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Effect of the ATPase inhibitor protein IF_1 on H^+ translocation in the mitochondrial ATP synthase complex

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ABSTRACT

The H^+ F_oF_1-ATP synthase complex of coupling membranes converts the proton-motive force into rotatory mechanical energy to drive ATP synthesis. The F_1 moiety of the complex protrudes at the inner side of the membrane, the F_o sector spans the membrane reaching the outer side. The IF_1 component of the mitochondrial complex is a basic 10 kDa protein, which inhibits the F_oF_1-ATP hydrolase activity. The mitochondrial matrix pH is the critical factor for the inhibitory binding of the central segment of IF_1 (residue 42–58) to the F_1-α/β subunits. We have analyzed the effect of native purified IF_1 the IF_1-(42–58) synthetic peptide and its mutants on proton conduction, driven by ATP hydrolysis or by $[K^+]$ gradients, in bovine heart inside-out submitochondrial particles and in liposome-reconstituted F_oF_1 complex. The results show that IF_1, and in particular its central 42–58 segment, displays different inhibitory affinity for proton conduction from the F_1 to the F_o side and in the opposite direction. Cross-linking of IF_1 to F_1-α/β subunits inhibits the ATP-driven H^+ translocation but enhances H^+ conduction in the reverse direction. These observation are discussed in terms of the rotary mechanism of the F_oF_1 complex.

Introduction

The F_oF_1-ATP synthase of coupling membranes converts the transmembrane proton-motive force into rotary mechanical energy, which in turn drives ATP synthesis from ADP and inorganic phosphate [1–3]. The mammalian mitochondrial ATP synthase, consists of 15 different subunits. Five (α_3, β_3, γ, δ, ε) constitute the F_1 catalytic moiety, which protrudes in the inner matrix space of mitochondria. Ten (ATP6 (a), F_oI-PVP (b), c_{10-14}, d, e, f, g, OSCP, F_6, A6L) belong to the F_o sector which spans the membrane from the inner to the outer side. The c and ATP6 (a) F_o subunits constitute a transmembrane proton channel [4,5]. The F_1 and F_o sector are structurally and functionally associated by a lateral stalk, the stator, and a central element, the rotor. ATP hydrolysis at the catalytic α/β sites, induces counter-clockwise rotation of the γ subunit in the central element, this drives rotation of the c subunit ring, resulting in proton translocation from the matrix to the outer space. H^+ translocation in the reverse direction, driven by transmembrane electrochemical proton gradient, induces clockwise rotation of the c-ring in F_o and γ in F_1, the successive contact of γ with the catalytic α/β sites drives ATP synthesis [6–8]. In mammalian mitochondria a 16th subunit is present, the IF_1 inhibitor protein. This is a 10 kDa basic protein which binds at the α/β catalytic sites and inhibits ATP hydrolysis. The binding of IF_1 to F_1 is pH dependent being optimal at pH's around neutrality [9,10]. When the aerobic proton motive force declines, like in ischemia or respiratory substrate deficiency, decrease of the matrix pH promotes binding of IF_1 to F_1, with prevention of ATP hydrolysis [11,12]. In conditions of normal generation of the respiratory electrochemical proton gradient, resulting in pH increase in the matrix space, IF_1 dissociates from the catalytic sites and ATP synthesis takes place normally.

Several lines of evidence show that the central segment Leu_{42}–Lys_{58} of bovine IF_1, highly conserved in mammals, binds to the α/β interface [13–15]. At the same time the C-terminal segment of IF_1 binds to the OSCP subunit in F_o in a pH independent fashion [15]. This contributes to anchor IF_1 to the F_oF_1 complex preventing its release when the proton motive force displaces the central part of the protein from the catalytic site [15].

In this paper the effect of native IF_1 and its synthetic residues 42–58 segment, on proton translocation from the F_1 side to the F_o side of the complex, and in the reverse direction, has been analyzed. The results show that in inside-out bovine heart submitochondrial particles, as well as in liposome reconstituted purified F_oF_1 complex, IF_1 and its synthetic 42–58 segment inhibit with different efficacy proton conduction from the F_1 to the F_o side and in the reverse direction. Chemical cross-linking of IF_1 to F_1 inhibits ATP driven H^+ translocation from the F_1 to the F_o side, but promotes H^+ translocation in the reverse direction. These observations

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are discussed in terms of the rotary mechanism of the F_oF_1 complex.

