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An Intrinsic ATPase Inhibitor Binds near the Active Site of Yeast Mitochondrial F_1-ATPase[1]

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An ATPase inhibitor and its stabilizing factor, the 9K protein, are regulatory factors of F_1F_o-ATPase. The binding sites for these factors on F_1 were examined using the zero length cross-linkers, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, and 1-ethyl-3-[3-dimethylamino)propyl]carbodiimide. The cross-linked products were analyzed by immunoblotting after SDS-polyacrylamide gel electrophoresis. The inhibitor and the 9K protein cross-linked to the α and β subunits of F_1, indicating that they interacted with both subunits. Peptide mapping and amino acid sequence analysis of the cross-linked products after weak acid hydrolysis showed that the inhibitor cross-linked to the Pro334-Asp363 region of the β subunit. Amino acid sequence analysis of the cross-linked peptide showed that the inhibitor binds to Asp363 of the β subunit. As this region contains the amino acid residues, including Tyr359, that are modified by nucleotide analogs and form the active site, the inhibitor probably binds to the catalytic site of F_1.

Key words: ATPase inhibitor, cross-linking, F_1ATPase, regulatory protein, subunit of F_1ATPase.

ATP synthase (F_1F_o-ATPase) in membranes of mitochondria, chloroplasts, and bacteria catalyzes ATP synthesis coupled to respiratory chain-linked proton transport across the membranes. The enzyme is composed of a catalytic sector, F_1, and an integral membrane sector, F_o, involved in proton transduction. The subunit stoichiometry of F_1 is known to be $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$, and F_1 has six nucleotide binding sites, which are classified as three catalytic and three noncatalytic sites according to their ability to exchange bound nucleotides with those in the external medium (for reviews see Refs. *1* and *2*).

The activity of mitochondrial ATP synthase is regulated by an intrinsic inhibitor protein (*3, 4*). The purified inhibitor binds to F_1-ATPase in an equimolar ratio (*5-7*) in the presence of Mg^{2+}-ATP (*8*), and completely inhibits the enzyme activity. The inhibitor is thought to be released from its binding site on the enzyme upon energization of the mitochondrial inner membrane, resulting in activation of synthesis of ATP by the enzyme (*7, 8*). When the membrane potential is lost, the inhibitor re-binds to the site and completely inhibits the ATP hydrolytic activity of the enzyme (*4*).

In yeast mitochondria, two protein factors, 9K and 15K proteins, have been shown to participate in regulation of F_1F_o-ATPase (*9, 10*). These two proteins function cooperatively to facilitate and stabilize the binding of the inhibitor to the enzyme. The structure of the 9K protein is similar to that of the inhibitor: both proteins consist of 63 amino acid residues, and about 50% of their residues are identical (*11*). The 9K protein, like the inhibitor, binds to F_1 in a molar ratio of 1:1, but inhibits the enzyme activity only partially (*12*).

Klein *et al.* (*5*) showed that the bovine heart inhibitor interacts with the β subunit of F_1. Jackson and Harris (*13*) found that the bovine inhibitor interacts with a peptide comprising residues Asp394-Met459 of the bovine β subunit. Previously, we reported that the inhibitor binds to F_1 at the interface of the α and β subunits (*14, 15*). In the present study, we determined the binding sites on yeast F_1 for the inhibitor and the 9K protein using the zero length cross-linkers, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC). The results showed that the inhibitor and the 9K protein interact with both the α and β subunits of F_1, and that the binding site of the inhibitor is located near the active site of the enzyme.

MATERIALS AND METHODS

Preparations—ATPase inhibitor (*16*), 9K protein (*16*), and F_1-ATPase (*17*) were purified from baker's yeast by the reported methods. Protein concentrations were deter-

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Abbreviations: AI, intrinsic ATPase inhibitor; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; F_1 or F_1ATPase, catalytic subunit of ATP synthase; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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mined by the method of Lowry *et al.* (*18*) with bovine serum albumin as a standard. The α, β, and γ subunits of yeast F_1 were separated by SDS-PAGE. After staining the gel with Coomassie Brilliant Blue R, the proteins were recovered by electro-elution as described (*19*). Specific antisera to the purified α, β, and γ subunits, the inhibitor and the 9K protein were raised in rabbits by the reported methods (*20*).

Cross-Linking of Proteins—Cross-linking of proteins was carried out essentially according to Klein *et al.* (*5*). Briefly, 60 μg of purified F_1 was incubated at 25°C with or without 5.7 μg of inhibitor protein (about 6 mol inhibitor/mol F_1) in a medium comprising of 0.25 M sucrose, 2 mM MgATP, and 10 mM MOPS, pH 6.5, as described previously (*7*). After 10 min, EEDQ at a final concentration of 1 mM was added and the incubation was continued for 7 min at 25°C. The reaction was stopped by the addition of ammonium acetate to a final concentration of 100 mM. Cross-linking of the 9K protein to F_1 was carried out similarly, by incubation for 20 min with 5 mM EDC instead of EEDQ.

Weak Acid Hydrolysis of Peptides—The subunits of F_1 and cross-linked products were separated by SDS-PAGE, and protein bands were eluted from the gel electrophoretically. After dialysis and lyophilization, the samples were treated with 70% formic acid at 30°C for 8 h. The reaction was stopped by the addition of 10 volumes of distilled water and the mixture was frozen in liquid nitrogen. Samples were lyophilized and dissolved in the sample buffer used for SDS-PAGE (*21*).

