Isolation of Supernumerary Yeast ATP Synthase Subunits e and i

CHARACTERIZATION OF SUBUNIT i AND DISRUPTION OF ITS STRUCTURAL GENE ATP18*

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Two subunits of the yeast ATP synthase have been isolated. Subunit e was found loosely associated to the complex. Triton X-100 at a 1% concentration removed this subunit from the ATP synthase. The N-terminal sequencing of subunit i has been performed. The data are in agreement with the sequence of the predicted product of a DNA fragment of Saccharomyces cerevisiae chromosome XIII. The ATP18 gene encodes subunit i, which is 59 amino acids long and corresponds to a calculated mass of 6687 Da. Its pI is 9.73. It is an amphiphilic protein having a hydrophobic N-terminal part and a hydrophilic C-terminal part. It is not apparently related to any subunit described in other ATP synthases. The null mutant showed low growth on nonfermentable medium. Mutant mitochondria display a low ADP/O ratio and a decrease with time in proton pumping after ATP addition. Subunit i is associated with the complex; it is not a structural component of the enzyme but rather is involved in the oxidative phosphorylations. Similar amounts of ATP synthase were measured for wild-type and null mutant mitochondria. Because 2-fold less specific ATPase activity was measured for the null mutant than for the wild-type mitochondria, we make the hypothesis that the observed decrease in the turnover of the mutant enzyme could be linked to a proton translocation defect through $F_0$.

The mitochondrial ATP synthase is the major enzyme responsible for aerobic synthesis of ATP. ATP synthase exhibits a tripartite structure consisting of a head piece (the catalytic sector), a base piece (the membrane sector), and a connecting stalk. However, the enzyme can be resolved into two parts: the first part is the catalytic sector called $F_1$, which is a watersoluble entity, composed of subunits $\alpha$, $\beta$, $\gamma$, $\delta$, and $\epsilon$, which retains the ability to hydrolyze ATP when in a soluble form; the second part is a detergent-soluble entity, called $F_0$, which is embedded in the membrane and is composed of hydrophobic subunits forming a specific proton pathway. The connecting stalk is composed of elements of both $F_1$ and $F_0$. When the two sectors are coupled, the enzyme functions as a reversible $\text{H}^+$-transporting ATPase or ATP synthase (1, 2). The model for energy coupling by $F_0$ ATP synthase that has gained the most general support is the binding change mechanism (3). This concept has been strengthened by the crystal structure of the major part of the bovine $F_1$ (4). The affinity changes for the substrates and products at the catalytic sites are coupled to proton translocation via the rotation of subunits that belong to $F_1$ and $F_0$ (for review, see Refs. 5–8). As a result, the Escherichia coli ATP synthase could be a rotary motor with a rotor that is composed of subunits $\gamma$ and $\epsilon$ and the dicyclohexylcarbodiimide binding protein oligomer, all other subunits being parts of the stator.

The E. coli ATP synthase and the bovine enzyme contain 8 and 16 different types of subunits, respectively (9). In the case of Saccharomyces cerevisiae, the ATP synthase is composed of at least 13 different kinds of subunits involved in the structure of the enzyme (10); the disruption of each of their structural genes leads to a lack of assembly of the complex. Additional subunits are involved in the regulation of the catalytic sector, such as the natural inhibitory peptide of the mitochondrial ATPase (11) and stabilizing factors in yeast (12, 13). Other additional subunits ($e$–$g$), which have been identified in the beef enzyme (9, 14) and found to be associated with the membranous sector (15), have their homologue in the yeast enzyme. With the exception of subunit f (16), the two other components, e and g, appear as being not essential for the structure of the mitochondrial enzyme, because their respective gene disruption does not significantly alter yeast growth on nonfermentable carbon sources (17–19). In this paper, we describe a new component of the yeast ATP synthase, the gene disruption of which leads to a decrease in oxidative phosphorylation yield.

