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Inhibitory and Anchoring Domains in the ATPase Inhibitor Protein IF_1 of Bovine Heart Mitochondrial ATP Synthase

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The inhibitor protein IF_1 is a basic protein of 84 residues which inhibits the ATPase activity of the mitochondrial F_oF_1-ATP synthase complex without having any effect on ATP synthesis. Results of cross-linking and limited proteolysis experiments are presented showing that in the intact F_oF_1 complex "in situ," in the inner membrane of bovine heart mitochondria, the central segment of IF_1 (residues 42–58) binds to the α and β subunits of F_1 in a pH dependent process, and inhibits the ATPase activity. The C-terminal region of IF_1 binds, simultaneously, to the OSCP subunit of F_o in a pH-independent process. This binding keeps IF_1 anchored to the complex, both under inhibitory conditions, at acidic pH, and noninhibitory conditions at alkaline pH.

KEY WORDS: H^+-ATP synthase; F_oF_1 complex; ATPase inhibitor protein.

INTRODUCTION

The mitochondrial F_oF_1-ATP synthase complex is responsible, like that of aerobic prokaryotes, for ATP synthesis driven by the transmembrane electrochemical proton gradient generated by electron flow along the respiratory chain. The complex is made up of two moieties, the catalytic sector F_1 and the transmembrane sector F_o. F_1 consists of 3α, 3β, 1γ, 1δ, 1ε subunits, the F_o of 10 subunits, each present in one copy except the c subunit which is present in 10–14 copies (Abrahams *et al*., 1994; Bianchet *et al*., 1998; Jones and Fillingame, 1998; Papa *et al*., 2000; Seelert *et al*., 2000; Stock *et al*., 1999). The two sectors are connected by two stalks (Karrasch and Walker, 1999; Pedersen *et al*., 2000), a central one made up of F_1 subunits γ, δ, ε, which contact the membrane embedded F_o-c oligomer, and a peripheral one whose main constituents are Fo-a, Fo-b (FoI-PVP), OSCP, and Fo-d subunits (Collinson *et al*., 1994; Xu *et al*., 2000). ATP synthesis is thought to be driven by $\Delta\mu H^+$ induced rotation of the central stalk (rotor) within the peripheral stalk which acts as a stator of a rotary motor. The central region of γ, coming sequentially in contact with the β subunits, induces conformational changes resulting in ATP displacement from the catalytic site, thus driving ATP synthesis (Abrahams *et al*., 1994; Bianchet *et al*.,1998; Boyer, 1997).

Under normal aerobic conditions oxidative phosphorylation covers, in mammalian tissues, up to 80% or more of the ATP requirement (Papa, 1996). Under ischemic conditions, in the absence of the respiratory proton gradient, the high catalytic activity could result in rapid hydrolysis of ATP produced in the cytosol by glycolysis. In mammalian tissues this is, however, prevented by the natural inhibitor protein IF_1 (Harris, 1984; Rouslin, 1987; Rouslin *et al*., 1990) which binds to the complex and inhibits ATP hydrolysis without having any effect on ATP synthesis. This differential effect is apparently due to displacement of IF_1 from the complex by the respiratory proton gradient, in particular the pH gradient (Harris, 1984).

Bovine IF_1 is a basic protein of 84 residues (Pullman and Monroy, 1963) with a well-conserved sequence in eukaryotes (Hashimoto *et al*., 1981; Lebowitz and Pedersen, 1993). It is absent in prokaryotes where an analogous counterpart of IF_1 may be represented by the ε subunit of the ATP synthase complex (Smith and Sternweiss, 1977).

Much work has been carried out to identify critical domains of IF_1 involved in the binding and the inhibitory

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action, and the subunits in the ATP synthase complex where the inhibitor binds. Harris *et al.* (Harris, 1984; Jackson and Harris, 1988) first suggested that an α-helical rod, encompassing residues 22–79 in bovine heart IF_1, represents the functional segment. Stout *et al*. (1993) showed that the synthetic peptide with the sequence Phe22-Lys46 of bovine IF_1 inhibited the ATPase activity in the soluble F_1. Papa *et al.* (1996, 2000) and van Raaij *et al.* (1996) found, however, that this peptide has no significant effect on the ATPase activity in the entire F_oF_1 complex. It is likely that removal of F_1 from the F_oF_1 complex uncovers site(s), otherwise occupied by F_o subunits, which can now interact with the 22–46 peptide. Other investigations indicated, on the other hand, that neither the last 10–20 residues of the C-terminus of IF_1 (Hashimoto *et al*., 1995; van Raaij *et al*., 1996) nor the first 13 N-terminal residues (van Raaij *et al*., 1996), are directly essential for the inhibitory activity of IF_1.

van Raaij *et al*. (1996) concluded from their studies with recombinant products of bovine IF_1 that the minimal inhibitory sequence consists of residues 14–47. Papa *et al*. (1996) have, on the other hand, shown that the synthetic peptide with the Leu42-Lys58 sequence of bovine IF_1 is equally effective, as the overall natural IF_1, in inhibiting the ATPase activity of both the F_oF_1 complex in the membrane and soluble F_1. The 42–58 segment has at positions 48, 49, 55, and 56 four histidines. Histidine chemical modification (Guerrieri *et al*., 1987; Panchenko and Vinogradov, 1985), site directed mutagenesis of His49 (Schnizer *et al*., 1996) and chemical substitution of His 48, 49, 55, 56 or of Lys 46, 47, 58 in the synthetic 42–58 peptide (Papa *et al*., 1996) show that these residues are directly responsible for the activity and pH dependence of IF_1. Cross-linking experiments have provided evidence showing that IF_1 binds to the β and α subunits (Jackson and Harris, 1988; Mimura *et al*., 1993), as well as to the γ and ε subunits of F_1 (Minauro-Sanmiguel *et al*., 2002).

Results are presented here showing that in the intact F_oF_1 in the membrane the central segment of IF_1 (residues 42–58) binds to the F_1 α and β subunits in a pH-dependent process and inhibits the ATPase activity. The C-terminal region binds, simultaneously, to the F_o-OSCP subunit in a pH-independent process, which anchors IF_1 to the F_oF_1 complex.

