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The Carboxyl-Terminal Region of the Yeast ATPase Inhibitor Is Indispensable for the Stability of the Protein in Mitochondria[1]

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The role of the carboxyl-terminal region of the yeast mitochondrial ATPase inhibitor was investigated. Three progressive C-terminal deletion mutants of the inhibitor were constructed: (i) Ile58→end; (ii) Ile51→end; and (iii) Gln43→end. The truncated inhibitor was detected in extracts of Ile58→end mutant yeast cells. For the Ile51→end mutant, the truncated inhibitor was only detected when the cells were grown on medium containing the membrane-permeable metal chelator, *o*-phenanthroline, which inhibits mitochondrial proteases. The most greatly truncated inhibitor protein, Gln43→end, was never detected even in the cells grown in the presence of the metal chelator. The rates of ATP synthesis and hydrolysis in the mutant mitochondria containing the Ile51→end inhibitor were similar to those in wild type control cells, while the Ile51→end inhibitor protein was degraded in the cells unless they were incubated in the presence of the chelator. These results indicate that the carboxyl-terminal region of the ATPase inhibitor is not involved in the its inhibitory action on the F_1F_o-ATPase, but is required for the stable conformation of the protein which is protected against degradation by proteases.

Key words: ATPase inhibitor (IF_1), ATP synthase, F_1F_o-ATPase, coiled-coil structure, mitochondria.

In energy-transducing membranes of bacteria, mitochondria, and chloroplasts, ATP synthase (F_1F_o-ATPase) catalyzes the synthesis of ATP utilizing the energy produced on proton flux through the membranes. The enzyme is composed of an integral membrane sector, F_o, and a catalytic sector, F_1. F_1 is water-soluble and exhibits ATPase activity on its own, and it has a particular subunit structure, $\alpha_3\beta_3\gamma\delta\varepsilon$. The α- and β-subunits are arranged alternately like the segments of an orange around the γ-subunit (*1*), and three catalytic sites are located at the interfaces between the α- and β-subunits (for a review see Ref. *2*).

Mitochondrial F_1F_o-ATPase is regulated by an intrinsic ATPase inhibitor protein (*3-8*). The inhibitor binds to the F_1 portion in a stoichiometry of 1:1 in the presence of Mg^{2+} and ATP (*9*), and completely inhibits the ATP-hydrolyzing activity of the enzyme, but not ATP synthesis during oxidative phosphorylation (*10*). Previously, we constructed a mutant yeast lacking the ATPase inhibitor, and showed that the protein is not required for oxidative phosphorylation but is important in that it maintains the cellular level of ATP by preventing ATP hydrolysis under de-energized conditions (*7*). It has been demonstrated by cross-linking experiments that the inhibitor binds to the α/β interface of F_1-ATPase and that the binding site is one of the three catalytic sites of the enzyme (*11*).

The ATPase inhibitor has been found in various eukaryotic cells, from yeast to mammals. The primary structures of the *Saccharomyces cerevisiae* (*12*), *Candida utilis* (*13*), cow (*14*), and rat (*15, 16*) proteins have been reported. The functional regions of the bovine ATPase inhibitor have been investigated by earlier workers. Dianoux *et al.* (*17*) showed that a proteolytic fragment of the inhibitor comprising residues Ser10-Asp84 exhibits the same specific activity as the native inhibitor, but another fragment containing Gly23-Asp84 is inactive. We previously reported that a fragment without the C-terminal sequence, Gly1-Asn51, also inhibits the F_1-ATPase, but ones without the N-terminal regions, Lys46-Asp84 and Glu52-Asp84, do not (*18*). Van Raaij *et al.* recently produced many kinds of deletion mutants of the bovine inhibitor and concluded that the minimal inhibitory sequence of the protein is Ala14-Lys47 (*19*).

