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The Region from Phenylalanine-17 to Phenylalanine-28 of a Yeast Mitochondrial ATPase Inhibitor Is Essential for Its ATPase Inhibitory Activity

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Mitochondrial ATP synthase (F_1F_o-ATPase) is regulated by an intrinsic ATPase inhibitor protein. In the present study, we investigated the structure-function relationship of the yeast ATPase inhibitor by amino acid replacement. A total of 22 mutants were isolated and characterized. Five mutants (F17S, R20G, R22G, E25A, and F28S) were entirely inactive, indicating that the residues, Phe17, Arg20, Arg22, Glu25, and Phe28, are essential for the ATPase inhibitory activity of the protein. The activity of 7 mutants (A23G, R30G, R32G, Q36G, L37G, L40S, and L44G) decreased, indicating that the residues, Ala23, Arg30, Arg32, Gln36, Leu37, Leu40, and Leu44, are also involved in the activity. Three mutants, V29G, K34Q, and K41Q, retained normal activity at pH 6.5, but were less active at pH 7.2, indicating that the residues, Val29, Lys34, and Lys41, are required for the protein's action at higher pH. The effects of 6 mutants (D26A, E35V, H39N, H39R, K46Q, and K49Q) were slight or undetectable, and the residues Asp26, Glu35, His39, Lys46, and Lys49 thus appear to be dispensable. The mutant E21A retained normal ATPase inhibitory activity but lacked pH-sensitivity. Competition experiments suggested that the 5 inactivated mutants (F17S, R20G, R22G, E25A, and F28S) could still bind to the inhibitory site on F_1F_o-ATPase. These results show that the region from the position 17 to 28 of the yeast inhibitor is the most important for its activity and is required for the inhibition of F_1, rather than binding to the enzyme.

Key words: ATPase inhibitor (IF_1), ATP synthase, F_1F_o-ATPase, mitochondria, regulation.

In aerobic organisms, most of the ATP required for energy-dependent reactions, such as biosynthesis, motility, and active transport, is synthesized by ATP synthase (F_1F_o-ATPase). The enzyme synthesizes ATP from ADP and inorganic phosphate using a respiratory chain-linked proton gradient across biomembranes. The enzyme is composed of two parts. a catalytic sector, F_1, and an integral membrane sector, F_o. F_1 consists of five types of subunits with the stoichiometry $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$. The α- and β-subunits are arranged alternately around the γ-subunit, and three catalytic sites are located at the α–β interfaces. Fo functions as a proton pathway and the energy produced on proton flux through this part is transmitted to F_1 as a rotation movement and used for ATP synthesis (for a review see Ref. *1*).

Mitochondrial F_1F_o-ATPase is regulated by a small basic protein, the ATPase inhibitor (*2*). The inhibitor binds to the enzyme in a 1:1 molar ratio in the presence of MgATP and completely inhibits its activity. The inhibitory action of the inhibitor to the enzyme is unidirectional (*3*). The inhibitor does not inhibit ATP synthesis of F_1F_o, but when the electrochemical gradient is lost, it completely inhibits the ATP hydrolyzing activity of the enzyme (*4, 5*). The action of the inhibitor also depends on pH: effective inhibition of F_1F_o-ATPase by the inhibitor requires a low pH (<7.0). Above pH 7.5, the activity sharply declines (*2*).

The inhibitor has been isolated from various eukaryotic cells from yeast to mammals, and the primary structures of *Saccharomyces cerevisiae* (*6*), *Candida utilis* (*7*), beef (*8*), rat (*9, 10*), mouse (*11*), and human (*12*) have been determined. The regulation of F_1F_o by the inhibitors seems to be achieved by a shared mechanism from yeast to mammals, because the amino acid sequences of the inhibitors are very similar, and the yeast inhibitor can inhibit bovine F_1-ATPase and *vice versa* (*13*).

The functional region of the bovine inhibitor was previously investigated using proteolytic fragments (*14, 15*), synthetic peptides (*16*), and deletion mutants (*17*) of the protein, and it was found that the minimal sequence which can inhibit F_1F_o-ATPase was Ala14–Lys47 (*17*). Recently, it was shown that the histidines at positions 48, 49, and 55 of the mammalian inhibitors are necessary to inactive the protein at high pH (*18, 19*). However, the yeast inhibitor lacks homologous histidine residues, and the mechanism of

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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, P/O ratio, phosphorus/oxygen ratio.

its pH-sensitivity is unknown.

