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Antipeptide antibodies to the carboxy terminal and the DCCD binding region of the human mitochondrial ATP synthase β-subunit

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Antibodies to defined epitopes on the human ATP synthase would provide a powerful tool in the definition of the subunit composition of the enzyme complex and in the characterization of any defect in its assembly in diseases associated with mitochondrial disorders. Antibodies have been thus raised against synthetic peptides, corresponding to two regions on the human ATP synthase β-subunit: the C-terminal region, and a region which includes the two dicyclohexylcarbodiimide (DCCD)-reactive glutamic acid residues suggested to be involved in the enzyme catalytic activity. The antibodies to the C-terminal peptide reacted with the ATP synthase β-subunit in ELISA, in Western immunoblotting and in immunohistochemical experiments, and had the ability to immunoprecipitate the enzyme complex. The antibodies to the DCCD-binding region peptide did not react to the ATP synthase β-subunit in its native configuration, although reacted well under Western immunoblotting conditions.

Introduction

Monoclonal antibodies to the various subunits of the ATP synthase (H^+-ATPase, EC 3.6.1.3) have been instrumental in the elucidation of the structure, function and assembly of this multimeric enzyme complex [1–3], which is the terminal enzyme in oxidative phosphorylation; this enzyme complex uses the transmembrane proton motive force generated by the electron transport chain for the synthesis of ATP. In particular, the use of monoclonal antibodies to the two major subunits of the yeast ATP synthase (α and β) as immunoprobes for defects in the assembly of the enzyme complex in various *mit*$^-$ mutants of the yeast *Saccharomyces cerevisiae* has resulted in the definition of the assembly pathway of the enzyme complex [4].

Antibodies to defined epitopes on the human ATP synthase would provide a powerful tool in the definition of the subunit composition of the enzyme complex, and in the characterization of its assembly defect in disease associated with mitochondrial disorders. Several monoclonal antibodies to the anti-yeast-ATP synthase, previously isolated in our laboratory, have been examined for their cross-reactivity to the human enzyme. However, while cross-reactivity can be clearly demonstrated by Western-immunoblotting, the affinity of the antibodies to the human subunit was found to be at least three orders of magnitude lower than their affinity to the yeast proteins. Thus, the anti-yeast-ATP synthase monoclonal antibodies are not useful for the study of the human enzyme complex.

In the present study, antibodies have been raised against synthetic peptides, corresponding to the sequence of two important regions on the human ATP synthase β-subunit. The first one of these peptides is of eight amino acid residues long, corresponding to the sequence of the carboxy terminal region of the human β-subunit [5]. The second peptide corresponds to a region between amino acid residues 186 and 202 on the β-subunit, and includes the two dicyclohexylcarbodiimide-reactive glutamic acid residues which have been suggested to be involved in the catalytic activity of the enzyme complex [6]. The antibodies to the carboxyl terminal react with the ATP synthase β-subunit in ELISA and in Western immunoblotting experiments, and have the ability to immunoprecipitate the enzyme complex. The antibodies to the DCCD-binding region, however, did not react to the ATP synthase β-subunit

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in its native configuration, although they reacted well under Western immunoblotting conditions.

Materials and Methods

Synthesis of Peptides. The amino acid sequences of the two peptides used in the present study are shown in Table 1. The first peptide, corresponding to the sequence of the carboxy terminal region of the human β-subunit [5], is eight amino acid residues long and was synthesized on an Applied Biosystems 430A peptide synthesizer employing conventional *t*-Boc chemistry [7,8]. The second peptide is of 18 amino acid residues, corresponding to a region between amino acid residues 186 and 202 of the β-subunit plus an extra lysine residue at amino-terminal end.

Production of antibodies. Synthetic peptides were coupled to either keyhole limpet haemocyanin (KLH; Calbiochem-Behring, U.S.A.) or bovine serum albumin (BSA; Sigma, U.S.A.) as an immunogenic carrier by using glutaraldehyde [9]. Rabbits were immunized with KLH-conjugated peptides (250 μg of peptide per rabbit), emulsified in Freund's complete adjuvant (total vol 1 ml per rabbit), for the primary injection. Booster injections were carried out at 2-weekly intervals after the primary injection, using incomplete adjuvant (125 μg peptide per rabbit). Anti-peptide antibodies in rabbit sera were assayed 1 week after each booster injection, by an enzyme-linked immunosorbent assay (ELISA) with the peptides (coupled to BSA) or crude F_1-ATPase as an antigen as described below. Two rabbits were immunized with each peptide.

