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Overexpression, Purification, and Characterization of Human and Bovine Mitochondrial ATPase Inhibitors: Comparison of the Properties of Mammalian and Yeast ATPase Inhibitors

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Mitochondrial ATP synthase (F_1F_0-ATPase) is regulated by an intrinsic ATPase inhibitor protein. In this study, we overexpressed and purified human and bovine ATPase inhibitors and their properties were compared with those of a yeast inhibitor. The human and bovine inhibitors inhibited bovine ATPase in a similar way. The yeast inhibitor also inhibited bovine F_1F_0-ATPase, although the activity was about three times lower than the mammalian inhibitors. All three inhibitors inhibited yeast F_1F_0-ATPase in a similar way. The activities of all inhibitors decreased at higher pH, but the magnitude of the decrease was different for each combination of inhibitor and ATPase. The results obtained in this study show that the inhibitory mechanism of the inhibitors was basically shared in yeast and mammals, but that mammalian inhibitors require unique residues, which are lacking in the yeast inhibitor, for their maximum inhibitory activity. Common inhibitory sites of mammalian and yeast inhibitors are suggested.

KEY WORDS: ATPase inhibitor (IF_1); ATP synthase; F_1F_0-ATPase; mitochondria; regulation.

INTRODUCTION

ATP synthase (F_1F_0-ATPase), which is a constituent of mitochondrial inner membranes and bacterial plasma membranes, catalyzes the terminal step of oxidative phosphorylation. The enzyme generates ATP from ADP and inorganic phosphate utilizing the energy produced on proton flux through the membranes. The enzyme consists of a catalytic sector, F_1, and an integral membrane sector, Fo. F_1 consists of five subunits with a stoichiometry of $\alpha_3\beta_3\gamma_1\delta_1$. 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 to the α subunits (Abrahams *et al.*, 1994). Fo part forms a proton channel, and the energy produced on proton flux through this part is transmitted to F_1 as a rotation movement of γ (Noji *et al.*, 1997; Sambongi *et al.*, 1999) and used for ATP synthesis (review: Boyer, 1997).

Mitochondrial F_1F_0-ATPase is regulated by a small basic protein called an ATPase inhibitor. The inhibitor is not required for ATP synthesis of F_1F_0, but binds to the F_1 portion of the enzyme in a 1:1 molar ratio and completely inhibits its ATP-hydrolyzing activity (Schwerzmann and Pedersen, 1986). The inhibitory action of the inhibitor is influenced by pH; effective inhibition of F_1F_0-ATPase by the inhibitor requires a low pH (<7.0). Above pH 7.5, the activity declines (Pullman and Monroy, 1963; Schwerzmann and Pedersen, 1986).

The inhibitor was first isolated from bovine heart mitochondria by Pullman and Monroy (1963) and homologous proteins have been isolated from various eukaryotic cells from yeast to mammals (Dianoux and Hoppe, 1987;

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Key to abbreviations: F_1F_0 or F_1F_0-ATPase, mitochondrial ATP synthase; F_1 or F_1-ATPase, catalytic part of F_1F_0-ATPase; GST, glutathione *S*-transferase; I_{50}, amount of the inhibitor required to inhibit 50% of the ATPase activity; IPTG, isopropylthio-β-D-galactoside; PBS, phosphate buffered saline.

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Frangione *et al.*, 1981; Higuti *et al.*, 1993; Ichikawa *et al.*, 1999; Lebowitz and Pedersen, 1993; Matsubara *et al.*, 1981; Yamada *et al.*, 1997). In bacteria, a homologous inhibitor protein has not been isolated, but the activity of their F_1F_0 is controlled by a regulatory subunit, ε (Hara *et al.*, 2001; Kato-Yamada *et al.*, 1999; Kuki *et al.*, 1988; Sternweis and Smith, 1980).

We previously investigated the residues required for the function of the yeast ATPase inhibitor by site-directed mutagenesis (Ichikawa *et al.*, 1998, 2000, 2001), demonstrating that the region from Phe 17 to Leu44 was involved in the inhibitory activity, and that five residues (Phe17, Arg20, Arg22, Glu25, and Phe28) in the region from Phe17 to Phe28 are essential (Ichikawa *et al.*, 2001) (Fig. 1). It was also shown that Glu21 of the inhibitor is required for its pH-sensitivity (Ichikawa *et al.*, 2001) (Fig. 1).

The functional region of the bovine inhibitor was also investigated using deletion mutants of the inhibitor, and the minimal sequence which can inhibit F_1F_0-ATPase has been defined as Ala 14-Lys47 (van Raaij *et al.*, 1996) (Fig. 1). Zanotti *et al.* (2000) reported that a synthetic peptide consisting of residues from Leu42 to Lys58 of the bovine inhibitor exhibited ATPase inhibitory activity. Furthermore, it was shown that the histidine residues at positions 48, 49, and 55 of the inhibitors from the cow and rat are required to inactivate the protein at high pH (Lebowitz and Pedersen, 1996; Schnizer *et al.*, 1996) (Fig. 1).

