Comparative Aspects of the Attachment of F$_1$-ATPase to Micrococcus lysodeikticus Membranes: Role of Ions and Subunits

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Purified adenosine triphosphatase (F$_1$-ATPase) (EC 3.6.1.3) from Micrococcus lysodeikticus membranes required divalent metal ions for its reattachment to membranes depleted of the enzyme. This requirement showed specificity: Mg$^{2+}$ ions were able to reconstitute a physiologically significant F$_1$-ATPase-membrane complex, whereas Zn$^{2+}$ ions produced one differing from the native and the Mg$^{2+}$-reconstituted ATPase-membrane complexes. Micrococcus lysodeikticus membranes contained a limited number of specific binding sites, which did not seem to be modified after ATPase release and membrane manipulation. The binding properties of F$_1$-ATPase preparations correlated well with their content of δ-subunit. The results suggested that F$_1$-ATPase molecules able to rebind to M. lysodeikticus membranes must contain at least two copies of the δ-subunit. The isolated subunits (particularly α- and β-subunits) showed a certain capacity for rebinding to the membranes, but an enzyme form consisting only of α- and β-subunits was unable to reattach to membranes. These results prove unambiguously that δ-, but not α- or any other polypeptide, was involved in the attachment of F$_1$-ATPase to M. lysodeikticus membranes, unlike other bacterial F$_1$-ATPases. These results were consistent with a subunit stoichiometry and arrangement: $\alpha_3 \beta_3 \gamma(1-2) \delta_2$-Mg$^{2+}$-membrane.

INTRODUCTION

The energy-transducing adenosine triphosphatases are complex enzyme systems involved in coupling the energy of oxidative phosphorylation and photophosphorylation to ATP synthesis. The water-soluble part of the complex, named F$_1$-ATPase or F$_1$ factor, has been the subject of intensive studies over recent years (Penefsky, 1979; Downie et al., 1979; Shavit, 1980; Cross, 1981). These F$_1$-ATPases have been characterized as oligomeric proteins usually made up of a maximum of five subunits designated as α, β, γ, δ, ε, according to their decreasing molecular weights. However, a sixth polypeptide has been reported in some mitochondrial F$_1$-ATPase preparations (Senior, 1973), while the absence of some (or all) of the lower molecular weight (minor) subunits γ, δ and ε in bacterial F$_1$-ATPases of BF$_1$ factors has been observed (Downie et al., 1979; Muñoz, 1982). These F$_1$-ATPases are structurally and functionally linked to a proton-translocating sector, F$_0$, which spans the membrane. Thus the F$_0$–F$_1$ complexes act as reversible proton pumps coupling the trans-membrane proton gradient to ATP synthesis. A role of the δ subunit in the binding of the F$_1$-ATPase to the membrane sector of the proton-translocating ATPase has been widely reported in the systems studied, namely chloroplasts (Nelson, 1976) and bacteria (Muñoz, 1982). Nevertheless, the situation is not unambiguous since the α and ε subunits are also involved in the binding of F$_1$-ATPase to membranes of Streptococcus faecalis

Abbreviations: BF$_1$-ATPase, bacterial F$_1$-ATPase; F$_1$-ATPase, water-soluble portion of the energy-transducing ATP synthetase-ATPase complex; F$_0$, membrane sector of the complex.
(Abrams et al., 1976a, b) and *Escherichia coli* (Sternweis, 1978), respectively. Although divalent cations play a role in the binding of F$_1$-ATPase to the membranes (Abrams & Smith, 1974; Muñoz, 1982), the specificity and mechanism of their role is not known (Muñoz, 1982).

We reported in previous work (Mollinedo et al., 1980; A. Mimbrera, unpublished observations) that purified *M. lysodeikticus* F$_1$-ATPase was composed of four subunits ($\alpha$, $\beta$, $\gamma$ and $\delta$). A role of the $\delta$-subunit in binding *M. lysodeikticus* F$_1$-ATPase to the membrane was already assigned in these studies. The present report describes that, in addition to the S-subunit, the binding of this F$_1$ shows a specific requirement for divalent cations. Furthermore, information on the subunit stoichiometry of the enzyme has been gained from the study of the binding properties.