We previously constructed progressive C-terminal deletion mutants of the yeast inhibitor and showed that the region Ile51–Lys63 is not involved in the its inhibitory action, but is required for the stable conformation of the protein which is protected against degradation *in vivo* (*20*) Recently, it was reported that the homologous region of the bovine inhibitor is required for dimer formation of the protein (*21*). We also constructed two mutants of Lys19 (Lys19→Asn and Lys19→Arg), and showed that the mutations decrease inhibitory activity at higher pH (*22*).

In the present study, we examined the role of the residues conserved in the primary structures of the proteins by site-directed mutagenesis and found that the region from position 17 to 28 of the yeast inhibitor is the most important for its activity.

MATERIALS AND METHODS

Construction of Mutant Yeasts—Saccharomyces cerevisiae strain D26 (**a** *trp1 leu2 his3 inh1::TRP1*), which contains a null mutation in the gene coding for the ATPase inhibitor (*5*), was used as the host for expression of the mutant ATPase inhibitors.

The mutated genes were obtained using an Altered Site II *in vitro* Mutagenesis Kit (Promega, USA) as described previously (*22*). The oligonucleotides used for mutagenesis are listed in Table I. The sequences of the mutants were verified with an ABI PRISM 310 DNA sequencer (PE Biosystems, USA). The mutated genes were cloned under the *GAL10* promoter of the YEp51 plasmid, and the inhibitor-deficient yeast D26 was transformed as described previously (*22*). The mutants obtained were named as shown in Table I.

The control strain, which harbored an expression plasmid containing the coding sequence of the wild-type inhibitor, was also constructed and named YC63 (*20*). The strain D26/YEp51, which harbored a YEp51 plasmid containing no coding sequence, was constructed and used as an inhibitor-deficient control

Measurement of CD Spectra—Samples containing 0.1 mg/ml (14 μM) ATPase inhibitor and 50 mM potassium phosphate buffer (pH 6.5) were placed in a quartz cuvette with a 1-mm path length, and spectra were recorded with a J-820 spectropolarimeter (Jasco Corporation, Tokyo) in the range of 190–250 nm. The α-helical content was calculated using the method of Chen *et al.* (*23*).

Other Procedures—The yeast cells were grown on a medium containing 2% peptone, 1% yeast extract, and 2% galactose for 18 h at 30°C as described previously (*22*) Wild-type and mutant ATPase inhibitors were extracted from yeast cells by heating as described (*20*). Purification of the mutant inhibitors by reverse-phase HPLC was performed as described previously (*22*). Submitochondrial particles were prepared from an inhibitor-deficient yeast strain, W3 (**a** *trp1 leu2 his3 inh1::TRP1 stf1::LEU2*) (*5*), using a reported method (*24*). The concentrations of the purified ATPase inhibitors were determined by the method of Lowry *et al.* (*25*) with bovine serum albumin as a standard. SDS–polyacrylamide gel electrophoresis was done by the method of Schagger and von Jagow (*26*).

TABLE I **Synthetic oligonucleotides for mutagenesis of the ATPase inhibitor.**

Mutation	Oligonucleotide sequence	Name
Phe17 → Ser	5'-GAGGATTCGTCTGTTAAAAGG-3' (21 mer)	F17S
Arg20 → Gly	5'-GTTTGTTAAAGGGGAAAGGGC-3' (21 mer)	R20G
Glu21 → Ala	5'-GTTAAAAGGGCAAGGGCCACG-3' (21 mer)	E21A
Arg22 → Gly	5'-TAAAAGGGAAGGGGCCACGGA-3' (21 mer)	R22G
Ala23 → Gly	5'-AGGGAAAGGGGCACGGAAGAC-3' (21 mer)	A23G
Glu25 → Ala	5'-AGGGCCACGGCAGACTTCTTC-3' (21 mer)	E25A
Asp26 → Ala	5'-GCCACGGAAGCCTTCTTCGTT-3' (21 mer)	D26A
Phe28 → Ser	5'-GAAGACTTCTCCGTTAGGCAG-3' (21 mer)	F28S
Val29 → Gly	5'-GACTTCTTCGGTAGGCAGCGT-3' (21 mer)	V29G
Arg30 → Gly	5'-CTTCTTCGTTGGGCAGCGTGA-3' (21 mer)	R30G
Arg32 → Gly	5'-CGTTAGGCAGGGTGAGAAGGA-3' (21 mer)	R32G
Lys34 → Gln	5'-GCAGCGTGAGCAGGAGCAACT-3' (21 mer)	K34Q
Glu35 → Val	5'-CGTGAGAAGGTGCAACTACGC-3' (21 mer)	E35V
Gln36 → Gly	5'-AGCGTGAGAAGGAGGGACTACGCCATTTGA-3' (30 mer)	Q36G
Leu37 → Gly	5'-GTGAGAAGGAGCAAGGACGCCATTTGAAAG-3' (30 mer)	L37G
His39 → Asn	5'-GCAACTACGCAATTTGAAAGAA-3' (22 mer)	H39N
His39 → Arg	5'-GCAACTACGCCGTTTGAAAGAA-3' (22 mer)	H39R
Leu40 → Ser	5'-CTACGCCATTCGAAAGAACAA-3' (21 mer)	L40S
Lys41 → Gln	5'-ACGCCATTTGCAAGAACAACT-3' (21 mer)	K41Q
Leu44 → Gly	5'-ATTTGAAAGAACAAGGGGAAAAACAACGAA-3' (30 mer)	L44G
Lys46 → Gln	5'-ACAACTGGAACAACAACGAAA-3' (21 mer)	K46Q
Lys49 → Gln	5'-AAAACAACGACAGAAGATTGA-3' (21 mer)	K49Q