However, the residues postulated in the studies on the mammalian inhibitors are lacking in the yeast inhibitor (Fig. 1). In this study, we overexpressed and purified the human and bovine ATPase inhibitors, and their inhibitory activities on both bovine and yeast F_1F_0-ATPase, pH-sensitivities of the activities, and their secondary structures were compared with those of the yeast inhibitor.

MATERIALS AND METHODS

Construction of the Expression Vector

The coding sequence of the human heart ATPase inhibitor was amplified by PCR from cDNA (Ichikawa *et al.*, 1999) using primers, 5′-GCG GAT CCG ATC AGT CCG AGA ATG TCG ACC-3′ and 5′-CGG AAT TCT TAA TCA TCA TGT TTT AGC ATT-3′. The product was cloned into the BamHI/EcoRI site of the pGEX-6P-1 (Amersham Pharmacia Biotech, England), and introduced into *Escherichia coli*, JM109. The resultant plasmid coded the mature form of the inhibitor of which the N terminus fused to glutathione *S*-transferase (GST). The fusion protein can be cleaved specifically at the fusion junction with PreScission™ Protease (Amersham Pharmacia Biotech), and the resultant inhibitor contains three extra amino acids (Gly-Pro-Leu-) at the N terminus.

The coding sequence of the bovine ATPase inhibitor was amplified from a bovine heart cDNA library (Clontech Laboratories Inc., USA, Cat. #: BL1017b) using primers, 5′-CCC GAA TTC ATG GGC TCG GAA TCG GGA GAT AAT GT-3′ and 5′-GCC AAG CTT AGT CGT CAT CCT CAC TCT GTT TTA GT-3′. The product was cloned under the tac promoter of the expression vector, pMK2 (Kitamura *et al.*, 1994), and introduced into *E. coli*,

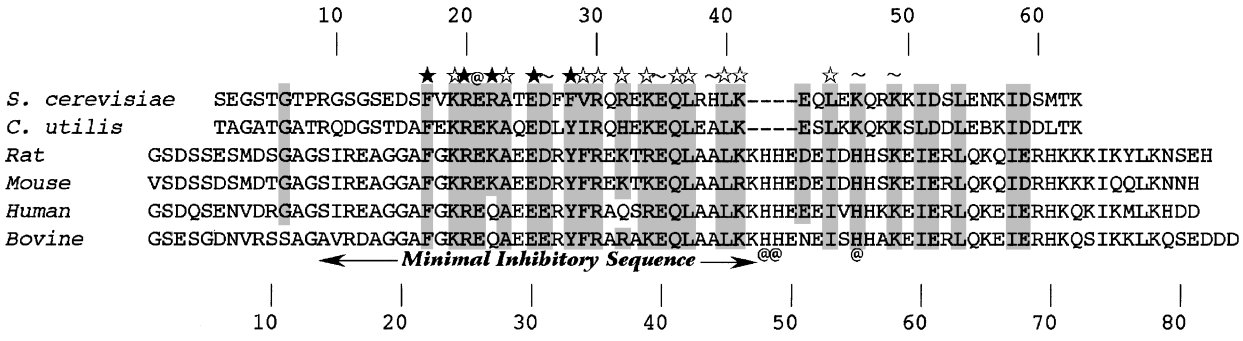

Fig. 1. Alignment of ATPase inhibitors. Primary structures of the inhibitors from *Saccharomyces cerevisiae* (Matsubara *et al.*, 1981), *Candida utilis* (Dianoux and Hoppe, 1987), rat (Higuti *et al.*, 1993; Lebowitz and Pedersen, 1993), mouse (Yamada *et al.*, 1997), human (Ichikawa *et al.*, 1999), and cow (Frangione *et al.*, 1981) are arranged. Identical and conservatively substituted residues are shaded. Black and white asterisks show residues which are essential and partially required for the inhibitory activity of the yeast inhibitor (Ichikawa *et al.*, 2000, 2001). Tildes show the residues that appear to be dispensable (Ichikawa *et al.*, 2001). Atmarks indicate the residues which are important for inactivation of the protein at high pH (Ichikawa *et al.*, 2001; Lebowitz and Pedersen, 1996; Schnizer *et al.*, 1996). The minimal inhibitory sequence of the bovine ATPase inhibitor (van Raaij *et al.*, 1996) is also shown.

JM109. The resultant plasmid coded the mature form of the inhibitor for which the import signal sequence was replaced by a single methionine residue.

The coding sequences of the inhibitors were verified with an ABI PRISM 310 Genetic Analyzer (PE Biosystems, USA).

Measurement of CD Spectra

Samples containing 0.1 mg/mL ATPase inhibitor and 50 mM potassium phosphate buffer (pH 6.5 or 8.2) 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 a computer program, JWSSE-480 (Jasco Corporation).

Preparation of Membranes Used for the Assay of the Inhibitors

Inverted membrane vesicles of *E. coli*, DK8, harboring a pBWU17 plasmid that overexpress the wild-type F_1F_0-ATPase (Moriyama *et al.*, 1991; Omote *et al.*, 1998) were kindly gifted from Dr Iwamoto-Kihara (Institute of Scientific and Industrial Research, Osaka University). Yeast submitochondrial particles were prepared from an inhibitor-deficient strain of *Saccharomyces cerevisiae*, W3 (**a** *trp1 leu2 his3 inh1::TRP1 stf1::LEU2*) (Ichikawa *et al.*, 1990), using reported methods (Takeshige *et al.*, 1976). The inhibitor-depleted particles of bovine heart mitochondria were prepared as described by Horstman and Racker (1970).