EXPERIMENTAL PROCEDURES

Biochemical Procedures—The S. cerevisiae strain D273-10B/A/H/U (MATa, met6, ura3, his3; Ref. 20) was the wild-type strain. Cells were grown aerobically at 28 °C in a complete liquid medium containing 2% lactate as a carbon source (21) and harvested in logarithmic growth phase. Mitochondria were prepared according to the methods of Lang et al., (22) and Guérin et al., (23). Protein amounts were determined according to the method of Lowry et al., (24) in the presence of 5% SDS using bovine serum albumin as standard. The specific ATPase activity was measured at pH 8.4 according to the method of Somlo (25). Oxygen consumption rates were measured as described by Rigoulet and Guérin (26). Variations of transmembrane potential ($\Delta \Psi$) were evaluated by measurement of fluorescence quenching of rhodamine 123 with an SFM25 Kontron fluorescence spectrophotometer (27).

Purification of the ATP Synthase—Immunoprecipitated samples of ATP synthase were prepared from 2 mg of mitochondrial proteins as described by Todd et al. (28). Polyclonal antibodies raised against the $\alpha$-subunit were added to the 100,000 × g supernatant of the 0.375% Triton X-100 extract. The immunoprecipitated materials were washed with increasing Triton X-100 concentrations. The final pellet was dried under vacuum and then dissolved in 20 μl of dissociation buffer devoid of reducing agent. A 10-μl aliquot of this sample was analyzed by

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Large amounts of ATP synthase were prepared according to a modification of a procedure by Arselin et al. (29). To a known volume of a mitochondrial suspension at 10 mg of protein/ml in 0.1 M Tris acetate, 1 mM ATP, 1 mM EDTA, pH 8.0, was added an equal volume of 0.75% (w/v) Triton X-100. After a 20-min incubation time at 4 °C, the sample was centrifuged at 100,000 × g for 15 min at 4 °C, and the obtained supernatant was diluted with an equal volume of the previous buffer and centrifuged at 300,000 × g for 5 h. The pellet dissolved in the minimal volume of 20 mM Tris acetate, 65 mM sucrose, 1 mM EDTA, 0.1% Triton X-100, pH 7.5, was submitted to gel permeation chromatography followed by an ion exchange chromatography (29).

**Purification of Subunits e and i**—The ATP synthase was concentrated by the centrifugation method described above, and the pellet was dissolved in 6 M guanidinium chloride, 0.1 M NaCl, 0.05 M Tris-HCl, pH 8.0. After centrifugation at 100,000 × g for 20 min, the supernatant was submitted to gel permeation chromatography on a Superdex 75 (HR 10/30) column (Amersham Pharmacia Biotech) eluted with the same buffer at a flow rate of 0.2 ml/min. Protein-containing fractions of relative molecular masses of 10 and 5 kDa were immediately submitted to reverse phase HPLC on a Vydac C18 column (15 × 0.46 cm). Elution was done by a linear acetonitrile gradient (24–71%) containing 0.1% trifluoroacetic acid that was developed for 40 min. Protein-containing fractions were dried under vacuum and stored at −20 °C.

**Other Biochemical Procedures**—SDS-PAGE was performed according to the method of Schägger and Von Jagow (30). The slab gel was then silver-stained according to the method of Ansorge (31). Western blot analyses were performed as described previously (29). The polyclonal antibodies anti-TIM11 raised against subunit e were kindly provided by Dr. Neupert (Institut für Physiologische Chemie der Universität München, München, Germany). ProBlott membranes were incubated with peroxidase-labeled antibodies and revealed with the ECL reagent of Amersham Pharmacia Biotech. Automated sequence analyses were performed with an Applied Biosystems model 491 liquid phase sequanator. The electrospray mass spectrum of the intact subunit i was obtained on an Autospec mass spectrometer (Fisons VG-Analytical) fitted with an electrospray source. Analysis conditions were described previously (16). Proteinolytic cleavage and peptide separation were as described by Arselin et al. (32).

