The γ subunit of F_1 and the PVP protein of F_o (F_oI) are components of the gate of the mitochondrial F_oF_1 H^+-ATP synthase

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The γ subunit of the F_1 moiety of the bovine mitochondrial H^+-ATP synthase is shown to function as a component of the gate. Addition of purified γ subunit to F_o-liposomes inhibits transmembrane proton conduction. This inhibition can be removed by the bifunctional thiol reagent diamide. Immunoblot analysis shows that the diamide effect is likely due to disulphide bridging of the γ subunit with the PVP protein of the F_o sector.

H^+-ATP synthase; Diamide; Thiol group; F_1; F_o

1. INTRODUCTION

The H^+-ATP synthase of coupling membranes (EC 3.6.1.34) is made up of three parts: (i) the catalytic sector of F_1 universally consisting of 5 subunits (α_3, β_3, γ, δ and ϵ); (ii) the H^+-translocating, membrane integral sector, F_o (with a variable number of subunits); (iii) the stalk [1] (gate or coupling sector) made up by some F_1 and F_o components [1-4]. In the mitochondrial enzyme the stalk includes also additional proteins such as OSCP [5], F_6 [6] and, possibly, the ATPase protein inhibitor [7].

Previous work from this laboratory has shown that the carboxyl region of the PVP protein (F_oI) [8], a membrane-integral component of F_o [9,10], extends in the stalk where it is covered by F_1 subunits [11]. The PVP protein is involved in the correct binding of F_1 to F_o and modulates the conformation, activity and sensitivity to oligomycin [11] and DCCD [12] of the transmembrane H^+ channel in F_o.

Observations are available in literature showing that in bacteria [13] and chloroplasts [14] the γ subunit of F_1, is involved in the gate function of the ATP synthase. In the chloroplast enzyme the γ subunit has two cysteines (that in position 91 is conserved in all the species examined [15]), whose oxidation to form a disulphide bond is involved in the light-dependent thioredoxin regulatory system [16].

It is shown, in this paper, that in the bovine-heart mitochondrial H^+-ATP synthase the γ subunit functions as a component of the gate. Addition of purified γ subunit to F_o-liposomes inhibits H^+ conduction. Treatment with diamide of submitochondrial particles (see also [17]) and F_o liposomes reconstituted with γ subunit induces a dramatic enhancement of H^+ conduction, apparently due to disulphide bridging of the γ subunit with the PVP protein.

2. MATERIALS AND METHODS

Chaps, oligomycin, valinomycin and asolectin were obtained from Sigma; SDS, acrylamide and *N,N'*-methylenebisacrylamide, goat anti-rabbit IgG labeled with peroxidase, horseradish peroxidase color development reagent from Bio-Rad; nitrocellulose membranes (0.45 μm pore size) from Schleicher and Schuell.

2.1. *Preparations*

Heavy bovine heart mitochondria were prepared as described in [18] and F_1 depleted urea particles (USMP) as in [19]. F_o was isolated by Chaps solubilization from USMP [12]. F_o subunits were isolated by preparative gel electrophoresis [20]. Purification of F_1 was performed by a modification [12] of the chloroform extraction procedure [21]. F_o-liposomes were prepared as in [22].

2.2. *Electrophoresis and immunoblot procedures*

SDS-PAGE and immunoblot analysis were performed as in [11,22]. PVP protein of F_o and γ subunit of F_1 were isolated as in [20] and were used for immunization of rabbits as in [11] and/or for reconstitution experiments.

2.3. *Assays*

H^+ translocation in submitochondrial particles and F_o-liposomes was analyzed potentiometrically, as in [22]. Proteins were estimated by a modified Lowry-Folin assay [23].

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Abbreviations: Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; SDS-PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis; USMP; urea-treated submitochondrial particles; DCCD, *N,N'*-dicyclohexylcarbodiimide; NEM, *N*-ethylmaleimide; PVP, protein (F_oI), OSCP, subunits of the membrane sector F_o

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3. RESULTS

In bovine heart submitochondrial particles oxidation of vicinal dithiols to disulphides by diamide results in stimulation of oligomycin-sensitive ATPase activity and passive H^+ conduction [17]. The enhancement of H^+ conduction appears to depend on the presence of F_1 subunits in the particles, being less pronounced in particles partially depleted of these subunits [17].

