Influence of the α -, β - and γ -subunits of the energy-transducing adenosine triphosphatase from *Micrococcus lysodeikticus* in the immunochemical properties of the protein and in their reconstitution studied by a radioimmunoassay method

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We developed a sensitive and specific radioimmunoassay of the energy-transducing adenosine triphosphatase (F_1 -ATPase, EC 3.6.1.3) of *Micrococcus lysodeikticus* and extended the assay to the α -, β - and γ -subunits of the enzyme. We isolated these subunits and studied cross-reactions. We found the immunochemical properties of α - and β -subunits to differ, and γ -subunit showed an intermediate behaviour between that of α - and β -subunits. Our findings indicate that each subunit of *M. lysodeikticus* F_1 -ATPase has its own identity and that conformational antigenic determinants and/or co-operative antigenic sites arise from subunit assembly. Equimolecular amounts of α - and β -subunits (up to three copies of each) reconstituted partially the immunochemical properties of the ATPase molecule, and addition of 2 mol of γ -subunit per mol of $\alpha_3\beta_3$ complex improved reconstitution. Our findings describe the first reconstitution of biological activity of this ATPase by assembly of the isolated subunits, and provide support for earlier proposals on the stoichiometry of the $\alpha_3\beta_3\gamma_2$ type for *M. lysodeikticus* F_1 -ATPase. The radioimmunoassay method affords opportunities to study the homologies between different energy-transducing ATPases and their constituent polypeptides before the primary structure of these complex proteins has been determined.

The water-soluble portions of the ATPase-ATP synthetase complex (EC 3.6.1.3) are called F_1 -ATPases or F_1 factors (Penefsky, 1974; Pedersen, 1975; Kozlov & Skulachev, 1977; McCarty, 1979; Downie *et al.*, 1979). They are oligomeric proteins of up to six subunits involved in energy transduction. The subunits are designated α , β , γ , δ and ϵ according to their molecular weights. The sixth polypeptide in some mitochondrial F_1 -ATPase preparations may be the natural inhibitor of the ATPase activity (Penefsky, 1974; Pedersen, 1975). The two major subunits, α and β , are stable and show molecular weights in the 50 000–60 000 range in all F_1 -ATPases (Pedersen, 1975; McCarty, 1979; Andreu & Muñoz, 1979). The minor subunits, γ , δ and ϵ , vary depending on the source and method of isolation of the F_1 factor. Their variability is

particularly striking in bacterial F_1 -ATPases or BF_1 factors, which have two to five subunits (Downie *et al.*, 1979).

It is of interest to know the relationships between the structural and functional properties of BF_1 factors. Some attempts to obtain information on this subject involved reconstitution of the activity of BF_1 -ATPase from isolated subunits (Yoshida *et al.*, 1977) or from subunit complexes (Vogel & Steinhart, 1976). Immunological techniques may be well-suited to study these relations, but they have rarely been applied. Thus Kanner *et al.* (1975) studied functions of the subunits of *Escherichia coli* BF_1 -ATPase by experiments with antibodies against subunits isolated in the presence of SDS. Yoshida *et al.* (1979) reported on the use of antibodies produced against subunits of the thermophilic bacterium *PS₃* isolated in urea or guanidine, and questioned the immunological significance of the antisera against subunits isolated in the presence of SDS. Previously, we prepared antibodies against α -, β - and γ -subunits of *Micrococcus lysodeikticus*

Abbreviations used: F_1 -ATPase, the water-soluble portion of the ATPase-ATP synthetase complex; BF_1 -ATPase or BF_1 factor, bacterial F_1 -ATPase; SDS, sodium dodecyl sulphate.

BF₁-ATPase isolated in urea and used them to determine the functional role of each subunit (Mollinedo *et al.*, 1980).

We now report on a specific, highly sensitive, radioimmunoassay for the BF₁-ATPase of *M. lysodeikticus* and its α -, β - and γ -subunits. This is, to our knowledge, the first report on a radioimmunoassay for an energy-transducing protein and some of its constituent polypeptides. The implications of this work are discussed.