Gel Electrophoresis and Immunoblotting—SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (*21*). The proteins in the gel were transferred to a nitrocellulose filter with a semi-dry blotter (LKB-Produkter AB, Bromma, Sweden), and bands reacting with the antisera were located with a biotinylated secondary antibody and the streptavidin-peroxidase complex as described previously (*4*). For amino acid sequence analysis, a large amount of F_1 (5 mg) was used for cross-linking with the inhibitor, and complexes were separated by preparative slab gel electrophoresis on a 1 cm thick gel (Ajinoki PES NA-P, Handa, Japan) according to the method of Oshima *et al.* (*22*).

Amino Acid Sequence Analysis—Proteins and peptides were sequenced with an Applied Biosystems model 492 gas-phase sequencer. About 100 pmol of protein was transferred electrophoretically to a polyvinylidene difluoride membrane from the gel after SDS-PAGE (*23*), and protein bands were stained with Coomassie blue and subjected to sequence analysis.

Materials—EEDQ was purchased from Wako Pure Chemicals, Osaka, and EDC from Dojin Laboratories, Kumamoto.

RESULTS

Cross-Linking of the Inhibitor-F_1 Complex with EEDQ—F_1 and the inhibitor-F_1 complex were cross-linked with EEDQ, which gives rise to an amide bond between adjacent carboxyl and amino groups, and the cross-linked products were analyzed by SDS-PAGE (Fig. 1). The pattern of the inhibitor-F_1 complex revealed two additional protein bands that reacted with the antibody against the inhibitor protein (Fig. 2). Their apparent molecular sizes, 66 and 62 kDa, were comparable to the calculated molecular masses of the inhibitor-α (62.8 kDa) and inhibitor-β (60.3 kDa) complexes, respectively. Moreover, the 66-kDa protein reacted with the anti-α antibody and the 62-kDa protein with the anti-β antibody. Thus the 66- and 62-kDa proteins are cross-linked products of the inhibitor-α and inhibitor-β subunits, respectively. No cross-linked product was observed when MgATP was omitted from the reaction medium, showing that the cross-linking was specific to the inactivated inhibitor-F_1 complex.

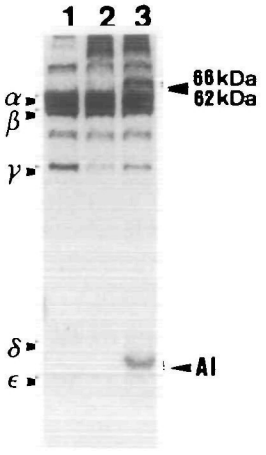

Fig. 1. **Cross-linking of the inhibitor-F_1 complex with EEDQ.** The F_1 and the inhibitor-F_1 complex were cross-linked as described under "MATERIALS AND METHODS." Samples (containing 2.2 μg of F_1) were electrophoresed in a 12% polyacrylamide gel and then stained with Coomassie Brilliant Blue R. Lane 1, F_1 (control); lane 2, cross-linking of F_1; lane 3, cross-linking of the inhibitor-F_1 complex. AI, ATPase inhibitor protein.

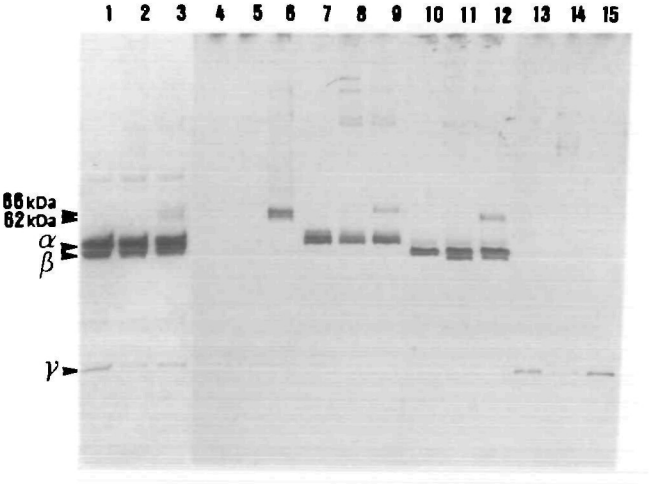

Fig. 2. **Immunocharacterization of cross-linked products.** The cross-linked products were separated on a linear gradient 10-20% polyacrylamide gel containing SDS. Proteins were stained with Coomassie Brilliant Blue R (lanes 1-3), or transferred to a nitrocellulose filter and stained with either the anti-inhibitor (lanes 4-6), anti-α (lanes 7-9), anti-β (lanes 10-12), or anti-γ (lanes 13-15) antibody, followed by the secondary antibody and the peroxidase complex. Lanes 1, 4, 7, 10, and 13, F_1 (control); lanes 2, 5, 8, 11, and 14, cross-linking of F_1; lanes 3, 6, 9, 12, and 15, cross-linking of the inhibitor-F_1 complex.

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Cross-Linking of the 9K Protein-F_1 Complex with EDC—The complex between the 9K protein and F_1 could be cross-linked with EEDQ, but many bands were generated on SDS-PAGE under the conditions used. Therefore, we used EDC to cross-link the 9K protein-F_1 complex. Cross-linking of the 9K protein-F_1 complex gave products of 62 and 59 kDa which reacted with the antibody against the 9K protein (Fig. 3). From their molecular sizes and reactivities with specific antibodies, these 62- and 59-kDa peptides were identified as cross-linked products of the 9K protein-α (63.7 kDa) and 9K protein-β (60.2 kDa) subunits, respectively. Thus the 9K protein also interacts with both the α and β subunits of F_1.

