Topological and Functional Relationship of Subunits F_1-γ and F_oI-PVP(b) in the Mitochondrial H^+-ATP Synthase†

Antonio Gaballo, Franco Zanotti, Angela Solimeo, and Sergio Papa*

Institute of Medical Biochemistry and Chemistry, University of Bari, Bari, Italy

Received June 16, 1998; Revised Manuscript Received August 14, 1998

ABSTRACT: Diamide treatment of the F_oF_1-ATP synthase in "inside out" submitochondrial particles (ESMP) in the absence of a respiratory $\Delta\mu H^+$ as well as of isolated F_o reconstituted with F_1 or F_1-γ subunit results in direct disulfide cross-linking between cysteine 197 in the carboxy-terminal region of the F_oI-PVP(b) subunit and cysteine 91 at the carboxyl end of a small α-helix of subunit F_1-γ, both located in the stalk. The F_oI-PVP(b) and F_1-γ cross-linking cause dramatic enhancement of oligomycin-sensitive decay of $\Delta\mu H^+$. In ESMP and MgATP particles the cross-linking is accompanied by decoupling of respiratory ATP synthesis. These effects are consistent with the view that F_oI-PVP(b) and F_1-γ are components of the stator and rotor of the proposed rotary motor, respectively. The fact that the carboxy-terminal region of F_oI-PVP(b) and the short α-helix of F_1-γ can form a direct disulfide bridge shows that these two protein domains are, at least in the resting state of the enzyme, in direct contact. In isolated F_o, diamide also induces cross-linking of OSCP with another subunit of F_o, but this has no significant effect on proton conduction. When ESMP are treated with diamide in the presence of $\Delta\mu H^+$ generated by respiration, neither cross-linking between F_oI-PVP(b) and F_1-γ subunits nor the associated effects on proton conduction and ATP synthesis is observed. Cross-linking is restored in respiring ESMP by $\Delta\mu H^+$ collapsing agents as well as by DCCD or oligomycin. These observations indicate that the torque generated by $\Delta\mu H^+$ decay through F_o induces a relative motion and/or a separation of the F_oI-PVP(b) subunit and F_1-γ which places the single cysteine residues, present in each of the two subunits, at a distance at which they cannot be engaged in disulfide bridging.

The ATP synthase[1] of coupling membranes consists of a globular catalytic moiety, F_1,[2] which protrudes out of the inner side of the membrane, a membrane integral proton translocating moiety, F_o, and a stalk which provides a structural and functional connection between F_1 and F_o (*1*−*4*).

The X-ray crystallographic analysis of the structure of the bovine mitochondrial F_1-ATPase (*5*) has provided a structural basis for the binding change, rotatory mechanism of the catalytic process in F_1, in which the F_1-γ subunit rotates in the central cavity of the F_1-α3β3 hexamer (*6*). Biochemical (*7*) and physical (*8*, *9*) evidence for a rotatory movement of the F_1-γ subunit relative to the α3β3 hexamer has been obtained. The structural organization and the mechanism of the proton translocating F_o sector (*10*−*12*), the mechanism by which the energy provided by transmembrane proton translocation through the F_o sector is transferred to F_1 to drive ATP synthesis, and how the reverse process, that is, ATP-driven uphill proton translocation, occurs are less understood. The solution of these problems, and in particular that of energy coupling in the ATP synthase, rests on the elucidation of the structural and functional organization of the components of the stalk.

The stalk is made up of interdigitated extensions of F_1 and F_o subunits which ensure transmission of the energy provided by a downhill flow of protons through the F_o domain in the membrane to the extrinsic F_1 domain where ATP is synthesized from ADP and Pi. The crystal structure of bovine mitochondrial F_1-ATPase shows a stem protruding about 40 Å from the center of the bottom of the spherical body. This consists of the extension of the C-terminal and N-terminal α-helices of the F_1-γ subunit which runs in the central cavity of the α3β3 hexamer. In the stem a third short α-helix, residues 73−90, of the F_1-γ subunit is identified. Cross-linking and mutational analysis show that in the *Escherichia coli* F_oF_1 complex the ϵ subunit (equivalent to the δ subunit of the mitochondrial F_1) is located in the stalk (*13*, *14*). The crystal structure of the mitochondrial F_1 shows, at the bottom of the sphere, that the stem is surrounded by alternating bundles of C-terminal α-helices of the 3 α and 3 β subunits. These are reached by F_o subunits contributing to the stalk, in which the central parts of the F_1-γ subunit

† This work was financially supported by Grants No. 97.01167.PF49 Biotechnology Project, Consiglio Nazionale delle Ricerche, Italy, and the National Project of Murst for Bioenergetics and Membrane Transport, Italy.

[1] Enzymes: ATP synthase (EC 3.6.1.34); glucose-6-phosphate dehydrogenase (EC 1.1.1.49); catalase (EC 1.11.1.6); hexokinase (EC 2.7.1.1.).

[2] Abbreviations: FCCP, carbonylcyanide *p*-(trifluoromethoxy)phenylhydrazone; Chaps, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; DCCD, *N*,*N*′-dicyclohexyl-carbodiimide; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; ESMP, submitochondrial particles prepared in the presence of EDTA; MgATP-SMP, submitochondrial particles prepared in the presence of Mg-ATP; F_o, 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_oI-PVP-(b), protein subunit of mitochondrial F_0; OSCP, oligomycin-sensitivity conferring protein.

10.1021/bi981422c CCC: $15.00
Published on Web 11/20/1998

and the ϵ subunit (in the *E. coli* enzyme) penetrate for most of its length (*14*).

In prokaryotic and eukaryotic ATP synthases F_o has three, conserved membrane intrinsic subunits: a and c which are essential for proton transport (*11*, *15*), and the b subunit in *E. coli*, which is the least conserved component and has counterpart(s) in the eukaryotic enzymes (*16*−*19*). The bovine mitochondrial F_o has seven additional constituents, that is, subunits OSCP, d, e, f, g, F_6, and A_6L (*20*, *21*). The F_o subunits contributing to the stalk in the mitochondrial ATP synthase have been identified with different approaches: (i) limited proteolysis of F_o and F_1 subunits (*16*, *17*, *22*−*24*); (ii) immunodetection by subunit specific antibodies raised against purified F_o subunits (*21*, *23*, *25*); (iii) cross-linking of F_o and F_1 subunits (*25*−*30*); and (iv) reconstitution from isolated subunits (*31*). Taken together, the results of these studies indicate that the carboxy-terminal region of F_oI-PVP-(b) subunit, OSCP, F_6, and subunit d contribute to the stalk in the mitochondrial F_oF_1-ATP synthase. It is thought that OSCP (equivalent to subunit δ of the *E. coli* enzyme) and F_oI-PVP(b) subunit are part, together with subunit a (ATPase 6), of the stator of the rotary motor, which holds the $\alpha 3\beta 3$ hexamer, while the γ and δ (ϵ in *E. coli*) constitute, together with the multiple copies of the F_o-c subunit, the rotor portion (*7*, *8*, *32*, *33*). In this paper a study based on disulfide cross-linking is presented of the topological relationship of the F_1-γ and F_oI-PVP(b) subunits in the stalk of the mitochondrial F_oF_1-ATP synthase and their functional interaction in the energy-linked H^+ translocation. It should be noted that cysteine residues in mitochondrial F_oF_1 subunits are very limited in number: C201 and C251 in F_1-α, C91 in F_1-γ, C18 in F_1-ϵ, C197 in F_oI-PVP(b), C118 in OSCP, C100 in d, C72 in f, and C64 in c (4).

