Disulfide cross-linking of subunits F_1-γ and F_0I-PVP(b) results in asymmetric effects on proton translocation in the mitochondrial ATP synthase.

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Abstract A study is presented on the effect of diamide-induced disulfide cross-linking of F_1-γ and F_0I-PVP(b) subunits on proton translocation in the mitochondrial ATP synthase. The results show that, upon cross-linking of these subunits, whilst proton translocation from the A side to the B F_1 side is markedly accelerated with decoupling of oxidative phosphorylation, proton translocation in the reverse direction, driven by either ATP hydrolysis or a diffusion potential, is unaffected. These observations reveal further peculiarities of the mechanism of energy transfer in the ATP synthase of coupling membranes.

Key words: H^+-ATP synthase; F_0; F_1; F_0I-PVP(b) subunit; F_1-γ subunit; H^+ translocation

1. Introduction

The X-ray crystallographic analysis of the F_1 moiety of the mitochondrial F_0F_1-ATP synthase [1], chemical [2,3] and optical observations [4,5] have provided evidence supporting the binding-change rotary mechanism in the ATP synthase of coupling membranes [6]. This mechanism envisages ATP synthesis as the result of ATP displacement from the three catalytic sites in F_1, successively, when they are hit by the γ subunit, whose N- and C-termini, forming a bent coiled-coil, are made to rotate in the central cavity of the F_1-α3β3 hexamer by downhill flux of protons through the F_0 membrane sector from the electropositive acid side (A) to the electronegative basic side (B, F_1 side) [6–8]. Conversely, ATP hydrolysis by inducing rotation of the γ subunit generates uphill proton translocation through F_0 from the B to the A side.

The stalk sector of ATP synthase, which connects F_1 to F_0 and couples catalysis in F_1 with proton translocation through F_0, is made up of the F_1 subunits γ, δ, ε and the F_0-b subunit [9], plus OSCP, F6, A6L and subunit d in the mitochondrial enzyme [10–12].

Contrary to a previous general view based on electron microscopy [13–16], according to which the stalk subunits are assembled in a single structure connecting the centers of F_1 and F_0, recent cross-linking results [17,18] and average analysis of electron microscopy images [19–21] indicate that, in addition to a central stalk, constituted by rotating γ and ε [3] in *Escherichia coli*, γ and δ in the mitochondrial complex, there is a second lateral stalk made up by F_0-b and δ subunit in *E. coli* [19], F_0I-PVP(b) and OSCP in mitochondria [20]. This lateral stalk would represent the stator of the rotary motor, holding the F_1 α3β3 hexamer attached to the F_0-a subunit [3,20].

We have recently found that in the mitochondrial F_0F_1 complex, in the absence of transmembrane $\Delta\tilde{\mu}H^+$, diamide induces disulfide cross-linking of Cys-197 in the C-terminal region of F_0I-PVP (b) with Cys-91 at the C-terminus of a short α-helix (residues 73–90) in the portion of the γ subunit protruding out of the α3β3 hexamer of F_1 and extending in the stalk [12,22]. This shows that, at least under resting conditions, these two protein domains are in direct contact. Cross-linking of F_0I-PVP(b) and γ subunits resulted in a marked enhancement of passive dissipation of the respiratory $\Delta\tilde{\mu}H^+$ with decoupling of oxidative phosphorylation [22]. Interestingly enough the respiratory $\Delta\tilde{\mu}H^+$ prevented the diamide-induced cross-linking of F_0I-PVP(b) and γ [22].

In this paper we have extended these studies to the effect of disulfide cross-linking of F_0I-PVP(b) and γ in the mitochondrial ATP synthase on the ATP-driven uphill proton translocation from the B F_1 side space to the A side. The results show that, unlike what was found for proton translocation from the A to the B side, cross-linking of the two subunits had no effect on the ATPase activity and proton translocation from the B to the A side, when the latter was driven by either ATP hydrolysis or a diffusion membrane potential.

