Biochimica et Biophysica Acta, 765 (1984) 258–267
Elsevier

BBA 41495

MONOCLONAL ANTIBODIES AGAINST SUBUNITS OF YEAST MITOCHONDRIAL H^+-ATPase

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(Received September 26th, 1983)
(Revised manuscript received February 10th, 1984)

Key words: Monoclonal antibody; ATPase; ATP synthetase; Mitochondrial ATPase; (Yeast)

Fourteen stable lines of myeloma-spleen cell hybrids producing antibodies against the mitochondrial H^+-ATPase have been isolated. One reacted with the α-subunit of the enzyme complex (M_r 56000), nine with the β-subunit (M_r 54000), and four with a 25 kDa subunit which has not been previously characterized. These antibodies are inhibitory or stimulatory or have no effect upon the enzyme activity. Two of the monoclonal anti-β-subunit antibodies were found to be particularly effective in immunoprecipitating intact H^+-ATPase complex.

Introduction

In recent years, the mitochondrial H^+-ATPase (ATP synthetase, EC 3.6.1.3) has been the subject of intensive investigations in many laboratories. The H^+-ATPase is the terminal enzyme in oxidative phosphorylation and catalyzes the synthesis of ATP when coupled to the mitochondrial electron transport chain (see Refs. 1 and 2 for recent reviews). The mechanism by which the proton motive force generated by electron transport chain reactions is utilized to drive the synthesis of ATP is one of the major unsolved problems of biochemistry.

The mitochondrial ATPase is assembled from hydrophobic protein subunits which are synthesized in the mitochondria, as well as subunits imported from the extra-mitochondrial cytoplasm [3]. In yeast, the enzyme complex consists of about ten different protein subunits which are organized into two functional sectors: the F_1 sector which contains the catalytic site for ATP synthesis and hydrolysis, and the F_0 sector which acts as a proton channel [4]. The F_1 is well characterized and has been shown to consist of five different subunits (α, β, γ, δ, ϵ) which are imported from the cytoplasm [5,6]. The subunit composition of the F_0 sector, on the other hand, is not well established, but two mitochondrial translation products have conclusively been shown to be part of this sector: subunit 6 (M_r 20000) and subunit 9 (M_r 7600). In addition, recent results from our laboratory suggest that the F_0 sector contains a third mitochondrially synthesized subunit which on SDS-polyacrylamide gel has the mobility of a 10 kDa protein [7].

Monoclonal antibodies have a specificity not previously available with polyclonal antisera, because the former recognize only one epitope on an antigen. For this reason, monoclonal antibodies against subunits of the H^+-ATPase complex would provide a powerful tool in the study of the structure, function and assembly of the enzyme complex. These antibodies can be used, for example, as specific probes to define the exact subunit composition of the enzyme complex, to study the function of individual subunits of the enzyme

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Abbreviation: ELISA, enzyme-linked immunosorbent assay.

complex, and to elucidate the structural organization of the H^+-ATPase subunits as well as the geometry of these subunits on the inner mitochondrial membrane. Monoclonal antibodies which bind to specific subunits of the H^+-ATPase without causing the dissociation of the enzyme complex could be used in the isolation of intact H^+-ATPase, and therefore would be extremely useful in the study of the assembly of the enzyme complex.

In this communication we describe the isolation and characterization of fourteen monoclonal antibodies directed against various subunits of the H^+-ATPase: one anti-α-subunit and nine anti-β-subunit antibodies, and four antibodies against a 25 kDa subunit. Two of the monoclonal anti-β-subunit antibodies were found to be particularly effective in immunoprecipitating intact H^+-ATPase complex.

Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium, foetal-calf serum, Freund's complete and incomplete adjuvant were obtained from Gibco (New York); poly(ethylene glycol) 4000 (gas chromatography grade) was from BDH (Victoria, Australia). aminopterin, hypoxanthine and thymidine were from Calbiochem (CA). Azaguanine was purchased from Sigma Chemical Co. (MO); Pristane (2,6,10, 14-tetramethylpentadecane) was from Aldrich (Milwaukee, WI); sheep antimouse immunoglobulin was from Sigma Silenus (Clayton, Australia). The mouse myeloma line, P3X63-Ag 8.653 was kindly provided by Dr. R.G.H. Cotton (Genetic Research Unit, Royal Children's Hospital, Parkville, Victoria, Australia).

