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Availability to monoclonal antibodies of antigenic sites of the α and β subunits in active, denatured or membrane-bound mitochondrial F_1-ATPase

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The binding of five monoclonal antibodies to mitochondrial F_1-ATPase has been studied. Competition experiments between monoclonal antibodies demonstrate that these antibodies recognize four different antigenic sites and provide information on the proximity of these sites. The accessibility of the epitopes has been compared for F_1 integrated in the mitochondrial membrane, for purified β-subunit and for purified F_1 maintained in its active form by the presence of nucleotides or inactivated either by dilution in the absence of ATP or by urea treatment. The three anti-β monoclonal antibodies bound more easily to the β-subunit than to active F_1, and recognized equally active F_1 and F_1 integrated in the membrane, indicating that their antigenic sites are partly buried similarly in purified or membrane-bound F_1 and better exposed in the isolated β-subunit. In addition, unfolding F_1 by urea strongly increased the binding of one anti-β monoclonal antibody (14 D_5) indicating that this domain is at least partly shielded inside the β-subunit. One anti-α monoclonal antibody (20 D_6) bound poorly to F_1 integrated in the membrane, while the other (7 B_3) had a higher affinity for F_1 integrated in the membrane than for soluble F_1. Therefore, 20 D_6 recognizes an epitope of the α-subunit buried inside F_1 integrated in the membrane, while 7 B_3 binds to a domain of the α-subunit well exposed at the surface of the inner face of the mitochondrial membrane.

Introduction

The ATPase-ATPsynthase (F_0-F_1) is essential for ATP synthesis in energy-transducing membranes, such as bacteria, chloroplasts and mitochondria. This complex contains two distinct parts: the F_0 part channelling the transfer of protons across the membrane, and the F_1 part containing the catalytic sites for ATP synthesis and ATP hydrolysis (for recent reviews, see Refs. 1–6).

F_1 can be solubilized and is made of five subunits organized according to a stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$. The mechanism of ATP synthesis, although studied extensively by many laboratories, is still controversial. A better understanding of the F_0-F_1 structure and of the fine changes of this structure occurring during catalysis is a requirement in order to elucidate this mechanism. Monoclonal antibodies (mAbs) may be powerful tools to reach this goal.

MAbs specific to the α- and β-subunits of F_1 were prepared for the first time in our laboratory for pig heart mitochondria [7,8]. These antibodies, as well as mAbs raised against the oligomycin-

Abbreviations: F_1, mitochondrial F_1-ATPase; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; RIA, radioimmunoassay.

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sensitivity-conferring protein [9], have been used: (i) to determine an $\alpha_3\beta_3$ stoichiometry in F_1 [7] and a ratio of two oligomycin-sensitivity-conferring proteins per F_1 in mitochondria (10); (ii) to identify essential epitopes on the β-subunit by studying interspecies cross-reactivity [9]; and (iii) to analyze the topography of the oligomycin-sensitivity-conferring protein in the mitochondrial membrane [11]. Other mAbs have been raised against α, β and a peptide of 25 kDa of the yeast mitochondrial F_0-F_1 complex [12], against the α and β subunits of *Escherichia coli* F_1 [13], against the α [14], β and γ subunits [15] of chloroplast F_1, against the coupling factor B of mitochondria [16], and against all subunits of *E. coli* F_1 [17]. Stoichiometries of 3α and 3β subunits have also been found for all species with these mAbs. Moreover, a correlation between inhibitory effects of mAbs and conservation of epitopes along the phylogenic scale has been stressed [17].

In the present paper, the binding of anti-α and anti-β mAbs to F_1 is used as a probe of the availability of domains of α- and β-subunits of F_1. It is shown that the accessibility of four distinct antigenic determinants to mAbs can be modulated by unfolding the protein, by integration of F_1 in the membrane or by the addition of nucleotides to soluble F_1. In addition, the binding curves of anti-α mAbs to soluble or membrane-bound F_1 suggest an asymmetry of the α-subunits.

Materials and Methods

Materials

[^{125}I]NaI was obtained from the Commissariat à l'Energie Atomique, France. Specific chemicals were purchased from the following sources: protein A-Sepharose; Pharmacia; protein A: I.B.F.; horseradish-peroxidase conjugated antimouse immunoglobulin sheep antibody; Biosys; IODO-GEN; Pierce. All other chemicals were of the highest purity available.

Methods

Biological preparations. Previously described procedures were used to obtain: pig-heart mitochondria [18]; F_1 [19]; purified β-subunit [20]; and electron-transport particles prepared according to Penin et al. [21], which are essentially inverted submitochondrial particles as checked according to Ref. 22. MAbs prepared according to Kohler and Milstein [23] have been previously characterized: 14 D_5, 19 D_3 and 5 G_{11} were specific of the β-subunit and 20 D_6 of the α-subunit [7]; 7 B_3 obtained from another fusion was specific of the α-subunit [8]. The monoclonal antibodies were purified from ascitic or culture supernatant fluid by affinity chromatography on protein A-Sepharose [24]. Purified mAbs or protein A were iodinated with ^{125}I in the presence of IODO-GEN [25], as described previously [7]. Protein concentration was estimated by the method of Lowry et al. [26]. The ATPase activity was measured as in Ref. 19.

