Requirement for Lysine-19 of the Yeast Mitochondrial ATPase Inhibitor for the Stability of the Inactivated Inhibitor-F$_1$Fo Complex at Higher pH

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The ATPase inhibitor is a regulatory subunit of mitochondrial ATP synthase. In this study, the role of Lys19 of the yeast ATPase inhibitor was examined by site-directed mutagenesis. Two amino acids (Gln and Glu) were substituted for the Lys19. The purified mutant inhibitor (Lys19→Gln) had similar ATPase inhibitory activity to that of the wild-type inhibitor at pH 6.5, but was less active at pH 7.4. ATP synthesis in mutant mitochondria was normally activated by the addition of ADP and succinate, but the inactivated ATPase complex in the mutant mitochondria was activated more readily than that in control cells by raising pH. These results show that Lys19 of the yeast ATPase inhibitor is not essential for ATPase inhibitory activity, but increases the stability of the inhibitor-F$_1$Fo complex at higher pH.

Key words: ATPase inhibitor (IF$_1$); ATP synthase; F$_1$Fo-ATPase; mitochondria

ATP synthase (F$_1$Fo-ATPase) catalyzes the terminal step of oxidative phosphorylation. The enzyme synthesizes ATP from ADP and Pi using the proton electrochemical gradient generated by the respiratory chain. The enzyme is composed of a catalytic sector, F$_1$, and an integral membrane sector, Fo, which functions as a proton channel. F$_i$ contains five kinds of subunit with a $\alpha_6\beta_6\gamma_1\delta_1\varepsilon_1$ stoichiometry, and three catalytic sites located on the interface between the $\alpha$- and $\beta$-subunits. The catalytic sites participate sequentially in a series of conformation changes during the catalytic cycles (for review see Ref. 1).

Mitochondrial ATP synthase contains a regulatory subunit named ATPase inhibitor. The inhibitor is not required for ATP synthesis, but is important to maintain the cellular level of ATP by preventing ATP hydrolysis when the proton electrochemical gradient is lost. The inhibitor protein binds to the F$_1$ portion of the enzyme in a 1:1 molar ratio in the presence of MgATP, and completely inhibits the ATP-hydrolyzing activity. We previously showed that the yeast ATPase inhibitor interacts with the $\alpha/\beta$ interface of F$_1$-ATPase.

The activity of the ATPase inhibitor is highly pH-dependent. Effective inhibition of F$_1$-Fo-ATPase by the inhibitor requires a low pH (<7.0). The secondary structure of the inhibitor has been investigated as a function of pH in which interconversion of the protein between the active and inactive states occurs, and it was shown that the inhibitor is mainly $\alpha$-helical above pH 7.0. However, below this pH, a conflicting proposal was made: a prominent decrease in $\alpha$-helical content in rat and buffalo inhibitors was reported and pH-dependent conformational change between active and inactive form of the protein was proposed, but no large structural change was detected in a bovine inhibitor.

The inhibitor was first isolated from beef heart mitochondria by Pullman and Monroy, and has since been found in various eukaryotic cells ranging from yeast to mammals. The primary structures of Saccharomyces cerevisiae, Candida utilis, bovine, rat, and mouse inhibitors have been reported and considerable sequence similarity among the proteins have been shown (Fig. 1). The functional regions of the bovine inhibitor has been investigated using proteolytic fragments and deletion mutants of the protein, and it was shown that the residues from Ala14 to Lys47 of the inhibitor are important in ATPase inhibitory activity. We recently showed that the C-terminal region of the yeast inhibitor containing the residues Ile51-Lys63 is not involved in its inhibitory action on the F$_i$-Fo-ATPase, but is important to the stability of the protein in vivo. Previously, the physiological function of the lysine