2. Materials and methods

2.1. Materials

Chemicals were purchased from the following companies: valinomycin, oligomycin, carbonylcyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), diamide and neutral red from Sigma Chemical Co.; ATP, phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase and NADH from Boehringer; 9-amino-6-chloro-2-methoxyacridine (ACMA) from Molecular Probes; acrylamide, *N*,*N*′-methylenebisacrylamide, sodium dodecyl sulfate (SDS), goat anti-(rabbit IgG) alkaline phosphatase conjugate, and AP color development reagent

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Abbreviations: FCCP, carbonylcyanide *p*-(trifluoromethoxy)phenylhydrazone; ESMP, submitochondrial particles prepared in the presence of EDTA; F_0, membrane integral sector of H^+-ATP synthase; F_1, catalytic sector of bovine heart H^+-ATP synthase; F_1-γ, protein subunit of mitochondrial F_1; F_0I-PVP(b), protein subunit of mitochondrial F_0; OSCP, oligomycin sensitivity-conferring protein; ACMA, 9-amino-6-chloro-2-methoxyacridine; Neutral red, 3-amino-7-dimethylamino-2-methylphenazine hydrochloride

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(5-bromo-4-chloro-3-indolyl phosphate, nitroblue tetrazolium) from Bio-Rad; nitrocellulose membrane (0.45 μm pore size) from Schleicher and Schüll, Biotech, Ltd. All other chemicals were of high-purity grade.

2.2. Preparations

Beef heart mitochondria and inside-out submitochondrial particles (ESMP) were prepared as described in [23,24]; K^+-ESMP were obtained with the same procedure as ESMP except that mitochondria were exposed to ultrasonic energy in the presence of 150 mM KCl.

2.3. Assays

Proton translocation was analyzed following potentiometrically, in ESMP, proton release induced by a diffusion potential (positive inside) imposed by valinomycin-mediated K^+ influx as described in [25] and, in K^+-ESMP, proton uptake induced by a diffusion potential imposed by valinomycin-mediated K^+ efflux in a medium lacking K^+. For the kinetic analysis of the proton translocation the potentiometric traces were converted into proton equivalents by double titration with standard HCl and KOH.

ATP-driven proton pumping was monitored following ACMA fluorescence quenching (excitation at 410 nm, emission at 490 nm) [26] or spectrophotometrically following neutral red pH indicator absorbance changes (530–590) [27]. The assay medium for fluorescence measurements contained 250 mM sucrose, 20 mM Tricine pH 7.4, 6 mM $MgCl_2$, 2 μg/mg valinomycin, 0.4 mg particles and 2 μM ACMA. The same medium, except that Tricine was replaced by 100 mM HEPES pH 7.4 and ACMA by 100 μM neutral red, was used for spectrophotometric analysis. In both cases the reaction was started by the addition of ATP.

The ATP hydrolase activity was measured with an ATP-regenerating system as described in [28].