Peptide Mapping of the β Subunit by Partial Hydrolysis with Acid—The primary structures of the β subunit (*24*) and the inhibitor protein (*4, 25*) of yeast have been reported. The β subunit of yeast F_1 has three aspartyl prolyl bonds; Asp134-Pro135, Asp333-Pro334, and Asp363-Pro364, while the inhibitor does not. As these aspartyl prolyl peptide bonds are selectively hydrolyzed by weak acid (*26*), we performed peptide mapping of the β subunit after treatment with 70% formic acid for 8 h at 30°C. As shown in Fig. 4, acid hydrolysis yielded four bands, 14.5, 17.6, 35.4, and 38.7 kDa (Fig. 4, lane 1). Amino acid sequence analyses revealed that the amino terminal residue of $F_1\beta$ used in this study was Ser20 of the reported sequence, and that the 38.7 kDa band contained two peptides of $F_1\beta$, one beginning with the amino terminus and the other with Pro135. The 14.5 kDa band also contained two peptides, one beginning with the amino terminal and the other with Pro364. The 35.4 and 17.6 kDa band materials were found to start at the amino terminal and Pro334, respectively. These results showed that four protein bands containing six different peptide fragments were generated on cleavage at one of the three aspartyl prolyl bonds in the β subunit (Fig. 5, a and b).

Peptide Mapping of the β Subunit Cross-Linked to the Inhibitor—On hydrolysis of the inhibitor-β cross-linked complex, the bands of 17.6 and 38.7 kDa were replaced by ones of two new peptides of 25.7 and 45.7 kDa, while the 35.4 kDa peptide remained unchanged (Fig. 4, lane 2). These new peptides were found to react with the anti-inhibitor antibody (Fig. 4, lane 4). From their apparent molecular sizes, 25.7 and 45.7 kDa, they could be cross-linked products between the inhibitor and the 17.6 kDa- and 38.7 kDa-peptides, respectively, of the β fragment (Fig. 5c).

Amino acid sequence analysis showed that the 45.7 kDa peptide was a cross-linked product of the inhibitor with the 38.7 kDa peptide of $F_1\beta$ composed of Pro135-Asn492, but not with that composed of Ser20-Asp363. The 25.7 kDa peptide was a cross-linked product between the inhibitor and the 17.6 kDa peptide fragment of $F_1\beta$, Pro334-Asn492 (Fig. 5c). No cross-linked product between the inhibitor and the 14.5 kDa fragment of $F_1\beta$ was obtained. Moreover, the 38.7 kDa peptide of the β subunit, composed of Ser20-Asp363, also gave no cross-linked product. These results strongly indicated that the weak acid did not attack aspartyl prolyl bond, Asp363-Pro364, of the β subunit cross-linked to the inhibitor, but cleaved one of the other two bonds, Asp134-Pro135 or Asp333-Pro334, generating four peptide fragments (Fig. 4, lane 2, and Fig. 5c).

Determination of the Cross-Linking Amino Acid Residue of the β Subunit—As peptide mapping of the β subunit cross-linked to the inhibitor suggested that the Pro334-Asp363 region of $F_1\beta$, possibly binds the inhibitor, we performed more than 30 cycles of amino acid sequencing of the cross-linked product, the 25.7 kDa peptide, between the inhibitor and the 17.6 kDa fragment of $F_1\beta$, using larger amounts of the peptide. As shown in Table I, sequencing up

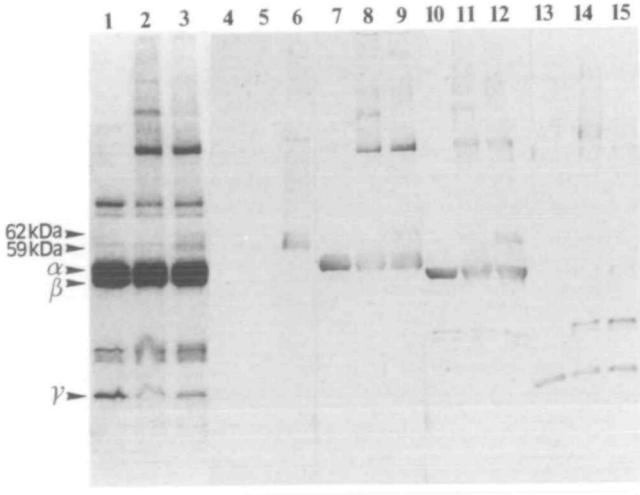

Fig. 3. **Cross-linking of the 9K protein-F_1 complex with EDC.** F_1 and the 9K protein-F_1 complex were cross-linked as described under "MATERIALS AND METHODS." The cross-linked products were separated in a linear gradient 10-20% polyacrylamide gel containing SDS and then stained with Coomassie Brilliant Blue R (lanes 1-3), or transferred to a nitrocellulose filter and subjected to immunostaining with either the anti-9K protein (lanes 4-6), anti-α (lanes 7-9), anti-β (lanes 10-12), or anti-γ (lanes 13-15) antibody. Lanes 1, 4, 7, 10, and 13, F_1 (control), lanes 2, 5, 8, 11, and 14, cross-linking of F_1; lanes 3, 6, 9, 12, and 15, cross-linking of the 9K protein-F_1 complex.