RESULTS

Construction of Mutant Yeasts—We previously constructed C-terminal deletion mutants of the yeast inhibitor and showed that the region from Ile51 to Lys63 of the protein was not involved in its inhibitory action on F_1F_o-ATPase (*20*). Therefore, we targeted the residues which were conserved in the region from positions 1 to 50 for the mutation analysis. A total of 22 mutants (listed in Table I) were constructed and expressed in the inhibitor-deficient yeast strain D26 (*5*) under the control of a *GAL10* promoter. Expression of the proteins in the mutant cells grown on the galactose medium was confirmed by immunoblotting (data not shown). All the mutants grew at a normal rate on the galactose medium. Respiration rate and growth yield of the mutant cells were also normal, indicating oxidative phosphorylation was normally activated in the mutant cells

Purification of the Mutant ATPase Inhibitors—Proteins were extracted from the mutant cells by heating, and the mutated inhibitors were purified from the proteins by Macro-Prep High S column chromatography Figures 1 a and b show typical chromatograms and SDS-PAGE profiles of the protein for the mutant (F17S), respectively. Most of the mutant inhibitors were purified in this step, and any samples containing small amounts of impurities (L37G, L40S, and L44G) were further purified by subsequent

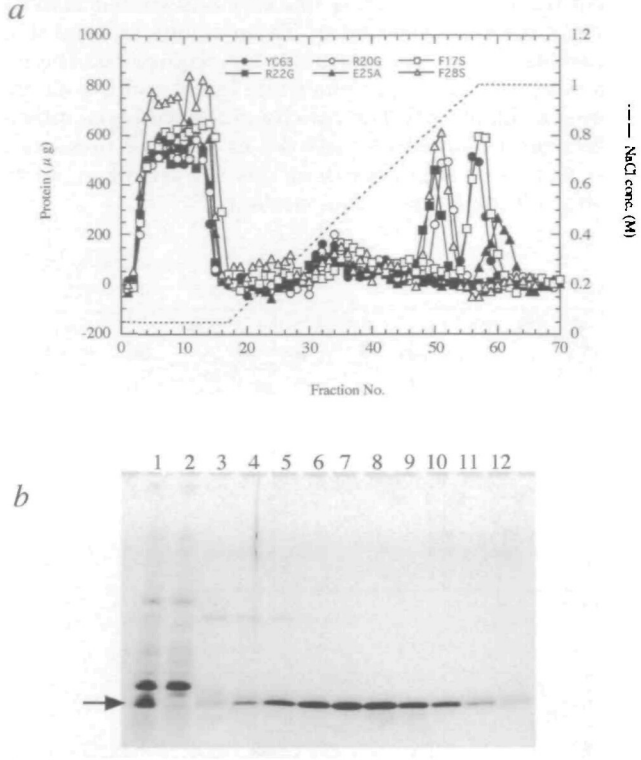

Fig 1 **Purification of wild-type and mutant ATPase inhibitors.** a· Separation of the inhibitors on a Macro-Prep High S column Proteins extracted from yeast cells by heating were put on a Macro-Prep High S column (1 5 cm × 2.5 cm, Bio-Rad Laboratories, USA) The starting buffer (40 ml) was 50 mM sodium acetate (pH 5 0), and the elution buffer (40 ml) contained 1 M NaCl in addition to the above Fractions of 2 ml were collected, and the proteins of each fraction were determined using the method of Bradford *et al* (*32*) ●, YC63 (wild-type), □, F17S; ○, R20R, ■, R22G, ▲, E25A, △, F28S b SDS-PAGE analysis of the fractions from the column for mutant F17S Aliquots (10 μl) from the heat extract and selected fractions were analyzed on a 15% polyacrylamide gel and stained with Coomassie Brilliant Blue R-250 Lane 1, proteins extracted from yeast cells by heat treatment, lane 2, mixture of fractions numbered from 3 to 17 (containing proteins which were not retained on the column), lane 3–12, fractions numbered from 53 to 62.

