Monoclonal antibodies to mitochondrial coupling factor B

Saroj Joshi*, Lakshmi Kantham*, Suresh Kaplay* and D. Rao Sanadi*+

*Department of Cell Physiology, Boston Biomedical Research Institute, 20 Staniford Street, Boston, MA 02114 and +Department of Biological Chemistry, Harvard Medical School, Boston, MA 02115, USA

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Two monoclonal antibodies (MAb I and IV) have been prepared which showed high and specific reactions towards bovine heart mitochondrial coupling factor B (F_B). Both have been identified as sub-type IgG_1 of mouse immunoglobulins. MAb I reacts with purified and functionally active F_B, alkylated or oxidized forms of F_B and even with peptides formed on digestion of F_B with trypsin. When used together, MAb I and IV reacted with F_B in immunoblots of normal and urea treated samples of mitochondria, submitochondrial particles, ammonia-EDTA extracted particles, and H^+-ATPase. Both MAbs inhibited F_B-stimulated ATP-dependent reverse electron flow activity when F_B was incubated with the antibody either before or after its addition to F_B-deficient AE-particles. Reactivity of MAb I towards F_B declined upon exposure of F_B to guanidine HCl while reactivity of MAb IV remained unaltered.

Coupling factor B *H^+-ATPase* *Mitochondria* *Monoclonal antibody*

1. INTRODUCTION

The mitochondrial H^+-ATPase consists of two parts, the hydrophobic membrane sector (F_0) and the more hydrophilic F_1, bearing the catalytic sites for ATP hydrolysis and synthesis. There is a close similarity between the F_1 subunits from different sources with respect to the number, size and function [1]. Unlike F_1, F_0 from different sources appears to be more variable in the number of subunits. While the bacterial F_0 is known to contain 3 subunits [2], the composition of eukaryotic F_0 remains unelucidated.

Antibodies to F_1 have proved to be useful for studying the role of F_1 subunits in the membrane. Authors in [3], using rabbit antiserum to F_1, found that F_1 complexes faced the matrix side of the mitochondrial inner membranes. Anti-F_1 serum has been successfully used to precipitate oligomycin sensitive ATPase from Triton X-100 solubilized submitochondrial particles [1]. The disposition of different subunits of F_1 has been studied by radioactive labeling of submitochondrial particles and mitochondria followed by immunoprecipitation of oligomycin-sensitive ATPase [1].

The elegant study in [4] using monospecific rabbit antisera to each of the different F_1 subunits showed that the β and γ subunits are required for ATP hydrolysis, whereas the δ and ϵ subunits are involved in the binding of F_1 to the membrane. By following a similar approach, it has been demonstrated [5] that the β-subunit, and small portions of α and γ are required for the hydrolytic activity of F_1-ATPase. All the observations made so far using polyclonal antisera have offered a general understanding of the behavior of individual subunits. More precise information regarding the structural domains of the subunits that are involved in either the catalysis or binding to a different subunit or membrane could be obtained by using monoclonal antibodies. The recent example is the determination of subunit stoichiometry

+ To whom correspondence should be addressed

Abbreviations: F_B, coupling factor B; AE-particles, ammonia-EDTA particles; ETP_H, electron transport particles; ELISA, enzyme-linked immunosorbent assay; PBS-Tween, 10 mM phosphate buffer, 0.9% NaCl and 0.05% Tween-20; MAb, monoclonal antibody

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of α and β subunits in the pig heart mitochondrial ATPase [6], where monoclonal antibodies have been used to illustrate the presence of more than two copies of α and β subunits per H^+-ATPase complex. There is a need for similar studies to be carried out on the F_0 components.

The role of F_B in beef heart mitochondrial H^+-ATPase has been clearly established [7–9]. It is an essential component of F_0, required for ATP synthesis but not for hydrolysis. To aid further studies on the mechanism of F_B action, we have obtained two lines of potent monoclonal F_B antibodies, some of the properties of which are presented here.

2. MATERIALS AND METHODS

The myeloma NS-1 cell lines were supplied by Dr Timothy Springer, Harvard Medical School. BALB/c mice were purchased from Charles River Breeding Station. Peroxidase conjugate of goat antimouse immunoglobulin was obtained from Cappell Laboratories. Mouse immunoglobulin subtype identification kit was obtained from Boehringer-Mannheim. All other chemicals were of reagent grade.

Isolation of beef heart mitochondria [10], ETP_H [11], F_0 [12], preparation of AE-particles [10], purification of F_B [10], assay of ATP-driven NAD^+ reduction by succinate [10], ELISA [13], and electrophoretic transfer of proteins to nitrocellulose paper and immunostaining [14] were carried out as described previously.