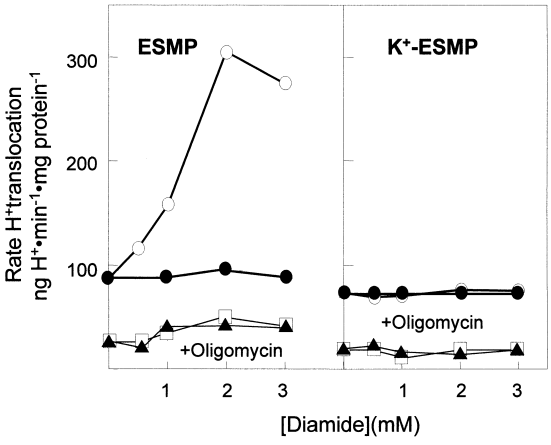

Fig. 1. Differential effects of diamide treatment on proton conduction in ESMP and K^+-ESMP. ESMP (3 mg/ml) were incubated in a medium containing: 200 mM sucrose, 30 mM KCl and 20 mM succinate (potassium salt) pH 7.4 in the presence or absence of a constant stream of N_2. K^+-ESMP (3 mg/ml) were incubated under the same conditions and in the same mixture with omission of KCl. Once anaerobiosis was reached or in the succinate-respiratory state, diamide was added at the concentrations indicated and after 2 min the reaction was stopped by dilution with the same medium and centrifugation at $105\,000 \times g$. ESMP and K^+-ESMP pellets were resuspended in the same medium. ESMP (1 mg/ml) treated with diamide in anaerobiosis (○) or in the succinate-respiratory state (●) were incubated for 2 min in 150 mM KCl. K^+-ESMP (1 mg/ml) treated with diamide in anaerobiosis (○) or in the succinate-respiratory state (●) were incubated for 2 min in 200 mM sucrose. H^+ release in ESMP and H^+ uptake in K^+-ESMP were initiated by the addition of 2 μg of valinomycin/mg particle protein. ESMP or K^+-ESMP treated with diamide in anaerobiosis (□) or succinate-respiratory state (▲) were incubated, in 150 mM KCl or 200 mM sucrose respectively, for 2 min with oligomycin 2 μg/mg particle protein before adding valinomycin. For other details see Section 2.

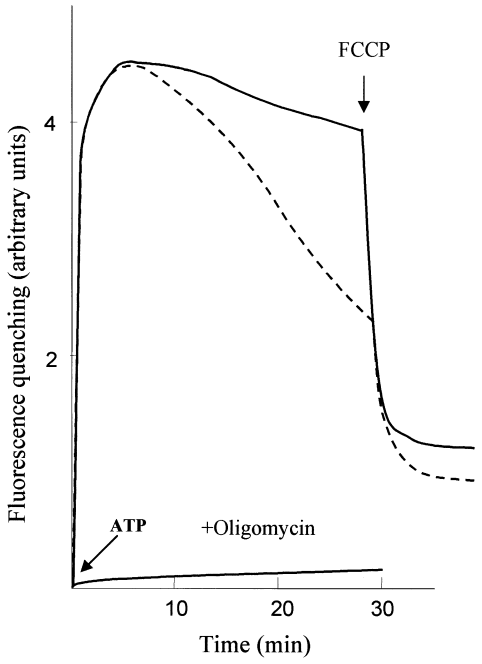

Fig. 2. Effect of diamide on the time course of ATP-driven ACMA fluorescence quenching in ESMP. The measurement of proton translocation driven by ATP hydrolysis was monitored by ACMA fluorescence quenching as described in Section 2. ESMP were preincubated for 2 min under a constant stream of N_2 in the medium containing KCl and succinate as described in the legend to Fig. 1 in the absence (continuous trace) or presence of 2 mM diamide (dashed trace). 10 μl of the suspension of preincubated ESMP (400 μg proteins) was added to 2 ml of the reaction mixture, supplemented with valinomycin, described in Section 2. The reaction was started by the addition of 0.2 mM ATP. Where indicated, oligomycin (2 μg/mg protein ESMP) and FCCP 2 μM were added. For other details see Section 2.

2.4. Electrophoretic analysis

Electrophoresis in 12–20% gradient SDS-PAGE was performed as in [29].

2.5. Immunochemical procedures

Polyclonal antibodies raised against SDS-denatured subunits γ and F_0I-PVP(b) were isolated from rabbit antisera and the IgG fraction was purified with caprylic acid and ammonium sulfate [22].

The SDS gel slabs were electrotransferred to nitrocellulose sheets and immunoblotting was performed with a goat anti-(rabbit IgG) alkaline phosphatase conjugate as indicator antibody [22].

2.6. Protein determination

Protein concentration was determined according to Lowry et al. [30].

3. Results

We have previously shown that, in inside-out inner membrane vesicles of bovine heart mitochondria (ESMP), in the anaerobic non-respiring state, or in the isolated F_1 and F_0, diamide-induced disulfide cross-linking of F_1-γ and F_0I-PVP(b) subunit results in promotion of proton conduction through F_0 from the A (inner space of inside-out ESMP) to the B space (outer, F_1 side in ESMP) [22].