Preparation of antigen and immunization schedule

Mitochondrial H^+-ATPase was isolated from a wild-type haploid strain of *Saccharomyces cerevisiae* (strain J69-1B) [8], essentially as described by Tzagoloff and Meagher [5]. The H^+-ATPase was suspended to a final concentration of 5–10 mg protein/ml in phosphate-buffered saline containing 0.17 M NaCl/3.4 mM KCl/8 mM Na_2HPO_4/1.47 mM KH_2PO_4. Female Balb/c mice (8–12 weeks old) were injected intraperitoneally with the above suspension (100 μg per mouse) emulsified with an equal volume of Freund's complete adjuvant. Incomplete adjuvant was used for the subsequent immunization (20 μg per mouse) which were carried out 4 and 6 weeks after the primary immunization. Serum titres for anti-ATPase antibodies were determined 7–12 days after the last immunization by an enzyme-linked immunosorbent assay (ELISA) with purified H^+-ATPase as an antigen as described below. Two mice with the highest titres were injected intraperitoneally for 4 consecutive days with purified H^+-ATPase in phosphate bufferd saline (20 μg protein per injection) and used for the fusion.

Construction of hybridoma cells

Fusion was carried out essentially as described by Stahli et al. [9]. Washed mouse spleen cells ($1.0 \cdot 10^8$) were fused with P3X63-Ag 8.653 myeloma cells ($5 \cdot 10^7$) which have been grown in Dulbecco's modified Eagle's medium containing 8-azaguanine. Poly(ethylene glycol) 4000 (50% solution in serum-free medium) was added dropwise to the mixture of spleen and myeloma cells under agitation over a 1-minute period at room temperature. The mixture was then left standing for 90 s before 5 ml of solution A, containing 11.0 mM glucose/5.4 mM KCl/0.14 mM NaCl/10 mM Na_2HPO_4/5.0 mM NaH_2PO_4/0.001% phenol red (pH 7.4), was slowly added (initially dropwise) with gentle shaking over a period of 5 min. After 10 min, cell clumps were dispersed by repeated gentle pipetting and diluted into 300 ml with Dulbecco's modified Eagle's medium containing 10 mM hypoxanthine/0.46 mM aminopterin/ 1.6 mM thymidine. 1 ml aliquots were distributed into 288 Costar Wells of 2 ml capacity, already containing about 10^5 peritoneal macrophages per well. The medium in each well was changed after 5 days of growth and thereafter as required as indicated by the cell density. After 2–3 weeks of growth, hybridoma supernatants were tested for the production of antibodies against the H^+-ATPase by an ELISA (see below). Positive hybridomas were cloned twice by limiting dilution on microtitre trays containing peritoneal macrophages [9].

Antibody class determination

The class of immunoglobulins produced by each hybridoma cell line was determined in a standard ELISA assay using alkaline phosphatase-labelled sheep antimouse IgG or IgM (Kirkegaard & Perry Laboratories, Inc., Gaithersberg, MD 20760, U.S.A.) as the second antibody.

Large-scale monoclonal antibody production

Hybridoma cells ($2–6 \cdot 10^6$) were injected intraperitoneally into Balb/c mice primed at least 2 days earlier with 0.5 ml pristane [10]. Ascites fluid was tapped after 10–14 days, clarified by centrifugation at $1000 \times g$ for 5 min and stored at −20°C. Monoclonal antibody Ig was purified from the ascites fluid on a Protein A-Sepharose column essentially according to Ey et al. [11]. Protein concentrations of column eluates were calculated from the absorbance of the solution at 280 nm, using an extinction coefficient (1% w/v; 1 cm) of 14 [11], and antibody titre was determined by ELISA. Purified IgG was aliquoted and stored at −20°C in phosphate-buffered saline (pH 7.4). Thawed aliquots were checked by an ELISA for activity prior to use.

Enzyme-linked immunosorbent assay (ELISA)

Poly(vinyl chloride) microtitre plates (U-shaped, Disposable Products, Oakleigh, Australia) were coated with 150 ng of purified H^+-ATPase in phosphate-buffered saline at 37°C overnight. Plates were washed four times in phosphate-buffered saline containing 1.5 mM $MgCl_2$/0.05% Tween-20/2.0 mM β-mercaptoethanol/20 mg per ml bovine serum albumin (buffer 1). Diluted mouse serum, hybridoma supernatants, or ascites fluid were then pipetted into the wells (50 µl per well) and incubated at 37°C. After 1 h incubation, plates were washed four times with buffer 1 as above. Alkaline phosphatase-labelled sheep anti-mouse Ig (New England Nuclear, Boston, MA), diluted 1000-times with buffer 1, was then added to each well (50 µl per well) and incubated for 1 h at 37°C. Plates were again washed four times in buffer 1, then twice with water followed by the addition of 50 µl of *p*-nitrophenyl phosphate (2 mg/ml in 100 mM Na_2CO_3/100 mM β-mercaptoethanol/1 mM $MgCl_2$) to each well. After a further incubation at 37°C for 1 h, the reaction was stopped by the addition of 0.1 ml of 0.5 M Na_2CO_3.