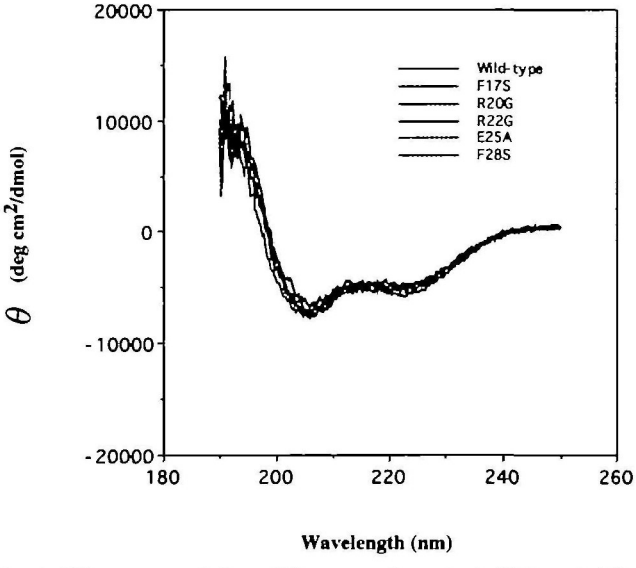

Fig 3 **CD spectra of the wild-type and mutant ATPase inhibitors.** Spectra for the 14 μM solution of the wild-type and mutant ATPase inhibitor in 50 mM potassium phosphate buffer (pH 6.5) were recorded as described under "MATERIALS AND METHODS"

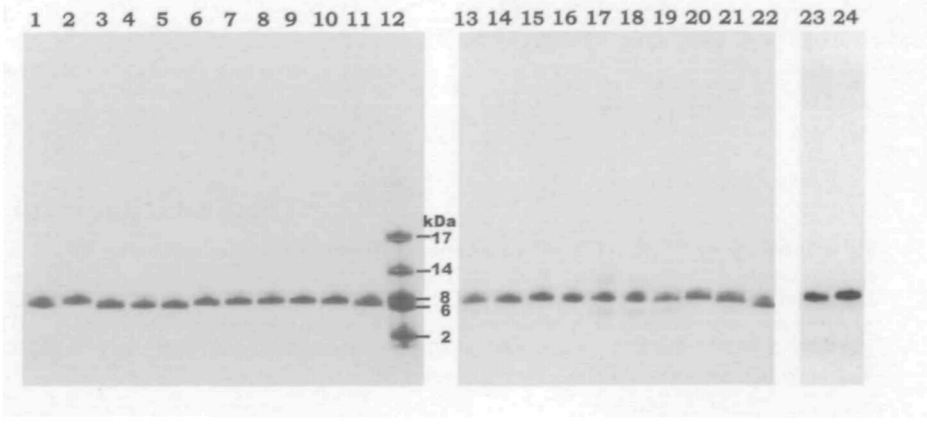

Fig 2 **SDS-PAGE analysis of purified inhibitors.** Approximately 1 μg of each inhibitor was electrophoresed on a 15% polyacrylamide gel and stained with Coomassie Brilliant Blue R-250 Lane 1, wild-type inhibitor, lane 2, F17S, lane 3, R20G, lane 4, E21A; lane 5, R22G; lane 6, A23G, lane 7, E25A; lane 8, D26A; lane 9, F28S, lane 10, V29G; lane 11, R30G, lane 12, molecular weight marker; lane 13, R32G, lane 14, K34Q, lane 15, E35V; lane 16, Q36G, lane 17, L37G, lane 18, L40S, lane 19, K41Q; lane 20, L44G, lane 21, K46Q, lane 22, H49Q, lane 23, H39N; lane 24, H39R

reverse-phase HPLC. Figure 2 shows the SDS-PAGE of purified inhibitors. The yields of the inhibitors were about 1–2 mg from 8 g of yeast cells.

The secondary structures of the purified inhibitors were analyzed by CD. All mutants had similar spectra to that of the wild-type control, which was previously shown (*22*). The spectra of wild-type and representative mutants are shown in Fig. 3. These results indicate that the mutations do not cause marked changes in the secondary structure of the protein. The α-helical contents of the mutant inhibitors were calculated to be 10–20 %.

