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# Structural and functional modifications induced by diamide on the $F_0$ sector of the mammalian ATP synthase

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In this report data are presented which firmly establish that by treating isolated $F_0$ with the thiol reagent diamide, two 25 kDa $F_0$ subunits react to form a dimer of 45 kDa apparent molecular mass. This dimerising effect is correlated to the impairment of the binding of $F_1$ to $F_0$, both at μM and mM diamide concentrations. Under the latter condition, modification of other $F_0$ subunits also occurs. Passive proton conductance through $F_0$, as well as its sensitivity to *N,N'*-dicyclohexylcarbodiimide, are affected at low diamide concentration. Thus perturbation of the cysteine residue of the 25 kDa $F_0$ subunit is sufficient for altering the ATP synthase proton channel.

ATP synthase; Mitochondria; SH group; Diamide; $F_0$; $F_1$

## 1. INTRODUCTION

Mitochondrial ATP synthase is a multisubunit complex made of two distinct moieties, the catalytic part $F_1$, and the proton-conductive, membrane-embedded sector $F_0$ [1].

Our recent work [2] has provided evidence for the crucial role of the $F_0$ 25 kDa subunit, called $F_0$1 [3] or b [4], which contains a single cysteine [4], in the interaction between $F_0$ and $F_1$. This was found by treating isolated $F_0$ with a thiol reagent, diamide. This led most strikingly to impaired binding of $F_1$ to $F_0$, indicating by the oligomycin insensitivity of ATPase activity. Most importantly, these effects could be correlated with loss of (monomeric) subunit $F_0$1, as revealed by SDS-PAGE [2].

Involvement of subunit $F_0$1 in the binding of $F_1$ to $F_0$ was undoubtedly proven [2], yet other effects of diamide on $F_0$ needed clarification. A new band, of approximately twice the molecular weight of $F_0$1 appeared in the SDS-PAGE of diamide-treated $F_0$. Could it be the dimerisation product of $F_0$1? Additionally, which of the other $F_0$ subunits bearing SH groups were affected by diamide, and what were the consequences of these modifications?

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*Abbreviations:* diamide, diazenedicarboxylic acid bis(dimethylamide); $F_1$, coupling factor 1 of ATP synthase; $F_0$, hydrophobic part of ATP synthase; OSCP, oligomycin-sensitivity-conferring-protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; AdNT, adenine nucleotide translocator; DCCD, *N,N'*-dicyclohexylcarbodiimide

In this paper it is demonstrated that, at all concentrations studied, diamide induces dimerisation of $F_0$1 while the aggregation products of other $F_0$ subunits appear only at mM concentrations. We show, however, that diamide-induced modification of $F_0$1 is the only factor responsible for the increased passive proton conductance of $F_0$ which is insensitive to DCCD.

## 2. MATERIALS AND METHODS

$F_0$ was isolated either by treating bovine heart ATP synthase [5] with 4 M urea (urea-$F_0$, see [2]) or by incubating the enzyme, isolated according to [6], with 3.5 M NaBr [7]. Contrary to urea-$F_0$, this NaBr-$F_0$ preparation did not lose its content of OSCP [7]; thus purified OSCP was not added to reconstitution of the ATP synthase. In [2] details for all the experimental procedures can be found, except for the second dimension electrophoresis. This was run as in [2] but the samples were treated as follows. Desired stained bands were punched out of the first gel and incubated at 37°C for 2 h in 2 ml of 10 mM Tris-acetate (pH 8.2) containing 4% SDS and, when needed, 3% β-mercaptoethanol. The gel slices were then inserted into the sample wells [8].

The concentration of diamide given in the text refers to the net concentration effectively present, after its rapid reaction with the 0.25 mM dithiotreitol present in the suspension medium (see legend to Table I).

Passive proton flow through $F_0$ in response to a valinomycin-induced potassium diffusion potential was measured in asolectin vesicles, which were pelletted at 100000 × *g* and suspended in 0.2 mM Tricine/NaOH (pH 8.3), 20 mM sucrose and 2.5 mM $MgSO_4$ [9]. Other experimental details were as in [2]. Protein concentration was determined according to [10]. All reagents were obtained from Sigma, except for Tricine and cholic acid (recrystallized from 70% ethanol) from Merck (Darmstadt, Germany), and polyacrylamide and bis-acrylamide from Bio-Rad.

