml. Acriflavine was added at a concentration of 12.5 &igrave;g/ml. Extract-

tion of the cells and immunoprecipitation of the F, inhibitor were carried out as described under "Materials and Methods.

<table>
<thead>
<tr>
<th>Labeling conditions</th>
<th>Maximal amount of radioactivity precipitable with 100 ml of antiserum</th>
</tr>
</thead>
<tbody>
<tr>
<td>No antibiotic</td>
<td>1448</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>18</td>
</tr>
<tr>
<td>Acriflavine</td>
<td>644</td>
</tr>
</tbody>
</table>

When extracts of subcellular fractions of S. cerevisiae were analyzed by double diffusion in Ouchterlony plates with inhibitor-antiserum, the strongest reaction developed with the mitochondrial extract, whereas no precipitation was detected with extracts of the 100,000 x g supernatant. This seems to indicate that the inhibitor is predominantly, if not exclusively, localized in the mitochondria. (d) Although the inhibitor pro-

Eberhard Ebner, unpublished results.
Yeast F1 Inhibitor

The yeast strains examined are listed under "Materials and Methods." The F1 inhibitor was determined in crude extracts (cf. Step 1 of the purification procedure). F1-ATPase activity was measured in the mitochondrial fraction.

<table>
<thead>
<tr>
<th>Strain</th>
<th>F1 inhibitor content of 1 ml of crude extracta</th>
<th>Specific activity of F1-ATPaseb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/g</td>
<td>μg/ml</td>
</tr>
<tr>
<td>Commercial bakers' yeast</td>
<td>0.8</td>
<td>19</td>
</tr>
<tr>
<td>D273-10B (&quot;grande&quot;)</td>
<td>0.7</td>
<td>15</td>
</tr>
<tr>
<td>D273-10B-1 (cytoplasmic pete)</td>
<td>0.1</td>
<td>2</td>
</tr>
<tr>
<td>Pet 936 (nuclear F1-deficient mutant)</td>
<td>0.2</td>
<td>3</td>
</tr>
</tbody>
</table>

a Approximately 1 ml of crude extract was obtained from 1-g cells (wet weight) in this particular experiment.

b For definition of unit, see "Materials and Methods." Determined by inhibition of F1-ATPase.

d Determined by immunoprecipitation.

The yeast strains examined are listed under "Materials and Methods." The F1 inhibitor was determined in crude extracts (cf. Step 1 of the purification procedure). F1-ATPase activity was measured in the mitochondrial fraction.

Acknowledgments — We wish to thank Dr. H. Lill, Boehringer Mannheim GmbH, for providing us with large amounts of mitochondrial fraction.

The yeast strains examined are listed under "Materials and Methods." The F1 inhibitor was determined in crude extracts (cf. Step 1 of the purification procedure). F1-ATPase activity was measured in the mitochondrial fraction.
A protein inhibitor of mitochondrial adenosine triphosphatase (F1) from Saccharomyces cerevisiae.

E Ebner and K L Maier


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