# The membrane in submitochondrial particles protects $F_1$-ATPase from trinitrobenzolsulphonate and dinitrofluorobenzole

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## 1. INTRODUCTION

It is widely believed that $F_1$-ATPase forms so-called knobs on the surface of the coupling membranes [1]. This knob is connected with the membrane by a fairly fine stalk and rises above its surface by tens of Å [2–4]. However, some data do not agree well with this model. In [5] a spin label covalently bound to $F_1$-ATPase of *Micrococcus lysodeikticus* was rapidly reduced by the respiratory chain localised in the hydrophobic phase of the membrane. In experiments with bilirubin [6], a photo-activated modifier of membrane proteins, β-subunits of ATPase were shown to react with other membrane components; phospholipids probably take part in this process [6]. The results in [7–9] testify to the fact that membrane in submitochondrial particles protects the active site of $F_1$-ATPase from hydrophilic inhibitors.

In this work the accessibility of isolated and membrane-bound $F_1$-ATPase to the hydrophilic modifying agent, TNBS, has been studied. From the results obtained it follows that the membrane screens a considerable part of the surface of $F_1$-ATPase (probably $> 1/3$). The results obtained with a more hydrophobic modifying agent, DNFB, testify to the fact that the part of the $F_1$-ATPase immersed in the membrane comes into contact with the inner membrane proteins and not with the phospholipid region of the membrane.

*Abbreviations*: DNFB, dinitrofluorobenzene; TNBS, trinitrobenzosulphonate

## 2. METHODS

Submitochondrial particles were obtained from beef heart mitochondria as in [10]. $F_1$-ATPase was isolated according to [11] or [12]. Before use $F_1$-ATPase was desalted on a Sephadex G-50 column (1 × 20 cm).

TNBS from Sigma and DNFB from Reanal were used.

The particles or $F_1$-ATPase were treated with 50 mM TNBS in a mixture containing 100 mM triethanolamine–$SO_4$ buffer (pH 8.3) and 250 mM sucrose for 5 min at 20–22°C. The reaction of the $F_1$-ATPase or particles with 50 mM DNFB was done in the same buffer for 10 min.

To stop the reaction, an 8–10-fold excess of β-alanine was added to the reaction mixture. The modified $F_1$-ATPase was separated from the TNBS- or DNFB-derivative of β-alanine by dialysis against 0.02 M phosphate buffer (pH 7.0). Modified submitochondrial particles were washed twice by centrifugation in 10 mM Tris–$SO_4$ buffer (pH 7.5) containing 250 mM sucrose, and then $F_1$ was isolated from these particles as in [12]; $F_1$ was additionally subjected to dialysis in a phosphate buffer as above.

When determining the extent of modification of $F_1$-ATPase, the extinction coefficient of the trinitrophenyl derivative of amino acids was taken to

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be equal to $1.1 \times 10^4$ $M^{-1}.cm^{-1}$ ($\lambda = 367$ nm) [13], for the dinitrophenyl derivative $1.6 \times 10^4$ $M^{-1}.cm^{-1}$ ($\lambda = 365$ nm) [14]. Spectral measurements were done in 0.02 M phosphate buffer (pH 7.0) containing 1% sodium dodecyl sulphate. The spectra of the control preparation of the enzyme of the same concentration were subtracted from the spectra of the modified factor $F_1$.

Treatment of the desalted factor $F_1$ (1–1.5 mg/ml) with 0.5% or 1% dimethylsuberimidate was carried out in 100 mM triethanolamine–$SO_4$ buffer (pH 8.3) containing 250 mM sucrose, for 60 min at 20–22°C. The cross-linked protein was dialysed against 30 mM triethanolalamine buffer.

Electrophoresis in 10% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulphate was done as in [15]. Electrophoresis in the absence of dissociating agents was performed in 5% polyacrylamide gels according to [16].

Protein was determined by the method in [17].

## 3. RESULTS AND DISCUSSION

In studying the accessibility of $F_1$-ATPase to TNBS and DNFB, we proceeded from the fact that both compounds mainly react with the amino groups of proteins, forming stained products with known extinction coefficients [13,14]. The results of determining the extent of modification of isolated and membrane-bound $F_1$-ATPase are given in table 1. When determining the extent of $F_1$-ATPase modification in particles, and enzyme was isolated as in [12]. According to electrophoretic data, isolated $F_1$-ATPase did not contain noticeable protein contamination (see fig. 1).

