α Subunit of Mitochondrial F$_1$-ATPase from the Fission Yeast

DEDUCED SEQUENCE OF THE WILD TYPE AND IDENTIFICATION OF A MUTATION THAT ALTERS APPARENT NEGATIVE COOPERATIVITY*

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The nuclear gene atp1 encoding the mitochondrial ATP synthase α subunit of the fission yeast Schizosaccharomyces pombe was sequenced. It contains a 1,608-base pair-long open reading frame interrupted by two introns of 175 and 269 base pairs, located near the 5'-end of the gene. The initiation site of transcription AAAC was located 60 nucleotides upstream of the translation initiation codon. The deduced polypeptide sequence contains a 27-amino acid residue presequence, presumably involved in mitochondrial targeting, preceding a mature protein of 509 amino acid residues. The atp1 alleles from mutant A2313 (Boutry, M., and Goffeau, A. (1982) Eur. J. Biochem. 125, 471–477) and its related phenotypic revertant R351 (Falson, P., Di Pietro, A., Darbouret, D., Jault, J. M., Gautheron, D. C., Boutry, M., and Goffeau, A. (1987) Biochem. Biophys. Res. Commun. 148, 1182–1186) were also cloned and sequenced. A single nonsense mutation CAA-TAA (Gln173-stop) in mutant A2313 became a missense mutation TAA-TTA (stop-Leucine) in revertant R351. Glutamine 173 is located in the first putative element of the nucleotide binding site. Its substitution by a leucine residue appears responsible for the lower enzyme affinity toward ADP and for the loss of cooperativity of F$_1$-ATPase activity.

The ATP synthase is the major ATP provider of the cell. It is a ubiquitous heterooligomeric protein associated with bacterial, mitochondrial, or chloroplast membranes. This enzyme is composed of two parts: Fo, integrated in the membrane and responsible for proton transfer, and the extrinsic F$_1$, involved in ATP synthesis when associated with its membrane counterpart but only able to hydrolyze ATP when separated from Fo (for reviews, see Godinot and Di Pietro, 1986; Cross, 1988). Stoichiometry of the F$_1$ moiety is α$_{3}$β$_{3}$γδε. The primary structure of each subunit is known for different bacterial, mitochondrial, or chloroplast membranes. This en-

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Materials and Methods

*Strains—The wild type S. pombe strain was 972h*. The phenotypic revertant strain R351 was derived from the S. pombe mutant strain A2313 (Boutry and Goffeau, 1982) and was described previously.
Wild-type and Mutated atp1 Gene of S. pombe

(Falson et al., 1987). Escherichia coli J3109 (recA1upsE44end- A1750 tyrB18A417A116 lac-proAB lacY::lacZD159I) and J71/18 (supE44 lacY::lacZD159I lacZD158I lacY::lacZD159I) strains were used to amplify plasmids.

**Enzymes and Reagents**—T4 DNA ligase, DNA polymerase I (Klenow fragment), T4 polynucleotide kinase, nuclease S1, kilobase sequencing system, and restriction enzymes were from Bethesda Research Laboratories. Synthetic oligonucleotides were from Eurogentec (Belgium). Novozone SP 234 was generously given by Novo (Denmark). Gene-Clean II was purchased from Biotage, Inc.

**Preparation of Nucleic Acids**—Yeast genomic DNA was prepared according to a published procedure (Davis et al., 1980) scaled up as follows. A 200-ml instead of a 5-ml culture was used. Cells were washed with cold distilled water, centrifuged, and suspended at 35 °C for 10 min in 20 ml of 1 M sorbitol, 25 mM EDTA, 50 mM diethritol, pH 8.0 (NaOH). After centrifugation, the pellet was washed in 1 ml sorbitol, spun again, and suspended in 10 ml of 1 M sorbitol, 0.1 M sodium citrate, pH 5.8 (HCl), 10 mM EDTA. Novozone (Beach and Nurse, 1981) was added at a final concentration of mg/ml, and the suspension was incubated at 35 °C for 1 h. After a 5-min centrifugation at 3,000 rpm ( rotor JA20, Beckman), the spheroplast pellet was suspended in 5 ml of 50 mM Tris-Cl, pH 7.4, 20 mM EDTA, and 0.5 ml of 10% sodium dodecyl sulfate was added. The suspension was incubated at 30 °C for 30 min. After centrifugation, synthetic oligonucleotides were from Eurogentec. Novozone SP 234 was generously given by Novo (Denmark). Gene-Clean II was purchased from Bio-TEK, Inc.

