ATP Synthase of Yeast Mitochondria

ISOLATION OF SUBUNIT \( j \) AND DISRUPTION OF THE ATP18 GENE

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The subunit composition of the mitochondrial ATP synthase from Saccharomyces cerevisiae was analyzed using blue native gel electrophoresis and high resolution SDS-polyacrylamide gel electrophoresis. We report here the identification of a novel subunit of molecular mass of 6,687 Da, termed subunit \( j \) (Su \( j \)). An open reading frame of 127 base pairs (ATP18), which encodes for Su \( j \), was identified on chromosome XIII. Su \( j \) does not display sequence similarity to ATP synthase subunits from other organisms. Database searches, however, identified a potential homolog from Schizosaccharomyces pombe with 51% identity to Su \( j \) of S. cerevisiae. Su \( j \), a small protein of 59 amino acid residues, has the characteristics of an integral inner membrane protein with a single transmembrane segment. Deletion of the ATP18 gene encoding Su \( j \) led to a strain (\( \Delta su j \)) completely deficient in oligomycin-sensitive ATPase activity and unable to grow on nonfermentable carbon sources. The presence of Su \( j \) is required for the stable expression of subunits \( 6 \) and \( f \) of the \( F_0 \) membrane sector. In the absence of Su \( j \), spontaneously arising rho\(^-\) cells were observed that lacked ubiquinol-cytochrome \( c \) reductase and cytochrome \( c \) oxidase activities. We conclude that Su \( j \) is a novel and essential subunit of yeast ATP synthase.

Yeast mitochondrial ATP synthase (1) is similar to the corresponding bovine enzyme (2, 3) regarding its polypeptide composition, but there are also differences. All components of the bovine catalytic sector of the ATP synthase (\( F_1 \)), i.e., subunits \( \alpha \), \( \beta \), \( \gamma \), \( \delta \), \( \varepsilon \), and the inhibitor protein (IF\(_1\)), have homologous counterparts in Saccharomyces cerevisiae (4–9). The structural similarity extends to subunits of the membrane sector (\( F_0 \)) and second stalk, i.e., subunit \( a \) (Su \( a \) or Su \( 6 \)) (10), Su \( b \) (11), Su \( c \) (proteolipid or Su \( 9 \)) (12), which is mitochondrialy encoded in yeast but nuclearly encoded in mammals, Su \( d \) (13), oligomycin sensitivity conferring protein (14), Su \( e \) (15), and Su \( f \) (16). In addition, a gene encoding a putative homolog of subunit \( g \) of the bovine ATP synthase has been identified in the yeast genome (Su \( g \)). The corresponding protein, however, has not been observed in the isolated ATP synthase yet. A yeast homolog of bovine subunit \( e \) has also been reported recently (17). Subunit \( e \), also known as Tim11, was originally reported as being a component of the mitochondrial inner membrane import machinery (18); however, it has subsequently been shown to be a membrane-bound subunit of the ATP synthase complex (17). Although in mitochondria it is associated with the ATP synthase complex and can be co-immunoprecipitated with subunits of the \( F_1 \) sector (17), subunit \( e \), like subunit \( g \), has not been identified yet in the purified ATP synthase enzyme. A yeast homologue to bovine subunit \( F_6 \) (19) so far has not been reported. Conversely, a novel subunit, subunit \( h \), has been found recently in yeast ATP synthase (20), which appears not to be related to any of the known subunits of bovine or other ATP synthases.

The experiments described in this paper focus on the reassessment of the polypeptide composition of the yeast ATP synthase using a different isolation technique, namely blue native electrophoresis (BN-PAGE). BN-PAGE is a microscale technique for the separation of the multiprotein complexes of oxidative phosphorylation directly from isolated mitochondrial membranes (21). Combined with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a second dimension, an overview on the protein subunits of all oxidative phosphorylation complexes is obtained in a two-dimensional gel (22). Using this two-dimensional electrophoretic technique, we observed in the ATP synthase the presence of a previously undetected protein in the 6–7-kDa range. N-terminal protein sequencing revealed it to be a novel subunit of the ATP synthase, subunit \( j \) (Su \( j \)). Su \( j \) is encoded by a gene termed here ATP18 and has no apparent bovine counterpart.

EXPERIMENTAL PROCEDURES

Materials—Aminocaproic acid (6-aminohexanoic acid) and imidazole were from Fluka, Tricine and Serva Blue G (Coomassie Blue G-250) were from Serva, and phenylmethylsulfonyl fluoride was from Sigma. Hydroxyapatite was prepared as described recently (23).