It follows from the data obtained (table 1) that the extent of modification of isolated $F_1$-ATPase is considerably higher than in the case of membrane-bound $F_1$-ATPase. This conclusion is true both of the hydrophilic modifying reagent, TNBS, as well as of DNFB, which is highly soluble in the lipid phase of the membrane. These results might well testify to the fact that most of the amino groups of $F_1$-ATPase in the particles are protected by other membrane proteins.

However, it has been shown that treatment of isolated $F_1$-ATPase with TNBS (fig. 2) or DNFB (not shown) leads to almost complete dissociation of the enzyme into subunits. Dissociation into subunits can lead to an increase in the number of amino groups that are accessible to the modifying reagents. To avoid TNBS- (or DNFB-) induced dissociation of $F_1$-ATPase into subunits, the enzyme was pretreated with a cross-linking reagent, dimethylsuberimidate. As shown in [18,19], dimethylsuberimidate-induced cross-linking of $F_1$-ATPase subunits prevents the dissociation of the enzyme into subunits at low temperatures or under pressure.

$F_1$-ATPase pretreated with 1% dimethylsuberi-

Table 1

The degree of modification of isolated and membrane-bound $F_1$-ATPase by TNBS and DNFB

| Preparation | mol TNBS bound/mol $F_1$-ATPase | mol DNFB bound/mol $F_1$-ATPase |
|---|---|---|
| Isolated $F_1$-ATPase | 100 (3) | 38 (2) |
| Isolated $F_1$-ATPase pretreated with dimethylsuberimidate | 30.0 ± 1.0 (9) | 14.5 (5) |
| $F_1$-ATPase in submitochondrial particles | 21.7 ± 1.2 (16) | 7.9 (6) |

For conditions see section 2. When determining the degree of modification of $F_1$-ATPase in particles, the modified $F_1$-ATPase was isolated as in [12]. The average values obtained in several experiments are given. The number of experiments, which equal the number of tested preparations of $F_1$-ATPase and particles is indicated in the brackets

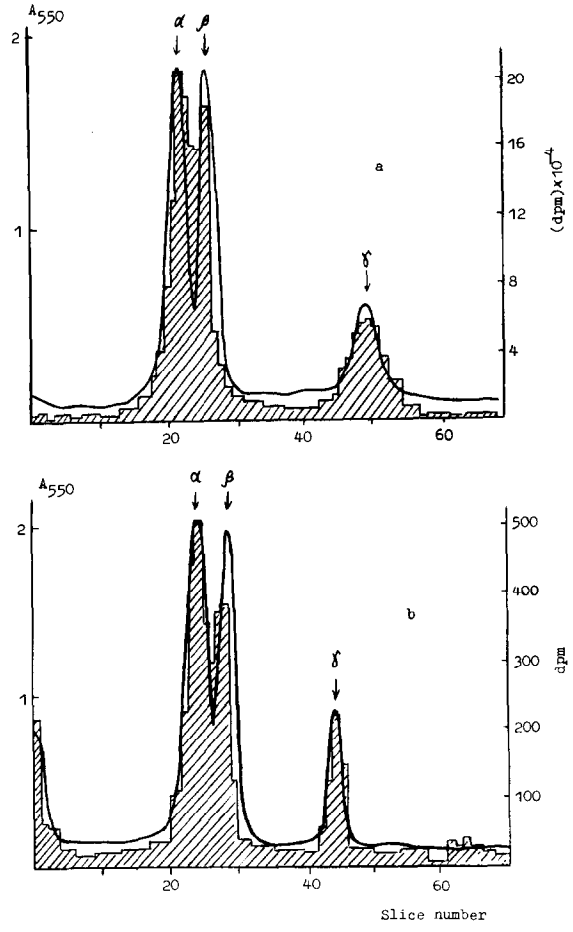


Fig. 1. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis of TNBS-modified $F_1$-ATPase treated with sodium [$^3$H] borohydride: (a) isolated $F_1$-ATPase was treated with TNBS and then with sodium [$^3$H]-borohydride; (b) submitochondrial particles were treated with TNBS; $F_1$-ATPase was then isolated according to [12] and treated with sodium [$^3$H]borohydride. For conditions see section 2 and the caption to table 2: (——) absorbance of the gel at 550 mm; (▨) radioactivity of the gel slices.