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Abbreviations: F$_1$Fo-ATPase, mitochondrial ATP synthase; F$_i$ or F$_1$-ATPase, catalytic subunit of F$_1$-Fo-ATPase; MOPS, 3-(N-morpholino) propanesulfonic acid; PVDF, polyvinylidene fluoride; CD, circular dichroism; RCR, respiratory control ratio; P/O ratio, phosphorus/oxygen ratio.
residues of the bovine ATPase inhibitor has been investigated by using chemical modification.\textsuperscript{19,20} Klein et al.\textsuperscript{19} labeled the bovine inhibitor with phenyl [\textsuperscript{14}C]isothiocyanate and showed that about half of the ten lysine residues of the inhibitor were not required for the ATPase inhibitory activity. Jackson and Harris\textsuperscript{20} showed that most of the lysine residues of the bovine inhibitor are not involved in binding to F\textsubscript{i}, but suggested that only Lys24 interacts with the enzyme. As shown in Fig. 1, the Lys24 of the bovine inhibitor is highly conserved in the primary structures of the inhibitors from 5 different species, and the residue seems to be important in biological activity of the protein.

In this study, we examined the role of homologous lysine residues of the yeast ATPase inhibitor at position 19 by site-directed mutagenesis and showed that the residue increases the stability of the inhibitor-enzyme complex at higher pH.

\section*{Materials and Methods}

\textit{Strains and growth conditions.} Saccharomyces \textit{cerevisiae}, strain D26 (a trp1 leu2 his3 inh1::TRP1), which contains a null mutation in the gene coding for the ATPase inhibitor\textsuperscript{20} was used as the host for expression of the wild-type and mutant form of the inhibitor. \textit{S. cerevisiae}, strain DKD-5D (a trp1 leu2 his3) was used as a wild-type control. The \textit{Excherichia coli}/\textit{S. cerevisiae} shuttle vector, YEp51,\textsuperscript{21} was used for the construction of the expression plasmids. The plasmid contained a 2-\mu m circle origin of replication, a \textit{GAL10} promoter, and a \textit{LEU2} gene as the selection marker.

Yeast cells transformed by various plasmids were grown with vigorous shaking at 30°C on a 0.67% yeast nitrogen base and 1% glucose with appropriate amino acids (20 mg/liter). The medium volume was then increased 10-fold by adding a rich medium containing 2% peptone, 1% yeast extract, and 2% galactose, and cells were further grown for 14-15 hours at 30°C.

\textit{Construction of expression plasmids.} The coding sequence of the wild-type ATPase inhibitor (258 base pairs) was amplified by PCR as described previously.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
Protein & Oligonucleotide sequence & Strain \\
\hline
Lys19→Gln & 5'-TTCGTTTTGTCTAAAAGGAAG-3' & K19Q \\
Lys19→Glu & 5'-TTCGTTTTGTTAAAGGAAG-3' & K19E \\
\hline
\end{tabular}
\caption{Synthetic Oligonucleotides for Mutagenesis of ATPase Inhibitor}
\end{table}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1}
\caption{Aligned Sequence of ATPase Inhibitors from Various Species. Identical and conservatively substituted residues are shaded. The arrow indicates the location of the lysine residue that was replaced by glutamate or glutamic acid using site-directed mutagenesis in this study.}
\end{figure}

\textit{Purification of wild-type and mutant ATPase inhibitors by reverse-phase HPLC.} Parts of the eluate from a Macro-prep CM column (about 0.5–1 mg protein) were put on the column of an Asahipak ODP-50 (6.0×250 mm, Asahi Chemical Industry Co., Ltd.) and proteins were eluted with a linear gradient of 0 to 100% acetonitrile in 0.1% trifluoroacetic acid at 25°C. The purified inhibitors were lyophilized and dissolved in water at a concentration of 1 mg/ml.