To summarize the results obtained by earlier workers, the C-terminal region from His48 to Asp84 is not involved in the interaction of the inhibitor with the active site of F_1-ATPase. However, the region forms a particular α-helical coiled-coil structure (*20*), which possibly mediates the interaction of the inhibitor protein with the enzyme. In the present study, we constructed three yeast mutants lacking the homologous C-terminal region of the yeast inhibitor and

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Abbreviations: F_1F_o-ATPase, mitochondrial ATP synthase; F_1 or F_1-ATPase, catalytic subunit of F_1F_o-ATPase; MOPS, 3-(morpholino)-propanesulfonic acid; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; PVDF, polyvinylidene fluoride.

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found that deletion of this region renders the protein susceptible to proteolysis in mitochondria.

MATERIALS AND METHODS

Strains and Growth Conditions—*Escherichia coli* strain JM109 was used for the propagation of plasmid DNA. *Saccharomyces cerevisiae* strain D26 (a *trp1 leu2 his3 inh1::TRP1*), which contains a null mutation in the gene coding for the ATPase inhibitor (*7*), was used as the host for expression of the wild-type and mutated forms of the inhibitor. *S. cerevisiae* strain DKD-5D (a *trp1 leu2 his3*) was used as a wild-type control. The *E. coli*/*S. cerevisiae* shuttle vector, YEp51 (*21*), was used for the construction of the expression plasmid. This multicopy vector contains the yeast *LEU2* gene as the selection marker, the 2-μm circle origin of replication and the *GAL10* promoter, which is induced on the addition of galactose.

Yeast cells transformed with various plasmids were grown with vigorous shaking at 30°C on 0.67% yeast nitrogen base and 1% glucose supplemented with appropriate amino acids (20 mg/liter). The medium volume was then increased 5-fold by adding a rich medium containing 2% peptone, 1% yeast extract, and 2% galactose, and the cells were further grown for 14–15 h at 30°C. The metal chelator, *o*-phenanthroline, was added to a final concentration of 5 mM 2 h prior to cell harvest.

Construction of Expression Plasmids—Deletion mutants of the yeast ATPase inhibitor were constructed from plasmid pAI-2 (*7*) by PCR using the oligonucleotide primers listed in Table I. The coding sequence of the wild-type ATPase inhibitor was also amplified as a control. The products were cloned into the *SalI*/*Hin*dIII site of the pUC119 plasmid and the sequences were verified with an ABI377 DNA sequencer (Perkin Elmer, USA). Then the fragments of mutated genes were cloned into the *SalI*/*Hin*dIII site under the *GAL10* promoter of YEp51. Yeast strain D26 was transformed with the plasmids according to Ito *et al.* (*22*).

Preparation of Cell Extracts—The protein extracts used for immunoblotting and for assaying of the ATPase inhibitor were prepared from yeast cells by heating as follows. Cells were washed once with water and then suspended to 0.2 g, wet weight, per ml distilled water. The cell suspensions were heated at 80°C for 5 min and then centrifuged at 10,000 rpm for 5 min at 0°C to remove denatured proteins. The supernatants were dialyzed and lyophilized, and then the proteins were dissolved in water at the concentration of 10 mg/ml.

Immunoblotting—SDS-polyacrylamide gel electrophoresis was performed by the method of Schägger and von Jagow (*23*). The proteins in the gel were transferred to a PVDF membrane using a semi-dry blotter (ATTO, Tokyo), and bands reacting with the antiserum were detected on a film (RX, Fuji Photo Film, Tokyo) using a chemiluminescent reaction kit (New England Biolabs, USA).

Other Procedures—Mitochondria were isolated from yeast cells by the method of Daum *et al.* (*24*), and ATP synthesis and hydrolysis were assayed as described previously (*7*). Protein concentrations were determined by the method of Lowry *et al.* (*25*). One unit of ATPase inhibitor activity is defined as the amount of inhibitor required to produce 50% inhibition of 0.2 unit of ATPase (*26*).

