J. Biochem. **138**, 201–207 (2005)
DOI: 10.1093/jb/mvi116

Mitochondrial ATP Synthase Residue βArginine-408, Which Interacts with the Inhibitory Site of Regulatory Protein IF_1, Is Essential for the Function of the Enzyme

Naoki Ichikawa*, Naomi Chisuwa, Mamiko Tanase and Mai Nakamura

Department of Food and Nutrition, Faculty of Human Life Science, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi, Osaka 558-8585

Received April 13, 2005; accepted May 29, 2005

Mitochondrial ATP synthase (F_1F_0-ATPase) is regulated by an intrinsic ATPase inhibitor protein, IF_1. We previously found that six residues of the yeast IF_1 (Phe17, Arg20, Glu21, Arg22, Glu25, and Phe28) form an ATPase inhibitory site [Ichikawa, N. and Ogura, C. (2003) *J. Bioenerg. Biomembr.* 35, 399–407]. In the crystal structure of the F_1/IF_1 complex [Cabezón, E. *et al.* (2003) *Nat. Struct. Biol.* 10, 744–750], the core residues of the inhibitory site interact with Arg408, Arg412 and Glu454 of the β-subunit of F_1. In the present study, we examined the roles of the three β residues by means of site-directed mutagenesis. A total of six yeast mutants were constructed: R408I, R408T, R412I, R412T, E454Q, and E454V. The βArg412 and βGlu454 mutants (R412I, R412T, E454Q, and E454V) could grow on a nonfermentable lactate medium, but the βArg408 mutants (R408I and R408T) could not. The ATPase activity of isolated mitochondria was decreased in R412I, R412T, E454Q, and E454V mutant cells, and undetectable in R408I and R408T cells. The subunits of F_1 (α, β, and γ) were detected in mitochondria from each mutant on immunoblotting, and the F_1F_0 complex was isolated from them. These results indicate that βArg408 is essential not for assembly of the F_1F_0 complex but for the catalytic activity of the enzyme. In the crystal structure of F_1, βArg408 binds to αGlu399 in the α_{DP}/β_{DP} pair and seems to be important for formation of the closed α_{DP}/β_{DP} conformation. IF_1 seems to disrupt this α_{DP}Glu399/β_{DP}Arg408 interaction by binding to β_{DP}Arg408, and to interfere with the change from the open α_{DP}/β_{DP} conformation to the closed conformation that is required for catalysis by F_1F_0-ATPase.

Key words: IF_1 (ATPase inhibitor protein), ATP synthase (F_1F_0-ATPase), mitochondria, regulation.

Abbreviations: AMP-PNP, 5′-adenylyl-imidodiphosphate; *ATP2* gene, the gene coding for the β-subunit of yeast ATP synthase; F_1F_0 or F_1F_0-ATPase, mitochondrial ATP synthase; F_1 or F_1-ATPase, catalytic part of ATP synthase; $(His)_6$ tag, hexahistidine tag; IF_1, mitochondrial ATPase inhibitor protein (regulatory subunit of ATP synthase); *INH1* gene, the gene coding for yeast IF_1; Ni-NTA, nickel-nitrilotriacetic acid; PVDF, polyvinylidene fluoride.

Mitochondrial ATP synthase (F_1F_0-ATPase) catalyzes the terminal step of oxidative phosphorylation. The enzyme synthesizes ATP from ADP and inorganic phosphate by utilizing the proton electrochemical gradient across the mitochondrial inner membrane. The enzyme is composed of a globular catalytic part, F_1, and an integral membrane part, F_0. F_1 consists of five subunits with a stoichiometry of $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$. The α- and β-subunits are arranged alternately around the γ-subunit as a central axis, and three catalytic sites are located on the β-subunits at the interfaces with the α-subunits (*1*). The three β-subunits have different conformations and the catalytic sites on the subunits exhibit different occupancies by nucleotides: the first site binds ADP, the second one binds ATP, and the third one binds no nucleotides (*1*). The three conformations are named β_{DP}, β_{TP}, and β_E, respectively (*1*). During catalysis, rotation of the central γ-subunit sequentially changes the conformation of the three βs, and the binding affinity of the catalytic sites for substrates and products. F_0 functions as a proton channel, and the energy produced on proton flux through it is transmitted to catalytic F_1 as rotational movement of the γ-subunit (*2*) (reviews Refs. *3* and *4*).

Mitochondrial F_1F_0-ATPase is regulated by an intrinsic ATPase inhibitor protein, IF_1 (*5*). IF_1 binds to the F_1 part of the enzyme in a 1:1 molar ratio in the presence of Mg^{2+} and ATP, and completely inhibits the ATP-hydrolyzing activity of the enzyme, but not the synthesis of ATP during oxidative phosphorylation. IF_1 is not required for the synthesis of ATP by F_1F_0, but is important for maintaining the cellular level of ATP by preventing its hydrolysis by the enzyme when the proton electrochemical gradient across the mitochondrial inner membrane is lost (*6*). The inhibitory action of IF_1 is greatly influenced by pH; effective inhibition of F_1F_0-ATPase requires a low pH (<7.0). Above pH 7.5, the activity sharply decreases (*5*) (reviews Refs. *7* and *8*).