Other Procedures

A yeast ATPase inhibitor was purified from the *S. cerevisiae* strain, YC63 (Ichikawa *et al.*, 1998), as described previously (Ichikawa *et al.*, 2001). Protein was measured by the methods of Lowry *et al.* (1951) with bovine serum albumin as a standard. I_{50} of the inhibitor was defined as the amount of the inhibitor required to inhibit 50% of the ATPase activity in the condition shown in Fig. 4.

RESULTS

Expression and Purification of the Human ATPase Inhibitor

Human ATPase inhibitor was overexpressed as a fusion protein of the GST in *E. coli* cells. The induced cells were disrupted by sonication and a soluble fraction of the cells was applied to a Glutathione Sepharose 4B column (Fig. 2(a)). Most of the fusion protein bound to the column and a large portion of the impurities were washed away (Fig. 2(a), lanes 2–5). The inhibitor was recovered from the column by on-column cleavage of the fusion protein (Fig. 2(a), lane 6). This fraction still contained marked amounts of impurities (Fig. 2(a), lane 6), and the inhibitor was further purified by subsequent Macro-prep High S column chromatography (Fig. 2(b)). The preparation of the inhibitor yielded a single band on SDS-PAGE gel (Fig. 2(b)). The yield of the human inhibitor from 1 L of the culture was about 0.4 mg.

Expression and Purification of the Bovine ATPase Inhibitor

The bovine ATPase inhibitor (mature form) was directly expressed in *E. coli* cells under the control of the *tac* promoter (Fig. 2(c)). The inhibitor was extracted from the cells by heating and purified with Macro-prep High S column chromatography. As shown in Fig. 2(d), this single chromatography step resulted in a single band of the inhibitor on the SDS-PAGE gel. The yield of the bovine inhibitor from 1 L of culture was about 2 mg.

CD Analysis of the Purified Inhibitors

The secondary structures of the purified inhibitors were analyzed by CD and compared to those of the yeast inhibitor which was previously overexpressed and purified (Ichikawa *et al.*, 2001). The spectra at pH 6.5 are shown in Fig. 3(a). The spectra of human and bovine inhibitors were very similar, indicating that both inhibitors had similar structures. The α-helical contents of the human and bovine inhibitors were calculated to be 40.2 and 48.7%, respectively. In contrast to mammalian inhibitors, the spectrum of the yeast inhibitor showed a helix of low content (Fig. 3(a)). The α-helical contents of the yeast inhibitor were calculated to be 16.8%.

Because the activities of the inhibitors are greatly influenced by pH, the structures were also analyzed at higher pH (pH 8.2) (Fig. 3(b)). As shown in Fig. 3(a) and (b), no significant changes in the spectra of the inhibitors were detected between pH 6.5 and 8.2. These results indicate that the secondary structures of the inhibitors are not affected by pH.