Fig. 1 presents a titration of the effect of diamide on H^+ conduction in submitochondrial particles partially depleted of F_1 subunits by urea (USMP) and in liposomes reconstituted with purified F_o (see Fig. 2). In USMP diamide caused, up to a critical concentration, a 3-fold stimulation of H^+ conduction. Higher concentrations of diamide started to depress H^+ conduction. In F_o liposomes, on the contrary, no enhancement of H^+ conduction was observed over a wide range of diamide concentrations. High concentrations of diamide, however, inhibited H^+ conduction (cf. [17,24]).

Immunoblot analysis with antisera against purified PVP protein and F_1 showed that, in USMP, the reaction with diamide caused a decrease of the bands of both the PVP protein and the γ subunit (Fig. 2). On the contrary, there was no detectable decrease of the PVP protein upon reaction of diamide with liposome-reconstituted with F_o, which showed no visible trace of F_1 subunits and in particular of γ subunit (Fig. 2).

In addition to the γ subunit and the PVP protein [25], also OSCP [26] and subunit c of mitochondrial F_o [27] have single cysteine residues. In the experiment shown in Table I, USMP, after treatment with diamide, was incubated with [^{14}C]NEM. The particles were then subjected to SDS-PAGE and radioactivity measured in the isolated bands. The results show that diamide caused a significant decrease of radioactive NEM in the γ and PVP bands. There was also a decrease of radioactive NEM in the band of OSCP and subunit c. Independent experiments showed, however, that diamide treatment did not affect at all the binding of [^{14}C]DCCD in subunit c (Table I), neither did it cause any decrease of the band of OSCP, detected by an antiserum raised against purified OSCP (not shown).

Fig. 3 shows that the enhancement of H^+ conduction induced in USMP by diamide treatment was correlated with a decrease of the antigenic bands of the γ subunit and of the PVP protein.

The experiment in Fig. 4 shows that addition of increasing concentrations of purified γ subunit to F_o-liposomes caused a progressive, marked inhibition of passive H^+ conduction (cf. [28]). Treatment of F_o-liposomes with diamide, which per se caused in controls about a 40% inhibition of H^+ conduction, dramatically enhanced H^+ conduction in the γ-supplemented F_o-liposomes (also in this case H^+ conduction was oligomycin sensitive). Immunoblot analysis (Fig. 4) showed that diamide treatment, which did not affect

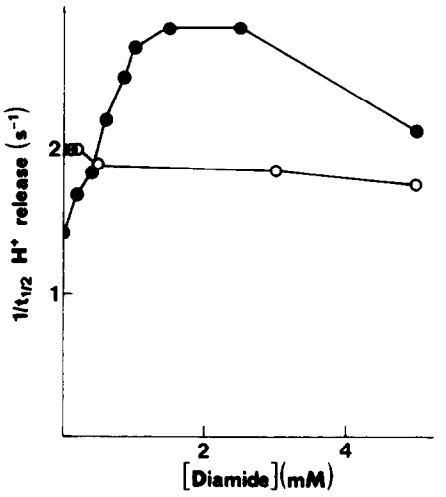

Fig. 1. Titration of the effect of diamide on H^+ conduction in USMP and F_o-liposomes. USMP (3 mg protein/ml) were incubated in a reaction mixture containing: 200 mM sucrose, 30 mM KCl, 0.5 μg valinomycin/mg particle proteins, 0.2 mg/ml catalase and 20 mM succinate (potassium salt), pH 7.5. Incubation was carried out in a stirred glass vessel under a constant stream of N_2 at 25°C . After anaerobiosis respiration driven H^+ translocation was activated by repetitive pulses of 1–3% H_2O_2 (5 μl/ml) and pH of the suspension was monitored potentiometrically. F_o-liposomes (0.7 mg protein/ml) were suspended in a mixture containing 0.15 M KCl. After 2 min, 2 μg valinomycin/ml was added and the H^+ release promoted by the K^+ diffusion potential was followed potentiometrically. Incubation procedure: (●—●) USMP, or (○—○) F_o-liposomes were preincubated for 2 min with diamide at the concentrations reported in the figure before the addition of H_2O_2 or of valinomycin.