Recently, Cabezón *et al.* (*9*) reported the crystal structure of the bovine F_1/IF_1 complex. In the structure, IF_1 binds at the α_{DP}/β_{DP} interface of F_1 and opens the cata-

*To whom correspondence should be addressed. E-mail: ichikawa@life.osaka-cu.ac.jp

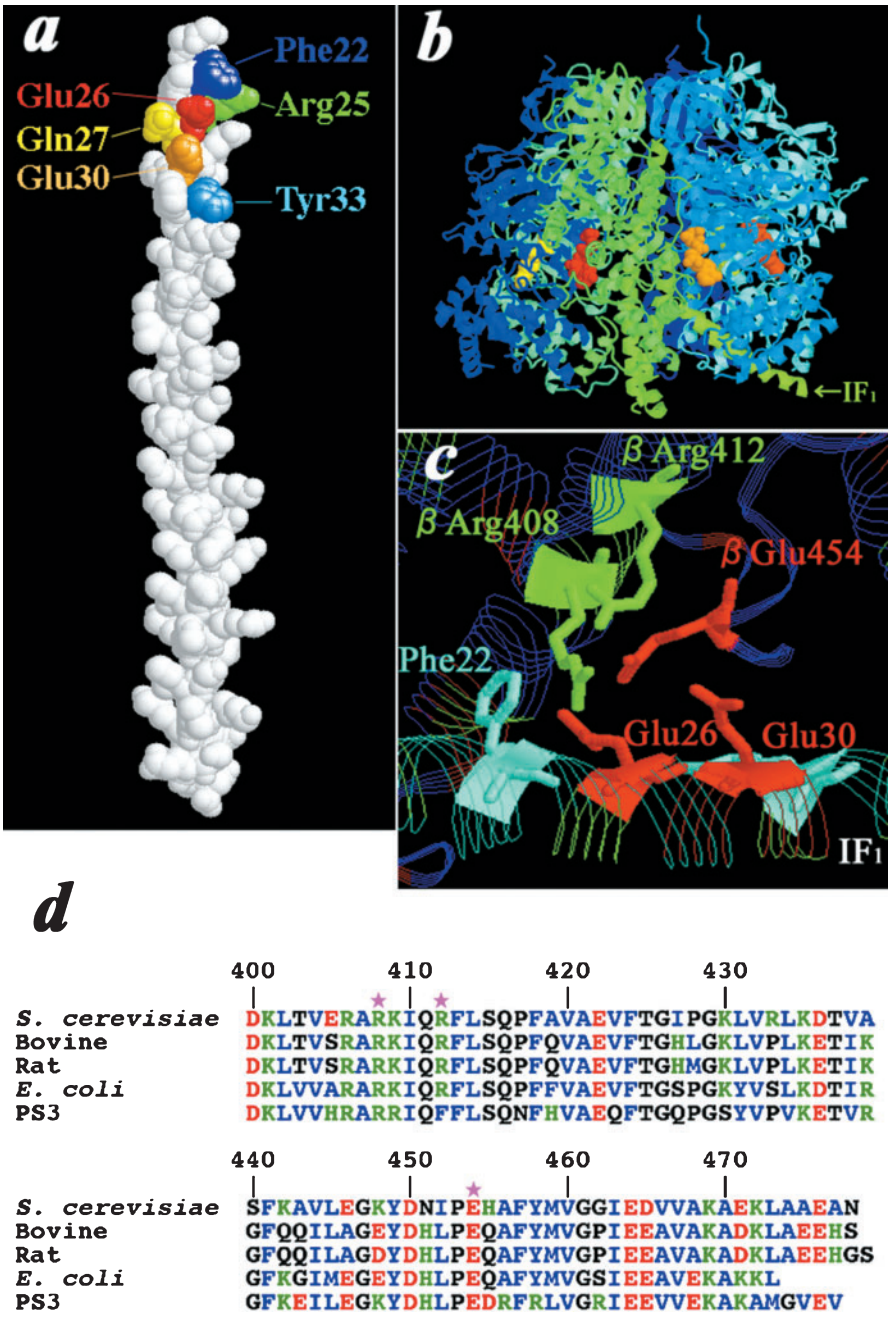

Fig. 1. **Interaction between F_1 and the inhibitory site of IF_1.** a: Inhibitory site of IF_1 determined by site-directed mutagenesis (*12*, *14*). The essential residues (Phe22, Arg25, Gln27, Glu30, and Tyr33) and Glu26, which is required for pH sensitivity, are indicated in the crystal structure of bovine IF_1 reported by Cabezón *et al.* (Ref. *13* and PDB #1GMJ). b: Crystal structure of the bovine F_1/IF_1 complex (Ref. *9* and PDB #1OHH). The position of IF_1 is indicated. c: Interface between the three core residues of the inhibitory site of IF_1 (Phe22, Glu26, and Glu30) and β_{DP}. d: Aligned sequences of the C-terminal regions of β from *Saccharomyces cerevisiae*, cow, rat, *Escherichia coli*, and thermophilic bacterium *Bacillus* PS3 (SWISS-PROT accession numbers, P00830, P00290, P10719, P00824, and P07677). Stars indicate the location of βArg408, βArg412, and βGlu454. Acidic (red), basic (green), and hydrophobic (blue) residues are colored.

lytic interface between α_{DP} and β_{DP}. The catalytic site of β_{DP} binds ATP (or AMP-PNP), and it was indicated that IF_1 stops the pre-hydrolysis step in the catalytic pathway of the enzyme.

We previously investigated the residues required for the function of the yeast IF_1 by means of site-directed mutagenesis (*10*–*12*), and identified five residues (Phe17, Arg20, Arg22, Glu25, and Phe28) essential for the ATPase inhibitory activity, and one residue (Glu21) required for pH-sensitivity (*12*). In the crystal structure of bovine IF_1 (*13*), the homologous residues (Phe22, Arg25, Glu26, Gln27, Glu30, and Tyr33) form a cluster on the surface of the α-helix (Fig. 1a), and the cluster defines the inhibitory site of IF_1 (*14*). The three core residues of the cluster (Phe22, Glu26, and Glu30) interact with three residues of β_{DP} (βArg408, βArg412, and βGlu454) in the crystal structure of the F_1/IF_1 complex (Fig. 1c). These three residues of β are highly conserved in the primary structure (Fig. 1d) and seem to be important for the catalysis and/or regulation of F_1F_0-ATPase.