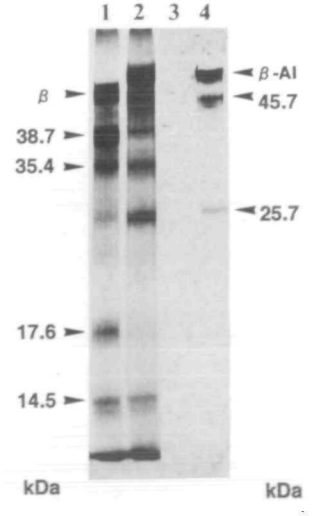

Fig. 4. **Cleavage of the inhibitor-β complex by formic acid.** The subunit and the inhibitor-β complex were purified by preparative gel electrophoresis and then treated with 70% formic acid as described under "MATERIALS AND METHODS." The samples (each 0.3 μg protein) were electrophoresed in a 12% polyacrylamide gel and then subjected to staining with silver (lanes 1 and 2), or immunostaining with the anti-inhibitor antibody (lanes 3 and 4). Lanes 1 and 3, β, lanes 2 and 4, inhibitor-β complex

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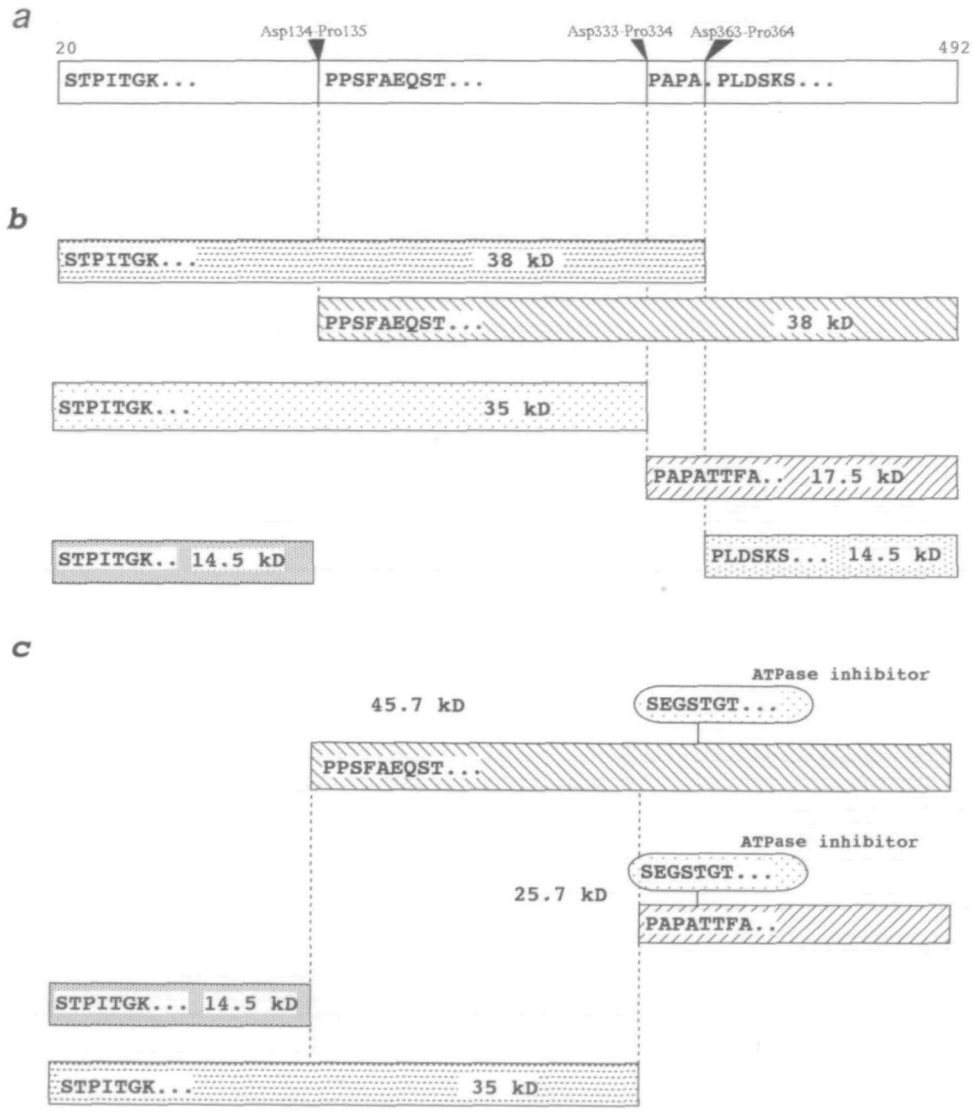

Fig. 5 **Acid-cleaved peptides of $F_1\beta$ and the $F_1\beta$-inhibitor complex.** a, cleavage sites of $F_1\beta$ with formate; b, molecular sizes and amino terminal sequences of $F_1\beta$ generated on formate treatment; c, peptides, and their amino terminal sequences, of the $F_1\beta$-inhibitor complex.

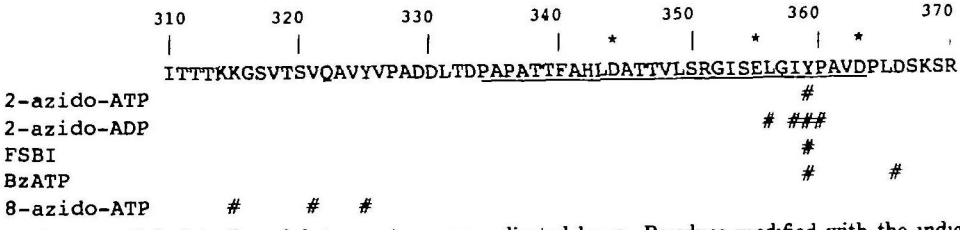

Fig. 6 **Amino acid residues in the β subunit that are modified with nucleotide analogs.** The amino acid sequence of yeast F_1 (region Ile310-Arg370) is shown. The Pro334-Asp363 region, that is considered to constitute part of the inhibitor binding site, is underlined Asp344, Glu355, and Asp363, which can be cross-linked to the inhibitor protein, are indicated by *. Residues modified with the indicated reagents (2) are indicated with # Abbreviations: FSBI, 5′-*p*-fluorosulfonylbenzoylinosine; BzATP, 3′-*o*-(4-benzoyl)benzoyl-ATP.

to the 29th cycle showed the identity of the sequence of $F_1\beta$, Pro334-Val362, the repetitive yield being more than 90%, although the sequence of the β subunit fragment was slightly different from the reported sequence (*24*). At the 30th cycle no PTH-amino acid was observed, and beyond this cycle, the sequence yield decreased abruptly. These results suggested that the amino acid at the 30th cycle, Asp363, could be cross-linked to the inhibitor. The yields of the inhibitor sequence were low, but its sequence was clearly seen up to the 10th cycle.