MATERIALS AND METHODS

Chemicals

Chemicals were purchased from the following Companies: valinomycin, oligomycin, were obtained from Sigma; acrylamide, *N*,*N*′-methylenebisacrylamide, sodium dodecyl sulfate (SDS), Goat anti (rabbit IgG)-alkaline-phosphatase conjugate, AP color development reagent (BCIP-5 bromo-4-chloro-3 indolyl phosphate) (NBT-nitroblue tetrazolium) were obtained from Bio-Rad; ATP, ADP, catalase, hexokinase, p^1,p^5 di(Adenosin-5-) pentaphosphate, glucose-6-phosphate deydrogenase, phosphoenolpyruvate, NADH, NADP, pyruvate kinase, lactate dehydrogenase, Trypsin, Trypsin inhibitor were obtained from Roche; 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysulfosuccinimide (NHS) were obtained from Pierce; nitrocellulose membrane (0.45-μm pore size) were obtained from Schleicher and Schüll Biotech, Ltd.; PVDF membrane (immobilon transfer; 0.45-μm pore size) was from Millipore; chemicals for synthesis and sequence proteins were obtained from Applied Biosystem; thioanisol and ethanediol were obtained from Fluka; *tert*-butylether were obtained from Carlo Erba; Sephadex™ G-50 Coarse was obtained from Amersham Pharmacia Biotech. All other chemical reagents were of analytical grade.

Preparations of Submitochondrial Particles, F_1 and F_1 Inhibitor Protein

Inside-out vesicles of the inner mitochondrial membrane were obtained by exposure of isolated beef-heart mitochondria (Low and Vallin, 1963) to ultrasonic energy in the presence of 4 mM EDTA at pH 8.5 (ESMP) or in the presence of 1 mM MgATP (MgATP-SMP) (Lee and Ernster, 1968). IF_1 depleted submitochondrial particles, were prepared removing the inhibitor protein by passing ESMP through a Sephadex™ G-50 column (S-SMP) (Racker and Horstmann, 1967). Native IF_1 was prepared from MgATP-SMP according to (Kanner *et al*., 1976). F_1 was purified by a modified (Guerrieri *et al*., 1989a) chloroform extraction procedure (Beechey *et al*., 1975).

The percentage of inversion of submitochondrial particles was found to range from preparation to preparation from 96–100% (Guerrieri and Papa, 1981).

Synthesis of Peptides

Synthetic peptides with conserved or mutated sequences of IF_1 were obtained by standard solid-phase method with a fully automatic peptide synthesizer (model 431A, Applied Biosystem) using the Fmoc chemistry (Kent, 1988). After synthesis the peptides were deprotected by incubation for 2 h at room temperature in 83% trifluoroacetic acid, 4% thioanisol, 2% ethanediol, 6% crystalline phenol. After cleavage the peptides were precipitated by 10 vol. of anhydrous *tert*-butylether, washed

four additional times with ether and lyophilized. The purity of the synthetic peptides was checked by reverse-phase HPLC on a Perkin–Elmer C-8/10 column with a linear gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid. The sequence of the synthetic peptides was verified by amino acid analysis performed with a fully automatic peptide sequencer (model 473A, Applied Biosystems). The repetitive yield of sequence analysis was higher than 90%.

Final purification of the synthetic peptides as well as of the IF_1 protein was performed by electroelution from SDS-PAGE in 10 mM Tris/HCl pH 7.4, 0.01% w/v SDS, 50% v/v glycerol. This procedure eliminated any residual trace of organic solvents.

Cross-Linking

Submitochondrial particles were diluted to a protein concentration of 2 mg of protein/mL in a medium containing 200 mM sucrose, 30 mM KCl, 20 mM K-Succinate, pH 6.7 to which 1-ethyl-3-[(3-dimethylamino) propyl]-carbodiimide (EDC) was added in the presence of *N*-hydroxysulfosuccinimide (NHS) at equimolar concentrations (Belogrudov *et al*., 1995). EDC, which activates carboxyl groups for the interaction with a nucleophile, results in zero-length cross-linking. EDC was used in the presence of NHS to improve the yield of the EDC-mediated cross-linking products (Staros *et al*., 1986). The reaction was allowed to proceed for 30 min at 21°C. The cross-linked particles were spun down at 100,000 × *g* for 20 min and the pellet suspended in the medium used for the assay procedures.

Trypsin Digestion

Trypsin digestion of submitochondrial particles was performed as described in Xu *et al.* (2000). Submitochondrial particles, were diluted to 2 mg/mL in a medium containing 0.25 M sucrose, 10 mM Tris/acetate, 1 mM EDTA, 6 mM $MgCl_2$, pH 7.6, and incubated for 20 min at 25°C with the amounts of trypsin reported in the legends to the figures. The reaction was stopped by the addition of five-fold excess (w/w) trypsin inhibitor. After 5-min incubation in ice, the soluble and particulate fractions were separated by centrifugation at 100,000 × *g* for 20 min and the pellet was suspended in the medium used for the assay procedures.

Electrophoresis

SDS-PAGE was performed on slab gel with linear gradient of polyacrylamide (12–20%) as in Zanotti *et al.* (1988). SDS-denatured subunits were collected by electroelution from SDS-PAGE in 10 mM Tris/HCl, pH 7.4, 0.01% w/v SDS, 50% v/v glycerol as in Zanotti *et al.* (1987).

Immunological Procedures

Polyclonal antibodies raised against SDS-denatured subunits α β, γ, δ, F_oI-PVP(b), OSCP and F_6 were isolated from antisera collected after the 4th to the 6th booster injection, of the respective subunits, in rabbits. The IgG fraction was purified by a combination of precipitations with caprylic acid and ammonium sulfate (McKinney and Parkinson, 1987). The transfer of proteins from SDS-PAGE to nitrocellulose was performed in 125 mM Tris/HCl pH 8.6, 192 mM glycine, 20% methanol (v/v) at about 100 mA for 1 h at room temperature in a semidry apparatus. After electrotransfer, the nitrocellulose sheets were blocked with 3% bovine serum albumin in 50 mM Tris/HCl pH 7.4, 0.9% NaCl (w/v) for 1 h at 37°C. Incubation with purified IgG fractions diluted in the same buffer, was carried out overnight at 4°C.

Immunoblotting was performed with a goat anti (rabbit IgG)-alkaline-phosphatase conjugate as indicator antibody for 1h at room temperature (Hensel *et al*., 1990). After each incubation step the protein excess was removed by extensive washing with 50 mM Tris/HCl pH 7.4, 0.9% NaCl (w/v). Densitometric analysis of immunodecorated blots was performed with a Camag TLC Scanner II at 590 nm. The quantity of antigen detected was evaluated from the computed peak areas.