## 3. RESULTS AND DISCUSSION

Table I shows the effect of increasing concentrations

of diamide on the $F_0$ extracted from the ATP synthase either with urea or NaBr. We measured the sensitivity of the ATP hydrolysis rate to oligomycin after reconstitution of the enzyme [2]. Aliquots of diamide-treated $F_0$ were in parallel subjected to electrophoresis for evaluation of the structural changes. These experiments show that both urea-$F_0$ and NaBr-$F_0$ were similarly affected by diamide. In either case, the oligomycin sensitivity of the hydrolytic activity decreased parallel to the disappearance of $F_0$1 subunit and to the increase of the 45 kDa band. The 45 kDa band has been postulated to be the diamide-cross-linked dimerisation product of $F_0$1 [2]. Two-dimensional SDS-PAGE of $F_0$, treated with $\mu$M and mM concentrations of diamide, was carried out to test this hypothesis.

Fig. 1 shows the first dimension SDS-PAGE of diamide-untreated (lanes 1 and 2) and diamide-treated urea-$F_0$ (lanes 3-8), performed in the absence of reducing agents [2]. Lanes 3-6 show that, when diamide was used in the $\mu$M range (52, 86 and 130 $\mu$M), disappearance of the 25 kDa subunit of $F_0$ was concomitant only to the increase of the approximately 45 kDa band (marked II in Fig. 1) (see Table I for the quantitative estimations).

The effect of diamide in a mM concentration (2.8 mM, lanes 7 and 8) was by far more dramatic. For example, there was also a clear diminution of $F_0$ subunits d and 4 and an appearance of at least two other bands: 55 (marked I) and 27 (marked III) kDa. The 55 kDa band appears approximately at the same position as the $\alpha$ subunit of contaminating $F_1$.

In order to identify the origin of the diamide-cross-linked new bands of Fig. 1, these were excised, treated with $\beta$-mercaptoethanol (to reduce the cross-links) and loaded on a second gel.

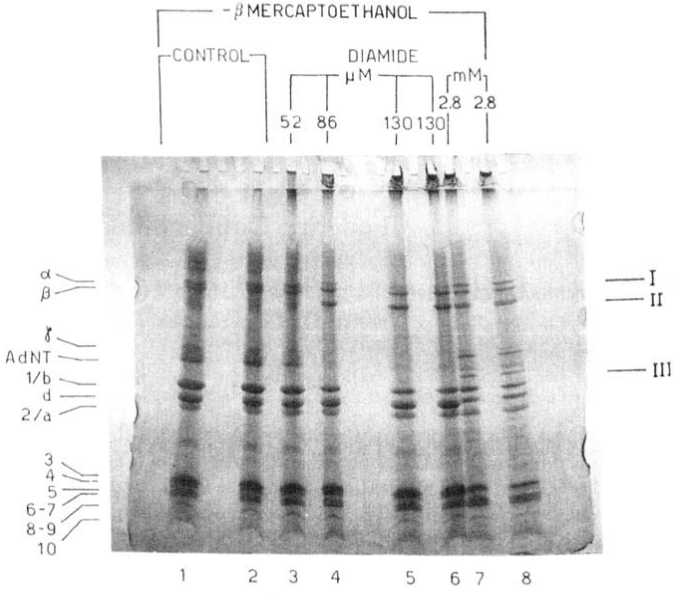


Fig. 1. SDS-PAGE of $F_0$ treated with increasing concentrations of diamide. Aliquots (approximately 0.150 mg) of urea-$F_0$ were concentrated by centrifugation (see legend to Table I) and suspended in 20 $\mu$l of 10 mM Tris-acetate (pH 8) containing 4% SDS, 20% glycerol and 1% pyronine. A 20 cm long linear gradient of 13-19% polyacrylamide gel, containing a 0-24% linear sucrose gradient, was used [2]. The following samples were examined. Untreated $F_0$ (lanes 1,2). $F_0$ treated with diamide at 52 $\mu$M (lane 3), 86 $\mu$M (lane 4), 130 $\mu$M (lanes 5,6) and 2.8 mM (lanes 7,8). $\alpha$, $\beta$ and $\gamma$ indicate the contaminating $F_1$ subunits, AdNT the adenine nucleotide translocator and numbers the bands found associated with $F_0$ [3]. In the 25-18 kDa region, the nomenclature of $F_0$ subunits according to [4,11] is also given. Roman letters indicate the bands appearing upon diamide treatment of $F_0$: I,II and III of apparent molecular weight of 55, 45 and 27 kDa, respectively.