Activity of Mutant Inhibitors Mutated on a Lysine Residue—The effects of mutation on the ATPase inhibitory activity were investigated with assays at pH 6.5, 7.2, and 8.0. The purified inhibitors were incubated at the indicated pH with submitochondrial particles isolated from inhibitor-deficient mutant yeast, and residual ATP-hydrolyzing activity was measured (Fig. 4).

Figure 4a shows the activity of the inhibitors mutated on a lysine residue (K34Q, K41Q, K46Q, and K49Q). All the mutants were fully active at pH 6.5. At pH 7.2, the activity of the K34Q and K41Q mutants decreased markedly, though the K46Q and K49Q mutants had similar activity to that of the wild-type control. These results indicate that the residues Lys46 and Lys49 are not required for the inhibitory activity of the protein, while Lys34 and Lys 41 are required at higher pH. The activity of the wild-type inhibitor decreased markedly at pH 8.0 as reported previously (*2*). A decrease in the activity at this pH was likewise observed in all the lysine-residue mutants.

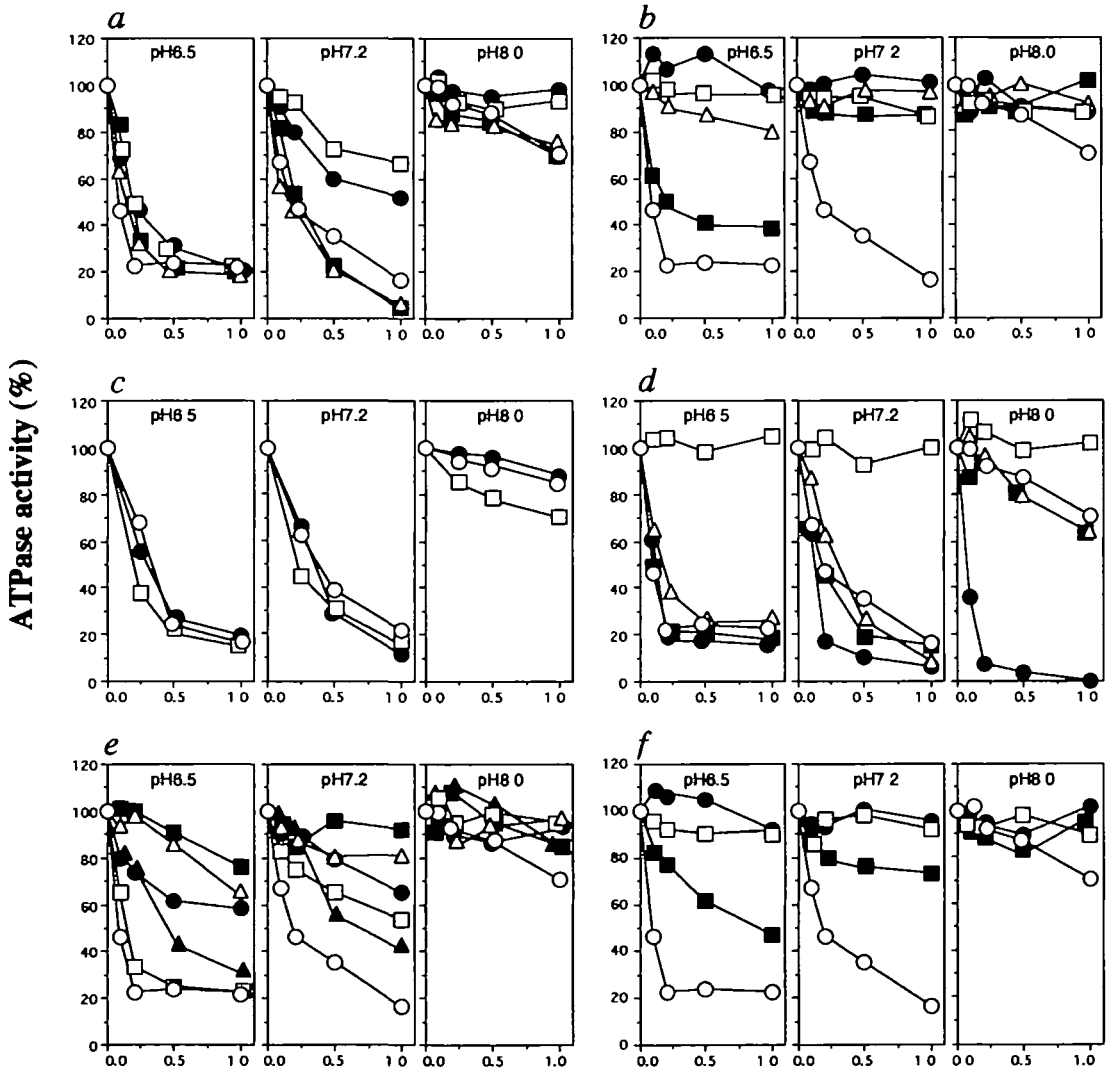

Fig 4. **Inhibition of F_1F_o-ATPase by mutant inhibitors.** The indicated amounts of purified ATPase inhibitors were incubated with submitochondrial particles (containing 0.2 units of F_1F_o-ATPase) at 25°C for 10 min in a medium containing 50 mM of a Tris-maleate buffer (pH 6.5, 7.2, or 8.0), 5 mM $MgSO_4$ and 5 mM ATP in a final volume of 50 μl, and the remaining ATPase activity was measured as described previously (*15*) a, ○, wild-type control, ●, K34Q, □, K41Q; ■, K46Q, △, K49Q b, ○, wild-type control, ●, R20G, □, R22G, ■, R30G, △, R32G c, ○, wild-type control, ●, H39N, □, H39R. d, ○, wild-type control; ●, E21A; □, E25A, ■,D26A, △, E35V. e, ○, wild-type control; ●, A23G, □, V29G, ■, *L37G*, △, *L40S*, ▲, *L44G* f, ○, wild-type control; ●, F17S, □, F28S; ■, Q36G.

Activity of Mutant Inhibitors Mutated on an Arginine Residue—As shown in Fig. 4b, two arginine residue mutants (R20G and R22G) were inactive at all examined pHs, indicating that residues Arg20 and Arg22 are essential for the activity of the inhibitor. The activity of the mutant R30G was slightly lower than the wild-type control at pH 6.5 The R32G mutant had some residual inhibitory activity at pH 6.5, but mutants R30G and R32G were both inactive at pH 7.2 and 8.0. These results show that Arg30 and Arg 32 are also involved in the inhibitory activity.