Amino Acid Sequence Analysis

About 200 pmol homogeneous protein bands were transferred to PVDF membranes (immobilon transfer) as in (Matsudaira, 1987). Proteins electroblotted onto PVDF membranes were stained with Coomassie blue and the bands cut. The membrane pieces were centered on a TFA-treated glass-fiber coated with polybrene and placed in the cartridge block of the sequencer. Protein were sequenced using an Applied Biosystems sequencer (model 477A) equipped with a on-line PTH analyser.

Assays

ATP Hydrolase Activity

The ATP hydrolase activity was measured with an ATP-regenerating system (Guerrieri *et al*., 1989b) in a

reaction mixture containing 200 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 10 U lactate dehydrogenase, 4 U pyruvate kinase, 1 mM phospho*enol*pyruvate, 0.1 mM NADH, 1 mM rotenone, 20 mM Tris/HCl, pH 7.4. Final volume 1 mL. The reaction was started by the addition of 1 mM ATP and followed by monitoring the oxidation of NADH at 340 nm with a Perkin–Elmer spectrophotometer.

Synthesis of ATP

The synthesis of ATP in S-SMP was measured according to Guerrieri *et al*. (1995). The ATP synthase activity of S-SMP amounted to ≈30 nmol ATP·min^{-1}·mg protein^{-1}.

Protein Determination

Protein concentration was determined according to Lowry *et al*. (1951).

RESULTS

Removal of IF_1 from the F_oF_1 ATP synthase complex in vesicles (S-SMP), obtained by passing through a sephadex column inside out-bovine heart submitochondrial particles prepared in the presence of MgATP (MgATP-SMP), resulted in a six- to eightfold enhancement of the rate of ATP hydrolysis (cf. Racker and Horstmann, 1967) (Fig. 1). The addition of the native IF_1-(1–84) protein or of the IF_1-(42–58) synthetic peptide, inhibited the ATPase activity of S-SMP back to the low activity observed in MgATP-SMP. In MgATP-SMP both peptides had no effect on the ATPase activity. Replacement of His or Lys with Ala in the IF_1-(42–58) synthetic peptide abolished its inhibitory activity. The data summarized in the Table I show that the IF_1-(42–58) synthetic peptide had the same temperature dependence, and the same, even higher inhibitory affinity as compared to the natural IF_1-(1–84) inhibitor protein. As observed for the natural IF_1-(1–84) protein, the synthetic IF_1-(42–58) segment exhibited a higher inhibitory affinity for F_oF_1 complex in S-SMP as compared to soluble F_1 (Table I) and did not exert, like the natural IF_1-(1–84) protein, any inhibitory activity on ATP synthesis in S-SMP (data not shown). Figure 2(A) shows that the IF_1-(42–58) synthetic peptide exhibited the same non-competitive inhibition kinetics as the natural IF_1-(1–84) protein. Figure 2(B) shows that treatment of MgATP-SMP with EDC, under conditions in which this reagent produces zero-length protein cross-linking, resulted in extensive inhibition of the oligomycin-sensitive ATPase activity. The same treatment of S-SMP with EDC did not cause, on the other hand, significant inhibition of the ATP hydrolase activity. Immunoblot analysis with specific antibodies showed that in Mg-ATP particles EDC produced cross-linking of IF_1 with both F_1-α and β subunits and F_o-OSCP (Fig. 3). EDC treatment resulted in a decrease of α and β subunits, OSCP and IF_1 in their original electrophoretic position, which was accompanied by their appearance in higher molecular weight cross-linking products. In particular the α and β subunits appeared together with the IF_1-(1–84) protein in a band of 62kDa. F_o-OSCP appeared together with the IF_1-(1–84) protein too, in a band of ∼32 kDa. Cross-linking of IF_1 with α and β subunits exhibited a pH dependence, in that it was evident at pH 6.7 but not at pH 7.6 (Fig. 3 compare lanes 2, 3 with lanes 4, 5). Cross-linking of IF_1 with OSCP was, on the other hand, pH independent.

Figure 4 shows results of a typical experiment in which S-SMP were reconstituted with the natural IF_1-(1–84) protein (compare lanes 1 and 3) or the synthetic

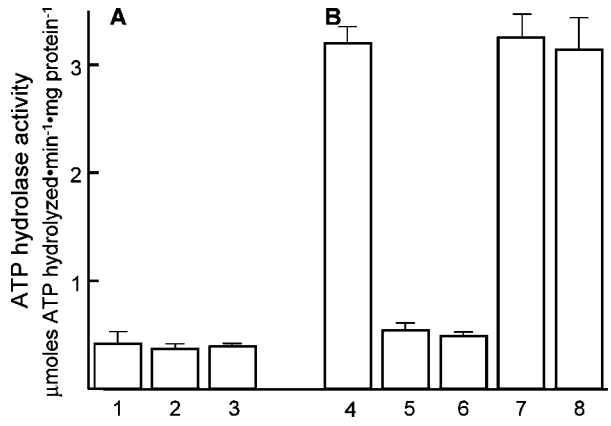

Fig. 1. Effect of isolated IF_1-(1–84) protein and IF_1-(42–58) synthetic peptide on ATP hydrolase activity in bovine heart submitochondrial particles. Preparation of MgATP-SMP and S-SMP was carried out as described under Materials and Methods section. (A) MgATP-SMP (2 mg of protein/mL) were incubated for 10 min at 21°C in a reconstitution mixture containing 200 mM Sucrose, 10 mM Tris/Acetate pH 6.7, 1 mM EDTA, 6 mM $MgCl_2$, and 1 mM Mg-ATP in the absence (Column 1) or in the presence of 1 μM isolated IF_1-(1–84) protein (Column 2) or 0.4 μM IF_1-(42–58) synthetic peptide (Column 3). (B) S-SMP (2 mg of protein/mL) were incubated as described in (A), in the absence (Column 4) or in the presence of 1μM isolated IF_1-(1–84) protein (Column 5), 0.4 μM IF_1-(42–58) synthetic peptide with the natural sequence (Column 6), 1μM IF_1-(42–58) synthetic peptide with [Ala48,49,55,56] (Column 7) or 1μM [Ala46,47,58] (Column 8) substitutions. In both experiments, at the end of the incubation the suspension was centrifuged at 100,000 × *g* for 20 min and the reconstituted particles were suspended in the same reconstitution medium. 50 μg particle proteins were then used to measure the ATP hydrolase activity as reported under Materials and Methods section. The data presented are mean ± SD of three independent experiments.