Yeast Strains and Growth Conditions—For construction of the \( \Delta su \) j:HIS3 yeast strain, introduction of the HIS3 gene resulting in a partial deletion and disruption of the ATP18/Su \( j \) gene was performed as follows. The HIS3 gene was amplified from the plasmid pFA6a-HIS3MX6 (24) using the following primers: S1, 5'-GTTTAACATAC-GACGAGCATATTGATGGATTGTACTGGAACATCGCCATGCCTACGCTGAGGCTGAGATTTAGAAGAAAGG-3'; and S2, 5'-GAAATTCCTCGCTGTTAAGGAGGAAGGGTACGACGATCGATGAAATTGATGGATTGTACTGGAACATCGCCATGCCTACGCTGAGGCTGAGATTTAGAAGAAAGG-3'.

Correct integration of the HIS3 marker into the ATP18/Su \( j \) locus was

\[ \text{GACGACAGATTAATTGATTGGATTGTACTGCCATGCGTACGCTGAGGCTGAGATTTAGAAGAAAGG-3'} \]

\[ \text{corresponding to nucleotides} \quad \text{+43 to +3 of the ATP18/Su j locus.} \]

The resulting polypeptide chain reaction product was transformed into the haploid yeast strain W303-1A using the lithium acetate method (25), and HIS3 positive clones were selected. Correct integration of the HIS3 marker into the ATP18/Su \( j \) locus was
confirmed by polymerase chain reaction using oligonucleotides that
primed upstream and downstream of the disrupted ATP18 gene.

The resulting yeast strain Δsu j, the corresponding wild-type W303-
1A, and Δsu f were grown in YPGal medium supplemented with 0.5%
lactate at 30 °C (26). Cells were harvested by centrifugation at 1,800
3 g, washed three times with sucrose buffer (250 m M sucrose, 5 m M
6-aminohexanoic acid, 10 m M Tris/HCl, pH 7.0), and used directly for
preparation of mitochondrial membranes.

Isolation of Mitochondrial Membranes—About 5 g (wet weight) of
sedimented cells, 5 ml of glass beads (0.25–0.5 mm), and 5 ml of sucrose
buffer were vortexed for 10 min in a 50-ml tube. After dilution with
sucrose buffer, the sedimented glass beads were removed, and the
supernatant was centrifuged for 20 min at 1,250 × g. Mitochondrial
membranes were then collected by centrifugation for 30 min at
18,000 × g, taken up with sucrose buffer at a protein concentration of
10–30 mg/ml, and stored at −20 °C.

For the analysis of the submitochondrial localization of Su j, intact
mitochondria were isolated according to previously published methods
(26).

Electrophoretic Techniques—BN-PAGE was performed as described
previously (22) with the following modifications. Mitochondrial mem-
branes (400 μg of protein) were sedimented by centrifugation for 10 min
at 100,000 × g. The pellet was suspended with 40 μl of 50 mM NaCl, 2
m M 6-aminohexanoic acid, 1 mM EDTA, 50 mM imidazole HCl, pH 7.0,
and 1.0 μl of 0.5 mM phenylmethylsulfonyl fluoride in Me2SO was added.
Membrane protein complexes were solubilized by the addition of Triton
X-100 (9.6 mgl from a 10% (w/v) stock solution, 2.4 g of Triton X-100/g of
protein). After centrifugation for 20 min at 100,000 × g, the superna-
tant was supplemented with 5 μl of a Coomassie Blue G-250 dye
suspension (5% Serva Blue G (w/v) in 750 mM 6-aminohexanoic acid)
and immediately applied to a 1.6-mm acrylamide gradient gel for ana-
lytical BN-PAGE (1-cm gel well, linear 4–13% acrylamide gradient gel
overlaid with a 4% sample gel).

For SDS electrophoresis, the Tricine-SDS-PAGE (27) or the Laemmli
system (28) was used. Two-dimensional electrophoresis (BN-PAGE/
Tricine-SDS-PAGE), staining, and densitometric quantification were
performed as described previously (29, 30).