Materials and methods

Chemicals

9-Amino-6-chloro-2-methoxyacridine (ACMA) was from Molecular Probes; 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (NHS) were from Pierce. All other chemicals were of high purity grade.

Preparations

Bovine heart sub-mitochondrial particles (SMP), with various degrees of IF_1 were obtained according to [16]. Native IF_1-(1–84) was isolated from MgATP-SMP according to [16].

Synthesis of peptides

Bovine heart IF_1 synthetic peptides, with conserved or mutated aminoacid sequence, were obtained by standard solid-phase method with a fully automatic peptide synthesizer (model 431A, Applied Biosystems) using Fmoc chemistry. The repetitive yield of sequence analysis was higher than 90% [17].

Final purification of the synthetic peptides was performed by electro-elution from SDS–PAGE in 10 mM Tris/HCl pH 7.4, 50% glycerol (v/v), thus eliminating any trace of organic solvent.

Isolation of F_oF_1 complex

S-SMP were diluted to 2 mg protein/ml with 0.15 M K_2HPO_4, 1 mM ATP, 25 mM EDTA, 0.5 mM DTT and 5% ethylene glycol (pH 7.9). After centrifugation at 105,000*g* for 45 min, the pellet was suspended and washed twice in the same buffer. The final pellet was suspended in TA buffer (50 mM Tricine, 1 mM ATP, 25 mM EDTA, 0.5 mM DTT and 5% ethylene glycol; pH 7.9) at a protein concentration of 4 mg/ml and incubated with 1% CHAPS for 10 min at 4 °C. After 1 h centrifugation at 105,000*g* the supernatant was layered on 20% sucrose in TA buffer (4 ml supernatant on 32 ml TA-sucrose) containing 0.2% CHAPS and centrifuged for 10 h at 27,500*g* at 2 °C. After centrifugation the central 21 ml were concentrated (4–5 times) with a Centricon YM-10 filter. The yield of purified F_oF_1 sector was about 8–9 mg protein/100 mg protein of S-SMP.

Preparation of F_oF_1 vesicles

For reconstitution experiments 3 mg F_oF_1-ATP synthase complex, was mixed with 30 mg acetone-washed sonicated asolectin in 1 ml 0.1 M phosphate buffer, pH 7.2 containing 1.6% potassium cholate, 0.8% potassium deoxycholate and 0.2 mM EDTA. The mixture was dialysed overnight against 0.1 M potassium phosphate buffer, pH 7.5, followed by a 3 h dialysis against 10 mM sodium Tricine buffer pH 7.5. Both dialysis media contained 0.25 mM EDTA and 2.5 mM $MgSO_4$.

Cross-linking

MgATP-SMP were diluted to a final concentration of 2 mg/ml in a medium containing 200 mM sucrose, 30 mM KCl, 20 mM K-succinate pH 6.5. EDC, which activates carboxyl groups for the interaction with a nucleophile, and NHS were added to MgATP-SMP at equimolar concentrations [18]. Reactions were 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 was suspended in a specific medium depending on the assay procedure.

Trypsin digestion

Trypsin digestion of MgATP-SMP was performed as reported in [15].

Assays

Proton conduction. Bidirectional proton conduction was analyzed in SMP, in F_oF_1-liposomes K^+-unloaded (F_oF_1-liposomes) or K^+-loaded (F_oF_1-K^+ liposomes) following potentiometrically the H^+ flow induced by the diffusion potential imposed by valinomycin-mediated K^+-flow in a K^+-containing or K^+-deprived medium as reported in "*Preparation of F_oF_1 vesicles*" sector. To minimize the electrode response time, a low-resistance and low-capacitance glass electrode connected to a MOS FET Electrometer Amplifier, model 604 Keithley, was used. By this system pH variations can be measured in stirred suspensions with resolution of 0.01 pH unit and overall rise time (10–90% change) of less than 0.3–0.4 s. Kinetic analysis of proton translocations was performed converting the potentiometric traces into proton equivalents by double titration with standard HCl and KOH.