The present results provide evidence showing that the F_1-γ and F_oI-PVP(b) subunits, components of the rotor and stator of the ATP synthase, respectively, are in direct contact and can form a disulfide bridge in the resting state of the enzyme. The respiratory $\Delta\mu H^+$ induces a relative motion and/or separation of the two subunits at a distance which prevents their disulfide cross-linking.

MATERIALS AND METHODS

Chemicals were purchased from the following companies: valinomycin, oligomycin, antimycin, nigericin, carbonylcyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), diamide, and Chaps from Sigma Chemical Co.; *N*,*N*′-dicyclohexyl-carbodiimide (DCCD) from Fluka; ^{14}[C]-DCCD (50Ci/mol) from Amersham; acrylamide, *N*,*N*′-methylenebisacrylamide, sodium dodecyl sulfate (SDS), goat anti (rabbit IgG)-alkaline-phosphatase conjugate, and AP color development reagent (BCIP-5 bromo-4-chloro-3 indolyl phosphate) (NBT-nitroblue tetrazolium) from Bio-Rad; catalase from Boehringer; and nitrocellulose membrane (0.45 μm pore size) from Schleicher and Schüll, Biotech, Ltd. All other chemicals were of high-purity grade.

Preparations. Inverted vesicles of the inner mitochondrial membrane were obtained by exposure of isolated beef heart mitochondria (*34*) to ultrasonic energy in the presence of EDTA at pH 8.5 (ESMP) or in the presence of 1 mM MgATP (MgATP-SMP) (*35*). Sequential treatment of ESMP with Sephadex chromatography (the F_1 inhibitor protein was removed) and urea (*36*) produced particles from which F_1 subunits were removed (USMP). The percentage of inversion of submitochondrial particles was found to range from preparation to preparation from 96% to 100% (*37*). F_o sector was isolated by Chaps solubilization from USMP (*38*). F_1 was purified by a modified (*38*) chloroform extraction procedure (*39*). The subunits F_1-α/β and γ, F_oI-PVP(b) and OSCP were isolated by electroelution from preparative gel electrophoresis (*40*). After purification, all proteins were solubilized in 50 mM Tris/HCl, pH 7.4, 0.01% SDS (w/v), 50% glycerol (v/v) at a protein concentration of approximately 0.5 μg/μL.

Reconstitution Experiments. Isolated F_1 subunits, obtained by electroelution of SDS−PAGE bands of F_1, or the entire F_1 sector at the concentrations described in the legend to the figures, were incubated 20 min with isolated F_o in 0.25 M sucrose, 10 mM Tris/acetate, pH 7.5, 1 mM EDTA, and 6 mM $MgCl_2$. The incubation was stopped by centrifugation at 105000*g* for 20 min.

Preparation of F_o Vesicles. F_o vesicles were prepared by the dialysis method (*41*). Three milligrams of F_o or F_o reconstituted with F_1 or isolated F_1 subunits was mixed with 30 mg of acetone-washed sonicated asolectin in 1 mL of 0.1 M potassium phosphate buffer, pH 7.2, containing 1.6% potassium cholate (w/v), 0.8% potassium deoxycholate (w/v), and 0.2 mM EDTA. The mixture was dialyzed overnight against 0.1 M potassium phosphate, 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 $MgSO_4$.

Measurement of Proton Conduction. Proton conduction in submitochondrial particles was analyzed by following potentiometrically either the anaerobic release of the respiratory proton gradient (*42*) or proton release from submitochondrial particles or F_o liposomes induced by a diffusion potential (positive inside) imposed by valinomycin-mediated K^+ influx (*40*). 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. With this system pH changes in stirred suspensions can be measured with resolutions of 0.01 pH unit and overall rise time (10%−90% change) of less than 0.3−0.4 s (*43*). For the kinetic analysis of the proton release from submitochondrial particles or F_o liposomes, the potentiometric traces were converted into proton equivalents by double titration with standard HCl and KOH.

Determination of P/O Ratios. ATP produced by oxidative phosphorylation in ESMP or Mg-ATP particles was directly converted to glucose 6-phosphate with the addition of glucose and hexokinase in the reaction mixture (see legend to Table 4). The respiratory activity was measured following O_2 uptake with a Clark oxygen electrode coated with a high-sensitivity membrane (YSI 5776) in a closed thermostatically controlled (25 °C) water-jacketed glass cell equipped with a magnetic stirrer. The O_2 concentration of the reaction mixture was determined with NADH (*44*). Glucose-6-phosphate was determined on extracts of the particle suspension with glucose-6-phosphate dehydrogenase (*45*).

Electrophoresis. SDS−PAGE was performed on slab gels with a linear gradient of polyacrylamide (12%−20%) as in ref *22*.