Subunit specificity of monoclonal antibodies

Subunits of purified H^+-ATPase (40–100 µg) were separated by electrophoresis on polyacrylamide gels in the presence of SDS [17]. The separated proteins were then transferred to nitrocellulose filters (0.22 or 0.45 µm pore size, obtained from Amicon, MA) electrophoretically at 60 V for 1 h essentially according to Vaessen et al. [13]. The transfer buffer comprised 25 mM Tris/192 mM glycine/20% methanol (v/v) at pH 8.3. A strip of the nitrocellulose filter was stained for 10–15 min in 0.1% (w/v) Amido black in the presence of methanol (45%, v/v) and acetic acid (10%, v/v), and destained in a solution containing acetic acid (7.5%, v/v)/methanol (40%, v/v). The rest of the filters were incubated in phosphate-buffered saline containing bovine serum albumin (8%) at 37°C for 1 h to saturate remaining protein-binding sites. The filters were then incubated in diluted ascites fluid (1 : 50 in phosphate-buffered saline) at 37°C for 16 h. After washing with three changes of phosphate-buffered saline, the filters were further incubated in 0.5 ml of phosphate-buffered saline containing ^{125}I-labelled sheep anti-mouse immunoglobulin (10^6 cpm/incubation) and 8% (w/v) bovine serum albumin for 2–3 h at room temperature with vigorous shaking. Sheep anti-mouse IgG was iodinated with ^{125}I according to Greenwood and Hunter [14]. The filters were then washed twice in 50 ml of phosphate-buffered saline containing Triton X-100 (1%, v/v) with 1.5 h incubation for each washing. The filters were dried and autoradiographed on prefogged Kodak X-R1 film at −80°C [12] overnight, using Ilford intensifying screens.

Determination of ATPase activity

A modification of the method of Pullman et al. [15] was employed to assay the ATPase activity. The basic incubation mixture contained the following in 1.0 ml: 50 µMol Tris-HCl (pH 8.0), 1 µMol ATP, 3 µMol $MgCl_2$, 5 µg antimycin A, 1 µMol phosphoenol pyruvate, 0.3 µMol NADH, 5 units lactate dehydrogenase and 2.5 units pyruvate kinase. The reaction was initiated by the addition of 10 µg of purified H^+-ATPase. Oxidation of

NADH at 28°C was followed spectrophotometrically at 340 nm. To determine the ability of the monoclonal antibodies to inhibit the enzyme activity, 100 μg purified monoclonal antibody Ig in 50 μl phosphate-buffered saline was added to 10 μg purified H^+-ATPase in 50 μl 4 mM Tris-acetate buffer (pH 7.5) containing 15% glycerol (v/v)/0.1% Triton X-100 (v/v). The mixture was then left on ice for 2 h prior to the assay of the ATPase activity.

Immunoprecipitation of H^+-ATPase complex with monoclonal antibodies

Yeast cells were harvested in late exponential phase, washed with ice-cold water and labelled with [^{35}S]sulphate (approx. 500 μCi/ml) in low sulphate medium at 28°C [16] for 4 h in the absence of antibiotics, or for 1 h in the presence of cycloheximide [17]. The medium was removed and the cells were washed twice with a solution containing 1% casamino acids/2 mg per ml sodium sulphate. Cells were then suspended in 13 mM Tris-HCl buffer (pH 7.4) containing 0.33 M sorbitol, 0.27 M mannitol, 0.7 mM EDTA and the proteinase inhibitors 10 mM *para*-aminobenzamidine-HCl, 10 mM ε-amino-*N*-caproic acid and 2 mM phenylmethylsulphonyl fluoride [17]. Mitochondria were then prepared essentially as described previously [18], and suspended in 4 mM Tris-acetate buffer (pH 7.5) containing 0.25% Triton X-100, 2 mM ATP and the above proteinase inhibitors (extraction buffer) at 6.25 mg protein per ml. After incubation at 0–4°C for 10 min, the suspension was centrifuged at 106000 × *g* for 20 min and aliquots (50 μl) of the supernatant were preincubated with 50 μl of CNBr-activated Sepharose-4B (with their active sites blocked with glycine) for 1 h at 4°C to absorb any materials which would bind non-specifically to the beads. After the removal of the Sepharose-4B the supernatant (50 μl) was incubated for 4 h at 4°C with 50 μg of monoclonal antibody IgG coupled to CNBr-activated Sepharose-4B beads. The beads were collected by centrifugation in an Eppendorf centrifuge and washed at least three times with the extraction buffer. All immunoprecipitates were suspended in 10 μl water and dissolved by the addition of 15 μl 0.6 M Tris-HCl buffer (pH 6.7)/10% SDS and boiled for 2 min. Small aliquots were removed for the determination of radioactivity and suitable aliquots were subjected to polyacrylamide gel electrophoresis as above [17].

