J. Biochem. **144,** 547–553 (2008)
doi:10.1093/jb/mvn100

Glutamic Acid in the Inhibitory Site of Mitochondrial ATPase Inhibitor, IF_1, Participates in pH Sensing in Both Mammals and Yeast

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Received June 26, 2008; accepted August 2, 2008; published online August 7, 2008

The mitochondrial ATPase inhibitor, IF_1, regulates the activity of F_1F_o-ATPase. The inhibitory activity of IF_1 is highly pH-dependent. The effective inhibition by IF_1 requires a low pH. Under basic conditions, its activity markedly declines. The importance of His49 in the pH dependence of bovine IF_1 is well-known. However, the residue is not conserved in yeast IF_1. We previously showed that Glu21 is required for the pH dependence of yeast IF_1, but the function of homologous Glu in mammalian IF_1 is not clear. In this study, we examined the requirement for Glu26 of bovine IF_1 (corresponding to Glu21 of yeast IF_1) regarding its pH dependence by amino acid replacement. Three mutant proteins, E26A, H49K and the double mutant E26A/H49K, were overexpressed and purified. All mutants retained their inhibitory activity well at pH 8.2, although wild-type IF_1 was ~10-fold less active at pH 8.2 than at 6.5. A covalent cross-linking study revealed that both wild-type IF_1 and the E26A mutant formed a tetramer at pH 8.2, although H49K and E26A/H49K mutants did not. These results indicate that, in addition to His49, Glu26 participates in pH sensing in bovine IF_1, and the mechanism of pH sensing mediated by Glu26 is different from the dimer–tetramer model proposed previously.

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

Abbreviations: F_1 or F_1-ATPase, catalytic part of ATP synthase; F_1F_0 or F_1F_0 -ATPase, mitochondrial ATP synthase; I_{50}, amount of IF_1 required to inhibit 50% of F_1F_0-ATPase; IF_1, regulatory subunit of ATP synthase (mitochondrial ATPase inhibitor protein).

Mitochondrial ATP synthase (F_1F_o-ATPase) catalyses the terminal step of oxidative phosphorylation in eukaryotic cells. The enzyme generates ATP from ADP and inorganic phosphate by coupling the energy of the proton electrochemical gradient across the mitochondrial inner membrane. 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 as a central axis, and three catalytic sites are located on the β-subunits at the interfaces to the α-subunits (*1*). F_o mediates the passage of protons from the intermembrane space into the matrix. This proton flux drives the rotation of the c-subunit ring in F_o, along with the γ-subunit, and causes cyclical conformation changes in the catalytic sites in F_1 (*2*).

A small basic protein, IF_1 (also called ATPase inhibitor protein), regulates mitochondrial F_1F_o-ATPase. IF_1 does not affect ATP synthesis during oxidative phosphorylation, but when the proton electrochemical gradient is lost, IF_1 binds to the F_1 part of the enzyme in a 1:1 stoichiometry and completely inhibits ATP hydrolysis by the enzyme. IF_1 was first isolated from bovine heart mitochondria (*3*), and homologous proteins have been found in various eukaryotic cells from yeast to mammals (Fig. 1).

Binding of IF_1 to F_1Fo-ATPase is highly pH-dependent (*3*, *4*). At an acidic pH (<7.0), the binding of IF_1 to F_1Fo is facilitated and the activity of the enzyme is effectively inhibited. However, above pH 7.5, the inhibitory activity sharply declines. The pH dependence has been observed in most IF_1s isolated from various eukaryotic cells (*3–6*) and seems to be a common characteristic of them.

In bovine IF_1, it has been well-established that the His49 residue is required for the pH dependence (*7*, *8*). Schnizer *et al*. (*7*) showed that the mutations of His49 abolish the inactivation of bovine IF_1 at a high pH. Furthermore, Cabezon *et al*. (*8*) proposed a model, whereby His49 regulates the interconversion between active dimeric and inactive tetrameric states of IF_1 in a pH-dependent manner. The His49 residue is located on the outside of the minimal inhibitory sequence and conserved in mammalian IF_1s but not in yeast and nematode IF_1s (Fig. 1).

Although His49 is not conserved in yeast IF_1, its inhibitory activity is also noticeably affected by the pH (*6*). We previously investigated the residues required for the function of the yeast IF_1 by site-directed mutagenesis, and found that the mutation of Glu21 abolished the

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pH dependence of the protein (*9*). This Glu21 residue is located in the inhibitory site of IF_1 and is well-conserved in the primary structures of IF_1s from yeast to mammals (Fig. 1). However, the function of the residue has not been examined except for in yeast.