Activity of Mutant Inhibitors Mutated on a Histidine Residue—The inhibitory activity of mutants on His39 is shown in Fig. 4c. The mutant H39N had similar inhibitory activity to that of the wild-type control (YC63) at all examined pHs. The activity of mutant H39R was also similar to the wild-type control at pH 6.5 and 7 2, but was slightly higher than the control at pH 8.0. Thus, His39 appears to be dispensable.

Activity of Mutant Inhibitors Mutated on an Acidic Residue—The effects of mutation on acidic residues are shown in Fig. 4d. The mutant E25A was completely inactivated at all pHs, indicating that Glu25 is essential for activity. The mutants D26A and E35V had similar activity to that of the wild-type control at all examined pHs, and the residues Asp26 and Glu35 thus appear to be dispensable. At pH 8.0, the activities of the mutants decreased in the same way as that of the wild-type control. The mutant E21A had similar inhibitory activity to that of the wild-type control at pH 6.5 and 7.2, but at pH 8.0 the mutant retained normal activity. These results show that Glu21 is required for the pH sensitivity of the protein.

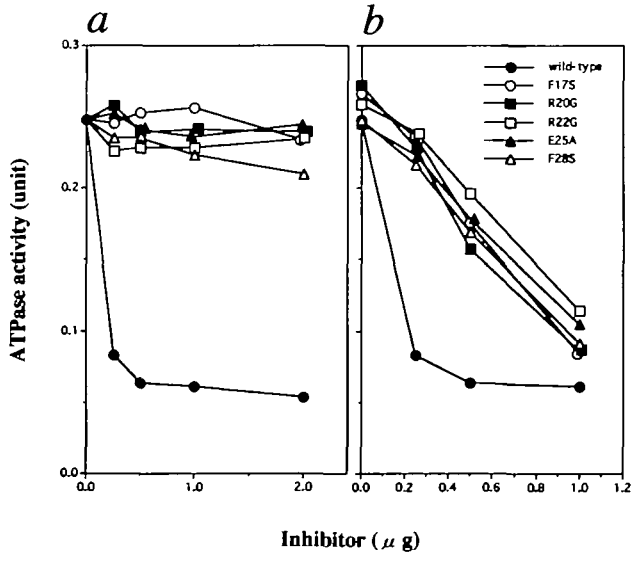

Fig. 5 **Competition of noninhibitory mutants with the wild-type inhibitor.** a ATPase inhibitory activity of the purified inhibitor The indicated amounts of ATPase inhibitors were incubated with submitochondrial particles at pH 6 5 in the same manner as in Fig 4, and the remaining ATPase activity was measured ●, wild-type ATPase inhibitor; ○, F17S; ■, R20G; □, R22G; ▲, E25A; △, F28S. b Competition of mutant inhibitors with the wild-type inhibitor. The indicated amounts of wild-type inhibitor were incubated with submitochondrial particles and 1 μg of mutant inhibitor under the same conditions, and the remaining ATPase activity was measured ●, control experiment without competitor; ○, F17S, ■, R20G, □, R22G, ▲, E25A, △, F28S