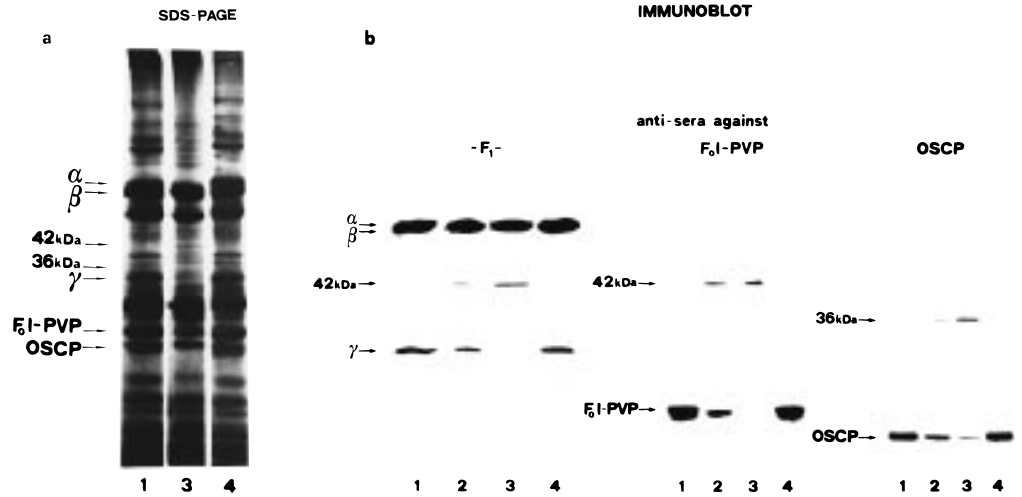

FIGURE 1: Coomassie blue stained SDS−PAGE slabs and immunoblot detection of cross-linking products of F_1-γ, F_oI-PVP(b), and OSCP subunits in ESMP. ESMP were prepared as described under Materials and Methods: lane 1, control ESMP; lanes 2 and 3, ESMP incubated with 2 mM diamide for 2 and 5 min respectively; and lane 4, ESMP incubated 5 min with 2 mM diamide were then treated for 30 min with 20 mM DTT. For diamide treatment, ESMP were suspended in a reaction mixture containing the following: 200 mM sucrose, 30 mM KCl, and 20 mM K-succinate, pH 7.4. Incubation was carried out at 25 °C in a thermostatically controlled glass vessel under a constant stream of N_2. Oxygen concentration was monitored polarografically. Once anaerobiosis was reached, 2 mM diamide was added and the incubation continued for 2 or 5 min. The reaction was stopped by the addition of 90% acetone (v/v). The washed pellets, obtained after centrifugation at 20000*g*, were solubilized in 2.3% SDS and 10 mM Tris-HCl, pH 6.8, and boiled for 3 min. (Panel a) Proteins (50 μg) were applied on SDS slab gels with a linear gradient of polyacrylamide (12%−20%) and detected by Coomassie-blue staining. (Panel b) The proteins of the SDS−PAGE slabs were electrotransferred to nitrocellulose and immunodecorated with purified IgG fractions (*46*) of rabbit antisera raised against purified F_1, F_oI-PVP(b) and OSCP, respectively. For other details see under Materials and Methods.

Immunological Procedures. Polyclonal antibodies raised against SDS-denatured subunits F_oI-PVP(b), OSCP, and γ were isolated from antisera collected after the 4th−6th booster injection in rabbits. The IgG fraction was purified by a combination of precipitations with caprylic acid and ammonium sulfate (*46*). 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 semi-dry 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 1 h at room temperature (*47*). After each incubation step the excess protein 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.

Protein Determination. Protein concentration was determined according to Lowry (*48*).

RESULTS

Diamide, which oxidizes vicinal thiol groups followed by formation of disulfide bridges (*49*), induces in the mitochondrial F_oF_1 complex cross-linking of F_1-γ and F_oI-PVP-(b) subunits, each one of them having a single cysteine residue (Cys197 in F_oI-PVP(b) and Cys91 in F_1-γ). Diamide treatment of submitochondrial particles (ESMP) caused, in fact, depending on the incubation time, partial or complete disappearance of the immunodetected bands of F_1-γ and F_oI-PVP(b) subunits, from their original position in the SDS−PAGE (Figures 1 and 3 and Table 1), and appearance in the gel of a new band (apparent Mw 42 kDa), which reacted with both anti-F_1 and anti F_oI-PVP(b) antibodies and, upon reductive cleavage of disulfide bonds with DTT, disappeared with reapparance of these two protein bands in their original position (Figure 1 and Table 1). This cross-linking product of F_1-γ and F_oI-PVP(b) was isolated from the gel, electrophoresed again, and immunodetected with both anti F_1 and anti F_oI-PVP(b) antibodies (Figure 2). Upon reduction with DTT the isolated cross-linking product released the F_1-γ and F_oI-PVP(b) subunits (Figure 2) (see also ref *28*).

The diamide treatment of ESMP resulted also in disulfide cross-linking of OSCP (a single cysteine at position 118) with another thiol-bearing subunit (Figures 1 and 3 and Table 1). Its cross-linking product detected by the anti-OSCP antibody migrated in the SDS−PAGE with an apparent Mw of 36 kDa, different from that of the cross-linking product of F_1-γ and F_oI-PVP(b) subunits, and did not cross-react with the polyclonal antibodies against these subunits. When a purified F_o preparation was treated with diamide, the F_oI-PVP(b) band was unaffected while that of OSCP was still decreased (Figures 3 and 4a) with the appearance of the cross-linking product which reacted with the antibody against OSCP but not with that against F_oI-PVP(b) (Figure 4a). It should be noted that the purified F_o preparation used did not contain any detectable trace of F_1-γ as shown by both Coomassie blue and silver staining (not shown) or immunodecoration with anti-F_1 antibody. The cross-linking product of OSCP obtained in the diamide-treated F_o preparation was electroeluted from the gel and, upon reduction with DTT, released the OSCP subunit (Figure 4b)

When purified F_o was reconstituted with purified F_1 or with the isolated F_1-γ, diamide treatment caused again, depending on the incubation time, partial or complete disappearance of the F_oI-PVP(b) and of the F_1-γ band

Table 1: Diamide-Induced Disulfide Cross-Linking of F_1-γ, FoI-PVP(b), and OSCP in ESMP and Associated Effects on Anaerobic Relaxation of the Respiratory Proton Gradient

expts			control	control + DTT	1st addition diamide	2nd addition DTT
A[a]	$1/t_{1/2}$ H^+ release (sec^{-1})		0.98	0.96	3.05	1.14
	immunoblot (arbitrary units) subunits:	F_1-γ	6.70	6.48	0.98	5.90
		F_oI-PVP(b)	5.80	5.90	0.96	5.40
		OSCP	7.21	6.94	0.86	6.15
B[b]	^{14}[C]-DCCD (cpm) 8 kDa c subunit		2450		2600	

[a] ESMP (3 mg of protein/mL) were incubated in a medium containing the following: 200 mM sucrose, 30 mM KCl, and 20 mM succinate (potassium salt), pH 7.4. Incubation was carried out under a constant stream of N_2 at 25 °C. Once anaerobiosis was reached, 2 mM diamide was added and, after 2 min, the reaction was stopped by dilution with the same buffer and centrifugation at 105000*g*. Control and diamide-treated ESMP pellets were suspended in the same incubation mixture (3 mg of protein/mL) in the presence or in the absence of 20 mM DTT supplemented with 0.5 μg of valinomycin/mg of ESMP protein and 13 000 units of catalase. Respiration-driven proton translocation was activated by repetitive pulses of 1%−3% H_2O_2 (5 μL/mL). The pH of the suspension was monitored potentiometrically as described under Materials and Methods. Then 50 μg of protein was washed with 90% acetone (v/v) and centrifuged at 20000*g*, and the pellet, solubilized in 2.3% SDS and 10 mM Tris/HCl, pH 6.8, was boiled for 3 min. Proteins separated by PAGE were electrotransferred to nitrocellulose and immunodecorated with anti F_oI-PVP(b), anti F_1-γ, and anti OSCP sera. Areas of γ, F_oI-PVP(b), and OSCP immunoreactive bands were determined as arbitrary units by densitometric analysis of immunoblots, as reported under Materials and Methods. [b] ESMP and ESMP treated with diamide were incubated for 30 min with 50 μM ^{14}[C]-DCCD in the same incubation mixture reported above (1 mg of protein/mL). The reaction was stopped by centrifugation at 105000*g*, and 300 μg of particles was subjected to SDS−PAGE. After staining and destaining, a slice of the 8 kDa c subunit band was treated with Beckman tissue solubilizer 450 and the radioactivity determined by liquid scintillation counting.