Materials and methods

Chemicals

[¹²⁵I]Iodide (16 mCi/ μ g) was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). Chloramine-T, KI, sodium metabisulphite, NaCl, urea and Tris were obtained from Merck (Darmstadt, Germany), Triton X-100 was from Calbiochem (San Diego, CA, U.S.A.), EDTA was from Fisher Scientific Co. (Fairlawn, NJ, U.S.A.) and specially pure SDS was from BDH Chemicals (Poole, Dorset, U.K.).

Purification of BF₁-ATPase and its subunits

Micrococcus lysodeikticus N.C.T.C. 2665 strain A was grown and harvested as described previously (Andreu *et al.*, 1973). The crude soluble BF₁-ATPase was obtained from the membranes by osmotic shock (Muñoz *et al.*, 1968; Mollinedo *et al.*, 1980). BF₁-ATPase was purified by preparative polyacrylamide-gel electrophoresis (Andreu & Muñoz, 1975), and its subunit composition was as that described previously (Mollinedo *et al.*, 1980).

BF₁-ATPase subunits (α , β and γ) were isolated as random-coil polypeptides by preparative gel electrophoresis of purified ATPase in the presence of 8M-urea, required to dissociate the enzyme fully (Andreu & Muñoz, 1975, 1979). In the preparative urea/polyacrylamide-gel-electrophoresis system, gels and upper buffer contained 8M-urea/1mM-dithiothreitol, whereas the lower chamber did not contain urea. Samples were treated with 10M-urea/5mM-dithiothreitol before electrophoresis (Andreu & Muñoz, 1975).

The urea/polyacrylamide-gel-electrophoresis system did not allow the isolation of the δ -subunit. This subunit was obtained by micro-preparative SDS/polyacrylamide-gel (10% acrylamide) electrophoresis (Mollinedo *et al.*, 1980).

Analytical gel electrophoresis

Non-dissociating and SDS/polyacrylamide-gel electrophoreses were performed in Tris/glycine buffer, pH 8.5 $\pm$ 0.2 (Andreu *et al.*, 1973). Gels (11 cm $\times$ 0.6 cm) of 7% acrylamide/0.18% *NN'*-methylenebisacrylamide (for non-dissociating polyacrylamide-gel electrophoresis) or 10% acrylamide/

0.28% *NN'*-methylenebisacrylamide (for SDS/polyacrylamide-gel electrophoresis) were used. Electrophoreses were run at room temperature, first at 2 mA/column (20 min) and then at 5 mA/column (2 h). In SDS/polyacrylamide-gel electrophoresis, gels and buffers contained 0.1% SDS and samples were pretreated with a protein/detergent ratio of 1:10 (w/w) at 85°C for 5 min. Gels were stained with Coomassie Brilliant Blue R250 (Andreu *et al.*, 1973) or sliced into 1 mm slices and their radioactivities subsequently counted in a Beckman Radioimmuno Analyzer.

Preparation of antisera

Male New Zealand White rabbits were immunized with purified BF₁-ATPase judged to be more than 95% pure by polyacrylamide-gel electrophoresis. Pure enzyme (0.4 mg) was incorporated in Freund's complete adjuvant to a final volume of 1 ml and was injected weekly subcutaneously into rabbits for a period of 3 weeks. Then 2 weeks later the rabbits were given a subcutaneous booster injection and were bled 1 week later from an ear vein.

Antisera were also raised against α -, β -, γ - and δ -subunits isolated in the presence of SDS and judged to be more than 92% homogeneous by SDS/polyacrylamide-gel electrophoresis. After clotting had occurred, sera were obtained, heated at 55°C for 30 min to inactivate complement and stored (Brody & Carlstrom, 1961).