Table I. Temperature and pH Dependence of the Inhibitory Effect of Purified IF1-(1-84) Protein and IF1-(42-58) Peptide Reconstituted in S-SMP on ATP Hydrolase Activity

	ATP hydrolase activity I_{50} μH inhibitor							
	Sephadex-SMP				Soluble F_1			
	pH 6.5		pH 7.5		pH 6.5		pH 7.5	
	21°C	37°C	21°C	37°C	21°C	37°C	21°C	37°C
Control								
+IF_1-1-(1–84) protien	0.57 ± 0.05	0.036 ± 0.004	2.87 ± 0.3	2.72 ± 0.3	1.20 ± 0.2	0.84 ± 0.1	ni	ni
+IF_1-1-(42–58) peptide	0.22 ± 0.04	0.009 ± 0.001	2.24 ± 0.1	2.12 ± 0.2	0.81 ± 0.3	0.66 ± 0.1	ni	ni

Note. ni: No inhibition detected. S-SMP (2 mg of protein/mL) or soluble F_1 (0.2 mg of protein/mL) were reconstituted with isolated IF1-(1–84) protein or IF1-(42–58) synthetic peptide, analyzed for hydrolysis of ATP, following the same experimental procedure reported in the legend to Fig. 1. Each I_{50} value for the inhibition of the ATP hydrolase activity was obtained from measurements carried out at the pH and temperature reported in the table. The ATP hydrolase activities of S-SMP and Soluble F_1, were 3.18 and 77.34 (μmoles ATP hydrolyzed · min^{-1}·mg $protein^{-1}$ respectively. The data reported in the table are means of 3 experiments ± SD.

IF_1-(42–58) peptide (compare lanes 1 and 5) (panel A). Treatment with EDC, which had no effect on the immunodetected levels of α and β subunits and OSCP in S-SMP, caused, when these were reconstituted with the IF_1-(1–84) protein, a decrease in the level of both α and β subunits and OSCP, but only of α and β subunits in S-SMP reconstituted with the IF_1-(42–58) peptide. EDC cross-linking products of IF_1 and F_oF_1 subunits were isolated by elution and concentration of proteins from gel slices in the position corresponding to ∼62 and ∼32 kDa. Immunoblot analysis of this material showed that while the natural IF_1-(1-84) protein was cross-linked to both α and β subunits and OSCP, the IF_1-(42–58) peptide was cross-linked only to α and β subunits (panel B).

Figure 5 shows the effect of pH on the binding and inhibitory activity of IF_1-(1–84) protein and IF_1-(42–58) synthetic peptide in S-SMP. Preincubation of S-SMP at pH 6.7 resulted in the binding of both peptides which remained sticked to S-SMP during washing (see Fig. 4) and exerted, when S-SMP were resuspended in the reaction medium, a pH-dependent inhibition of the ATPase activity (specific inhibitory effect at pH 6.7). When S-SMP were preincubated at pH 7.6, only the IF_1-(1–84) protein appeared to bind to S-SMP and exert the pH-dependent inhibitory effect.

Identification of the IF_1 segment(s) responsible for the binding of the inhibitor to the F_oF_1 complex was obtained by exposing MgATP–SMP to limited trypsin proteolytic digestion.

Incubation of MgATP–SMP with trypsin at the concentration of 7.5 μg/mg particle protein resulted in a limited cleavage of the α and β subunits (the first 15 and 7

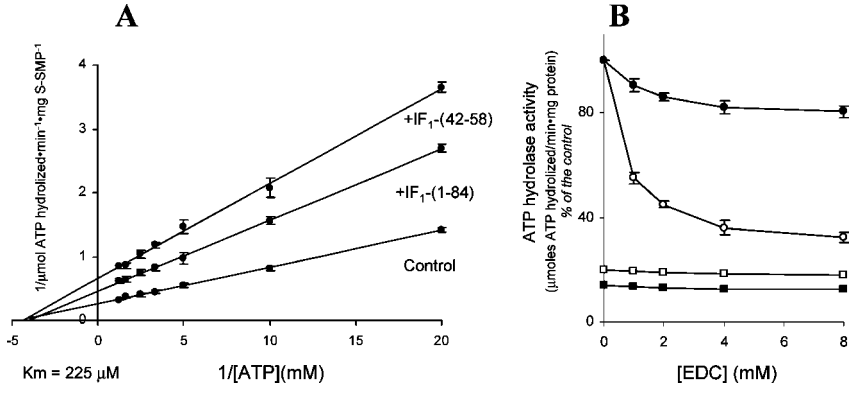

Fig. 2. Inhibition kinetics of ATP hydrolysis in S-SMP reconstituted with purified IF_1-(1–84) protein and IF_1-(42–58) synthetic peptide (Panel A) and effect of EDC on the ATP hydrolase activity of MgATP-SMP and S-SMP (Panel B). A - Isolated IF_1-(1–84) protein and IF_1-(42–58) synthetic peptide, were reconstituted with S-SMP at the concentrations of 0.4 and 0.2 μM respectively. B - MgATP-SMP or S-SMP (2 mg of protein/mL) were treated with EDC at the concentrations reported in the figure: ■ MgATP-SMP or □ S-SMP were incubated for 2 min with oligomycin (2 μg/mg particle protein) before the addition of 1 mM Mg-ATP. For experimental procedures see under Materials and Methods section and the legend to Fig. 1. The data reported are means ± SD of three independent experiments.