For production of monoclonal antibodies, BALB/c mice were immunized intraperitoneally by 4 weekly injections of 50 μg F_B purified to the penultimate step [10] mixed with an equal volume of Freund's adjuvant.

Two mice with the highest antibody titer to highly purified F_B, as determined by ELISA, were selected for hybridoma production and received another injection. Fusion experiments were performed 3 days after the last injection. Spleen cells were fused with NS-1 myeloma cells as in [15]. Briefly, spleen and myeloma cells mixed in a ratio of 4:1 were fused in the presence of 50% (v/v) polyethylene glycol 1600. The fused cells were dispersed in hypoxanthine/aminopterin/thymidine medium in microtiter wells and maintained until macroscopic colonies were formed. Supernatants from such wells were screened for antibodies to F_B by ELISA. Wells that contained antibodies to F_B were cloned by the method of limiting dilution. Antibody containing ascitic fluid was generated by injecting pristane-primed BALB/c mice with 1–2 $\times 10^6$ hybridoma cells per mouse.

For immunoglobulin subtype identification F_B-MAb complex on ELISA plate was treated with type specific rabbit antisera, i.e., specific to α, γ, γ_{2a}, γ_{2b}, γ_3, μ heavy chains and $\varkappa$ and λ light chains of mouse immunoglobulins. Further reaction was carried out as for the regular ELISA technique, using peroxidase conjugated goat antiserum to rabbit immunoglobulins.

3. RESULTS AND DISCUSSION

Twenty antibody producing colonies were identified by ELISA and the best 4 were cloned. The ascitic fluids of MAb I and MAb IV were found to be most potent. The lower limit of sensitivity by ELISA was seen at 1:2000000 and 1:250000 dilutions of MAb I and IV, respectively (fig.1). The binding of the two antibodies was additive when used in subsaturating amounts. The titers with MAb II and III were low. Both MAb I and IV reacted strongly with a polypeptide corresponding in mobility to F_B in immunoblots whereas the MAb II and III reacted very poorly, if at all.

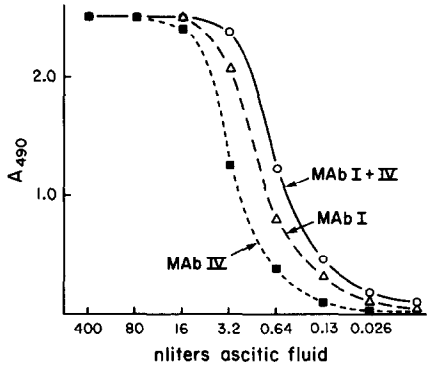

Fig.1. Immunoreactivity of MAb I and IV with F_B. ELISA plates were coated with 200 ng purified F_B per well in 200 μl of 0.05 M Na-carbonate buffer and further reactions were carried out with serially diluted ascitic fluids as in [13]. The amount of ascitic fluid (MAb I or IV) indicated in the figure was taken in 200 μl PBS-Tween buffer.

MAb I and IV have been identified by ELISA to be mouse immunoglobulin subtype IgG_1. Both monoclonals were found to contain the $\varkappa$ light chain.

3.1. *Reaction of monoclonal antibodies with different forms of isolated F_B*

F_B has been shown to have a reactive dithiol that is required for its activity [16]. F_B appears to undergo gross conformational changes when treated with -SH alkylating agents, such as *N*-ethylmaleimide or -SH oxidising agents such as copper–*o*-phenanthroline, that are reflected in altered mobilities on SDS-polyacrylamide gel electrophoresis (SDS–PAGE) [7].

Reduced, alkylated and oxidized forms of F_B moved with a mobility corresponding to M_r values of 17700, 22000 and 16400, respectively, on SDS–PAGE, but these changes did not affect the reactive epitope on the protein or its accessibility to MAb I [7]. MAb I reacted also with some of the peptides of F_B formed on partial digestion with trypsin (fig.2), indicating that MAb I is a highly sensitive and specific probe to follow F_B. It is interesting to note that trypsin activity was not completely inhibited by soyabean trypsin inhibitor in the presence of SDS (see fig.2, lane 7).

Spot tests for antigen-antibody reactivity were carried out by incubating 36 mU partially purified F_B in 35 μl of different denaturants for 5 h at room temperature and spotted directly on the nitrocellulose paper. Immunostaining was carried out with either MAb I or IV as described in section 2. When the spot test was carried out with different samples of denatured F_B, MAb I showed considerably reduced reactivity towards F_B treated with 1.0% SDS, 1.0% β-mercaptoethanol, but MAb IV appears to react nearly as well. Treatment of F_B with 7.0 M urea, 1.0% β-mercaptoethanol did not affect the reaction with either MAb. MAb I reacted poorly with F_B treated with 5.0 M guanidine HCl, 1.0% β-mercaptoethanol whereas the reaction with MAb IV was not affected (not shown).