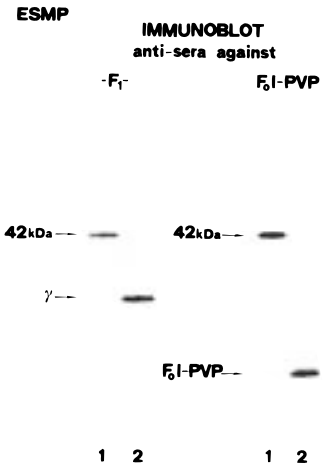

FIGURE 2: Immunodetection of the disulfide cross-linking product of F_1-γ and F_oI-PVP(b) subunits induced by diamide treatment of ESMP. ESMP (300 μg) treated with 2 mM diamide for 5 min, in the same experimantal conditions reported in the legend to Figure 1, were subjected to SDS−PAGE. A piece of the gel slabs was cut between the α/β and γ subunits, and proteins were electroeluted from it in buffered 50% glycerol as described in ref *40*. Electroeluted proteins (lanes 1) and electroeluted proteins treated for 30 min at 25 °C with 20 mM DTT (lanes 2) were subjected to a second electrophoretic run followed by electrotransfer to nitrocellulose and immunodecoration with antisera against F_1 and F_oI-PVP(b) subunits.

(Figure 3). It can be noted that the addition of F_1-α/β to F_o was, on the other hand, ineffective in this respect; furthermore the addition of F_1 or of its subunits had no effect on the diamide-induced decrease of the immunodetected band of OSCP (Figure 3). Diamide treatment of ESMP did not cause any cross-linking of F_o-c subunits (a single cysteine at position 64), between them or another subunit, as shown by the fact that it did not decrease the amount of [^{14}C]-DCCD-labeled original band of apparent Mr 8000 of this subunit (Table 1).

The results presented in Tables 1 and 2 show that diamide-induced cross-linking of F_oI-PVP(b) and F_1-γ caused, both in ESMP (see also Figure 5) and in F_o reconstituted with F_1 or with isolated F_1-γ, a dramatic enhancement of the oligomycin-sensitive proton conduction in F_o. Diamide treatment of F_o, which induced cross-linking of OSCP but left F_oI-PVP(b) unaffected, had practically no effect on oligomycin-sensitive proton conduction in F_o liposomes (Table 2 and Figure 5). It should be noted that in the experiments presented in Figures 1−5 and Tables 1 and 2, submitochondrial particles (ESMP) and F_o vesicles were treated with diamide in the absence of transmembrane $\Delta\mu H^+$ (*28*). Figure 5 shows that, when ESMP respiring with succinate as substrate were treated with diamide, no enhancement of the passive back-flow of protons took place. The immunoblot results presented in Figure 6 show that, under these conditions, diamide treatment of ESMP did not induce the cross-linking of F_oI-PVP(b) with F_1-γ and of OSCP with another component of F_o, as it did when ESMP were treated with diamide in anaerobiosis. Suppression of diamide-induced cross-linking of F_1 and F_o subunits and the resulting enhancement of proton conduction is not merely due to the presence of oxygen, but this effect requires respiratory activity, as shown by the reappearance of F_1-γ, F_oI-PVP(b), and OSCP cross-linking, upon inhibition of respiration by antimycin A (Figure 6). Suppression of diamide-induced cross-linking of F_1 and F_o subunits was in fact dependent on the $\Delta\mu H^+$ generated by respiration and its dissipation through the proton channel in F_o, as shown by restoration of cross-linking in respiring ESMP by FCCP (or nigericin), which abolishes the respiratory $\Delta\mu H^+$, and by DCCD and oligomycin which inhibit proton conduction in F_o (Table 3).

In another set of experiments the effect of diamide treatment of submitochondrial particles on proton pumping and ATP synthesis supported by succinate respiration was examined. Oxygen pulses of succinate supplemented ESMP or MgATP particles result in respiration-linked proton uptake. The efficiency of respiration-linked proton pumping can be evaluated from the extent of proton uptake at the respiring steady state and the H^+/e^- ratio, which is calculated from the ratio of the initial rate of proton release ensuing upon anaerobiosis and the steady-state respiratory rate measured just before anaerobiosis is reached (*50*). The results of these experiments are summarized in Table 4. Treatment of ESMP or MgATP-SMP with diamide, both in the respiring state (non cross-linking conditions) and in anaerobiosis (cross-linking conditions), had no effect on the rate of succinate respiration and the extent of respiratory proton uptake.

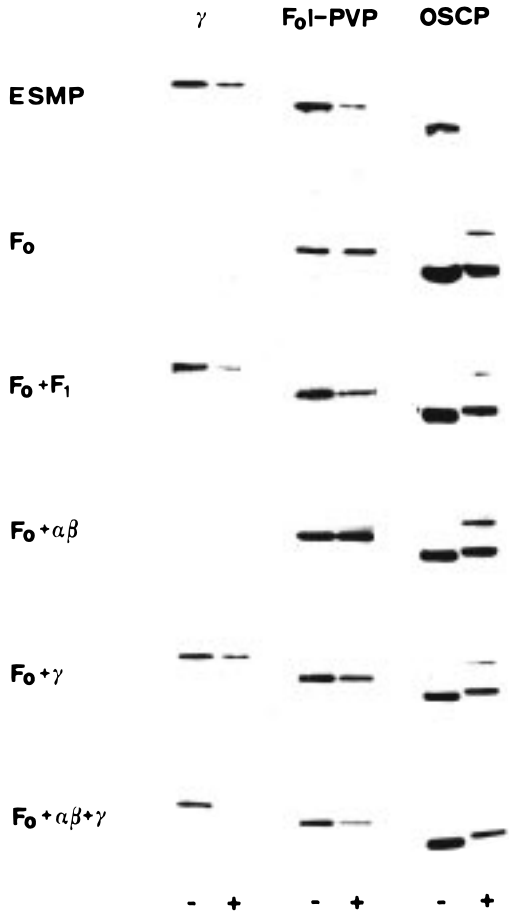

FIGURE 3: Immunoblot analysis of disulfide cross-linking of ATP synthase subunits in ESMP and in F_o liposomes. ESMP, F_1, F_1 subunits, and F_o sector were prepared as described under Materials and Methods. Where indicated, isolated subunits or F_1 was added to F_o at the concentration of 4 μg/mg or 0.5 mg/mg of protein F_o, respectively. The incubation was stopped by centrifugation at 105000*g* to eliminate the excess of F_1 or F_1 subunits. The binding to F_o of added α/β and γ subunits was directly verified by immunoblots of the precipitated pellet. F_o and F_o reconstituted with F_1 or F_1 subunits were incorporated in liposomes by the dialysis method described under Materials and Methods. ESMP and F_o liposomes were treated with diamide in the succinate mixture described in the legend to Figure 1, after the suspension was made anaerobic by respiratory activity under a constant stream of N_2 at 25 °C. Once anaerobiosis was reached, 2 mM diamide was added and the incubation continued for 2 min. The reaction was stopped by the addition of 90% acetone (v/v). The washed pellets, obtained after centrifugation at 20000*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, anti-FoI-PVP(b), and anti-OSCP sera. For other details see under Materials and Methods.