Isolation of ATP Synthase, Separation of Subunits, and N-terminal
Sequencing—All steps were performed at 4 °C, and the pH values of all
buffers were adjusted to 4 °C unless otherwise indicated. Mitochondrial
membranes from the W303-1A strain (50 mg of protein) were washed
with a 4-fold volume of buffer 1 (50 mM NaCl, 2 mM 6-aminohexanoic
acid, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 mM imidaz-
ode HCl, pH 7.0) and collected by centrifugation for 60 min at 100,000 ×
g. The pellet was homogenized in 2.35 ml of buffer 1, and 0.6 ml of
Triton X-100 (20% w/v) was added (2.4 g of Triton X-100/g of protein).
Proteins were identified by direct Edman degradation or after deformylation (*), except Su 6, which was identified by Western blotting. The sequence of Su d was obtained without decylation, although the protein was reported to be acetylated (†). Small letters in the sequences indicate amino acids that were not identified. The masses of the mature proteins do not include N-terminal modifications. AA, number of amino acids.

| Band in SDS gel | Assignment | Gene | N-terminal sequence | Mature protein | Mass |
|-----------------|------------|------|---------------------|----------------|------|
| 1               | Su 9 oligomer | ATP9 | MQLVLA(*) | 76 | 7,759 | P00841 |
| 2               | Su α | ATP1 | ASTRAQPTEV | 510 | 54,952 | P07251 |
| 3               | Su β | ATP2 | ASAAPSTPI | 478 | 51,294 | P00850 |
| 4               | Su γ | ATP3 | ATLEKEVR | 278 | 30,614 | P38077 |
| 5               | Su 4 or Su b | ATP4 | MSGTEKQTD | 209 | 23,249 | P05626 |
| 6               | Su 5 or OSCP | ATP5 | ASKAAAAPPV | 195 | 20,870 | P09457 |
| 7               | Su 6 or Su a | ATP6 | No sequence | 249 | 27,856 | P00854 |
| 8               | Su d | ATP7 | sLNKSAANKL(*) | 173 | 19,677 | P30902 |
| 9               | Su δ | ATP16 | AKAAASAGSL | 138 | 14,553 | Q12165 |
| 10              | Su h | ATP14 | DVIQDLLYRE | 92 | 10,408 | Q12349 |
| 11              | Su f | ATP17 | VSTLIPPPK | 95 | 10,565 | Q06405 |
| 12              | Inhibitor protein | INH1 | sEgStGFGPRG | 63 | 7,383 | P30197 |
| 13              | Su ε | ATP15 | sNwPAG1 | 61 | 6,611 | P21306 |
| 14              | Su j | ATP18 | MLKKFPTTLPKYY | 59 | 6,687 | P81450 |
| 15              | Su 8 | ATP8 | MPQLVPEFY(*) | 48 | 5,822 | P00856 |

After centrifugation for 60 min at 100,000 × g, the supernatant was adjusted to 150 mM Na⁺ phosphate and loaded onto a 3-ml hydroxyapatite column equilibrated with buffer 2 (0.05% Triton X-100, 2 mM 6-amino-hexanoic acid, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 150 mM Na⁺ phosphate, pH 7.7). Hydroxyapatite-bound ATP synthase was washed at room temperature with 1 column volume of buffer 2 (0.1% Triton X-100, 333 mM 6-aminohexanoic acid, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 300 mM Na⁺ phosphate, pH 7.3) and eluted with buffer 4 (0.1% Triton X-100, 333 mM 6-aminohexanoic acid, 200 mM Na⁺ phosphate, pH 7.3). One ml of the fraction with highest ATP hydrolysis activity (about 30% of the total yield, 0.5 mg of total protein) was supplemented with 200 mM 6-aminohexanoic acid and loaded onto a 3-mm-thick preparative gel for BN-PAGE. The major blue band comprising ATP synthase, which was visible during BN-PAGE, was excised and cut into 4 pieces. A stack of these 4 pieces was processed by Tricine-SDS-PAGE in a second dimension and electroblotted onto Immobilon P membranes (30). The transferred proteins were sequenced directly using a 473A protein sequencer (Applied Biosystems) or after incubation in a 1:1 (v/v) mixture of trifluoroacetic acid and methanol (24 h at 37 °C) for deformylation (31). The precipitated proteins were sequenced directly using a 473A protein sequencer (Applied Biosystems) or after incubation in a 1:1 (v/v) mixture of trifluoroacetic acid and methanol (24 h at 37 °C) for deformylation (31).

Catalytic Activities—Oligomycin-sensitive ATP hydrolysis was measured at 25 °C using an assay coupled to the oxidation of NADH. Before the test, the buffer 0.25 mM NADH, 1 mM phosphoenolpyruvate, 2.5 units/ml lactate dehydrogenase, and 2 units/ml pyruvate kinase were added to the test buffer (250 mM sucrose, 50 mM KCl, 5 mM MgCl₂, 2 mM NaCl, 0.5 mM NADH, 0.05 mM Tris/HCl, pH 7.5). The reaction was started with protein without detergent and stopped by the addition of 25 μg of oligomycin from a 5 mg/ml stock solution in Me₂SO.