DISCUSSION

Previously, we found that the bovine ATPase inhibitor interacted with both the α and β subunits of F_1 (*14, 15*). In the present study, we observed that both the yeast inhibitor and the 9K protein, another regulatory factor for F_1F_o-ATPase, also bound to the α and β subunits. No interaction between these proteins and the smaller F_1 subunits, γ, δ, and ε, was observed. Since both the inhibitor and the 9K

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TABLE I. **Amino acid sequence analysis of the cross-linked 25.7-kDa peptide.**

Cycle	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
	(pmol)																
A	0.0	**24.1**	0.0	**25.0**	0.0	0.0	0.0	**21.1**	0.0	0.0	0.0	**17.0**	0.7	0.0	0.0	0.0	0.0
D	0.0	0.0	0.0	1.2	0.0	0.0	0.0	0.0	0.0	0.0	**17.7**	3.0	2.1	1.4	1.4	2.2	2.3
E	0.0	7.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	1.2	0.1	0.6
F	0.0	0.0	0.0	6.7	0.0	0.0	**29.8**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
G	0.0	0.0	6.5	0.0	0.0	3.2	0.0	0.0	0.0	0.3	0.0	2.6	2.2	0.0	1.9	3.2	5.2
H	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**11.2**	0.8	0.0	0.0	0.1	0.2	0.0	0.1	0.2
I	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.5	0.7	2.9	9.3	3.8	4.3	5.0	4.7
K	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.3	0.0	0.0	0.0
L	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**16.2**	0.0	0.0	0.0	0.0	0.0	**11.9**	1.8
P	**57.3**	5.9	**36.0**	0.0	0.0	0.0	0.0	1.8	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.6	1.1
Q	0.0	0.0	0.0	0.0	0.0	0.0	6.7	0.5	0.0	0.0	0.2	1.4	1.9	1.5	0.8	2.6	2.8
R	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.4	1.2	0.4	0.8	2.7	1.3	2.1	2.0	2.0
S	0.5	0.0	2.0	0.2	0.0	0.0	0.8	2.1	0.8	0.2	0.0	1.1	1.1	0.3	0.0	0.6	**3.2**
T	0.0	0.0	0.0	0.0	**8.2**	**6.0**	1.0	0.0	2.1	0.0	0.0	0.0	**3.5**	**2.8**	0.0	0.4	0.4
V	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.4	0.5	1.7	3.1	**17.8**	5.0	4.9
Y	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.3	1.5	1.5	1.7
$F_1\beta$ Reported	Pro	Ala	Pro	Ala	Thr	Thr	Phe	Ala	His	Leu	Asp	Ala	Thr	Thr	Val	Leu	Ser
$F_1\beta$ ATPase inhibitor	^{334}Pro-(Ser)-	-Ser-Glu-	Pro-Gly-	Ser-(Ser)-	Thr-Thr-	Ser-Gly-	Phe-(Thr)-	Ala-Pro-	His-Arg-	Leu-(Gly)-	Asp-(Ser)-	Ala-Gly-	Ser-(Ser)-	Ser-(Glu)-	Val-Asp-	Leu-Ser-	Ser Phe

Cycle	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
	(pmol)																
A	0.0	0.0	0.0	0.5	2.0	1.5	0.7	2.3	1.7	1.6	**6.6**	3.2	1.7	1.1	1.2	0.9	0.1
D	1.7	3.8	1.8	2.7	2.8	2.5	1.5	0.7	0.1	0.1	0.0	0.0	**0.0**	0.0	0.0	**0.0**	0.0
E	0.0	0.0	0.0	0.2	**3.9**	2.4	1.4	1.4	1.1	0.8	0.5	0.0	0.0	0.0	0.1	0.4	0.0
F	0.7	1.5	0.7	1.6	1.6	1.2	1.9	2.5	2.5	1.8	1.4	0.8	0.4	0.1	0.1	0.2	0.0
G	4.8	**6.3**	6.4	4.3	2.5	3.2	**7.2**	4.1	3.9	2.9	1.7	0.0	0.8	1.8	0.8	0.4	0.0
H	0.4	0.3	0.7	0.7	0.4	0.3	0.2	0.3	0.4	0.2	0.1	0.1	0.1	0.0	0.0	0.0	0.0
I	6.5	7.2	**12.2**	7.0	5.0	3.4	3.1	**6.5**	4.4	2.8	2.1	1.0	0.9	0.6	0.2	1.0	0.0
K	0.3	3.9	1.0	1.5	0.8	0.0	0.7	0.5	0.3	0.5	0.0	0.0	0.0	0.0	0.4	0.5	0.0
L	0.0	0.5	0.0	0.2	0.2	**6.7**	5.2	2.9	1.2	1.8	0.6	0.0	0.0	0.0	**0.9**	1.0	0.0
P	1.3	1.8	1.0	1.7	1.9	1.6	2.0	1.3	2.9	**5.8**	3.4	1.7	0.7	**1.6**	2.1	1.2	0.0
Q	2.6	1.3	1.2	2.7	1.7	2.6	2.0	2.0	1.9	1.7	1.4	0.0	0.1	1.1	0.6	0.1	0.0
R	**12.1**	5.1	3.1	3.1	2.6	2.8	2.0	1.0	0.8	0.4	0.0	0.6	0.6	0.0	0.0	0.0	0.0
S	1.1	1.4	0.0	**2.0**	0.0	0.9	0.6	0.8	0.3	0.4	0.0	0.0	0.0	0.0	0.1	0.0	0.0
T	0.1	0.0	0.0	0.1	0.0	0.0	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.8
V	4.9	4.0	4.2	5.2	6.8	5.0	3.3	4.1	2.7	3.3	2.4	1.9	4.1	2.1	0.0	0.0	0.0
Y	1.7	2.0	1.8	1.9	1.8	1.7	1.7	1.3	**5.4**	4.7	3.3	1.6	0.6	0.2	0.0	0.0	0.0
$F_1\beta$ Reported $F_1\beta$	^{351}Arg Arg-	Gly Gly-	Ile Ile-	Ser Ser-	Glu Glu-	Leu Leu-	Gly Gly-	Ile Ile-	Tyr Tyr-	Pro Pro-	Ala Ala-	Val Val-	X Asp-	^{364}Pro Pro-	Leu Leu-	X Asp-	X Ser