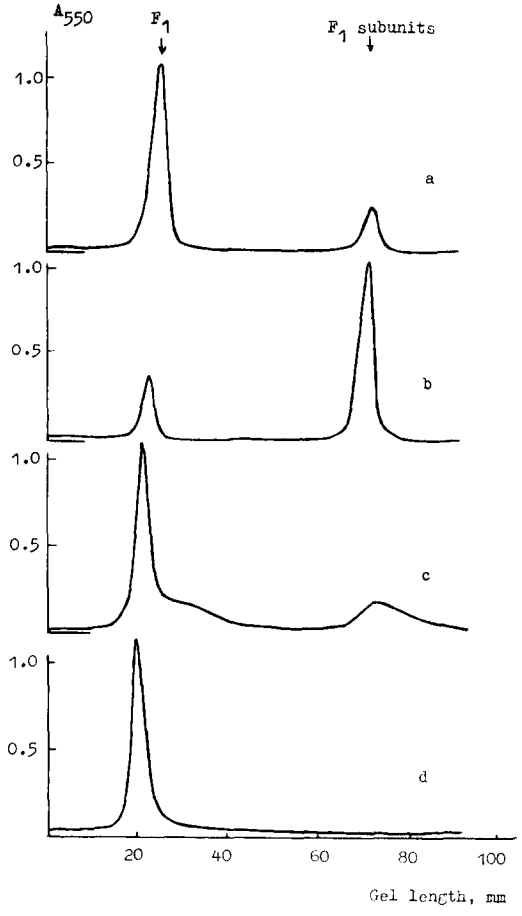


Fig. 2. Electrophoresis of $F_1$-ATPase in the absence of dissociating agents: a, control preparation; b, $F_1$-ATPase treated with TNBS; c, $F_1$-ATPase pretreated with 0.5% dimethylsuberimidate and then treated with TNBS; d, $F_1$-ATPase pretreated with 1% dimethylsuberimidate and then treated with TNBS. For the conditions of treatment with dimethylsuberimidate and TNBS see section 2. Electrophoresis was carried out for 14 h at a current of 4 mA in a tube. 60 μg of factor $F_1$ were placed on each gel. The gels were stained with amido black and scanned at 550 nM.

midate did not dissociate into subunits as a result of TNBS (fig. 1) or DNFB (not shown) treatment. Since dimethylsuberimidate reacts with the amino groups of proteins. $F_1$-ATPase pretreated with dimethylsuberimidate should have fewer amino groups accessible to TNBS and DNFB than the enzyme that had not been subjected to dimethylsuberimidate treatment. However, the isolated $F_1$-ATPase pretreated with dimethylsuberimidate still binds more TNBS or DNFB molecules than $F_1$-ATPase in submitochondrial particles (table 1). This result suggests that a considerable part of the surface of $F_1$-ATPase ($\geq 1/3$, judging from the data in table 1) is shielded by membrane proteins and thereby protected from the water-soluble TNBS and the lipid-soluble DNFB.

Schneider et al. [20] showed that both isolated $F_1$-ATPase and ATPase in submitochondrial particles have similar accessibility to another hydrophilic modifying reagent, diazobenzosulphonate. The difference between the results of [20] and those of the present paper may be explained by the different specificity of the modifying reagents used in these two studies. Another difference in the conditions of the experiments with TNBS and diazobenzosulphonate was that in our experiments a considerably larger number of amino acid residues of $F_1$-ATPase was subjected to modification than in [20]. It is obvious that the greater the number of amino acid residues entering into the reaction with the modifying reagent, the more objective is the information on the accessibility of the molecule of the enzyme to the given modifying reagent.