### METHODS

**Solubilization of F$_1$-ATPase and production of depleted membranes.** *Micrococcus lysodeikticus* strain A was grown and harvested as described (Muñoz et al., 1969). Membranes were obtained from protoplasts prepared by using lysozyme, and by subsequent osmotic shock (Muñoz et al., 1969) carried out in the presence of 10 mM-MgCl$_2$ (Mollinedo et al., 1980). Membranes were subsequently washed four times with 30 mM-Tris/HCl (pH 7.5) in the absence of divalent cations. The F$_1$-ATPase was released into solution by two subsequent washings of membranes with 3 mM-Tris/HCl (pH 7.5) and centrifugation at 35000 g for 30 min at 4°C. About 60% of the enzyme appeared in the supernatant fluid of the fifth and sixth washes. The membrane residues after release of the enzyme (referred to hereafter as depleted membranes) were frozen as a pellet and used in the reconstitution experiments. They contained a small amount of bound ATPase activity (less than 4% of the ATPase units).

**Purification of the F$_1$-ATPase and its subunits.** The F$_1$-ATPase was purified from the crude soluble fraction by preparative PAGE (Andreu & Muñoz, 1975). The $\alpha$, $\beta$- and $\gamma$-subunits were isolated by preparative gel electrophoresis of purified F$_1$-ATPase in the presence of 8 M-urea (Andreu & Muñoz, 1975; Andreu et al., 1976). Purified F$_1$-ATPase and subunits appeared to be homogeneous (>$95\%$) as judged by analytical gel electrophoresis. The urea-PAGE system did not allow the isolation of the $\delta$-subunit. This subunit was obtained by micro-preparative SDS-PAGE (Mollinedo et al., 1980). However, this purification method may result in severe modifications in conformation and properties of the $\delta$-subunit as deduced from the loss or change of its immunological properties (Larraga et al., 1981).

Analytical gel electrophoresis in non-dissociating and dissociating conditions was carried out as described (Andreu et al., 1973).

Molar proportions of the subunits in the different enzyme preparations were calculated from the staining intensities of each subunit in the PAGE patterns obtained in presence of SDS. Gels were stained with either Coomassie brilliant blue R250 (Fairbanks et al., 1971) or Coomassie Brilliant blue G-250 (Dietzel et al., 1972) and scanned at 575 nm in a Gilford 2400 spectrophotometer equipped with a 2410S linear transport. In spite of the limitations of the method, the use of two staining procedures and the application to various ATPase preparations made the results obtained statistically significant. Stoichiometries were also calculated from SDS patterns of iodinated ATPase (see below).

**Analytical procedures.** ATPase activity was measured by the liberation of P$_i$, as described by Muñoz et al. (1968a, b). One unit of enzyme activity is defined as that amount which liberates 1 $\mu$mol P$_i$ min$^{-1}$ at 37°C under the experimental conditions described (Muñoz et al., 1968b). Protein concentration was determined by the Lowry method with bovine serum albumin as standard.

**Iodination of the enzyme and its subunits.** F$_1$-ATPase and its $\alpha$, $\beta$- and $\gamma$-subunits were iodinated by a modification of the chloramine-T procedure (Greenwood et al., 1963) as previously reported (Larraga et al., 1981). This labelling method did not change the relative mobility in PAGE or the molecular state of any of the subunits of the enzyme and of the whole F$_1$-ATPase.

**Binding of F$_1$-ATPase to depleted membranes.** The procedure for measuring rebinding of F$_1$-ATPase to depleted membranes was as reported (Mollinedo et al., 1980), with the following modifications. Pure $[^{125}]$IF$_1$-ATPase (20000 c.p.m.; specific activity: $1.9 \times 10^4$ c.p.m. $\mu$g$^{-1}$) was mixed with depleted membranes (500 $\mu$g protein) in 200 $\mu$l 30 mM-Tris/HCl (pH 7.5) and in the presence of varying amounts of divalent cations (as chlorides) as indicated in the respective figures. The mixture was vigorously shaken for 5 min, incubated for 15 min at 37°C and then centrifuged at 43000 g for 30 min. Pellet and supernatant were collected and the amount of BF$_i$ factor bound to the membranes was determined as the percentage of radioactivity in the sediment. The same procedure was applied in rebinding experiments of defective F$_1$-ATPases, previously labelled with $^{125}$I. In saturation experiments, the amount of F$_1$-ATPase was varied in assays carried out as above.