protein bind to F_1 in a molar ratio of 1:1 (*5-7*), it is likely that these proteins bind to the interfaces of the α and β subunits of the enzyme. As the primary structures of the inhibitor and the 9K protein are highly homologous (*11*), and their binding is competitive (*12*), they may bind to the same site on F_1.

Klein *et al.* (*5*) postulated that the inhibitor interacts directly with the β subunit of bovine F_1, but does not interact with the α one. The amino acid sequences of the inhibitors from yeast and bovine heart are very similar (*25*), and the yeast inhibitor can inhibit bovine F_1-ATPase, and *vice versa* (*27*). These facts suggest that the bovine and yeast inhibitors bind to homologous sites on the F_1-ATPases. Since the inhibitor protein possibly binds to the catalytic sites of F_1, which have been shown to be located on the interfaces of the α and β subunits (*28-30*), it is highly likely that it interacts with not only the β subunit but also the α one.

Although F_1-ATPase contains trimeric $\alpha\beta$ pairs, the inhibitor or 9K protein binds to F_1 in a molar ratio of 1:1 (*5-7, 12*). This observation can be explained by assuming an asymmetric structure of F_1. Nucleotide binding has been shown to result in a conformational change of F_1 and has been suggested to cause the prominent asymmetry of the enzyme (*31, 32*). As Mg^{2+}-ATP is required for the binding of the inhibitor and the 9K protein to F_1 (*7, 12*), a single binding site for the inhibitor and the 9K protein can be formed when F_1 assumes an asymmetric structure on the binding of Mg^{2+}-ATP.

Peptide mapping showed that the inhibitor binds to the Pro334-Asp363 region of the $F_1\beta$ subunit. Jackson and Harris (*13*) isolated a CNBr fragment of the cross-linked product of the bovine inhibitor-F_1 complex by SDS-PAGE, and concluded that the inhibitor binds to a peptide compris-

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ing residues Asp394-Met459 (corresponding to Asp408-Met473 of the yeast enzyme) of the β subunit. If homologous sequences bind to the inhibitor, our results indicate that the Pro320-Asp349 peptide of the bovine β subunit should bind to the inhibitor. We have no explanation for this discrepancy at present. However, in the present study no cross-linking of the inhibitor with two 14.5 kDa fragments including the peptide, Pro364-Asn492, was observed. Furthermore, amino acid sequence analysis of the 45.7 kDa fragment of the cross-linked product showed that the inhibitor bound to the $F_1\beta$ fragment of Pro135-Asn492, but not to Ser20-Asp363. These results suggested that Asp363 was cross-linked, resulting in prevention of cleavage at Asp363-Pro364 on acid-treatment. The 25.7 kDa peptide of the cross-linked product was identified as the peptide fragment of $F_1\beta$, Pro334-Asn492, on amino acid sequence analysis. As shown in Table I, the observed sequence, PAPATTFAHLDATTVL..., was slightly different from the reported sequence, PSPSTTFAHLDASSVL.... (*24*). This discrepancy cannot be explained, but the present sequence completely agrees with that of $F_1\beta$ from other sources such as ox (*33*). No PTH-amino acid was observed at the 30th cycle of Edman degradation, suggesting modification of the amino acid residue by cross-linking. The yield of the inhibitor sequence should be the same as that of $F_1\beta$, since the inhibitor bound to F_1 in the ratio of 1:1, but it was very low. In ox, Jackson and Harris reported that the N-terminus of the inhibitor is blocked and only one peptide sequence, $F_1\beta$, is present in the inhibitor-F_1 complex. Unlike the bovine inhibitor, however, the N-terminus of the yeast inhibitor is neither blocked nor multiple, as described for the bovine inhibitor (*34*). The present results showed two peptide sequences, those of the inhibitor and the $F_1\beta$ fragment, in the inhibitor-F_1 cross-linking peptide, although the yield of the inhibitor sequence was low. The reason for the low yield of the inhibitor sequence is unclear, but it may be due to its intramolecular cross-linking or multiple cross-linking sites, including the N-terminus.

