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ATP Synthase Complex from Beef Heart Mitochondria

ROLE OF THE THIOL GROUP OF THE 25-kDa SUBUNIT OF F_O IN THE COUPLING MECHANISM BETWEEN F_O AND F_1*

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In order to assess the role of thiol groups in the F_O part of the ATP synthase in the coupling mechanism of ATP synthase, we have treated isolated F_O, extracted from beef heart Complex V with urea, with thiol reagents, primarily with diazenedicarboxylic acid bis-(dimethylamide) (diamide) but also with Cd^{2+} and *N*-ethylmaleimide. F_OF_1 ATP synthase was reconstituted by adding isolated F_1 and the oligomycin-sensitivity-conferring-protein (OSCP) to F_O. The efficiency of reconstitution was assessed by determining the sensitivity to oligomycin of the ATP hydrolytic activity of the reconstituted enzyme.

Contrary to Cd^{2+}, incubation of diamide with F_O, before the addition of F_1 and OSCP, induced a severe loss of oligomycin sensitivity, due to an inhibited binding of F_1 to F_O. This effect was reversed by dithiothreitol. Conversely, if F_1 and OSCP were added to F_O before diamide, no effect could be detected. These results show that F_1 (and/or OSCP) protects F_O thiols from diamide and are substantiated by the finding that the oligomycin sensitivity of ATP hydrolysis activity of isolated Complex V was also unaltered by diamide.

Gel electrophoresis of F_OF_1 ATP synthase, reconstituted with diamide-treated F_O, revealed that the loss of oligomycin sensitivity was directly correlated with diminution of band F_O 1 (or subunit b). Concomitantly a band appeared of approximately twice the molecular weight of subunit F_O 1. As this protein contains only 1 cysteine residue (Walker, J. E., Runswick, M. J., and Poulter, L. (1987) *J. Mol. Biol.* 197, 89–100), the effect of diamide is attributed to the formation of a disulfide bridge between two of these subunits.

These results offer further evidence for the proposal, based on aminoacid sequence and structural analysis, that subunit F_O 1 of mammalian F_O is involved in the binding with F_1 (Walker *et al.* (1987)).

***N*-Ethylmaleimide affects oligomycin sensitivity to a lesser extent than diamide, suggesting that the mode of action of these reagents (and the structural changes induced in F_O) is different.**

It is well known that sulfydryl groups play an important role in the maintenance of the integrity of biological membranes (1) and in particular of the mitochondrial inner membrane (for a review see 2, 3). Indeed, modification of mitochondrial membrane thiol groups can lead to malfunction of a number of integral membrane proteins; for example, the carriers for phosphate and adenine nucleotides are severely impaired when mitochondria are treated with alkylating agents such as maleimide derivatives (2, 4–6).

As for the mitochondrial ATP synthase, the enzyme complex which catalyses the synthesis and hydrolysis of ATP, it has been reported that thiol groups, belonging to F_O[1] (7), are involved in the coupling mechanism between F_O, the membrane integrated sector, and F_1, the hydrophilic part of the enzyme bearing the catalytic sites (8). What has emerged so far is the presence of functionally important pairs of contiguous thiol groups (dithiols). Their location and role are, however, still controversial. In fact it is not yet clear whether they are uniquely located on the F_O subunit known as factor B (9) or are also elsewhere on F_O (10). There is also uncertainty whether oxidation of the dithiols of factor B enhances (11) or inhibits (11, 12) proton flow through the membrane sector.

The effect of oxidizing the vicinal thiols of F_O by means of specific agents such as diamide (for a discussion of the reaction mechanism see "Results and Discussion") has been studied using inverted inner mitochondrial membranes with F_1 either bound or intentionally displaced (10–12). Similar investigations have also been made on ATP synthases purified by different methods (10, 12). In some cases the results have been compared with those obtained on F_O extracted from ATP synthases and then inserted into liposomes (11, 12). However, the information obtained cannot be treated with confidence, since artifactual changes may occur during extraction of the ATP synthase or purification of F_O, in ways which may differ with the actual method used.

Because of this difficulty, we have studied the effect of oxidizing thiols of F_O, with diamide, by incubating F_O with the reagent either before or after the reconstitution of a complete F_OF_1 ATP synthase. This was achieved by adding F_1 and OSCP to F_O. Then the coupling mechanism of the enzyme complex, reconstituted under the two different conditions, was tested by assaying the sensitivity of the F_1-mediated hydrolysis of ATP to oligomycin, using the fact that oligomycin, by binding to F_O, exerts an inhibitory effect on F_1 (13).

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[1] The abbreviations are: F_O, hydrophobic part of ATP synthase; diamide, diazenedicarboxylic acid bis-(dimethylamide); NEM, *N*-ethylmaleimide; DTT, dithiothreitol; Tricine, *N*-tris(hydroxymethyl)-methylglycin; DCCD, *N,N'*-dicyclohexylcarbodiimide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide-gel electrophoresis; F_1, coupling factor 1 of ATP synthase; OSCP, oligomycin-sensitivity-conferring protein; Complex V, ATP synthase isolated according to Ref. 20); F_OF_1 ATP synthase, ATP synthase reconstituted from isolated F_O, F_1, and OSCP.

This paper shows that if F_O is treated with diamide, before the addition of F_1 and OSCP, this leads to a severely decreased sensitivity to oligomycin of the F_1-mediated hydrolysis of ATP. Under these conditions, the perturbation of the structure of F_O is such that F_1 no longer binds to the membrane sector. On the other hand, binding of F_1 and/or OSCP to F_O, prior to the addition of diamide, protects F_O from reaction with the reagent. SDS-PAGE of F_OF_1 ATP synthase (reconstituted with diamide-treated F_O) shows that subunit F_O 1 (named according to Ref. 14) (or subunit b, according to Ref. 15), which contains only one thiol group (15), partly disappears, possibly because it dimerizes in the presence of diamide. Therefore, this subunit of F_O is likely to be involved in the structural link with F_1, as suggested for mammalian (15, 47) and for the homologous bacterial (16, 17) and yeast (18) subunit.