ATP-driven proton pumping. ATP hydrolysis-dependent proton pumping was monitored following ACMA fluorescence quenching (excitation at 410 nm, emission at 490 nm) [19]. 0.4 mg of S-SMP proteins were incubated in a medium containing 250 mM sucrose, 20 mM Tricine pH 7.4, 6 mM $MgCl_2$, 2 μg/mg protein valinomycin and 2 μM ACMA. Reaction was started by the addition of ATP at the indicated concentrations.

ATP hydrolase activity. ATPase activity was measured by an ATP-regenerating system as described in [16].

ATP synthase activity. S-SMP were incubated in a medium containing 200 mM sucrose, 10 mM K-succinate, 3 mM $MgCl_2$, 1 mM K–EDTA, 10 mM K-Pi at the indicated pH, 20 mM glucose, 5 U hexokinase and 300 μM Ap5A. MgADP (300 μM) was then added and the respiratory rate in state 3 was allowed for 5 min. S-SMP suspensions were treated with equal volume of 28% perchloric acid (v/v). After centrifugation at 20,000*g*, the supernatant was cooled in ice, neutralized with 60% KOH (w/v) and clarified by centrifugation. The neutralized suspension was added to a mixture containing 1 mM $MgCl_2$, 150 mM Tris/HCl (pH 7.4) and 7 U glucose-6-phosphate dehydrogenase. NADP (1 mM) was added and glucose-6-phosphate amount was determined following NADPH formation (wavelength 340 nm, $\varepsilon = 6.22\ mM^{-1}$).

Protein determination

Protein concentration was determined according to Lowry [20] using BSA as standard.

Statistical analysis

Statistical analysis was performed by Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

Results

The impact of endogenous IF_1 on proton translocation in the F_oF_1 complex was examined in three different preparations of bovine heart submitochondrial particle: MgATP particles, which retain high amount IF_1, EDTA particles with low residual content of IF_1 and S-SMP particles devoid of IF_1 by treatment with Sephadex™-G50 [16]. The results presented in Fig. 1 show that the pro-

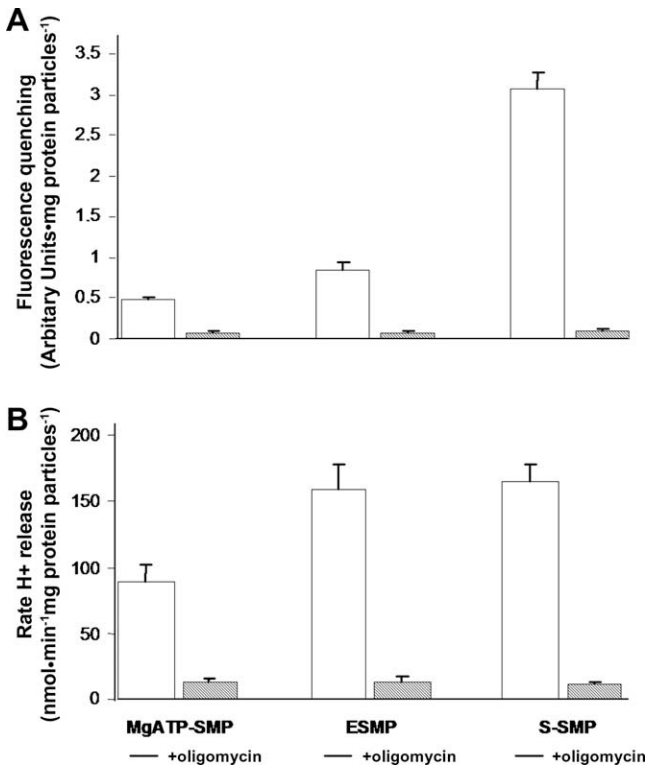

Fig. 1. H^+ conduction in SMP with different content of IF_1 protein. (A) Measurement of proton conduction driven by ATP hydrolysis was monitored by ACMA fluorescence quenching. SMP with different content of IF_1 (1 mg protein/ml) were incubated for 10 min at 21 °C in a medium containing 200 mM sucrose, 10 mM Tris/HCl pH 6.7, 1 mM K–EDTA, 6 mM $MgCl_2$. An aliquot of the suspension containing 0.4 mg of SMP was added to 2 ml of the reaction mixture. Reaction was started by the addition of 0.2 mM ATP. Data were expressed as Arbitrary Units of fluorescence quenching. (B) SMP with different content of IF_1 (0.4 mg protein/ml) were incubated for 2 min at 21 °C in a medium containing 0.15 M KCl, 1 mM Tris/HCl, pH 6.7. Measurement of passive H^+ release, induced by K^+ uptake, was initiated by the addition of valinomycin (2 μg/mg particle protein) and followed potentiometrically. Where indicated, oligomycin (1.5 μg/mg protein) was added to the respective incubation medium. For other experimental details, see under Materials and methods. Data are means ± SD of three independent experiments.