RESULTS

Construction of C-Terminal Deletion Mutants of the ATPase Inhibitor—It has been indicated by CD spectral analysis and prediction of the secondary structure from the primary sequence that the bovine ATPase inhibitor has an α-helical coiled-coil structure in its C-terminal region (*19, 20*). The yeast inhibitor was also confirmed to have a coiled-coil structure in a homologous region (*20*), in which leucine and isoleucine residues are repeated four times every seven residues starting at Leu37 (Fig. 1). A similar sequence pattern is often found in the regions of many kinds of protein which have a coiled-coil structure. In the present study, three progressive C-terminal deletion mutants of the inhibitor (Ile58→end, Ile51→end, and Gln43→end) were constructed, one to three of the Leu (Ile) residues arranged regularly being eliminated. The mutants were named YC57 (Ile58→end), YC50 (Ile51→end), and YC42 (Gln43→end), respectively. A control strain, which harbors the expression plasmid containing the coding sequence of the wild-type inhibitor, was also constructed and named YC63. All the mutants grew at a normal rate on a galactose medium.

Expression of the Truncated ATPase Inhibitors—Expression of the truncated ATPase inhibitors in the mutant cells grown on the galactose medium was detected by immunoblotting. As shown in Fig. 2a, bands reacting with antibodies against the ATPase inhibitor were detected on the electrophoregrams of extracts of DKD-5D (wild-type), YC63 and YC57, but not for YC50 and YC42 even after overexposure of the film. The inhibitory activities of the extracts were enzymatically assayed using purified F_1-ATPase (Fig. 3a), and the results coincided with those obtained on immunoblotting. The specific inhibitory activities of the extracts of DKD-5D and YC63 were 25 and 125 units/mg, respectively, indicating that the inhibitor was overproduced in YC63, because of the greater efficiency of the *GAL10* promoter.

In order to examine the possibility that the truncated inhibitor proteins were degraded after translation in YC50 and YC42, we added *o*-phenanthroline, a membrane-permeable metal ion-chelator, to the growth medium, which has been demonstrated to strongly inhibit the metal-dependent proteolysis in yeast mitochondria (*27, 28*). As shown

TABLE I. **Synthetic oligonucleotides used for amplification of the coding sequences of the wild-type and mutant inhibitors.**

Protein	Oligonucleotide sequence	Strain
N-terminal	5′-CCGGTCGACATGTTACCACGTTCAGCATTA-3′	
Wild-type	5′-CCCAAGCTTATTTGGTCATCGAGTCAATTT-3′	YC63
Ile58→end	5′-CCCAAGCTTATTTATTTTCCAAAGAATCAA-3′	YC57
Ile51→end	5′-CCCAAGCTTACTTCTTTCGTTGTTTTTCCA-3′	YC50
Gln43→end	5′-CCCAAGCTTATTCTTTCAAATGGCGTAGTT-3′	YC42

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in Fig. 2b, a band of the truncated inhibitor protein was detected for the extract of YC50 when the cells were grown on the medium containing the chelating agent. ATPase inhibitory activity was also detected in the mutant cells (Fig. 3b). However, neither a truncated protein nor inhibitory activity was detected in YC42 cells.

ATP-Hydrolysis and -Synthesis in Mitochondria Isolated from Mutant Yeast Cells—We previously reported that the addition of an uncoupler, such as CCCP, induces ATP-hydrolyzing activity in mitochondria isolated from inhibitor-deficient mutant yeast cells, whereas this kind of ATP hydrolysis was not observed in normal mitochondria isolated from wild type yeast cells, in which the inhibitor protein immediately binds to F_1F_o-ATPase upon de-energization of the mitochondrial membranes (*7, 8*). Figure 4a shows that the ATP hydrolysis induced by CCCP was observed in mitochondria from D26 but not in those from YC50 and wild type cells. The ATPase complex with the truncated inhibitor also dissociated normally on the addition of succinate, and then the synthesis of ATP commenced, as observed in wild type mitochondria (Fig. 4b), indicating normal functioning of the truncated inhibitor protein in mitochondria. Moreover, the inactivated ATPase complex readily dissociated on an increase in pH to 7.4 or an increase in ionic strength in the mutant mitochondria similarly to the process observed in wild type mitochondria (Fig. 5, a and b).