Cd^{2+}, another, but more hydrophilic, dithiol modifier, is without effect under all conditions, while NEM, a monothiol reagent, affects only slightly the sensitivity to oligomycin of the reconstituted ATP synthase. This latter piece of data, together with electrophoretic pattern of diamide-treated F_O, point to the different structural alterations induced by (and thus to the probably different mode of reaction of) NEM and diamide.

EXPERIMENTAL PROCEDURES

Methods

Preparations of Complex V, F_O, F_1, and OSCP—ATP synthase (Complex V) was prepared from beef heart mitochondria, isolated as in Ref. 19, according to Stiggall *et al.* (20). F_1 was prepared as in Ref. 21. Pig heart OSCP was a kind gift of the Laboratory of Biology and Technology of Membranes of Centre National de la Recherche Scientifique, University C. Bernard, Lyon and bovine heart OSCP was prepared according to Ref. 22. F_O was extracted in presence of 4 M urea (23). F_O, suspended in 0.25 M sucrose, 50 mM Tris-acetate (pH 7.5) at a concentration of 5–15 mg/ml, was frozen in liquid nitrogen and stored at −80 °C. Unless otherwise stated, all preparations of F_O employed were derived from Complex V.

Reconstitution of F_O into Asolectin Vesicles—For reconstituting F_O into phospholipid vesicles, the dialysis method described in Ref. 24 was followed but with some modifications.

30 mg/ml (1 mg of phospholipid phosphorous/ml) of partially purified soybean lipids (asolectin) were sonicated in 10 mM Tricine/NaOH (pH 8), 7.5 mM DTT, 0.2 mM EDTA, and 0.5% sodium cholate. Sodium deoxycholate was omitted and sodium cholate was 0.5% instead of 1.6% because it was found that addition of liposomes prepared in their combined presence or with 1.6% sodium cholate only, inhibited the activity of free F_1 in solution (though to different extents), perhaps because of incomplete removal of the detergents at the recommended concentrations (24). This problem did not arise with liposomes prepared in the presence of 0.5% sodium cholate, when no inhibition was observed.

2 mg/ml of isolated F_O were added to the vesicles which were then dialyzed against 10 mM Tricine/NaOH (pH 8), 2.5 mM $MgSO_4$, 0.25 mM DTT, and 0.2 mM EDTA at 4 °C, with changes of the medium after 1 and 4 h from the start of the dialysis. The dialysate was frozen in liquid nitrogen and stored at −80 °C.

Reconstitution of F_OF_1 ATP Synthase in the Presence and Absence of Diamide or NEM—All incubations were carried out at 30 °C. Dialyzed F_O-containing liposomes (in 10 mM Tricine/NaOH (pH 8), 2.5 mM $MgSO_4$, 0.25 mM DTT, 0.2 mM EDTA) were incubated (except otherwise indicated) for 15 min in the presence and absence of 2 mM diamide. The amount of diamide added was 0.25 mM more than specified here and throughout the text to allow for its very rapid reaction with the 0.25 mM DTT present in the suspension medium. F_1 and OSCP were then added to the mixtures, which were incubated for 20 min. The total volume of the additions to F_O-containing liposomes was usually less than 12% of the latter, thus the composition of the incubation medium was virtually that of the dialysis medium. Aliquots were then transferred into tubes containing oligomycin (in ethanol). The final concentration of oligomycin used in all experiments was 6 μg/mg total protein (final ethanol concentration, 2% v/v). All samples were incubated for a further 10 min. The final volume of the incubation mixtures varied from 35 to 45 μl. When NEM (2.4 mM) was used, in the presence or absence of diamide, the same procedure was followed.

Where indicated, 7 mM DTT was added to the samples and allowed to incubate for a further 10 min; alternatively, F_1 or OSCP, before being added to untreated F_O, was incubated for 15 min with 2.5 mM diamide. In these latter cases, 0.25 mM DTT was not present in the incubation mixtures.

When samples were to be used also for SDS-PAGE, the conditions for reconstitution differed, as detailed later.

Unless otherwise specified, the final protein concentrations/ml were: 1.65 mg of F_O, 0.45 mg of F_1, 0.1 mg of OSCP. Phospholipids were present at a concentration of 1 mg of phospholipid phosphorous/ml.

Throughout the text, the term F_OF_1 ATP synthase will be used to mean the ATP synthase reconstituted by adding together isolated F_O, F_1, and OSCP, as described here.

Incubation of F_OF_1 ATP Synthase with Diamide—F_O-containing liposomes, F_1 and OSCP, were incubated together at 30 °C for 30 min, after which 2 mM diamide was added and the incubation continued for a further 15 min. Otherwise the procedure was as described above.

Reconstitution of Complex V into Asolectin Vesicles and Incubation with Diamide—Asolectin vesicles, prepared in the presence of 0.5% sodium cholate and dialyzed subsequently, as described above, were added to Complex V. This procedure was chosen because Complex V can be reconstituted directly (and more efficiently) as already reported in Ref. 20. The final Complex V concentration was 1.6 mg of protein/ml, that of phospholipids 1 mg of phospholipid phosphorous/ml. The volume of Complex V added to the vesicles was minimal, thus here also the incubation medium was the dialysis medium. This mixture was incubated for 30 min at 30 °C. Aliquots (approximately 35 μl) were then incubated for 2 or 30 min in the presence and absence of 1.8 mM diamide. The protein concentration of Complex V in presence of diamide was 1.5 mg/ml. Oligomycin (in ethanol) was then added at a concentration of 6 μg/mg protein and incubated as described above (final ethanol concentration, 2% v/v).

When Complex V had to be run on gel, the conditions were as described later.

Determination of Unbound F_1—F_O-containing liposomes were incubated for 15 min in the presence or absence of 2.1 mM diamide. When present, DTT (9 mM) was incubated with F_O at this stage. F_1 and OSCP were then added and incubated as described previously. Aliquots of the mixtures were assayed for ATP hydrolytic activity (see later) in the presence and absence of oligomycin while the rest was centrifuged for 10 min at 10,000 × *g*, at 25 °C. The supernatant was collected and its volume determined. The pellet was then suspended in a same volume of 10 mM Tricine/NaOH (pH 8.0), 2.5 mM $MgSO_4$, 0.25 mM DTT, 0.2 mM EDTA. Both the supernatant and the pellet suspension were incubated for a further 10 min at 30 °C (the latter with and without oligomycin) before being assayed for ATPase activity.