Preparation of mitochondria. Human placenta were obtained from the Royal Women Hospital and the St. Vincent's Hospital, Melbourne, within 1–2 h of delivery and transported in ice to our laboratory in Monash University. Bovine hearts were obtained from a local abattoir and collected immediately after the animal was slaughtered. Human heart and liver were obtained from an organ donor within 3 h post-mortem.

Placental mitochondria were isolated by the method of Hare et al. [10] in the presence of proteinase inhibitors, ϵ-amino-*n*-caproic acid (5 mM), *p*-aminobenzamidine·HCl (5 mM) and phenylmethylsulfonyl fluoride (2 mM). Human and bovine heart mitochondria were isolated essentially as described by Smith [11] except that all buffers contained 5 mM β-mercaptoethanol [12]. Human liver mitochondria were isolated as previously described [13], except that all buffers contained the proteinase inhibitors phenylmethylsulfonyl fluoride (2 mM), ϵ-amino-*n*-caproic acid (5 mM) and *p*-aminobenzamidine·HCl (5 mM).

Yeast mitochondria were isolated essentially as described previously [14] from a wild-type haploid strain J69–1B (*a, ade1 his* [*rho*$^+$]) of *Saccharomyces cerevisiae*.

Preparation of submitochondrial particles. Submitochondrial particles from human placenta, human liver, human heart and bovine heart were prepared by a sonication method [12]. Mitochondria (300 mg) were suspended in a 10 ml (final volume) of 20 mM Tris·HCl buffer (pH 7.5) containing 0.25 M sucrose and 1 mM dithiothreitol with the help of a glass tissue-homogenizer. The suspension was centrifuged at 30000 × *g* for 30 min at 4°C, and the pellet was resuspended in 10 ml of the above buffer. The suspension was then sonicated using a MSE ultrasonic disintegrator at setting 2 for 3 × 1 min with 2 min intervals. After a low-speed centrifugation of the mixture for 15 min at 30000 × *g* at 4°C to remove intact mitochondria, the supernatant was centrifuged at 140000 × *g* in a Beckman 70.1 Ti rotor, for 5 h at 4°C to pellet the submitochondrial particles. The pellet was suspended in 10 ml of 20 mM Tris·HCl buffer (pH 7.5) containing 0.25 M sucrose, 1 mM EDTA and 5 mM β-mercaptoethanol.

Yeast submitochondrial particles were isolated as described by Tzagoloff and Meagher [15].

Isolation of crude F_1-ATPase. F_1-ATPase preparations from human placenta, human liver, human heart, bovine heart and yeast were prepared essentially as described by Beechey et al. [16] and Walker et al. [12]. Submitochondrial particles were suspended at a protein concentration of 30 mg/ml in a 20 mM Tris·HCl buffer (pH 7.5) containing 0.25 M sucrose, 1 mM EDTA and 5 mM β-mercaptoethanol. Analytical-grade neutralized chloroform (0.5 vol) was added and the mixture was shaken vigorously for 10–30 s at room temperature. After breaking the emulsion by low-speed centrifugation in a MSE bench centrifuge at room temperature for 5 min, the aqueous layer was collected and stirred under a stream of nitrogen to remove the residual chloroform. The aqueous layer was further

TABLE 1

Synthetic peptides employed in the production of antibodies to the ATP synthase β-subunit

Synthetic peptide	Sequence	Position (amino acid residue)	Region
1	$[NH_3]$-K-L-A-E-E-H-S-S-[COOH]	475 to 482	C-terminal
2	$[NH_3]$-K-G-V-G-E-R-T-R-E-G-N-D-L-Y-H-E-F-I-[COOH][a]	186 to 202	DCCD binding

[a] An extra lysine has been added at the amino-terminal end to allow the conjugation of this peptide to the immunogenic carries by glutaraldehyde.

centrifuged at 140000 × *g* for 45 min at 20°C. F_1-ATPase was collected from the yellowish supernatant. A modified method of Lowry et al. [17] was used to estimate protein concentration, using bovine serum albumin (BSA) for the construction of the standard curve.