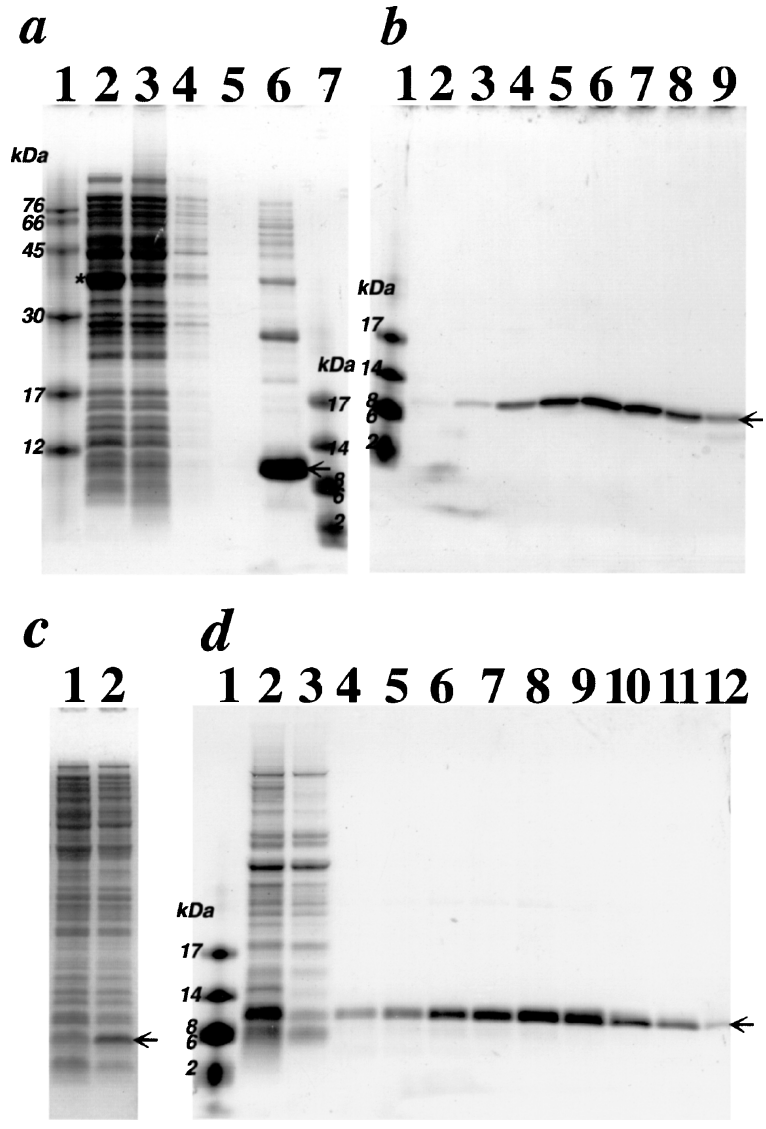

Fig. 2. Expression and purification of the inhibitors. (a) Separation of the human inhibitor with a Glutathione Sepharose 4B column. JM109 cells containing the expression plasmid that coded the GST-inhibitor fusion protein were grown in 1 L of rich medium at 37°C and induced with 1 mM IPTG for 2 h. The cells were harvested by centrifugation and washed once with PBS. The cells (4-g wet weight) were resuspended in 50 mL of PBS and sonicated on ice by using a Branson Sonifier 250D at a power setting of 5 for a total of 90 s in a duty of 50%. The lysate was centrifuged at 9000 rpm for 5 min at 4°C and the supernatant was loaded onto the column. Following washing, on-column cleavage of the fusion protein, and elution of the inhibitor were performed using a Bulk GST Purification Kit and PreScission™ Protease (Amersham Pharmacia Biotech). Aliquots of the different stages of the preparation were separated on a 15% polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. Lanes 1 and 7, molecular-weight markers; lane 2, extract before loading on the column; lane 3, flow-through; lanes 4 and 5, wash; lane 6, elute of the column after on-column cleavage of the fusion protein. The asterisk and arrow show the position of the GST-inhibitor fusion protein and the inhibitor, respectively. (b) Purification of human inhibitor with the Macro-prep High S column. The proteins obtained from the Glutathione Sepharose 4B column were dialyzed against a 50 mM sodium acetate buffer (pH 5.0), and separated on a Macro-prep High S column (Bio-Rad Laboratories, USA) as described previously (Ichikawa *et al.*, 2001). The pure inhibitor was eluted at 0.8 M on a linear gradient of 0–1 M sodium chloride in a 50 mM sodium acetate buffer (pH 5.0). Lane 1, molecular-weight markers; lanes 2–9, fractions of the purified inhibitor. The arrow shows the position of the inhibitor. (c) Expression of bovine ATPase inhibitor in *E. coli*. JM109 cells containing the expression plasmid were grown on a rich medium with (lane 2) or without (lane 1) IPTG (1 mM). The cells were harvested and lysed in a loading buffer of SDS-PAGE, and the aliquots were analyzed on 15% polyacrylamide gels. The arrow shows the position of the inhibitor. (d) Purification of the bovine inhibitor by Macro-prep High S column. The cells were induced with 1 mM IPTG for 3 h in 1 L of rich medium, and harvested by centrifugation. A 2.6 g of cells (wet weight) was obtained. Proteins were extracted from the cells by heating (80°C for 5 min), and separated on the column, as described above. The inhibitor eluted at about 0.8 M NaCl. Lane 1, molecular-weight markers; lane 2, extract before loading on the column; lane 3, flow-through; lanes 4–12, fractions of the purified inhibitor. The arrow shows the position of the inhibitor.