Antibody Production—Antisera against the C-terminal region of Su j were raised in rabbits against a chemically synthesized peptide CRFARGGKPVEVD that had been coupled to activated ovalbumin (Pierce).

Miscellaneous—Hypotonic swelling and carbonate extraction of mitochondria were performed as described previously (32, 33). Protein determination was performed according to Bradford (34) and a Lowry protocol in the presence of SDS (35).

## RESULTS

Isolation of ATP Synthase and Analysis Protein Subunit Composition—The ATP synthase binds stronger to hydroxyapatite than most other mitochondrial proteins of yeast. Therefore hydroxyapatite chromatography is an efficient technique for its isolation. After this first purification step, some of the known ATP synthase subunits can already be recognized in SDS-PAGE (Fig. 1A, lanes 3–5). The same fractions of the hydroxyapatite column were also applied to a gel for BN-PAGE. The S. cerevisiae ATP synthase (Fig. 1B, lanes 3–5) is slightly smaller than the bovine ATP synthase that was loaded in parallel. BN-PAGE was then repeated on the preparative scale using the hydroxyapatite fraction with the highest amount of ATP synthase complex (Fig. 1B, lane 4). The band of ATP synthase was excised, and the subunit composition of this

![FIG. 2. Sequence and subunit mitochondrial localization of Su j from S. cerevisiae and comparison to a S. pombe homolog.](image-url)
complex was analyzed further by SDS-PAGE. N-terminal protein sequencing of the resolved proteins (Fig. 1C) showed the presence of known subunits of the ATPase and one additional protein that was termed subunit j, Su j. To exclude the possibility that this protein may represent a contamination of the ATP synthase, a two-dimensional resolution of the sample from BN-PAGE, was revealed after resolution by SDS-PAGE in a second dimension (not shown). BHM, bovine heart mitochondria.

Subunit j could not be removed from the ATP synthase isolated by hydroxyapatite chromatography by adding 7 g of Triton X-100/g of protein and application to BN-PAGE. After the addition of Triton X-100 and 2 M urea and application to BN-PAGE, most of the ATP synthase was dissociated into the individual subunits. The residual fraction of holo-ATP synthase still contained Su j (data not shown).

Polypeptide Composition of ATP Synthase—Direct Edman degradation of the proteins transferred to Immobilon P or after deborynation (Su 8 and Su 9) confirmed the presence of known subunits of ATP synthase. The mature subunits α and β were found to be 4 and 14 amino acids, respectively, shorter than described in protein data bases (Table I). The presence of subunit 6 was confirmed by Western blotting and subsequent immunodecoration using a specific antiserum. The novel Su j protein was used to raise antibodies against Su j. Su j was sensitive to added protease in intact mitochondria (Fig. 2C). Because no streaking of this protein was observed, we conclude that Su j is a true constituent of the ATP synthase complex.

Subunit j is a protein anchored to the inner membrane by a single transmembrane domain at its N terminus and has an N-in-C-out orientation.

Deletion of the ATP18 Gene Leads to Spontaneously Arising Rho− Cells—To test whether the presence of Su j is essential for the activity of the F1F0-ATP synthase, the gene encoding Su j was deleted. The resulting yeast strain Δsu j was respiratory incompetent, as it could no longer grow on the nonfermentable carbon source glycerol, in contrast to its isogenic wild-type strain (Fig. 3). Enzymatic measurement of the F1F0-ATP synthase activity confirmed the loss of oligomycin-sensitive ATPase activity in isolated Δsu j in contrast to the wild-type control (results not shown); therefore Su j seemed to be an essential subunit of the yeast F1F0-ATP synthase. However, comparison of the mitochondrial proteins of the Δsu j and Δsu f strains by BN-PAGE (Fig. 4) and two-dimensional resolution (not shown) revealed that not only the ATP synthase but also cytochrome oxidase (complex IV) and ubiquinol-cytochrome c reductase (complex III) were below the limit of detection (<10% as compared with wild-type W303-1A). The reduced levels of complex III and IV can be explained by the fact that a spontaneous transition of the Δsu j cells to the rho− state was observed.3 Interestingly a similar formation of rho− cells was observed in the Δsu f strain, as reported by Spannagel et al. (16).

Su j is essential for growth on nonfermentable carbon sources. Yeast strains Δsu j and corresponding isogenic wild-type W303-1A grown on YPD (glucose-containing) medium were resuspended in sterile water at a concentration of 10 A578/ml. A dilution series was generated by serially diluting this suspension 10-fold each time. 2 μl of each of the resulting dilutions were spotted onto a YPG (glycerol-containing) plate (spots 1–5) and were incubated at 30 °C for 2 days.