gressive removal of IF_1 from submitochondrial particles, resulted in a parallel increase of the oligomycin-sensitive ATP driven proton uptake by the particles, i.e. proton translocation from the F_1 side, exposed to the external space in the inside-out submitochondrial particles, to the F_o-side now reaching the inner space (Fig. 1A). Removal of IF_1 resulted in smaller enhancement of proton release from the particles, i.e. proton translocation from the inner F_o side to the outer F_1 side, driven by valinomycin-induced K^+ uptake by the submitochondrial particles (Fig. 1B).

The results summarized in Table 1 show that the addition to S-SMP, deprived of endogenous IF_1 [16], of the entire purified IF_1 protein or its synthetic 42–58 segment [15] inhibited, in a pH dependent fashion (the inhibition exhibited at pH 6.5 practically vanished at pH 8.0) ATP hydrolysis and H^+ uptake (H^+ flow from the F_1 to the F_o side). Both the entire IF_1 and the IF_1-(42–58) segment, were less effective in inhibiting H^+ release from submitochondrial particles driven by K^+ gradient (H^+ flow from the F_o to the F_1 side) and did not exert any inhibitory activity on ATP synthesis driven by succinate supported respiration. The inhibitory activity of the IF_1-(42–58) segment on ATP hydrolysis and H^+ uptake by the particles was practically abolished by replacement of histidine residues 48, 49, 55, 56 or lysine residues 46, 47, 58 with alanines.

Lysine replacement with alanines did not abolish the inhibitory activity of the synthetic peptide on H^+ release from S-SMP.

The effect of purified IF_1 native protein and its 42–58 synthetic segment on proton translocation was then tested in liposome reconstituted, purified F_oF_1 complex. Studies by electron microscopy and Fourier image processing on two-dimensional crystals of the H^+-ATP synthase complex from bovine heart mitochondria [21] and co-reconstitution in liposomes of the isolated bovine complex with bacteriorhodopsin show unidirectional F_oF_1 incorporation into the lipid bilayer [22], with the hydrophobic moiety (F_o) spanning the membrane and the relatively hydrophilic F_1 moiety, protruding in the outer medium [23]. Due to the proteoliposome impermeability, added IF_1 and its synthetic segment can exert their inhibitory effect only on the F_oF_1 complex incorporated with the F_1 moiety and the IF_1 docking site exposed at the outer liposomal surface, i.e. the same orientation of the inside-out submitochondrial particles. Oligomycin sensitive H^+ uptake from the outer F_1 to the inner F_o side, and H^+ release in the reverse direction were driven by valinomycin-induced K^+ release and K^+ uptake, respectively. The results presented in Figs. 2 and 3 show that both H^+ uptake and H^+ release, were inhibited by IF_1 as well as by the 42–58 IF_1 synthetic segment. It can, however, be noted that in the case of H^+ uptake the inhibitory affinity of the IF_1-(42–58) segment (I_{50} = 0.1 μM) was higher that of the entire IF_1 (I_{50} = 0.47 μM) and even 20 times higher that its inhibitory affinity for inhibition of H^+ release (I_{50} = 1.97 μM). In the case of H^+ release the I_{50} of the IF_1-(42–58) segment was, in fact, higher that the I_{50} of the entire IF_1. Replacement in the synthetic IF_1-(42–58) segment of histidine residues 48, 49, 55, 56 or of lysine residues 46, 47, 58 with alanines abolished completely its inhibitory activity on H^+ uptake (Fig. 2B). H^+ release by the proteoliposomes was, however, still inhibited, as already observed in S-SMP (Table 1), when lysine residues 46, 47, 58 were replaced by alanine (Fig. 3B).