Coating of F_1 to microtitration plates. The wells of microtest plates were coated with 50 µl F_1 (80 µg/ml in 0.1 M sodium phosphate buffer, pH 7.5) and air dried. The 96-wells microtest plates used were either flexible (Falcon 3912) for RIA or rigid (Nunclon Delta) for ELISA. To improve the coating of F_1, 50 µl 80% acetone in water (v/v) was added to each well. After drying, the remaining non-specific binding sites were saturated with serum albumin: three successive washings were made by filling the wells with 10 mM sodium phosphate buffer/150 mM NaCl (pH 7.2) containing 1% bovine serum albumin, incubating the plates for 10 min and emptying the wells by flicking the plates. The plates containing solid phase F_1 could be kept at −20°C for several weeks before use.

Competition among mAbs for binding to antigenic determinants of F_1. The ability of binding of one α- or β-specific mAb in the presence of a saturating concentration of another mAb was tested by radioimmunoassay. In preliminary experiments the saturating amount of mAbs was determined as follows: serial dilutions of mAb (purified or 50% ammonium sulfate precipitate of ascitic fluid) were incubated overnight with F_1 coated to the wells of flexible plates. After three washings with 10 mM sodium phosphate buffer/150 mM NaCl (pH 7.2) containing 1% bovine serum albumin, 50 µl sheep antibody to mouse immunoglobulin (diluted 1 : 300 in 10 mM sodium phosphate buffer/150 mM NaCl) were incubated in each well for 1 h at 37°C. After three more washings, 50 µl of ^{125}I-labeled protein A

($2 \cdot 10^5$ cpm per well) were incubated for 1 h. The wells were washed six times, dried, cut out and counted in a γ-counter (Packard). The dilution of mAb giving the maximal binding to solid phase F_1 was used for subsequent competition studies. The competition between antibodies for binding to solid phase F_1 was then tested as described in Table I by first incubating one mAb at saturating concentration (protecting antibody) and then measuring the binding of another mAb labeled with ^{125}I (tested mAb).

Competitive ELISA. Competition between binding of soluble antigens (F_1, β-subunit, electron-transport particles) and solid phase F_1 (F_1 coated to microtitration plates) to a given mAb was made as follows: after preincubation of the studied antigen with the mAb, the mixture was added to microtitration wells containing solid phase F_1. Only the mAbs not bound to soluble antigen could react with coated F_1 and be titrated afterwards with the second antibody. For these experiments, limited concentrations of mAb corresponding to 60–80% of the maximal binding to coated F_1 were used. The mAbs were preincubated in the presence of various concentrations of competing antigens for 2 h at 30°C. For competitions with F_1 or β, the buffer A contained 30 mM Tris-base/192 mM glycin/0.5 mM EDTA (pH 8.0)/5% glycerol/0.02% Tween 20; when ATP + $MgCl_2$ were aded, EDTA was omitted. For competitions with electron-transport particles, the buffer B contained 0.25 M sucrose, 30 mM Tris-base, 192 mM glycin, 0.5 mM $MgSO_4$ (pH 8.0), 0.02% Tween 20. The mixture of mAb and competing antigen (50 μl) was transferred to the 96-wells rigid plate coated with F_1. After a 3 h-incubation at 37°C, the plates were washed three times with 10 mM sodium phosphate buffer/150 mM NaCl containing 0.05% Tween 20 and incubated for 1 h at 37°C with 50 μl of peroxidase conjugated-antimouse immunoglobulin sheep antibody (diluted 1/300 in sodium phosphate buffer/NaCl). The plates were washed three times with 10 mM sodium phosphate buffer/150 mM NaCl/0.05% Tween 20.

The peroxidase substrate (100 μl 0.25 mM 2,2′-azinobis (3-ethylbenzthiazolinesulfonic acid)/ 0.18 mM H_2O_2/0.1 mM sodium phosphate, pH 6.8) was then incubated for 30 min at room temperature with gentle shaking. The color intensity of the plates was measured with a Dynatech microplate reader at 410 nm.

When the competition was studied with denatured F_1, F_1 was pretreated with 8 M urea: the stock solution of F_1 (5 mg protein per ml 100 mM Tris-SO_4/5 mM EDTA/50% glycerol, pH 8.0) was diluted with an equal volume of 25 mM Tris-base/192 mM glycin (pH 8.0). Crystals of urea were added to obtain a final concentration of 8 M. The mixture was heated for 5 min at 100°C and diluted 4-fold in buffer A. Verification was made that the presence of 2 M urea (which corresponds to the highest concentration of solutions tested containing F_1 unfolded by urea) did not significantly modify the interaction between F_1 and the various mAbs.