The Pro334-Asp363 region contains Tyr359 corresponding to bovine Tyr345 and *Escherichia coli* Tyr331. These tyrosine residues can be modified with nucleotide analogs (for a review see Ref. *2*), such as 2-azido-ATP (*35-37*), 2-azido-ADP (*38*), 5′-*p*-fluorosulfonylbenzoylinosine (*39*), and 3′-*O*-(4-benzoyl)benzoyl-ATP (*40*) (Fig. 6), and have been considered to be a part of the active site of F_1. Amino acid residues which can be modified with 8-azido-ATP (*41*) are also located near the region. Thus, the binding site of the inhibitor is considered to be situated near or at the active site of F_1.

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REFERENCES

1. Amzel, L.M. and Pedersen, P.L. (1983) Proton ATPases: Structure and mechanism. *Annu. Rev. Biochem.* **52**, 801-824
2. Futai, M., Noumi, T., and Maeda, M. (1989) ATP synthase (H^+-ATPase): Results by combined biochemical and molecular biological approaches. *Annu. Rev. Biochem.* **58**, 111-136
3. Pullman, M.E. and Monroy, G.C. (1963) A naturally occurring inhibitor of mitochondrial adenosine triphosphatase. *J. Biol. Chem.* **238**, 3762-3769
4. Ichikawa, N., Yoshida, Y., Hashimoto, T., Ogasawara, N., Yoshikawa, H., Imamoto, F., and Tagawa, K. (1990) Activation of ATP hydrolysis by an uncoupler in mutant mitochondria lacking an intrinsic ATPase inhibitor in yeast. *J. Biol. Chem.* **265**, 6274-6278
5. Klein, G., Satre, M., Dianoux, A.C., and Vignais, P.V. (1980) Radiolabeling of natural adenosine triphosphatase inhibitor with phenyl(^{14}C)isothiocyanate and study of its interaction with mitochondrial adenosine triphosphatase. Localization of inhibitor binding sites and stoichiometry of binding. *Biochemistry* **19**, 2919-2925
6. Gomez-Fernandez, J.C. and Harris, D.A. (1978) A thermodynamic analysis of the interaction between the mitochondrial coupling adenosine triphosphatase and its naturally occurring inhibitor protein. *Biochem. J.* **176**, 967-975
7. Hashimoto, T., Negawa, Y., and Tagawa, K. (1981) Binding of intrinsic ATPase inhibitor to mitochondrial ATPase—Stoichiometry of binding of nucleotides, inhibitor, and enzyme. *J. Biochem.* **90**, 1151-1157
8. Horstman, L.L. and Racker, E. (1970) Partial resolution of the enzymes catalyzing oxidative phosphorylation. *J. Biol. Chem.* **245**, 1336-1344
9. Schwerzmann, K. and Pedersen, P.L. (1986) Regulation of the mitochondrial ATP synthase/ATPase complex. *Arch. Biochem. Biophys.* **250**, 1-18
10. Okada, Y., Hashimoto, T., Yoshida, Y., and Tagawa, K. (1986) Existence of stoichiometric amounts of an ATPase inhibitor and two stabilizing factors with mitochondrial ATP synthase in yeast. *J. Biochem.* **99**, 251-256
11. Matsubara, H., Inoue, K., Hashimoto, T., Yoshida, Y., and Tagawa, K. (1983) A stabilizing factor of yeast mitochondrial F_1F_o-ATPase-inhibitor complex: Common amino acid sequence with yeast ATPase inhibitor and *E. coli* ε and bovine δ subunits. *J. Biochem.* **94**, 315-318
12. Hashimoto, T., Yoshida, Y., and Tagawa, K. (1987) Binding properties of 9K protein to F_1-ATPase: A counterpart ligand to the ATPase inhibitor. *J. Biochem.* **102**, 685-692
13. Jackson, P.J. and Harris, D.A. (1988) The mitochondrial ATP synthase inhibitor protein binds near the C-terminal region of the $F_1\beta$ subunit. *FEBS Lett.* **229**, 224-228
14. Mimura, H., Hashimoto, T., Yoshida, Y., Ichikawa, N., and Tagawa, K. (1993) Binding of an intrinsic ATPase inhibitor to the interface between α- and β-subunits of F_1F_oATPase upon deenergization of mitochondria. *J. Biochem.* **113**, 350-354
15. Hashimoto, T., Yamamoto, Y., Yoshida, Y., and Tagawa, K. (1995) Cleavage of bovine mitochondrial ATPase inhibitor with endopeptidases, and binding of the resulting peptides to the interface between the α- and β-subunits of F_1ATPase. *J. Biochem.* **117**, 641-647
16. Tagawa, K., Hashimoto, T., and Yoshida, Y. (1986) Isolation and characterization of an inactivated complex of F_1F_o-ATPase and its inhibitory factors from yeast in *Methods in Enzymology* (Fleischer, S. and Fleischer, B., eds.) Vol. 126, pp. 504-512, Academic Press, New York
17. Takeshige, K., Hess, B., Bohm, M., and Zimmermann-Telschow, H. (1976) Mitochondrial adenosine triphosphatase from yeast *Saccharomyces cerevisiae*. Purification, subunit structure and kinetics. *Hoppe-Seyler's Z. Physiol. Chem.* **357**, 1605-1622
18. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275
19. Bernstein, M., Hoffmann, W., Ammerer, G., and Scheckman, R. (1985) Characterization of a gene product (Sec53p) required for protein assembly in the yeast endoplasmic reticulum. *J. Cell Biol.* **101**, 2374-2382
20. Samblook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, pp. 18.3-18.10, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
21. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
22. Oshima, T., Sakane, M., Yamazaki, T., and Soda, K. (1990) Thermostable alanine dehydrogenase from thermophilic *Bacillus sphaericus* DSM462. Purification, characterization and kinetic