The subsequent release of F$_1$-ATPase bound to the membrane was measured radiochemically or by determination of the ATPase units after different solubilization treatments, as specified in the respective tables.
Attachment of BFₐ-ATPase to membranes

RESULTS

Effect of divalent ions in the binding of F₁-ATPase to depleted membranes

These experiments were performed with an F₁-ATPase preparation composed of α-, β-, γ- and δ-subunits with the stoichiometry 3·00 : 3·00 : 1·11 : 2·37. In these experiments, there was a large excess of membrane binding sites with respect to F₁-ATPase (see below).

The addition of differential metal ions yielded different degrees of attachment (Fig. 1). The concentrations of Ca²⁺ and Mg²⁺ necessary to achieve 50% binding were 3–5 times higher than those of Zn²⁺ and Cu²⁺. Other cations such as Co²⁺, Hg²⁺, Ni²⁺ and Mn²⁺ behaved in accordance with their ligand affinity (see below).

A basal binding (about 20%) was observed with no divalent ion added. This binding might be due to residual divalent cations in the depleted membranes. However, the treatment of the membranes with EDTA did not affect this residual binding. EDTA does not totally remove the divalent cations bound to purified F₁-ATPase (Mollinedo et al., 1981). It is not surprising that a similar situation can be found in membranes.

Specificity of divalent cations in F₁-ATPase rebinding to the membranes

To explore the physiological significance of the enzyme-membrane complexes obtained in the different experimental conditions, the solubilization of ATPase from the reconstituted F₁-membranes was compared to that of native membranes. Two types of F₁-ATPase–membrane complexes were considered in these reconstitution–dissociation experiments. One was formed in the presence of Mg²⁺ ions (affinity by oxygen ligands), the other produced with Zn²⁺ ions (affinity for sulphur and nitrogen ligands). The solubilization procedure previously described (Muñoz et al., 1969) was first applied (Table 1). The four washes with 30 mM-Tris/HCl (pH 7·5) released 10–15% of the ATPase bound to native and Mg²⁺-F₁-membrane complexes while they did not solubilize any enzyme from the Zn²⁺-F₁-membranes. The amount of ATPase solubilized by ‘osmotic shock’ (3 mM-Tris/HCl, pH 7·5) compared well for native and Mg²⁺-F₁-membranes. However, EDTA, which solubilized only low amounts of ATPase (10–20%) from native membranes and Mg²⁺-F₁-membrane complexes, was highly effective in solubilizing ATPase (>50%) from its Zn¹⁺-membrane complex. EGTA behaved like EDTA; urea was effective in ATPase solubilization from native membranes and Mg²⁺-F₁-membrane complexes, while LiCl was ineffective.

Thus the F₁-ATPase from native membranes and from enzyme–membranes reconstituted with the addition of Mg²⁺ ions showed similar qualitative and quantitative properties, suggesting that the enzyme molecule links to the membrane in both cases in a similar, or identical, way.
Table 1. Release of F$_1$-ATPase from native membranes and from the BF$_1$-membrane complexes reconstituted in the presence of Mg$^{2+}$ and Zn$^{2+}$ ions

Native F$_1$-ATPase–membrane and the BF$_1$–membrane complexes obtained in the presence of 10 mM-Mg$^{2+}$ or 2 mM-Zn$^{2+}$, taken in each case as 100% ATPase bound, were treated for 15 min at room temperature as indicated below. The solubilization of F$_1$-ATPase was measured radiochemically as described in Methods. Results are expressed as the mean from three different experiments. ND, Not determined.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>F$_1$-ATPase release from membranes (%)</th>
<th>Native Mg$^{2+}$-reconstituted</th>
<th>Zn$^{2+}$-reconstituted</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 mM-Tris/HCl (pH 7-5)</td>
<td>13-6</td>
<td>13-7</td>
<td>0</td>
</tr>
<tr>
<td>3 mM-Tris/HCl (pH 7-5)</td>
<td>70-0</td>
<td>50-0</td>
<td>10-7</td>
</tr>
<tr>
<td>1-5 mM-EDTA</td>
<td>15-2</td>
<td>20-7</td>
<td>55-1</td>
</tr>
<tr>
<td>1-5 mM-EGTA</td>
<td>16-5</td>
<td>23-5</td>
<td>ND</td>
</tr>
<tr>
<td>1 M-LiCl</td>
<td>0-0</td>
<td>8-5</td>
<td>ND</td>
</tr>
<tr>
<td>1-5 M-Urea</td>
<td>100</td>
<td>56-9</td>
<td>ND</td>
</tr>
</tbody>
</table>

