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A Protein Inhibitor of Mitochondrial Adenosine Triphosphatase (F_1) 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 NH_2 -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 F_1 -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 (F_1 -ATPase) by binding to the F_1 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 F_1 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 F_1 inhibitor. Furthermore, the biosynthesis of the mitochondrial ATPase system has been intensively investigated in *Saccharomyces cerevisiae* (23-25) and it would seem desirable to characterize the ATPase inhibitor in a similar fashion. Finally, the F_1 inhibitor can be obtained in radioactive form (13) and may serve as a specific probe for F_1 protein.

This communication describes the F_1 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 eGmbH, 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 F_1 inhibitor: the "grande" strain D273-10B (αPET^+) (26) and two "petite" mutants derived from it, the cytoplasmic petite mutant $\alpha D273-10B-1$ (αPET^-) (16) and the nuclear F_1 -deficient mutant pet 936 (αpet^+) (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 μ mol of MgATP and 400 μ mol 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 μ mol 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

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taken that the total amount of NaOH consumed during the measurement did not exceed 10 μ mol.

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 μ mol of titratable H^+ /min under the above described assay conditions. According to the calculation of Chance and Nishimura (31) this corresponds to 1.18 μ mol of P_i 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 Electrofocusing

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° 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 crushed in a Teflon-glass homogenizer. 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 per cent ampholine, pH range 3 to 10, was used. The cathode was at the top of the column. The protein sample was added to the dense solution prior to mixing the gradient. Electrolysis was carried out at 300 V for 2 days until the 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 5–8° in a Spinco model E ultracentrifuge. Rotor speed was varied between 18,000 and 32,000 rpm. The initial inhibitor concentrations for the analytical runs ranged from 0.95 to 3.0 mg/ml. The samples were dissolved in 67 mM NaK P_i , 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 (300 μ g each) were hydrolyzed with 6 N HCl at 110° for 24, 48, and 96 h in sealed, evacuated ampules. Hydrolysates were analyzed by the procedure of Spackman *et al.* (34). Cysteic acid and methionine sulfone were determined after performic acid oxidation according to Moore (35). The NH_2 -terminal 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 F_1 inhibitor were prepared according to Mason *et al.* (27). For immunoprecipitation of F_1 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° 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 elution.

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 0.9 cm) immersed in boiling water (98°). The effluent was collected in a glass beaker immersed in a cooling water bath (15°) and slowly stirred to allow rapid cooling to approximately 30°. Slow stirring of the boiled suspension was continued overnight in the cold room (+4°). The slurry was centrifuged in a Sorvall G-3 rotor (Sorvall, Inc., Norwalk, Connecticut) for 5 min at 5000 rpm ($5000 \times g_{max}$). 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.

A column of CM-cellulose (Servacel CM23, Serva, Heidelberg, Germany, 5×80 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 $5,000 \times g$ for 10 min and suspended in 25 ml of distilled water. The suspension was homogenized and the pH was adjusted to 7 with 1 M Tris base. The slightly turbid solution was centrifuged at $27,000 \times g$ for 10 min and the precipitate was 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 (2.5×15 cm) equilibrated with 10 mM NaK P_i , 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 NaK P_i , pH 7. Fractions of 4 ml each were collected and their absorbance at 220 nm was measured. The peak eluting at approximately 0.3 M P_i 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 (2.5×80 cm) of Sephadex 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 F_1 -ATPase (specific activity 60 units $\times$ mg $^{-1}$) was provided by Dr. E. Agsteribbe (Biozentrum Basel). Hydroxyapatite prepared according to O. Levin (39) was a gift of H. Hinze (Biochemisches Institut Freiburg). Kallikrein trypsin inhibitor from Bayer, Werk Elberfeld was a gift of Dr. Schmidt-Kastner (Leverkusen).