Determination of H^+-ATPase activity. H^+-ATPase activity was assayed by a modification of the method of Pullman [18]. The basic incubation mixture (1 ml) consists of 50 mM Tris-HCl (pH 8.0), 3.3 mM $MgCl_2$, 2 mg antimycin A, 1 mM phospho*enol*pyruvate, 5 units of lactate dehydrogenase, 2.5 units of pyruvate kinase, 1 mM ATP, and 0.3 mM NADH. The reaction was initiated by the addition of 10–50 μl of the sample to be measured. Oxidation of NADH was followed spectrophotometrically at 340 nm at a constant temperature of 28°C. The sensitivity of the H^+-ATPase to oligomycin was measured by the addition of 10 μl of 1 mg/ml oligomycin solution. Enzyme activity was expressed in terms of mmol ATP hydrolyzed/min per mg protein, which is equal to the mmol of NADH oxidized/min per mg enzyme protein.

Enzyme linked immunosorbent assay (ELISA). Antigen (1 to 1000 ng per well in 50 μl phosphate-buffered saline, PBS, containing 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4 and 0.14 M NaCl) was incubated in the wells of a microtitre plate at room temperature overnight. The wells were then washed three times in PBS containing 1 mM $MgCl_2$ and 0.05% (v/v) Tween-20 (washing buffer). After washing, 5% BLOTTO (5 g skim milk in 100 ml PBS, 150 μl per well) was added to block the remaining active sites in the well, followed by an incubation for 1 h at 37°C. After three times washing with the washing buffer, rabbit antiserum (in various dilutions) was pipetted into the wells (50 μl per well) and incubated at 37°C for 1 h. The wells were washed three times, and β-galactosidase-linked donkey anti-rabbit Ig (Amersham International, U.K.) diluted 500-times with washing buffer, was added to the wells (50 μl/well). After 1.5 h of incubation at 37°C, the microtitre plate was washed three times with washing buffer, and 100 μl of a substrate solution containing 4-nitrophenylgalactoside (0.9 mg/ml), 0.1 M $MgCl_2$ and 0.1 M β-mercaptoethanol in PBS was added into each well. After a further 1 h incubation at 37°C, the color developed was read in a Titertek plate reader (Multiskan MCC) at 405 nm.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was carried out in a Bio-Rad Miniprotean II gel apparatus, on 0.75 mm thick 12% slab gels in the presence of 0.1% sodium dodecyl sulphate (SDS; BDH, Poole, U.K.), essentially as described by Laemmli [19] and Studier [20]. Protein samples to be separated electrophoretically (10–15 μg) were solubilized by boiling for 4 min at 100°C, in a 62.5 mM Tris-HCl solubilizing buffer (pH 6.7) containing 2% (w/v) SDS, 10% (v/v) glycerol, 0.00125% (w/v) Bromophenol blue and 5% (v/v) 2-β-mercaptoethanol. Electrophoresis was carried out at 200 volts (constant voltage) until the dye front reached the bottom of the gel. The separated proteins were visualized essentially as described by Merril et al. [21] employing a silver staining method.