In this study, we examined the requirement for Glu26 of bovine IF_1, corresponding to Glu21 in yeast IF_1, for its pH-dependent activation–inactivation using amino acid replacement. Three mutant proteins (E26A, H49K and the double mutant E26A/H49K) were expressed in *Escherichia coli* cells, purified and characterized. The results indicated that, in addition to His49, Glu26 participates in the pH dependence of bovine IF_1. We suggest the mechanism of pH sensing of IF_1 mediated by Glu26.

MATERIALS AND METHODS

Yeast Strains and Methods—A yeast strain that expresses E21A mutant IF_1 under the control of a *GAL10* promoter was previously constructed (*9*). The control strain, YC63, is reported in ref. (*10*). The IF_1-deficient yeast strain, D26, was constructed previously (*11*). Wild-type and mutant yeast IF_1 proteins were purified as described in ref. (*9*). Yeast mitochondria (*12*) and sub-mitochondrial particles (*13*) were isolated by the reported methods.

Construction of E. coli Strains that Express Mutated Bovine IF_1 Proteins—The coding sequence of bovine IF_1 (*14*) 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′ (*6*). The mutated genes E26A and H49K were constructed by the PCR-based method (15), using internal mutagenic primers 5′-TTC GGA AAA AGA GCC CAG GCC GAA GAG-3′ and 5′-CTC TTC GGC CTG GGC TCT TTT TCC GAA-3′ or 5′-TTG AAG AAA CAC AAA GAA AAT GAG ATC-3′ and 5′-GAT CTC ATT TTC TTT GTG TTT CTT CAA-3′. Primers that were the same as those applied in the construction of the H49K mutant were used for amplification of the double-mutated gene E26A/H49K from the template E26A DNA. These PCR products were cloned under the *tac* promoter of the expression vector pMK2 (*16*), and introduced into *E. coli* JM109 cells. The resultant plasmids encoded the mature form of IF_1s for which the import signal sequence was replaced by a single methionine residue. The coding sequences of the plasmids were verified by sequencing analyses.

Purification of Wild-type and Mutated Bovine IF_1 Proteins—JM109 cells transformed with the plasmids (see above) were grown on a rich medium containing 50 μg/ml ampicillin for 2 h at 37°C. After the addition of 1 mM isopropyl-β-D-thiogalactopyranoside, the cells were grown for a further 3 h. The cells were harvested by centrifugation (9,000 r.p.m., 10 min, 2°C) and suspended in H_2O. The IF_1 proteins were extracted by heating and purified with Macro-Prep High S column chromatography (Bio-Rad Laboratories, Inc., USA), as described previously (*9*). Purified proteins were measured by the method of Lowry *et al*. (*17*).

Assay of ATPase Activity—Samples (10 μl) containing ATPase were transferred to 0.5 ml of an ATPase assay system consisting of 50 mM Tris maleate (pH 7.8), 5 mM $MgSO_4$ and 5 mM ATP, and incubated at 25 or 37°C. The reaction was stopped by the addition of 3 ml of stop solution consisting of 6% perchloric acid and 0.2% SDS, and then the formation of inorganic phosphate from ATP was measured, as described below.

The principle of the determination of inorganic phosphate was the same as that described by Allen (*18*). A 0.2 ml of reducing reagent containing 2% amidol and 8% sodium bisulfite and 0.2 ml of 4% ammonium molybdate solution were added to the samples after the addition of the stop solution (see above), in that order. After 5 min, absorbance at 660 nm was measured. The concentration of phosphate present was read from a standard curve established with known amounts of pure potassium phosphate.

One unit of ATPase was defined as the amount of the enzyme forming 1 μmol of phosphate per minute. I_{50} was defined as the amount of IF_1 required for 50% inhibition of 0.1 U of F_1F_o-ATPase in each condition.