In this study, we examined the roles of the conserved residues of yeast $F_1\beta$ by means of site-directed mutagenesis, and found that βArg408, which interacts with the inhibitory site of IF_1, is essential for the catalytic activity of F_1F_0-ATPase.

MATERIALS AND METHODS

Yeast Strains—A *Saccharomyces cerevisiae* strain lacking the gene coding for the β-subunit of F_1-ATPase (geno-

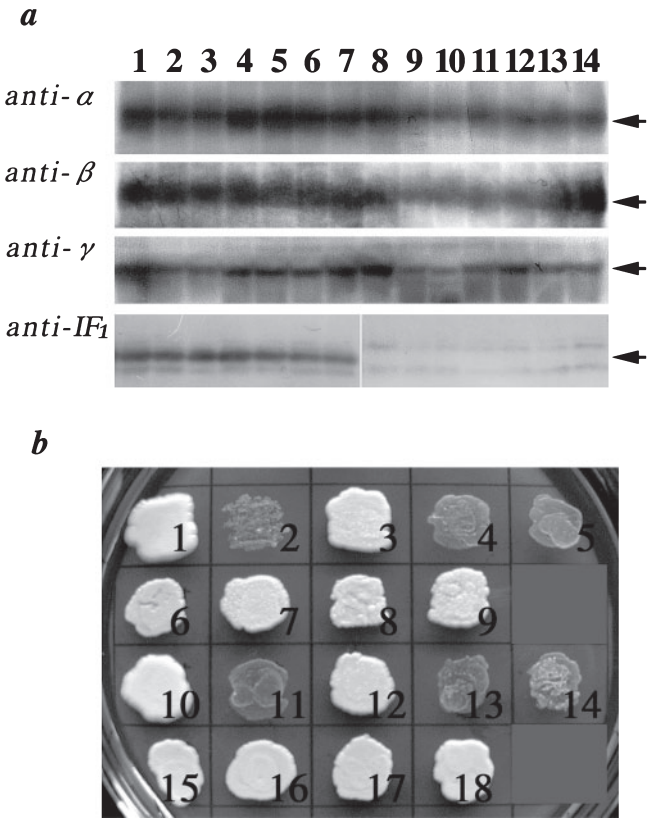

Fig. 2. **Properties of mutant yeast cells.** a: Immunodetection of the subunits of F_1 and IF_1 in mutant yeast cells. Mutant yeast cells were grown aerobically on a medium containing 1% yeast extract, 2% peptone, and 2% galactose. Mitochondria were isolated from the cells according to Daum *et al.* (*21*), and the proteins (30 μg each) were separated on a 12.5% polyacrylamide gel and then transferred to a PVDF membrane. Subunits of F_1 (α, β, γ) and IF_1 were detected with specific antibodies. Arrows indicate the bands of the proteins. Lane 1, WT; lane 2, R408I; lane 3, R408T; lane 4, R412I; lane 5, R412T; lane 6, E454Q; lane 7, E454V; lanes 8–14, IF_1-deficient versions of lanes 1–7. b: Growth phenotypes of mutant yeasts. The mutant cells were cultured on a medium containing 1% yeast extract, 2% peptone, 2% lactate, and 0.1% galactose at 30°C for 5 days. 1, BY4741; 2, BY4741(β^-); 3, WT; 4, R408I; 5, R408T; 6, R412I; 7, R412T; 8, E454Q; 9, E454V; 10–18, IF_1-deficient versions of 1–9.

type: **a** *his3 leu2 met15 ura3 atp2::kanMX4*) was obtained from the *Saccharomyces* Genome Deletion Project (http://sequence-www.stanford.edu/group/yeast_deletion_project/deletions3.html and Ref. *15*, the record number of the strain being 6924). We called this strain BY4741(β^-). A mutant strain lacking both β and IF_1 was constructed by disruption of the *INH1* gene in BY4741(β^-) cells using a *URA3* selection marker. The parent strain, BY4741 (**a** *his3 leu2 met15 ura3*), was also used as a control in the growth test.

Construction of Yeast Strains That Express Mutated β—The numbering of amino acid residues of the yeast $F_1\beta$ used in this study is the same as the bovine system (*16*) (Fig. 1d). The yeast mutants that express mutated β were named as follows, (wild-type residue) (residue number) (mutant residue), where the residues were given a single-letter code.

We used a previously constructed expression system for N-terminus $(His)_6$-tagged β (*17*) to express the mutant βs in this study. Site-directed mutagenesis was performed by a PCR-based method (*18*). A total of six mutants were constructed: R408I (AGA→ATA); R408T (AGA→ACA); R412I (AGA→ATA); R412T (AGA→ACA); E454Q (GAA→CAA); and E454V (GAA→GTA). *Bam*HI–*Sma*I mutagenized fragments (392 bp, corresponding to βPro350-C-terminus of β) were ligated into a YCp type shuttle vector, pKO313/mtH6-ATP2 (*17*), which was treated with the same enzyme. The sequences of the mutants were verified with an ABI PRISM 310 DNA sequencer (PE Biosystems, USA). The resultant plasmids contained the *GAL1* promoter and coding regions of the mutant βs fused to the import signal sequence of the ATPase inhibitor (*6*), and a $(His)_6$ tag at the N-terminus. The yeast cells lacking β [BY4741(β^-)] or both β and IF_1 (see above) were transformed with the plasmids. A previously constructed strain that expresses $(His)_6$-tagged normal β (*17*) was named WT and used as the control.