Iodination of antigens

BF₁-ATPase and its α -, β - and γ -subunits were iodinated by a modification of the chloramine-T procedure (Greenwood *et al.*, 1963). The reaction mixture (final volume 300 μ l) contained TNE buffer (100 mM-Tris/2.5 M-NaCl/10 mM-EDTA, pH 7.2), 0.05% Triton X-100, 10 μ g of the antigen, 1 mCi of [¹²⁵I]iodide and 10 μ g of chloramine-T. After a gentle vortex-mixing at 22°C for 1 min, the reaction was stopped by addition of 120 μ g of sodium metabisulphite and 16 μ g of KI. The iodinated antigens were separated from the unreacted [¹²⁵I]iodide by gel filtration on a column (0.9 cm $\times$ 15 cm) of Sephadex G-50 for BF₁-ATPase and its α - and β -subunits, and a column (same dimensions) of Sephadex G-25 for the γ -subunit. The columns were previously equilibrated with TNE buffer containing 0.05% Triton X-100. Fractions were monitored for ¹²⁵I, and those containing the iodinated antigen were dialysed against 1 litre of 30 mM-Tris/HCl buffer, pH 7.5 (three changes), and were stored at -20°C.

The antiserum against δ -subunit isolated in the presence of SDS did not give any precipitation reaction with BF₁-ATPase or its δ -subunit isolated in the presence of SDS. Moreover, the δ -subunit isolated in the presence of SDS did not form a precipitate with anti-BF₁-ATPase sera. The apparent

lack of antigenic properties of the δ -subunit isolated in the presence of SDS, as prepared by us, hindered its further use as an antigen.

The molecular state of the antigens was checked by SDS/polyacrylamide-gel electrophoresis (see the Results section).

Radioimmunoassay of BF_1 -ATPase and its α -, β - and γ -subunits

Radioimmunoassay was performed in borosilicate glass tubes (10 mm $\times$ 75 mm). The reaction mixture (final volume 200 μ l) contained TNE buffer and 0.05% Triton X-100. In addition, each tube received 20 μ l of antiserum (diluted 3200-fold for anti- BF_1 -ATPase and anti- β -subunit, 1600-fold for anti- α -subunit and 800-fold for anti- γ -subunit). Different amounts of non-iodinated antigens (3 to 15 000 ng) were added, followed by the addition of labelled antigens (20 000 c.p.m.) to each tube. After a gentle vortex-mixing, the mixture was incubated for 3 h at 37°C, followed by an additional incubation at 4°C for 18 h. Then 50 μ l of a 10% suspension of formalin-treated *Staphylococcus aureus* was added, and the mixture was incubated for 20 min at 22°C with constant shaking. After the addition of 1 ml of TNE buffer containing 0.05% Triton X-100, the precipitate was collected by centrifugation at 5000g for 20 min, the supernatant was then carefully removed and the radioactivity in each tube was counted in a Beckman Radioimmuno Analyzer.

Statistical calculations

Data as the means of a minimum of two determinations each were corrected for non-specific binding. The binding curves were adjusted by a linear-least-squares-regression computer program (Chard, 1978). Although every radioimmunoassay has its own error (Chard, 1978), and this is illustrated in some examples given in the present paper (see Figs. 2 and 4), the standard curves were calculated as the means of several experiments in order to make the slope values more reliable.

Results

Labelling and molecular state of BF_1 -ATPase of *M. lysodeikticus* and its α -, β - and γ -subunits

Fig. 1 shows the protein and radioactivity profiles of BF_1 -ATPase as well as of the isolated α -, β - and γ -subunits in SDS/polyacrylamide-gel (10% acrylamide) electrophoresis. The correspondence between the profiles was good, indicating no change in the relative mobility and/or molecular state of any of the subunits of *M. lysodeikticus* BF_1 -ATPase after the iodination reaction. The α -, β - and γ -subunits gave average specific radioactivities of 1.23, 1.35 and 4.5 mCi/ μ mol respectively. Purified iodinated

Fig. 1. Purity and molecular state of BF_1 -ATPase of *M. lysodeikticus* and its subunits

Samples were analysed by SDS/polyacrylamide-gel (10% acrylamide) electrophoresis before being labelled with 125 I to test their purity and after this labelling to check the preservation of their molecular integrity. (a) 40 μ g of purified BF_1 -ATPase (70 000 c.p.m.); (b) 25 μ g of purified α -subunit (50 000 c.p.m.); (c) 25 μ g of purified β -subunit (55 000 c.p.m.); (d) 8 μ g of purified γ -subunit (50 000 c.p.m.). —, Protein staining (A_{575}); O, radioactivity. For other details see the Materials and methods section.