Fig. 1. **Alignment of IF_1 proteins.** Amino acid sequences of IF_1s from bovine, humans, rats, *Caenorhabditis elegans*, *Candida utilis* and *Saccharomyces cerevisiae* are aligned (UniProt IDs, ATIF1_BOVIN, ATIF1_HUMAN, ATIF1_RAT, ATIF2_CAEEL, ATIF_PICJA and ATIF_YEAST). Identical and conservatively substituted residues are shaded. Regions required for the ATPase inhibitory activity are indicated by arrows [minimal inhibitory sequence of bovine IF_1 (*23*) and inhibitory site of *S. cerevisiae* IF_1 (*9*)]. *Spades* and *hearts* indicate bovine His49 and yeast Glu21, respectively. These residues are required for inactivation of the proteins at a high pH. Bovine Glu26 investigated by amino acid replacement in this study is indicated by a *club*.

Other Procedures—MALDI-TOF/MS was carried out with Shimadzu AXIMA-CFR plus (Shimadzu Corporation, Kyoto) using sinapic acid as a matrix. The IF_1-depleted submitochondrial particles from bovine heart muscle for the assay of IF_1 were prepared as described (*19*). Dimethyl suberimidate (*20*) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka). The polyacrylamide gels used for SDS–PAGE were purchased from ATTO Corporation (Tokyo).

RESULTS

Glu21 of Yeast IF_1 is Required for pH-dependent Inactivation of the Protein—We previously constructed a yeast strain that expresses mutant IF_1 of which Glu21 is replaced by Ala (E21A) (*9*). The mutant IF_1 was purified from the yeast cells and its ATPase inhibitory activities under acidic (pH 6.5) and basic (pH 8.2) conditions were compared with that of the wild-type control (Fig. 2A and B). As shown in Fig. 2A, the activity of wild-type IF_1 markedly declines at pH 8.2 in comparison with that at pH 6.5. In contrast, the activity of the E21A mutant at pH 8.2 was similar to that at pH 6.5 (Fig. 2B).

Furthermore, the ATPase activity of mitochondria isolated from the cells was also examined (Fig. 2C). The ATPase activity of the wild-type control was sharply activated above pH 7.5, but that of the E21A mutant was low level through pH 6.5–8.0.

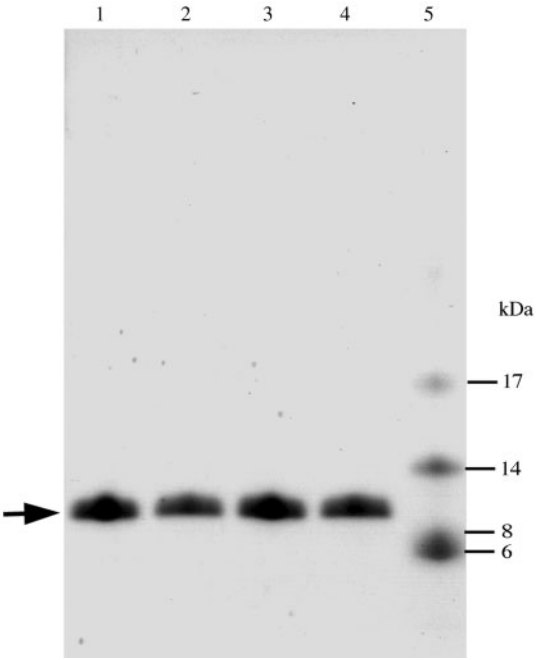

Fig. 3. **SDS–PAGE analysis of purified IF_1 proteins.** Approximately 0.5 μg of the proteins was electrophoresed on a 15% polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. Lane 1, wild-type IF_1; lane 2, E26A; lane 3, H49K; lane 4, E26A/H49K; lane 5, molecular weight markers.