Treatment of ESMP with diamide in anaerobiosis resulted in an increase of the rate of proton back-flow and enhancement of the steady-state H^+/e^- ratio for proton pumping from 1.2 to 3.0, which is the upper limit for proton pumping with succinate as respiratory substrate (*51*). The remarkable enhancement of the H^+/e^- ratio is consistent with the increase in the rate of proton back-flow through F_o, which prevents decoupling of redox-driven proton pump by transmembrane ΔpH (*51*−*53*). This finding suggests, incidentally, that the entry mouth of the proton channel of the F_oF_1 complex in the membrane can get very close to the output mouth of the proton pumps in respiratory complexes so as to drain effectively, under the prevailing experimental conditions

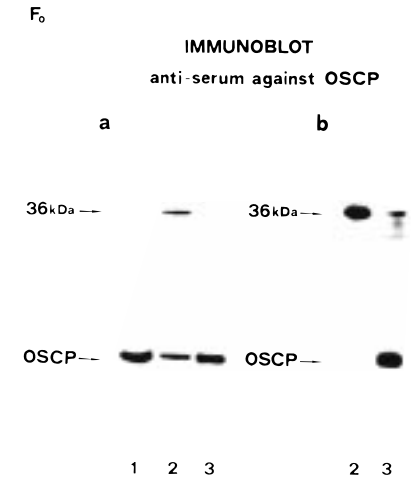

FIGURE 4: Immunodecoration of the cross-linking product of OSCP subunit induced by diamide treatment of F_o liposomes. Purified F_o was prepared as described under Materials and Methods. F_o was incorporated in liposomes by the dialysis method described under Materials and Methods. F_o liposomes were treated for 5 min with 2 mM diamide in the succinate mixture as described in the legend to Figure 1. The reaction was stopped by the addition of 90% acetone (v/v). After centrifugation at 20000*g*, the washed pellet was solubilized in 2.3% SDS and 10 mM Tris-HCl, pH 6.8, and boiled for 3 min. (Panel a) Proteins (50 μg) were subjected to gel electrophoresis, electrotransferred to nitrocellulose, and immunodecorated with anti-OSCP serum: lane 1, control; lane 2, F_o liposomes treated with diamide; lane 3, diamide-treated F_o liposomes incubated 30 min with 20 mM DTT. (Panel b) F_o liposomes (200 μg), treated with diamide, were subjected to SDS−PAGE. A piece of the gel slab was cut between the α/β and γ subunits, and the proteins were electroeluted as described in the legend to Figure 2. Electroeluted proteins (lane 2) and electroeluted proteins treated 30 min with 20 mM DTT (lane 3) were subjected to a second electrophoretic run followed by electrotransfer to nitrocellulose and immunodecoration with anti-OSCP serum.

Table 2: Effect of Diamide-Induced Disulfide Cross-Linking of F_o and F_1 Subunits on Proton Conduction in ESMP and F_o Liposomes

	H^+ release (nmol·min^{-1}·mg of protein^{-1})			
	without oligomycin		with oligomycin	
additions	− diamide	+ diamide	− diamide	+ diamide
ESMP	174.4	610.0	31.4	98.3
F_o liposomes	760.1	625.1	112.4	78.4
+ F_1	484.1	1348.9	50.3	104.3
+ $\alpha\beta$	706.9	582.0	101.3	74.3
+ γ	440.3	1423.1	52.7	112.9
+ $\alpha\beta$ + γ	408.4	795.3	52.3	96.4

[a] Preparation of ESMP, F_1, F_o, and F_1 subunits, reconstitution procedures, diamide treatment, and incorporation into liposome vesicles were carried out as described under Materials and Methods and in the legend to Figure 3. For measurement of H^+ conduction, ESMP (1 mg of protein/mL) and F_o liposomes (0.4 mg of protein/mL) were suspended in 0.15 M KCl, and passive H^+ release induced by K^+ uptake was initiated by the addition of valinomycin (2 μg/mg of particles protein). Where indicated, submitochondrial particles and F_o liposomes were incubated for 5 min with 1.0 μg of oligomycin/mg of particles protein before the addition of 0.15 M KCl and valinomycin. For other details see under Materials and Methods.

used, the respiratory $\Delta\mu H^+$. Although the coupling efficiency of redox-linked proton pumping was, under these conditions, increased, the coupling efficiency for the utilization of respiratory $\Delta\mu H^+$ by the F_oF_1 complex to synthesize ATP was decreased by the F_1-γ and FoI-PVP(b) cross-linking induced by diamide, as shown by the decrease of the P/O ratio. Internal control experiments show, clearly, that

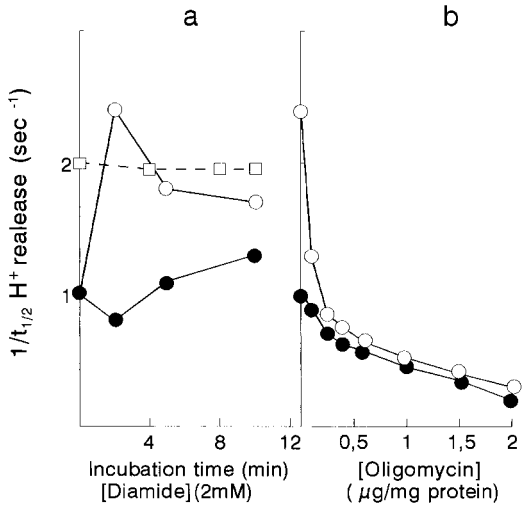

FIGURE 5: Effect on proton conduction of diamide treatment of ESMP and F_o liposomes. (Panel a) ESMP were treated with diamide for the times indicated in the figure in the succinate mixture described in the legend to Table 1 after the suspension was made anaerobic by respiratory activity under a constant stream of N_2 at 25 °C (○). ESMP, respiring with succinate in the mixture described in the legend to Table 1, were incubated with diamide (●). At the time indicated in the figure the reaction was stopped by dilution with the same standard medium and the suspension centrifuged at 105000*g*. In both cases the ESMP pellet was suspended in the succinate reaction mixture containing valinomycin and catalase (see legend to Table 1) and respiration-driven proton translocation was activated by repetitive pulses of H_2O_2. F_o liposomes were treated with diamide and suspended in 0.15 M KCl (□); H^+ release was initiated by the addition of 2 μg of valinomycin/mg of F_o. (Panel b) Before starting respiration with H_2O_2 pulses in the presence of catalase, ESMP (●) and ESMP treated for 2 min in anaerobiosis with 2 mM diamide (○) were incubated for 2 min with oligomycin at the concentrations reported in the figure. For other details see the legend to Table 1 and under Materials and Methods.