Results

The isolation of hybridomas-producing monoclonal antibodies against yeast H^+-ATPase

A number of immunization schedules were initially tested for their efficiency to induce ATPase antibody production in mice. The schedule described in the Materials and Methods produced the highest serum titres and was therefore the one used for fusion. However, reasonable high titres (more than 1 : 500) could also be obtained by a shorter immunization schedule involving a primary injection of 100 μg antigen per mouse, followed by three weekly booster injections with 20 μg antigen per mouse.

Of 14 Balb/c mice immunized with purified H^+-ATPase, two with the highest titre (1 : 4000) were used for fusions. Spleen cells obtained from the two mice were fused with P3X63-Ag 8.653 myeloma cells and plated into two lots of 288 Costar wells as discussed in Materials and Methods. After 2 weeks, 260 wells from the first fusion (90%) and 60 wells from the second fusion (21%) showed sufficient growth of hybridoma cells to assay for antibody activity. The ability of these hybridomas to synthesize anti-ATPase-antibody was first screened by using an enzyme-linked immunosorbent assay, with purified ATPase complex as an antigen. Ten of the hybridoma-containing wells of the first fusion (4%) and four wells of the second fusion (7%) were found to contain anti-ATPase-antibodies. Cells from the positive wells were expanded and subcloned twice by limited dilution as described in Materials and Methods to ensure monoclonality. On the second subcloning, all wells derived from each of the original 14 clones were found to contain hybridoma cells producing anti-ATPase-antibodies. This result indicates that the anti-ATPase secreting subclones obtained from the initial 14 hybridomas are now monoclonal.

To obtain larger quantities of antibodies, cloned cells were expanded in culture and injected into pristane-primed mice for ascites fluid production. Monoclonal antibodies were then purified from

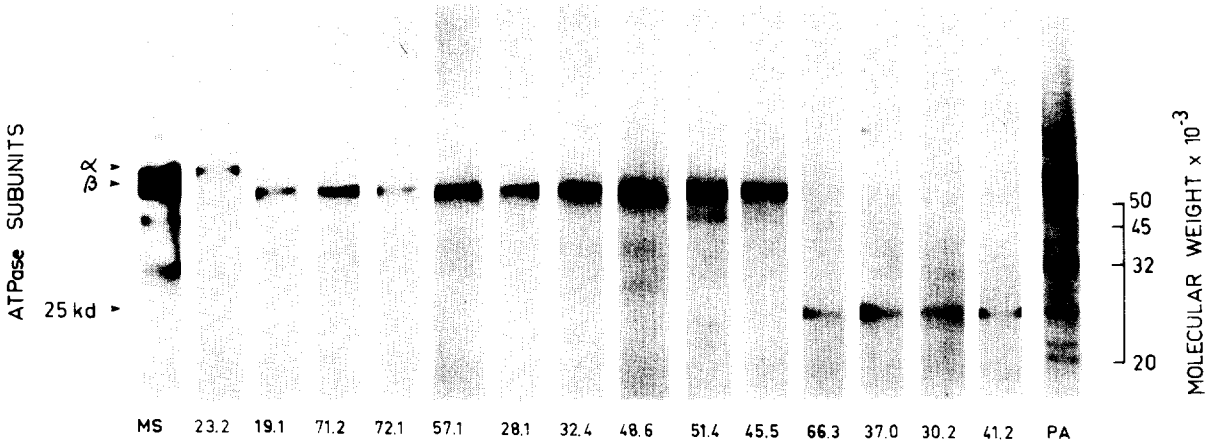

Fig. 1. The specificity of the monoclonal anti-H^+-ATPase antibodies. Glycerol gradient purified H^+-ATPase was electrophoresed on 10% polyacrylamide gels in the presence of SDS, as previously described [17]. The dye-front was run off the gel to obtain a better separation of the α-subunit from the β-subunit. Protein bands were then transferred electrophoretically from the gel to nitrocellulose filter [13]. A strip of the nitrocellulose filter was stained by 0.1% (w/v) Amido black. The rest of the filter strips were incubated with diluted serum or ascites fluids at 37°C for 16 h. Monoclonal antibodies bound to the ATPase subunits were then detected by using ^{125}I-labelled sheep antimouse immunoglobin as described in Materials and Methods. MS is the autoradiogram of the mitochondrial H^+-ATPase immunoblotted with a mouse serum against yeast H^+-ATPase. Similar patterns of immunoblottings were obtained with three other mice antisera. The other autoradiograms are of the ascites fluids tested, as follows: RH 23.2, RH 19.1, RH 71.2, RH 72.1, RH 57.1, RH 28.1, RH 32.4, RH 48.6, RH 51.4, RH 45.5, RH 66.3, RH 37.0, RH 30.2, RH 41.2 PA is an Amido-black-stained profile of the purified mitochondrial H^+-ATPase.

the ascites fluid by chromatography on Protein A-Sepharose column [11]. All of the antibodies secreted by different hybridoma were found to be of the IgG class.