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 F_1 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 alkali-

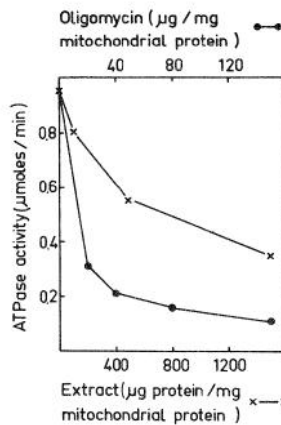


FIG. 1 (left). Inhibition of mitochondrial ATPase activity by an extract prepared from boiled yeast suspension and by oligomycin. ATPase activity was determined in the pH-stat as described under "Materials and Methods" except that the initial volume of the reaction mixture was 2.5 ml. The reaction was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

FIG. 2 (right). Electrophoresis and electrofocusing of purified F_1 inhibitor from yeast in polyacrylamide gels. *a*, cationic disc electrophoresis, pH 2.7 (7.5% acrylamide, 20 μ g of inhibitor); *b*, cationic disc electrophoresis, pH 2.7 (15% acrylamide, 10 μ g of inhibitor); *c*, electrophoresis in the presence of 0.2% sodium dodecyl sulfate, pH 9 (15% acrylamide, 6 μ g of inhibitor); *d*, electrofocusing in 7.5% acrylamide, 1% ampholine, pH range 3 to 10 (30 μ g of inhibitor). Wires denote the position of the tracking dye: *a* and *b*, methylene blue; *c*, bromphenol blue.

line conditions. At pH 9, approximately 20% more inhibitory activity as well as protein could be extracted than at pH 5.5. The alkaline extraction was, however, not used for the preparation of the inhibitor since the inhibitor did not bind to CM-cellulose at alkaline pH. The inhibitory activity of the crude extract proved to be nondialyzable through Spectrapor cellulose casing (molecular weight cut-off 3500), was trypsin-sensitive, and could be precipitated with trichloroacetic acid. It, therefore, seemed to represent proteinaceous material.

The inhibitory protein could be purified by conventional fractionation methods. Table I summarizes the results of the purification procedure which has been detailed under "Materials and Methods." Based on protein content, an 80-fold purification was achieved; the yield was approximately 25%. While this may appear to be a modest purification, it should be recalled that approximately 98 to 99% of the total cell protein had already been eliminated by the boiling procedure used in Step 1.

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 electrofocusing in polyacrylamide gels (Fig. 2). When the gels were sliced prior to staining and were extracted with buffer, inhibitory activity was found to coincide with the protein band. We conclude that the purified inhibitor is homogeneous in gel electrophoresis and gel electrofocusing.

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 molec-

Step	Volume ml	Total protein mg	Specific activity ^a units/mg	Purification fold	Yield %
1. Extract from boiled yeast suspension	8000	9500	1	(1)	(100)
2. Chromatography on CM-cellulose	500	52	50	50	30
3. Chromatography on hydroxypapatite	20	26	80	80	25

^a For definition of unit, see "Materials and Methods."

ular 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 NH_2 -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 Satre *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 CF_1 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 $47 \pm 6\%$. The F_1 inhibitor, therefore, seems to be considerably more hydrophilic than an "average" protein.

The 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 F_1 inhibitor from yeast contains compounds other than amino acids. The determination of the NH_2 -terminal amino acid was performed by dansylation.¹ 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 dansyllic 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 NH_2 -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

¹ The abbreviation used is: dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

TABLE II
Amino acid composition of F_1 inhibitors from yeast and beef heart

Amino acid	Saccharomyces cerevisiae ATPase inhibitor			Extrapolated	Beef heart ^a ATPase inhibitor	Candida utilis ^b ATPase inhibitor
	Residues					
	Hours of hydrolysis					
	24	48	96			
	μmol				residues/mol	
Lysine	11.9	13.9	12.7	8	11	10
Histidine	1.5	1.7	1.6	1	6	1
Arginine	10.6	10.7	11.2	7	9	3
Aspartic acid	8.1	8.3	8.5	5	8	8
Threonine	6.2	6.0	5.6	4		5
Serine	9.7	9.0	8.1	6	6	3
Glutamic acid	21.7	20.8	22.4	14	20	13
Proline						1
Glycine	6.2	6.5	6.4	4	6	3
Alanine	1.7	1.6	1.6	1	11	6
Cysteine ^c					ND ^d	
Valine	3.6	4.1	3.2	2	2	1
Methionine	1.4	1.4	1.4	1		
Isoleucine	2.9	3.1	2.9	2	4	2
Leucine	6.3	6.0	6.5	4	5	7
Tyrosine					1	1-2
Phenylalanine	8.2	7.4	8.0	5	2	2
Tryptophan ^c					ND ^d	ND ^d
Total number of amino acids				64	91	66-67
Minimal molecular weight				7,400	10,500	7,500

^a Data taken from Ref. 7. Hydrolysis was for 24 h.

