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Exploration of δ-subunit interactions in beef heart mitochondrial F_1-ATPase by monoclonal antibodies

Joël Lunardi, Alain Dupuis, Yveline Frobert*, Jacques Grassi* and Pierre V. Vignais

Laboratoire de Biochimie, Département de Recherche Fondamentale, Centre d'Etudes Nucléaires, 85X, 38041 Grenoble Cédex and **Section de Pharmacologie et d'Immunologie, Département de Biologie, Centre d'Etudes Nucléaires de Saclay, 91191 Gif-sur-Yvette, France*

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Three monoclonal antibodies (mAbs) recognizing distinct epitopes on the δ-subunit of beef heart mitochondrial F_1-ATPase were studied for their reactivity towards the δ-subunit both in isolated F_1 and in the F_0-F_1 complex of submitochondrial particles. Two of the antibodies termed mAb δ195 and mAb δ239 had free access to δ in F_1 and the F_0-F_1 complex. Partial hindrance was observed for the third antibody mAb δ22. By a double antibinding assay, it was found that the binding sites for mAb δ195 and mAb δ239 were close to each other and possibly overlapping. Mapping studies conducted with the isolated δ-subunit showed that mAb δ195 and mAb δ239 interacted with the N-terminal portion of δ extending from Ala-1 to Met-16, whereas mAb δ22 interacted with the fragment spanning Ser-17–Glu-68. It was concluded that the Ala-1–Met-16 segment of the δ-subunit in F_1 and the F_0-F_1 complex is freely accessible from the outside, whereas the Ser-17–Glu-68 segment of δ is partially hidden, possibly as a result of interactions with other subunits.

Monoclonal antibody; ATPase, F_1-

1. INTRODUCTION

Monoclonal antibodies (mAbs) have recently been used to explore the structural organization and the function of the various subunits of F_1-ATPases isolated from mitochondria of pig heart [1,2], beef heart [3], and yeasts [4,5], and from chloroplasts [6] and *Escherichia coli* [7–10]. Except in the case of *E. coli* F_1, where the small subunits γ, δ and ϵ have been explored, all other studies have been carried out with mAbs directed against the major subunits, namely α and β. In experiments aimed at characterizing the topography of the small mitochondrial F_1 subunits, we have prepared mAbs against the δ-subunit of beef heart mitochondria. We have explored the accessibility of the specific epitopes to these mAbs in F_1 and the F_0-F_1 complex, and mapped these epitopes on the δ-subunit.

Correspondence address: J. Lunardi, Laboratoire de Biochimie, Département de Recherche Fondamentale, Centre d'Etudes Nucléaires, 85X, 38041 Grenoble Cédex, France

Abbreviations: mAb, monoclonal antibody; OSCP, oligomycin sensitivity conferring protein; F_1, catalytic sector of the ATPase complex; AChE, acetylcholinesterase

2. MATERIALS AND METHODS

F_1 purified from beef heart submitochondrial particles [11] was dissociated into an $\alpha\gamma\delta\epsilon$ complex and the β-subunit. The $\alpha\gamma\delta\epsilon$ complex, free of the β-subunit, was recovered by DE-52 chromatography [12]. The $\alpha\gamma\delta\epsilon$ complex was supplemented with a fraction of F_1 enriched in $\delta\epsilon$ subunits [13], and injected into the foot pad of three high responder Biozzi's mice with complete Freund's adjuvant. Two booster injections were given after three and six weeks. The mouse with the highest antibody titer to $\alpha\gamma\delta\epsilon$ complex received a last intravenous boost three days before fusion. Preparation of hybridomas, immunoenzymatic screening of culture supernatants, mAbs production by ascitic fluids and determination of the mAb classes and subclasses were performed as described [14]. mAbs were screened using biotinylated F_1 subunits reacting via avidin interaction with biotinylated acetylcholinesterase [14].