**Isolation of atp1 from Different Strains**—Genomic libraries of BamHI digests of the wild-type and revertant R351 nuclear DNA were constructed in the plasmid pTZ18U. Cloning experiments were carried out as described by Sambrook et al. (1989). The probe was a 432-base pair CiaI fragment containing the NH2-terminal coding sequence of atp1 (see Fig. 1), labeled either by nick translation or random priming (Sambrook et al., 1989). atp1 of the A2313 mutant was isolated directly from amplified genomic DNA as described by Sambrook et al. (1989). The amplified DNA was purified on a denaturating acrylamide-urea gel. Labeled DNA was purified on a phenol-chloroform extraction and ethanol precipitation as described by Sambrook et al. (1989). DNA was hybridized under high-stringency conditions to a filter containing 32P-labeled probe (20,000 cpm) or probe (20,000 cpm) hybridized with 50 ng of either RNA prepared for the wild type or rRNA as a control. After incubation at 42 °C for 10 h in 10 mM Pipes, pH 6.4 (HCl), 100 mM NaCl, and 80% (v/v) formamide, the hybridization mixture was diluted with 85-fold in 30 mM sodium acetate, pH 4.6, 250 mM NaCl, 1 mM ZnSO4, and 20 μg/ml salmon sperm DNA, containing 150 ng of nucleases S1 and S2 and digested at 30 °C for 30 min. Protected fragments were separated on a 0.4% acrylamide sequencing gel and revealed by autoradiography. A size scale was made by primer extension and dideoxy sequencing with the same primer (527).

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**Determination of NH2-terminal Protein Sequence**—About 225 μg of purified F-ATPase (Falson et al., 1989) was dissolved in the sample buffer (Laemmli, 1970), heated at 100 °C for 5 min, and loaded on a 10% sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, the upper band corresponding to the α subunit was transferred onto a glass microfiber membrane soaked in poly(4-vinyl-N-methlypyrrolidinium)iodide as described by Vandekerckhove et al. (1980). Transfer was carried out in 50 mM Tris, 50 mM boric acid, pH 8.3 (HCl), for 7 h at 4 V/cm. The membrane was then washed in 10 mM sodium borate, pH 8.0 (HCl), 25 mM NaCl and dried. The fixed polypeptide was stained with a solution of fluorescine (1 mg/600 μl of acetone) and located under UV light. The membrane area containing the protein was cut and placed in the reaction chamber of an automated sequencing apparatus (Applied Biosystems model 477A) coupled with a phenylthiohydantoin amino acid analyzer (Applied Biosystems model 120A). The analytical peptide sequence was described by Hewick et al. (1981), and the gradient applied on the reverse phase column was described by Hunkapiller and Hood (1983).

**Computer Aid and Analysis**—DNA and protein analysis was made with the software PCGENE 6.00 of A. Bairach provided from IntelliGenetics, Inc./Genoff, s.a. (Switzerland). Alignment of homologous sequences was made according to the program CLUSTAL developed by Corpet (1988). Protein secondary structure analysis was carried out with the software ANTHEFROT (Deiàgge et al., 1988).

**RESULTS AND DISCUSSION**

**Isolation of the atp1 Genomic Sequence**—The previously isolated genomic clone pMal (Boutry et al., 1984) was suspected not to contain the whole atp1 transcription promoter since transformants of the mutant A2313 by this clone only recovered a moderate growth on a respiratory substrate. Therefore this initial clone was used to screen a S. pombe BamHI genomic library and to isolate a clone containing the whole gene included in a 5.2-kb insert (Fig. 1). The atp1 sequence was determined on both strands either from subclones or with synthetic oligonucleotides. More than 97% of the gene was thus sequenced on both strands, and when not, at least two sequences were obtained in the same orientation. **Structural Organization of atp1**—Fig. 1 shows the general structure of atp1 as determined from nucleotide sequence data of the cloned segment. The coding sequence was identified by homology with sequences from other organisms. However, interruption of the open reading frame by stop codons (positions 236 and 565) strongly suggested the presence of inter-
in frame by a stop codon at -33.