Use of competitive binding curves to estimate the number of β-subunits of F_1 accessible to the monoclonal antibodies, using an isolated β-subunit as a standard. Berzofsky and Schechter [27] have proposed a mathematical analysis for the binding of a ligand to an homogeneous antibody in the presence of a competitor. For example, for the binding of a protein, they have shown that, in this type of competition curves, the total concentration of the soluble protein which reduces by 50% the binding of the antibody to the protein bound to the solid support, corresponds to a value

$$[X] = \frac{1}{K_X} + \frac{A}{2}$$

where A is the concentration of antibody binding sites and K_X the affinity of the antibody for the protein. In order to use this equation to compare the binding of one mAb to F_1 and to the isolated β-subunit, it is necessary to assume that K_X, the affinity of the mAb for the β-subunit, is the same when the β-subunit is isolated as when it is integrated in F_1. Under these conditions, the total soluble protein concentration ([β] or [F_1]), corresponding to 50% binding of the antibody, will be directly proportional to the concentration of antibody binding sites. When a log scale is used, the slope of the binding curve is proportional to the affinity of the competitor (here soluble protein vs. bound protein) [27,28]. Therefore, it is reasonable to assume that the affinity of one mAb for F_1 and for the β-subunit is identical when the curves are

parallel. In such a case, the number of antibody binding sites on F_1 can be estimated by comparison of the midpoint of the curves obtained with the isolated β-subunit and with F_1.

Titration of F_1 present in the mitochondrial membrane. The total amount of F_1 present in submitochondrial particles was measured by quantitative immunotitration using monoclonal antibodies as described previously [10]. It was found that the electron-transport particles used in the present work contained 0.39 nmol F_1 per mg of mitochondrial protein [29]. Taking into account an M_r of 380000 for F_1 [1], the electron-transport particles contain 15% of F_1.

Results

Analysis of the proximity between antigenic determinants of α and β in F_1. To determine whether the various mAbs recognize distinct or overlapping sites on F_1, the binding of each labeled mAb was tested in the presence of saturating amounts of each other mAb (protecting mAb). Table I shows that, when the labeled mAb is tested in the presence of the same unlabeled mAb, 11–18% of the binding observed in the absence of protecting mAb are measured. Therefore, a percentage of ^{125}I-labeled mAb binding lower than 20% cannot be considered as corresponding to a different antigenic determinant. Moreover, a percentage of binding higher than 60% can be considered as corresponding to a different antigenic determinant [30]. The results presented in Table I can be analyzed on the basis of these limits. The presence of 7 B_3 (anti-d), as the protecting mAb, does not significantly decrease the binding of any other labeled anti-α or anti-β mAbs (63 to 82%), suggesting that it recognizes a determinant distinct from the others. The presence of 20 D_6 (anti-α) slightly hinders the binding of both the anti-α 7 B_3 (50 %) and the anti-β 19 D_3 (56%), but it does not significantly decrease the binding of other anti-β mAbs 5 G_{11} and 14 D_5. The presence of 14 D_5 (anti-β) does not prevent the binding of anti-α mAbs (7 B_3, 69% and 20 D_6, 82%), while it slightly hinders the binding of anti-β mAbs 19 D_3 and 5 G_{11} (45% and 59% of binding, respectively). The presence of 19 D_3 (anti-β) does not significantly diminish the binding of anti-α mAbs and anti-β 14 D_5 (70–80% of binding), but it strongly decreases the binding of 5 G_{11} (only 7% of binding). The presence of 5 G_{11} (anti-β) does not prevent the

TABLE I

COMPETITION BETWEEN VARIOUS MONOCLONAL ANTIBODIES FOR BINDING TO ANTIGENIC SITES OF MITOCHONDRIAL F_1-ATPase

The wells of flat-bottom microtest flexible plates were coated with F_1 and saturated with bovine serum albumin as described in Materials and Methods. In preliminary experiments, the dilution of purified mAb necessary to saturate the wells was tested by measuring the maximal amount of antibody that could be retained by F_1 coated to the wells. For competition experiments, each well received 50 μl of the saturating concentration of the protecting mAb estimated in the preliminary experiment. After overnight incubation, 50 μl of the ^{125}I-mAb under test were added to each well and incubated for 4 h at room temperature with gentle shaking. Each well was then emptied, washed 6 times with 10 mM sodium phosphate buffer/150 mM NaCl containing 1% bovine serum albumin, cut out and counted. Five wells were run for each mAb combination. Each value represents the average of at least two experiments. For each mAb, five wells were used to measure the maximal binding (i.e., without protecting mAb) and five wells to determine the background (i.e., without F_1). The counts corresponding to the maximal binding obtained for the various mAbs were respectively: 37700 for 7 B_3, 64600 for 20 D_6, 46130 for 14 D_5, 19800 for 19 D_3 and 4300 cpm for 5 G_{11}. The average value of the backgrounds was 150 cpm. The Greek character in parenthesis indicates the F_1 subunit recognized by the corresponding mAb.

Protecting mAb	Tested ^{125}I-mAb Percentage of binding of tested labeled mAb in the presence of protecting mAb				
	7 B_3	20 D_6	14 D_5	19 D_3	5 G_{11}
7 B_3 (α)	12	72	82	63	68
20 D_6 (α)	50	18	62	56	70
14 D_5 (β)	69	82	11	45	59
19 D_3 (β)	70	80	70	16	7
5 G_{11} (β)	78	50	50	36	18

binding of 7 B_3, while it decreases the binding of other mAbs, barely for 20 D_6 and 14 D_5 (50%) and significantly for 19 D_3 (36%).