Biotinylation of F_1 subunits was performed as follows. An aliquot of 400 μl of the $\alpha\gamma\delta\epsilon$ complex enriched with δ and ϵ (1 mg/ml) subunits was incubated with 0.1 mM biotin *N*-

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hydroxysuccinimide ester (IBF France) in 1 M sodium carbonate buffer, pH 9.1. After 30 min at room temperature, the reaction was stopped by addition of 10 μl of 0.5 M ammonium acetate, pH 7.1, and the biotinylated proteins were separated from free biotin by dialysis. To check the effectiveness of biotinylation, the reacted subunits were separated by SDS-polyacrylamide gel electrophoresis and electrotransferred onto a nitrocellulose sheet (0.45 μm, Schleicher and Schüll). The transferred proteins were then reacted with an avidin-peroxidase complex and then revealed with diaminobenzidine in the presence of nickel and cobalt salts [15].

Screening of mAbs with the biotinylated F_1 subunits was performed in two steps. In a first step, the biotinylated subunits were reacted with mAbs previously immobilized on a solid-phase coated with anti-mouse antiglobulins. In a second step, biotinylated AChE was linked to the immobilized system by means of avidin [14], and the bound AChE was revealed by a colorimetric assay based on the use of Ellman's reagent [16]. This method proved to be sensitive and convenient for extensive screening. Positive clones were further characterized by Western blot using unmodified F_1. Absence of reactivity of the preimmune serum against the biotinylated F_1 subunits was verified. The mAbs were recovered from the ascitic fluids by precipitation with 50% saturated ammonium sulfate and further purified by affinity chromatography, using a column of protein A-Sepharose, equilibrated with a 0.14 M Na phosphate buffer, pH 8.1. mAbs of the IgG_1 and IgG_2 classes were eluted with a 0.14 M Na phosphate buffer of pH 6.0 and 4.3, respectively, and the eluates were neutralized by dropwise addition of 1 M NaOH [17]. When used in enzymatic assays, purified mAbs were freed of P_i by centrifugation-filtration on short columns of Sephadex G50 [18]. Other determinations pertaining to protein concentration [19], total ATPase activity and oligomycin-sensitive ATPase activity [20] were carried out as described. Reconstitution of oligomycin-sensitive ATPase activity was performed by adding F_1 in the presence of OSCP to urea-extracted submitochondrial particles, devoid of F_1 [21]. In brief, F_1 was first incubated for 30 min at room temperature with the different mAbs at concentration ratios of F_1 to mAb ranging from 0.2 to 2 (mol/mol), and the immunoreacted F_1 was rebound to urea-treated particles in the presence of OSCP [20].

For mapping studies, the δ-subunit was cleaved at methionyl residues by treatment with a 10-fold excess of CNBr in 80% formic acid under nitrogen for 5 h at 37°C. Fragmentation by the V8 protease was achieved by incubation with the enzyme (1:50, w/w) in 100 mM ammonium bicarbonate for 15 h at 37°C. F_1 subunits and fragments of the δ-subunit were separated by SDS-polyacrylamide gel electrophoresis as described in [22] and [23], respectively. Treatment of the gels to remove SDS, electrophoretic transfer to nitrocellulose and immunochemical detection were as described in [24].

3. RESULTS AND DISCUSSION

Injection of mice or rabbits with mitochondrial F_1 resulted in the production of antibodies predominantly directed against the β-subunit (unpublished). As we were interested in mAbs directed against the minor F_1 subunits and particularly the δ-subunit, immunization of mice was carried out with a subunit mixture of beef heart F_1 deprived of the β-subunit and enriched in δ- and ϵ-subunits.