Measurement of Proton Conductance through F_O—Proton flow through F_O was measured at 25 °C with a pH electrode, by following proton release from F_O-containing liposomes induced by a diffusion potential (positive inside) imposed by valinomycin-mediated potassium influx, as described in Ref 25.

Dialyzed F_O-containing liposomes were pelletted at 100,000 × *g* for 15 min and then suspended in 20 mM sucrose, 2.5 mM $MgSO_4$, 0.2 mM Tricine/NaOH (pH 8.3) (24). 75 μl of this suspension, containing 0.14 mg of F_O and 0.087 mg of phospholipid phosphorous, were incubated at 30 °C for 13 min in the presence and absence of 2 mM diamide. The samples were then immediately transferred to a pH electrode cell containing 0.9 ml of the suspending medium plus 0.16 M KCl. 2 mM diamide was also present in those assays where diamide-treated F_O was added. Proton release from F_O-containing liposomes was initiated by addition of 2 μl of 1 mg/ml ethanol solution of valinomycin. When indicated, the incubation in the presence and absence of diamide was prolonged for 10 min in the presence and absence of 50 μM of a freshly prepared DCCD (in ethanol). When the effect of DCCD on proton release was tested, DCCD (50 μM) was also present in the pH electrode cell. In all cases, the final maximum ethanol concentration was 0.7% (v/v).

Gel Electrophoresis—A modified (14) Laemmli method (26) was used for SDS-PAGE of F_OF_1 ATP synthase in which F_O had been incubated in the presence or absence of diamide. In order to compare, in the same sample, the effect of the reagent on both the oligomycin

sensitivity and the structure of the enzyme, gels were run with aliquots of F_OF_1 ATP synthase whose oligomycin sensitivity had been previously assayed. High concentrations of lipids interfere with a good resolution of the gel; hence F_O-containing liposomes had to be replaced by stock F_O, which was diluted to 3.7 mg/ml with a medium of the same composition as the dialysis medium (see before). F_1 and OSCP were added so as to maintain the same protein weight ratio used with F_O inserted into liposomes. Incubation steps and oligomycin concentration were as described previously. When present, DTT (10 mM) was incubated after the addition of F_1 and OSCP. At the end of the final incubation (with or without diamide), aliquots were tested for oligomycin sensitivity while the rest of the incubation was centrifuged, for 10 min at room temperature, in a Beckman bench centrifuge run at the maximal speed. The pellets were suspended in the dialysis medium (without DTT) to a final volume of 10–15 μl and combined with an equal volume of 10 mM Tris acetate (pH 8.2) containing 4% SDS and 0.1 mM EDTA. 2–3 μl of a saturated pyronine solution (in 80% glycerol, v/v) were also added. As the effect of diamide had to be ascertained, β-mercaptoethanol was omitted even from the control samples. Electrophoresis was performed in a 20-cm long, linear gradient 13–19% polyacrylamide gel with a 1-cm 5% stacking gel. 15% sucrose was included in the stacking gel, whereas the running gel contained a 0–24% linear sucrose gradient. Slab gels were run at 12 mA/slab for 15 h at temperatures equal to or higher than 23 °C. Gels were stained with 0.25% Coomassie Blue in 45% methanol and 9% acetic acid and destained electrophoretically in 5% methanol and 7.5% acetic acid.

When Complex V was used, here also asolectin vesicles were not added. Protein concentration was 3.7 mg/ml. The composition of the medium, the incubation periods, oligomycin sensitivity assay, and the preparation of gel samples were as detailed above.

For spectral measurements of Coomassie Blue-stained spots, at 570 nm, a Shimadzu Dual-wavelength Chromato Scanner CS-930 was used.

ATP Hydrolysis and Protein Assays—The release of ADP was monitored spectophotometrically, at 340 nm, by measuring NADH oxidation in a coupled lactate dehydrogenase-pyruvate kinase reaction and ATP regenerating system, at 30 °C (20). When oligomycin-treated samples were assayed, 2 μg of oligomycin/ml were also added to the cuvette. The final ethanol concentration was 0.2% (v/v). Assays were initiated by adding 9 μg of F_OF_1 ATP synthase or 2 μg of purified F_1 or 12 μg of Complex V/ml of reaction mixture.

Protein concentration was determined according to Lowry *et al.* (27) with crystalline bovine serum albumin as standard.

Materials

Soybean phospholipids (asolectin) were obtained from Sigma and partially purified by washing with acetone followed by extraction into ethylether. They were stored in the dark, at −80 °C. Tricine, EDTA, and cholic acid were obtained from Merck (Darmstadt, Federal Republic of Germany, F.R.G.). Cholic acid was recrystallized in 70% ethanol. Polyacrylamide was from Bio-Rad. Diamide was purchased from Sigma. The enzymes for the ATP hydrolysis assay and phosphoenolpyruvate were purchased from Boehringer (Mannheim, F.R.G.). All other reagents, including oligomycin, NEM, and DCCD, were obtained from Sigma.

RESULTS

Reconstitution of F_OF_1 ATP Synthase from Isolated F_O, F_1, and OSCP—When F_O, extracted from beef heart Complex V in the presence of urea, was incubated in molar excess with purified F_1 and OSCP, the resulting F_OF_1 ATP synthase had a sensitivity to oligomycin equal to that of the original Complex V preparation (Table I). This established that the conditions employed for isolation and reconstitution gave rise to a tightly coupled F_OF_1 ATP synthase, with a virtually unaltered turnover of F_1 (23, 28). (F_O needed to be present in molar excess over F_1 so as to ensure complete binding of the latter (23, see also Table II).)

As already reported by Galante *et al.* (23), the F_O prepared from Complex V after electrophoresis by SDS-PAGE showed some contamination by F_1 subunits (see legend on Table IV) and also by the 21-kDa band of OSCP (not shown). However, these F_1 subunits had no hydrolytic activity and if purified OSCP was not added to F_O and F_1 sensitivity to oligomycin was not recovered (data not shown). Maximal sensitivity to oligomycin was attained when F_O, F_1 and OSCP were incubated together at a protein weight ratio of 16.5:4.5:1. Our reconstitution experiments were carried out using either purified pig heart OSCP or beef heart OSCP, the former being able also to restore completely oligomycin sensitivity.