SDS-PAGE and Immunoblotting—The polyacrylamide gels used for SDS-PAGE were purchased from ATTO corporation (Tokyo). The proteins separated on gels were transferred to PVDF membranes (Immobilon-P., Millipore, USA) using a semi-dry blotter (AE-6675., ATTO Corporation, Tokyo), and bands reacting with antibodies were detected on film (RX-U., Fuji Photo Film, Tokyo) using a Phototope Star Western Blot Detection Kit (New England Biolabs, USA). Antibodies specific for the α (*19*), β (*19*), and γ (*19*) subunits of yeast F_1 and yeast IF_1 (*20*) were raised in rabbits previously.

Other Procedures—Mitochondria were isolated from yeast cells by the method of Daum *et al.* (*21*). Submitochondrial particles were prepared as described previously (*22*). Protein was measured by the method of Lowry *et al.* (*23*) with bovine serum albumin as a standard. The yeast IF_1 was purified as described previously (*12*). Aurovertin B was purchased from Sigma-Aldrich, USA. Three-dimensional images of the proteins were generated with RasMol computer software (version 2.6) on a Power Macintosh G4 computer (Apple Computer, Inc., USA).

RESULTS

Construction of Mutant Yeasts—As described above in detail, six mutants as to the β-subunit of yeast F_1-ATPase were constructed, and named R408I, R408T, R412I, R412T, E454Q, and E454V, respectively. We previously constructed an expression system for β, the N-terminus of which is fused to a $(His)_6$ tag (*17*), and used it to express the mutant βs in this study. The tagged mutant βs were expressed under the control of the *GAL1* promoter in yeast cells lacking a functional copy of the endogenous *ATP2* gene. All the mutant cells were viable on galactose medium, and expression of the βs in mitochondria was confirmed by immunoblotting (Fig. 2a, lanes 1–7). The IF_1, the α- and γ-subunits of F_1-ATPase were also detected in the mitochondria (Fig. 2a, lanes 1–7).

To examine the ability of each mutant β to function in mitochondria, the mutant cells were examined for growth on a medium containing a nonfermentable carbon source, lactate (Fig. 2b, 1–9). The βArg412 and βGlu454 mutants (R412I, R412T, E454Q, and E454V) could grow on the medium (Fig. 2b, 6–9), indicating that the residues are

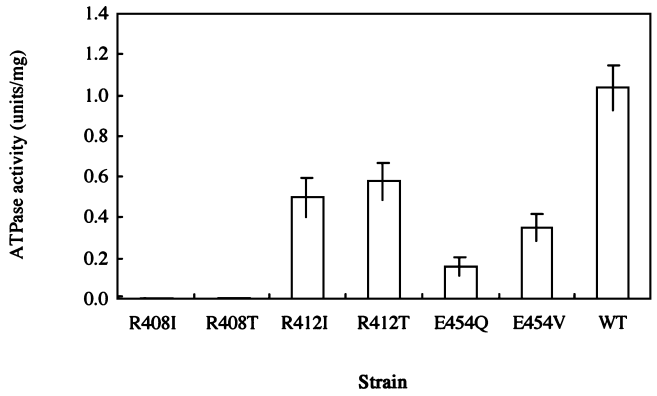

Fig. 3. **ATPase activity of isolated mitochondria.** Mutant yeast cells (IF_1-deficient) were grown on a medium containing 1% yeast extract, 2% peptone, and 2% galactose. Mitochondria were prepared from the cells and then assayed for ATPase activity as described previously (*17*). The values indicated are from three measurements with standard deviations.

not essential. Neither βArg408 mutant (R408I and R408T) could grow on the medium (Fig. 2b, 4 and 5), indicating that they lack the ability to produce ATP through F_1F_0. This defect could be attributable to: (i) dysfunction or disassembly of F_1F_0, or (ii) irreversible inhibition of F_1F_0 by IF_1. We constructed IF_1-deficient versions of the mutants (Fig. 2a, lanes 8–14) and examined their growth on lactate medium (Fig. 2b, 10–18). As shown in Fig. 2b, 13 and 14, growth of the IF_1-deficient versions of the βArg408 mutants on the medium was not recovered, indicating that the growth defect of the mutants is not due to inhibition of F_1F_0 by IF_1.

ATPase Activity of Isolated Mitochondria—Mitochondria were isolated from the mutant cells (IF_1-deficient versions), and then the effects of mutations on the ATP-hydrolyzing activity of F_1F_0-ATPase were examined (Fig. 3). In the βArg408 mutants (R408I and R408T), the activity was not detected, indicating that the residue is essential for the function of F_1F_0. The activities of the βArg412 mutants (R412I and R412T) decreased partially (47% and 55% of the wild-type control level), indicating that the residue is not essential. The activities of the βGlu454 mutants (E454Q and E454V) decreased to 14% and 32% of the control level. βGlu454 is not essential but seems to be important.

Inhibition of F_1F_0-ATPase from Mutant Yeast Cells by IF_1—F_1F_0 from the βArg412 and βGlu454 mutants (R412I, R412T, E454Q, and E454V) still had ATP-hydrolyzing activity, and so we prepared submitochondrial particles from the mutant cells and examined the IF_1-sensitivity of their F_1F_0-ATPase (Fig. 4). In the βArg412 mutants (R412I and R412T), F_1F_0 was well inhibited by IF_1 (Fig. 4b). In the βGlu454 mutants (E454Q and E454V), the sensitivity was markedly decreased (Fig. 4c). βGlu454 seems to be important for the inhibitory action of IF_1.

The inhibitory activity of IF_1 is greatly reduced at higher pH (*5*). Glu21 of yeast IF_1 (corresponding to bovine Glu26), which is close to βArg412 and βGlu454 in the crystal structure of the F_1/IF_1 complex (Fig. 1c), is required for the pH-dependency of IF_1 (*12*). To determine if βArg412 and βGlu454 are also involved in the pH-dependency of IF_1, the inhibitory activity of IF_1 toward the mutant F_1F_0 was measured at higher pH. The activity of IF_1 was decreased for all mutant F_1F_0s at higher pH (pH 8.0) as well as the wild-type control (Fig. 4), indicating that βArg412 and βGlu454 are not required for the pH-dependency of IF_1.