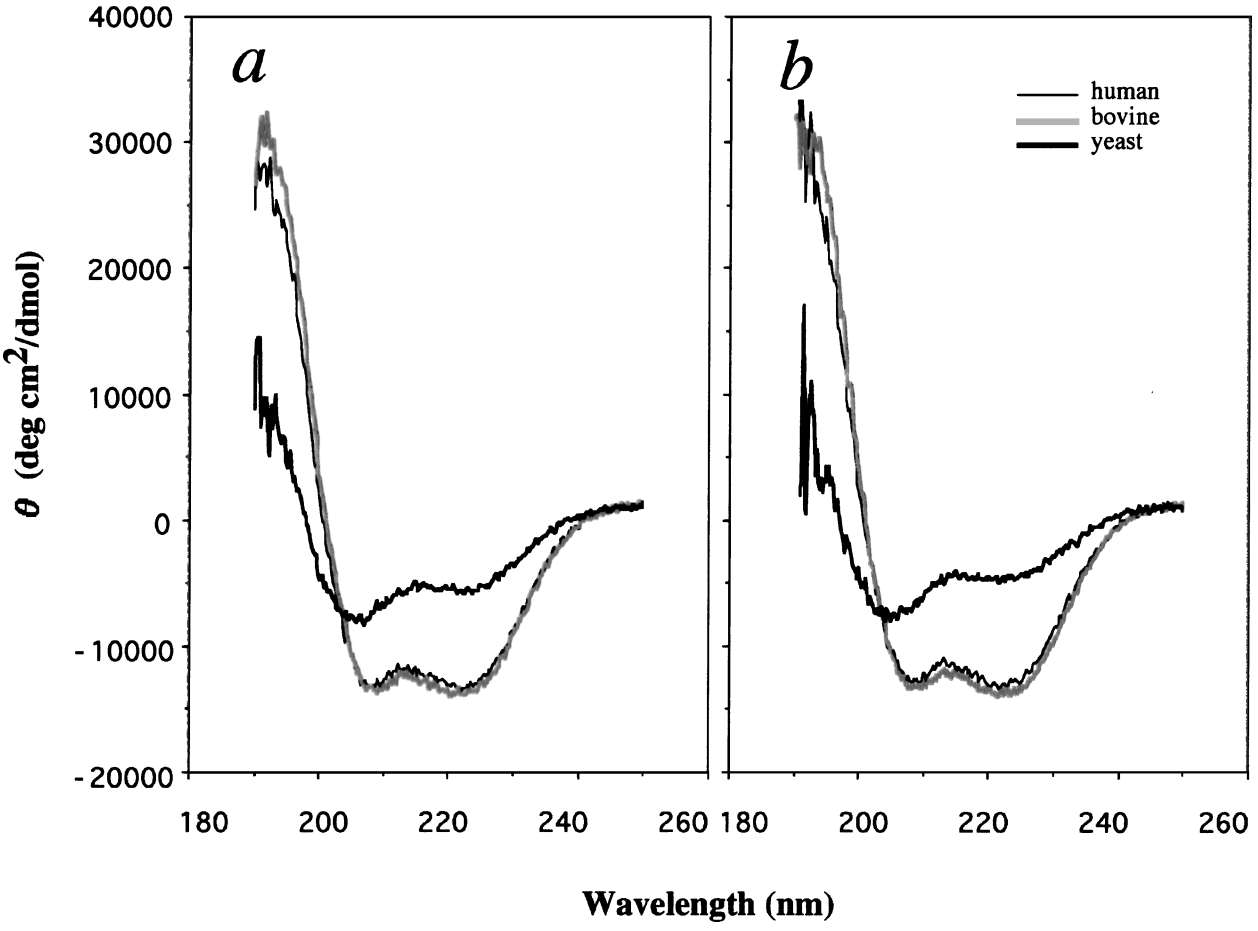

Fig. 3. CD Spectra of the purified inhibitors. Spectra for the 0.1 mg/mL solution of the inhibitors in 50 mM potassium phosphate buffer (pH 6.5 (a) or 8.2 (b)) were recorded as described in Materials and Methods section.

Inhibition of the Bovine Heart F_1F_0-ATPase by the Inhibitors

The mammalian and yeast inhibitors were tested for their ability to inhibit the F_1F_0-ATPase from both bovine and yeast. The results for the bovine ATPase are shown in Fig. 4(a). The human and bovine inhibitors inhibited the bovine ATPase in a similar way. The yeast inhibitor also inhibited the bovine ATPase (Fig. 4(a)), although the activity was lower than the mammalian inhibitors. The I_{50} values of the human and bovine inhibitors were 3.1 and 2.4 times lower than that of the yeast inhibitor (Table I).

Inhibition of the Yeast F_1F_0-ATPase by the Inhibitors

All three inhibitors inhibited the yeast F_1F_0-ATPase in a similar way (Fig. 4(b) and Table I), indicating that the activity of the mammalian inhibitors on yeast ATPase are comparable to that of the yeast inhibitor.

Effects of the Inhibitors to *E. coli* F_1F_0-ATPase

The activities of the inhibitors on the F_1F_0-ATPase in the plasma membrane of *E. coli* were also examined. As shown in Fig. 4(c) and (f), none of these inhibitors inhibited the enzyme. These results show that the mitochondrial ATPase inhibitor cannot inhibit bacterial F_1F_0-ATPase.

pH-Sensitivities of the Inhibitors

As shown in Fig. 4(d) and (e), the activities of all inhibitors decreased at higher pH (pH 8.2). However, the magnitudes of the decrease were different for