Effect of Preincubating F_O with Diamide on the Inhibition of ATP Hydrolytic Activity by Oligomycin in the Reconstituted F_OF_1 ATP Synthase—Incubation of diamide with F_1 alone

TABLE I

Effect of diamide on the oligomycin sensitivity of ATP hydrolytic activity of reconstituted F_OF_1 ATP synthase and Complex V

F_O-containing liposomes were incubated for 15 min in the absence and presence of 2 mM diamide, then reconstituted with F_1 and OSCP, and assayed spectrophotometrically for the oligomycin sensitivity of ATP hydrolysis rate, as described under "Experimental Procedures." The concentration of added DTT was 7 mM. F_1* signifies F_1 treated with 2.5 mM diamide for 15 min before being added to F_O. When diamide (2 mM) was added after F_O, F_1, and OSCP had been incubated together, here also diamide was incubated for 15 min. Complex V was reconstituted into asolectin vesicles as under "Experimental Procedures" and, when added, diamide concentration was 1.8 mM and was incubated with the enzyme for 2 or 30 min, after which the ATP hydrolysis rate was assayed spectrophotometrically. Temperature was 30 °C throughout.

Average specific activity and % oligomycin sensitivity of 14 preparations of F_OF_1 ATP synthase (using three different batches of either F_O and F_1) were 10.6 ± 2.1 μmol ATP hydrolyzed/min × mg total protein and 93.3 ± 4.7, respectively. When diamide-treated F_O was used, 18 preparations of F_OF_1 ATP synthase (using three different batches of either F_O and F_1) had an average % oligomycin sensitivity of 46.7 ± 9.5 (the average specific hydrolytic activity being the same as with untreated F_O). Average specific activity of three preparations of F_1, used in the type of experiments shown here, was 60 ± 8.5 μmol ATP hydrolyzed/min × mg protein.

System	First addition	Second addition	Third addition	ATP hydrolysis rate	Oligomycin sensitivity
				μmol ATP hydrolyzed × (min × mg total protein)$^{-1}$	%
F_O	F_1 and OSCP			14.5	95
	F_1* and OSCP			15	95
	Diamide	F_1 and OSCP		15	30
	Diamide	F_1 and OSCP	DTT	14.5	90
	F_1 and OSCP	Diamide		14	95
Complex V				15	98
	Diamide			15	98

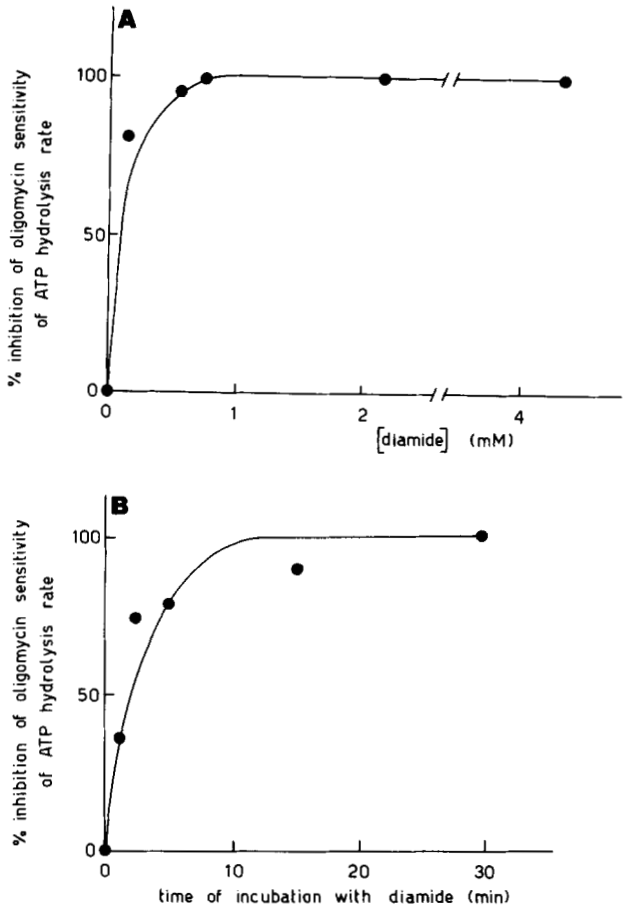

FIG. 1. **Dose response (*A*) and time dependence (*B*) of the effect of diamide on the oligomycin sensitivity of ATP hydrolytic activity of F_OF_1 ATP synthase.** *A*, F_O-containing liposomes were incubated, for 15 min at 30 °C, with different concentrations of diamide and then reconstituted with F_1 and OSCP, as described under "Experimental Procedures." The sensitivity to oligomycin of ATP hydrolysis rate was then assayed at 340 nm. The % inhibition of oligomycin sensitivity was calculated taking as 100% value the sensitivity to oligomycin found after incubation of F_O with 4.3 mM diamide. Data points have been fitted by a regression analysis to an exponential function ($y = 1-e^{-6.36x}$). 50 and 99% of maximal inhibitory effect were obtained at 0.11 and 0.7 mM diamide, respectively. *B*, F_O-containing liposomes were incubated for different time periods with 2.1 mM diamide, at 30 °C, and then reconstituted with F_1 and OSCP, as described under "Experimental Procedures." Then the oligomycin sensitivity of F_OF_1 ATP synthases was assayed at 340 nm. The % inhibition of oligomycin sensitivity was calculated taking as 100% value the sensitivity to oligomycin found after 30 min of incubation of F_O with diamide. Data points have been fitted by regression analysis to an exponential function ($y = 1-e^{-0.324x}$). 50 and 99% of maximal inhibitory effect were obtained after 2 and 13 min of incubation time, respectively.

prior to the reconstitution step did not affect the catalytic properties of F_1 (under the conditions employed by us) (Table I), nor was F_OF_1 ATP synthase activity diminished by using OSCP pretreated with diamide (not shown).