Degradation of the Truncated ATPase Inhibitor in a Mutant Yeast—Since the truncated ATPase inhibitor (Ile51→end) was only detected when cells were grown in the presence of *o*-phenanthroline, which inhibits metalloprotease in mitochondria (Figs. 2 and 3). The degradation process was examined in detail in the mutant cells. Cells were preincubated in a medium containing *o*-phenanthroline, and then transferred to a medium containing 0.5% yeast extract, 2% glucose, 10 mM $MgSO_4$, 5 mM $CaCl_2$, 10 mM $MnCl_2$, and 4 mg/ml cycloheximide. From the cells grown, the inhibitor protein was extracted and analyzed by immunoblotting. A gradual but significant decrease in the intensity of the band was observed for the mutant inhibitor (Ile51→end), while the protein of the wild-type yeast did not change (Fig. 6a). The results of assaying of the ATPase-inhibitory activity were also consistent with the

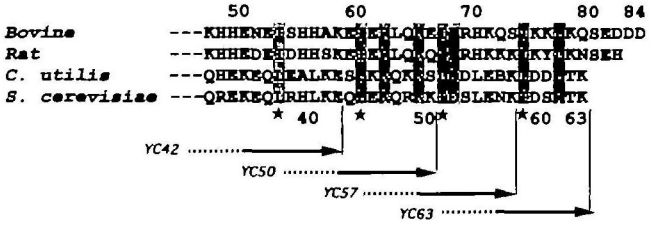

Fig. 1. **Aligned sequences of the carboxyl-terminal regions of the ATPase inhibitors from various species.** Identical and conservatively substituted residues are shaded. Asterisks denote 4 repeats of (iso)leucine residues in the region that is predicted to be involved in coiled-coil formation. The arrows indicate the carboxyl-terminals of the truncated inhibitors in the mutants.

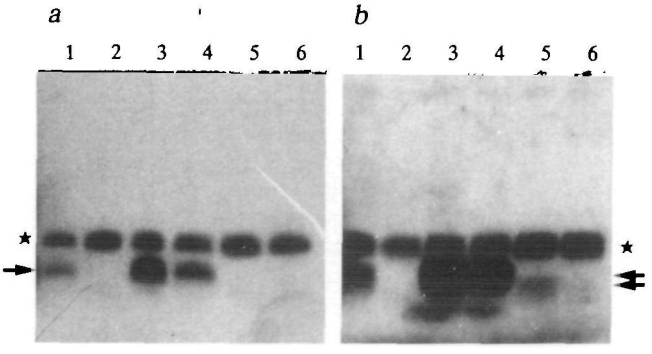

Fig. 2. **Immunoblots of yeast cell extracts.** Yeast cells were grown for 2 h at 30°C in the presence (b) or absence (a) of 5 mM *o*-phenanthroline as described under "MATERIALS AND METHODS." Cell extracts were separated on a 15% polyacrylamide gel, followed by transfer to a PVDF membrane. The ATPase inhibitor bands were detected with antiserum against the protein. Arrows indicate the normal and truncated ATPase inhibitors. Asterisks indicate nonspecific bands which reacted with the chemiluminescent substrate. Lane 1, DKD-5D; lane 2, D26 harboring YEp51; lane 3, YC63; lane 4, YC57; lane 5, YC50; lane 6, YC42.