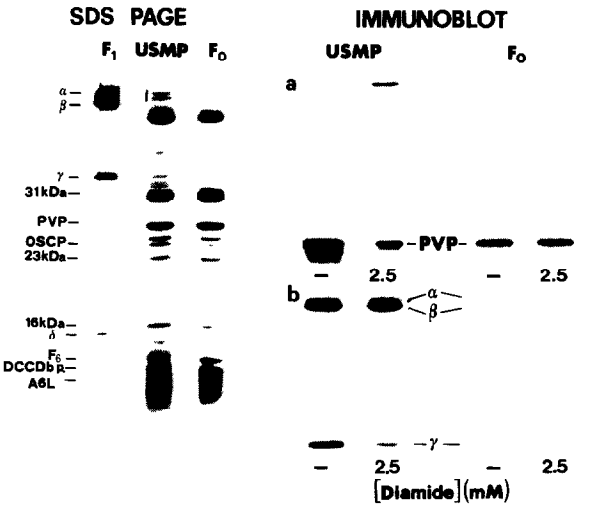

Fig. 2. SDS-PAGE of purified F_1, F_o and USMP and immunoblot analysis of bands of PVP and F_1 subunits in USMP and F_o after diamide treatment. For SDS-PAGE 15 μg of purified F_1, 25 μg of F_o and 50 μg of USMP were applied on SDS slab gels with a linear gradient of polyacrylamide (14–20%) and detected by Coomassie blue staining. For immunoblot analysis 100 μg of USMP and 25 μg of purified F_o, were subjected to SDS-PAGE, then protein bands were electro-transferred to nitrocellulose and tested with anti-PVP (a) or anti F_1 serum (b).

Table I

Effect of diamide on the binding of [^{14}C]NEM and [^{14}C]DCCD on subunits of H^+-ATP synthase

USMP (3 mg/ml) were incubated for 30 min with 3 mM [^{14}C]NEM or 30 μM [^{14}C]DCCD in 0.20 M sucrose, 20 mM K-succinate and 30 mM KCl (pH 7.4). Where indicated, a 2 min pretreatment with 2.5 mM diamide was carried out. The reaction was stopped by centrifugation at 105 000 × g and 300 μg of samples were subjected to SDS-PAGE. After staining and destaining slices of the protein bands were treated with Beckman tissue solubilizer 450 at 60°C for 12 h and radioactivity determined by liquid scintillation counting. The % reported in the Table indicate the relative change of cpm in each protein band after diamide treatment.

Additions	Subunits H^+-ATP synthase			
	γ (cpm %)	PVP (cpm %)	OSCP (cpm %)	c (cpm %)
[^{14}C]NEM	496	625	305	550
Diamide + [^{14}C]NEM	315 −37	420 −33	280 −8	445 −19
[^{14}C]DCCD				2450
Diamide + [^{14}C]DCCD				2600 +6

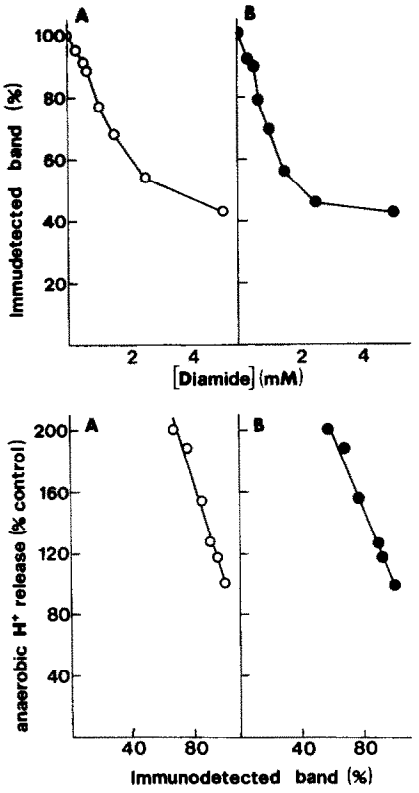

Fig. 3. Relationship between anaerobic H^+-release and % of immunoreactive bands of PVP and γ protein after diamide treatment of USMP. For diamide treatment see legend to Fig. 1. 100 μg of particle proteins were subjected to SDS-PAGE followed by immunoblot analysis with a specific serum anti-PVP (A) or anti-F_1 (B) as described in section 2. Immunodecorated blots were analyzed by densitometry using a Camag TLC scanner at 590 nm. The percent of antigen detected was evaluated from the computed peak areas taking as 100% the peak areas in untreated USMP. The rate of passive H^+ release from USMP was measured as in Fig. 1, the reciprocal value of $t_{\frac{1}{2}}$ of this process in untreated USMP was taken as 100%. (○—○) immunodetected PVP band; (●—●) immunodetected γ band.

the PVP band in control F_o-liposomes (see Fig. 2), produced, when purified γ subunit was added, a decrease of the bands of both the PVP protein of F_o and of γ subunit.