Table I shows that when F_O was incubated for 15 min in the presence of 2 mM diamide, followed by the addition of F_1 and OSCP, the sensitivity to oligomycin of the reconstituted enzyme was diminished by 70%, whereas the hydrolytic activity in the absence of the inhibitor was unaffected. In contrast, if diamide was added after F_O, F_1, and OSCP had been incubated together, there was no effect on the sensitivity to oligomycin. Even a more prolonged (30 min) incubation had no effect (data not shown). Thus, those thiol groups of F_O, which are accessible to diamide in the isolated protein, are protected from the reagent when F_1 and OSCP are already bound to F_O.

The inactivating effect by diamide could be completely reversed by adding excess DTT to F_O, after both its incubation with diamide and the subsequent addition of F_1 and OSCP, thus restoring the sensitivity of the reconstituted system toward oligomycin (Table I). The same results were obtained if DTT (which has no effect by itself on the ATP synthase) was added to F_O before reconstitution (data not shown).

The dose response and the time dependence of the inhibition by diamide was studied with the following results. The maximal inhibition with F_O was obtained at a diamide concentration of around 0.7 mM (Fig. 1*A*) and the incubation time of 15 min was sufficient for the maximal effect of 2.1 mM diamide (Fig. 1*B*).

Diamide was equally effective whether the pH of the medium was reduced from 8 to 7.5 or 7 (data not shown).

The effect of diamide was also studied on F_O extracted with urea from the ATP synthase purified according to Serrano *et al.* (29). This F_O, incubated with diamide, behaved in the same way as the F_O from Complex V (data not shown), despite the fact that the control-reconstituted enzyme had only about 70% sensitivity to oligomycin and a high contamination by the adenine nucleotide translocator.

Effect of Diamide on the Oligomycin Sensitivity of ATP Hydrolysis of Complex V—The protective effect of F_1 and/or OSCP on diamide-sensitive thiols of F_O has also been shown by treating Complex V with diamide. No change was found on either the rate of ATP hydrolysis or on its oligomycin sensitivity, even when the incubation with diamide was prolonged for 30 min (Table I).

The same results were obtained using the ATP synthase purified as in (29) (not shown).

Diamide Treatment Prevents the Binding of F_1 to F_O—One possible reason for the loss of oligomycin sensitivity of F_OF_1 ATP synthase, reconstituted with diamide-treated F_O, is that diamide induces a change in the structure of F_O so that it no longer binds F_1. Centrifugation of the mixture, reconstituted as described above, would then result in the appearance of F_1-mediated ATPase activity of the supernatant. Table II shows that indeed ATPase activity is found in the supernatant when diamide-treated F_O induces the partial loss of oligomycin sensitivity and that DTT restores the original sensitivity.

The results of Table II also exclude that the gravitational force used in the centrifugation might have provoked a spurious adhesion of F_1 to the vesicles.

Effect of Diamide on H^+ Release from F_O Liposomes Induced by Valinomycin-mediated K^+ Influx in the Presence and Absence of DCCD—The same batch of F_O used in the experiments of Table I was also used to measure the H^+ release from F_O-containing liposomes in response to a valinomycin-induced membrane potential (positive inside). Table III shows that addition of DCCD, an inhibitor of proton flux through F_O, decreased by 80% the K^+-mediated H^+ release. It is also shown that incubation for 13 min of F_O liposomes with 2 mM diamide led to a stimulation of H^+ release by the liposomes. However, if DCCD was added after the F_O-containing liposomes had been treated with diamide, the inhibition obtained was only 50%. This finding may indicate that the conformational change consequent on the reaction with diamide is such as to distort the DCCD-binding site, either directly or indirectly (see also "Discussion"). The increased conductivity of diamide-treated F_O and the lack of inhibition by DCCD are at variance with previous reports (11, 12) on this type of measurement. We have no ready explanation for such conflicting results.

TABLE II

Determination of unbound F_1

F_O-containing liposomes were incubated for 15 min in the absence and presence of 2.1 mM diamide before the addition of F_1 and OSCP, as detailed under "Experimental Procedures." When indicated, incubation of diamide-treated F_O with DTT (9 mM) was carried out before the addition of F_1 and OSCP. After the assay for the oligomycin sensitivity of ATP hydrolysis rate, the mixtures were centrifugated for 10 min at 10,000 × g, at 25°C. The supernatants and pellets (after resuspension) were then assayed for ATP hydrolysis rate and oligomycin sensitivity. These values, found before the centrifugation, are given as μmol of ATP hydrolyzed/min × mg total protein and % of remaining activity after addition of oligomycin, respectively. The same values of the supernatant and of the pellet fractions, obtained upon centrifugation, are expressed as % of the activities of the F_OF_1 synthases measured prior to centrifugation. When F_1 alone was added to phospholipid vesicles, it was added at the same F_1/phospholipid ratio as that used in the reconstitution experiments. SN, supernatant.

	Values prior to centrifugation		% of total starting activity after centrifugation		
	ATP hydrolysis rate	Oligomycin sensitivity	ATP hydrolysis rate		Oligomycin sensitivity
			SN	Pellet	Pellet
F_OF_1 ATP synthase	10	95	3	85	95
F_O (+ diamide)F_1 ATP synthase	10.5	45	45	55	75
F_O (+ diamide + DTT)F_1 ATP synthase	10	90	4	85	90
PL vesicles + F_1	55		98	1	

TABLE III

Effect of diamide on proton conductance of F_O

Dialyzed F_O-containing liposomes (pelletted and resuspended in a sucrose/$MgSO_4$/Tricine-NaOH medium (pH 8.3)), were incubated for 13 min at 30°C in the absence and presence of diamide (2 mM) (and for further 10 min if 50 μM DCCD was also present). Proton conductance of F_O was measured at 25°C in a pH electrode cell, containing additionally 0.16 M KCl, by following proton release from F_O-containing liposomes, in response to a potassium diffusion potential (positive inside) induced by addition of valinomycin. The proton release is expressed as the reverse of the $t_{1/2}$ of the reaction.