3.2. *Reactivity of monoclonal antibodies with membrane preparations*

As small an amount as 50 ng F_B could be detected on immunoblots when both MAb I and IV, diluted to 1:2000 in PBS-Tween were used together, reaching a sensitivity somewhat greater than the silver staining method. When 80 μg mitochondria, ETP_H, F_B-deficient AE-particles, H^+-ATPase, and F_B were immunoblotted in a similar way, one specific immunoreactive band corresponding to isolated F_B was evident in all these preparations (fig.3). However, the intensity

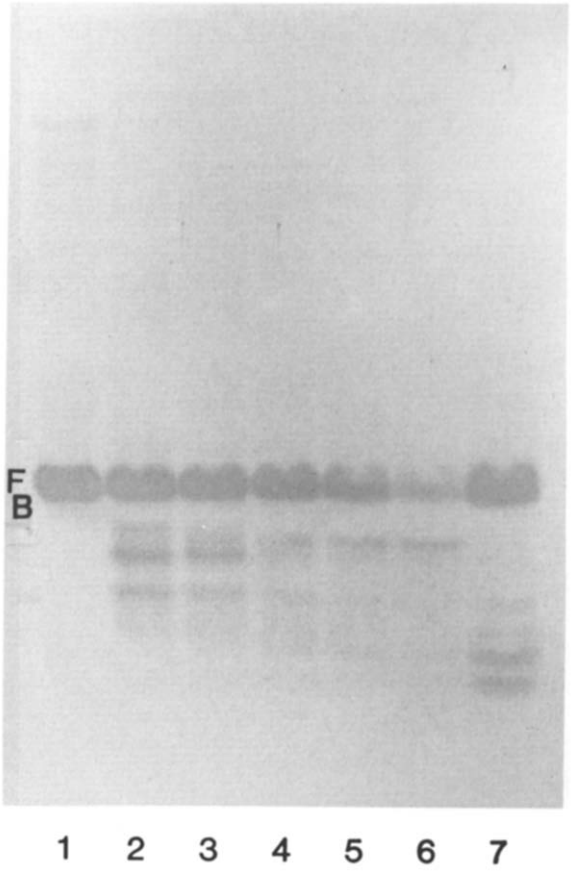

Fig.2. Immunoreactivity of MAb I with tryptic peptides from F_B. Partially purified F_B (0.2 units factorB in 50 μl) was incubated with 0, 2, 4, 12, 24 and 50 μg trypsin for 15 min at 30°C and the reaction was stopped by the addition of a 5-fold excess of soybean trypsin inhibitor in 1–12 μl (nos 1–6). To one sample of F_B, 250 μg inhibitor was added before the addition of 50 μg trypsin (no.7). The reaction mixture (40 μl) was dispersed in 40 μl digestion mixture (0.18 M Tris-Cl, pH 6.8, 2.8% SDS, 7% β-mercaptoethanol, 28% glycerol), and 20-μl aliquots from each were loaded on the gel and electrophoresis and immunoblotting were carried out as in [14]. F_B digestion in sample 7 indicates that the inhibitor does not inactivate trypsin in the presence of SDS.