**Cloning and Sequencing of the ATP18 Gene**—The yeast gene ATP18 was amplified by polymerase chain reaction from yeast genomic DNA. Two oligonucleotides, 5'-GAAGTCTTCTTGAATTCCGTG-3' (104,804–104,804, Crick strand, chromosome XIII) and 5'-CATGGATCCATACTGGCA-3' (103,388–103,408, Watson strand, chromosome XIII) were used. The 5' oligonucleotide contained an EcoRI site. A BamHI site was created by the 3' oligonucleotide. Polymerase chain reaction amplification resulted in a 1402-bp EcoRI-BamHI DNA fragment that was inserted into an EcoRI-BamHI-cleaved pUC18 vector. After cloning, the 1402-bp EcoRI-BamHI DNA fragment was inserted into an M13 mp9 vector for sequencing as described by Sanger et al. (33). The oligonucleotide 5'-CATGGATCCATACTGGCA-3' (103,388–103,408, Watson strand, chromosome XIII) was used. This fragment was cloned into the pRS316 vector. The N-terminal part of the protein is blocked, because sequencing gave very low yields of amino acid. However, the following VNVLR amino acid residues were detected in cycles 3–7. Trypsin cleavage of the protein resulted in a few peptides that were purified by reverse phase HPLC (not shown). One of these peptides had the sequence NLEDPNIFEFV, a sequence that is found in subunit e at the positions 72–82. Confirmation of the identification of this subunit was brought by Western blot analysis. Subunit e migrated as a broad band on SDS-PAGE at the same level as subunit f (Figs. 1 and 2). When using low concentrations of Triton X-100 (0.1–0.375%), this protein remained associated with the yeast ATP synthase, but it was released in the presence of 1% Triton X-100 (Fig. 2, B and C). Subunit e is not essential to the structure of the enzyme, because it has been reported that inactivation of the gene encoding for subunit e results in slow growth and a decrease in mitochondrial respiration (17). This behavior is in agreement with the weak association of the subunit with the complex we have shown.

Reverse phase HPLC of the 5-kDa Superdex fraction gave two main peaks (Fig. 1C). The first peak was identified as the e-subunit because of its retention time (32 min) and by Western blot analysis (not shown). The second peak, named subunit i and showing a retention time of 37 min, was analyzed. Like the e-subunit, its relative molecular mass on SDS-PAGE was in the range of 5 kDa. It was poorly stained by the silver-staining technique, which explains its lack of detection in purified ATP synthase (Fig. 1D, lane 1). The N-terminal part of the protein as a component of the mitochondrial ATP synthase (14, 18). This protein was absent in our previous preparations. The concentration of 1% (w/v) Triton X-100 that had been previously used to wash the yeast ATP synthase immunoprecipitates was found to remove components migrating in SDS-PAGE in the relative molecular mass range of 10 kDa. The same was true in the case of our previous purification procedure for isolating large amounts of enzyme. In the experimental procedure, there was an ultrafiltration step, the aim of which was to concentrate the solubilized ATP synthase (29). This step also increased the detergent concentration and, as a consequence, removed loosely bound proteins. Thus, to keep the Triton X-100 concentration at 0.1%, we modified the purification procedure. To remove insoluble proteins, the crude 0.375% Triton X-100 extract was centrifuged. The supernatant was then diluted to obtain a detergent concentration of 0.1%, and centrifugation at 300,000 × g for 5 h made it possible to pellet the complex. Finally, the pellet was dissolved in a buffer containing 0.1% Triton X-100, and the ATP synthase was purified by molecular sieving and ion exchange chromatography (29). Purification of supernumerary subunits was achieved by two chromatographic steps. Gel permeation chromatography under denaturing conditions led to two well separated peaks containing proteins of relative molecular masses of 5 and 10 kDa (Fig. 1A). Reverse phase HPLC of the 10-kDa fraction made it possible to separate three components (Fig. 1B), which were analyzed by the Tricine-SDS-PAGE of Schägger and Von Jagow (30); this procedure allows an efficient separation of proteins with molecular masses that are in the range of 3–10 kDa. By contrast with what was observed with the Laemmli method (36), subunits 4 and 6 were not resolved, and the δ-subunit migrated more slowly than subunit h (Fig. 1D). The first two peaks (Fig. 1B) were identified as subunits h and f by their retention times (28 and 36 min, respectively), by SDS-PAGE analysis (Fig. 1D, lanes 2 and 3, respectively) and by Western blotting (not shown). The third component, showing a retention time of 39 min, migrated at the same relative molecular mass as subunit f on SDS-PAGE (Fig. 1D, lane 4). From amino acid sequencing of the whole protein, we assume that the protein is blocked, because sequencing gave very low yields of amino acid. However, the following VNVLR amino acid residues were detected in cycles 3–7. Trypsin cleavage of the protein resulted in a few peptides that were purified by reverse phase HPLC (not shown). One of these peptides had the sequence NLEDPNIFEFV, a sequence that is found in subunit e at the positions 72–82. Confirmation of the identification of this subunit was brought by Western blot analysis. Subunit e migrated as a broad band on SDS-PAGE at the same level as subunit f (Figs. 1 and 2). When using low concentrations of Triton X-100 (0.1–0.375%), this protein remained associated with the yeast ATP synthase, but it was released in the presence of 1% Triton X-100 (Fig. 2, B and C). Subunit e is not essential to the structure of the enzyme, because it has been reported that inactivation of the gene encoding for subunit e results in slow growth and a decrease in mitochondrial respiration (17). This behavior is in agreement with the weak association of the subunit with the complex we have shown.