Characterization of monoclonal antibodies by immunoblotting

As shown in Fig. 1, out of fourteen monoclonal antibodies tested, one (RH 23.2) reacted with the α-subunit of the F_1 sector (M_r 56000). Six of the other antibodies (RH 19.1, RH 71.2, RH 72.1, RH 57.1, RH 28.1 and RH 32.4) reacted with the β-subunit which has an apparent M_r 54000. RH 48.6 reacted with the β-subunit, but in addition trace amount of this antibody was also found to bind to a 37 and a 28 kDA polypeptides. Similarly, the autoradiograms of RH 51.4 and 45.5 which was found to be also anti-β-antibodies, shows the binding of these antibodies to a 47 kDa polypeptide. Three of the monoclonal antibodies, RH 66.3, RH 30.2 and RH 41.2, are against a polypeptide with an apparent M_r 25000 that has been shown previously to copurify with the ATPase complex but has not been well characterized [19,20]. Another antibody, RH 37.0 was also found to react against the 25 kDa polypeptide but unlike the other antibodies, RH 37.0 also reacts, in tracer amounts, with a 70 kDa polypeptide, the significance of which is not yet known.

Effect of monoclonal antibodies on the mitochondrial ATPase activity

Antibodies produced by different hybridomas were tested for their effect on the mitochondrial ATPase activity. Preliminary analysis of these monoclonal antibodies established that all three types of antibodies are represented in our collection: stimulatory, inhibitory and null antibodies. In order to establish the kinetics of the antibody effects on ATPase activity, a representative of each type was investigated in more detail. As shown in Fig. 2, a progressive stimulation and inhibition of enzyme activity by cloned RH 72.1 and RH 57.1, respectively could be demonstrated with time. The maximal inhibition and stimulation were achieved after about 60 min of incubation. The maximal inhibition was 90% for monoclonal antibody RH 57.1, while the maximal stimulation observed for

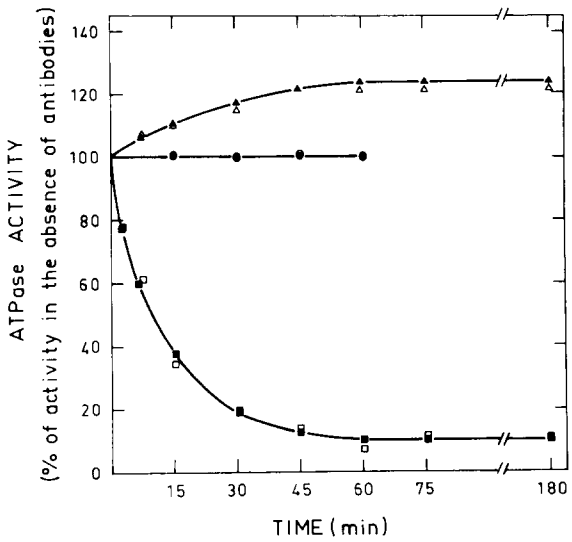

Fig. 2. Effects of monoclonal antibodies RH 57.1, RH 72.1 and RH 37.0 on the mitochondrial ATPase activity. Purified yeast ATPase (10 μg in 50 μl) was incubated at 0°C with 100 μg (in 50 μl) of purified monoclonal antibodies for the indicated length of time. ATPase activity was then measured as described in the Material and Methods. Results presented are of two sets of experiments (indicated by open and closed symbols) and are expressed as percentage of inhibition of the ATPase activity. RH 57.1 (■, □); RH 72.1 (▲, △); RH 37.0 (●, ○).

antibody RH 72.1 was 25%. Clone RH 37.0 had no effect on the enzyme activity for the period of investigation.

The stimulatory and inhibitory effects of the other monoclonal antibodies were accordingly determined after 60 min incubation of the antibodies with the H^+-ATPase. The results are summarized in Table I. Most of the monoclonal antibodies (ten) do not significantly affect the ATPase activity. However, in addition to RH 57.1, RH 45.5 was also found to inhibit the ATPase activity, although by only 36%. Furthermore, beside RH 72.1, which reacts with the β subunit, RH 23.2, an anti α-antibody was also found to stimulate the enzyme activity by 25%.

Precipitation of H^+-ATPase complex with monoclonal anti-β antibodies

Preliminary screening of our antibodies collection indicate that our monoclonal antibodies vary to a great extent in their ability to immunoprecipitate intact H^+-ATPase complex from a triton

TABLE I

EFFECT OF ANTIBODIES ON H^+-ATPase ACTIVITY

The effect of the monoclonal antibodies on the ATPase activity is expressed as mean ± S.D. of three separate experiments.