To determine the accessibility of individual subunits of $F_1$-ATPase to TNBS, $F_1$-ATPase isolated from particles treated with TNBS was incubated with sodium [$^3$H]borohydride as in [21]. This treatment leads to the radioactive labelling of the trinitrophenyl derivatives of the amino acid residues.

Table 2

The degree of modification by TNBS of subunits of isolated and membrane-bound $F_1$-ATPase

| Object | mol TNBS bound to $\alpha$, $\beta$ and $\gamma$-subunits/mol $F_1$-ATPase | | |
|---|---|---|---|
| | $\alpha$ | $\beta$ | $\gamma$ |
| Isolated $F_1$ATPase | 45 | 24 | 21 |
| $F_1$-ATPase in particles | 11 | 7 | 3 |

$F_1$-ATPase modified in particles by TNBS was isolated according to [12]. Treatment of TNBS-modified $F_1$-ATPase with 5 mM sodium [$^3$H]borohydride was carried out for 1 h at 22°C in 100 mM sodium phosphate buffer (pH 9.0) containing 1% sodium dodecyl sulphate. To determine the degree of the radiolabelling of the subunits, $F_1$-ATPase was subjected to gel electrophoresis (fig. 2). Gels were stained with amido black, the protein bands corresponding to the ATPase subunits were eluted by 1% sodium dodecyl sulphate, and eluted radioactivity was measured. The data show the average values obtained with 6 gels. The level of the radioactive labelling of $F_1$-ATPase, which was not treated with TNBS was <1% of that obtained for the membrane-bound $F_1$-ATPase pretreated with TNBS. Other details are given in section 2

The results of these experiments are given in fig. 1 and table 2: when submitochondrial particles were treated with TNBS, 1.5-times more molecules of TNBS bound to the $\alpha$-subunits of ATPase then to the $\beta$-subunits. This result makes it look as if the $\alpha$-subunits of membrane-bound $F_1$-ATPase are more accessible to TNBS than the $\beta$-subunits. However, this does not appear to be the case since TNBS treatment of isolated $F_1$-ATPase also results in more extensive modification of the $\alpha$-subunits than the $\beta$-subunits (fig. 1, table 2). The data in the literature on the content of lysine in the subunits of $F_1$-ATPase are contradictory. In [22] both types of subunits had approximately the same number of lysine residues, while, according to [16] the content of lysine in the $\alpha$-subunits is twice as high as that in the $\beta$-subunits. From the results obtained it follows that the $\alpha$-subunits have more lysine residues than the $\beta$-subunits or the lysine residues of the $\alpha$-subunits have a higher capacity to react with TNBS than the lysine residues in the $\beta$-subunits. An unexpectedly high ability to react with TNBS was demonstrated by the $\gamma$-subunit of isolated $F_1$-ATPase. It is known that the stoichiometry of the subunits in mitochondrial $F_1$-ATPase corresponds to the formula: $\alpha_3\beta_3\gamma\delta\epsilon$ [22,23]. The lysine content (calculated per mole of the subunit) in the $\gamma$-subunit is approximately the same as that of the $\beta$-subunit [16,22]. Thus, it may be expected that in the experiment with isolated $F_1$-ATPase $\beta$-subunits should bind at least 3- or (taking into account the $M_r$-value of the subunits) 5-times more TNBS molecules than the $\gamma$-subunit. However, as can be seen from the data in table 2 and fig. 1, the $\gamma$-subunit binds approximately the same number of TNBS molecules as the $\beta$-subunits. Comparing the results obtained with isolated and membrane bound $F_1$-ATPase (table 2), it could be concluded that the membrane protects the $\gamma$-subunit from TNBS more than it does the $\alpha$- and $\beta$-subunits. The conclusion that the $\gamma$-subunit of $F_1$-ATPase is shielded greatly by the mitochondrial membrane is also in good agreement with data showing that the membrane protects the $\gamma$-subunit from trypsin [24].

Thus, the results of this paper testify to the fact that a considerable part of the $F_1$-ATPase surface is in contact with other proteins of the mitochondrial membrane. It seems likely that the $\gamma$-subunit

of $F_1$-ATPase comes into contact with the mitochondrial membrane proteins to a greater extent than the $\alpha$- and $\beta$-subunits.

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