In conclusion, among the antibody pairs tested, a significant reciprocal inhibition is observed in only one case: 5 G_{11} and 19 D_3. This indicates common or overlapping epitopes. In addition, a reciprocal inhibition at the limit of the significance is observed between 14 D_5 and 5 G_{11}.

Competition studies

In the above experiments, the binding of mAbs was studied with F_1 immobilized on microtitration plates. In such a case, variations in the accessibility of the epitopes related to conformational changes of the antigen under different conditions were unlikely. In all further experiments, the binding of mAbs to F_1 was first carried out in solution

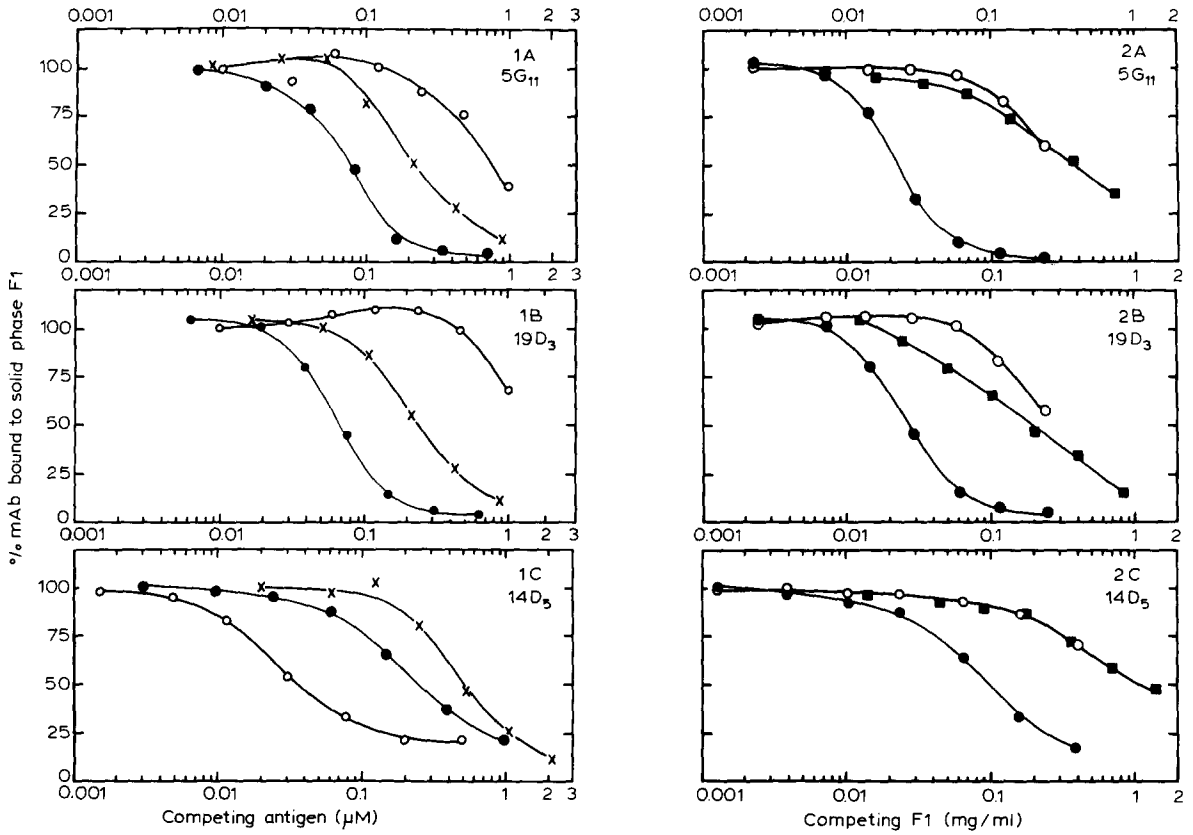

Fig. 1. Comparison of the binding of anti-β subunit mAbs to purified β-subunit, to soluble F_1 and to F_1 unfolded with urea. Limiting concentrations of mAb were preincubated in solution with the indicated concentrations of soluble F_1 (●), urea-treated F_1 (○) or purified β-subunit (×). After a 2-h incubation at 30°C, 50 μl of each sample was added to the wells of microtitration plates coated with F_1. The amounts of mAb bound to solid phase F_1 was measured by ELISA as described in Materials and Methods. The results are expressed as percentages of the maximal amount of mAb bound to solid phase F_1 measured simultaneously under the same conditions, except that the competing antigen was omitted from the preincubation medium. The M_r of F_1 and β were taken as 380000 [1] and 51300 [37], respectively.