\textit{Measurement of CD spectra.} The samples containing 2 \(\mu\)M ATPase inhibitor were placed in a quartz cuvette with a 10-mm path length and spectra were recorded with a J-720 spectropolarimeter (Jasco Corporation, Tokyo) in a range between 200–250 nm. The \(\alpha\)-helical content was calculated from the relationship: \textsuperscript{21} \(f_\alpha = (\Theta_{222} + 2340)/30300\), where \(f_\alpha\) is the \(\alpha\)-helical content (%) and \(\Theta_{222}\) is the mean residue ellipticity in deg·cm\(^2\)/dmol. Protein was measured by the methods of Lowry \textit{et al.}\textsuperscript{24} with bovine serum albumin as a standard.

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\textit{Other procedures.} SDS-polyacrylamide gel electrophoresis was done by the methods of Schägger
and von Jagow\cite{25} or Laemmli.\cite{26} Immunoblotting was done as described previously\cite{19} using a chemiluminescent reaction kit (New England Biolabs, Inc., USA). Mitochondria were isolated from yeast cells by the methods of Daum \textit{et al.},\cite{27} and ATP synthesis,\cite{28} oxygen consumption,\cite{29} P/O ratio,\cite{30} and respiratory control ratio\cite{31} were measured as described previously. One unit of ATPase inhibitor is defined as the amount of inhibitor required to produce 50\% inhibition of 0.2 units of ATPase.

**Results**

\textit{Construction of the mutant yeasts}

As described above in detail, site-directed mutation of the conserved Lys19 of the yeast ATPase inhibitor was constructed. Two amino acids (Glu and Gln) were substituted for the Lys19. The mutants were named K19E (Lys19→Glu) and K19Q (Lys19→Gln), respectively. The control strain, which harbored the expression plasmid containing the coding sequence of the wild-type inhibitor, was constructed earlier and named YC63.\cite{18} All the mutants grew at a normal rate on a galactose medium.

Expression of the inhibitor protein in mutant cells grown on the galactose medium was confirmed by immunoblotting. As shown in Fig. 2, a protein reacting with antibody against the ATPase inhibitor were detected in electrophogram of extracts from

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**Fig. 2.** Immunoblots of Yeast Cell Extracts.

Proteins were extracted from normal and mutant yeast cells by heating as described previously.\cite{19} The extracts were electrophoresed on a 15\% polyacrylamide gel and stained with Coomassie brilliant blue R (a), or transferred to PVDF membrane and detected with antiserum against the ATPase inhibitor (b). Arrows show normal and mutant ATPase inhibitors. Asterisk indicates nonspecific bands which reacted with the chemiluminescence substrate. Lane 1, purified ATPase inhibitor; lane 2, DKD-5D (wild-type); lane 3, D26 harboring YEp51 (inhibitor deficient strain); lane 4, YC63; lane 5, K19E; lane 6, K19Q; lane 7, molecular weight marker.

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**Fig. 3.** Separation of Wild-type and Mutant ATPase Inhibitors on a Macro-prep CM Column.

About 50 mg of proteins extracted from yeast cells by heating were put on a macro-prep CM column (1.5 x 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 protein concentration of each fraction was measured using the methods of Lowry \textit{et al.} \cite{24}

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**Fig. 4.** SDS-PAGE Analysis of Purified Inhibitors.

The inhibitors purified from wild-type and mutant yeast cells were electrophoresed on a 15\% polyacrylamide gel and stained with Coomassie brilliant blue R. Lane 1, wild-type ATPase inhibitor; lanes 2 and 4, molecular weight marker; lane 3, mutant ATPase inhibitor (Lys19→Gln).
the mutant cells. The inhibitor proteins were overproduced in YC63 and the mutant cells, because of a greater efficiency of the GAL10 promoter.