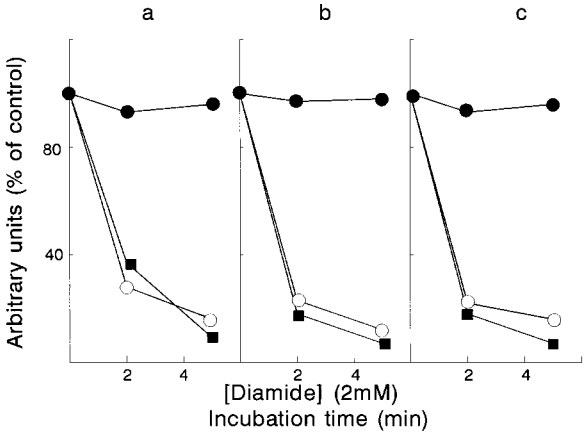

FIGURE 6: Effect of respiration on diamide-induced disulfide cross-linking of F_1-γ, F_oI-PVP(b), and OSCP in ESMP. ESMP (3 mg of protein/mL) were incubated with 2 mM diamide in the succinate mixture reported in the legend to Table 1 for the time given in the figure in anaerobiosis (○), in succinate-respiring state (see legend to Figure 2) (●), and in aerobiosis in the presence of 1.0 μg of antimycin A/mg of particle protein to inhibit succinate-supported respiration (■). A 50 μg protein sample of ESMP was treated as described in the legend to Figure 1 for densitometric analysis of immunodecorated bands of F_oI-PVP(b) (a), F_1-γ (b), and OSCP (c). The percentage of antigen detected in the bands was evaluated from the computed peak areas taking as 100% the peak areas in untreated ESMP. For other details see the legend to Table 1 and under Materials and Methods.

diamide was without any of these effects when added to respiring ESMP, conditions in which cross-linking of F_oF_1 subunits is prevented. The effects produced by the anaerobic treatment of ESMP with diamide are also observed in MgATP particles. In these particles, which retain a better capacity for oxidative phosphorylation, as compared to ESMP, anaerobic treatment with diamide caused a more definite decrease of the P/O ratio, although the enhancing effect of diamide treatment on the rate of proton back-flow and on the H^+/e^- ratio for proton pumping was smaller, probably due to the preservation of a tighter native asset of the F_oF_1 complex in these particles.

DISCUSSION

The F_oI-PVP(b) subunit, present in one copy in the mitochondrial F_oF_1-ATP synthase (*54*), (in the *E. coli* enzyme there are two copies of the corresponding b subunit (*11*)), is essential for the binding of the F_1 head piece to the F_o membrane sector (*16*−*18*, *22*, *23*, *31*). The carboxy-terminal region of F_oI-PVP(b) protrudes out of the membrane integral part of F_o , where it is anchored, at the lateral side of the bundle of c subunits, by two hydrophobic helices of the N-terminal region, and reaches the surface of the F_1 complex (*4*, *17*, *31*, *54*). OSCP in the mitochondrial (*54*) and the homologous F_1-δ in the *E. coli* ATP synthase (*55*) apparently extend from the stalk, where they interact with the C-terminal region of the b subunit, all along the surface of F_1. The F_1-γ subunit, which penetrates with its C and N termini the central cavity of the $\alpha 3\beta 3$ hexamer of F_1, constitutes with its large central lobe, of which a short lateral α-helix (residues 73−90) is identified in the F_1 crystal (*5*), a large part of the stalk.

It has been proposed that the two b subunits in *E. coli* and the single copy of F_oI-PVP(b) subunit in the mitochondrial enzyme, together with F_1-δ in *E. coli*, and the corresponding OSCP in mitochondria, constitute a stator attached to the surface of the $\alpha 3\beta 3$ hexamer while the γ and ϵ subunits, in *E. coli*, and γ and δ in mitochondria represent, together with the bundle of c subunits, the rotor of the ATP synthase rotary motor (*3*, *8*, *33*, *55*). According to the rotatory model, proton flow through a channel at the interface of subunit a and c subunits generates a torque which drives the rotor and stator in the opposite direction (*33*, *56*). Our finding that cross-linking of the F_1-γ and F_oI-PVP(b), through disulfide cross-linking of Cys 91 of γ and Cys 197 in F_oI-PVP(b), and hence their relative immobilization results in opening of the matrix mouth of the proton channel of F_o with rapid dissipative diffusion through it of the respiratory proton gradient "decoupled" from ATP synthesis, is consistent with and provides support to the view that the F_oI-PVP-(b) and F_1-γ are components of the stator and rotor element, respectively.

It seems worth noting here that Cys91 of the mitochondrial F_1-γ is conserved in the chloroplast F_1-γ subunit (Cys89), which has, in addition 3 other cysteines (Cys199, Cys205, Cys322) (*57*). It has been found that internal cross-linking of Cys89 with one of the other three cysteine residues in the chloroplast F_1-γ caused a pronounced increase in proton conduction (*58*).

One important aspect of the rotary motor of ATP synthase is represented by the topological organization of the stator and the rotor in the stalk. Contrary to a general view of the

Table 3: Effect of FCCP, DCCD, Nigericin, and Oligomycin on Diamide-Induced Disulfide Cross-Linking of F_1-γ, F_oI-PVP(b), and OSCP in Respiring ESMP[a]

	immunoblot (% of control)					
	anti γ serum		anti F_oI-PVP(b) serum		anti OSCP serum	
additions	− diamide	+ diamide	− diamide	+ diamide	− diamide	+ diamide
ESMP-AS[b]	100	29	100	24	100	23
ESMP-RS[c]	100	94	100	97	100	94
ESMP-RS + FCCP	97	30	99	27	96	20
ESMP-RS + DCCD	98	28	98	24	98	18
ESMP-RS + nigericin	99	26	97	20	99	24
ESMP-RS + oligomycin	100	28	100	27	100	22

[a] ESMP (3 mg of protein/mL) were incubated in anaerobiosis or in the succinate respiring state in the experimental conditions given in the legend to Figure 5. Where indicated 1 μM FCCP, 0.5 mM DCCD, 0.5 μg of nigericin/mg of particles protein, or 1 μg of oligomycin/mg particles protein were added. The incubation time was 2 min, except with DCCD which was 30 min. ESMP were then incubated with 2 mM diamide for 2 min and treated for immunodecoration with antibodies. Immunodecoration of SDS−PAGE of ESMP with antibodies and densitometric analysis was carried out as described in the legend to Table 1 and under Materials and Methods. The percentage of antigen detected in the bands was evaluated from the computed peak areas taking as 100% the peak areas in diamide-untreated ESMP. For other details see legend to Table 1 and under Materials and Methods. [b] ESMP-AS: submitochondrial particles incubated in anaerobiosis. [c] ESMP-RS: submitochondrial particles incubated in the succinate-respiring state.