	H^+ release	%
	$1/t_{1/2}(s^{-1})$	
F_O	0.082	100
F_O + 50 μM DCCD	0.017	20
F_O + 2 mM diamide	0.195	235
F_O + 2 mM diamide + 50 μM DCCD	0.100	120

SDS-PAGE of F_OF_1 ATP Synthase, Reconstituted with Diamide-treated F_O, Shows a Diminution of F_O 1 and the Appearance of a Band of Apparent Molecular Mass of 45 kDa—Fig. 2 shows the SDS-PAGE patterns of the F_OF_1 ATP synthase reconstituted with untreated F_O (*A*) or with F_O incubated with 0.02 mM diamide (*B*). Several are the alterations induced by diamide, also when compared with the pattern of Complex V treated with the reagent (see later) (not shown). These appear to be the diminution of bands F_O 1, F_O 4 (which, in the control, is the shoulder associated with F_O 3), that of the sum total of the area of F_1 subunits, and the appearance of a band of apparent molecular mass of 45 kDa (marked with an *arrow* in Fig. 2*B*). (The nomenclature of the bands associated with F_O is given according to Montecucco *et al.* (14) and that of the 18–25-kDa region also according to Refs. 15 and 48).

It can be noted that, though β-mercaptoethanol was absent, the resolution of the gels of Fig. 2 was always sufficiently high to reveal a band closely associated with OSCP which, according to Walker *et al.* (15), is subunit d of F_O. This protein was also affected by diamide (see later). Finally, from Fig. 2*B* it can be seen that the band, with the same molecular weight as that of the purified adenine nucleotide translocator, is fully removed by diamide.

Fig. 2*C* shows that the effects of 4.3 mM diamide are completely reversed by DTT (see legend of Table IV for the details of this incubation).

Electrophoresis of Complex V treated with diamide (0.005–4.3 mM) did not show any diminution of F_O 1 (or/and appearance of the 45-kDa band) even at the highest concentration of the reagent. The only proteins affected were the adenine nucleotide translocator (yet present only in a very low amount) and subunit d of F_O (50% decrease at 4.3 mM diamide) (not shown).

Diminution of Band F_O 1 Is Correlated with the Loss of Oligomycin Sensitivity, Possibly because F_O 1 Dimerizes in the Presence of Diamide—Table IV shows that when the reconstitution of F_OF_1 ATP synthase was carried out with F_O treated with different amounts of diamide, there was a direct relation between the % of disappearance of subunit F_O 1 and the loss of oligomycin sensitivity of the reconstituted enzyme. It also shows that the decreased area of F_O 1, whose molecular mass is close to 25 kDa (15), could be accounted for (though not always proportionally) by the area of the band of approximately twice its molecular mass (45 kDa). It has recently been reported that subunit F_O 1 (or subunit b (15)) contains only one —SH group (15). In view of this piece of information, our results suggest that the appearance, in the presence of diamide, of the 45-kDa band is due to the formation of a disulfide-linked dimer between two F_O 1 subunits. The apparently lower molecular weight of the dimer, with respect to double the mass of F_O 1, can be explained by a nonidentical electrophoretic movement of the two proteins. The intensity of the staining of the postulated dimer band does not always quantitatively match the loss of intensity of the F_O 1 band. However, this could simply arise from the nonproportional staining of the polypeptide in the monomeric and dimeric forms.

Table IV also shows that, in the presence of diamide, diminution of the sum total of the area of F_1 subunits roughly followed the loss of oligomycin sensitivity (note that, as detailed in the legend, in calculating the diamide-affected area of F_1 subunits, the contamination by these subunits, as present in isolated F_O, was taken into account). This result qualitatively supports the finding that alteration of F_O 1 by diamide prevents the binding of F_1 to F_O (as shown in Table II), the unbound F_1 remaining in the supernatant during the pelleting of the gel samples (see "Experimental Procedures").

As expected from the data of Tables I and II, the effects of diamide were reversed by DTT.