The experiments summarized in Fig. 1 show that anaerobic diamide treatment of ESMP results in marked enhancement of the oligomycin-sensitive proton translocation from the A to

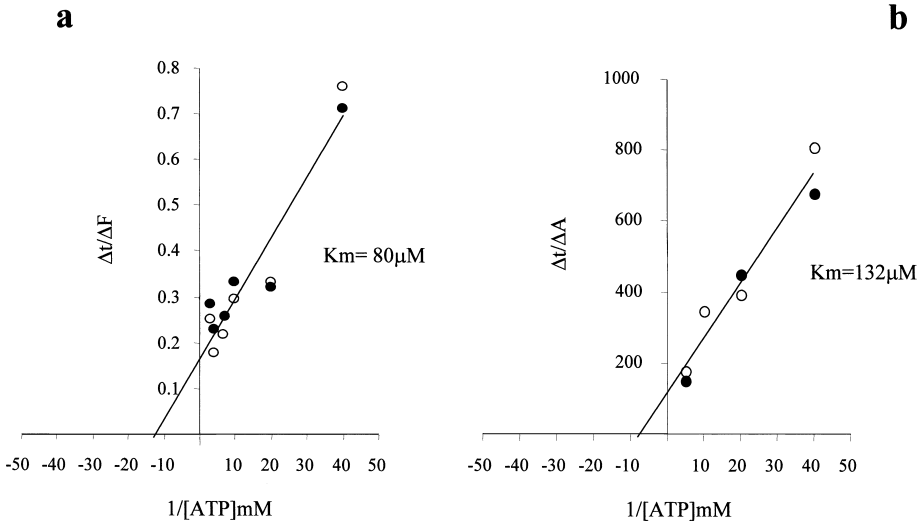

Fig. 3. Lineweaver–Burk plots of ATP-driven proton translocation in ESMP. For anaerobic treatment of ESMP with 2 mM diamide, see the legend to Fig. 1. H^+ translocation driven by ATP hydrolysis at the given concentrations of ATP was monitored by ACMA fluorescence (panel a) and neutral red absorbance (panel b) under the conditions described in the legend to Fig. 2 and in Section 2. Symbols: (●) ESMP, 0.2 mg/ml; (○) diamide-treated ESMP, 0.2 mg/ml. The best fitting line was obtained by the method of least squares. For other details see Section 2.

the B space induced by a positive membrane potential inside the vesicles, generated by valinomycin-mediated K^+ diffusion from the B to the A space. It can be noted that incubation of ESMP with diamide, in the respiratory state, conditions under which no cross-linking of F_1-γ and F_0I-PVP(b) occurs [22], has no effect on proton conduction. Oligomycin-sensitive proton translocation through F_0 in the opposite direction from the B to the inner A space, induced by a K^+ diffusion potential of the opposite sign, is, in contrast, unaffected by diamide treatment.

Fig. 2 shows the time course of ATP-driven proton pumping, sensitive to oligomycin, in ESMP, monitored by ACMA fluorescence. It can be seen that anaerobic treatment of ESMP with diamide has no effect on proton translocation from the B (F_1 side) to the A space driven by ATP hydrolysis, under conditions in which the treatment accelerates the subsequent back flow of protons from the A to the B side. The same pattern is observed when proton translocation is monitored by the absorbance changes of neutral red, used to follow the pH in the internal space (not shown). Fig. 3 shows Lineweaver–Burk plots of initial rates of ATP-driven proton translocation in ESMP monitored by ACMA fluorescence (panel a) or neutral red absorbance (panel b). Both plots, whose linearity excludes problems arising from possible non-linearity of probe response to pH changes [31], show that diamide treatment has no effect on the V_{max} and apparent K_m for proton translocation driven by ATP hydrolysis. Furthermore, direct spectrophotometric analysis of the kinetics of ATP hydrolysis shows that diamide treatment has no effect on the apparent V_{max} and K_m of this process (Fig. 4). Eadie–Hofstee plot analysis, which allowed the resolution of the three phases of the kinetics of ATP hydrolysis in ESMP [32], revealed that diamide treatment had practically no effect on the V_{max} and K_m values of the three phases (results not shown).