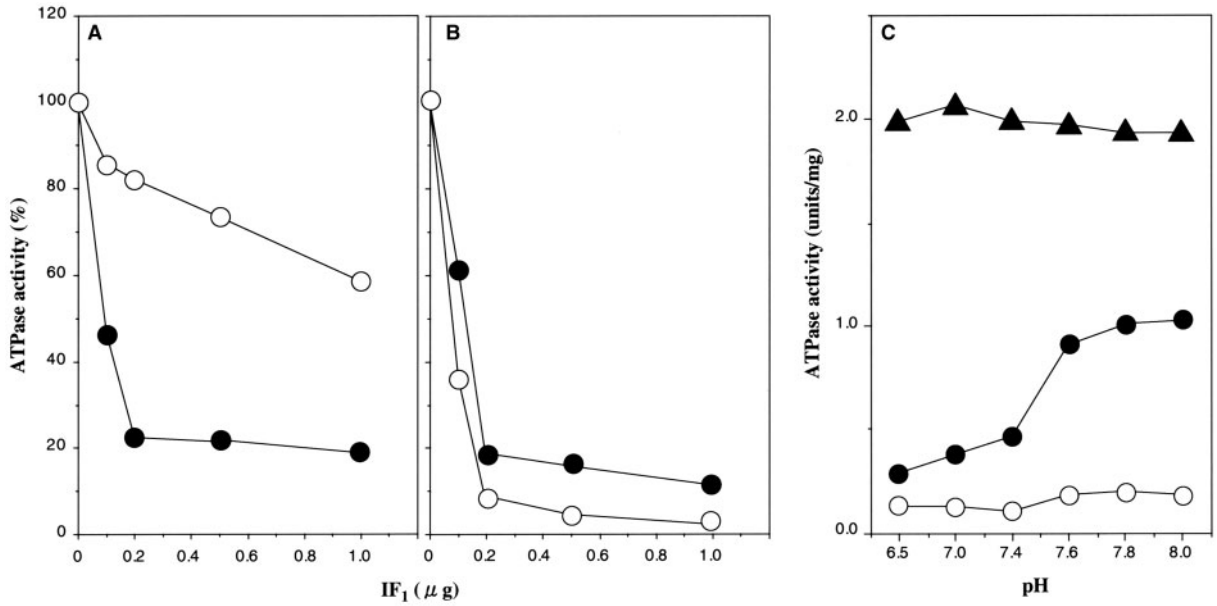

Fig. 2. **Glu21 of yeast IF_1 is required for pH dependence of the protein.** (A and B) Inhibition of F_1F_o-ATPase by wild-type and E21A mutant yeast IF_1s at pH 6.5 and 8.2. The indicated amounts of IF_1 proteins were incubated with the IF_1-deleted submitochondrial particles [containing 0.2 U of F_1Fo-ATPase, prepared from IF_1-deleted mutant yeast cells (*11*)] to give a total volume of 50 μl in 50 mM Tris buffer, 5 mM $MgSO_4$ and 5 mM ATP. The buffer used in the mixture was Tris maleate (pH 6.5) or Tris SO_4 (pH 8.2). After incubation for 10 min at room temperature, the remaining ATPase activity was measured at 25°C. (A) Wild-type IF_1; (B) E21A mutant IF_1. Solid circles, pH 6.5; open circles, pH 8.2. (C) ATPase activity in isolated mitochondria at various pH. Mitochondria isolated from the control and mutant yeast cells were incubated in 50 mM Tris buffer. The buffer used in the mixture was Tris maleate (pH 6.5–7.4) or Tris SO_4 (pH 7.6–8.0). After incubation for 1 h at room temperature, the ATPase activity was measured at 25°C. Solid circles, wild-type control [YC63 cells (*10*)]; open circles, E21A mutant yeast (*9*); solid triangles, IF_1-deleted mutant yeast (*11*). These mutant yeasts can grow at a normal rate on galactose medium (*9*, *11*).

These results indicate that the Glu21 of yeast IF_1 is required for the pH dependence of the protein.

Expression and Purification of Mutated Bovine IF_1 Proteins—To examine the participation of Glu26 of bovine IF_1, corresponding to Glu21 in yeast IF_1, in pH dependence, we constructed the E26A mutant of bovine IF_1. For comparison, H49K and the double mutant E26A/H49K were also constructed. These mutant proteins were overexpressed in *E. coli* cells under the control of the *tac* promoter.

The proteins were extracted from *E. coli* cells by heating, and purified by Macro-Prep High S column chromatography. Wild-type and H49K IF_1s were purified in this step, whereas E26A and E26A/H49K IF_1s, containing small amounts of impurities, were further purified by repeat chromatography under the same conditions. Fig. 3 shows SDS–PAGE analyses of the purified proteins. The preparation of the proteins yielded single bands on the gel (Fig. 3, lanes 1–4). The yields of wild-type, E26A, H49K and E26A/H49K IF_1s were 1.6, 0.2, 1.0 and 0.2 mg per 0.5 litres of culture, respectively.

Since the masses of the proteins determined by MALDI-TOF/MS were the same as the calculated values except for the molecular weight of methionine (Table 1), the N-terminus of all purified proteins lacked the translational initiator methionine.