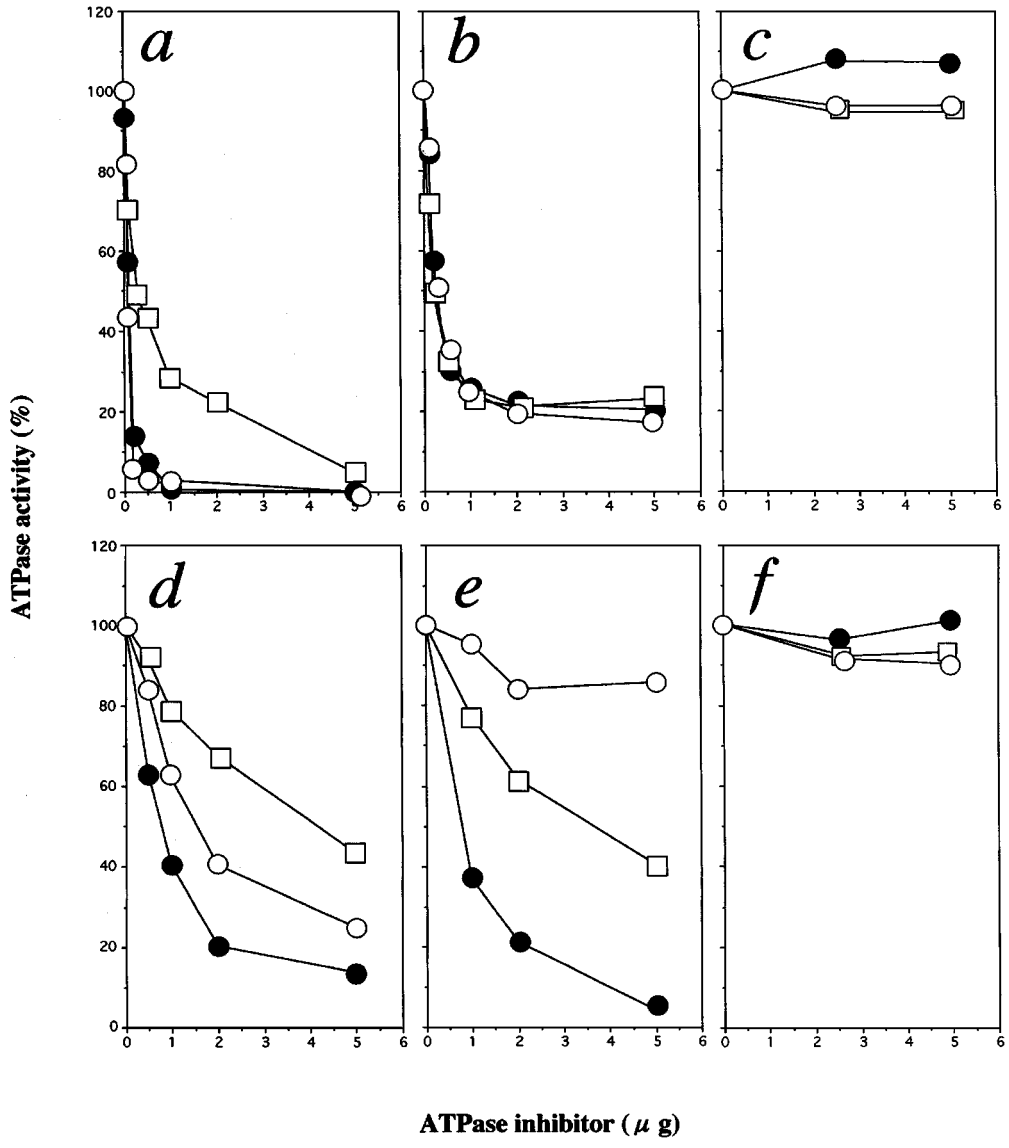

Fig. 4. Inhibition of F_1F_0-ATPase from various organisms by the inhibitors. The indicated amounts of human (○), bovine (●), and yeast (□) inhibitors were incubated with the inhibitor-depleted submitochondrial particles prepared from a bovine heart (a and d) or *inhl*$^-$ mutant yeast (b and e), or inverted membrane vesicles of *E. coli* (c and f) (each containing 0.2 units of F_1F_0-ATPase), in a medium containing 50 mM Tris-maleate buffer (pH 6.5), 5 mM $MgSO_4$, and 5 mM ATP (a, b, and c) or 50 mM $TrisSO_4$ buffer (pH 8.2), 5 mM $MgSO_4$, and 5 mM ATP (d, e, and f) in a final volume of 50 μL. After 10 min at 25°C, remaining ATPase activity was measured at 25°C, as described previously (Ichikawa *et al.*, 2001).

each individual inhibitor and F_1F_0 (Fig. 4 and Table I). At pH 8.2, the I_{50} values of the human, bovine, and yeast inhibitors on bovine F_1F_0 increased about 22.7-, 8.3-, and 16.4-fold, respectively. On yeast F_1F_0, the value of the bovine and yeast inhibitors increased 3.0- and 14.5-fold at pH 8.2, and in the same condition, the human inhibitor was almost inactive (Fig. 4(e)). These results show that the pH-sensitivity of the inhibitor is highly dependent on the origin of the inhibitor and the F_1F_0-ATPase.

DISCUSSION

In this study, we overexpressed and purified human and bovine ATPase inhibitors and compared the properties of the inhibitors with those of the yeast inhibitor.

Table I. Amount of Inhibitor Required to Inhibit 50% of the ATPase Activity in the Condition in Fig. 4 (I_{50})

Origin of the F_1F_0 and inhibitor	Amount of inhibitor (I_{50}) (μg) pH 6.5	pH 8.2	I_{50} (pH 8.2)/ I_{50} (pH6.5)
Bovine F_1F_0			
Human inhibitor	0.07	1.59	22.7
Bovine inhibitor	0.09	0.75	8.3
Yeast inhibitor	0.22	3.6	16.4
Yeast F_1F_0			
Human inhibitor	0.23	>5[a]	—
Bovine inhibitor	0.24	0.72	3.0
Yeast inhibitor	0.21	3.05	14.5

[a] Substantial inhibitory activity not detected in this condition.