3.1. *Characterization of anti-δ mAbs*

In a first step, using the AChE-avidin-biotin ELISA system described in [14], twenty clones producing anti-F_1 mAbs were selected for study. These clones were cultured and injected intraperitoneally

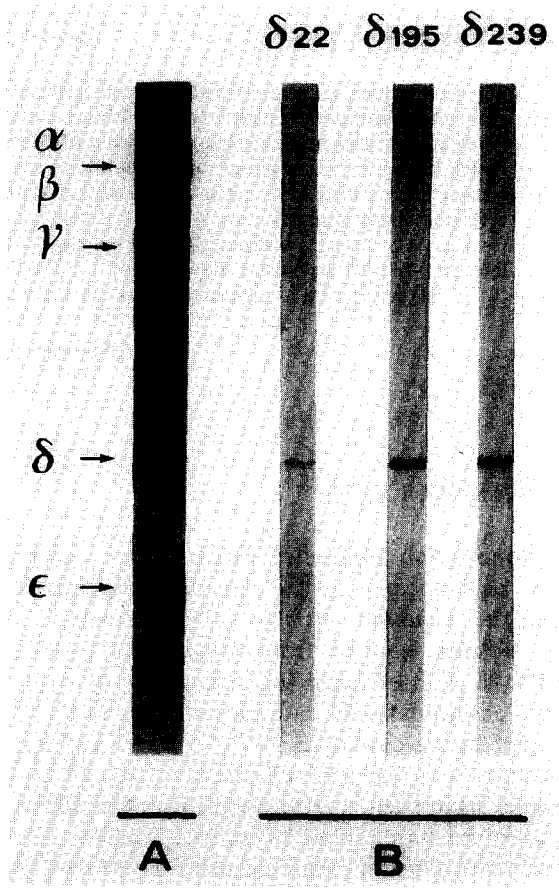

Fig.1. Specificity of the monoclonal anti-δ antibodies. After SDS-polyacrylamide gel electrophoresis of F_1 (5 μg per track), the separated subunits were transferred onto nitrocellulose and incubated with a 10000-fold diluted solution of mAb initially at 0.4 mg/ml. After an hour of incubation at room temperature, the bound mAbs were revealed, using a peroxidase conjugated goat anti-mouse immunoglobulin. Lanes: A, Coomassie blue staining of the gels; B, immunocharacterization of the transferred F_1 subunits tested with mAb δ22, mAb δ195 and mAb δ239. Blotting and immunodetection are described in section 2.

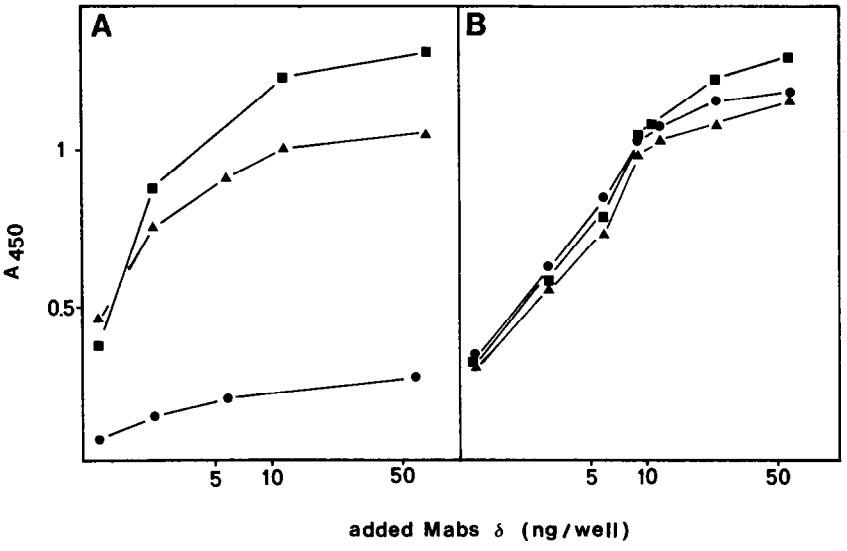

Fig.2. Immunoreactivity of mAb δ22, δ195 and δ239 with F_1 and isolated δ, assayed by ELISA. ELISA plates (NUNC) were coated with 500 ng of purified F_1 (panel A) or 60 ng of purified δ-subunit (panel B) per well in 150 μl of phosphate buffer. Reactions were further carried out with purified mAbs added in 150 μl of phosphate buffer as indicated. (●) mAb δ22; (▲) mAb δ195; (■) mAb δ239. Bound antibodies were revealed using a peroxidase conjugated anti-mouse immunoglobulin and tetramethylbenzidine as peroxidase substrate.