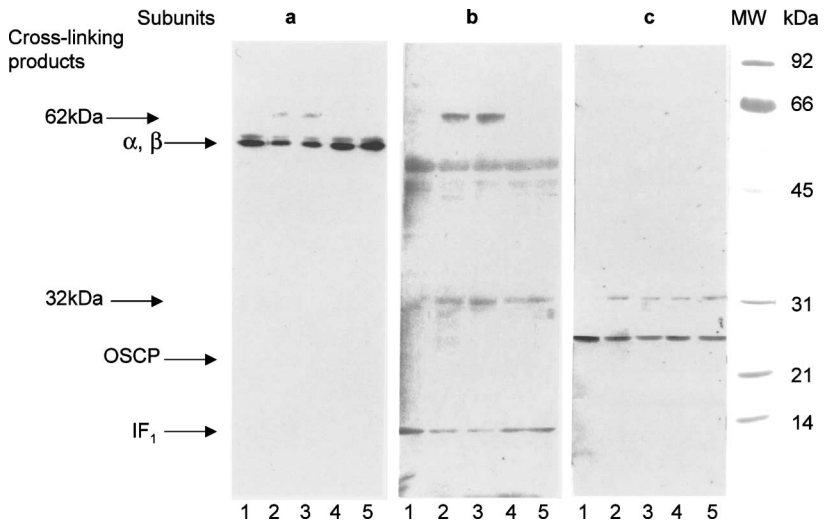

Fig. 3. Immunoblot analysis of EDC-induced cross-linking products of IF_1 with α and β subunits and OSCP in MgATP-SMP. MgATP-SMP were treated with EDC as described under Materials and Methods section. After centrifugation at 100,000 × *g* for 20 min, the pellets were suspended in the same medium of the cross-linking reaction. Fifty micrograms of submitochondrial particle proteins, solubilized by 3 min boiling in 2.3% SDS (w/v) and 10 mM Tris/HCl, pH 6.8 were subjected to SDS-PAGE on a linear 12–20% polyacrylamide gradient, electrotransferred to nitrocellulose, and immunodecorated with purified IgG fractions of rabbit antisera raised against α and β subunits (a), IF_1 (b), OSCP (c). Molecular weight standards were electrotransferred to nitrocellulose and detected with Ponceau S. For other details see under Materials and Methods section. Control MgATP-SMP (Lane 1), MgATP-SMP treated with EDC at the concentrations of 2 and 4 mM at pH 6.7 (Lane 2 and 3 respectively) and pH 7.6 (Lane 4 and 5). For other experimental details see under Materials and Methods section.

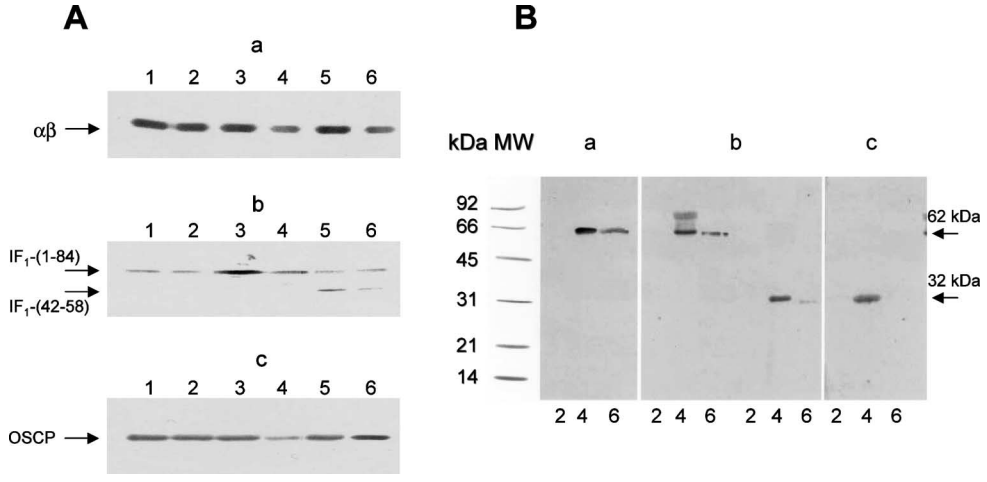

Fig. 4. Immunoblot analysis of EDC induced cross-linking products of α and β subunits and OSCP in S-SMP reconstituted with isolated IF_1-(1–84) protein and IF_1-(42–58) synthetic peptide. S-SMP (2 mg of protein/mL) were incubated in the reconstitution mixture in the presence of isolated IF_1-(1–84) protein or IF_1-(42–58) synthetic peptide at saturating concentrations of 5 and 2 μM respectively, as reported in the legend to Fig. 1. After centrifugation, pellets were suspended in the cross-linking medium, pH 6.7, in the presence of 4 mM EDC and incubated as reported under Materials and Methods section. (A) Fifty micrograms of submitochondrial particle proteins solubilized in SDS were subjected to SDS-PAGE, electrotransferred to nitrocellulose and immunodecorated with purified IgG fractions of rabbit antisera against α and β subunits (a), IF_1 (b), and OSCP (c) as reported in the legend to Fig. 4. (B) SDS-solubilized proteins (300 μg) were subjected to SDS-PAGE. The region of the gel above the α and β subunits band, containing the 62-kDa cross-linking product and the region between the α and β subunits and OSCP bands, containing the 32-kDa cross-linking product, were cut. Proteins were electroeluted in a buffer containing 50% glycerol as described in (Zanotti *et al.*, 1987). The electroeluted proteins were subjected to a second electrophoretic run followed by electrotransfer to nitrocellulose and immunodecoration with purified IgG fractions of rabbit antisera raised against α and β subunits (a), IF_1 (b) and OSCP (c). Molecular weight standards were electrotransferred to nitrocellulose and detected with Ponceau S. Lane 1 - Control S-SMP. Lane 2 - S-SMP treated with EDC. Lane 3 - S-SMP reconstituted with isolated IF_1-(1–84) protein. Lane 4 - S-SMP reconstituted with isolated IF_1-(1–84) protein treated with EDC. Lane 5 - S-SMP reconstituted with IF_1-(42–58) synthetic peptide. Lane 6 - S-SMP reconstituted with IF_1-(42–58) synthetic peptide treated with EDC.

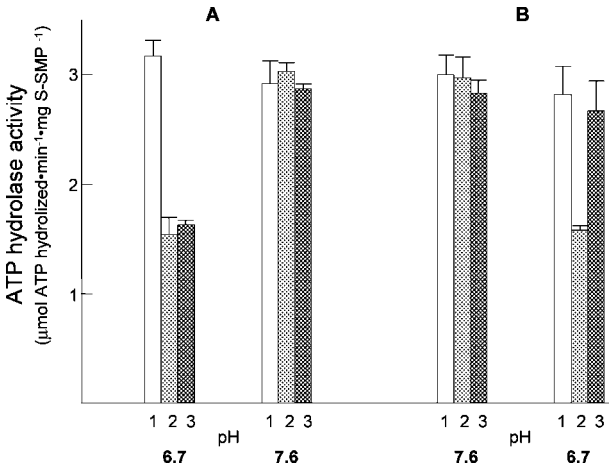

Fig. 5. Effect of pH on reconstitution of S-SMP with isolated IF_1-(1–84) protein and IF_1-(42–58) synthetic peptide. S-SMP (2 mg of protein/mL) were subjected to the reconstitution as reported in the legend to Fig. 1, at pH 6.7 (A) and 7.6 (B), with IF_1-(1–84) protein or IF_1-(42–58) synthetic peptide, at the concentrations of the respective I_{50} (see Table I). After centrifugation at 100,000 × *g* for 20 min, each pellet was subdivided in two aliquots which were suspended in the same reconstitution medium at pH 6.7 or 7.6 as reported in the figure. After 10 min incubation at the temperature of 21°C, 50-μg particle protein were used to measure the ATP hydrolase activity as reported in the legend to Fig. 1 and under Materials and Methods section. Column: 1. Control S-SMP; 2. S-SMP reconstituted with isolated IF_1-(1–84) protein; 3. S-SMP reconstituted with IF_1-(42–58) synthetic peptide. The data reported are means ± SD of three independent experiments.