Table 1. **Masses of the purified IF_1 proteins measured by MALDI-TOF/MS.**

IF_1	Mass (Da)	
	Measured (error)	Calculated values except for the molecular weight of Met
Wild-type	9,582.4 (+ 1.0)	9,581.4
E26A	9,524.4 (+ 1.0)	9,523.4
H49K	9,573.2 (+ 0.8)	9,572.4
E26A/H49K	9,514.9 (+ 0.5)	9,514.4

Effect of pH on Inhibitory Activity of the Mutated Bovine IF_1 Proteins—The effects of pH on the inhibition of F_1F_o-ATPase by the mutated bovine IF_1s were investigated at pH 6.5 and 8.2 (Fig. 4A–D and Table 2). At pH 6.5, the inhibitory activities of all mutant IF_1s were comparable with that of the wild-type control (Fig. 4A–D). At pH 8.2, the activity of the wild-type IF_1 decreased markedly (Fig. 4A), but H49K and E26A/H49K mutants retained a similar activity to that at pH 6.5 (Fig. 4C and D). The E26A mutant was also very active at pH 8.2 (Fig. 4B). The requirement of wild-type IF_1 to

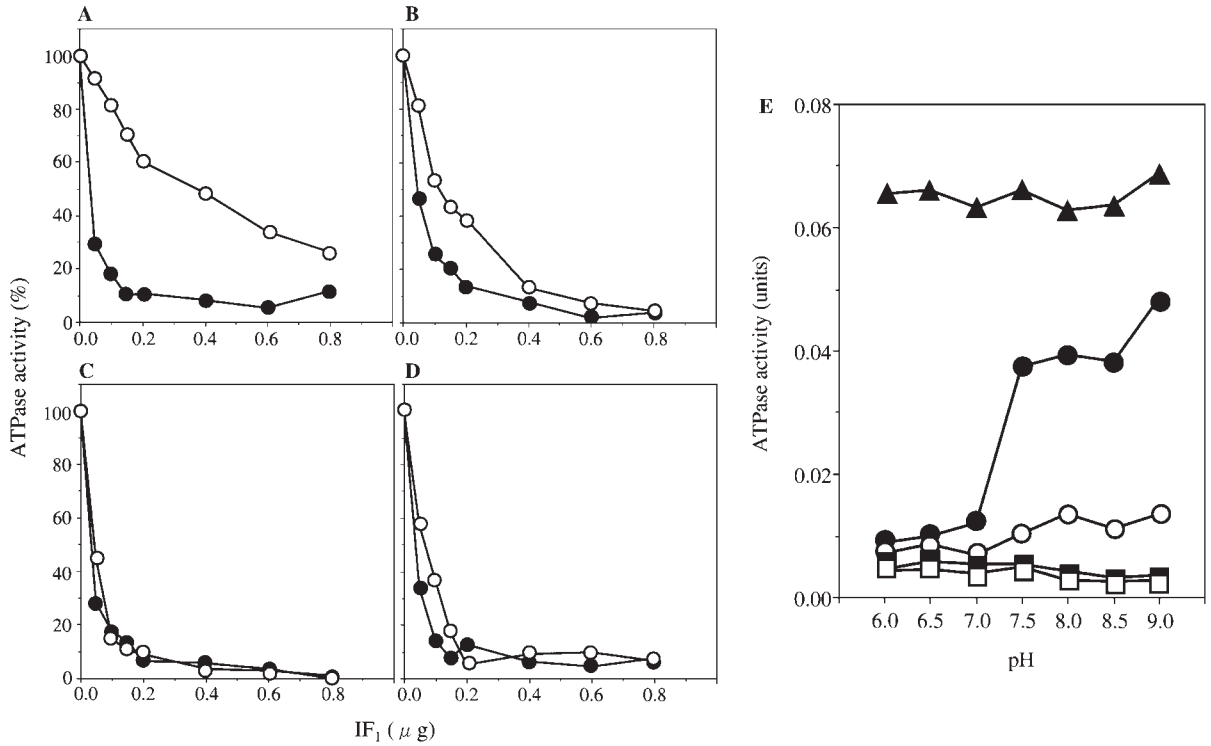

Fig. 4. **Inhibition of F_1F_o-ATPase by wild-type and mutant bovine IF_1 proteins.** (A–D) Inhibition of F_1Fo-ATPase by the IF_1 proteins at pH 6.5 or 8.2. IF_1-depleted submitochondrial particles isolated from bovine heart (containing 0.1 U of F_1F_o-ATPase) were incubated with the indicated amounts of IF_1 proteins to give a total volume of 50 μl in 50 mM Tris buffer, 0.5 mM $MgSO_4$ and 0.5 mM ATP. The buffer used in the mixture was Tris maleate (pH 6.5) or Tris SO_4 (pH 8.2). After incubation for 10 min at room temperature, the remaining ATPase activity was measured at 37°C. (A) Wild-type IF_1; (B) E26A; (C) H49K; (D) E26A/H49K. Solid circles, pH 6.5; open circles, pH 8.2. (E) Inhibition of F_1F_o-ATPase by the IF_1 proteins at pH 6.0–9.0. Submitochondrial particles were incubated with 0.4 μg of IF_1 to give a total volume of 50 μl in 50 mM Tris buffer, 0.5 mM $MgSO_4$ and 0.5 mM ATP. The buffer used in the mixture was Tris maleate (pH 6.0–7.0) or Tris SO_4 (pH 7.5–9.0). After incubation for 10 min at room temperature, the remaining ATPase activity was measured at 37°C. Solid circles, wild-type IF_1; open circles, E26A; solid squares, H49K; open squares, E26A/H49K; solid triangles, control without any IF_1 proteins.