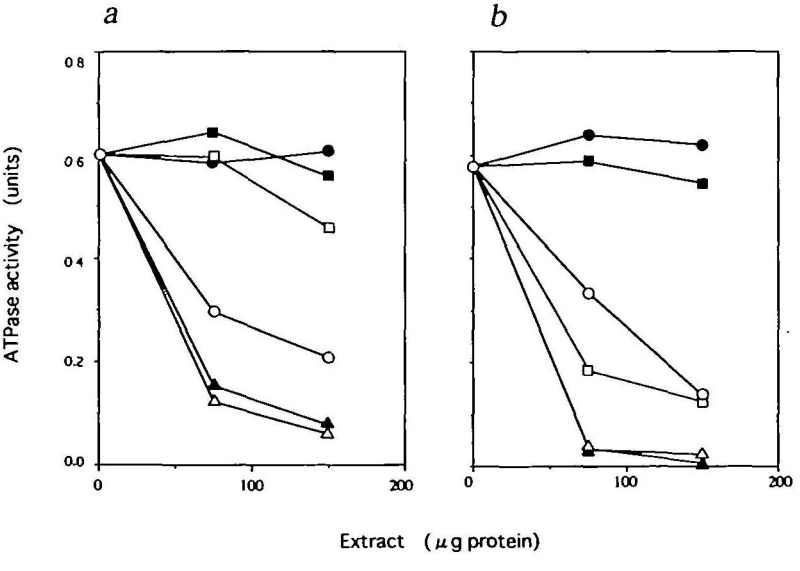

Fig. 3. **Inhibition of F_1-ATPase by cell extracts.** F_1-ATPase was incubated with cell extracts in a medium comprising 35 mM MOPS (pH 6.5), 5 mM ATP, and 5 mM $MgSO_4$. After incubation for 30 min at 25°C, ATPase activities were assayed at 25°C by measuring the formation of inorganic phosphate from ATP as described (*18*). Yeast cells were grown in the presence (b) or absence (a) of 5 mM *o*-phenanthroline. ○, DKD-5D; ●, D26 harboring YEp51; △, YC63; ▲, YC57; □, YC50; ■, YC42.

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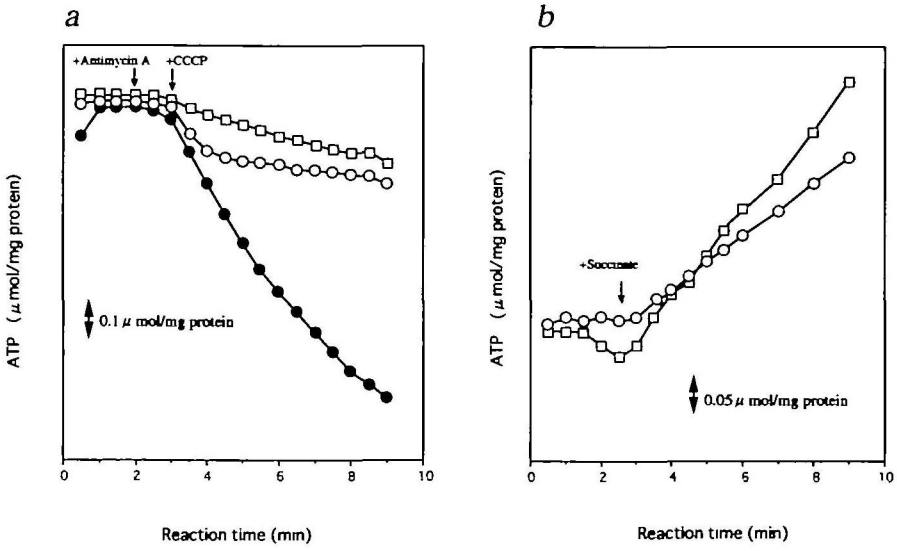

Fig. 4. **Uncoupler-induced ATPase activity and ATP synthesis in isolated mitochondria.** a: Mitochondria from cells grown in the presence of *o*-phenanthroline were incubated at 25°C in a medium containing 0.3 M mannitol, 10 mM Tris-maleate (pH 6.5), 0.2 mM EDTA, 5 mM potassium phosphate, 0.05% bovine serum albumin, 5 mM succinate, and 2 mM ATP. ATP hydrolysis was started by the addition of CCCP (0.5 μM), and then the amount of ATP hydrolyzed was measured as described (7). Antimycin A (1 μg/ml) and CCCP (0.5 μM) were added at the times indicated. ○, DKD-5D; ●, D26 harboring YEp51; □, YC50. b: Mitochondria from the wild-type and YC50 were incubated in the medium described in a, except for 2 mM ADP instead of ATP. ○, DKD-5D; □, YC50.