Clone	ATPase protein subunit specificity	Inhibition of ATPase activity (% inhibition by 100 μg IgG)
RH 57.1	β	90 ± 2
RH 45.5	β + 47 kDa	36 ± 2
RH 28.1	β	2 ± 3
RH 19.1	β	4 ± 3
RH 71.2	β	8 ± 4
RH 48.6	β + 37 kDa + 28 kDa	7 ± 5
RH 51.4	β + 47 kDa	−6 ± 1
RH 32.4	β	−3 ± 3
RH 37.0	25 ± 70 kDa	0 ± 1
RH 66.3	25 kDa	2 ± 3
RH 41.2	25 kDa	8 ± 4
RH 30.2	25 kDa	3 ± 1
RH 23.2	α	−25 ± 2
RH 72.1	β	−25 ± 3

extract of yeast mitochondria. Two of the monoclonal anti-β-antibodies, RH 48.6 and RH 51.4, were found to be effective. The H^+-ATPase immunoprecipitated by these antibodies was found to contain fourteen polypeptides when analysed on SDS-polyacrylamide gel (Fig. 3). However, only ten of the polypeptides observed are probably genuine subunits of the ATPase complex; the other four polypeptides could be shown to be aggregation or breakdown products of one of these subunits. Thus, the polypeptide which has an apparent M_r of 61 000 can be attributed to the α subunit of the F_1 sector, because this protein band was also observed when purified α subunit was electrophoresed on a similar polyacrylamide gel (Orian, J.M. and Marzuki, S., unpublished data). The polypeptide with an apparent M_r of 47 000 and 37 000 are the breakdown products of the β-subunit described in the previous section, because these polypeptides react with some of the monoclonal anti-β-antibodies. The band with the mobility of 29 kDa is probably an α-subunit breakdown product, described previously by Ryrie and Gallagher [19]. The intensity of this band (and also the 47 and 37 kDa bands) varied from one immunoprecipitate to another and was greatly re-

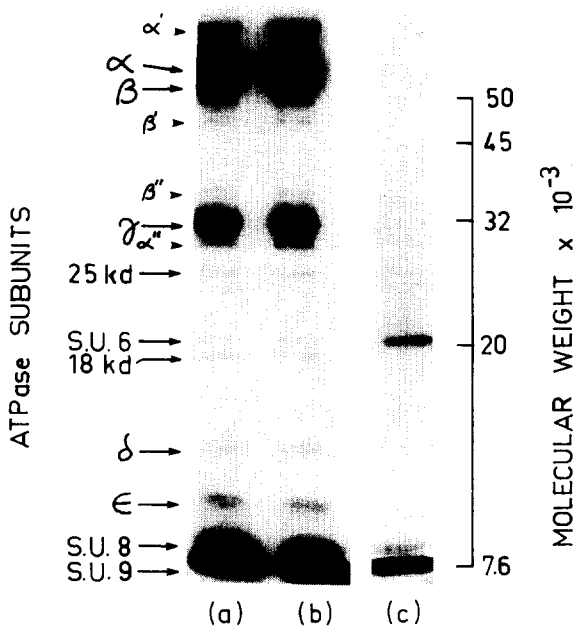

Fig. 3. Polyacrylamide gel electrophoresis of the H^+-ATPase complex precipitated by monoclonal anti-β-antibodies. Yeast cells were labelled with [^{35}S]sulphate at 28°C for 4 h in the absence of antibiotics or for 1 h in the presence of cycloheximide [17] as described in Materials and Methods. Submitochondrial particles were isolated, and H^+-ATPase was extracted with 0.25% (w/v) Triton X-100 [17]. The H^+-ATPase was then isolated from the Triton extract by precipitation with monoclonal anti-β-antibody. The immunoprecipitate was analysed by SDS-polyacrylamide gel electrophoresis [17]. (a) Autoradiogram of H^+-ATPase immunoprecipitated by RH 48.6 from cell labelled in the absence of antibiotics. (b) Similar to (a), but immunoprecipitated by RH 51.4. (c) Autoradiogram of H^+-ATPase immunoprecipitate by RH 48.6 from cells labelled in the presence of cycloheximide. A similar pattern was observed when RH 51.4 was used instead of RH 48.6. The mobility of the 18 kDa polypeptide appears to vary slightly in different experiments. In some cases it runs almost together with subunit 6 of even slightly behind it [7].

duced by the addition of proteinase inhibitors to the solutions used throughout the mitochondrial isolation and the immunoprecipitation steps.