Fig. 5 shows that the cross-linking of the F_0I-PVP(b) and F_1-γ subunits, induced by diamide treatment of anaerobic particles, revealed by the disappearance of their immunodetected bands in the native positions and appearance of immunoreactivity for both in the cross-linking product with an

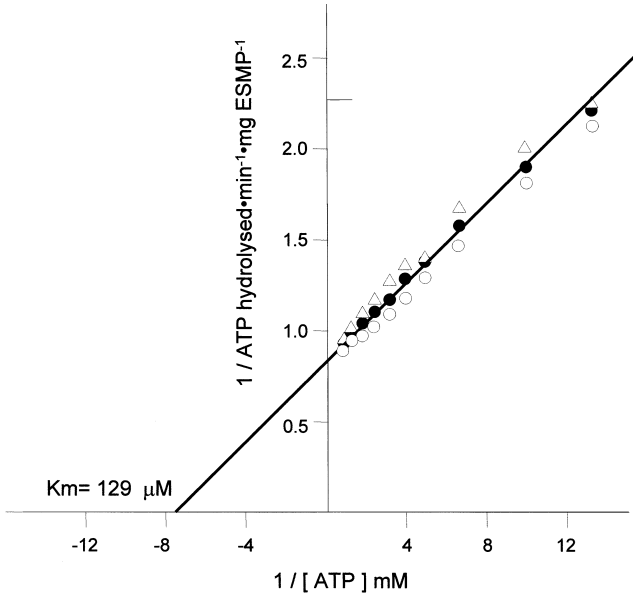

Fig. 4. Double reciprocal plots of ATP hydrolysis in ESMP. For treatment of succinate-respiring or anaerobic ESMP with 2 mM diamide see the legend to Fig. 1. (●) ESMP (0.2 mg/ml); (△) succinate-respiring diamide-treated ESMP (0.2 mg/ml); (○) anaerobic diamide-treated ESMP (0.2 mg/ml) were kept for 2 min in a reaction mixture containing: 200 mM sucrose, 10 mM Tris–acetate pH 6.7, 1 mM K-EDTA, 6 mM $MgCl_2$. An aliquot of the suspension containing 50 μg particle proteins was then added to 1 ml of the reaction mixture containing: 200 mM sucrose, 50 mM KCl, 6 mM $MgCl_2$, 10 U lactate dehydrogenase, 20 mM Tris–HCl pH 7.4, 0.1 mM NADH, 1 mM phosphoenolpyruvate, 4 U pyruvate kinase, 1 mM rotenone. The reaction was started by the addition of ATP at the given concentrations and followed by monitoring the oxidation of NADH at 340 nm. For other details, see Section 2.