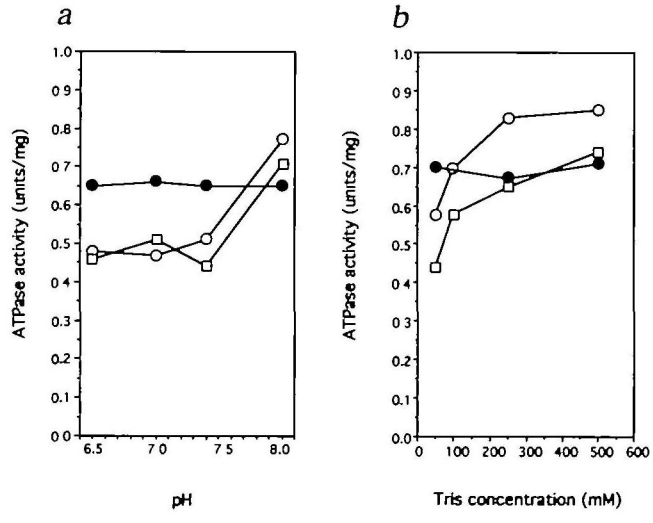

Fig. 5. **Effects of pH and ionic strength on the ATPase activity in isolated mitochondria.** Mitochondria isolated from cells grown in the presence of *o*-phenanthroline were preincubated with Tris-maleate (50 mM), changing the pH as indicated (a), or with increasing concentrations of Tris-maleate (pH 7.4) (b) at 25°C for 30 min, and them ATPase activities were assayed under the conditions described (*18*). ○, DKD-5D; ●, D26 harboring YEp51; □, YC50.

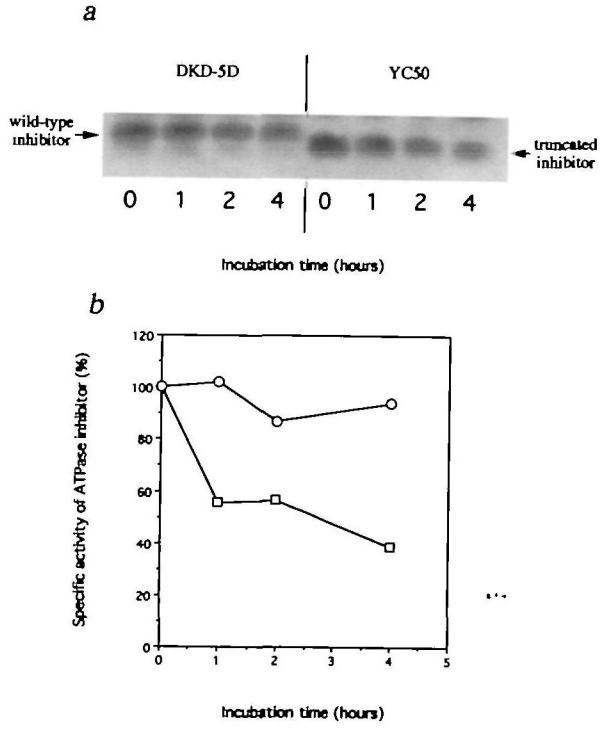

Fig. 6. **Degradation of the truncated ATPase inhibitor in mitochondria.** Yeast cells were grown in a medium containing 5 mM *o*-phenanthroline. The cells were washed twice with water and then incubated in a medium containing 0.5% yeast extract, 2% glucose, 10 mM $MgSO_4$, 5 mM $CaCl_2$, 10 mM $MnCl_2$, and 4 mg/ml cycloheximide at 30°C. At the times indicated, 1.6 g cells were taken and the inhibitor protein was extracted by the heat treatment described under "MATERIALS AND METHODS" to determine the amount in cells. a: The wild-type and truncated ATPase inhibitors were detected by immunoblotting, as described in Fig. 2. b: Specific activity of the ATPase inhibitors in the extracts were determined by the reported method (*18, 26*). ○, DKD-5D; □, YC50.

results obtained on immunoblotting (Fig. 6b). The activity of YC50 decreased to 40% of the initial level after 4 h, while no significant change was observed for the wild-type control.

DISCUSSION

In the present study, the possible role of the coiled-coil structure in the C-terminal region of the yeast ATPase inhibitor was examined by characterization of three kinds of truncated mutant. Since one truncated mutant inhibitor (Ile51→end) inhibits soluble and membrane-bound ATPase like the wild-type control does, the sequence from Ile51 to Lys63 of the yeast ATPase inhibitor does not seem to be essential for the ATPase inhibitory activity of the protein. The results were consistent with previous reports on the bovine ATPase inhibitor (*18, 19*), and indicated that the C-terminal sequence of the inhibitor, which forms a particular secondary structure, coiled coil, is not involved

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in the inhibitory-binding site at the α/β interface of F_1-ATPase. But the mutant inhibitor (Ile51→end) was degraded in the cells, indicating that the region is required for the formation of a stable conformation or complex which is protected against degradation by proteases.