βArg412 (corresponding to *E. coli* βArg398) is known as a binding site for aurovertins B and D, and the substitution of this residue confers resistance against the drugs to F_1F_0-ATPase (*24–26*). Thus, we examined the effect of aurovertin B on the activity of the mutant F_1F_0-ATPase by using submitochondrial particles. F_1F_0 from βGlu454 mutant cells was inhibited by aurovertin B as well as the

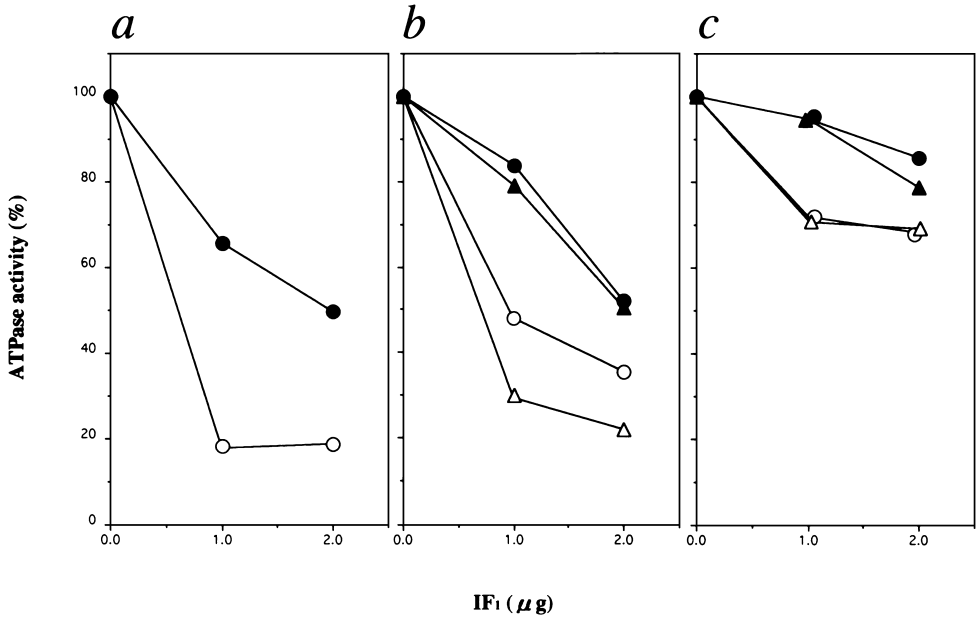

Fig. 4. **Inhibition of F_1F_0-ATPase from mutant yeast cells by IF_1.** Submitochondrial particles (0.3 mg) prepared from control and mutant cells (IF_1-deficient versions) were incubated with the indicated amounts of IF_1 in a medium containing 50 mM Tris-maleate (pH 6.5), 5 mM $MgSO_4$, and 5 mM ATP (open circles and open triangles), or 50 mM TrisSO_4 (pH 8.0), 5 mM $MgSO_4$, and 5 mM ATP (solid circles and solid triangles), in a final volume of 50 μl. After 5 min at 25°C, the remaining ATPase activity was measured at 25°C as described previously (*17*). a: WT. b: R412I (open and solid triangles), and R412T (open and solid circles). c: E454Q (open and solid triangles), and E454V (open and solid circles). The specific activities at 100% of WT, R412T, and E454Q submitochondrial particles were 1.30, 0.76, and 0.72 units/mg, respectively.