The isolated regulatory subunit of the ATP synthase, the

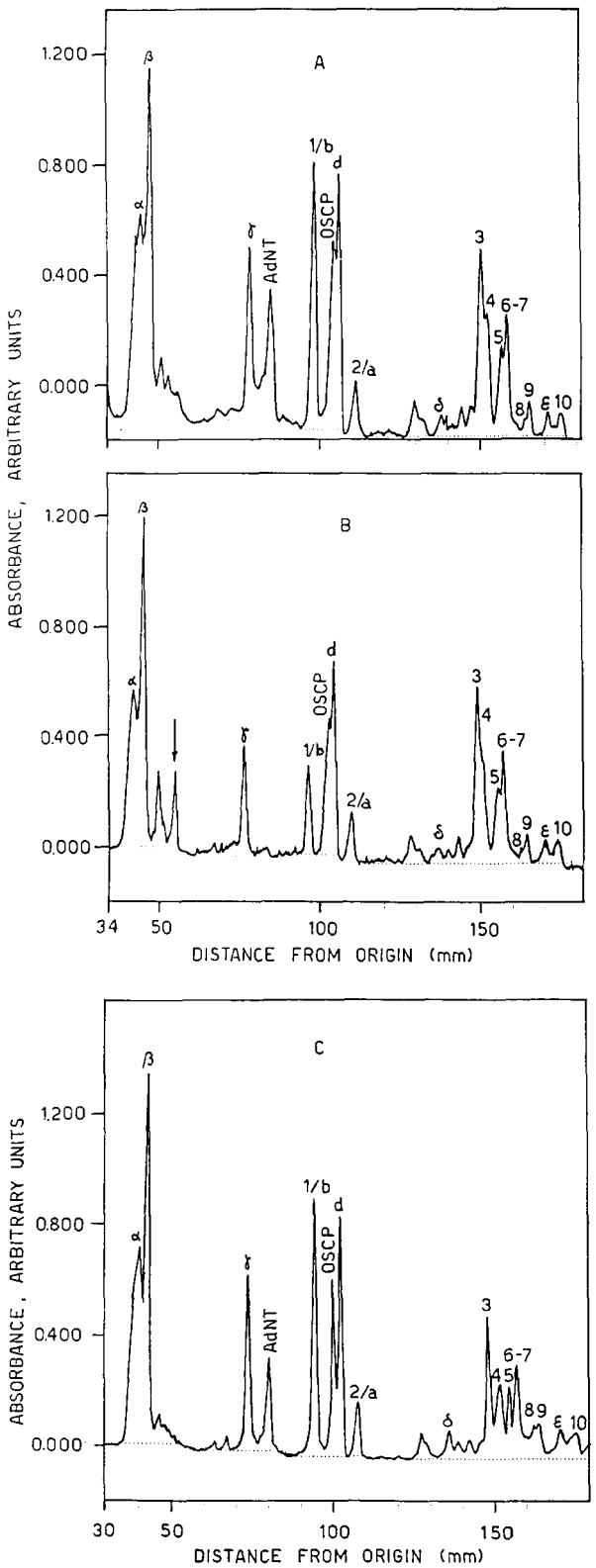

FIG. 2. **Effects of diamide on SDS-PAGE pattern of F_OF_1 ATP synthase.** Stock F_O (3.7 mg/ml) was incubated 15 min at 30 °C in the absence (*A*) and presence (*B*) of diamide (0.02 mM) before F_1 and OSCP additions. Fig. 2*C* is the pattern obtained in the presence of 4.3 mM diamide and 10 mM DTT, as detailed in the legend of Table IV. Incubation and reconstitution procedures and preparation of gel samples are described under "Experimental Procedures." and in the legend of Table IV. Electrophoresis was run on slab gels, containing 13–19% polyacrylamide and 0–24% sucrose linear gradients (see "Experimental Procedures"). β-Mercaptoethanol was omitted in all gels. As expected, in the presence of DTT the resolution of the bands is higher. Coomassie Blue staining patterns were read at 570 nm. *Greek letters* indicate the subunits of F_1 while *numbers* the bands found associated with F_O, in order of decreasing molecular weight, according to Montecucco *et al.* (14). In the 18–25-kDa region, the nomenclature of F_O subunits according to Walker *et al.* (15, 48) is also given. *AdNT*, adenine nucleotide translocator. The positions of AdNT, OSCP, F_O 4, and F_O 5 have been determined by use of the isolated AdNT, OSCP, inhibitor protein, and F_6, respectively. For the meaning of the *arrow* see text.

TABLE IV

Effect of different concentrations of diamide on the oligomycin sensitivity and SDS-PAGE of F_OF_1 ATP synthase reconstituted with diamide-treated F_O.

The same batch of F_OF_1 ATP synthase, reconstituted with F_O incubated with different concentrations of diamide (detailed in the table), was used for determining oligomycin sensitivity and for SDS-PAGE, as described under "Experimental Procedures." When present, DTT was 10 mM and was added after F_O (treated with 4.3 mM diamide), F_1, and OSCP had been incubated together. % diminution of oligomycin sensitivity of the enzyme, reconstituted with diamide-treated F_O, is calculated with respect to the value found in the absence of the reagent (which, in this experiment, was of 90%). Calculation of the % decrease of the areas of F_O 1 and of F_1 in the presence of diamide was performed by taking as 100% the ratio between the area of these proteins and that of band F_O 2 found in the control (without diamide). F_O 2 was chosen as internal reference in view of its lack of —SH residues (48) (see also Ref. 31). The decrease of F_1 is measured as the decrease of the sum total of the subunits of added F_1. However, as F_O itself contained contaminations by these subunits (20–25% with respect to added F_1), this value was subtracted from that of F_1 found in the gels. The recovery of the 45-kDa band was expressed as % of the diminished area of F_O 1 in the presence of diamide. Data with 0.02 mM diamide were taken from Fig. 2*B*.

Diamide addition	Decrease of oligomycin sensitivity	Decrease of F_O 1	Recovery of the 45-kDa band	Decrease of F_1
mM	%	%	%	%
0.005	20	15	100	0
0.02	55	60	65	35
1.1	85	75	60	60
4.3	90	80	55	80
4.3 plus DTT	0	0	0	0

so-called inhibitor protein (10–10.5 kDa (30)), has the same position as band F_O 4 that reacts with diamide. The inhibitor protein does not contain —SH groups (30), therefore there is also present an as yet unidentified protein, with the same molecular weight as the inhibitor protein but containing —SH groups, modified by diamide. As F_O 4 is closely associated with F_O 3, the exact evaluation of its decrease in the presence of diamide was not possible. On a qualitative ground, however, there seemed to be no direct correlation between changes of F_O 4 and loss of oligomycin sensitivity. Such a correlation was also not seen with subunit d (not shown).

Reactivity of Diamide with Mono or Vicinal Thiols: Comparison with the Effect of NEM on Isolated F_O—In a recent paper by Yagi and Hatefi (32), treatment of isolated F_O with *p*-chloromercuribenzoate, prior to addition of F_1 and OSCP, resulted in a reversible inhibition of the hydrolytic activity of the reconstituted enzyme. As the oligomycin sensitivity was unaffected, it follows that the lesion induced by this type of monothiol reagent is different from that induced by diamide.

This conclusion leads to a particularly important point not frequently discussed in the literature, *i.e.* the reaction mechanism of diamide. In fact from the literature it generally appears that in its reaction with proteins diamide is a dithiol oxidant (10–12, 33,34). The (few) reports on this point however are rather conflicting. On the one hand, the proposed reaction scheme of diamide (35), together with the experimental evidence reported (36, 37), suggest that stable adducts

TABLE V

Effect of NEM, in the presence or in the absence of diamide, on the oligomycin sensitivity of ATP hydrolysis rate of F_OF_1 ATP synthase

F_O-containing liposomes were incubated 15 min with either diamide or NEM. An aliquot was then withdrawn from the different incubations and treated 15 min with NEM, if diamide was already present, or with diamide, if NEM was already present. The final concentrations of diamide and NEM were 2.15 and 2.4 mM, respectively, in all cases. Addition of F_1 and OSCP, treatment with oligomycin, and assay for the oligomycin sensitivity of ATP hydrolysis rate were performed as described under "Experimental Procedures." DTT, when present, was 6.5 mM and was added after reconstitution with F_1 and OSCP and incubated for 10 min. The final volume was 45 μl, protein concentrations/ml were 1.33 mg of F_O, 0.33 mg of F_1, 0.06 mg of OSCP, and the phospholipid concentration was 0.66 mg of phospholipid phosphorous/ml. Temperature was 30° C throughout. Average specific activity of the reconstituted enzyme in the absence and in the presence of thiol reagents was 9.8 ± 0.6 μmol of ATP hydrolyzed/min × mg total protein.