**Purification of the ATPase inhibitors**

Wild-type and mutant ATPase inhibitors were extracted from yeast cells by heat treatment, and partially purified by macro-prep CM column chromatography. Figure 3 shows typical chromatograms. Peak I contains wild-type ATPase inhibitor. The mutant inhibitor (Lys19→Gln) was eluted a little earlier than the wild-type inhibitor (Fig. 3, Peak II). The mutant inhibitor (Lys19→Glu) was not retained on the column, indicating that the mutation influenced the charge on the surface of the protein. The wild-type and mutant (Lys19→Gln) inhibitors were further purified by subsequent reverse-phase HPLC. The final preparation of the inhibitors yielded single bands after SDS-polyacrylamide gel electrophoresis (Fig. 4). The yields of purified inhibitors were about 0.5 mg from 12 g of yeast cells.

**Inhibitory activity of the mutant inhibitor**

The purified ATPase inhibitors were tested for their ability to inhibit the F1-ATPase. As shown in Fig. 5(a), the mutant inhibitor (Lys19→Gln) retained similar ATPase inhibitory activity to that of the wild-type inhibitor when the proteins were preincubated in acidic pH (pH 6.5). But, at pH 7.4, the activity of the mutant inhibitor (Lys19→Gln) decreased to a level 2-fold less than that of the wild-type inhibitor (Fig. 5(b)). The specific inhibitory activities of the wild-type and mutant inhibitors were 7936 and 6369 unit/mg (pH 6.5), and 6329 and 3021 unit/mg (pH 7.4), respectively.

**CD analysis of the inhibitor**

Previously, pH-dependent changes in the helical content of rat6,7 and buffalo9 inhibitors were reported. Thus, the secondary structure of the inhibitor purified in this study was analyzed by CD as a function of pH in a range from 6.0 to 8.0. But, as shown in Fig. 6, significant changes in secondary structure were not detected in either wild-type or mutant inhibitors. The α-helical contents of the wild-type and mutant(Lys19→Gln) inhibitors were calculated to be 11% and 12%, respectively.

**ATP-synthesis and -hydrolysis in mitochondria isolated from mutant yeast cells**

As shown in Table 2 and Fig. 7, respiration and ATP synthesis in mutant mitochondria was normally activated by the addition of succinate and ADP. These results indicate that the inhibitor-F1 complex in mutant mitochondria was normally dissociated during oxidative phosphorylation. The bands showing a reaction with antibodies against the α and β subunits of the F1 were detected in the extracts of

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![ATPase Inhibitor (μg)](image)

Fig. 5. Inhibition of F1-ATPase by Wild-type and Mutant ATPase Inhibitors.

The indicated amounts of ATPase inhibitors were incubated with 0.4 units of F1-ATPase at 25°C for 15 min in a medium containing 35 mM Tris-maleate buffer (pH 6.5 (a) or pH 7.4 (b)), 5 mM MgSO4, and 5 mM ATP, in a final volume of 50 μl. For the assay of ATPase activity, a sample of 10 μl of the mixture was added to 0.5 ml of the assay system, that consisted of 50 mM Tris-maleate (pH 7.4), 5 mM MgSO4, and 5 mM ATP. Formation of inorganic phosphate from ATP was then measured as described previously.18 ○, wild-type inhibitor; ●, mutant (Lys19→Gln) inhibitor.

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**Fig. 6. CD Spectra of Wild-type and Mutant ATPase Inhibitors.**

The CD spectra of wild-type (○) and mutant (●) ATPase inhibitors were measured over the range of pH 6.0 to 8.0. The secondary structures of the inhibitors were not significantly affected by the pH change.

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**Fig. 7. ATP-synthesis and -hydrolysis in mitochondria isolated from mutant yeast cells.**

As shown in Table 2 and Fig. 7, respiration and ATP synthesis in mutant mitochondria was normally activated by the addition of succinate and ADP. These results indicate that the inhibitor-F1 complex in mutant mitochondria was normally dissociated during oxidative phosphorylation.
Figure 6. CD Spectra of the Wild-type and Mutant ATPase Inhibitors.
Spectra for the 2 μM solution of the wild-type (a) and mutant (Lys19→Gln) b) ATPase inhibitor in 50 mM potassium phosphate buffer (pH 6.0 (--), pH 7.0 (-----), and pH 8.0 (--------)) were recorded as described under “Materials and Methods”.