Table 4: Effect of Diamide on H^+/e^- Ratio and P/O in Anaerobiosis and in the Succinate-Respiring State in ESMP and in MgATP-SMP[a]

	respiration rate ng O/(min mg)	extent H^+ uptake ng of H^+/mg	V H^+ ng of H^+/(mg min)	H^+/e^-	ATP synthesis nmol of ATP/(mg min)	P/O
ESMP	115 ± 9.6	2.8 ± 0.1	69.5 ± 5.8	1.22 ± 0.20	29.3 ± 2.3	0.26 ± 0.04
ESMP-AS + diamide[b]	129 ± 12.9	3.0 ± 0.1	195.0 ± 19.5	3.09 ± 0.61	20.6 ± 1.3	0.16 ± 0.02
ESMP-SRS + diamide[c]	114 ± 8.8	2.7 ± 0.2	71.9 ± 5.5	1.27 ± 0.19	30.3 ± 1.1	0.27 ± 0.03
MgATP-SMP	103 ± 7.8	2.7 ± 0.1	34.0 ± 2.6	0.67 ± 0.10	71.4 ± 4.1	0.70 ± 0.09
MgATP-AS + diamide[d]	114 ± 8.3	2.8 ± 0.1	46.8 ± 3.4	0.83 ± 0.12	25.6 ± 3.2	0.23 ± 0.04
MgATP-SRS + diamide[e]	95 ± 5.9	2.7 ± 0.1	30.3 ± 1.9	0.64 ± 0.08	63.2 ± 3.8	0.67 ± 0.08

[a] ESMP (3 mg of protein/mL) or MgATP-SMP (3 mg of protein/mL) was incubated in the succinate reaction mixture described in the legend to Figure 1, in the thermostatically controlled glass cell at 25 °C, kept for the respiring state open and under a gentle stream of N_2 for the anaerobic treatment. Anaerobic or respiring particles were incubated for 2 min with 2 mM diamide. To measure respiration-driven proton uptake and the following anaerobic relaxation of the respiratory proton gradient, we supplemented the particles suspension, kept in the closed glass cell, with valinomycin and catalase and activated respiration with pulses of 1%−3% H_2O_2 (5 μL/mL) as described in the legend to Table 1. To measure P/O ratios of oxidative phosphorylation, we incubated the particles (1 mg of protein/mL) in a mixture containing the following: 200 mM sucrose, 10 mM K-succinate, 3 mM $MgCl_2$, 1 mM EDTA, 10 mM potassium phosphate, pH 7.4, 20 mM glucose, 5 units hexokinase, and 300 μM p^1, p^5 Di (Adenosin-5-) pentaphosphate (to inhibit adenylate kinase). MgADP (300 μM) was added, and the respiratory rate in state 3 was measured for 5 min. To measure the ATP synthesized in this interval, 500 μL of the submitochondrial particles suspension was treated with 500 μL of 28% perchloric acid (w/v). After centrifugation at 20000*g*, the supernatant was cooled in ice and neutralized with 60% KOH (w/v), then cleared by centrifugation. The neutralized suspension (700 μL) was added to a mixture containing the following: 1 mM $MgCl_2$, 150 mM Tris/HCl, pH 7.4, and 7 units glucose-6 phosphate dehydrogenase. NADP (1 mM) was added and glucose-6-phosphate was determined following spectrophotometric NADP reduction at 340 nm. The values reported in the Table are the means of 4 experiments ± S.E.M. [b] ESMP-AS: submitochondrial particles incubated in anaerobiosis. [c] ESMP-RS: submitochondrial particles incubated in the succinate-respiring state. [d] MgATP-SMP-AS: submitochondrial particles incubated in anaerobiosis. [e] MgATP-SMP-RS: submitochondrial particles incubated in the succinate-respiring state.

structure of the F_oF_1-ATP synthase, based on electron microscopy studies (*1*, *13*, *59*−*61*) according to which the F_oI-PVP(b), γ, OSCP (δ in *E. coli*), δ (ϵ in *E. coli*) and other mitochondrial supernumerary subunits are assembled in a single stalk, it has recently been proposed, on the basis of cross-linking data in the *E. coli* F_oF_1 complex, that the b and δ subunit of the proposed stator in *E. coli* (F_oI-PVP(b) and OSCP in mitochondria) constitute a second lateral stalk separate from the γ and ϵ (δ in mitochondria) rotor which constitute the central stalk (*3*, *33*, *55*). Recent average analysis of electron microscopy images of *E. coli* F_oF_1-ATP synthase shows, in addition to the fatter central stalk, a lateral thin structure which is considered to represent a second stalk made up by the F_o-b and F_1-δ subunits (*62*).

The present finding that, in the absence of a respiratory $\Delta\mu H^+$, diamide induces disulfide bridging of Cys 197 in the carboxyl-terminal region of F_oI-PVP(b) with Cys 91 at the C-terminus of the lateral short α-helix (residues 73−90) of γ shows that these two domains, at least in the resting enzyme in the absence of a transmembrane $\Delta\mu H^+$, are in direct contact rather then being separated as it would be suggested by the recent electron microscopy images of the *E. coli* F_oF_1-ATPase (*62*). The prevention of diamide-induced disulfide cross-linking of F_oI-PVP(b) and γ subunit, when the $\Delta\mu H^+$ generated by respiration relaxes through the F_o channel, provides evidence showing that the torque generated by the proton flow generates a relative motion and/or a separation of these two subunits which places the two cysteine residues at a distance at which they can no longer be engaged in disulfide bridging.

In the resting enzyme diamide also induces disulfide cross-linking of OSCP with another component of F_o. This is not subunit c as shown by the lack of diamide effect on the level of the [^{14}C]-DCCD label of the band of Mr 8000 of this subunit. The two possible candidates are subunits d or f of F_o, both of which have a single cysteine residue. Cross-linking of OSCP with either of these two subunits, which is also prevented by proton flow through F_o, has no significant effect on proton conduction in F_o as, in fact, expected when cross-linking occurs between two components of the stator element of the rotary motor.