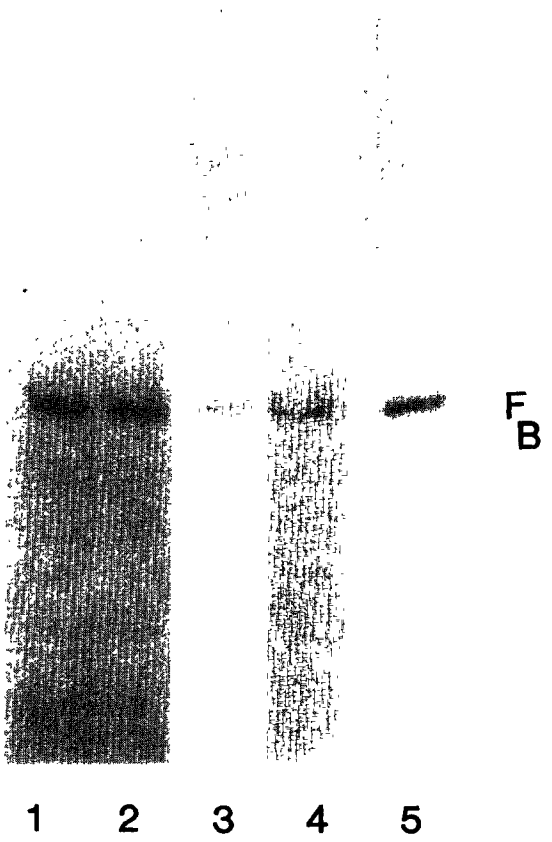

Fig.3. Reactivity of membrane bound F_B to MAb I in immunoblots. Mitochondria (1), ETP_H (2), AE-particles (3), H^+-ATPase (8 mg/ml) (4), and F_B (1.3 μ/ml) (5) were mixed with an equal volume of digestion mixture (see fig.2). Aliquots of 20 μl from each sample containing 80 μg membrane protein in 1–4 and 13 mU F_B in 5 were loaded on the gel. Electrophoresis and immunoblotting were carried out as in [14]. Ascitic fluids of MAb I and IV were diluted 1:1000-fold, mixed in equal volumes and used for immunostaining.

of the reactive band in the H^+-ATPase preparation was not as high as expected although the sample contained approx. 2 μg F_B [13], an amount well in excess of the binding capacity of the nitrocellulose paper. Treatment of these preparations with 50 mM *N*-ethylmaleimide, 1 mM copper *o*-phenanthroline, 6 M urea, trypsin, phospholipase A_2, phospholipase *C*, or lipase did not improve the intensity of the immunoreactive band. It is conceivable that in membrane preparations, the epitope recognized by these monoclonal antibodies is mostly shielded.

3.3. *Effect of MAb on F_B-stimulated ATP driven NAD^+ reduction by succinate, i.e., reversed electron flow activity*

The standard assay for F_B involves measurement of the stimulation of reversed electron flow activity of AE-particles [10]. Treatment of F_B with MAb I or IV prior to its addition to AE-particle abolished its ability to stimulate the AE-particle. When 10-μl aliquots (15–35 mU F_B activity) of partially purified F_B (n = 5) were incubated with 2 μl of either MAb I or MAb IV ascitic fluid for 2 min at room temperature before addition of AE-particles, 70–80% loss in F_B activity was observed. The reaction was specific since the same amount of ascitic fluid, which showed no reaction to F_B in immunoblots, did not have any effect on F_B activity.

Inhibition caused by MAb I and IV was concentration and time dependent (table 1). The extent of inhibition was affected only a little if the antibody

Table 1

Inhibition of F_B-stimulated ATP-driven NAD^+ reduction activity of AE-particles by MAb

Ascitic fluid (μl)	μmol NADH · min^{-1} · ml^{-1}				
	Control	2 min	1 h	3 h	1 h[a]
MAb I					
1	2.4	1.9	1.2	0.7	1.3
5	2.4	1.2	0.5	0.1	0.7
MAB IV					
1	2.4	1.9	1.2	1.1	1.9
5	2.4	1.6	0.7	0.5	0.8

[a] 10 μl of F_B (2.4 U/ml) was first added to the AE-particles (0.5 mg contained in 25 μl) and incubated for 1 min at 38°C. To this mixture 10 μl of appropriately diluted ascitic fluid was added, keeping the effective concentration same as in the experimental samples, incubated for 1 h in ice and assayed as usual

F_B (75 μl of 2.4 U/ml) was incubated in ice with 1 or 5 μl ascitic fluid and 10-μl aliquots were assayed after 2 min, 1 h and 3 h incubation as described in section 2. The F_B assays were by its stimulation of AE-particle activity in catalyzing ATP-dependent NAD^+ reduction by succinate

was added to F_B before or after the addition of AE-particles (table 2). The same levels of ascitic fluid did not show any effect on the particle activity. Results indicated that loss of factor B activity was accompanied by failure of binding of F_B to AE-particle (not shown). Whether the antibody interferes in the binding of F_B to AE-particle or promotes dissociation of reconstituted F_B molecule from AE-particles is not yet clear. Whether the antibodies change the conformation of F_B to cause inactivation is being further investigated.

MAb I and IV did not have any effect on the activity of ETP_H in the ATP-dependent reduction of NAD^+ by succinate. This is anticipated from previous similar data with rabbit antiserum to F_B [17] and indicates that the F_B molecule is well shielded in the H^+-ATPase complex.

MAb I failed to react with F_B denatured with guanidine HCl in the spot reaction on nitrocellulose paper, whereas MAb IV reactivity was unaffected. It is possible that these two Abs are directed against different sites on F_B or MAb I reaction is affected by the tertiary structure of F_B to a greater extent than the MAb IV reaction.

F_B has proved to be a poor immunogen and several attempts in our laboratory to make potent rabbit antiserum have failed. The MAb I and IV have a high titer. This property and the specificity of MAbs in general to one particular epitope make them valuable for studying both the functional and structural aspects of F_B. Preparation of MAbs to F_B has been reported for the first time here.

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