Table 1
pH dependent effect of isolated or synthetic IF_i in S-SMP.

pH	Rate H^+ uptake Fluorescence quenching arbitrary units mg^{-1}		ATP hydrolase μmol ATP hydrolyzed min^{-1} mg S-SMP^{-1}		Rate H^+ release Passive H^+ conduction nmol min^{-1} mg^{-1}		ATP synthesis activity nmol ATP min^{-1} mg S-SMP^{-1}	
	6.5	8.0	6.5	8.0	6.5	8.0	6.5	8.0
Control	3.25	2.86	3.25	3.05	118.4	95.9	22.0	21.8
IF1-(1–84) (0.5 μM)	1.22	2.80	1.15	2.90	90.9	71.4	21.5	21.0
IF1-(42–58) (0.5 μM)	0.47	2.63	0.17	2.95	68.7	51.8	21.2	20.4
[Ala 48,49,55,56] IF1-(42–58) (1.0 μM)	2.83	2.37	3.05	2.87	100.6	84.4	18.8	20.0
[Ala 46,47,58] IF1-(42–58) (1.0 μM)	2.89	2.43	2.93	2.80	43.8	34.5	22.4	21.8

S-SMP were reconstituted, at the indicated pH, with isolated IF_i-(1–84) or with IF_i-(42–58) synthetic peptides, at the indicated concentrations. For measurement of proton conduction driven by ATP hydrolysis or initiated by valinomycin-induced K^+ uptake, see legend to Materials and methods.
ATP hydrolysis—Aliquot containing 50 μg of S-SMP protein was added to 1 ml of the ATPase assay mixture (200 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 10 U lactate dehydrogenase, 20 mM Tris/HCl, 0.1 mM NADH, 1 mM PEP, 4 U pyruvate kinase and 1 mM Mg-ATP) and followed by monitoring the oxidation of NADH (wavelength 340 nm, $\varepsilon = 6.22\ mM^{-1}$). *ATP synthesis*—In S-SMP (1 mg protein/ml) was measured as described under Materials and methods. Data are means of three independent experiments ± SD.