into mice primed with incomplete Freund's adjuvant. The specificity of the ascites fluids against the F_1 subunits was tested by Western blotting, followed by visualization of the immune complex by the peroxidase method (cf. section 2). Under these conditions, only three out of the twenty ascitic fluids were found to immunoreact with the δ-subunit. The mAbs contained in these fluids were purified by affinity chromatography and their immunoreactivity was again tested by Western blot (fig.1).

The three mAbs were referred to as mAb δ22, mAb δ195 and mAb δ239. The first mAb corresponded to the IgG_{2k} isotype and the other two to the IgG_{1k} isotype. All of them were highly specific for the beef heart δ-subunit, and showed no reactivity towards the corresponding subunits from *Saccharomyces cerevisiae* F_1 and *Escherichia coli* F_1. Their accessibility to specific epitopes in the δ-subunit within isolated F_1 or the F_0-F_1 complex in submitochondrial particles was assayed by ELISA, and compared to their reactivity towards isolated δ. Whereas the three mAbs reacted with the same efficiencies with isolated δ, mAb δ195 and mAb δ239 displayed a much higher reactivity against F_1 than mAb δ22 did (fig.2). The same observation holds for the F_0-F_1 complex (not shown).

These results indicate that the epitopes of isolated δ which are recognized by mAb δ195 and

Table 1

ELISA additivity assay to test simultaneous binding of mAbs on epitopes of the δ-subunit

mAb	Absorbancy at 450 nm	Theoretical sum of absorbancies	Additivity index
mAb δ22	0.380	–	–
mAb δ195	0.835	–	–
mAb δ239	0.870	–	–
mAb δ22 + mAb δ195	1.140	1.215	87
mAb δ22 + mAb δ239	1.120	1.250	79
mAb δ195 + mAb δ239	1.220	1.705	43

Experimental conditions are as described in fig.2. The dilution used for each purified mAb corresponded to the lowest concentration at which saturation of δ was achieved. The theoretical sum of absorbancies (*A*) is the sum of absorbancies obtained for each mAb alone. The additivity index AI was calculated as described in section 3

mAb δ239 are also accessible in the δ-subunit associated to the other subunits within F_1 or the F_0-F_1 complex. In contrast, the epitope recognized by mAb δ22 is partially hindered in isolated F_1 and the F_0-F_1 complex. The free access of mAb δ195 and mAb δ239 to their epitopes on the δ-subunit in the F_1-F_0 complex indicates that these epitopes are not sequestered or hidden between the F_1 and F_0 sectors.

The three mAbs affected neither the F_1-ATPase activity, nor the oligomycin-sensitive ATPase activity of the F_0-F_1 complex of submitochondrial particles. Even when incubated with isolated F_1 for 30 min at a mAb to F_1 ratio of 4, they did not impair to any extent the reconstitution of the oligomycin-sensitive ATPase activity. The epitopes recognized by these mAbs are therefore not involved in the catalytic events of ATP hydrolysis; neither do they contribute to the binding of F_1 to F_0.