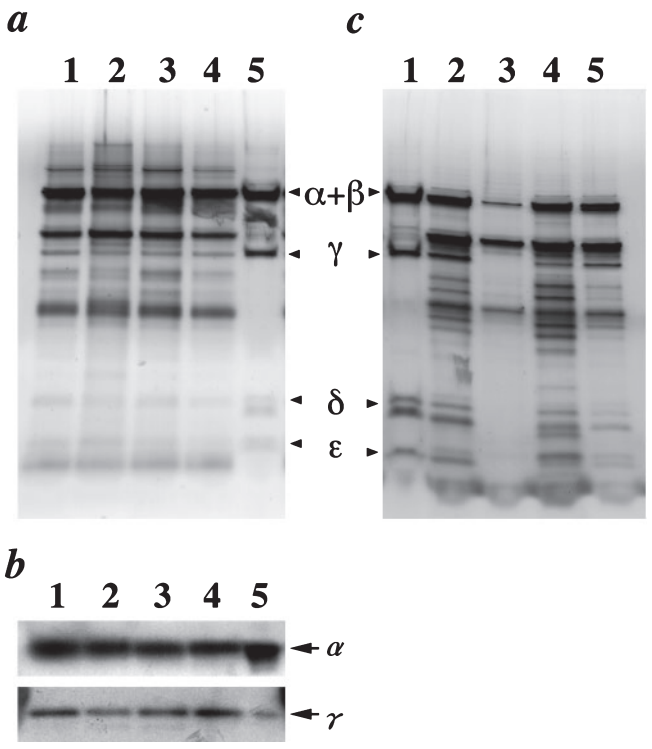

Fig. 5. **Isolation of F_1F_0 and F_1 from mutant mitochondria.** a: Isolation of F_1F_0 with Ni-NTA agarose. Submitochondrial particles (20 mg) were suspended in 2 ml of 0.25 M sucrose, 10 mM $TrisSO_4$ (pH 7.4), 2 mM ATP, 0.2% Triton X-100, and 10 mM imidazole, and then incubated on ice for 30 min. The suspension was centrifuged at 50,000 rpm for 15 min at 2°C, and the supernatant was loaded onto a Ni-NTA agarose column packed with 0.3 ml of resin (Qiagen K.K., Japan) and equilibrated with the above buffer. Proteins were eluted with imidazole. F_1F_0 was eluted at 40–80 mM imidazole. The F_1F_0 (130 ng) was analyzed on a 15% polyacrylamide gel and stained with silver. Lane 1, WT; lane 2, R408T; lane 3, R412T; lane 4, E454V; lane 5, purified F_1. b: Immunoblots of a. α (upper) and γ (bottom) of F_1F_0 were detected with specific antibodies. Lane 1, WT; lane 2, R408T; lane 3, R412T; lane 4, E454V; lane 5, purified F_1. c: Isolation of F_1 with Ni-NTA agarose. F_1 was extracted from submitochondrial particles by chloroform treatment and isolated using a column of Ni-NTA agarose as described previously (*17*). The F_1 (180 ng) was analyzed on a 15% polyacrylamide gel and stained with silver. Lane 1, purified F_1; lane 2, WT; lane 3, R408T; lane 4, R412T; lane 5, E454V.

wild-type control, but the enzyme from βArg412 mutants was insensitive to the drug (data not shown).

Isolation of F_1F_o from Mutant Mitochondria—As indicated above, F_1F_0-ATPase activity was lacking in mitochondria from the βArg408 mutants. This deficiency seemed to be due to a loss of the catalytic activity of F_1F_0-ATPase or impaired assembly of the subunits of the enzyme. In this study, mutated βs were expressed in an N-terminus $(His)_6$-tagged form, and we tried to isolate the assembled F_1F_0 complex from mutant mitochondria by metal affinity chromatography to examine the formation of the F_1F_0 complex in the mutant mitochondria.

Proteins were solubilized from the submitochondrial particles using Triton X-100 and applied to a Ni-NTA agarose column, and the eluates were analyzed by SDS-PAGE (Fig. 5a) and immunoblotting (Fig. 5b). Fractions obtained from the mutants (Fig. 5a, lanes 2–4) exhibited a similar band profile to that from the control strain (Fig. 5a, lane 1) on SDS-PAGE. All fractions gave bands for subunits of F_1F_0-ATPase [α+β, γ, δ, and ε (Fig. 5a), or α and γ, (Fig. 5b)], indicating that the fractions contained the enzyme. The fact that the α-, γ-,δ-, and ε-subunits of F_1F_0-ATPase were isolated with the $(His)_6$-tagged βs and Ni-NTA agarose indicates that the mutant βs can assemble into the F_1F_0 complex.

Isolation of F_1 from Mutant Mitochondria—We also tried to isolate the F_1 complex from mutant mitochondria using a column of Ni-NTA agarose. Proteins were extracted from the submitochondrial particles by chloroform treatment and then applied to an agarose column (Fig. 5c). Fractions obtained from R412T and E454V mutant cells contained all the subunits of F_1 (α, β, γ, δ, and ε) (Fig. 5c, lanes 4 and 5). But, only a small amount of the α+β band, and no bands for γ, δ, and ε were detected for the fraction from R408T mutant cells (Fig. 5c, lane 3). The F_1 of R408T seems to become disassembled or denatured during extraction or isolation of the enzyme.

DISCUSSION

In the present study, we examined the roles of the three residues of the β-subunit of yeast F_1F_0-ATPase that interact with the inhibitory site of the regulatory protein IF_1.

βArg408 mutants (R408I and R408T) could not grow on a lactate medium (Fig. 2b), and the mitochondria isolated from the mutant cells showed no ATPase activity (Fig. 3). These results indicate that βArg408 is essential for the function of F_1F_0-ATPase. Because the F_1F_0 complex was isolated from the mutant cells (Fig. 5, a and b), βArg408 is required for the catalytic activity of F_1F_0 rather than assembly of the enzyme complex.

As opposed to F_1F_0, the F_1 complex could not be isolated from the R408T mutant cells (Fig. 5c). The isolation involved treatment with chloroform. Chloroform is known to be a denaturing agent. βArg408 interacts with αGlu399 in the α_{DP}/β_{DP} pair in the crystal structure of F_1 (*1*) (Fig. 6a, see below), and seems to contribute to the stability of the enzyme complex. Thus, the F_1 in the βArg408 mutant may be sensitive to the agent. βArg408 may be required for the stability of the F_1 complex.

βArg412 (corresponding to *E. coli* βArg398) is known as a binding site for aurovertins B and D (*24–26*), and mutants with replacement of this residue have been well characterized in *Escherichia coli* (*24*, *25*). Lee *et al.* isolated three mutants [βR398H (*24*), βR398C (*25*), and βR398W (*25*)] and reported that their mutations conferred F_1F_0-ATPase resistance to aurovertin and had little, if any, effect on the activity of the enzyme (*24*, *25*). These results are consistent with our results. βArg412 is not essential for the function of F_1F_0-ATPase.

The ATPase activities of E454Q and E454V were markedly decreased (Fig. 3), and so residue Glu454 seems to be important. Because both F_1F_0 and F_1 were isolated from E454V (Fig. 5), this residue is important not for assembly but for the catalytic activity of the enzyme.