inhibit 50% of the ATPase at pH 8.2 was 10-fold higher than that at pH 6.5 (Table 2). In contrast, that of E26A, H49K and E26A/H49K mutants was 2.6, 1.3 and 1.8-fold, respectively (Table 2).

Measurements of the inhibitory activities over a wide range of pH are shown in Fig. 4E. The inhibitory activity of the wild-type IF_1 markedly declined above pH 7.0. H49K and E26A/H49K mutants showed no change through pH 6.0–9.0. The inhibitory activity of the E26A mutant slightly decreased above pH 7.0, but a large portion of F_1Fo-ATPase was inhibited by this mutant protein through pH 6.0–9.0.

Table 2. **Amount of IF_1 required to inhibit 50% of F_1F_o-ATPase (I_{50}).**

IF_1	I_{50} (μg)		I_{50} (pH 8.2)/I_{50} (pH 6.5)
	pH 6.5	pH 8.2	
Wild-type	0.035	0.370	10.6
E26A	0.045	0.115	2.6
H49K	0.035	0.045	1.3
E26A/H49K	0.038	0.067	1.8

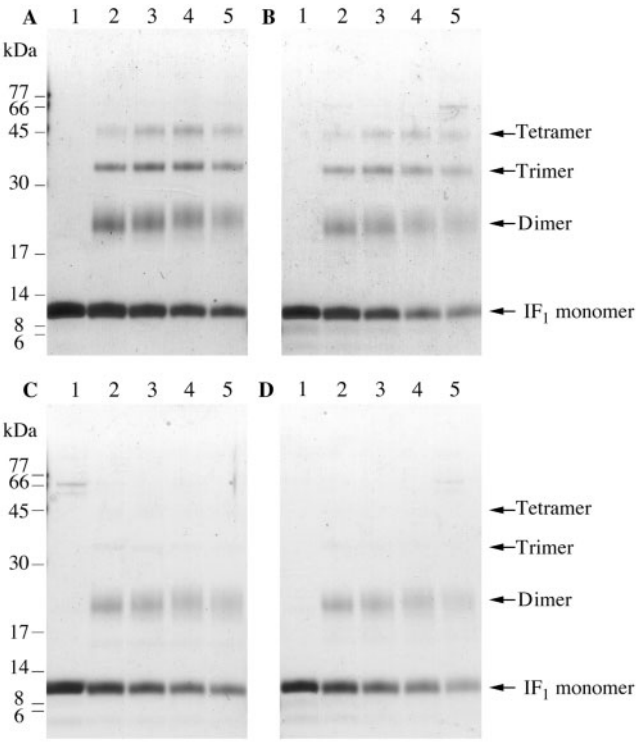

Fig. 5. **Covalent cross-linking of wild-type and mutant bovine IF_1 proteins with dimethyl suberimidate.** Cross-linking of the IF_1 proteins was performed using the cross-linking reagent dimethyl suberimidate according to the method described previously (*8*). Aliquots were sampled at various times from each reaction solution and sufficiently mixed with sample buffer of SDS–PAGE to stop the cross-linking reactions. These mixtures were analysed on 15% polyacrylamide gels and stained with Coomassie Brilliant Blue R-250. Lane 1, samples prior to the addition of the cross-linking reagent; lane 2, reaction time was 30 min; lane 3, 60 min; lane 4, 90 min; lane 5, 180 min. (A) Wild-type IF_1; (B) E26A; (C) H49K; (D) E26A/H49K. The molecular weights are shown on the left in kilo Daltons. Oligomers are indicated by arrows on the right.