^b Data taken from Ref. 13. Hydrolysis was for 48 h.

^c Determined by performic acid oxidation (35).

^d Not determined.

^e Determination by spectral analysis.

nm can be detected. This agrees with the absence of tyrosine which had already been found by amino acid analysis (cf. Table II).

From the absorption peak between 240 and 270 nm some saw-toothed projections appear to emerge which probably represent the vibrational fine structure of the phenylalanine band (42). Similar absorption spectra have been reported for pervalbumins (43) and the proteinase B inhibitor from *S. cerevisiae* (44).

As can be seen in Fig. 3, a 0.2 mM inhibitor solution in water exhibits an absorbance of less than 0.1 at 280 nm. Since a 0.2 mM tryptophan solution has an optical density of 0.5 at 280 nm, one can conclude that the inhibitor contains less than 0.2 mol of tryptophan/mol of protein. Quenching of the tryptophan absorption does not seem likely since boiling of the inhibitor in the presence of sodium dodecyl sulfate does not increase the absorbance at 280 nm.

Other Properties—The isoelectric point of the inhibitor was determined by isoelectric focusing (see "Materials and Methods") to be 9.05. This value correlates with the high content of basic amino acids of the protein (cf. Table II). Measurements of carbohydrate with the phenol-sulfuric acid method (45) yielded less than 0.5 mol of glucose equivalents/mol of inhibitor. The activity of the purified inhibitor was sensitive to trypsin and to the yeast endoproteases A and B. On the other hand, the inhibitory activity was stable against yeast exoprotease C, pancreatic RNase, micrococcal nuclease, snake venom phosphodiesterase, phospholipase A, neuraminidase, and lysozyme.

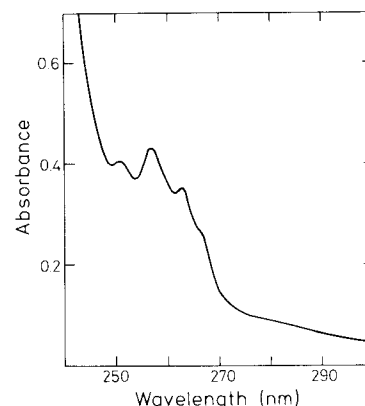


FIG. 3. Ultraviolet absorption spectrum of purified inhibitor. Inhibitor, 1.5 mg, was dissolved in 1 ml of water and the spectral absorbance against water as a reference was measured.

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 sedimented 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_1 -ATPase activity. This idea is further supported by the fact that highly purified soluble F_1 -ATPase is inhibited with similar efficiency as the ATPase activity of submitochondrial particles. This demonstrates that the inhibitor binds to the F_1 molecule itself.

The action of the F_1 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 histones, or the polyamines, spermine and spermidine. Conversely, the yeast F_1 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

Biosynthesis—Rabbit antiserum to yeast F_1 inhibitor is able to precipitate the inhibitor from crude extracts prepared by heating. As was shown with extracts from cells labeled with [³H]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-

² B. Puschendorf, personal communication.

TABLE III

Interaction between submitochondrial particles and inhibitor

Submitochondrial particles (2.5 mg) were suspended in 1 ml of distilled water and mixed with the indicated amount of inhibitor. The pH of the mixture was 6.7. After standing for 5 min at 25°, the mixture was centrifuged for 2 min at $8000 \times g$ in a model 3200 Eppendorf centrifuge. F_1 -ATPase inhibitory activity of the supernatant and enzyme activities of the sedimented particles were assayed as described under "Materials and Methods."

Inhibitor added	Inhibitor content of supernatant	Enzymic activities of sedimented particles			Total inhibitory activity recovered after centrifugation
		ATPase	Cytochrome c oxidase	Succinate cytochrome c reductase	
units ^a	units ^a		units ^a		%
		2.5	1.05	0.45	
0.9	0.1	1.6	0.75	0.37	89
9.0	1.3	0.4	0.9	0.37	87

^a For definition of units, see "Materials and Methods."

itor protein is completely suppressed (*cf.* Table IV). In the presence of acriflavine, a potent inhibitor of mitochondrial protein synthesis (47), incorporation of [3 H]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_1 inhibitor (like F_1 -ATPase itself (25)) is synthesized by cytoplasmic ribosomes.