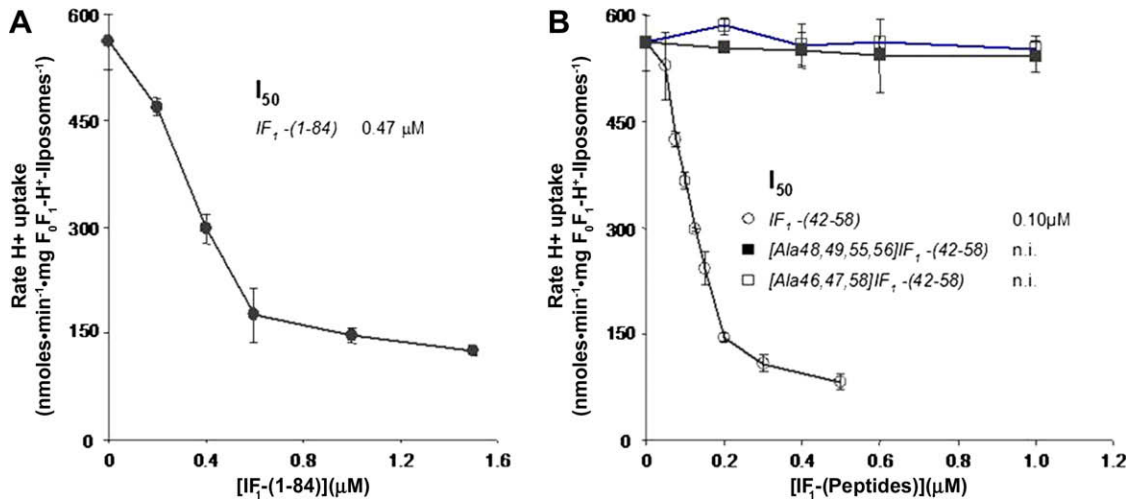

Fig. 2. On H^+ conduction induced by valinomycin-mediated K^+ release in F_oF_1-K^+ liposomes. Effect of isolated IF_1-(1–84) or synthetic IF_1-(42–58) peptides. Isolated native IF_1-(1–84) protein or synthetic IF_1-(42–58) with conserved and chemical mutated sequence, were incubated for 10 min at 21 °C with IF_1-depleted F_oF_1 complex in a medium containing: 200 mM sucrose, 10 mM Tris/acetate pH 6.5, 1 mM K–EDTA and 6 mM $MgCl_2$. Reconstitution was stopped by centrifugation at 100,000*g* for 20 min and the resulting pellet was suspended in the assay medium. (A) F_oF_1-K^+ liposomes reconstituted with native IF_1-(1–84) protein. (B) F_oF_1-K^+ liposomes reconstituted with synthetic IF_1-(42–58) peptides with conserved and chemical mutated sequence. F_oF_1-K^+ liposomes (0.4 mg protein/ml) reconstituted with native IF_1-(1–84) (●), IF_1-(42–58) (○), [Ala46,47,58]-IF_1-(42–58) (□) or [Ala48,49,55,56]-IF_1-(42–58) (■) at the indicated concentrations, were suspended in a medium deprived of K^+ and containing 200 mM sucrose and 1 mM Tris/HCl, pH 6.7. After 2 min, valinomycin (2 µg/mg protein) was added and the H^+ uptake promoted by the K^+ release was determined potentiometrically from the initial rate of pH changes upon addition of valinomycin. For other experimental details, see under Materials and methods. Data are means ± SD of three independent experiments. Oligomycin inhibited by more than 80% H^+ uptake (data not shown).

Treatment of Mg-ATP submitochondrial particles with the cross-linker EDC, resulted in marked depression of H^+ uptake by the particles but in equally effective stimulation of H^+ release (Fig. 4). Controlled treatment of Mg-ATP submitochondrial particles with trypsin, under conditions in which it resulted in limited proteolytic cleavage of the carboxyl-terminus of IF_1 [15], had no effect on H^+ uptake and release from the particles neither it affected the EDC induced inhibition of H^+ uptake and stimulation of H^+ release from the particles (Fig. 4).

Discussion

Previous results on EDC cross-linking and limited proteolysis of the F_oF_1 ATP synthase complex in submitochondrial particles have provided evidence showing that in the complex "in situ" in the inner membrane of bovine heart mitochondria, the central segment of IF_1 (residues Leucine42–Lysine58) binds to the α/β subunits of the catalytic sector F_1 in a pH dependent process and this binding results in inhibition of the ATP hydrolase activity [15]. The C-termi-