These results indicate that the pH dependence of the E26A IF_1 is very small, and, in addition to His49, Glu26 also plays an important role in the pH dependence of bovine IF_1.

Covalent Cross-linking of IF_1 Proteins—Purified bovine IF_1 predominantly forms a tetramer at pH 8 and a dimer below pH 6.5 (*8*). Previously, Cabezon *et al.* (*8*) proposed that the dimer is active, the tetramer is inactive and His49 mediates the pH-dependent interconversion between active dimeric and inactive tetrameric forms of IF_1. The E26A mutant IF_1 retained inhibitory activity at pH 8.2, and so we investigated the oligomeric states of wild-type and mutant IF_1 proteins at pH 8.2 using the cross-linking reagent dimethyl suberimidate (Fig. 5).

The cross-linked products from wild-type IF_1 contained the monomer, dimer, trimer and tetramer, indicating that IF_1 forms tetramers at pH 8.2 (Fig. 5A). H49K and E26A/H49K mutants only formed dimers (Fig. 5C and D), indicating that His49 is required to form tetramers, as reported previously (*8*). The cross-linked products of the E26A mutant contained monomers to tetramers equally to wild-type IF_1. These results indicate that the E26A IF_1 forms a tetramer at pH 8.2 in the same manner as wild-type IF_1, and that the tetramer of the mutant IF_1 remains active to inhibit F_1Fo-ATPase.

DISCUSSION

We previously found that Glu21 of yeast IF_1 is required for the pH dependence of the protein (*9*). In the present study, we constructed three mutants of bovine IF_1, E26A, H49K and a double mutant E26A/H49K, and examined the requirement for Glu26, corresponding to Glu21 in yeast IF_1, in pH dependence.

As shown in Fig. 4B and E, the mutation of Glu26 markedly decreased the pH dependence of bovine IF_1. These results indicate that glutamic acid at this position is not only required for the pH dependence of yeast IF_1 but also for that of mammalian IF_1.

The importance of Glu26 in pH dependence is also indicated by the investigation of MAI-1 protein, one of the IF_1 family proteins in *Caenorhabditis elegans* (*21*). The primary structure of MAI-1 is very similar to IF_1, but the protein lacks a mitochondrial import signal sequence and seems to be a cytosolic protein (*21*). The inhibitory activity of *C. elegans* IF_1 (also called MAI-2), of which Glu26 is conserved (Fig. 1), was as pH-dependent as bovine and yeast IF_1 (*21*). However, the activity of MAI-1, of which Glu26 is replaced by Gly, was pH-independent (*21*). Thus, Glu26 appears to be a general residue required for the pH sensing of IF_1 proteins. In addition to Glu26, His49 appears to also develop into a pH sensor in mammalian IF_1s.

Previously, Cabezon *et al.* (*8*) proposed a model, whereby the IF_1 dimer is active, the tetramer is inactive and the interconversion between active dimeric and inactive tetrameric forms is pH-dependent. The mechanism of pH dependence mediated by Glu26 seems to be different from the dimer–tetramer model, because E26A IF_1 can form a tetramer at pH 8.2 (Fig. 5B) in spite of its inhibitory activity at this pH (Fig. 4E).