BF_1 -ATPase yielded a specific radioactivity of 260 μ Ci/ μ mol in non-dissociating conditions, but in the presence of Triton X-100, which was necessary to improve labelling and molecular dispersion of BF_1 -ATPase (results not shown). The proportions between specific radioactivities of the subunits and of BF_1 -ATPase are consistent with the molecular weights of each component (Andreu & Muñoz, 1979).

Fig. 2. Radioimmunoassay of *M. lysodeikticus* BF_1 -ATPase and competition effect of its α -, β - and γ -subunits

The radioimmunoassay was performed as described in the Materials and methods section. Results are expressed as percentage of ^{125}I -labelled BF_1 -ATPase bound to rabbit anti- BF_1 -ATPase serum. O, BF_1 -ATPase; $\blacktriangle$, α -subunit; $\square$, β -subunit; $\bullet$, γ -subunit.

Radioimmunoassay of BF_1 -ATPase

Fig. 2 shows a standard curve for the radioimmunoassay of *M. lysodeikticus* BF_1 -ATPase. Binding of ^{125}I -labelled ATPase to the anti- BF_1 -ATPase antibodies was inhibited progressively by increasing amounts of non-labelled BF_1 -ATPase and α -, β - or γ -subunits. A 70% binding was obtained with 22 ng of BF_1 -ATPase, 220 ng of γ -subunit, 460 ng of α -subunit and 625 ng of β -subunit respectively. On a molar basis, the sensitivity of the reaction between the anti- BF_1 -ATPase antibodies and BF_1 -ATPase molecule was 100 times that of the α - and γ -subunits and over 500 times that of the β -subunit. These results suggest a co-operative effect in the immunogenic properties of the subunits in whole BF_1 -ATPase and/or conformational antigenic determinants imposed by the BF_1 -ATPase macromolecule.

The sensitivities of the assay were 3 ng of BF_1 -ATPase and 20, 55 and 65 ng of γ -, α - and β -subunits respectively, and the upper limits of the assay were 1000 ng for BF_1 -ATPase and 10000 ng for each of its subunits. The slopes of the curves differed significantly, but not as a consequence of simple dilution effects.

Cross-reaction of α -, β - and γ -subunits with BF_1 -ATPase

These experiments were aimed to detect antigenic similarities and differences of the α -, β - and γ -subunits as elicited by anti- BF_1 -ATPase antibodies. The results appear in Fig. 3. As expected, BF_1 -ATPase was the most effective ligand to

Fig. 3. Binding of ^{125}I -labelled α -subunit (a), β -subunit (b) and γ -subunit (c) of *M. lysodeikticus* BF_1 -ATPase to anti- BF_1 -ATPase serum and the effect of non-labelled ATPase and its α -, β - and γ -subunits on this binding

Details are given in the Materials and methods section. O, ATPase; $\blacktriangle$, α -subunit; $\square$, β -subunit; $\bullet$, γ -subunit.

displace iodine-labelled subunit from anti- BF_1 -ATPase antibodies. In addition, each subunit was most effective in displacement of itself, as iodine-labelled tracer, from the antibody. There were differences between the slopes of curves, particularly between those for the α - and γ -subunits (see Figs. 3a and 3c). The γ -subunit occupied a position between the α - and β -subunits (see Figs. 3a and 3b, and also Fig. 2), except for displacement of iodine-labelled γ -subunit (Fig. 3c). The data support the contention

that the γ -subunit is not contaminated substantially with α -subunit (see Fig. 3a showing that non-labelled γ -subunit at low concentration competes with ^{125}I -labelled α -subunit more strongly than does the non-labelled α -subunit itself), although they do not rule out the possibility of contamination with β -subunit.