REFERENCES

1. Gogol, E. P., Lucken, U., and Capaldi, R. A. (1987) *FEBS Lett. 219*, 274−278.
2. Cox, G. B., Devenish, R. J., Gibson, F., Howitt, S. M., and Nagley, P. (1992) *Molecular Mechanism in Bioenergetics* (Ernster, L., Ed.) pp 283−315, Elsevier, Amsterdam, The Netherlands.
3. Ogilvie, I., Aggeler, R., and Capaldi, R. A. (1997) *J. Biol. Chem. 272*, 16652−16656.
4. Papa, S., Xu, T., Gaballo, A., and Zanotti, F. (1998) in *Frontiers of Cellular Bioenergetics: Molecular Biology, Biochemistry and Physiopathology* (Papa, S., Guerrieri, F., and Tager, J. M., Eds.) Plenum Press, London, New York (in press).
5. Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) *Nature 370*, 621−628.
6. Boyer, P. D. (1997) *Annu. Rev. Biochem. 66*, 717−749.
7. Duncan, T. M., Bulygin, V. V., Zhou, Y., Hutcheon, M. L., and Cross, R. L. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 10964−10968.
8. Sabbert, D., Engelbrecht, S., and Junge, W. (1996) *Nature 381*, 623−625.
9. Noji, H., Yasuda, R., Yoshida, M., and Kinosita, K. (1997) *Nature 386*, 299−302.
10. Schneider, E., and Altendorf, K. (1987) *Microbiol. Rev. 51*, 477−497.
11. Fillingame, R. H. (1996) *Curr. Opin. Struct. Biol. 6*, 491−498.
12. Howitt, S. M., Rodgers, A. J. W., Hatch, L. P., Gibson, F., and Cox, G. B. (1996) *J. Bioenerg. Biomembr. 28*, 415−420.
13. Capaldi, R. A., Aggeler, R., Turina, P., and Wilkens, S. (1994) *Trends Biochem. Sci. 19*, 284−289.
14. Wilkens, S., Dahlquist, F. W., McIntosh, L. P., Donaldson, L. W., and Capaldi, R. A. (1995) *Nat. Struct. Biol. 2*, 961−967.
15. Schneider, E., and Altendorf, K. (1985) *Eur. J. Biochem. 153*, 105−109.
16. Houstek, J., Kopecky, J., Zanotti, F., Guerrieri, F., Jirillo, E., Capozza, G., and Papa, S. (1988) *Eur. J. Biochem. 173*, 1−8.
17. Papa, S., Guerrieri, F., Zanotti, F., Houstek, J., Capozza, G., and Ronchi, S. (1989) *FEBS Lett. 249*, 62−66.
18. Walker, J. E., Lutter, R., Dupuis, A., and Runswick, M. J. (1991) *Biochemistry 30*, 5369−5378.
19. Herrmann, R. G., Steppuhn, J., Herrmann, G. S., and Nelson, N. (1993) *FEBS Lett. 326*, 192−198.
20. Collinson, I. R., Runswick, M. J., Buchanan, S. K., Fearnley, I. M., Skehel, J. M., van Raaij, M. J., Griffiths, D. E., and Walker, J. E. (1994) *Biochemistry 33*, 7971−7978.
21. Belogrudov, G. I., Tomich, J. M., and Hatefi, Y. (1996) *J. Biol. Chem. 271*, 20340−20345.
22. Zanotti, F., Guerrieri, F., Capozza, G., Houstek, J., Ronchi, S., and Papa, S. (1988) *FEBS Lett. 237*, 9−14.
23. Heckman, C., Tomich, J. M., and Hatefi, Y. (1991) *J. Biol. Chem. 266*, 13564−13571.
24. Collinson, I. R., Fearnley, I. M., Skehel, J. M., Runswick, M. J., and Walker, J. E. (1994) *Biochem. J. 303*, 639−645.
25. Papa, S., Guerrieri, F., Zanotti, F., Fiermonte, M., Capozza, G., and Jirillo E. (1990) *FEBS Lett. 272*, 117−120.
26. Dupuis, A., Issartel, J. P., Lunardi, J., Satre, M., and Vignais, P. V. (1985) *Biochemistry 24*, 728−733.
27. Archinard, P., Godinot, C., Comte, J., and Gautheron, D. C. (1986) *Biochemistry 25*, 3397−3404.
28. Zanotti, F., Guerrieri, F., Capozza, G., Fiermonte, M., Berden, J., and Papa, S., (1992) *Eur. J. Biochem. 208*, 9−16.
29. Belogrudov, G. I., Tomich, J. M., and Hatefi, Y. (1995) *J. Biol. Chem. 270*, 2053−2060.
30. Watts, S. D., Zhang, Y., Fillingame, R. H., and Capaldi, R. A. (1995) *FEBS Lett. 368*, 235−238.
31. Collinson, I. R., van Raaij, M. J., Runswick, M. J., Fearnley, I. M., Skehel, J. M., Orriss, G. L., Miroux, B., and Walker, J. E. (1994) *J. Mol. Biol. 242*, 408−421.
32. Zhou, Y., Duncan, T. M., and Cross, R. L. (1997) *Proc. Natl. Sci. U.S.A. 94*, 10583−10587.
33. Junge, W., Lill, M., and Engelbrecht, S. (1997) *Trends Biochem. Sci. 22*, 420−423.
34. Low, H., and Vallin, J. (1963) *Biochim. Biophys. Acta 69*, 361−374.
35. Lee, C. P., and Ernster, L. (1968) *Eur. J. Biochem. 3*, 391−400.
36. Racker, E., and Hostman, L. L. (1967) *J. Biol. Chem. 242*, 2547−2551.
37. Guerrieri, F., and Papa, S. (1981) *J. Bioenerg. Biomembr. 13*, 393−400.
38. Guerrieri, F., Capozza, G., Houstek, J., Zanotti, F., Colaianni, G., Jirillo, E., and Papa, S. (1989) *FEBS Lett. 250*, 60−66.
39. Beechey, R. B., Hubbard, S. A., Linnett, P. E., Mitchell, A. D., and Munn, E. A. (1975) *Eur. J. Biochem. 242*, 2547−2551.
40. Zanotti, F., Guerrieri, F., Che, Y. W., Scarfò, R., and Papa, S. (1987) *Eur. J. Biochem. 164*, 517−523.
41. Okamoto, M., Sone, H., Hirata, H., Yoshida, H., and Kagawa, Y. (1977) *J. Biol. Chem. 252*, 6125−6131.
42. Pansini, A., Guerrieri, F., and Papa, S. (1978) *Eur. J. Biochem. 92*, 545−551.
43. Papa, S., Guerrieri, F., and Rossi Bernardi, L. (1978) *Methods in Enzymology* LV, 614−627.
44. Estabrook, R. W. (1967) *Methods in Enzymology* X, 41−47.
45. Papa, S., Tager, J. M., Guerrieri, F., and Quagliariello, E. (1969) *Biochim. Biophys. Acta 172*, 184−186.
46. McKinney, M. M., and Parkinson, A. (1987) *J. Immunol. Methods 96*, 271−278.
47. Hensel, M., Deckers-Hebestreit, G., Schmid, R., and Altendorf, K. (1990) *Biochim. Biophys. Acta 1016*, 63−70.
48. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
49. Kosower, E. M., Corea, W., Kinon, B. J., and Kosower, W. S. (1972) *Biochim. Biophys. Acta 264*, 39−44.
50. Papa, S., Guerrieri, F., Simone, S., Lorusso, M., and Larosa, D. (1973) *Biochim. Biophys. Acta 292*, 20−38.
51. Papa, S., Lorusso, M., and Capitanio, N. (1994) *J. Bioenerg. Biomembr. 26*, 609−618.
52. Cocco, T., Lorusso, M., Di Paola, M., Minuto, M., and Papa, S. (1992) *Eur. J. Biochem. 209*, 475−481.
53. Capitanio, N., Capitanio, G., Demarinis, D. A., De Nitto, E., Massari, S., and Papa, S. (1996) *Biochemistry 35*, 10800−10806.
54. Collinson, I. R., Skehel, J. M., Fearnley, I. M., Runswick, M. J., and Walker, J. E. (1996) *Biochemistry 35*, 12640−12646.
55. Wilkens, S., Dunn, S. D., Chandler, J., Dahlquist, F. W., and Capaldi, R. A. (1997) *Nat. Struct. Biol. 4*, 198−201.
56. Elston, T., Wang, H., and Oster, G. (1998) *Nature 391*, 510−513.
57. McCarty, R. E., and Nalin, C. M. (1986) *Plant Physiol. 19*, 576−583.
58. Moroney, J. V., and McCarty, R. E. (1979) *J. Biol. Chem. 254*, 8951−8955.
59. Fernandez-Moran, H. (1962) *Circulation 26*, 1039−1065.
60. Lucken, U., Gogol, E. P., and Capaldi, R. A. (1990) *Biochemistry 29*, 5339−5343.
61. Walker, J. E., and Collinson, I. R. (1994) *FEBS Lett. 346*, 39−43.
62. Wilkens, S., and Capaldi, R. A. (1998) *Nature 393*, 29.

BI981422C