Direct experimental localization of both introns was obtained by nuclease S1 protection assays, as shown in Fig. 3. The 5’-end of the radioactive probe was located 47 nucleotides downstream of the 3’-end of the second intron. Consequently, three bands were expected with a predictable size of 47 and 60 nucleotides for two of them. These correspond to the protected regions of the second and third exons, respectively (Fig. 3, lower panel).

As shown in Fig. 3, three main spots were revealed S1, S3, and S4. The major band of the protected regions of the second and third exons, respectively (Fig. 3, lower panel). The deduced amino acid sequence is numbered from the 1st methionine. The mitochondrial targeting sequence is underlined.

FIG. 1. atp1 structure and sequencing strategy. The figure represents the BamHI genomic insert of pJG75. The coding sequence is shown by closed boxes separated by two introns (shaded boxes). Large arrows indicate the extent and orientation of the nucleotide sequence obtained for each subclone; numbered arrows indicate sequences obtained from synthetic primers, as defined under "Materials and Methods."
efficiency. Our present data show that such splicing occurs effectively in vivo, at least for atp1.

A broad search for putative promoter sequences upstream of the transcription start site (Breathnach and Chambon, 1982) allowed us to identify a putative TATA box at −182 (Fig. 2, dotted-underlined sequence); TATATAXG was located 124 nucleotides upstream of the transcription start site. This observation is not consistent with the proposal that in S. pombe (Russell, 1989) as well as in higher eukaryotes (Breathnach and Chambon, 1982), TATA box elements are located closer to the transcription initiation site. On the contrary, such a situation has been reported for S. cerevisiae (Russell, 1983). Sixty-seven nucleotides downstream of the 3'-end of the coding sequence (Fig. 2, position 2122) is located the consensus hexanucleotide AATAAA involved in the 3'-end processing of eukaryotic mRNA (Proudfoot and Brownlee, 1976; Montell et al., 1983).

The codon bias index (Bennetzen and Hall, 1982) was calculated according to the codon usage table proposed recently for S. pombe (Russell, 1989). Surprisingly, an intermediate value of 0.50 was obtained, predicting that atp1 would not be highly expressed. As a comparison, we calculated the codon bias index value of S. cerevisiae atp1 (nucleotide sequence available from Takeda et al., 1986), and a value of 0.57 was found. The lower codon bias index value obtained for atp1 of S. pombe is in good agreement with the observation of Russell and Hall (1983) that the codon usage bias for genes of equivalent function is less severe in S. pombe than in S. cerevisiae.

Deduced Precursor and Mature Protein Sequences of α Subunit—The predicted protein encoded by atp1 is composed of 536 amino acid residues (Fig. 2), accounting for a calculated molecular weight of 58,587, which is consistent with the size of the in vitro synthesized product of hybrid selected RNA (Boutry et al., 1984). Assignment of the atp1 product to the ATP synthase α subunit was achieved by primary structure comparison with known α subunits; the identities found (not shown) were between 54% (E. coli) and 74% (S. cerevisiae). As atp1 is a nuclear gene encoding a mitochondrial protein, we could expect the presence of a mitochondrial targeting peptide (Von Heijne et al., 1989) in the NH2-terminal region of the precursor. Thus, as described under “Materials and Methods,” we sequenced the 20 1st-amino acid residues of the NH2-terminal region of the mature α subunit. We identified Ala69 of the precursor (Fig. 2) as the 1st residue of the mature subunit. The latter consists of 509 amino acid residues with a calculated molecular weight of 55,577. This molecular weight is consistent with gel electrophoresis estimation (Jault et al., 1989). However, the deduced isoelectric point of 8.8 is significantly different from the observed one, 7.3 (not shown), indicating that the experimental determination is biased probably by a strong association with the γ subunit3 (Williams et al., 1984; Williams and Pedersen, 1986). The targeting peptide (underlined in Fig. 2) displays the general features reported for mitochondrial targeting sequences: no acidic residue and enrichment in basic, hydrophobic, and polar amino acids (Von Heijne et al., 1989). Secondary structure analysis (Garnier et al., 1978) and estimation of the amphiphilicity (Eisenberg et al., 1982) of the 50 1st residues of the precursor α subunit revealed two amphiphilic structured segments: an α-helix Met1-Leu10 and a short β-sheet Ile17-Leu23. The β-sheet is followed by a coil Lys32-Gly36 and then followed by an α-helix Tyr24-Arg36. These data suggest that the first predicted α-helix probably interacts with mitochondrial membranes in the early steps of targeting. Such a hypothesis is supported

3 P. Falson, unpublished results.
by the recent results of Lemire et al. (1989) showing that an amphiphilic NH2-terminal α-helix is required for targeting and of Endo et al. (1989) showing that the interaction of the α-helix with micelles increases the level of β-helix in the NH2-terminal moiety of the presequence. A further hypothesis is that the cleavage of presequence is probably favored by the presence of a coil between the amphiphilic β-sheet and the large α-helix.