Table 2. Respiration in Mutant Mitochondria

<table>
<thead>
<tr>
<th>Strain</th>
<th>State 3 (nmol O₂/min/mg)</th>
<th>RCR</th>
<th>P/O</th>
</tr>
</thead>
<tbody>
<tr>
<td>YCG3</td>
<td>30.7</td>
<td>1.6</td>
<td>1.22</td>
</tr>
<tr>
<td>K19E</td>
<td>27.3</td>
<td>1.5</td>
<td>1.26</td>
</tr>
<tr>
<td>D26/YEp51</td>
<td>28.8</td>
<td>1.4</td>
<td>0.75</td>
</tr>
</tbody>
</table>

The CD spectra of mutant mitochondria, as well as those of the control cells, indicated that the mutant and the control cells contained similar amounts of F₁Fo-ATPase (Fig. 7). The ATPase in the mutant mitochondria was activated more readily than that in YCG3 by raising pH (Fig. 8). These results show that Lys19 of the yeast ATPase inhibitor increases the stability of the inactivated inhibitor-F₁Fo complex at higher pH.

Discussion

In this study, we examined the physiological role of Lys19 of the yeast ATPase inhibitor by site-directed mutagenesis. The purified mutant inhibitor (Lys19→Gln) still had inhibitory activity to F₁ATPase at pH 6.5, indicating that the positive charge of Lys19 is not absolutely necessary for binding to F₁. However, the ATPase in the mutant mitochondria was activated more readily than that in the wild-type control by raising the pH, indicating that Lys19 of the yeast ATPase inhibitor increases the stability of the inactivated inhibitor-F₁Fo complex at higher pH.

The effects of mutation on pH sensitivity are attributable to one of two possible mechanisms: (i) the mutation affects the pH-dependent interconversion between the highly helical inactive form and unfolded active form, which was reported for rat⁶,⁷ and buffalo⁸ inhibitor, or (ii) Lys19 of the inhibitor is directly involved in inhibitor-F₁ interaction and the
mutation decreases the affinity of the protein at higher pH. To test the first possibility, we analyzed the mutant inhibitor by CD. But, significant changes in secondary structure were not detected in either the wild-type or mutant ATPase inhibitors and this possibility could be ruled out.

Previously, the secondary structure of the inhibitor isolated from mammalian mitochondria was investigated by CD and it was shown that the protein is highly α-helical.6-9 The yeast inhibitor was also predicted to have a 68% α-helical structure using the method of Chou and Fasman,30 but the inhibitor isolated in this study had only 11-12% of the helix. This discrepancy may be explained by the denaturing of the protein in the process of purification, i.e., heat extracting and reverse-phase chromatography. Since the inhibitor isolated in this study had normal ATPase inhibitory activity and pH dependency in spite of its lower helicity, it is highly likely that the α-helical structure of the inhibitor is not directly correlated to the activity of the protein. This result is consistent with the observation of van Raaij et al.17 that the α-helicity of the bovine inhibitor is associated with the C-terminal part of the inhibitor, which is not required for the activity.

The second possibility is supported by the results of Jackson and Harris.20 They investigated the concealed residues of the inhibitor-F$_i$ complex using a differential trace-labeling method, and suggested that Lys24 of the bovine ATPase inhibitor contacts F$_i$. Thus, it is likely that the homologous Lys19 of the yeast inhibitor also directly interacts to F$_i$-ATPase. Previously, Gomez-Fernandez and Harris20 suggested that binding of inhibitor to F$_i$ is mainly due to hydrophobic interaction. It is probable that the Lys19 of the yeast ATPase inhibitor participates in the inhibitor-enzyme interaction along with the hydrophobic parts of the protein and the positive charge of the residue increase the stability of the inhibitor-F$_i$ complex.

References

Role of Lysine-19 of the Yeast ATPase Inhibitor