The mammalian and yeast inhibitors are very similar in primary structure and were expected to have similar conformation. However, the CD Spectra shown in Fig. 3 reveal that the secondary structures of the purified inhibitors are significantly different. The contents of the α-helix of the yeast inhibitor were much lower than those of the mammalian inhibitors, although both inhibitors had similar inhibitory activity on yeast F_1F_0-ATPase (Fig. 4(b)). Recent NMR studies (Gordon-Smith *et al.*, 2001) also showed that a fragment of the bovine inhibitor consists of residues 10–48, which still have sufficient inhibitory activity (van Raaij *et al.*, 1996), is near or at a random coil in solution (Gordon-Smith *et al.*, 2001), while the X-ray structure of the complete inhibitor is highly helical (Cabezón *et al.*, 2001). These results indicate that the low helical contents of the purified yeast inhibitor and fragment do not directly affect their inhibitory activity. The structures of the inhibitor and the fragment may change to the helical form after binding to F_1F_0-ATPase.

Previously, it was shown that the histidine residues at positions 48, 49, and 55 of the bovine and rat inhibitors were important for its pH-sensitivity (Lebowitz and Pedersen, 1996; Schnizer *et al.*, 1996). However, the residues are lacking in the yeast inhibitor (Fig. 1), and we examined the pH-sensitivity of the inhibitors' effect on both bovine and yeast F_1F_0-ATPase. The inhibitory activities of all the inhibitors decrased at higher pH, but the magnitude of the decreases were different according to the origin of the inhibitor and the ATPase (Fig. 4 and Table I). These results suggest that the pH-sensitivities of the inhibitors are not defined by only conserved residues, but also involve unique residues from each inhibitor and F_1F_0-ATPase. Because the pH-sensitivity is not an essential factor for the release of the inhibitor from F_1F_0 and the activation of ATP synthase (Ichikawa *et al.*, 2001), the natural residues required may not be stringently conserved.

As shown in Fig. 4(e), the inhibitory activities of human and bovine inhibitors on yeast F_1F_0-ATPase at pH 8.2 were significantly different, although the primary and secondary structures of the inhibitors are very similar (Figs. 1 and 3). This effect may be caused by the minor amino acid substitution between the human and bovine inhibitors. In fact, single amino acid replacement of the yeast and mammalian inhibitors by site-directed mutagenesis causes marked changes in the activities at high pH (Ichikawa *et al.*, 2001; Lebowitz and Pedersen, 1996; Schnizer *et al.*, 1996). Therefore, the minor amino acid substitution on the human and bovine inhibitors (for example, position 37 or 51; see Fig. 1) may affect the activities of the inhibitors at high pH.

Previously, it was shown that the bovine inhibitor inhibits purified yeast F_1-ATPase even better than the yeast inhibitor (Cabezón *et al.*, 2002). These results are inconsistent with our results that the activity of the yeast inhibitor on yeast submitochondrial ATPase was comparable to that of the mammalian inhibitors (Fig. 4(b)). This discrepancy may be due to the action of the mitochondrial inner membrane proteins that bind to the inhibitor (Ichikawa *et al.*, 2002; Lopez-Mediavilla *et al.*, 1993) or facilitate and stabilize the binding of the inhibitor to F_1F_0-ATPase (Hashimoto *et al.*, 1984).

As shown in Fig. 4(c), yeast and mammalian inhibitors did not inhibit *E. coli* F_1F_0-ATPase. In bacterial F_1F_0, homologous proteins of the mitochondrial ATPase inhibitor have not been found, but the enzyme contains a regulatory subunit called ε (Kato-Yamada *et al.*, 1999; Sternweis and Smith, 1980). The carboxyl-terminal α-helix domain of ε is not required for the function and assembly of the F_1F_0 complex, but is required for its inhibitory activity (Kuki *et al.*, 1988). Recent studies showed that the basic residues at the ε domain interact with the acidic DELSEED region of the β and inhibit the enzyme in a "ratchet" or "clutch" manner (Hara *et al.*, 2001). The primary structures of the mitochondrial ATPase inhibitors are not similar to the C-terminal region of the ε, and the inhibitor requires not only basic residues, but also acidic (yeast Glu25; see Fig. 1) or aromatic (yeast Phe17 and Phe28) residues for its activity (Ichikawa *et al.*, 2001). Thus, the regulatory mechanism of mitochondrial F_1F_0-ATPase by the inhibitor appears to be different from that of the bacterial enzyme by ε.