The location of the essential residue, βArg408, in the crystal structure of F_1 (*1*) is shown in Fig. 6a. In the structure, the active site interfaces of α_{DP}/β_{DP}, α_{TP}/β_{TP}, and α_E/β_E are tightly closed, partly open, and fully open, respectively (*1*). During catalysis, 120° rotation of γ raises

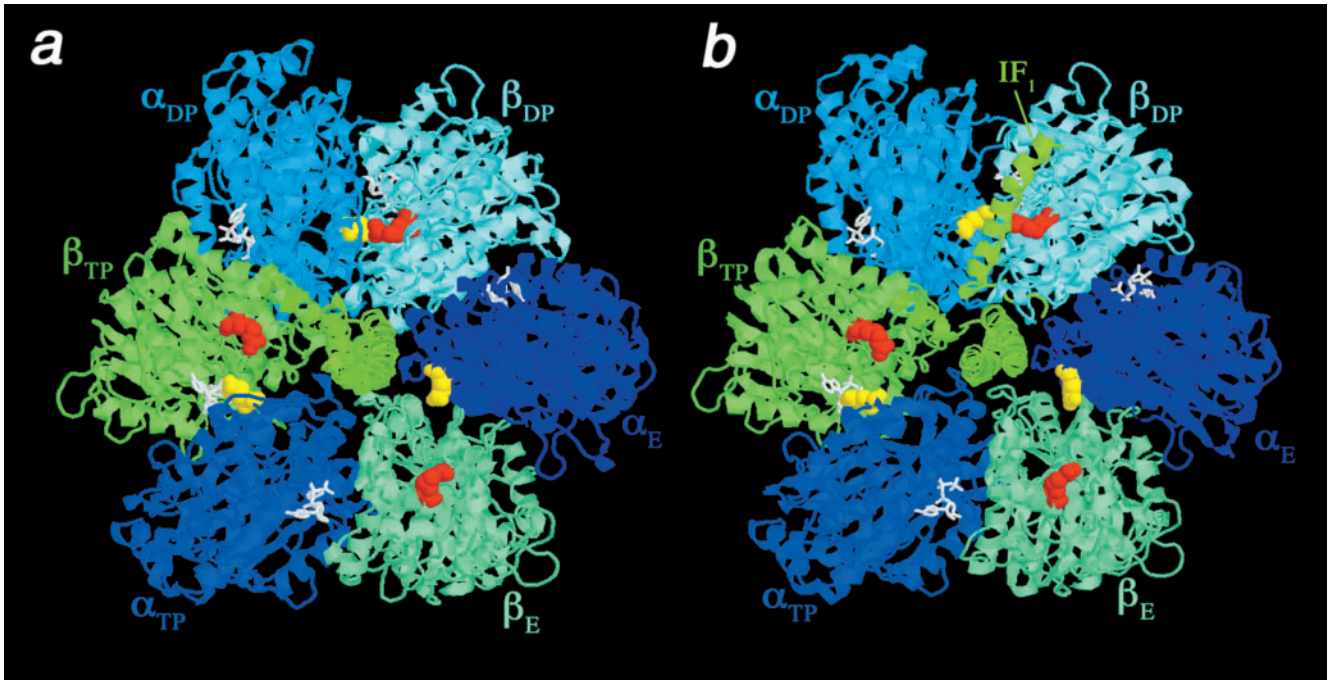

Fig. 6. **Positions of βArg408 and αGlu399 in F_1 and F_1/IF_1.** The crystal structures of F_1 (Ref. *1* and PDB #1BMF) (a) and F_1/IF_1 (Ref. *9* and PDB #1OHH) (b) viewed from the membrane side are presented. βArg408 and αGlu399 are colored red and yellow, respectively.

the interconversion of the sites such that the fully open α_E/β_E becomes a tightly closed α_{DP}/β_{DP}, the tightly closed α_{DP}/β_{DP} becomes a partly open α_{TP}/β_{TP}, and the partly open α_{TP}/β_{TP} becomes a fully open α_E/β_E. βArg408 (Fig. 6a, red) binds to αGlu399 (Fig. 6a, yellow) in the α_{DP}/β_{DP} pair, but has no counterpart in α_{TP}/β_{TP} or α_E/β_E. Thus, the binding of βArg408 to αGlu399 seems to be required for a tightly closed α_{DP}/β_{DP} to form. Mutations of βArg408 seem to impair the formation of this closed α_{DP}/β_{DP}, which is required for the catalytic cycle of the enzyme.

The location of IF_1 in the crystal structure of F_1/IF_1 (*9*) is shown in Fig. 6b. In the structure, IF_1 is interposed between α_{DP}Glu399 and β_{DP}Arg408, and opens the catalytic interface between α_{DP} and β_{DP}. Previously, Cabezón *et al*. (*9*) suggested that the binding of IF_1 prevents the closure of the α_{DP}/β_{DP} interface and prevents the approach of the guanidino group of α_{DP}Arg373 (αArg376 in *E. coli*), which is essential for catalysis (*27*), to γ-phosphate groups of a nucleotide bound to β_{DP}. Our results also support this model. The core residues of the inhibitory site of IF_1 interact with the βArg408 in β_{DP}, as shown in Fig. 1c. It is probable that IF_1 disrupts the α_{DP}Glu399-β_{DP}Arg408 interaction by covering β_{DP}Arg408, and interferes with the change from the open α_{DP}/β_{DP} conformation to the closed conformation that is required for catalysis by F_1F_0-ATPase.