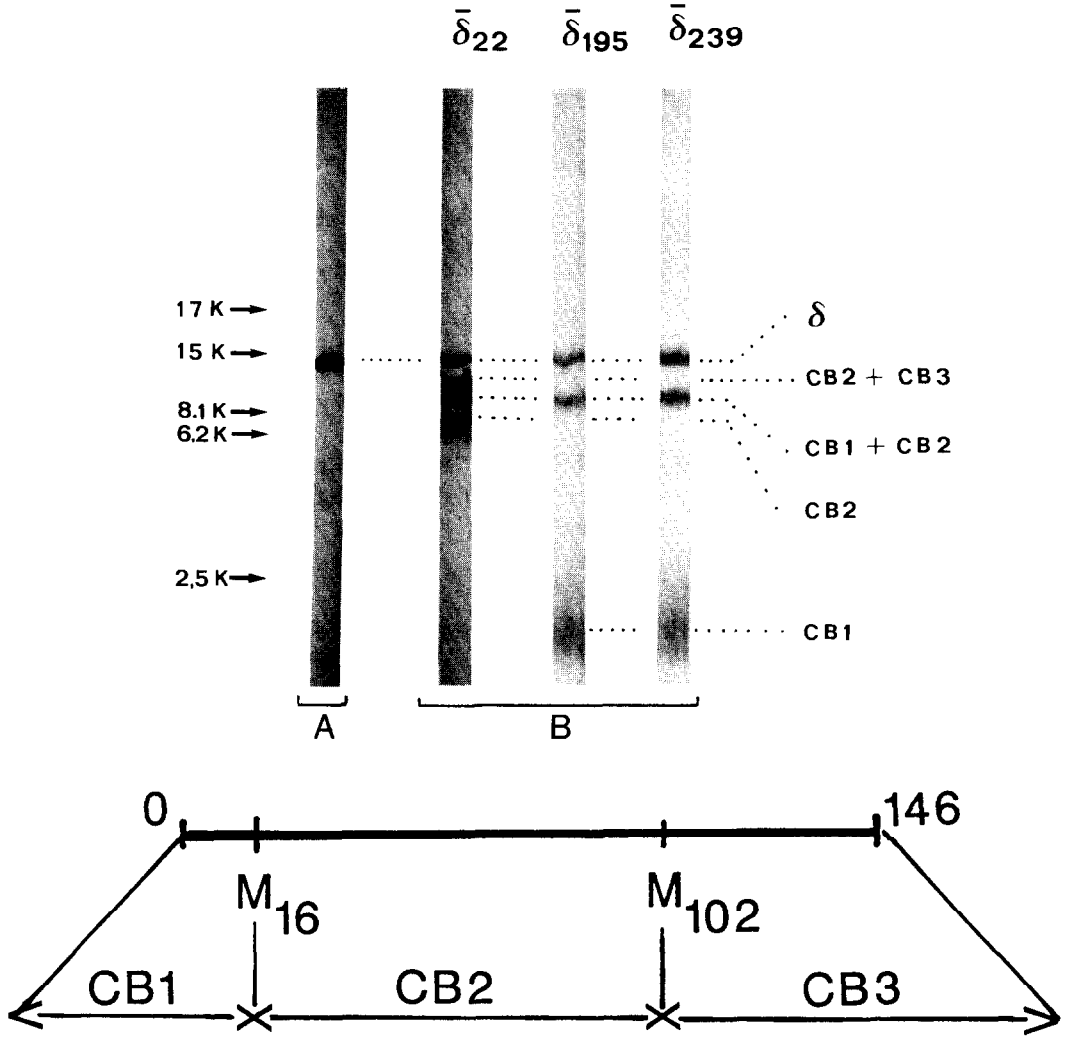

Fig.3. Immunoreactivity pattern of the peptides obtained by CNBr cleavage of the isolated subunit. CNBr fragments of the δ-subunit were separated by SDS-polyacrylamide gel electrophoresis and then electrotransferred onto nitrocellulose. Lane A corresponds to the control δ-subunit incubated with a mixture of mAbs δ22, δ195 and δ239. The three B lanes correspond to δ cleaved with CNBr and then reacted with each of the three mAbs. The molecular masses of reference proteins are designated by arrows. A schematic representation of the cleavage sites of the δ-subunit (146 amino acid residues) is given below the blots.