The fact that the most largely truncated mutant inhibitor protein (Gln43→end) was not detected in the cell extracts may raise the question of whether its precursor protein cannot be transported into mitochondria after translation in the cytoplasm because of its small molecular size. However, this possibility may be ruled out by the experimental results for gene fusion obtained by Hurt *et al.*, *i.e.* the signal peptide located at the N-terminus of the precursor protein is solely responsible for the binding of the protein to the mitochondrial membrane and its translocation across the membrane (*29*). Moreover, it has been demonstrated that synthetic peptides made up of only 22-34 amino acid residues are capable of being imported into mitochondria (*30-32*). So, it is highly likely that the precursor protein of the truncated mutant inhibitor undergoes normal processing in mitochondrial membranes, and that the mature mutant protein is degraded by mitochondrial proteases, although no experiment was carried out on the translocation of precursor proteins in the present study.

A coiled-coil structure is often found in fibrous proteins, such as keratin and tropomyosin. Such a structure is also found in several eukaryotic transcription factors, such as Jun, Fos and GCN4, and is known to be composed of a leucine zipper (*33*). The leucine zipper consists of 4 or 5 repeats of leucine residues every seven amino acid residues, and the hydrophobic surface of the secondary structure formed facilitates dimer formation (*34*). The C-terminal region of the ATPase inhibitor contains four repeats of the leucine sequence, and therefore is highly likely to form the coiled-coil structure which can play an important role in the interaction of the regulatory protein with other factor(s). The truncated inhibitor (Ile58→end) of YC57, which lacks one of the four (iso)leucine residues in the coiled-coil structure, was stable, while the greatly truncated inhibitor protein (Gln43→end) of YC42, which lacks three leucine residues, was never detected in any cell extracts. The intermediately truncated mutant inhibitor (Ile51→end) of YC50, lacking two leucine residues, which was detected only in cells grown in the presence of *o*-phenanthroline, seems to interact very loosely with the receptor protein and to gradually undergo proteolytic attack in mitochondria. The unstableness of the truncated inhibitor proteins is supported by the observation of Hu *et al.* that at least two, generally three, of the four leucines in the GCN4 leucine zipper are required for the function (*35*). The truncated proteins, which fail to be assembled, are probably degraded by metal- and ATP-dependent proteolytic enzymes in yeast mitochondria (*28, 36*), and also by several other species of protease which have been isolated and characterized (*37-39*).

Recently, van Raaij *et al.* (*19*) showed that the inhibitor purified from bovine heart forms a monodisperse aggregate of approximately 66 kDa, but that mutants without the coiled-coil region do not. If the inhibitor forms such as aggregate *in vivo* as well, it could be resistant to proteolysis.

We reported earlier that yeast F_1F_o-ATPase *in situ* contains two other regulatory factors (9- and 15-kDa proteins) which facilitate the formation of the inhibitor-enzyme complex (*40*). So, the inhibitor and the two protein factors possibly interact with the F_o sector of F_1F_o-ATPase (*40, 41*). Since the 9-kDa protein was also predicted to have a coiled-coil structure on analysis with a computer program (data not shown), the secondary structure seems to play an important role in the interaction with the three regulatory proteins in F_1F_o-ATPase.

The role of the coiled-coil structure in the C-terminal region of the ATPase inhibitor also seems to be the case for the swine inhibitor proteins. Lopez-Mediavilla *et al.* (*42*) showed that the ATPase inhibitor isolated from pig heart binds not only to its inhibitory-binding site at F_1 but also to a non-inhibitory site which is a membrane protein with a molecular size of approximately 5-6 kDa. The C-terminal region of the inhibitor is probably required for the binding to the receptor protein in the mitochondrial inner membrane, and the proteins form a stable complex which is protected against degradation by proteases.

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