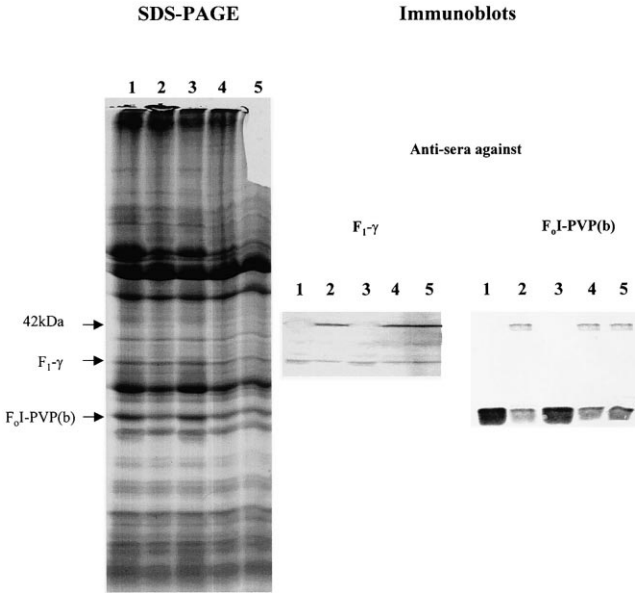

Fig. 5. Cross-linking of F_0I-PVP(b) and γ subunits induced by diamide treatment in ESMP. ESMP, 3 mg/ml, were incubated in the reaction mixture described in the legend to Fig. 1 under a constant stream of N_2. Once anaerobiosis was reached, 2 mM diamide or 2 mM ATP was added and the incubation continued for 2 min. After treatment with diamide or ATP, 2 mM ATP or 2 mM diamide respectively was added. After 4 min the reaction was stopped by the addition of 90% cold acetone (v/v). The washed pellets, obtained after centrifugation at 20 000×*g*, were solubilized in 2.3% SDS and 10 mM Tris–HCl pH 6.8 and boiled for 3 min. Proteins (50 μg) were subjected to gel electrophoresis, electrotransferred to nitrocellulose, and immunodecorated with anti-F_1-γ and anti-F_0I-PVP(b) sera. For other details see Section 2. Lane 1, control ESMP; lane 2, diamide-treated ESMP; lane 3, ATP-treated ESMP; lane 4, ESMP treated first with diamide and then with ATP; lane 5, ESMP treated first with ATP and then with diamide.

apparent MW of 42 kDa, is not prevented or reversed by ATP hydrolysis.

4. Discussion

Diamide-induced disulfide cross-linking of F_0I-PVP(b) and F_1-γ, considered to represent separate components of the stator and rotor of the ATP synthase motor respectively, results, as expected, in promotion of diffusion through F_0 from the A side to the B, F_1 side of the respiratory proton gradient and decoupling of oxidative phosphorylation [22]. The lack of any apparent effect of diamide-induced cross-linking of these two subunits on the ATPase activity and ATP-driven proton translocation from the B to the A side was, on the same grounds, unexpected. The finding that proton translocation from the B to the A side is unaffected by diamide treatment of ESMP, also when driven by a K^+ diffusion potential, in the absence of catalytic activity of F_1, shows that the promotion of H^+ release from the F_0 channel into the B space, caused by F_0I-PVP(b) and F_1-γ cross-linking, does not affect proton translocation in the reverse direction from the B to the A side. It can be mentioned, in this connection, that mutation of Leu-156 to Arg in the F_0-a subunit (ATPase 6) in NARP patients has been found to be associated with decoupling of oxidative phosphorylation, in the absence of any effect on proton pumping driven by ATP hydrolysis [33]. It is conceivable that there are different rate-limiting steps in the two directions of proton translocation through F_0.

Chemical cross-linking [2,3] and photochemistry experiments [4,5] have provided definite evidence showing that ATP hydrolysis promotes rotation of γ and ε [34], relative to the three β subunits with generation of a torsional force [35]. This and related observations have led to the proposal, still to be proven, that the rotation of γ and ε causes, in turn, rotation of the ring of subunits c relative to subunit a in F_0, with associated proton translocation, at the c–a interface, from the B to the A side [2,7,35–39].

The mechanism by which rotation of γ will make the c-ring rotate during ATP-driven proton pumping and by which $\Delta\tilde{\mu}H^+$-driven rotation of the c-ring induces rotation of γ during ATP synthesis, is not known. It can be speculated that electrostatic, protolytic interactions between polar residues in the hydrophilic loop of c with polar residues in γ and ε are involved in the coupling process [35–41].

The present results show that when the Cys-91 region of γ, which lies under the DELSEED region of the β subunit [1], is immobilized by disulfide cross-linking to the Cys-197 region of F_0I-PVP(b), ATP hydrolysis and the resulting energy-linked proton pumping from the B to the A side are unaffected. It might be possible that in the cross-linked enzyme, at least, the electrostatic interactions elicited by ATP hydrolysis directly promote injection of protons into the B side of the F_0 channel and their translocation to the A side, with possible promotion of rotation of the c-ring. It may be recalled that very recently Birkenhager et al. have obtained results which suggest a fixed interaction of γ and ε with only part of the c subunits [42].

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