The failure of chelating agents (EDTA, EGTA) to solubilize this BF$_1$ factor was striking and raised some doubts about the involvement of Mg$^{2+}$ in the binding of the enzyme to the membrane. However, the putative participation of divalent cations in the formation and/or stability of the F$_1$–membrane complex was further supported by the fact that enzyme solubilization by low ionic strength buffer was prevented by the addition of traces of divalent cations.

Quantitative analysis of F$_1$-ATPase binding to depleted membranes

The quantitative aspects of ‘in vitro’ attachment were studied by adding variable amounts of F$_1$-ATPase to a constant amount of depleted membranes in the presence of 20 mM-Mg$^{2+}$. The reattachment of the F$_1$-ATPase to depleted membranes followed a saturation-type curve. Saturation was achieved when the amount of bound enzyme was about 100 µg bound F$_1$-ATPase (mg membrane protein)$^{-1}$. This value is consistent with previous estimates of the amount of F$_1$-ATPase bound to native M. lysodeikticus membranes (Muñoz et al., 1969).

Owing to the δ-defective character of the ATPase molecules (see below), a large excess beyond this amount was required in the assays to attain saturation of binding. Maximal rebinding of the enzyme practically restored the original specific activity of the membranes-bound ATPase. This suggested that all the binding sites in the native membranes were fully occupied, a view supported by the inability of additional enzyme to rebind to the native membranes when these were mixed with a large excess of free F$_1$-ATPase.

Reattachment of different F$_1$-ATPase forms to depleted membranes

In previous work evidence supporting the role of the δ-subunit in the binding of M. lysodeikticus F$_1$-ATPase to the membranes has been presented (Mollinedo et al., 1980; Mimbrera et al., 1983). Figure 2 illustrates the attachment of two F$_1$-ATPase preparations with distinct subunit stoichiometries to depleted membranes. There was a good correlation between the content of δ-subunit in these preparations and the extent of binding. Thus the larger the content of δ-subunit in the enzyme, the higher was the reattachment of the F$_1$-ATPase to depleted membranes. This result was independent of the presence of divalent cations. These findings indicate not only an essential role of the δ-component in the binding capacity of BF$_1$ factor to the membrane, but also support the notion that this protein in its membrane-bound state may have at least two copies of the δ-subunit per molecule of enzyme. In this context, it is worth noting the variability in the content of δ-component found in this enzyme, ranging from 0 to 2 copies per mol enzyme, depending on the solubilization and purification procedure. The possible indirect role of other subunits, i.e. the γ-subunit, to facilitate binding may also be considered (see Discussion).
Fig. 2. Binding in the presence of Mg\(^{2+}\) of F\(_1\)-ATPase preparations differing by their δ-subunit content: one δ-subunit (○) and two δ-subunits (●) per α, β, γ. For experimental details see Methods.

Fig. 3. Binding of isolated α-, β- and γ-subunits of F\(_1\)-ATPase to M. lysodeikticus depleted membranes and effect of Mg\(^{2+}\) concentrations. For experimental details see Methods. Results are expressed as percentages of \(^{125}\)I-labelled subunits (about 20000 c.p.m. of each subunit were added in the experiments; specific radioactivities: α and β, \(5 \times 10^4\) c.p.m. µg\(^{-1}\); and γ, \(3 \times 10^4\) c.p.m. µg\(^{-1}\)) bound to the membranes (500 µg membrane protein) at each Mg\(^{2+}\) concentration in 200 µl 30 mM-Tris/HCl, pH 7.5. [Subunits δ showed a behaviour intermediate between those of α- and β-subunits and that of γ-subunit, although these results (not shown) should be taken with caution owing to the difficulties arising from the procedure used for the purification of this subunit (see Methods)]. ○, α-Subunit; Δ, β-subunit; □, γ-subunit.