Fig. 2. Decrease of the binding of 3 anti-β subunit mAbs to F_1 upon addition of MgATP or insertion of F_1 in the membrane. Limiting concentrations of mAb (adequately diluted, see Materials and Methods) were preincubated either with ETP (■) or with the indicated concentrations of F_1 in the absence (●) or presence (○) of 1 mM ATP + 1 mM $MgCl_2$. The electron-transport particles contain the indicated concentration of F_1, as calculated from Penin et al. [29]. Other experimental conditions as described in Fig. 1.

to allow for the detection of eventual conformational changes of F_1. Then, the remaining free mAbs was titrated by their binding to an excess of F_1 coated to the plates (see Materials and Methods). The concentration of competing antigen in solution capable of reducing by 50% the binding of mAbs to coated F_1 is defined as $K_{1/2}$.

Anti-β mAbs

Influence of the treatment of F_1 with urea on the binding of mAbs. Fig. 1A shows that, when 5 G_{11} is preincubated with F_1, the value of $K_{1/2}$ is about 0.07 μM. If F_1 has been treated with 8 M urea before the preincubation with 5 G_{11}, the value of $K_{1/2}$ is increased to 0.8 μM. Similar results are observed with 19 D_3 (Fig. 1B). Therefore, urea treatment of F_1 decreases the binding of both 5 G_{11} and 19 D_3. On the contrary, the $K_{1/2}$ value for 14 D_5 is lower for urea-treated F_1 (0.034 μM) than for F_1 (0.24 μM), indicating that the unfolding of F_1 enhances the binding of 14 D_5 (Fig. 1C).

Binding of mAbs to F_1 and to an isolated β-subunit. Fig. 1A and B shows that both mAbs 5 G_{11} and 19 D_3 exhibit titration curves for the purified β-subunit parallel to that of F_1. The experiment was repeated four times with 5 G_{11}. The $K_{1/2}$ values obtained when soluble F_1 and β are used as competing antigen were 0.071 ± 0.002 μM and 0.201 ± 0.027 μM, respectively. The ratios of $K_{1/2}$ for β and F_1 were calculated in each experiment. The mean of these radios was 2.78 ± 0.33 mol of β accessible to 5 G_{11} in each mol of F_1 (four experiments) and 3.2 mol of β accessible to 19 D_3 in each mol in F_1 (two experiments).

When the binding of 14 D_5 to F_1 and to purified β-subunit are compared (Fig. 1C), the titration curves are not parallel. Under these conditions, the concentrations of antibody binding sites in F_1 cannot be directly calculated from the binding curve of 14 D_5 to β (see below in Discussion).

Changes in the binding of mAbs to F_1 in the presence of nucleotides. Correlation with changes of ATPase activity. Fig. 2 shows that the presence of the substrate MgATP (1 mM) during the preincubation of soluble F_1 with all anti-β mAbs drastically decreases their binding to soluble F_1. Indeed, a $K_{1/2}$ higher than 0.23 mg/ml is obtained for F_1 preincubated in the presence of MgATP in the case of either 5 G_{11} or 19 D_3 instead of 0.021 mg/ml and 0.024 mg/ml, respectively, for 5 G_{11} and 19 D_3 binding to F_1 in the absence of MgATP. The $K_{1/2}$ value for 14 D_5 binding to F_1 raises up to 1 mg/ml in the presence of MgATP instead of 0.1 mg/ml in the absence of MgATP. Therefore, the presence of 1 mM MgATP decreases the binding of all three anti-β-mAbs. Verification was made that the presence of 1 mM MgATP did not change the binding of these mAbs to solid phase F_1 in the absence of any competing F_1.

F_1 is notorious for progressively falling apart into subunits when diluted in the absence of nucleotides [31]. The experiments described here involve incubations of F_1 lasting up to 5 h. In order to determine the extent of inactivation of F_1 occurring during these incubations, the ATPase activity of F_1 has been measured under the conditions used for competition studies, except that the addition of mAb was omitted. In the absence of nucleotides, only about 6% of the initial ATPase activity of soluble F_1 was recovered at the end of the experiment. On the contrary, in the presence of MgATP about 80% of the initial ATPase activ-

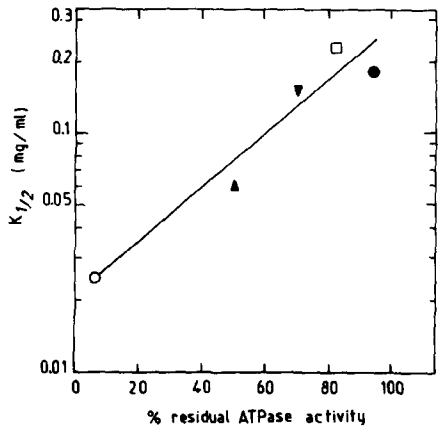

Fig. 3. Variations of the $K_{1/2}$ values for 5 G_{11} binding to F_1 in the presence of different nucleotides as a function of residual ATPase activity after a 5-h incubation. $K_{1/2}$ values expressed as mg F_1 per ml preincubation medium was determined as described in fig. 1A, except that 5 G_{11} was preincubated with F_1 either in the absence (○) or the presence of nucleotides: 1 mM GTP (▲), 5 mM GTP (▼), 1 mM ATP (●) or 1 mM ATP + 1 mM $MgCl_2$ (□). To measure the ATPase activity, F_1 was incubated at a concentration of F_1 of 0.037 mg per ml in buffer A (see Materials and Methods) supplemented with nucleotides. The residual activity was estimated by comparing the ATPase activity at the beginning and at the end of a 5-h incubation.

ity was remaining. These percentages were similar whatever F_1 concentration (within the range tested for competition experiments). The inactivation of F_1 could also be partly slowed down by the presence of either ATP in the absence of Mg^{2+}, or ADP or else GTP, although GTP was less efficient. Fig. 3 shows that the residual ATPase activity measured at the end of the incubation is roughly proportional to the $K_{1/2}$ value measured for the binding of 5 G_{11} to F_1, indicating that the value of $K_{1/2}$ increases with the percentage of active F_1.