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The molecular mass of the predicted protein is 6687.5 Da. This is in agreement with the measured mass of 6687.1 Da. The amino acid residues was identified and named ATP18 (Fig. 3). A primer of 177-bp DNA sequence encoding for a protein containing 59 amino acid residues (37). From these data, a 1402-bp DNA fragment was amplified by the polymerase chain reaction from our wild-type yeast strain D273-10B/A/H/U, cloned in a pUC18 vector, and sequenced. A 177-bp DNA sequence encoding for a protein containing 59 amino acid residues was identified and named ATP18 (Fig. 3). The molecular mass of the predicted protein is 6687.5 Da. This value is in agreement with the measured mass of 6687.1 ± 2 Da (data not shown) obtained from the electrospray mass spectrum of subunit i. Therefore, subunit i is devoid of a cleavable leader sequence. Sequencing showed that the first amino acid residue of subunit i was a methionine. Because there is no other initiating codon upstream from the gene, and because a stop codon is present at -18 bp upstream from the ATG, the first amino acid residue is the initiating methionine. Subunit i is a basic protein with a calculated pi of 9.73 and is amphipathic. It contains a hydrophobic N-terminal domain (residues 30–50), thus suggesting a possible α-helix transmembranous domain. The C-terminal part of the molecule (residues 30–50) is hydrophilic. A similar DNA sequence has been reported for Schizosaccharomyces pombe chromosome I.2 When comparing the hypothetical protein of S. pombe and subunit i of S. cerevisiae, a 51% similarity was found.

**Phenotypic Analysis of the Null Mutant ΔATP18**—The ATP18 gene was disrupted. The resulting strain (ΔATP18) displayed the same doubling time as that of the wild-type strain with a fermentable carbon source, whereas it showed a slow growth with an oxidative carbon source (Table I). With lactate as carbon source, a 2-fold lesser amount of matter was formed by the mutant strain than by the wild-type strain. This result also points to a defect in the oxidative phosphorylations. In addition, at stationary phase, with lactate as a carbon source, a recovery of the doubling time was measured with the complemented strain, thus showing that the ATP18 gene is likely involved in the oxidative phosphorylations. The cytochrome content of cells grown with lactate as a carbon source, a 2-fold lesser amount of matter was formed by the mutant strain than by the wild-type strain. This result also points to a defect in the oxidative phosphorylations. The cytochrome content of cells grown with lactate as a carbon source, a 2-fold lesser amount of matter was formed by the mutant strain than by the wild-type strain.

**Fig. 1.** Purification of subunits e and i. A, gel permeation chromatography on a Superdex 75 column of yeast ATP synthase subunits solubilized by 6 M guanidinium chloride (see "Experimental Procedures"). B, reverse phase chromatography of the 10-kDa peak from A, C, reverse phase chromatography of the 5-kDa peak from A. D, SDS-PAGE of 32 μg of ATP synthase (lane 1), subunit h (lane 2), subunit f (lane 3), subunit e (lane 4), and subunit i (lane 5). The slab gel was silver-stained. su., subunit; oscp, oligomycin sensitivity-conferring protein.