The F\(_1\)-ATPase rebinding levels to depleted membranes achieved with different enzyme preparations defective or modified in some subunits (Table 2) confirmed previous assumptions (Mollinedo et al., 1980) concerning the pivotal role played by the δ-component in enzyme binding. In contrast, the major subunits (α and β) or any of their fragments do not seem to play a role in these binding properties. The coupling function assigned to the δ-subunit of M. lysodeikticus correlates well with the relatively greater hydrophobicity of that polypeptide as compared to that of the α-, β- and γ-subunits of the enzyme (F. Mollinedo, unpublished).

Table 2. Effect of Mg\(^{2+}\) and subunit stoichiometry of F\(_1\)-ATPase forms on the re-attachment of the enzyme to depleted membranes of M. lysodeikticus

<table>
<thead>
<tr>
<th>Enzyme form</th>
<th>Subunit composition</th>
<th>F(_1)-ATPase rebinding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−Mg(^{2+})</td>
<td>+Mg(^{2+})</td>
</tr>
<tr>
<td>1</td>
<td>α(_3) β(_3) γ δ(_2)</td>
<td>18.5</td>
</tr>
<tr>
<td>2</td>
<td>α(_3) β(_3) γ δ(_2)</td>
<td>9.5</td>
</tr>
<tr>
<td>3</td>
<td>α(_3) β(_3) γ δ(_2)</td>
<td>10.3</td>
</tr>
<tr>
<td>4</td>
<td>α(_3) β(_3) γ δ(_2)</td>
<td>7.2</td>
</tr>
<tr>
<td>5</td>
<td>α(_3) β(_3) γ δ(_2)</td>
<td>7.2</td>
</tr>
</tbody>
</table>

* These values did not increase with increasing Mg\(^{2+}\) concentrations.
Binding of purified subunits of F\textsubscript{1}-ATPase other than \textit{\delta} to depleted membranes

The effect of Mg\textsuperscript{2+} concentration on the binding of the isolated \textit{\alpha}-, \beta- and \gamma-subunits to depleted membranes was examined (Fig. 3). The \textit{\alpha}- and \beta-subunits showed a high binding capacity that could be explained by their partially hydrophobic nature (Andreu & Muñoz, 1979). The \gamma-subunit showed a very low binding capacity.

Treatment of F\textsubscript{1}-ATPase from \textit{M. lysodeikticus} with the chelating agent EDTA for 24 h allowed the production of an enzyme preparation defective in the minor subunits \gamma and \delta (F. Mollinedo, unpublished; see also Table 2). A comparison between the binding to the membrane of this enzyme form and that of the native enzyme and of the \textit{\alpha} and \beta isolated subunits (compare Figs 1, 2, 3 and Table 2) revealed that, in contrast to the relatively high binding capacity shown by the major subunits in their purified state, the complex (\textit{\alpha}\textit{\beta}) was unable to attach to membranes (Table 2).

From these results it seems reasonable to conclude that there are binding sites for the membrane in the purified \textit{\alpha}- and \beta-subunits, but only those located in the \delta-subunit remain effective in the binding of the whole \textit{M. lysodeikticus} F\textsubscript{1}-ATPase to the membrane.

DISCUSSION

This work confirmed that the \delta-subunit was required for the attachment of F\textsubscript{1}-ATPase from \textit{M. lysodeikticus} to the membranes. One of the most important developments from these results was their application in further exploration of the subunit stoichiometry of this BF\textsubscript{1}. The failure to obtain 100\% binding for reputedly pure ATPase preparations was striking, although saturation of the binding sites in the membrane could be achieved. This defective binding may have as its molecular basis the fact that native \textit{M. lysodeikticus} F\textsubscript{1}-ATPase contains at least two copies of \delta-subunits per mol enzyme. This notion agrees with previous observations based on cross-linking studies of this BF\textsubscript{1} (Muñoz et al., 1980). It is well established that the \delta-polypeptide is loosely bound to the rest of the F\textsubscript{1}-ATPase in most coupling factors studied. Mild treatments lead to its dissociation from the enzyme and this may explain the variable presence or the absence of \delta-subunit in distinct F\textsubscript{1}-ATPases depending on the source and on the solubilization and purification procedures. This applies particularly to \textit{M. lysodeikticus} ATPase, since enzyme forms lacking \delta-subunits were easily obtained (Andreu et al., 1973).