REFERENCES

1. Abrahams, J.P., Leslie, A.G.W., Lutter, R., and Walker, J.E. (1994) Structure at 2.8 Å resolution of F_1-ATPase from bovine heart mitochondria. *Nature* **370**, 621–628
2. Noji, H., Yasuda, R., Yoshida, M., and Kinosita Jr, K. (1997) Direct observation of the rotation of F_1-ATPase. *Nature* **386**, 299–302
3. Boyer, P.D. (1997) The ATP synthase—A splendid molecular machine. *Annu. Rev. Biochem.* **66**, 717–749
4. Senior, A.E., Nadanaciva, S., and Weber, J. (2002) The molecular mechanism of ATP synthesis by F_1F_0-ATP synthase. *Biochim. Biophys. Acta* **1553**, 188–211
5. Pullman, M.E. and Monroy, G.C. (1963) A naturally occurring inhibitor of mitochondrial adenosine triphosphatase. *J. Biol. Chem.* **238**, 3762–3769
6. Ichikawa, N., Yoshida, Y., Hashimoto, T., Ogasawara, N., Yoshikawa, H., Imamoto, F., and Tagawa, K. (1990) Activation of ATP hydrolysis by an uncoupler in mutant mitochondria lacking an intrinsic ATPase inhibitor in yeast. *J. Biol. Chem.* **265**, 6274–6278
7. Schwerzmann, K. and Pedersen, P.L. (1986) Regulation of the mitochondrial ATP synthase/ATPase complex. *Arch. Biochem. Biophys.* **250**, 1–18
8. Harris, D.A. and Das, A.M. (1991) Control of mitochondrial ATP synthesis in the heart. *Biochem. J.* **280**, 561–573
9. Cabezón, E., Montgomery, M.G., Leslie, A.G.W., and Walker, J.E. (2003) The structure of bovine F_1-ATPase in complex with its regulatory protein IF_1. *Nat. Struct. Biol.* **10**, 744–750
10. Ichikawa, N., Fukuda, M., Hashimoto, T., and Tagawa, K. (1998) The carboxyl-terminal region of the yeast ATPase inhibitor is indispensable for the stability of the protein in mitochondria. *J. Biochem.* **124**, 886–891
11. Ichikawa, N., Fujisaka, R., and Kuribayashi, R. (2000) Requirement for lysine-19 of the yeast mitochondrial ATPase inhibitor for the stability of the inactivated inhibitor-F_1F_0 complex at higher pH. *Biosci. Biotechnol. Biochem.* **64**, 89–95
12. Ichikawa, N., Karaki, A., Kawabata, M., Ushida, S., Mizushima, M., and Hashimoto, T. (2001) The region from phenylalanine-17 to phenylalanine-28 of a yeast mitochondrial ATPase inhibitor is essential for its ATPase inhibitory activity. *J. Biochem.* **130**, 687–693
13. Cabezón, E., Runswick, M.J., Leslie, A.G.W., and Walker, J.E. (2001) The structure of bovine IF_1, the regulatory subunit of mitochondrial F-ATPase. *EMBO J.* **20**, 6990–6996
14. Ichikawa, N. and Ogura, C. (2003) Overexpression, purification, and characterization of human and bovine mitochondrial

ATPase inhibitors: comparison of the properties of mammalian and yeast ATPase inhibitors. *J. Bioenerg. Biomembr.* **35**, 399–407

15. Winzeler, E.A., Shoemaker, D.D., Astromoff, A., Liang, H., *et al.* (1999) Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. *Science* **285**, 901–906
16. Walker, J.E., Fearnley, I.M., Gay, N.J., Gibson, B.W., Northrop, F.D., Powell, S.J., Runswick, M.J., Saraste, M., and Tybulewicz, V.L.J. (1985) Primary structure and subunit stoichiometry of F_1-ATPase from bovine mitochondria. *J. Mol. Biol.* **184**, 677–701
17. Ichikawa, N. and Mizuno, M. (2004) Functional expression of hexahistidine-tagged β-subunit of yeast F_1-ATPase and isolation of the enzyme by immobilized metal affinity chromatography. *Protein Expr. Purif.* **37**, 97–101
18. Higuchi, R. (1989) Using PCR to engineer DNA in *PCR Technology* (Erlich, H.A. ed.) pp. 61–70, Stockton Press, New York
19. Ichikawa, N., Yoshida, Y., Hashimoto, T., and Tagawa, K. (1996) An intrinsic ATPase inhibitor binds near the active site of yeast mitochondrial F_1-ATPase. *J. Biochem.* **119**, 193–199
20. Hashimoto, T., Yoshida, Y., and Tagawa, K. (1983) Binding properties of an intrinsic ATPase inhibitor and occurrence in yeast mitochondria of a protein factor which stabilizes and facilitates the binding of the inhibitor to F_1F_0-ATPase. *J. Biochem.* **94**, 715–720
21. Daum G., Böhni, P.C., and Schatz, G. (1982) Import of proteins into mitochondria. Cytochrome b_2 and cytochrome *c* peroxidase are located in the intermembrane space of yeast mitochondria. *J. Biol. Chem.* **257**, 13028–13033
22. Ichikawa, N., Nakabayashi, K., and Hashimoto, T. (2002) A yeast mitochondrial ATPase inhibitor interacts with three proteins that are easy to dissociate from the mitochondrial inner membrane. *J. Biochem.* **132**, 649–654
23. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275
24. Lee, R.S.-F., Pagan, J., Satre, M., Vignais, P.V., and Senior, A.E. (1989) Identification of a mutation in *Escherichia coli* F_1-ATPase β-subunit conferring resistance to aurovertin. *FEBS Lett.* **253**, 269–272
25. Lee, R.S.-F., Pagan, J., Wilke-Mounts, S., and Senior, A.E. (1991) Characterization of *Escherichia coli* ATP synthase β-subunit mutations using a chromosomal deletion strain. *Biochemistry* **30**, 6842–6847
26. Van Raaij, M.J., Abrahams, J.P., Leslie, A.G.W., and Walker, J.E. (1996) The structure of bovine F_1-ATPase complexed with the antibiotic inhibitor aurovertin B. *Proc. Natl Acad. Sci. USA* **93**, 6913–6917
27. Nadanaciva, S., Weber, J., Wilke-Mounts, S., and Senior, A.E. (1999) Importance of F_1-ATPase residue α-Arg-376 for catalytic transition state stabilization. *Biochemistry* **38**, 15493–15499