Activity of Mutant Inhibitors Mutated on a Neutral Residue—The effects of mutation on neutral residues are shown in Fig. 4, e and f. The mutant A23G was only slightly active at both pH 6.5 and 7.2, and was inactive at pH 8.0. The activity of mutants L37G and L40S markedly decreased at pH 6.5 and was lost at pH 7.2 and 8.0. The activity of mutants Q36G and L44G also decreased at pH 6.5 and 7.2. These results show that the residues Ala23, Gln36, Leu37, Leu40, and Leu44 are involved in the inhibitory activity of the protein. The activity of the V29G mutant was normal at pH 6.5, but decreased at 7.2, indicating that the Val29 is required at higher pH. The mutants F17S and F28S were inactive at all pHs, indicating that the aromatic residues Phe17 and 28 are essential.

Competition of Noninhibitory Mutants with the Wild-Type Inhibitor—To test the ability of five inactive mutant inhibitors (F17S, R20G, R22G, E25A, and F28S) to bind at the inhibitory site on F_1F_o-ATPase, the competition of the mutants with the wild-type inhibitor was examined As shown in Fig. 5, all five mutants similarly interfered with the action of the wild-type inhibitor. About 1 μg of the mutant inhibitors was required for the 50% suppression of the action of 0.5 μg of the wild-type inhibitor, indicating that the binding affinity of the mutant inhibitors to F_1F_o decreased to less than half of that of wild-type inhibitor. These results suggest that the mutant inhibitors can still interact with the inhibitory site on F_1F_o-ATPase in spite of their inactive nature, though their affinity is somewhat diminished.

DISCUSSION

In the present study, we isolated and characterized 22 mutants of the yeast ATPase inhibitor to elucidate the structure-function relationship of the protein. We identified 5 essential residues (Phe17, Arg20, Arg22, Glu25, and Phe28), 10 residues which are not essential but involved in its activity at pH 6 5 or 7.2 (Ala23, Val29, Arg30, Arg32, Lys34, Gln36, Leu37, Leu40, Lys41, and Leu44), and one residue that is required for the pH sensitivity of the protein (Glu21). The requirements of the residues in the yeast inhibitor found in this study are shown in Fig. 6.

Previously, it was shown that mutation of histidine residues in the mammalian ATPase inhibitor at positions 48, 49, and 55 causes loss of pH-sensitivity of the protein, and models of the interconversion between the active and inactive forms of the inhibitor, regulated by protonation and deprotonation of the residues, have been proposed (*18, 19, 21*). However, the yeast inhibitor does not have homologous residues. The yeast protein contains only one histidine residue, at position 39, and in order to establish the role of this residue, we constructed two mutants of His39 (H39R and H39N). The effects of these mutations were slight or undetectable (Fig. 4c), indicating that the yeast inhibitor lacks regulation by histidine residues. Our results (Fig. 4d) showed that, in yeast, Glu21 is important in terms of the pH-sensitivity of the protein, rather than histidine residues. Homologous glutamic acid may also be involved in the pH-sensitivity of mammalian inhibitors, because the residue is highly conserved in the primary structures of the protein isolated from other species (Fig. 6).

It has been believed that the inactive nature of the inhibitor at high pH is important for the release of the protein