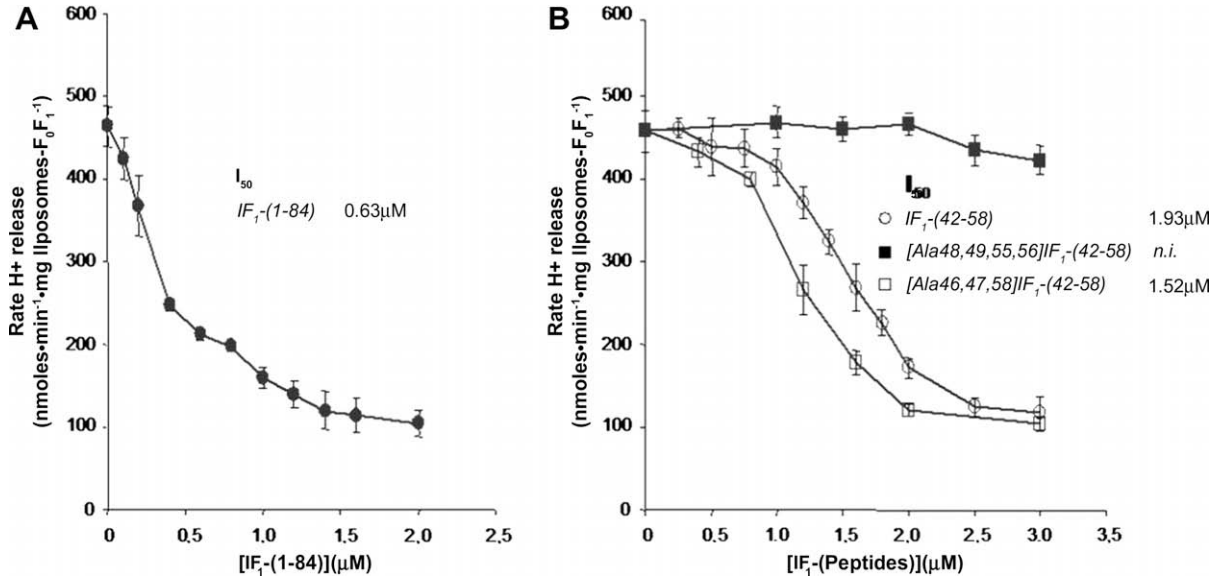

Fig. 3. On H^+ conduction induced by valinomycin-mediated K^+ uptake in F_oF_1 liposomes. Effect of isolated IF_1-(1–84) or synthetic IF_1-(42–58) peptides. (A) F_oF_1 liposomes reconstituted with native IF_1-(1–84) protein. (B) F_oF_1-K^+ liposomes reconstituted with synthetic IF_1-(42–58) peptides with conserved and chemical mutated sequence. F_oF_1 liposomes (0.4 mg of protein/ml) reconstituted with native IF_1-(1–84) (●), IF_1-(42–58) (○), [Ala46,47,58]-IF_1-(42–58) (□) or [Ala48,49,55,56]-IF_1-(42–58) (■) at the indicated concentrations, were suspended in a medium containing: 150 mM KCl and 1 mM Tris/HCl, pH 6.7. After 2 min, valinomycin (2 µg/mg protein) was added and the H^+ uptake promoted by the K^+ release was determined potentiometrically from the initial rate of pH changes upon addition of valinomycin. For other experimental details, see under Materials and methods. Data are means ± SD of five independent experiments. Oligomycin inhibited by more than 80% H^+ release (data not shown).