Table I

Effect of diamide on the ATP synthase

| Diamide addition (mM) | Decrease of oligomycin sensitivity (%) | | Decrease of $F_0$1 band (%) | | Recovery of 45 kDa band (%) | |
|---|---|---|---|---|---|---|
| | Urea-$F_0$ | NaBr-$F_0$ | Urea-$F_0$ | NaBr-$F_0$ | Urea-$F_0$ | NaBr-$F_0$ |
| 0.052 | 25 | 25 | 20 | 30 | 90 | 80 |
| 0.086 | 55 | 35 | 50 | 35 | 90 | 80 |
| 0.130 | 60 | 45 | 65 | 45 | 85 | 80 |
| 2.8 | 75 | 50 | 70 | 65 | 65 | 60 |

200 $\mu$g of urea- or NaBr-$F_0$ (see Methods) were incubated for 15 min at 30°C in 100 $\mu$l of 10 mM Tricine/NaOH (pH 8), 2.5 mM $MgSO_4$, 0.25 mM dithiotreitol, 0.2 mM EDTA in the absence or presence of different concentrations of diamide. Aliquots were either concentrated by centrifugation for SDS-PAGE (see legend to Fig. 1) or further incubated with purified $F_1$ and OSCP (the latter protein was added only to urea-$F_0$; see Methods) to reconstitute the ATP synthase at final protein concentrations of 1.65, 0.45 and 0.1 mg/ml, respectively. Aliquots were incubated or not incubated with 6 $\mu$g/ml oligomycin for 10 min and tested for ATP hydrolysis rate [2]. The percentage diminution of oligomycin sensitivity was calculated relative to the value found in the absence of diamide which was of 90% for either preparation. For other experimental details such as the calculations of SDS band areas see [2].

Fig. 2 (lanes 4 and 7) shows such an experiment with the only new band (i.e. the 45 kDa protein) found after treatment of $F_0$ with 86 and 130 $\mu$M diamide (lanes 4 and 6 of Fig. 1). Subunits $F_0$1 (lanes 5 and 8) and $F_0$2 (or a [11]) (lanes 6 and 9), excised from the same gel, were also present in Fig. 2. $F_0$2 was regarded as the internal standard for the recovery of the bands in the second gel, as it does not contain SH groups [11] and may not be affected by diamide [2]. The reduced 45 kDa band migrated at the same position as $F_0$1 (compare lanes 4 and 7 with lanes 5 and 8). The sum of the $\beta$-mercaptoethanol-reduced $F_0$1 (lane 4 or 7) with the $F_0$1 which was not cross-linked after the addition of the reagent (lane 5 or 8) indeed gave rise to a high recovery of the original untreated $F_0$1 (lane 2).

Untreated subunits $F_0$1 and $F_0$2 were also incubated with $\beta$-mercaptoethanol and subjected to the second electrophoresis (lanes 2 and 3 of Fig. 2, respectively). A band (lane 1 of Fig. 2) with the same molecular mass as the dimeric $F_0$1 was present in $F_0$ in the absence of diamide (see lanes 1 and 2 of Fig. 1). This latter band