Binding of mAbs to F_1 integrated in the membrane. When F_1 is integrated in the membrane (electron-transport particles), the total amount of membrane bound F_1 necessary to bind 50% of each anti-β mAb is the same as that observed with active F_1 preincubated with mgATP (Fig. 2A, B and C). This indicates that membrane-bound F_1 is as accessible to the three anti-β mAbs as active F_1. The ATPase activity of membrane-bound F_1 was also checked during the competition experiments, it was slightly increased from 3 to 4 μmol ATP hydrolyzed per min per mg protein.

Anti-α mAbs

Influence of the treatment of F_1 with urea on the binding of mAbs. Fig. 4A shows that the $K_{1/2}$ value of 7 B_3 is slightly higher for urea-treated F_1 (0.17 μM) than for untreated F_1 (0.076 μM), suggesting a diminution of the binding of 7 B_3 to urea treated F_1. On the contrary, for 20 D_6, the treatment of F_1 with urea decreases the value of $K_{1/2}$ by about 5-fold (0.0024 μM instead of 0.013 μM for untreated F_1) (Fig. 4B). This means that the unfolding of F_1 increases the binding of 20 D_6. Besides, when 20 D_6 is preincubated with untreated F_1, a plateau corresponding to about 25% of mAb bound to solid-phase F_1 is observed. In contrast, the binding of 20 D_6 to solid phase F_1 can be completely inhibited by urea-treated F_1 at concentrations as low as 0.02 μM.

Binding of mAbs to F_1 integrated in the membrane. Fig. 5B shows that the $K_{1/2}$ value for 20 D_6 binding of membrane bound F_1 (electron-transport particles) is about 50-times higher than the value observed with soluble F_1 (0.28 mg/ml instead of 0.006 mg/ml). On the contrary, this value

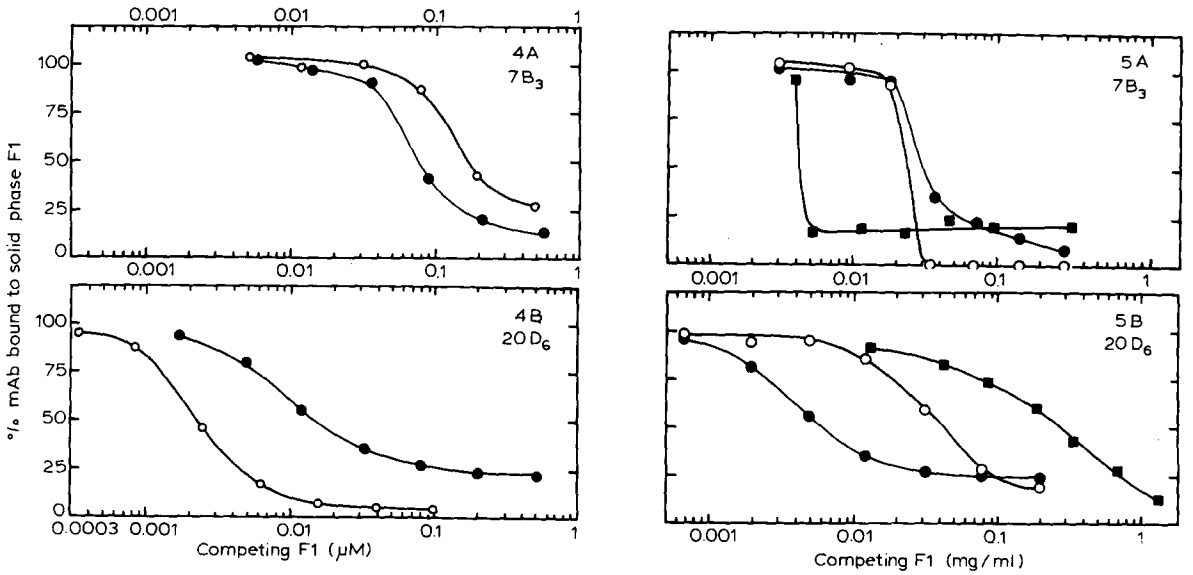

Fig. 4. Effects of unfolding of F_1 with urea on the binding of 2 anti-α subunit mAbs. Conditions as described in Fig. 1. Active F_1, ●; urea-treated F_1, O.