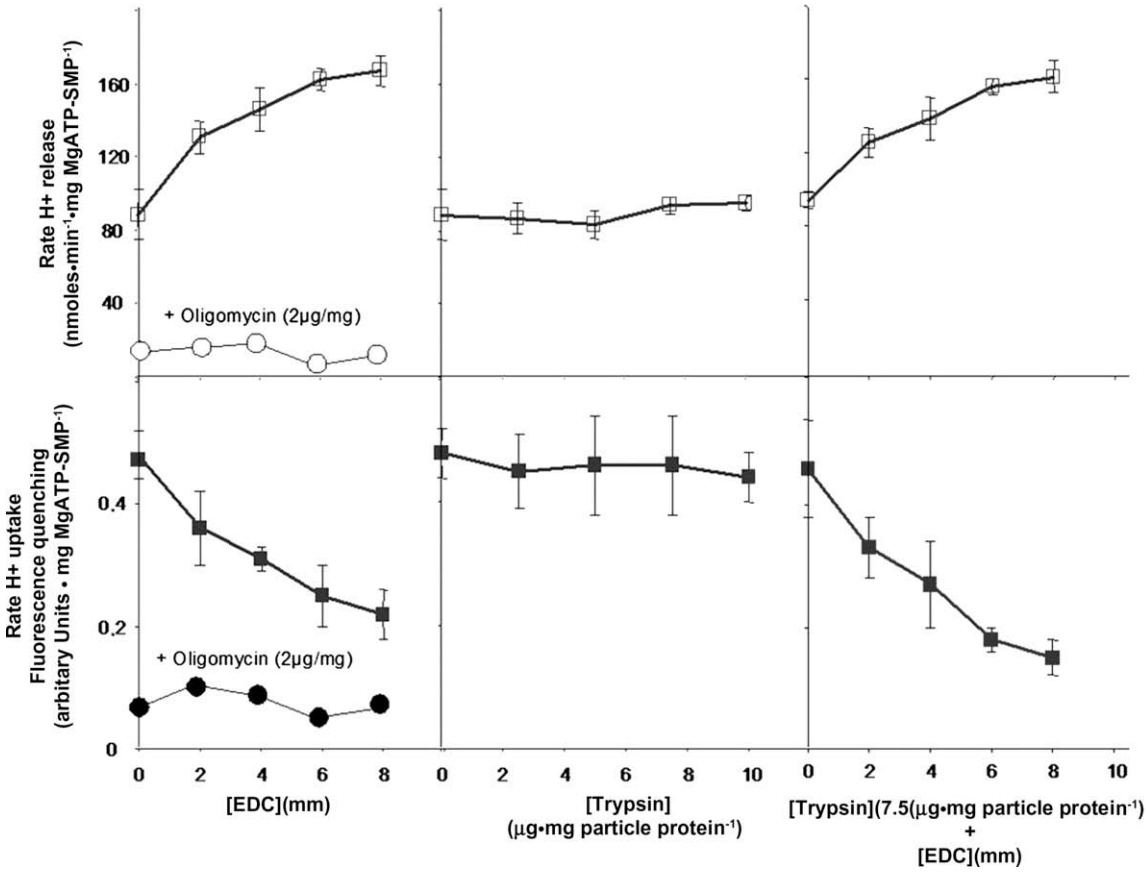

Fig. 4. Effect of the EDC cross-linker on the H^+ conduction in MgATP-SMP and trypsin-digested MgATP-SMP. Were indicated MgATP-SMP (2 mg/ml) were treated at 25 °C for 20 min with increasing trypsin concentrations as reported in [23]. Control or trypsin-digested (7.5 µg trypsin/mg protein) MgATP-SMP, were treated with EDC, as reported under Materials and methods, at the indicated concentrations. After centrifugation at 100,000*g* for 20 min, pellets were suspended in the same medium used for cross-linking reaction. H^+ uptake and release was measured as described in the legends of Figs. 2 and 3, respectively. For other details see under Materials and methods. Data are means ± SD of five independent experiments.

nal region of IF_1 was shown to bind also to the OSCP subunit of the F_o moiety in a pH independent process [15]. The present results show that IF_1 exerts a powerful inhibitory activity on H^+ traslocation in the ATP synthase complex in the native inner mitochondrial membrane or in the purified liposome reconstituted state, in the direction from the F_1 to the F_o side, either when driven by ATP hydrolysis or by a transmembrane proton gradient. IF_1 inhibits also H^+ translocation in the reverse direction, i.e. from the F_o to the F_1 side, but in this case it is less effective. The central IF_1-42–58 segment is directly responsible for the IF_1 inhibitory activity exerted on H^+ translocation [10,14,15]. This synthetic peptide exhibits a higher inhibitory affinity on H^+ translocation from the F_1 to F_o side than the entire IF_1 protein. The segment presents, however, a much lower inhibitory affinity for the inhibition of H^+ translocation from the F_o to F_1 side. Conserved histidines 48, 49, 55, 56 and lysines 46, 47, 58 are essential for the inhibitory effect of the 42–58 domain of IF_1 on H^+ translocation from the F_1 to the F_o side, as shown by the complete loss of the activity when these residues were replaced by alanines. Residue replacement showed however that the histidines but not the lysines were essential for inhibition of H^+ translocation from the F_o to the F_1 side. These observations provide evidence showing that the docking site for the IF_1-42–58 inhibitory segment in the F1-α/β subunits, likely in the region of the conserved 394–404 DELSEED sequence in the C-terminus of the β subunit, exists in different conformations in the ATP-driven H^+ translocation from the F_1 to F_o side and in the reverse H^+ translocation from the F_o to F_1 side. Even more striking is the finding that EDC treatment of MgATP particles results in inhibition of ATP driven H^+ translocation from the F_1 to F_o side, but in stimulation of reverse H^+ translocation from the F_o to F_1 side. Previous observations have shown that EDC produces cross-linking of the C-terminal IF_1 segment to OSCP and of the central inhibitory IF_1 segment to F_1-α/β subunits [15]. The opposite effects of EDC on H^+ translocations in the two directions were still present when the C-terminal region of IF_1 was cleaved off by limited tryptic digestion [24]. It is thus evident that cross-linking of the inhibitory region of IF_1 with F_1 α/β subunits is responsible for EDC inhibition of ATP-driven H^+ translocation from the F_1 to F_o side and stimulation of H^+ translocation, driven by the K^+ gradient, from the F_o to F_1 side, i.e. conditions similar to those of ATP synthesis. These observations are consistent with the rotary mechanism of the F_oF_1 complex. Cross-linking of the IF_1-(42–58) inhibitory domain to the α/β subunits blocks the hydrolytic catalysis and the resulting rotation of the central γ/c element with suppression of H^+ translocation from the F_1 to F_o side. On the other hand, when the inhibitory domain of IF_1 is cross-linked to the α/β subunits, H^+ translocation driven by the membrane potential from the F_o to F_1 side, might still induce rotation of the central γ/c element but in this case the H^+ flow can take place uncoupled from the catalytic step(s) at the F_1-α/β subunits site(s), blocked by the inhibitor protein.

Acknowledgments

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