Even under apparently optimal conditions, we were unable to obtain 100\% rebinding of the F\textsubscript{1}-ATPase preparations to membranes of \textit{M. lysodeikticus}. It is likely that we were measuring average binding abilities or micro-heterogeneous preparations. We cannot then rule out the possibility that more than two copies of the \delta-subunit or the co-operation with more than one copy of the \gamma-subunit, or with other polypeptide and undefined component(s) would be required for completing molecules of this BF\textsubscript{1} able to re-attach to the membrane. As a matter of fact, there is also evidence suggesting that \textit{M. lysodeikticus} ATPase may contain more than one copy of the \gamma-subunit (Andreu & Muñoz, 1979; A. Mimbrrera, unpublished results).

The present results have shown unambiguously that insertion of \textit{M. lysodeikticus} F\textsubscript{1}-ATPase into BF\textsubscript{1}-depleted membranes requires divalent cations. Specific ions, \textit{i.e.} Mg\textsuperscript{2+}, seem to be involved in that interaction. In this context it has been reported that Mg\textsuperscript{2+} facilitated the reconstitution of F\textsubscript{0}-F\textsubscript{1}-ATPase complexes with lipids and membranes (Abrams & Baron, 1968; Sone et al., 1977; Eytan & Matheson, 1976) and it has been claimed that Mg\textsuperscript{2+} plays a specific role in the reconstitution of pig heart mitochondrial F\textsubscript{0}F\textsubscript{1}-ATPase with liposomes. However, the mechanisms of this role are poorly understood. A plausible explanation is that Mg\textsuperscript{2+} and/or physiologically related cations like Ca\textsuperscript{2+} (see Fig. 1) act as a cationic bridge between anionic sites (peptide oxygen ?) in the enzyme and the membrane. Definitive answers to this complex problem must await structural analysis of the protein by three dimensional crystallography as has been carried out, for example, with a protein containing bacteriochlorophyll (Matthews, 1982). Nevertheless, the present results suggest some ideas about the possible interactions involved in F\textsubscript{1}-ATPase binding. It is tempting to suggest that \textit{M. lysodeikticus} F\textsubscript{1}-ATPase is held to the membrane by a complex set of interactions: electrostatic (see above); hydrogen bonds...
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directly or through water molecules (remember the effect of urea on solubilization, see Table 2); and hydrophobic interactions (see above).

This work has also demonstrated that re-attachment of F,-ATPase to M. lysodeikticus membranes can be induced by cations other than Mg2+ in an artificial manner, compelling caution in the interpretation of reconstitution experiments.

The reattachment of the ATPase to the membrane in the presence of Mg2+ provides evidence in favour of a reliable reconstitution of the native membrane-bound enzyme. At saturation, the amount of bound enzyme represented about 10% of the total membrane protein, a value which is consistent with previous estimations (Muñoz et al., 1969). The reconstituted BF,-membrane complexes possessed a specific activity similar to that of native membranes. Furthermore, native membranes were unable to bind more enzyme even when a large excess of it was added. This suggested that the depleted membranes had not undergone any significant change during solubilization, manipulation and storage and, therefore, that the sites occupied after reconstitution were the same as those in native membranes.

The molecular characteristics of the binding of M. lysodeikticus F,-ATPase to the membranes showed striking similarities to those of Streptococcus faecalis F,-ATPase (Abrams et al., 1976a, b) with regard to the involvement of the δ-subunit and Mg2+ ions. However, differences stemmed from the lack of involvement of the α-subunits of M. lysodeikticus ATPase, whereas those of S. faecalis F,-ATPase played a role in its binding (Leimgruber et al., 1978). This difference can now be explained by the excess of δ-subunit of the molecule of M. lysodeikticus ATPase as compared with S. faecalis F,.

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