N-terminal residues were removed respectively, (see ref. Walker *et al*., 1985; Xu *et al*., 1996; Xu *et al*., 1998), F_o-d and F_o-OSCP. F_1-γ, F_1-δ, F_oI-PVP(b) and F6 were, on the other hand, untouched by trypsin (Fig. 6(A)). IF_1 samples isolated from control and trypsin treated MgATP–SMP showed that the trypsin digested a substantial part of IF_1 to products with Mr ranging between 10 and 6.8 kDa. Elution of the proteolytic products and their aminoacid sequencing showed that all retained the N-terminal sequence of the native IF_1 protein (Fig. 6(B)). Thus trypsin-digested IF_1 from the carboxy-terminus. Immunoblot analysis of EDC cross-linking products showed that whilst the residual amount of α and β subunits remaining after trypsin digestion were still cross-linked by EDC to the proteolytic products of IF_1, the residual amount of OSCP was not any longer cross-linked to these IF_1 products, as instead observed for the undigested control sample (Fig. 7).

Figure 8 shows that limited proteolytic digestion of MgATP–SMP carried out with small amounts of trypsin resulted in a substantial enhancement of the ATPase activity measured at pH 7.6 (Fig. 8(B)). On the contrary no effect was exerted by trypsin treatment on the ATPase activity measured at pH 6.7 (Fig. 8(A)). Thus cleavage of the carboxyl-terminal segments, which eliminates pH-independent IF_1 binding to OSCP, left unaffected pH-dependent binding of IF_1 to the α and β subunits.

DISCUSSION

The present investigation shows that in the intact F_oF_1 complex "in situ" in the inner mitochondrial membrane the synthetic central segment of IF_1 binds to the α and β subunits of F_1 in a pH-dependent process which results in inhibition of the ATPase activity. The C-terminal region of IF_1 binds, simultaneously, to the OSCP subunit in a pH-independent process. This binding keeps IF_1 anchored to the complex, both under inhibitory conditions, at acidic pH, and noninhibitory conditions at alkaline pH.

The segment of IF_1 which binds to the α and β subunits and inhibits the ATPase activity can be identified on the basis of our results with the IF_1-(42–58) residue sequence, but it can also be more extended in particular towards the N-terminus (cf. Mimura *et al*., 1993; van Raaij *et al*., 1996). Studies with proteolytic fragments of IF_1 (Hashimoto *et al*., 1995) and genetic deletions in the recombinant protein (van Raaij *et al*., 1996) indicated that the last 10–20 residues of the C-terminus are not directly essential for the inhibitory activity. The same seems to apply to the first 13 residues of the N-terminus (van Raaij *et al*., 1996).

The IF_1-(42–58) synthetic peptide binds to the F_1-α and β subunits in the F_oF_1 complex in the membrane and inhibits the ATPase activity with the same, even higher, affinity, the same temperature dependence (Table I, see also Zanotti *et al*., 2000), the same kinetics (Fig. 2) and in a stable way (see Zanotti *et al*., 2000) as the natural IF_1-(1-84) protein (49, 22). Like the natural IF_1-(1–84) protein, the IF_1-(42–58) synthetic peptide exerts inhibitory effect on the oligomycin sensitive ATPase activity only after removal of endogenous IF_1 from the F_oF_1 complex in the inner mitochondrial membrane and has no effect on ATP synthesis.

Replacement of K46, K47, H48, and H49 in the IF_1-(42–58) peptide, results like in the case of the IF_1-(1–84) natural protein in loss of the activity. NMR analysis shows that the inhibitory activity of the IF_1-(42–58) synthetic peptide, which is likely to bind with its K46K47H48 residues to the last three residues of the conserved DELSEED sequence (Runswick and Walker, 1983) at position 398–404 in the C-terminus of the β-subunit at the bottom of F_1 facing the F_o sector (Runswick and Walker, 1983; Stock *et al*., 1999) is associated with its high conformational flexibility (de Chiara *et al*., 2002).

The natural IF_1-(1–84) protein, and the IF_1-(42–58) peptide inhibit more effectively the ATPase activity of