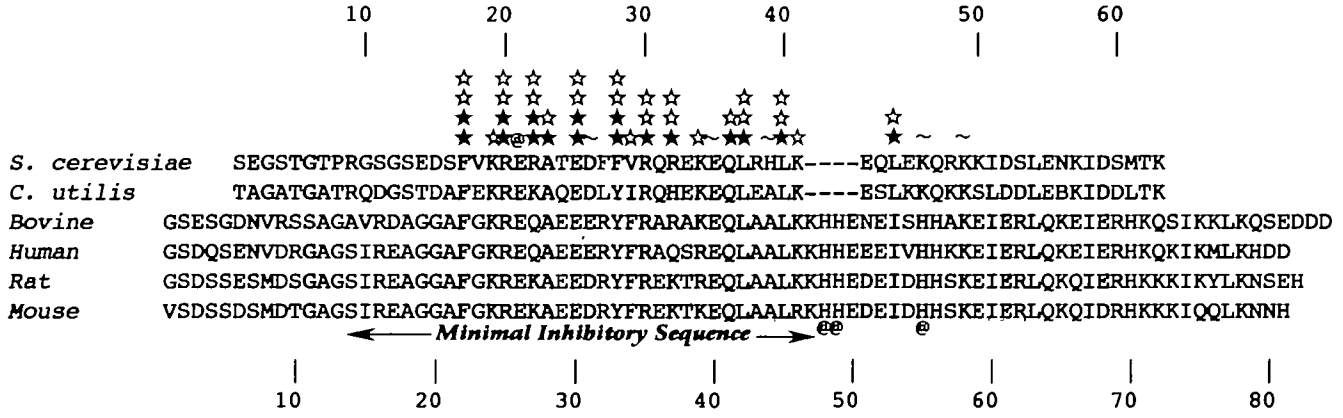

Fig 6 **Residues required for the activity of the ATPase inhibitor.** The sequences of ATPase inhibitors from six species are aligned Identical and conservatively substituted residues are shaded Asterisks show the important residues for ATPase inhibitory activity Black and white asterisks (★ and ☆) show requirements at pH 6 5 and 7 2, respectively Vertical stacks of two asterisks show essential residues and single asterisks show residues which are not essential, but important, at that pH Tildes (~) show the residues that appear to be dispensable The minimal inhibitory sequence of the bovine ATPase inhibitor is shown by an arrow Atmarks (@) denote residues which are important for inactivation of the protein at high pH Previously reported results on Lys19 of the yeast inhibitor (*22*) are also indicated

from F_1 to active ATP synthesis when the proton gradient is generated across the mitochondrial inner membrane by the action of the respiratory chain. However, the ATP synthesis in the E21A mutant cells did not seem to be inhibited by the protein, in spite of its inhibitory activity at high pH, because the rate of growth and respiration of the cells were normal. Moreover, mitochondria were isolated from E21A and control cells and state 3 respiration, respiratory control, and P/O ratios were investigated, but no effect of the mutation was detected (data not shown). These observations suggest that the pH-sensitivity of the inhibitor is not important for the release of the protein from the inhibitory site on F_1F_o-ATPase, and that the release is mainly regulated by other factor(s) which have previously been suggested (*3*) (*i.e.,* the change in the state of the transmembrane electrochemical gradient and the subsequent conformation change of F_1). We recently suggested that the F_1F_o molecule itself converts between phosphorylating and non-phosphorylating forms corresponding to respiration rates of mitochondria, and that the inhibitor binds preferentially to the non-phosphorylating form of the enzyme (*27*). The inhibitor may recognize the conformation of the non-phosphorylating form of F_1F_o-ATPase.

Previously, the role of lysine residues in the bovine inhibitor was investigated using chemical modification, and it was shown that most of the residues are not important for the function of the protein (*28, 29*). Our results also showed that the mutations on lysine residues of the yeast inhibitor did not cause any marked decrease in its activity at pH 6.5. The effects of the mutation were only detected at pH7.2 on the K34Q and K41Q mutants, indicating that Lys34 and Lys41 are required only at higher pH. The lysine residues of the ATPase inhibitor are not absolutely necessary for its function.

As shown in Fig. 6, the residues involved in the ATPase inhibitory activity of the yeast ATPase inhibitor were located at a region from position 17 to 44. The region coincides with the minimal inhibitory sequence of the bovine ATPase inhibitor, which was previously found by van Raaij *et al.* (*17*), indicating that the residues are also important in the mammalian ATPase inhibitor.

The essential residues (Phe17, Arg20, Arg22, Glu25, and Phe28) are concentrated in the region from position 17 to 28, and the importance of residues for the inhibitory activity decreases with distance from this region (Fig. 6). Thus, the region Phe17–Phe28 seems to be the active center of the protein. Furthermore, the competition experiments suggested that the five inactive mutants (F17S, R20G, R22G, E25A, and F28S) could still interact with the inhibitory site on F_1F_o-ATPase despite their inactivity. These results shows that the region is the most important for inhibitory activity and is required for the inhibition of F_1, rather than binding to the enzyme.

From the results of cross-linking experiments, we previously suggested that the binding site of the ATPase inhibitor is situated near or at the active site of F_1-ATPase (*30, 31*). It is likely that the five essential residues, Phe17, Arg20, Arg22, Glu25, and Phe28, interact near the site and stop the catalytic cycle of F_1F_o-ATPase. Identification of the sites on F_1F_o-ATPase which interact with the residues will allow elucidation of the regulatory mechanism of the enzyme by the ATPase inhibitor protein.

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