A peptide corresponding to the C-terminal region of the protein was used to raise antibodies against Su j. Su j was localized to mitochondria by immunostaining. It was inaccessible to added protease in intact mitochondria (Fig. 2C). Disruption of the outer membrane by hypotonic swelling rendered Su j sensitive to the added protease. In addition, Su j was resistant to alkaline extraction and therefore most likely is an integral membrane protein (Fig. 2C).

In summary, Su j is a protein anchored to the inner membrane by a single transmembrane segment at its N terminus and has an N-in-C-out orientation.

Su j is required for the stable expression of F0 sector subunits. Mitochondria (50 μg of protein) isolated from the Δsu j strain and corresponding isogenic wild-type strain W303-1A were subjected to SDS-PAGE and analyzed by Western blotting for the presence of marker proteins, as indicated. CCPO, cytochrome c oxidase; Mge1p, a matrix-localized soluble protein.

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sequence on chromosome XIII was identified with the potential to encode for a protein of 59 amino acid residues (Fig. 2A). This gene encoding for Su j was termed ATP18. We identified a potential homolog in Schizosaccharomyces pombe, a hypothetical protein of 6.8 kDa. This potential Su j homolog in S. pombe was 51% identical to the Su j of S. cerevisiae. The hydropathy plots for both proteins were very similar and suggested them to be membrane proteins with a single transmembrane domain (Fig. 2B).

Using the obtained N-terminal sequence of Su j, a search for an open reading frame corresponding to a 6.5-kDa protein in the yeast genome data base was performed. A 177-base pair sequence on chromosome XIII was identified with the potential to encode for a protein of 59 amino acid residues (Fig. 2A). This gene encoding for Su j was termed ATP18. We identified a potential homolog in Schizosaccharomyces pombe, a hypothetical protein of 6.8 kDa. This potential Su j homolog in S. pombe was 51% identical to the Su j of S. cerevisiae. The hydropathy plots for both proteins were very similar and suggested them to be membrane proteins with a single transmembrane domain (Fig. 2B).

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F1 sector was reduced in the core of the ATP synthase (Fig. 5). In the absence of Suj, subunits 6 and f of the F0 sector were not detectable. The α-subunit of the F1 sector was reduced in the Δsu j strain. Levels of other mitochondrial marker proteins, such as cytochrome c peroxidase and Mge1p, were not altered in the absence of Su j.

DISCUSSION

The subunit composition of the yeast mitochondrial F1F0-ATP synthase was analyzed using the combined techniques of BN-PAGE and high resolution Tricine-SDS-PAGE. We present evidence here for the existence of a novel ATP synthase subunit, Su j. A homologue in the purified bovine ATP synthase complex has so far not been reported. The presence of an open reading frame in S. pombe with 51% amino acid sequence identity to the S. cerevisiae Su j suggests that Su j represents a general component of eukaryotic ATP synthases.

The novel Su j protein appears to represent a bona fide subunit of the ATP synthase. Su j purified with the ATP synthase after BN-PAGE. As this technique resolves proteins for the structure and function of ATP synthase.

Deletion of the gene encoding Su j (ATP18) gave rise to a respiratory-deficient phenotype and loss of measurable oligomycin-sensitive ATP synthase activity.

The tight binding of subunit j to the isolated yeast ATP synthase raises the question as to why subunit j was not previously identified in other ATP synthase preparations. The previous use of SDS gels probably did not yield sufficient resolution of the smaller subunits of the complex. As shown here, the use of high resolution Tricine-SDS-PAGE has optimized the separation of the yeast ATP synthase subunits in the molecular mass range of Su j. Furthermore, although we used mild solubilization with Triton X-100 and BN-PAGE as a one-step procedure, subunits g and e (Tim11) or a potential homologue of bovine subunit F6 were not found in association with the ATP synthase complex. Notably, recent variation of the conditions for protein solubilization and BN-PAGE led to the isolation of an ATP synthase with three more bound proteins, including subunits e (Tim11) and subunit g. The analysis of the role of these proteins for the structure and function of ATP synthase will be discussed separately (37).

In conclusion, we demonstrate here that Su j is an integral membrane protein, spanning the membrane once in an N_in–C_out orientation. The membrane association of Su j is compatible with it being a subunit of the F0 sector of the ATP synthase. We are currently investigating the association of Su j with other known F0 sector subunits.

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