**Fig. 2.** Subunit e is loosely associated to the yeast ATP synthase. A, SDS-PAGE of ATP synthase. The enzyme was purified in the presence of 0.1% Triton X-100. Lane 1, 16 μg; lane 2, 32 μg. B, SDS-PAGE of ATP synthase immunoprecipitated with 90 mM NaCl, 20 mM Tris acetate, pH 7.5, and increasing Triton X-100 concentrations. Only a part of the silver-stained slab gel is shown. Lane 1, subunit h; lane 2, 0.1% Triton X-100; lane 3, 0.375% Triton X-100; lane 4, 1% Triton X-100. C, wild-type mitochondria (2 mg of mitochondrial protein) were solubilized with 0.375% Triton X-100 in a final volume of 0.4 ml. After centrifugation at 100,000 × g for 20 min at 4 °C, 0.1-ml aliquots of the supernatant were incubated with 0.1% Triton X-100 (lanes 1 and 4), 0.375% Triton X-100 (lanes 2 and 5), and 1% Triton X-100 (lanes 3 and 6). The 0.1% Triton X-100 sample was obtained by dilution with the extraction buffer devoid of detergent. The samples were incubated for 30 min at 4 °C and then centrifuged at 300,000 × g for 1 h to pellet the enzyme. The 300,000 × g supernatants (lanes 4–6) were precipitated by 0.3 M trichloroacetic acid and centrifuged at 10,000 × g for 10 min, and the pellets were washed twice with cold acetone before solubilization with 50 μl of dissociation buffer. The 300,000 × g pellets (lanes 1–3) were dissolved in the same volume of dissociation buffer. A volume of 10-μl aliquots of each dissociated sample was submitted to a Western blot analysis. Polyclonal antibodies raised against subunit e (dilution, 1:10,000) were used to probe the membrane. su., subunit; oscp, oligomycin sensitivity-conferring protein.

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2 D. Harris and R. Squares, unpublished results, EMBL accession number Z89753.
null mutant mitochondria (data not shown). We verified that ATP addition did not induce a proton leak with the null mutant mitochondria (data not shown), as in the case of a strain having a mutation in the hydrophobic part of subunit 4 (39).

The oligomycin-sensitive ATPase activity of both of these two strains was measured at pH 8.4 (25) either from freshly isolated mitochondria or from frozen and thawed mitochondria (Table II). After freezing and thawing of mitochondria, we have previously shown that there is a 2-fold increase in the wild-type mitochondrial ATPase activity (20). In these experimental conditions, this ATPase activity increase corresponds to the release of the natural inhibitory peptide of the mitochondrial ATPase.3 Null mutant mitochondria displayed a 30–37% oligomycin-insensitive ATPase activity compared with a 9–13% for the wild type. It is clear that under these experimental conditions, null mutant mitochondria showed an uncoupling between F1 and F0. It is to be pointed out that the mitochondrial ATPase activity (for review, see Ref. 38). As expected, KCN addition by inhibiting proton pumping mediated by the respiratory chain collapsed the ΔΨ, and subsequent ATP addition promoted a fluorescent quenching of the dye that was dicyclohexylcarbodiimide- (Fig. 4) and oligomycin-sensitive (not shown), thus reflecting the proton pumping mediated by the ATPase. In the case of null mutant mitochondria, there was also an ATP-dependent fluorescent quenching of the dye, but it showed a slow and significant decrease with time. This unexpected result could correspond to a loss of activity of the mutant enzyme. The ATP-dependent fluorescent quenching of null mutant mitochondria was also oligomycin-sensitive (not shown).

Wild-type and null mutant mitochondria were prepared by the protoplast method. The uncoupled respiration rate of null mutant mitochondria with NADH as substrate was 85% of that of the wild type (Table II), which correlates with the decrease in cytochrome oxidase amount measured in the cells. Although a similar state 4 was obtained for both kinds of mitochondria, isolated null mutant mitochondria displayed a low respiratory rate in the presence of ADP (state 3), leading to a significant difference in respiratory control (Table II). The ADP/O ratio of −1 calculated for the null mutant mitochondria indicates a lower efficiency of oxidative phosphorylations compared with the wild type.