In order to investigate whether the inhibitor of F_1 -ATPase is synthesized in a coordinate fashion with F_1 , we have measured the F_1 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_1 -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 bakers' 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_1 -ATPase in the mutants is thus accompanied by a lowered content of F_1 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 accidentally inhibits F_1 -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_1 -ATPase. (c)

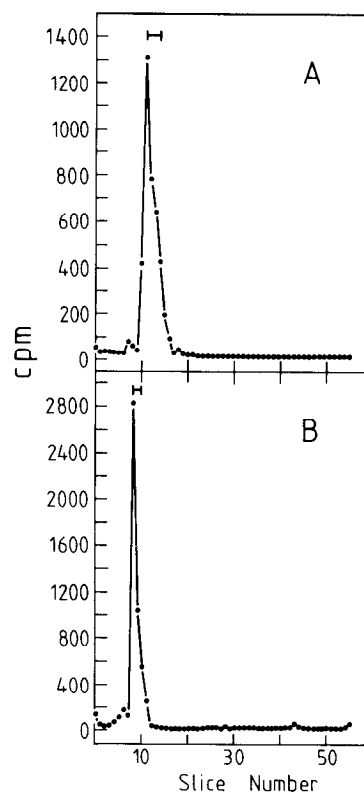


FIG. 4. Electrophoretic analysis of immunoprecipitates of radioactive F_1 inhibitor from crude extracts of the normal laboratory strain D273-10B. Extraction of the labeled cells by brief heat treatment, immunoprecipitation of the F_1 inhibitor, and analysis of the immunoprecipitates in 20% acrylamide gels containing sodium dodecyl sulfate were performed as outlined under "Materials and Methods." Prior to electrophoresis 35 μ g of purified inhibitor were added to each gel. A, cells grown in the presence of radioactive leucine, as described earlier (38). B, cells pulse-labeled with radioactive leucine according to Groot *et al.* (47): 2 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 30 min in the presence of 25 μ Ci/ml of L-[4,5- 3 H]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_1 inhibitor in presence and absence of antibiotics

Yeast cells were pulse-labeled with L-[4,5- 3 H]leucine as detailed in the legend of Fig. 4. The concentration of cycloheximide was 100 μ g/ml. Acriflavine was added at a concentration of 12.5 μ g/ml. Extraction of the cells and immunoprecipitation of the F_1 inhibitor were carried out as described under "Materials and Methods."

Labeling conditions	Maximal amount of radioactivity precipitable with 100 μ l of antiserum
No antibiotic	1448
Cycloheximide	18
Acriflavine	644

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 $100,000 \times g$ supernatant.³ This seems to indicate that the inhibitor is predominantly, if not exclusively, localized in the mitochondria. (d) Although the inhibitor pro-

³ Eberhard Ebner, unpublished results.

TABLE V

Inhibitor content and specific activity of F_1 -ATPase in different strains of *Saccharomyces cerevisiae*

The yeast strains examined are listed under "Materials and Methods." The F_1 inhibitor was determined in crude extracts (*cf.* Step 1 of the purification procedure). F_1 -ATPase activity was measured in the mitochondrial fraction.

Strain	F_1 inhibitor content of 1 ml of crude extract ^a		Specific activity of F_1 -ATPase
	units ^b	μg^c	
Commercial bakers' yeast	0.8	19	1.5
D273-10B ("grande")	0.7	15	2.0
D273-10B-1 (cytoplasmic petite)	0.1	2	0.4
Pet 936 (nuclear F_1 -deficient mutant)	0.2	3	0.2

^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 F_1 -ATPase.

^c Determined by immunoprecipitation.

tein described here is synthesized on cytoplasmic ribosomes, there is some evidence that its accumulation is subject to mitochondrial control. For example, pulse-labeling of the inhibitor *in vivo* with radioactive leucine was partially suppressed by acriflavine (*cf.* Table IV) and the inhibitor could not be found in extracts prepared from a cytoplasmic petite mutant.

In view of the proposed function for the F_1 inhibitor in controlling the backflow of energy from ATP to the mitochondrial inner membrane, its absence in the cytoplasmic petite mutant seems to make sense, since the mutant has lost a functional respiratory system. As a result, energy can only be fed into its mitochondrial system via cytoplasmically generated ATP (fermentative ATP). It will be of interest to learn how the mitochondrial genetic system controls the accumulation of the F_1 inhibitor.

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