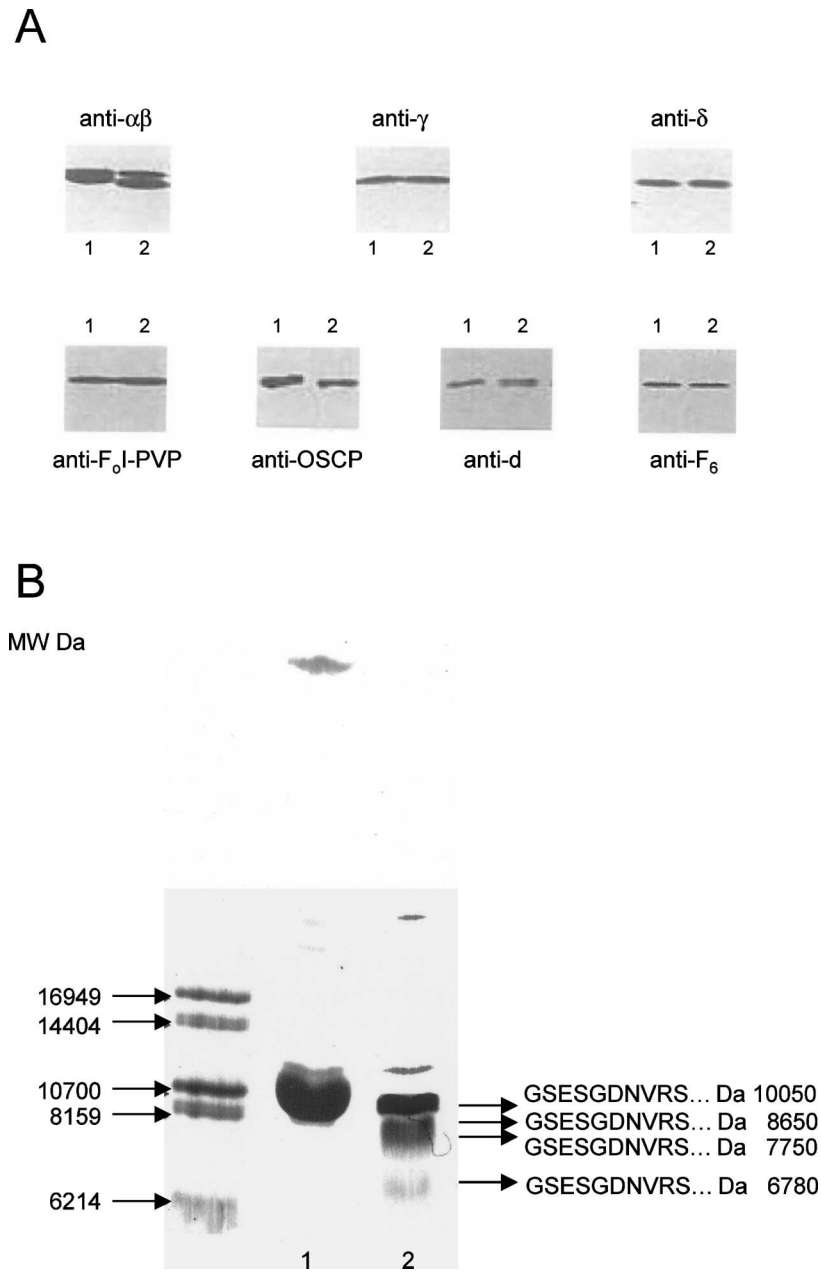

Fig. 6. Immunoblot analysis of trypsin digestion of F_oF_1 subunits (Panel A) and sequence analysis of the trypsin digestion products of IF_1 protein in MgATP-SMP (Panel B). MgATP-SMP (2 mg of protein/mL) were digested at 25°C as described under Materials and Methods section, with trypsin at the concentration of 7.5 μg/mg particle proteins for 20 min. A—25 μg of the submitochondrial particle proteins were subjected to SDS-PAGE on a linear 12–20% polyacrylamide gradient, electrotransferred to nitrocellulose, and immunodecorated with rabbit antisera against α and β, γ, δ, F_oI-PVP(b), OSCP, d, and F_6 subunits. For other details, see the legend to Fig. 3 and under Materials and Methods section. Lane 1—control particles; lane 2—trypsin-digested particles. **B -** IF_1 was isolated from control and trypsin-digested MgATP-SMP as described by Kanner *et al*. (1976). Twenty micrograms of isolated IF_1 were subjected in duplicate to a linear 12–20% gradient SDS-PAGE. One part of the gel was stained with Coomassie Blue, the other part transferred to a PVDF membrane. Each protein band was cut and sequenced as described under Materials and Methods sections. Lane 1. isolated IF_1 from MgATP-SMP. Lane 2. isolated IF_1 from trypsin-digested MgATP-SMP. The N-terminal amino acid sequence of each protein band, is presented in the figure.