Five of the polypeptides precipitated could be identified to be the F_1 subunits by comparison of their mobility in SDS-polyacrylamide gel to that of the isolated F_1 subunits (α, β, γ, δ and ε: M_r 56000, 54000, 31000, 14000 and 12000, respectively) (Fig. 3a and b). Three of the other subunits, with molecular weights of 20, 10 and 7.6 kDa, are synthesized by the mitochondria, as shown by immunoprecipitation of H^+-ATPase from cells which have been labelled with [^{35}S]sulphate in the presence of cycloheximide (so that only mitochondrial translation products were labelled) (Fig. 3c). Prior to labelling, the cells were preincubated in the presence of chloramphenicol which inhibits mitochondrial protein synthesis, allowing the accumulation of the cytoplasmically synthesized subunits of the H^+-ATPase [17]. These subunits could apparently be assembled into the F_0F_1-ATPase during the labelling period, together with the ^{35}S-labelled F_0-subunits synthesized upon the removal of the chloramphenicol (and the addition of cycloheximide), as indicated by the ability of the monoclonal anti-β-antibodies to precipitate the ^{35}S-labelled mitochondrially synthesized subunits (Fig. 3c). The identity of the other two polypeptides (M_r 18000 and 25000) have not been established, but since they reproducibly copurify with the β-subunit, it appears that these polypeptides are genuine subunits of the H^+-ATPase complex. Furthermore, immunoprecipitate obtained with monoclonal antibodies to the 25 kDa polypeptide contained a number of the H^+-ATPase subunits described above (data not shown) although some subunits appear to be missing presumably due to the instability of the enzyme complex (see Discussion).

Discussion

Fourteen stable hybridoma lines producing monoclonal antibodies against subunits of the yeast mitochondrial H^+ATPase have been isolated in the present study. Nine reacted with the β-subunit of the enzyme complex (M_r 54000), one with the α-subunit (M_r 56000), and four with the 25 kDa subunit. Although this observation indicates that the β-subunit of the yeast F_1 sector is the polypeptide which appears to be the most antigenic in mice and is consistent with the fact that the β-subunit is one of the major subunits of the enzyme complex, it should be noted that the initial screening of the hybridomas after fusion was done by an ELISA assay on coated plates only. This procedure may alter the antigenic structure of the enzyme complex and therefore might be somewhat selective. When anti-sera against yeast H^+-ATPase from four different mice were analysed for the

subunit specificities of their antibodies, however, only antibodies against the α-. β- and γ-subunits could be detected (Fig. 1). This observation is in contrast to the finding that rabbit antisera to bovine heart H^+-ATPase complex contain mostly anti-F_0-subunit antibodies [21]. It appears that a wide variation exists in the antigenicity of yeast and bovine heart H^+-ATPase subunits in different experimental animals.

Since it appears that the F_0-subunits of yeast H^+-ATPase are not very antigenic in our strain of mice, a different approach would have to be adopted to produce monoclonal anti-F_0-subunits antibodies which would be very useful as probes to study the geometry of the F_0-subunits in the mitochondrial inner membrane and the involvement of the individual F_0-subunits in the formation and regulation of the H^+-channel activity of the F_0-sector.

The observation that only one of the monoclonal antibodies was directed against the α-subunit is quite surprising in view of the fact that this subunit is the largest ATPase subunit and according to the current model of the ATPase complex [22], it should be as exposed as the β-subunit. It appears that the α subunit is less antigenic in mice than the β-subunit in our preparations of H^+-ATPase. Four antibodies were found to react with a 25 kDa protein which copurifies with the ATPase complex. This polypeptide has been observed in different preparations of the yeast ATPase complex [19,20]. However, although Todd and Douglas [22] have shown that the 25 kDa protein cross-linked to other subunits of ATPase complex (α, β and subunit 9) in the presence of the reversible cross-linking reagent, dithiobis(succinimidyl) propionate, so far there is no definitive evidence as yet that this protein is indeed a functional subunit of the enzyme complex. The availability of monoclonal antibodies against the 25 kDa protein described herein, would certainly be very useful in the elucidation of the function of this protein in the ATPase complex.

There is a wide range of effects exerted by monoclonal anti-β on the activity of the mitochondrial ATPase. For example, 100 μg of purified RH 57.1 inhibits the enzyme activity by 90%, while the same amount of RH 72.1 stimulates the same acticity by 25%. The β-subunit, which is one of the larger subunits in the complex, plays an important role in the functioning of the ATPase [4,23]. It has been shown that this subunit contains the catalytic sites for the hydrolysis and synthesis of ATP, and there are three copies of β-subunit per ATPase complex [4,23]. This subunit binds inhibitors of F_1 activity, such as aurovertin [24] and citreoviridin [25]. The collection of anti-β monoclonal antibodies reported in this communication would be very useful in the study of the tertiary structure as well as the function of this subunit, in particular its catalytic site.