4. DISCUSSION

The facts presented provide direct evidence showing that in the mitochondrial H^+-ATP synthase the γ subunit of F_1 functions as a component of the gate for the H^+ channel in F_o. The γ subunit of H^+-ATP synthase is known to be essential for assembly and catalytic activity of F_1 [2,13]. The overall amino acid conservation in the γ subunit is low [15]. There are, however, three regions rather well conserved, i.e. those from 18–28, 88–92 and 250–299 [15]. Genetic evidence indicates that in *E. coli* the first portion in the amino-terminal region of γ subunit represents a hydrophobic domain important for assembly of the F_1 sector [13]. The carboxyl-region from 251–299, is relatively rich in acidic and basic residues [15]. It is possible that this segment of the γ subunit is in close contact with the carboxyl-terminal region of the PVP subunit. These segments of the two proteins can be supposed to interact through salt-bridges, involved in the controlled transfer of H^+ from the catalytic domain in F_1 to the F_o channel. The conserved cysteine-91 in the γ subunit and the cysteine-197 in the PVP protein are apparently in vicinal position as indicated by the observation that diamide induces disulphide bridging of the two subunits. This modification apparently alters the gate of the proton channel with dramatic enhancement of H^+ conduction. It would be interesting to verify whether the oxidation state of the two vicinal cysteines in the γ subunit and the PVP proteins may have some

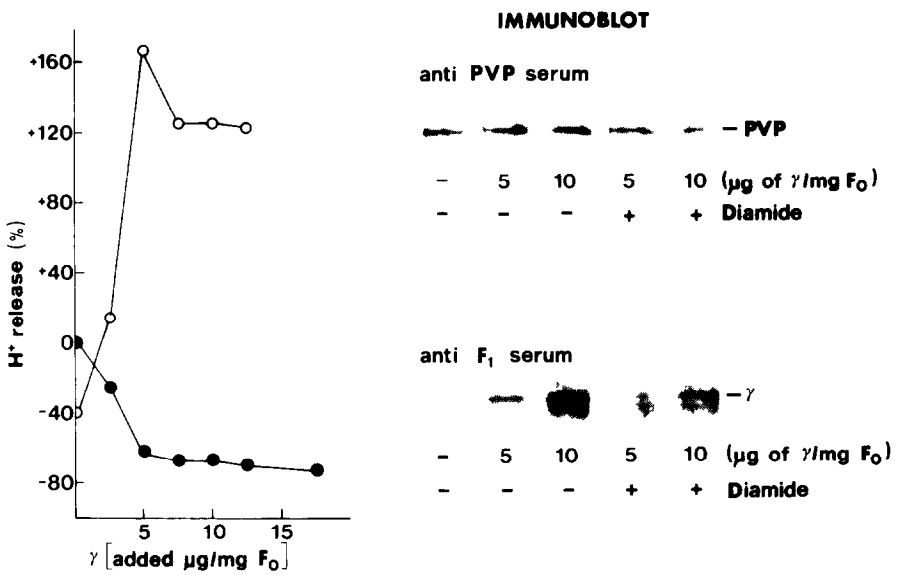

Fig. 4. Control of H^+ conduction in F_o-liposomes by γ subunit. Effect of diamide. Symbols: (●—●), purified γ subunit was added, at the concentrations reported in the figure, to F_o-liposomes and incubation was carried out at 25°C for 10 min before valinomycin addition. (○—○) F_o-liposomes reconstituted with γ subunit, at the concentrations reported in the figure, were treated with diamide (2.5 mM) for 2 min before valinomycin addition. Passive H^+ release was initiated by the addition of valinomycin as described in Fig. 1. The changes in the rate of this process (measured as $1/t_{\frac{1}{2}}$), caused by addition of γ subunit and treatment by diamide, are expressed as % decrease or increase with respect to the value exhibited by control F_o-liposomes. After valinomycin pulses, F_o-liposomes containing 50 μg of protein were washed in 90% acetone, centrifuged at 20000 × g and the pellet was solubilized in a mixture containing 2.3% sodium dodecylsulphate, 10 mM Tris-Cl, pH 6.8, and boiled for 3 min. After electrophoresis, the protein was transferred to nitrocellulose for immunoblot analysis. For other details see section 2.

regulatory role such as that played in chloroplasts by the two cysteines of the γ subunit [16].