To clarify the properties of the β -subunit, we determined whether a mixture of α - and β -subunits behaves like γ -subunit in the radioimmunoassay of $\text{BF}_1\text{-ATPase}$. In previous experiments, a solution of β -subunit ($7.36\text{ }\mu\text{g}$ in $2\text{ }\mu\text{l}$) was titrated with increasing amounts of α -subunit. Progressive changes in the slope of the curve were detected to approach the slope obtained with $\text{BF}_1\text{-ATPase}$ ($n_{\text{ATPase}} = 1.57$, $n_{\alpha} = 1$, $n_{\beta} = 0.89$, $n_{\gamma} = 0.92$) as the molar proportion neared 1:1 ($7.12\text{ }\mu\text{g}$ of α -subunit). Thus a binding curve with slope similar to that for native $\text{BF}_1\text{-ATPase}$ was obtained (Fig. 4) by using equimolar proportions of α - and β -subunits as ligand. A quantitative analysis of these data indicates that 100% displacement of $\text{BF}_1\text{-ATPase}$ by α -subunits plus β -subunits was obtained by 350–400 ng of the mixture and 50% displacement was obtained by 40 ng. Taking into account that the average molecular weight of the subunits is 60 000, it can be estimated that six copies of the subunits are present in these conditions. Since the subunits are in equimolar proportion, this supports an $\alpha_3\beta_3$ stoichiometry. These observations imply the reconstitution of the immunochemical properties of whole $\text{BF}_1\text{-ATPase}$ of *M. lysodeikticus*. To explore reconstitution further, we added γ -subunit to the $\alpha_3\beta_3$ complex. Addition of γ -subunit enhanced recon-

Table 1. Competition of α -, β - and γ -subunits of *M. lysodeikticus* $\text{BF}_1\text{-ATPase}$ with the whole ATPase molecule in radioimmunoassay

The results are taken from the experiments illustrated in Figs. 2, 3 and 4. Percentages of competition express the ability of each subunit to compete at 70% binding with $\text{BF}_1\text{-ATPase}$ in the radioimmunoassay and are calculated, as inverse proportion, from the different amount of each ligand required to obtain this binding, taking as 100% that of $\text{BF}_1\text{-ATPase}$.

Subunit(s)	Competition (%)
α	4.8
β	3.5
γ	10.5
$\alpha + \beta^*$	34.5 ± 2.5
$\alpha + \beta + \gamma^\dagger$	57.4

* Equimolar proportion 3:3; average of three experiments.

† Molar proportions of the subunits 3:3:2.

Fig. 5. Radioimmunoassay of α -subunit (a) and β -subunit (b) of *M. lysodeikticus* $\text{BF}_1\text{-ATPase}$ showing the competition effects of $\text{BF}_1\text{-ATPase}$ and its three larger subunits

Results are expressed as percentages of ^{125}I -labelled α - or β -subunits bound to their respective antisera. O, $\text{BF}_1\text{-ATPase}$; $\blacktriangle$, α -subunit; $\square$, β -subunit; $\bullet$, γ -subunit. For other details see the Materials and methods section.

Fig. 4. Effect of mixtures of α -, β - and γ -subunits on the binding of ^{125}I -labelled $\text{BF}_1\text{-ATPase}$ to anti- $\text{BF}_1\text{-ATPase}$ serum

Details are given in the Materials and methods section. ATPase (O), α -subunit ($\blacktriangle$) and β -subunit ($\square$) are shown as controls; ($\triangle$), $\alpha + \beta$ -subunits (1:1 molar ratio); ($\blacklozenge$), $\alpha + \beta + \gamma$ -subunits (3:3:2 molar proportions).

stitution, in that 60% competition for native BF_1 -ATPase and similar slope was obtained at a subunit proportion of $\alpha_3\beta_3\gamma_2$. This proportionality is consistent with the stoichiometry proposed for *M. lysodeikticus* BF_1 -ATPase (Muñoz *et al.*, 1981).