Comparison of the primary structures of the mature α subunits from different species reveals the presence in S. pombe of 2 tryptophan residues (217 and 284 in Fig. 2) which are conserved in S. cerevisiae but not in other species in which they are replaced by tyrosine or phenylalanine residues. These tryptophans are responsible for the intrinsic fluorescence observed recently with wild-type F1-ATPase from S. pombe and which could be possibly modulated by single amino acid substitutions of the α or β subunits (Di Pietro et al., 1989). In addition, the S. pombe α subunit contains a great number of cysteine residues. This observation is consistent with the particular F1-ATPase sensitivity to thiol modifiers reported previously (Falson et al., 1986).

Isolation and Sequencing of the Mutant and Revertant atp1 Genes—The mutant strain A2313 was characterized by the lack of immunodetectable α subunit and by no ATPase activity in a mitochondrial fraction (Boutry and Goffeau, 1982). The coding region of the mutant atp1 allele was obtained by direct amplification using the polymerase chain reaction from genomic DNA. Polymerase chain reaction experiments were carried out as described under "Materials and Methods." Two synthetic oligonucleotides (primers 785 and 1276, Fig. 1) of 24 mer each were used to amplify a DNA fragment of 2.1 kb. Fig. 4 shows that a DNA band of the expected size was obtained without any contaminating fragment. The amplified fragment was inserted in a pBluescript SK+ vector as follows. A HindIII cut (indicated in Fig. 1) generated two shorter fragments of 0.8 and 1.3 kb which were ligated to the vector. After transformation, we isolated several positive clones containing both fragments. No random mutation introduced during the amplification step was observed.

The revertant atp1 of the R351 strain was cloned from a BamHI library, as described for the wild-type gene. The isolated mutant and revertant atp1 genes were fully sequenced using synthetic oligonucleotides as described under "Materials and Methods." Total sequencing revealed no more than a single or a double mutation in atp1 of the mutant and revertant strains, respectively.

In the mutant gene, cytosine 1042 was converted into thymidine (Fig. 5), creating a stop codon TAA instead of CAA which encodes glutamine 173 of the mature α subunit (Gln200 of the precursor protein in Fig. 1). Thus, it appears that the mutation leads to the synthesis of only one-third of the protein. Since no α subunit was immunodetected (Boutry and Goffeau, 1982), this suggests either that the truncated subunit is not integrated into the complex or that the shortened polypeptide does not contain any epitope recognized by the polyclonal antibodies used for the immunodetection. The same single mutation was observed in the revertant atp1 followed by a second one modifying adenine 1043 into a thymidine and leading to the replacement of the stop codon TAA by TTA, which encodes leucine. Such a double mutation restores the capacity of the revertant strain to synthesize an entire subunit that was actually observed by electrophoretic analysis (Falson et al., 1987). Recently, several mutants of the E. coli α subunit have been obtained by in vitro random mutagenesis (Pa gan and Senior, 1990). Three of them were due to nonsense mutations: Gln272* (Chothia, 1975) whereas each of them has a different hydrophobic constant, 0.36 (glutamine) and 1.34 (leucine) (Sassagawa et al., 1982), indicating that the disappearance of glutamine suppresses potential hydrogen bonds and that the leucine residue locally induces a new hydrophobic environment. Secondary structure analysis (Garnier et al., 1978; Deléage et al., 1988) revealed that the coil + turn region containing the mutation is not dramatically modified but probably shortened. This glutamine residue is conserved in all α subunits described up to date. It is located in the segment 170-GDRQTTGKT-177, corresponding to the common "glycine-rich loop" GXGXGXG, found in all nucleotide-binding proteins such as adenylate kinase, EF-Tu, Ras-P21, and the F1 β subunit (Walker et al., 1982; Fry et al., 1986). It is thus the first report of a nondirected mutation on the α subunit affecting this loop, which is assumed to interact...
with the phosphate chain of the nucleotide. Near the glutamine residue, lysine 175 (176 in *S. pombe*) was modified by site-directed mutagenesis and found to play a critical role in either nucleotide binding in *E. coli* (Rao et al., 1988) or in subunit assembly in *Thermophilic bacterium PS3* (Yohda et al., 1988). More recently, an *E. coli* mutant in which alanine 177 (178 in *S. pombe*) was replaced by a valine residue was obtained, and preliminary results indicated a partial impairment of catalytic turnover (Pagan and Senior, 1990).