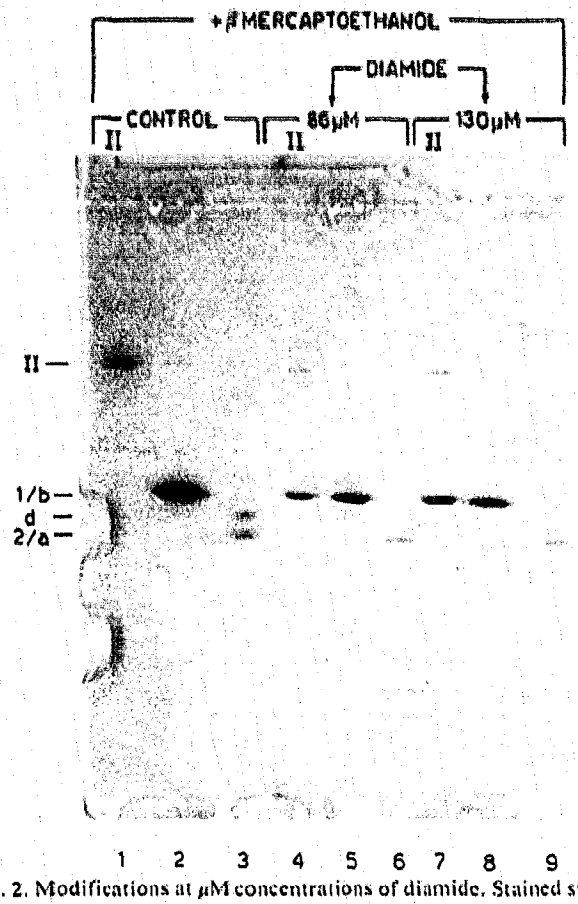


Fig. 2. Modifications at μM concentrations of diamide. Stained single bands (approximately 4 mm in width) of the first dimensional gel (see Fig. 1) were punched out, incubated in sample buffer in the presence of β-mercaptoethanol (see section 2) and loaded on the second gel. The first three lanes are bands excised from the untreated $F_0$ gel: a band with the same mass of band II (lane 1), $F_0$1 (or b; lane 2), $F_0$2 (or a, contaminated by subunit d; lane 3). Lanes 4–6 are bands excised from the gel of $F_0$ treated with 86 μM diamide: 45 kDa band (II) (lane 4), $F_0$1 (lane 5) and $F_0$2 (lane 6). Lanes 7–9 were obtained as above, except that $F_0$ was treated with 130 μM diamide: 45 kDa band (II) (lane 7), $F_0$1 (lane 8), $F_0$2 (lane 9).

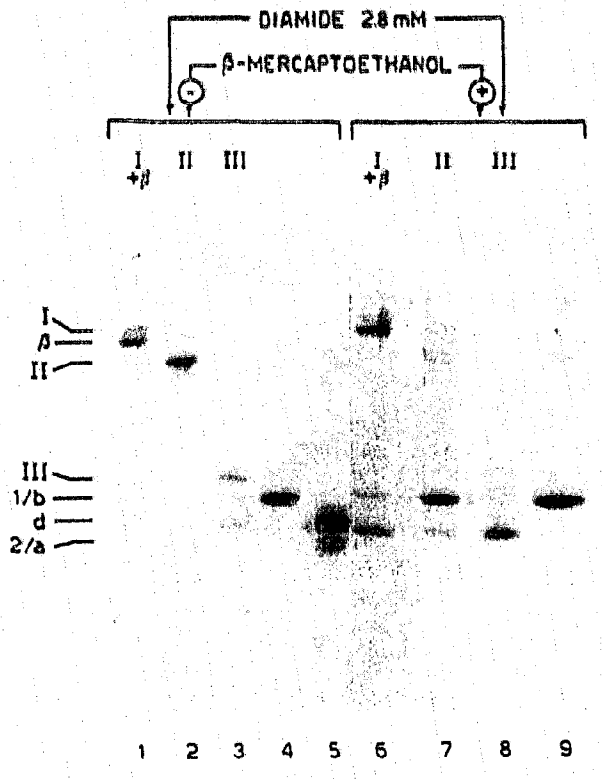


Fig. 3. Modifications at mM concentration of diamide. Stained bands of the first dimension gel of $F_0$ incubated with 2.8 mM diamide (lanes 7 and 8 of Fig. 1) were punched out and incubated in the sample buffer in the absence (lanes 1–5) or presence (lanes 6–8) of β-mercaptoethanol, before being loaded onto the second gel. Excised bands were: band I (contaminated by $F_1$ β-subunit), lanes 1 and 6; band II (45 kDa), lanes 2 and 7; band III, lanes 3 and 8; $F_0$1 (or b), lane 4 and 9; subunits d and $F_0$2 (or a), lane 5.

was not, however, affected by β-mercaptoethanol and probably is an as yet unknown contamination.