REFERENCES

[1] Gogol, I.P., Lucken, V. and Capaldi, A. (1987) FEBS Lett. 219, 274–278.
[2] Senior, A.E. (1988) Physiol. Rev. 68, 177–232.
[3] Papa, S., Guerrieri, F., Scarfò, R. and Zanotti, F. (1987) in: Perspectives of Biological Energy Transduction (Mukohata, Y., Morales, M.F. and Fleischer, S. eds) pp. 431–440, Academic Press, Japan.
[4] Papa, S. (1989) in: Organelles of Eukaryotic Cells: Molecular Structure and Interactions (Tager, J.M., Azzi, A., Papa, S. and Guerrieri, F. eds) pp. 9–26, Plenum Press, New York.
[5] Pringle, M.J., Keanneally, M.K. and Joshi, S. (1990) J. Biol. Chem. 265, 7632–7637.
[6] Joshi, S. and Pringle, M.J. (1989) J. Biol. Chem. 264, 15548–15551.
[7] Papa, S., Guerrieri, F., Zanotti, F. and Capuano, F. (1989) in: Molecular Basis of Membrane-Associated Diseases (Azzi, A., Drahota, Z. and Papa, S. eds) pp. 347–358, Springer-Verlag, Berlin Heidelberg.
[8] Papa, S., Guerrieri, F., Zanotti, F., Houstek, J., Capozza, G. and Ronchi, S. (1989) FEBS Lett. 249, 62–66.
[9] Montecucco, C., Dabbeni-Sala, F., Friedl, P. and Galante Y.M. (1983) Eur. J. Biochem. 132, 189–194.
[10] Zanotti, F., Guerrieri, F., Capozza, G., Houstek, J., Ronchi, S. and Papa, S. (1988) FEBS Lett. 237, 9–14.
[11] Houstek, J., Kopecky, J., Zanotti, F., Guerrieri, F., Jirillo, E., Capozza, G. and Papa, S. (1988) Eur. J. Biochem. 173, 1–8.
[12] Guerrieri, F., Capozza, G., Houstek, J., Zanotti, F., Colaianni, G., Jirillo, E. and Papa, S. (1989) FEBS Lett. 250, 60–66.
[13] Futai, M., Noumi, T. and Maeda, M. (1989) Annu. Rev. Biochem. 58, 111–136.
[14] Schumann, J., Richter, M.L. and McCarty, R.E. (1985) J. Biol. Chem. 260, 11817–11823.
[15] Walker, J.E., Fearnley, I.M., Gay, N.J., Gibson, B.W., Northrop, F.D., Powell, S.J., Runswick, M.J., Saraste, M. and Tybulewicz, V.L.J. (1985) J. Mol. Biol. 184, 677–701.
[16] Mills, J.D. (1984) in: H^+-ATPase (ATP synthase): Structure, Function Biogenesis. The F_oF_1 Complex of Coupling Membranes (Papa, S., Altendorf, K., Ernster, L. and Packer, L. eds) pp. 349–358, ICSU Press, Adriatica Editrice.
[17] Zanotti, F., Guerrieri, F., Scarfò, R., Berden, J. and Papa, S. (1985) Biochem. Biophys. Res. Commun. 132, 985–990.
[18] Low, H. and Vallin, I. (1963) Biochim. Biophys. Acta 69, 361–364.
[19] Racker, E. and Horstmann, L.L. (1967) J. Biol. Chem. 242, 2547–2551.
[20] Zanotti, F., Guerrieri, F., Che, Y.W., Scarfò, R. and Papa, S. (1987) Eur. J. Biochem. 164, 517–523.
[21] Beechey, R.B., Hubbard, S.A., Linnett, P.E., Mitchell, A.D. and Munn, E.A. (1975) Biochem. J. 148, 533–537.
[22] Guerrieri, F., Kopecky, J. and Zanotti, F. (1989) in: Organelles in Eukaryotic Cells (Tager, J.M., Azzi, A., Papa, S. and Guerrieri, F.) pp. 197–208, Plenum Press, New York.
[23] Hess, H.H., Lees, M.B. and Derr, J.E. (1978) Anal. Biochem. 85, 295–300.
[24] Huang, Y., Pringle, M.J. and Sanadi, D.R. (1985) FEBS Lett. 192, 83–87.
[25] Walker, J.E., Runswich, M.J. and Poulter, L. (1987) J. Mol. Biol. 197, 89–100.
[26] Walker, J.E., Gay, N.J., Powell, S.J., Kostina, M. and Dyer, M.R. (1987) Biochemistry 26, 8613–8619.
[27] Gay, N.J. and Walker, J.E. (1985) EMBO J. 4, 3519–3524.
[28] Pet, S. and Brusilow, W. (1989) J. Biol. Chem. 264, 2640–2644.