Fig. 5. Modifications of the binding of two anti-α subunit mAb to F_1 upon addition of MgATP or insertion of F_1 in the membrane. Conditions are the same as in Fig. 2. Electron-transport particles, ■; soluble F_1 preincubated in the absence (●) or presence (O) of 1 mM ATP+1 mM $MgCl_2$.

for 7 B_3 binding to F_1 present in the electron-transport particles (0.004 mg/ml) is much lower than that observed for soluble F_1 (0.027 mg/ml), as shown in Fig. 5A. These results indicate that, while the epitope recognized by 20 D_6 is less accessible in the membrane than in soluble F_1, the epitope of 7 B_3 is better exposed in the membrane than in soluble F_1. However, in spite of the higher reactivity of 7 B_3 with the electron-transport particles, a plateau corresponding to about 20% of mAb bound to solid phase F_1 is observed even for very high concentrations of membrane bound F_1 as competing antigen (Fig. 5A).

Changes in the binding of mAbs to F_1 in the presence of MgATP. The presence of 1 mM MgATP decreases the binding of anti-α 20 D_6 to soluble F_1 as in the case of the anti-β mAbs: the $K_{1/2}$ value is about 0.045 mg/ml of active soluble F_1 in the presence of MgATP, while 0.006 mg/ml was sufficient in the absence of Mg ATP (Fig. 5B). In the case of 7 B_3 (Fig. 5A), the addition of 1 mM MgATP barely decreases the $K_{1/2}$ value of soluble F_1. However, the presence of MgATP completely inhibits the binding of 7 B_3 to solid phase F_1 at a F_1 concentration of 0.035 mg/ml, while in the absence of MgATP this total inhibition is not yet reached at 0.3 mg F_1/ml. The curve obtained with 7 B_3 in the absence of MgATP is biphasic.

Discussion

Proximity of antigenic sites

In experiments where the binding of two mAbs to a protein are compared pairwise, the antigenic determinants recognized by these mAbs can be considered as identical or overlapping when the presence of one of them prevents the binding of the other and vice-versa. The results presented here show that two anti-β mAbs (5 G_{11} and 19 D_3) recognize identical or overlapping epitopes on F_1. In addition, the other anti-β mAb (14 D_5) occupies a site different from the two others, but closer to the site of 5 G_{11} than that of 19 D_3. The sites of the two anti-α mAbs are not close to each other. Indeed, although 20 D_6 inhibits 50% of the binding of 7 B_3, there is no reciprocity.

In conclusion, at least four distinct antigenic determinants can be detected with these mAbs on F_1, two on α-subunits (20 D_6 and 7 B_3) and two on β-subunits (14 D_5 and 5 G_{11} or 19 D_3). The close proximity between the epitopes of 5 G_{11} and 19 D_3 demonstrated by the experiments made with immobilized F_1 is further supported by the experiments made with soluble F_1. Indeed, both mAbs behave in a similar manner in all experiments performed. This conclusion is further supported by recent studies on the localization of the antigenic sites of the anti-β mAbs on the β-subunit [32]. These experiments have shown that 14 D_5 recognizes the large N-terminal formic acid cleavage product of the β-subunit and the cyanogen bromide cleavage product corresponding to the sequence spanning Glu 168 to Met 200 in the bovine heart enzyme. On the contrary, both 5 G_{11} and 19 D_3 recognize a 12 kDa, C-terminal acid formic cleavage product of the β-subunit [32].

Availability of antigenic sites on the β-subunit and on F_1

The titration curves obtained for 5 G_{11} and 19 D_3 using F_1 as a competing antigen are parallel to those obtained with the β-subunit. According to Berzofsky and Schechter [27] allows this behavior us to calculate that about 2.8 and 3.2 mol of β-subunit per mol of F_1 are accessible to 5 G_{11} and 19 D_3, respectively. Since F_1 contains 3 β-subunits [1,7], it means that 5 G_{11} and 19 D_3 can bind as well to all three β-subunits of purified F_1 as to the isolated β-subunit. However, since these experiments were performed in the absence of MgATP, the ATPase activity of F_1 was drastically reduced during the incubation. This is very likely due to a dissociation of F_1 into subunits [31]. On the contrary, in the presence of MgATP, the ATPase activity of F_1 was essentially preserved and the availability of the epitopes was much lower as shown by the increase in $K_{1/2}$-values. This availability is inversely proportional to the percentage of F_1 maintained in an active conformation. All these results indicate that the epitope corresponding to 5 G_{11} and 19 D_3 is available at the surface of the β-subunit, but becomes at least partly buried when the β-subunits are complexed with the other subunits to form an active F_1.

In a previous work [9], we have shown that 5 G_{11} and 19 D_3 recognize the β-subunits of all tested species including bacteria, chloroplasts, yeast and mammalian mitochondria. In addition,

both mAbs can, under specific conditions, inhibit ATP hydrolysis or ATP synthesis [9,33]. Dunn et al. [17] have suggested that the most conserved regions of the ATPase lie in the interior rather than on the surface of F_1. Our results show that at least one well-conserved epitope located on the C-terminal sequence of β and related to the enzyme activity lies on the surface of the β-subunit and becomes buried in active F_1.