System	First addition	Second addition	Third addition	Fourth addition	Oligomycin sensitivity
					%
F_O	F_1 and OSCP				90
	Diamide	F_1 and OSCP			52.5
	Diamide	NEM	F_1 and OSCP		50
	Diamide	NEM	F_1 and OSCP	DTT	90
	NEM	F_1 and OSCP			70
	NEM	Diamide	F_1 and OSCP		70
	NEM	Diamide	F_1 and OSCP	DTT	75

with cysteine residues in proteins are unlikely to be formed. On the other hand, Fleer *et al.* (38) have suggested that diamide could form a thiolester type product or/and induce the formation of a bridge between a thiol and a lysine or histidine amino group.

In order to try to clarify, albeit indirectly, the reaction mechanism of diamide, we have looked at the possible protective effect of diamide on the action of a monothiol reagent such as NEM and also on any protection exerted by NEM on diamide-sensitive thiols. From Table V, it appears that neither NEM, added after diamide, nor diamide, added after NEM, can increase the loss of oligomycin sensitivity provoked by the addition of the first reagent. As expected, DTT was able to increase the sensitivity to oligomycin to the control value only in that experiment where diamide was added first.

It thus seems that the thiol groups sensitive to diamide can react with NEM and also, as derivatization of thiols by NEM produces a milder damage than diamide, that diamide does not form stable adducts with cysteine residues but probably induces disulfide bonds. This conclusion is strengthened by the SDS-PAGE of Fig. 2*B*, where dimerization of subunit F_O 1 probably occurs. It is of course not possible to exclude that dimerization of subunit F_O 1 by diamide can also be due to a thiol-amino cross-link (38).

The experiments reported here and those of Yagi and Hatefi (10, 32), however, point to the existence, in F_O, of various classes of mono- and di-thiols, which react preferentially with a particular type of thiol reagent, and also that derivatization or oxidation of thiols induces a different conformational change in F_O which is reflected in a different type of functional alteration.

DISCUSSION

In order to assess the importance of thiol groups, belonging to mitochondrial F_O, in the assembly of F_O, F_1, and OSCP, we have treated a batch of F_O, extracted with urea from purified Complex V, with thiol reagents before and after the reconstitution of an F_OF_1 ATP synthase, achieved by adding purified F_1 and OSCP to F_O.

It seems clear that the reaction of F_O thiols with diamide leads to a severe perturbation of F_O functions, as revealed by the enhanced H^+ conductance (Table III) and by the impaired binding of F_1 to F_O (Table II), which consequently diminishes the sensitivity to oligomycin of the ATP hydrolytic activity of the enzyme (Table I).

Treatment of isolated F_O with diamide resulted in the formation of a band of approximately twice the molecular weight of subunit F_O 1, possibly because of the formation of a disulfide bridge between two molecules of subunit F_O 1 (Fig. 2*B*, Table IV). These results show the importance of the cysteine residue of subunit F_O 1 (15) for the structure and function of the enzyme. However, if DTT can reduce S–N as well as S–S bonds, the induction by diamide of the S–N bond between two F_O 1 subunits cannot be discarded (38). Nonetheless the data of Table V, which indicate that the diamide-sensitive – SH groups are also reactive with NEM, are best explained by the former interpretation.

Cd^{2+} is a dithiol modifier, more hydrophilic than diamide. Treatment of F_O, before or after the addition of F_1 and OSCP, with two different concentrations of Cd^{2+} (120 μM, 1.2 mM), did not alter any activity of the reconstituted enzyme (data not shown). The failure of Cd^{2+} to react with cysteines of F_O 1 subunits could indicate that these residues are not exposed on the surface of the F_O.

Subunit F_O 1 is homologous with subunit b of *Escherichia coli* (15) and with subunit 4 of yeast (15, 18), the role of which is to bind F_1 (16–18). Our results support the prediction, based on amino acid sequencing and structural analysis, that the mammalian F_O counterpart, subunit F_O 1, is also involved in the binding between F_O and F_1 ((15) and also the results reported in Ref. 47). Moreover, if the interpretation of our results is correct, it can also be concluded that this subunit is present at least in two copies, as is bacterial subunit b (39).

The disappearance of bands F_O 4 and subunit d and the loss of oligomycin sensitivity are poorly correlated. Thus, these proteins seem not to be involved in the binding with F_1, though the possibility cannot be discarded that they are involved in the increased conductance and lower DCCD binding of diamide-treated F_O (Table III), together with F_O 1 itself. In *E. coli* it has been shown that the formation of adducts with subunit b cysteine residues can indeed destabilize the proton channel (40).

The results presented show that F_O is quite a flexible protein: addition of DTT after diamide quickly restores the

original F_O structure capable of binding F_1 (Tables I, II, IV, and Fig. 2).

In our view, it is particularly interesting that F_1 has a protective effect on F_O thiols (though it cannot be excluded *a priori* that OSCP as well participates in such protection, either directly or indirectly via F_1 (41–43)). Table I convincingly shows that no modification occurs when diamide is incubated with the reconstituted F_OF_1 ATP synthase or with the purified Complex V. This could be taken to indicate that F_1, when binding to F_O, drastically perturbs the structure of F_O, in analogy with the conformational change occurring in F_1 upon binding to F_O (42, 43). Such an interpretation is compatible with the report that DCCD sensitivity of F_O in submitochondrial particles, with a full complement of F_1, is twice that if F_1-depleted particles (44, 45). The diamide-sensitive thiols could be separated as a consequence of F_1 binding and therefore no longer react with the reagent. The alternative explanation that these thiols are hidden from diamide upon binding of F_1 is equally possible.

Not all conformational changes of F_O can, however, protect F_O from diamide. For instance, that change subsequent to oligomycin binding (the inhibitor having been added to F_O before diamide) did not protect F_O from the reaction with diamide (data not shown).

It has been claimed that factor B is a component of F_O and that it has a vicinal dithiol group essential for energy coupling but not for the binding of F_1 to F_O (46). Thus, according to our results, we exclude an involvement of factor B and agree with Yagi and Hatefi (10), who already have suggested that other vicinal thiols of F_O, different from those of factor B, are important for a proper assembly, and therefore function, of the mitochondrial ATP synthase.

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