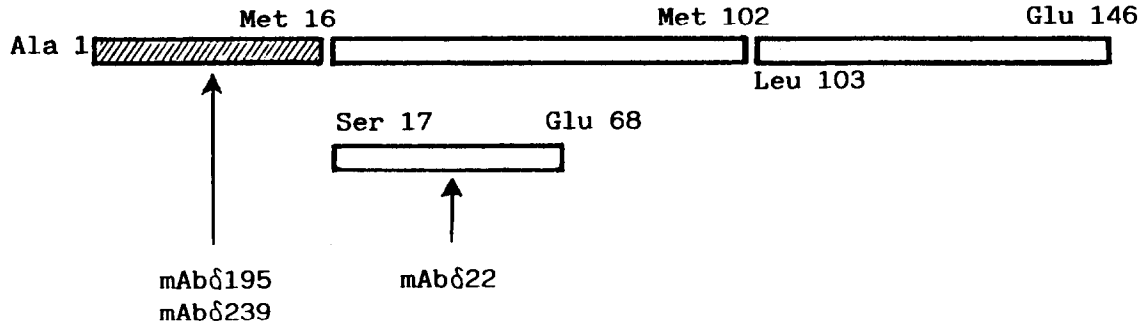

Fig.4. Scheme illustrating the reactivity of mAb δ22, mAb δ195 and mAb δ239 against different segments of the δ-subunit. The hatched segment, Ala-1–Met-16, corresponds to the readily accessible portion of the δ-subunit in F_1 or the F_0-F_1 complex (for details see text).

3.2. *Mapping of the epitopes of the δ-subunit reacting against mAb δ22, mAb δ195 and mAb δ299*

To test whether the epitopes recognized by the three anti-δ mAbs were spatially separated or overlapping, an ELISA double antibinding assay was applied as described in [25]. This method relies on the estimation of the number of epitopes on the antigen simultaneously available to a pair of antibodies. The additivity index, AI, is expressed for a pair of antibodies by the formula

$$AI = \left(\frac{2A_{1+2}}{A_1 + A_2} - 1\right) \times 100$$

where A_1, A_2 and A_{1+2} are the absorbancies observed in ELISA for the first antibody alone, the second antibody alone, and the two antibodies together. If antibodies bind to distinct sites, AI is expected to be equal or close to 100%. This is indeed the case for the pairs mAb δ22-mAb δ195 and mAb δ22-mAb δ239, but not for the pair mAb δ195-mAb δ239 (table 1). In other words, the epitope recognized by mAb δ22 differs from those recognized by mAb δ195 and mAb δ239. On the other hand, the epitopes recognized by mAb δ195 and mAb δ239 are probably close to each other, if not overlapping.

Further mapping data were obtained by determination of the immunoreactivity of peptides resulting from cleavage of the δ-subunit. CNBr cleavage at the two methionyl residues, Met 16 and Met 102, present in beef heart δ [26] generated three peptides spanning Ala-1–Met-16, Ser-17–Met-102, and Leu-103–Glu-146 and referred to as CB1, CB2 or CB3, respectively (fig.3). Both mAb δ195 and mAb δ239 reacted against CB1, showing a close proximity or possibly an overlapping of the two respective epitopes, in agreement with the result of the additivity test. mAb δ22 reacted against CB2 and the Ala-1–Glu-68 fragment obtained by digestion of δ by the *Staphylococcus aureus* V8 protease. By combining the results of enzymatic and chemical cleavages, the epitope reacting against mAb δ22 was therefore localized more precisely between Ser-17 and Glu-68 (fig.4).

In summary, the present results show that the N-terminal portion of the δ-subunit is freely accessible in isolated δ and also in δ associated to the other subunits of the F_1 sector, either in F_1 or the F_0-F_1 complex. On the other hand, the portion of the δ chain spanning Ser-17–Glu-68 is partially hidden in F_1 or the F_0-F_1 complex, probably because of the steric hindrance generated by contact with the other subunits of F_1. The epitopes of the δ-subunit recognized by the three mAbs do not seem to be involved in the catalytic events of ATP hydrolysis.

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