Table 1 summarizes some of the quantitative results.

Similar results were obtained in supplementary experiments with radioimmunoassays developed for the different subunits, as illustrated in Fig. 5 for α - and β -subunits. The low titre of the anti- γ -subunit serum made difficult the comparison in quantitative terms of the radioimmunoassay of this subunit because of the displacement to the left of the curves. These experiments showed BF_1 -ATPase to compete up to 100% with each anti-subunit serum, as should be expected. In agreement with experiments described above, the γ -subunit showed behaviour intermediate between that of α - and β -subunits, but closer to that of the α -subunit. On the other hand, BF_1 -ATPase was able to compete 300% with α -subunit, 150% with γ -subunit and 400–500% with β -subunit in its own radioimmunoassay (see Fig. 3). These findings are consistent with the stoichiometric and immunological characteristics outlined above.

Discussion

Radioimmunoassay has contributed to progress in the detection of biologically active molecules (Chard, 1978). Most of the early applications concerned measurements of hormones and some enzymes (Bauer *et al.*, 1972). The use of radioimmunoassays has increased recently, especially in the study of enzymes and regulatory proteins (Wallace & Cheung, 1979; Mallie & Garvey, 1979; Chafouleas *et al.*, 1979). However, only a few studies have used this sensitive and specific technique to analyse proteins at the molecular level.

The present paper is the first report on a radioimmunoassay of an energy-transducing protein. The assay used has potential applications for the study of the complex oligomeric F_1 -ATPase system. This method can be used advantageously, because of its specificity, sensitivity and speed, to compare the similarities between different energy-transducing F_1 -ATPases and the homologies between the subunits of a given F_1 -ATPase even before the primary structures of these proteins have been determined. This matter is of obvious importance, as the resolution of the structure of F_1 -ATPases is now being approached from a crystallographic point of view (Amzel & Pedersen, 1978; Wakabayashi *et al.*, 1977).

Some of these applications were explored in the present work. In particular, the role of the subunits in the immunochemical properties of the BF_1 -

ATPase molecule was studied. The findings indicate that the α -, β - and γ -subunits contribute to the antigenic properties of *M. lysodeikticus* BF_1 -ATPase, but that additional antigenic determinants, probably conformational, arise from their proper folding and assembly into the BF_1 -ATPase molecule.

Our previous attempts to reconstitute enzymic properties of BF_1 -ATPase were unsuccessful (Andreu & Muñoz, 1975, 1979). In the present work, however, we were able to reconstitute the immunological properties of the BF_1 -ATPase of *M. lysodeikticus*. The radioimmunoassay method enabled us to study reconstitution by different criteria. Our findings support the stoichiometry $\alpha_3\beta_3\gamma_2$ recently proposed for this bacterial F_1 -ATPase (Muñoz *et al.*, 1981), thus providing indirect but additional support for this controversial proposal.

The present findings confirmed also the individuality and specificity of the α -, β - and γ -subunits of this bacterial F_1 -ATPase. It is to be noted that the sensitivity of the method used would allow the concentration of either the BF_1 -ATPase or its main subunits in the membrane to be measured. This provides another use of the assay, namely to detect changes in the concentration of the enzyme or of its subunits as a function of the growth condition of the organism. The method may also be of use in studies on biosynthesis.

We have not yet examined the role of δ -subunit in the immunochemistry of *M. lysodeikticus* BF_1 -ATPase because we lack a method to purify it and preserve its antigenic properties. We found purification of δ -subunit by SDS/polyacrylamide-gel electrophoresis to result in loss or change of its immunological properties. Hopefully, this problem can be overcome in future work.

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