Two of the monoclonal anti-β-antibodies RH 48.6 and RH 51.4 were found to be very effective in the immunoprecipitation of intact H^+-ATPase complex. The complex brought down by the monoclonal antibodies contained ten polypeptides considered to be true components of the complex. Four other polypeptides revealed by SDS-polyacrylamide gel elctrophoresis are probably aggregation or break-down products of α- and β-subunits. Five of the H^+-ATPase polypeptides could be identified as F_1 subunits. Three of the other polypeptides are mitochondrially synthesized and two of these have been firmly established to be the subunits of the F_0 sector: subunit 6 and 9 with an M_r of 20 000 and 7600, respectively [26]. The third polypeptide which on SDS-polyacrylamide gel has a mobility of a 10 kDa protein has in the past been the subject of conflicting reports because its presence varies in different preparations of yeast ATPase complex [19,20,26–28]. However, the following observations indicate that this protein is indeed a subunit of the yeast H^+-ATPase.

Firstly, unlike the previous polyclonal antibody immunoprecipitate [20,26,27], the 10 kDa mitochondrial translation product was always found to be associated with ATPase immunoprecipitate obtained with monoclonal anti-β-antibodies RH 48.6 and RH 51.4. It is possible that the polyclonal antibodies used in the previous studies [20,26,27] causes the dissociation of this polypeptide from the enzyme complex, resulting in the variability of the amount of the 10 kDa protein associated with the subunits which are antigenic (this will be discussed in more detail).

Secondly, our laboratory has recently isolated several *mit*$^-$ mutants of yeast, which as a result of a mutation in the mtDNA, cannot synthesize the

10 kDa polypeptide [29]. Mitochondria isolated from these mutants still have some ATPase activity, but show little or no oligomycin sensitivity indicating, that the F_1 sector of the H^+-ATPase complex is associated with a defective F_0 sector. The mutations in these strains have been mapped on to a previously unidentified reading frame which is located between the *oli2* and the *oxi3* regions of the mtDNA coding for ATPase subunit 6 and cytochrome oxidase subunit I, respectively [29,30]. The amino acid composition of the polypeptide predicted by the sequence of the reading frame is similar to that of the purified 10 kDA mitochondrial translation product [31,32].

The two monoclonal anti-β-antibodies discussed above are of a special interest because of their potential usefulness in the study of the assembly of the yeast H^+-ATPase. This enzyme complex is not very stable and it is difficult to immunoprecipitate the complex in an intact form.. Immunoprecipitates obtained with anti-sera against purified F_1-ATPase frequently do not contain the mitochondrially synthesized F_0-subunits 6, 8 or 9 [27]. The best immunoprecipitate of assembled yeast H^+-ATPase reported to date is that of Todd et al. [20] obtained with the use of the polyclonal anti-β antiserum. This immunoprecipitate contains all of the cytoplasmically synthesized subunits observed in this communication but lacks the mitochondrially synthesized subunit 8. In addition, the analysis of the amount of subunit 9 associated with various immunoprecipitates obtained with the polyclonal anti-β-antibodies indicate that this subunit might be partially dissociated during the extraction and immunoprecipitation of the complex.

The reason for the apparent inability of polyclonal anti-F_1-subunits antibodies to bring down reproducibly some of the F_0-subunits is not clear. One possible explanation is that the binding of an antibody molecule to certain antigenic determinations might induce conformational changes in the subunit(s) concerned, resulting in the dissociation of the F_0-subunits from the enzyme complex. Consistent with this hypothesis is the observation that our monoclonal antibodies RH 57.1 and RH 45.5 (which inhibit the ATPase activity) immunoprecipitate only some of the subunits of the H^+-ATPase (data not shown). The binding of monoclonal antibodies RH 51.4 and RH 48.6, on the other hand, do not appear to affect the stability of the enzyme complex. The enzyme complex precipitated with monoclonal antibody RH 48.6 was found to retain an ATPase activity which is sensitive to inhibition by oligomycin (84% inhibited by 10 μg/ml oligomycin, data not shown).

Preliminary investigations in our laboratory indicate that these antibodies are even capable of immunoprecipitating defective ATPase complex from *mit*$^-$ mutants which lack subunits 6, 8 or 9 as the result of mutations in their structural genes [7]; the defective ATPase complex in these mutants are much less stable than that of the wild-type strain. We are currently investigating the subunit composition of the defective ATPase complex to define the nature of the defect in the H^+-ATPase assembly process, associated with the inability of the mitochondrial mutants to synthesize one or more of its subunits. Such information would give us a better understanding of the mechanism of assembly of the mitochondrial H^+-ATPase complex.

Acknowledgements

We would like to thank Professor A.W. Linnane for his support and interest in this project. The valuable technical advice given by Dr. R.G.H. Cotton and Ms. Kerry Fowler of the Royal Children's Hospital, and Dr. B. Adler of Microbiology Department of Monash University at the initial stage of this investigation is gratefully acknowledged.

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