The modified properties of the *S. pombe* partial revertant strain and of its purified mitochondrial F$_1$ATPase were described previously (Falson et al., 1987; Di Pietro et al., 1989) and are summarized in Table I. (i) The growth of the strain in either fermentative or oxidative conditions was still partially impaired. (ii) The mutation made the enzyme more resistant to azide inhibition and markedly decreased its affinity for ADP. (iii) A lower amount of endogenous nucleotides, almost 1 mol/mol of enzyme, was titrated after incubation in the presence of magnesium. (iv) The most dramatic effect was the loss of apparent negative cooperativity whereas the maximal rate of ATP hydrolysis was not markedly decreased. Such an alteration was deduced from double-reciprocal plots of ATP hydrolysis versus ATP concentration in conditions under which ATP concentration (0.03–3 mM) was much higher than that of the enzyme. A Hill number of 1.0 was found for the mutant instead of 0.7 for the wild type. Consistent with this was the observation that bicarbonate had no activating effect.

**Which Role for the α Subunit?—** As these results show strongly a modified interaction between the enzyme and ADP, it was proposed that the lower enzyme affinity for ADP means that during ATP hydrolysis the step of ADP release is no longer limiting (Di Pietro et al., 1989). The mutation presented here is located in a region proposed by Senior and coworkers (for the E. coli enzyme) to be a nucleotide-binding domain (Maggio et al., 1987, 1988), and the modified enzyme affinity observed here is in agreement with this hypothesis. However, the ATPase activity being largely restored, it is clear that glutamine 173 of the α subunit has no direct catalytic role. This more likely suggests that binding of adenine nucleotide to the α subunit may control ATP hydrolysis, resulting in an apparent negative cooperativity of ATP hydrolysis. Similar effects, i.e. loss of negative cooperativity and increased dissociation constant for ADP, were obtained in the *T. bacterium* PS3 by another α subunit mutation: substitution of the aspartic acid residue 261 (Asp$^{70}$ in *S. pombe*) by an asparagine residue (Yohda et al., 1988). Both types of results suggest a regulatory role for the α subunit. Residues Gin$^{73}$ and Asp$^{70}$ in *S. pombe* are located in two segments of the α subunit, homologous to adenylate kinase segments (Fry et al., 1986), as indicated below.

X-ray crystallography and NMR spectroscopy (Fry et al., 1986) have shown that both segments of adenylate kinase are spatially located in the same region, the first one interacting with the phosphate chain of ATP and the second one interacting with magnesium. As the effects of mutation Gin$^{73}$ → Leu and Asp$^{70}$ (270 in *S. pombe*) → Asn are quite similar, it is tempting to propose that these residues and their respective segments are spatially close together, as in adenylate kinase.

In conclusion, the results presented here show clearly that isolation of mutant and selection of related revertants are a powerful method to study yeast ATP synthase. Indeed, such a procedure was also used for the *E. coli* enzyme (Senior et al., 1984) although identification and localization of reversion have not been reported yet. Further isolation of other revertants from the A2313 and other mutants is in progress to obtain new data on the ATP binding site of the α subunit.

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**Table I**

| Effects of Gin$^{73}$ → Leu substitution on growth and purified F$_1$ATPase properties | Wild type | R351 revertant |
|---|---|---|
| Growth yield | 100% | 82% |
| Glucose | 100% | 48% |
| Purified F$_1$ATPase | 100% | 82% |
| ATPase activity | 1.0 | 1.0 |
| Hill number | 200 | 200 |
| Bicarbonate activation | yes | no |
| K$_M$ ADP (μM) | 450 | 800 |
| Leu (μM) | 35 | 206 |
| Endogenous nucleotides | 3.6 | 2.8 |

* Values were obtained from Di Pietro et al. (1989).

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