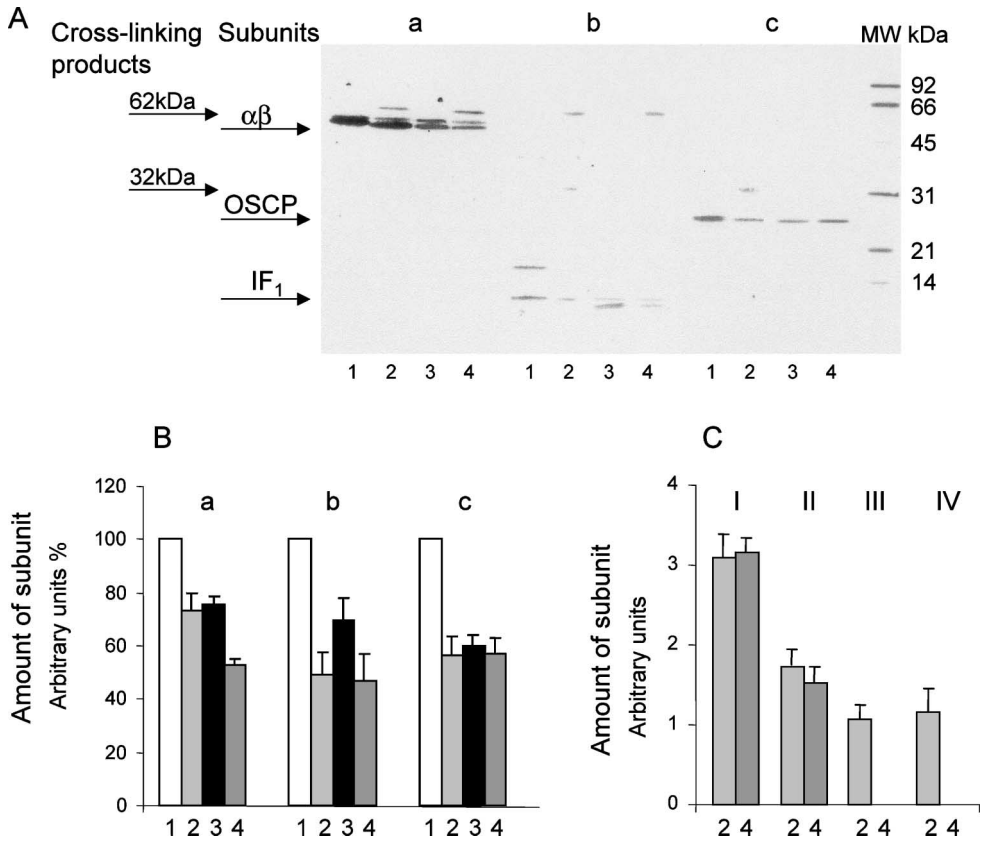

Fig. 7. Immunoblot densitometric analysis of EDC induced cross-linking between IF_1, α and β subunits and OSCP from control and trypsin-digested MgATP-SMP. MgATP-SMP (2 mg of protein/mL) were incubated in the absence or in the presence of 7.5μg trypsin per mg submitochondrial particles and/or of 4mM EDC. For the experimental procedures see legends to Figs. 3 and 6 and under Materials and Methods section. The pellets obtained after centrifugation at 100,000 $\times$ *g* for 20 min, were suspended in a medium containing 200 mM sucrose, 30 mM KCl, 20 mM K-succinate, pH 6.7. An aliquot of the suspension containing trypsin-digested MgATP-SMP, was treated with 4 mM EDC as described in the legend to Fig. 3. After 30 min, the suspension was centrifuged and the pellet was suspended in the same medium of the cross-linking reaction. A—50 μg submitochondrial particle proteins, solubilized by 3-min boiling in 2.3% SDS (w/v) and 10 mM Tris/HCl, pH 6.8 were then subjected to SDS-PAGE on a linear 12–20% polyacrylamide gradient, electrotransferred to nitrocellulose and immunodecorated with purified IgG fractions of rabbit antisera raised against α and β subunits (a), IF_1 (b), OSCP (c). Molecular weight standards were electrotransferred to nitrocellulose and detected with Ponceau S. For other details see under Materials and Methods section. B—The immunodetected subunit bands, obtained as shown in Panel A, were scanned using a VersaDoc Imaging System from BIO-RAD. The areas of the bands are the means $\pm$ SD of three different experiments. The data are expressed as percentage of control bands. C—Relative amounts of cross-linking products immunodetected with purified IgG fractions of rabbit antisera raised against α/β subunits (I) (Mr $\approx$ 62 kDa), IF_1 (II) (Mr $\approx$ 62 kDa) and (III) (Mr $\approx$ 32 kDa), OSCP (IV) (Mr $\approx$ 32kDa). The data presented are means $\pm$ SD of three different experiments. Columns: 1—MgATP-SMP; 2— EDC-treated MgATP-SMP; 3—trypsin-digested MgATP-SMP; 4— trypsin-digested MgATP-SMP treated with EDC.

the F_oF_1 complex as compared to soluble F_1. This indicates that the F_o subunit(s) contribute to shape the natural site of IF_1 binding. EDC cross-linking and limited proteolytic cleavage of IF_1 show that the C-terminal region of IF_1 binds in a pH-independent process to OSCP. Evidence has also been produced that IF_1 binds also to the F_1-γ and ε subunits (Minauro-Sanmiguel *et al.*, 2002). Simultaneous binding of IF_1 to α and β subunits and OSCP, which are components of the stator, and to F_1-γ and ε subunits, which are components of the rotor, can provide a firm interaction of IF_1 with the F_oF_1 complex. This might interfere with rotation of the central stalk thus blocking the rotary mechanism of catalysis (Minauro-Sanmiguel *et al.*, 2002). The pH-independent binding of IF_1 to OSCP might also prevent IF_1 loss from the complex at alkaline pH, set up in the matrix by respiration, conditions under which the binding at the inhibitory site in the β and α subunits is unlaced.

X-ray crystallographic analysis has shown that "in vitro" the isolated bovine IF_1 protein is at pH 6.7 a dimeric association by formation of an antiparallel α-helical coiled coil between the C-terminal regions of

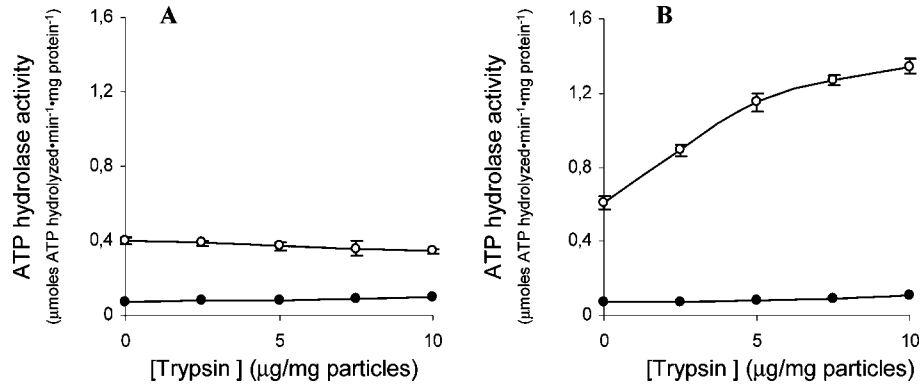

Fig. 8. Effect of pH on ATP hydrolase activity of trypsin-digested MgATP-SMP. MgATP-SMP (2 mg of protein/mL) were digested with trypsin at the concentrations reported in the figure. After 20 min, the digestion was stopped by the addition of the trypsin inhibitor. Control and trypsin-digested MgATP-SMP obtained after centrifugation, were suspended in the medium at pH 6.7 (A) or at pH 7.6 (B). After 10-min incubation, 50 μg of each sample were used to measure the ATP hydrolase activity as reported in the legend to Fig. 1 and under Materials and Methods section. Symbols: MgATP-SMP (2 mg of protein/mL) treated with trypsin at the concentrations reported in the figure; MgATP-SMP were incubated with oligomycin (2 μg/mg particle protein) for the last 2 min of the 10-min incubation before the addition of 1 mM Mg-ATP. The data presented are means $\pm$ SD of four independent experiments.

monomers (Cabezon *et al*., 2001). At alkaline pHs dimers assemble into inactive higher oligomers by forming antiparallel coiled coils in the N-terminal regions. Recently X-ray crystallographic analysis of soluble F_1-IF_1 complex has shown that the 4–40 and 4–47 N-terminal residues of two IF_1 molecules bind respectively to the α-β interface of two F_1 associated to form a dimer (Cabezon *et al*., 2003). Hence it has been concluded that the N-terminal segments of an IF_1 dimer bind to two F_1 moieties associated in a dimer and inhibit the ATPase activity. The sequence of IF_1 from residue 48 to the C-terminal residue 84 was, however, not resolved in the crystal. Results have also been presented indicating that F_1 dimers can be formed in the mitochondrial membrane, which are inactive regardless of the binding of IF_1 (Tomasetig *et al*., 2002).

We would like to propose, alternatively, that each single IF_1 molecule binds to one F_oF_1 complex, with the central segment bound at the inhibitory site at the α/β interface and the carboxy-terminal region bound to F_o-OSCP. "In vivo" the high binding affinities of these IF_1 segments to the respective docking sites in F_1 and F_o will prevent the formation of dimers and higher order oligomers which, otherwise, form spontaneously in relatively higher concentrated solutions of purified IF_1 (Cabezon *et al*., 2000; Dominguez-Ramirez *et al*., 2001).

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