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mechanism. *Eur. J. Biochem.* **260**, 15458-15465
23. Moos, M., Jr., Nguyen, N.Y., and Liu, T.-Y. (1988) Reproducible high yield sequencing of proteins electrophoretically separated and transferred to an inert support. *J. Biol. Chem.* **263**, 6005-6008
24. Takeda, M., Vassarotti, A., and Douglas, M.G. (1985) Nuclear genes coding the yeast mitochondrial adenosine triphosphatase complex. Primary sequence analysis of *ATP2* encoding the F_1-ATPase β-subunit precursor. *J. Biol. Chem.* **260**, 15458-15465
25. Matsubara, H., Hase, T., Hashimoto, T., and Tagawa, K. (1981) Amino acid sequence of an intrinsic inhibitor of mitochondrial ATPase from yeast. *J. Biochem.* **90**, 1159-1165
26. Landon, M. (1977) Cleavage at aspartyl-prolyl bonds in *Methods in Enzymology* (Hirs, C.H.W. and Timasheff, S.N., eds.) Vol. 47, pp. 145-149, Academic Press, New York
27. Klein, G., Satre, M., and Vignais, P. (1977) Natural protein ATPase inhibitor from *Candida utilis* mitochondria. Binding properties of the radiolabeled inhibitor. *FEBS Lett.* **84**, 129-134
28. Williams, N. and Coleman, P.S. (1982) Exploring the adenine nucleotide binding sites on mitochondrial F_1-ATPase with a new photoaffinity probe, 3′-*O*-(4-benzoyl)benzoyl adenosine 5′-triphosphate. *J. Biol. Chem.* **257**, 2834-2841
29. Noumi, T., Tagaya, M., Miki-Takeda, K., Maeda, M., Fukui, T., and Futai, M. (1987) Loss of unisite and multisite catalyses by *Escherichia coli* F_1 through modification with adeosine tri- or tetraphosphopyridoxal. *J. Biol. Chem.* **262**, 7686-7692
30. Schafer, H.-J., Rathgeber, G., Dose, K., and Kagawa, Y. (1989) Photoaffinity cross-linking of the coupling factor 1 from *Micrococcus luteus* by 3′-arylazido-8-azido-ATP. *FEBS Lett.* **253**, 264-268
31. Melese, T. and Boyer, P.D. (1985) Derivatization of the catalytic subunits of the chloroplast ATPase by 2-azido-ATP and dicyclohexylcarbodiimide. *J. Biol. Chem.* **260**, 15398-15401
32. Boyer, P.D. (1987) The unusual enzymology of ATP synthase. *Biochemistry* **26**, 8503-8507
33. Walker, J.E., Fearnley, I.M., Gay, N.J., Gibson, B.W., Northrop, F.D., Powell, S.J., Runswick, M.J., Saraste, M., and Tybulewicz, V.L.J. (1985) Primary structure and subunit stoichiometry of F_1-ATPase from bovine mitochondria. *J. Mol. Biol.* **184**, 677-701
34. Runswick, M.J., Walker, J.E., Gibson, B.W., and Williams, D.H. (1986) The frayed N-terminal of the inhibitor protein of bovine mitochondrial F_1-ATPase. *Biochem. J.* **235**, 515-519
35. Cross, R.L., Cunningham, D., Miller, C.G., Xue, Z., Zhou, J.-M., and Boyer, P.D. (1987) Adenine nucleotide binding sites on beef heart F_1 ATPase: Photoaffinity labeling of β-subunit Tyr-368 at a noncatalytic site and β Tyr-345 at a catalytic site. *Proc. Natl. Acad. Sci. USA* **84**, 5715-5719
36. Xue, Z., Miller, C.G., Zhou, J.-M., and Boyer, P.D. (1987) Catalytic and noncatalytic binding sites of chloroplast F_1 ATPase. Photoaffinity labeling and peptide sequencing. *FEBS Lett.* **223**, 391-394
37. Wise, J.G., Hicke, B.J., and Boyer, P.D. (1987) Catalytic and noncatalytic nucleotide binding sites of the *Escherichia coli* F_1ATPase. Amino acid sequences of β-subunit tryptic peptides labeled with 2-azido-ATP. *FEBS Lett.* **223**, 395-401
38. Garin, J., Boulay, F., Issartel, J.P., Lunardi, J., and Vignais, P.V. (1986) Identification of amino acid residues photolabeled with 2-azido[α-^{32}P]adenosine diphosphate in the β-subunit of beef heart mitochondrial F_1-ATPase. *Biochemistry* **25**, 4431-4437
39. Bullough, D.A. and Allison, W.S. (1986) Inactivation of the bovine heart mitochondrial F_1-ATPase by 5′-*p*-fluorosulfonylbenzoyl[^{3}H]inosine is accompanied by modification of tyrosine 345 in a single β subunit. *J. Biol. Chem.* **261**, 14171-14177
40. Admon, A. and Hammes, G.G. (1987) Amino acid sequence of the nucleotide binding region of chloroplast coupling factor 1. *Biochemistry* **26**, 3193-3197
41. Hollemans, M., Runswick, M.J., Fearnley, I.M., and Walker, J.E. (1983) The sites of labeling of the β-subunit of bovine mitochondrial F_1-ATPase with 8-azido-ATP. *J. Biol. Chem.* **258**, 9307-9313

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