As shown in Fig. 4(b), the mammalian and yeast inhibitors inhibited yeast F_1F_0-ATPase in a similar way. The yeast inhibitor also inhibited the bovine F_1F_0-ATPase (Fig. 4(a)). These results indicate that the mechanism of the inhibition of mitochondrial F_1F_0 by the inhibitor is

basically shared in yeast and mammals. From the results of site-directed mutagenesis, we previously showed that the residues from Phe 17 to Leu44 of the yeast inhibitor are involved in its inhibitory activity (Ichikawa *et al.*, 2001). Especially, the five residues essential for the inhibitory activity (Phe 17, Arg20, Arg22, Glu25, and Phe28) and one residue required for pH-sensitivity (Glu21) are concentrated at a region from Phe 17 to Phe28 (Fig. 1) (Ichikawa *et al.*, 2001). If the region forms an α-helix, these six residues form clusters on the surface of the putative helix (Fig. 5(a)). Furthermore, recent X-ray studies (Cabezón *et al.*, 2001) showed that the homologous residues of the bovine inhibitor (Phe22, Arg25, Glu26, Gln27, Glu30, and Tyr33; see Fig. 1) also form clusters on the surface of the helix (Fig. 5(b)). Because the five essential residues of the yeast inhibitor are involved in the inhibition of F_1F_0-ATPase rather than binding to the enzyme (Ichikawa *et al.*, 2001), the clusters appear to be inhibitory sites of the inhibitors.

Because the mammalian inhibitors inhibited the bovine ATPase more strongly than the yeast inhibitor (Fig. 4(a) and Table I), any residues lacking the yeast inhibitor also appear to contribute to the activity of the mammalian inhibitor. These results are consistent with previous results reported by van Raaij *et al.* (1996) that residues 10–21 and 47–56 of the bovine inhibitor, which are lacking in the yeast inhibitor, are also involved in its inhibitory activity or stabilization of the inhibitor-F_1F_0 complex. The results obtained in this study indicate that these residues of the mammalian inhibitor are not essential, but are required for the maximum inhibition of mammalian F_1F_0-ATPase.

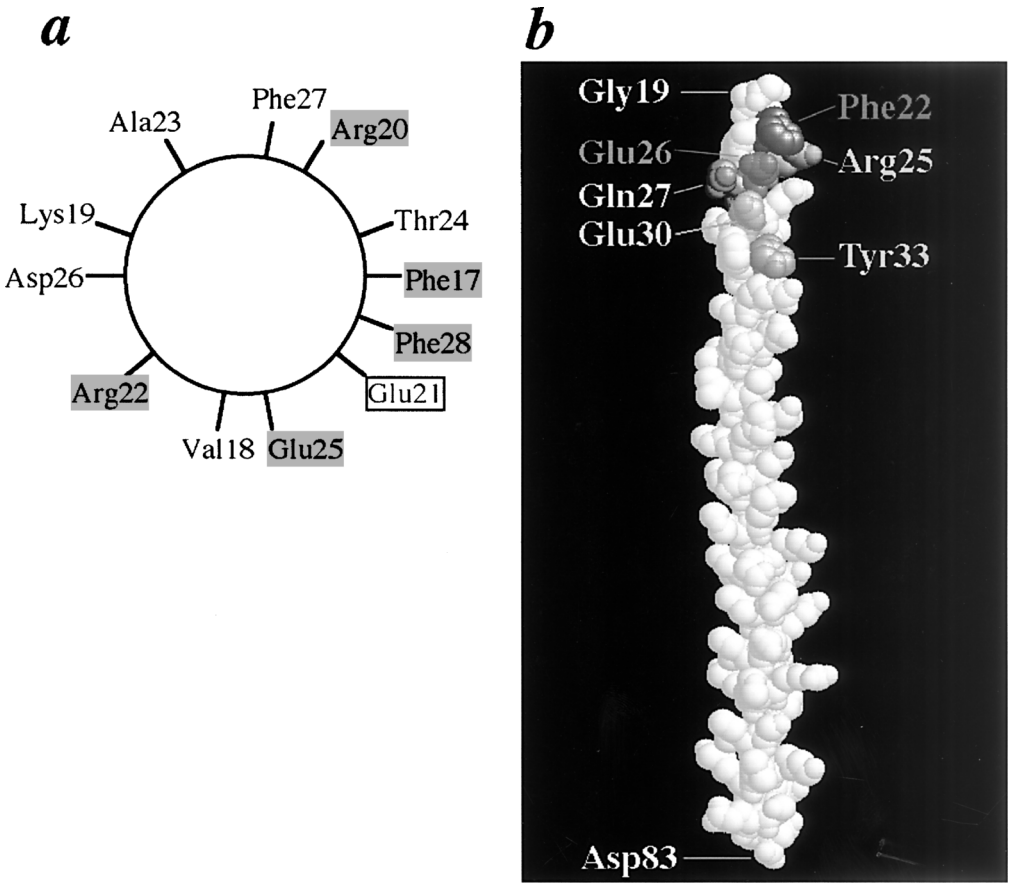

Fig. 5. Putative inhibitory sites of the yeast and bovine inhibitors. (a) Positions of residues 17–28 of the yeast ATPase inhibitor in a putative α-helical wheel. Essential residues (Phe 17, Arg20, Arg22, Glu25, and Phe28) and Glu21 which are required for pH sensitivity are indicated. (b) Position of the homologous residues of the bovine ATPase inhibitor in an X-ray structure reported by Cabezón *et al.* (2001). The positions of Phe22, Arg25, Glu26, Gln27, Glu30, and Tyr33 (corresponding to yeast Phe17, Arg20, Glu21, Arg22, Glu25, and Phe28) are indicated. This figure was generated by RasMol computer software (version 2.6) on Power Macintosh G4 computer (Apple Computer, Inc., USA).

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