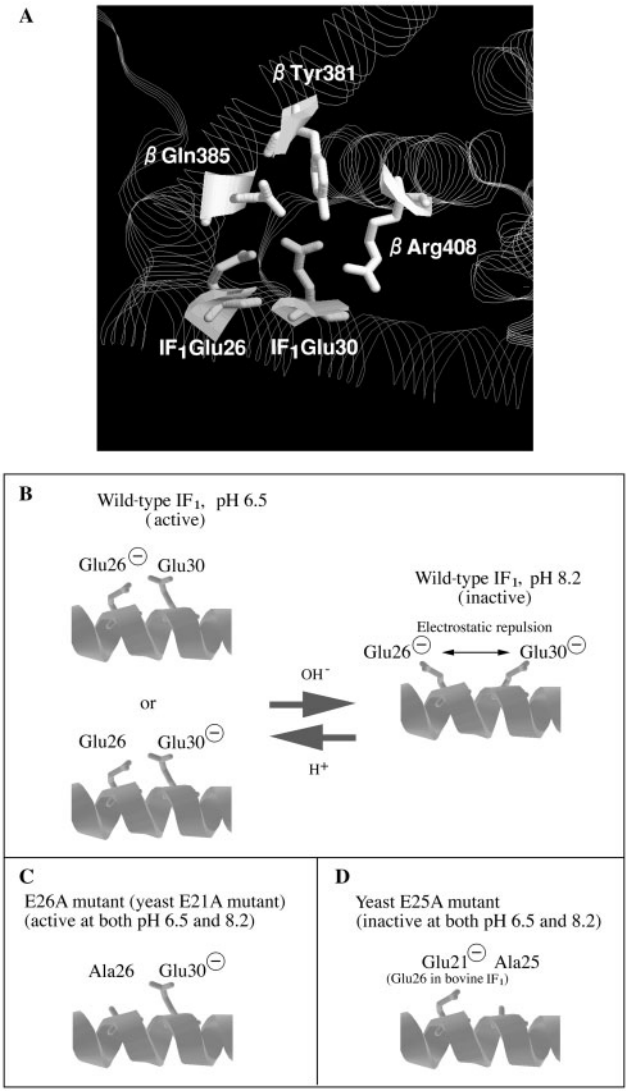

Fig. 6. **Model of interconversion between active and inactive states of IF_1 regulated by dissociation of carboxyl group of Glu26 and Glu30.** (A) Interface between the Glu26-Glu30 region of IF_1 and β-subunit of F_1 in the crystal structure of the bovine F_1–IF_1 complex reported by Gledhill *et al*. (*22* and PDB # 2v7q). Glu30 (corresponding to Glu25 of yeast IF_1) interacts with Tyr381, Gln385 and Arg408 of the β-subunit. This image was generated with RasMol computer software (version 2.6) on a Power Macintosh G4 computer (Apple computer, Inc., USA). (B–D) Scheme describing the structural and functional changes of wild-type bovine IF_1, the bovine E26A mutant (corresponding to the yeast E21A mutant) and yeast E25A mutant. See text for details.

The mechanism of pH sensing of IF_1 mediated by Glu26 may be derived from the X-ray-based structure of the bovine F_1–IF_1 complex recently reported (*22*). In the structure, Glu26 of IF_1 has no direct contact with the F_1-ATPase and its side chains are exposed in the aqueous phase (*22*). However, Glu26 is located near Glu30 of IF_1 (Fig. 6A). The side chain of Glu30 directly interacts with the β-subunit of F_1 (*22*) and seems to be important in the F_1–IF_1 interaction. In fact, homologous Glu25 of yeast IF_1 is essential for the ATPase inhibitory activity (amino acid replacement of the residue abolishes the inhibitory activity) (*9*). Because the side chain of Glu26 is located near that of Glu30, dissociation of the carboxyl group of Glu26 may affect the conformation or direction of the side chain of Glu30 and may modulate the activity of IF_1 protein.

Thus, we propose the model of pH-dependent interconversion between the active and inactive state of IF_1 mediated by Glu26 as below (Fig. 6B–D). (i) Wild-type IF_1 at pH 6.5 (Fig. 6B, left). Either the carboxyl group of Glu26 or Glu30 dissociates. The side chain of Glu30 maintains the conformational fit to interact with F_1-ATPase. (ii) Wild-type IF_1 at pH 8.2 (Fig. 6B, right). Carboxyl groups of both Glu26 and Glu30 dissociate. Electrostatic repulsion between the carboxyl groups changes the conformation or direction of the side chain of Glu30. As a result, interaction between IF_1 and F_1 is destabilized and the inhibitory activity declines. (iii) E26A mutant (yeast E21A mutant) (Fig. 6C). Glu26 is replaced by neutral Ala. Conformation of the Glu30 side chain is not affected by pH and can interact with F_1 at both pH 6.5 and 8.2. (iv) Yeast E25A mutant (Fig. 6D). Glu25 (bovine Glu30) that is essential for the inhibitory activity is replaced and the activity is abolished.

ACKNOWLEDGEMENTS

We thank Tomomi Shimonaka, technical staff of Osaka City University, for performing MALDI-TOF/MS analyses of IF_1 proteins.

CONFLICT OF INTEREST

None declared.

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