Proton-pumping activities of the mitochondrial preparations were measured in the same conditions as those used for the respiration rate measurements (Fig. 4). In the presence of ethanol, wild-type mitochondria displayed a fluorescent quenching of rhodamine 123, which was transiently decreased with addition of 50 μM ADP, thus reflecting a decrease in the transmembranous ΔΨ attributable to a proton influx through F0 during ADP phosphorylation. After ADP consumption, the ΔΨ increased, and finally carbonyl CCCP addition produced a reversal of the fluorescent quenching corresponding to the collapse of the Δψ. In the case of null mutant mitochondria, ADP addition induced a lower decrease in fluorescent quenching of the dye. In addition, the time during which ADP was consumed was significantly increased. These two points have to be correlated with the observed low ADP/O ratio of null mutant mitochondria. Modifications of the transmembrane ΔΨ mediated by the ATPase proton-pumping activity were analyzed after energizing mitochondria by ethanol, an activation step that is necessary to remove the natural inhibitory peptide of the mitochondrial ATPase, which otherwise would inhibit the

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Oxidative phosphorylation measurements and ATPase activities of wild-type and null mutant mitochondria

D273–10B/A/H/U (wild-type) and null mutant (ΔATP18) mitochondria were prepared from yeast cells grown with 2% lactate as a carbon source. Five and two different mitochondrial preparations were made from wild-type and null mutant cells, respectively. Results are the average of four different experiments and are presented with the S.D. Oligomycin (6 μg/ml) and CCCP (3 μM) were added where indicated. Respiration rates were obtained with NADH as substrate.

| Mitochondria          | Respiration rates (nmol of O/min/mg of protein) | Respiratory control (nmol of Pi/min/mg of protein) | ATPase activity (nmol of Pi/min/mg of protein) |
|-----------------------|-----------------------------------------------|--------------------------------------------------|-----------------------------------------------|
|                       | CCCP State 4 State 3                            |                                                  | −Oligomycin +Oligomycin                       |
| Fresh wild type       | 1615 ± 200 395 ± 110 1020 ± 120                 | 2.7 ± 0.6 1.6 ± 0.1                               | 3635 ± 155 470 ± 20                           |
| ΔATP18                | 1380 ± 130 365 ± 10 645 ± 6                     | 1.77 ± 0.04 1.01 ± 0.06                           | 1885 ± 70 710 ± 65                            |
| Frozen and thawed wild type | 10 645 10 120                                    | 1.77 ± 0.04 1.01 ± 0.06                           | 5750 ± 170 485 ± 70                           |
| ΔATP18                | 110 1020                                            |                                                  | 2230 ± 260 630 ± 80                           |

Yeast ATP Synthase Subunit i

We report here that, as shown previously (18), subunit e is associated with the yeast ATP synthase and that, in addition, this subunit is easily removed from the complex by Triton X-100. Therefore, this protein is not essential to the structure of the enzyme. Another component of the ATP synthase, named subunit i, is also described. So far, such an additional protein has not been identified in the beef enzyme. The absence of subunit i in the null mutant does not alter the structure or the assembly of the yeast ATP synthase. We have observed that polyclonal antibodies raised against the α-subunit made it possible to precipitate the wild-type and the null mutant ATP synthase from mitochondrial 0.375% Triton X-100 extracts, and that the obtained immunoprecipitates showed similar patterns of subunits on SDS-PAGE (not shown).

Disruption of the ATP18 gene showed that this subunit is essential for oxidative phosphorylations. However, the efficiency of the phosphorylating system is decreased with inactivation of the ATP18 gene, and this probably originates from a reduced turnover of the enzyme. It has been described that mutants in F0 often display pleiotropic effects such as low oxygen consumption and low cytochrome oxidase activity (40–44). The experimental data give insight into a mechanistic defect that is expressed during ATP synthase function rather than a deficiency caused by a decrease in the cytochrome oxidase amount. ON the basis of all the data presented in this paper, we conclude that the null mutant ATP synthase, devoid
of the supernumerary subunit i, shows in vitro a defect in proton translocation through F₀ during ATP synthesis and ATP hydrolysis. In vivo, this alteration leads to a slow growth of mutant cells and the production of a low amount of matter on nonfermentable carbon sources. Experiments are in progress to elucidate the involvement of subunit i in the activity of the ATP synthase, especially at the proton translocation level, and to investigate the relationships of subunit i with other known components of the yeast complex.

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