The effects induced by unfolding of F_1 with urea provide other information on the topology of the antibody binding sites. Urea treatment increases by more than one order of magnitude the concentration of soluble F_1 necessary to bind 50% of the mAbs 5 G_{11} and 19 D_3. Similarly, it has been shown previously [7] that a treatment of F_1 with SDS decreased the reactivity of these two antibodies. This experiment suggested that 5 G_{11} and 19 D_3 were mainly conformational antibodies. Urea and/or SDS destroy the secondary and tertiary structure of F_1, which is necessary to observe an optimal binding of 5 G_{11} and 19 D_3. It can be concluded that these structures are sufficiently maintained in the isolated β-subunit to preserve the binding capacity of 5 G_{11} and 19 D_3. The higher affinity of 5 G_{11} and 19 D_3 for F_1 as compared to F_1 denatured by SDS and urea can be explained by a distortion of the linear sequence epitope by unfolding. If the epitopes comprise amino-acid residues very distant in the sequence, but juxtaposed only on the protein surface by folding, urea or SDS treatments would completely destroy the epitopes. Therefore, 5 G_{11} and 19 D_3 recognize a fragment of the primary sequence of β able to change its conformation by folding.

In the case of the anti-β mAb, 14 D_5, the binding curves of F_1 incubated in the absence of nucleotide and of the isolated β-subunit are not parallel, indicating that the epitopes are not exposed in the same way. This might indicate that F_1 incompletely dissociated is still interacting with some subunits, rendering the epitope less accessible to 14 D_5 than in the case of the isolated β-subunit. Contrary to what is observed for 19 D_3 and 5 G_{11}, urea treatment of F_1 increases the binding of 14 D_5. This means that the epitope recognized by 14 D_5 must be at least partially buried in the β-subunit.

Active F_1 in the presence of ATP and F_1 integrated in the membrane give similar binding curves for the 3 anti-β mAbs. This indicates that the epitopes exposed at the surface of F_1 in the presence of ATP and on the electron-transport particles have the same affinity for the anti-β mAbs. Therefore, the conformation of β in active electron-transport particles is similar to that of F_1 maintained in its active form by preincubation with MgATP.

Availability of the antigenic sites of the α-subunit in soluble or membrane-bound F_1

The epitope corresponding to the anti-α 20 D_6 is optimally exposed when F_1 is completely dissociated by urea treatment, less accessible in F_1 incubated in the absence of nucleotide and even less in active F_1. Therefore, this epitope must be at least partly located on the surface of α-subunit in an area interacting with other subunits. This area is not as easily accessible when F_1 is maintained in its active form by the presence of nucleotide as in inactive F_1.

When F_1 is integrated in the mitochondrial membrane the accessibility of the epitope recognized by 20 D_6 is further decreased by a factor of 6 in comparison to active F_1. This epitope either lies on the surface of F_1 which is in contact with the membrane, or is further masked inside F_1 due to a conformational change occurring when F_1 binds to the membrane.

The only mAb which binds to a very-well-exposed antigenic site of F_1 is the anti-α 7 B_3. Contrarily to all other mAbs tested, the affinity of this antibody becomes higher and higher when the conformation of F_1 becomes closer to the 'in situ' conformation, that is in membrane-bound F_1.

The behavior of these mAbs recognizing two distinct sites suggests a conformation of α different in electron-transport particles and in active F_1.

Apparent heterogeneity of the α-subunits in soluble or membrane-bound F_1

The binding curves of 20 D_6 to untreated F_1 in the presence or absence of ATP show plateau values at significantly less than 100% binding of these antibodies. The presence of such plateaus indicates a heterogeneity of either the antibody or the antigen [27,34]. Since the antibodies are monoclonal [8], the heterogeneity must be due to the

antigen. After urea treatment of F_1, the plateau observed for 20 D_6 disappears. This result suggests that there is a heterogeneity between the three α-subunits of soluble F_1, and that, upon urea treatment, these three α-subunits become equivalent. However, it cannot be excluded that this heterogeneity might be induced by the binding of a first mAb to F_1 which might decrease the affinity of a second mAb to another α-subunit of the same F_1 molecule. For the anti-α 7 B_3, although very low concentrations of electron-transport particles can decrease by 50% the binding of the antibody, a complete inhibition of the binding is never reached (plateau at less than 100%) even when the electron-transport particles concentration is increased by more than 100 times. As discussed above, this type of behavior is characteristic of a heterogeneity in the molecular structure of the binding sites. This indicates that the α-subunits present at the surface of the electron-transport particles appear heterogeneous. On the contrary, the α-subunits accessible at the surface of active F_1 appear homogeneous for the binding of 7 B_3, since no plateau is observed.

The apparent heterogeneity of the α-subunits is in agreement with the conclusions of Amzel and Pedersen [1], who have shown by single crystal X-ray diffraction studies that the three α- and β-subunits are not structurally equivalent. The heterogeneous behavior of the α-subunits has also been recently proposed in the case of chloroplast F_1 [35] and of yeast F_1 [36] to explain heterogeneous modification of the α-subunits by chemical reagents. Our results suggest that the heterogeneity of the α-subunits also exist in membrane-bound F_1.

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