Fig. 3 shows the second dimension SDS-PAGE of selected bands from the first electrophoresis of 2.8 mM diamide-treated $F_0$ (lanes 7 and 8 of Fig. 1), not incubated (lanes 1–5) or incubated (lanes 6–9) with β-mercaptoethanol. These are: band I (lanes 1 and 6), band II (lanes 2 and 7), band III (lanes 3 and 8), $F_0$1 (lanes 4 and 9) and $F_0$ subunits d and 2 (lane 5). Under reducing conditions, band II gave rise to both $F_0$1 and subunit d (lane 7), while bands I and III, though to different extents, mainly to subunit d (lanes 6 and 8). Any of these did not appear to contain $F_0$ subunit 4, whose intensity anyhow markedly decreased with 2.8 mM diamide (lanes 7 and 8 of Fig. 1), suggesting that aggregation products of $F_0$4 may have very high molecular weights and may not enter the gel.

Table II

Effect of diamide on passive proton flow through $F_0$

| | $H^+$ release ($1/t_{1/2}$ ($s^{-1}$)) | % |
|---|---|---|
| $F_0$ | 0.075 | 100 |
| $F_0$ + DCCD | 0.045 | 60 |
| $F_0$ + oligomycin | 0.045 | 60 |
| $F_0$ + 130 μM diamide | 0.105 | 140 |
| $F_0$ + 130 μM diamide + DCCD | 0.100 | 135 |
| $F_0$ + 130 μM diamide + oligomycin | 0.080 | 105 |
| $F_0$ + 2.8 mM diamide | 0.105 | 140 |
| $F_0$ + 2.8 mM diamide + DCCD | 0.095 | 125 |
| $F_0$ + 2.8 mM diamide + oligomycin | 0.080 | 105 |

0.125 mg NaBr-$F_0$ reconstituted in liposomes (in 100 μl of the suspending medium; see Methods) was incubated in the absence or presence of different diamide concentrations for 15 min at 30°C. The incubation was prolonged for 15 min in the absence or presence of 50 μM of a freshly prepared DCCD or of oligomycin (1 μg/mg protein) (both in ethanol). The mixture was then transferred to a pH electrode cell containing 1 ml of the Tricine buffer plus 0.16 M KCl at 25°C. Proton release from $F_0$-containing liposomes was initiated by addition of valinomycin and is expressed as the reverse of the $t_{1/2}$ of the reaction. Unspecific proton leakage in liposomes was not subtracted from that of $F_0$-containing liposomes. Other details were as in [2].

The data shown so far definitively show that diamide induces dimerisation of the $f_o1$ subunits and that this $F_o$ subunit is present at least in two copies. Modification of cysteine-197 [4] of $F_o1$ may thus be responsible for the lack of interaction between $F_o$ and $F_1$ at any diamide concentration.

The native redox state of this thiol group, and not the modification of other $F_o$ subunits occurring only at mM concentrations of diamide, was found to be equally important for the passive proton conductance through $F_o$ reconstituted in liposomes. Table II shows that the maximal increase of the proton flow through NaBr-$F_o$ was reached already at those $\mu$M concentrations of diamide in which $F_o1$ was the only affected subunit (Table I and Fig. 1). In addition, at the same low diamide concentrations the DCCD-block of proton flow was readily lost, while the oligomycin effect was slightly more evident (Table II). One may infer that the distorted $F_o1$ affects more the DCCD-binding site than that for oligomycin.

A recent report [12] on the effect of millimolar diamide concentrations on the isolated $F_o$ proposes that a disulphide bridge forms between the 25 kDa subunit of $F_o$ and the $\gamma$-subunit of $F_1$, rather than between two 25 kDa subunits. However, contrary to [12], we directly prove here that the latter phenomenon clearly occurs at those micromolar concentrations of the reagent (Fig. 2) in which binding of $F_1$ to $F_o$ is hampered (Table I). Interestingly, we too did consider an involvement of the cysteine-containing $\gamma$-subunit of $F_1$. This hypothesis was, however, abandoned already in [2], because of the lack of correlation between the $\gamma$-subunit disappearance and the studied functional parameters. Thus, if a discrepancy exists, it may be ascribed to structural differences of $F_o$ that has been prepared with different methods.

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