Western immunoblotting. Western immunoblotting experiment was performed essentially as described previously [1]. The separated proteins on polyacrylamide gel were electrophoretically transferred onto nitocellulose filters (Schleicher and Schuell, Dassel, Germany) at 60 V for 1 h, in a transfer buffer containing 25 mM Tris, 192 mM glycine and 20% methanol at pH 8.3 [22]. Following the transfer, a strip of filter was stained if required [staining procedure was 10–15 min in 0.1% (w/v) Amido black in the presence of methanol (45%, v/v) and acetic acid (10%, v/v), followed by destaining in a solution containing acetic acid (7.5%, v/v) and methanol (40%, v/v)]. The rest of the filters were incubated in phosphate-buffered saline (PBS; 10 mM NaH_2PO_4 (pH 8.2) and 150 mM NaCl) containing 5% (w/v) Carnation skim milk (5% BLOTTO) for 30 min at 37°C to saturate remaining protein-binding sites. The filters were then incubated in 5% BLOTTO (approx. 250 μl/strip of filter in sealed plastic bag) containing the antipeptide antibodies (1:100 in 5% BLOTTO, v/v) at room temperature for 2 h in a rotating table. The filter were further washed from excess antibodies three times, each for 10 min, with 5% BLOTTO. Antibodies bound to the antigens on the filters were detected with the use of horseradish peroxidase-labelled anti-rabbit Ig, at 1:100 dilution in 5% BLOTTO, by incubation for 1 h at room temperature on a rotating table. After washing three times with 5% BLOTTO, the color development was initiated by the addition of a solution containing 3 mg/ml 4-chloro-1-naphthol in methanol, diluted 6 × in PBS (pH 7.4), and 0.02% (v/v) H_2O_2, and an incubation for 15–30 min in the dark.

Coupling of antibodies to cyanogen-bromide (CNBr)-activated Sepharose 4B. Antipeptide antibodies against the carboxy terminal region of the human ATP synthase β-subunit were coupled to CNBr-activated Sepharose 4B essentially as described previously [1]. Freeze-dried CNBr-activated Sepharose was swollen for 15 min in a solution containing 1 mM HCl. The swollen Sepharose beads were washed with 200 ml of the same solution, followed by washing with the coupling buffer (5 ml) consisted of 0.1 M $NaHCO_3$ and 0.5 M NaCl (pH 8.3). Swollen beads were then suspended in 5 ml of the coupling buffer, and mixed with antibodies solution (5–10 mg protein/ml of beads suspension). After 2 h of gentle mixing at room temperature, the Sepharose 4B beads were washed with the coupling buffer (200 ml). The residual active groups on the Sepharose beads were blocked by the addition of the

coupling buffer solution containing 0.2 M glycine (pH 8.0) and gentle mixing for a further 17 h at room temperature. Finally, the beads were washed alternately with 0.1 M acetate buffer solution (pH 4.0) and the coupling buffer (pH 8.3) to ensure that no unbound antibodies remain attached to the beads. The washing cycle of low and high pH is essential, since protein desorption occurs only when the pH is shifted.

Immunoprecipitation of mitochondrial ATP synthase. Human liver mitochondria were suspended in a 0.02 M Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose and 5% (w/v) of octyl glucopyranoside at a protein concentration of 5 mg/ml, and were held at 4°C for 30 min to solubilize membrane proteins. Protein antigens were immunoprecipitated from the detergent extract with the antipeptide antibodies coupled to CNBr-activated Sepharose 4B as previously described [1]. The immunoprecipitates were washed three times with 0.02 M Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose and 1.25% (w/v) of octyl glucopyranoside. Proteins bound to the conjugated antibodies were released with 50 μl of 0.1 M glycine-HCl (pH 2.5) and immediately adjusted to pH 8 with 1–2 μl of 2 M Tris solution (pH 11). Small aliquots (10–15 μl) were then analyzed by SDS-polyacrylamide gel electrophoresis and a silver staining method [21] was used to visualize the protein bands on the gel.

Immunohistochemistry. Serial dilutions of protein A purified antipeptide antibodies were incubated for 20 min at 20°C with 5 μm tissue cryo-section mounted on glass slides. The sections were washed twice with PBS, incubated with fluorescein isothiocyanate conjugated sheep anti-rabbit immunoglobulin F(ab')$_2$ fragments (Silenus Laboratories, Melbourne, Australia), washed again and mounted for examination by fluorescence microscopy using transmitted illumination with narrow band blue (495 nm) excitation.

Results and Discussion

Description of synthetic peptides

Two peptides were synthesized for the purpose of the present study. The first peptide represents the carboxy terminal of the human β-subunit, and is eight amino acid residues long (Table I). The primary consideration in choosing the carboxy terminal region was that in general the C-terminus of a protein is relatively free to rotate [23], thus likely to be accessible to antibodies. Furthermore, it is expected that the binding of antibodies to this region would result in only a minimal effect on the conformation of the β-subunit.

The second peptide, which is 18 amino acid residues long, corresponds to a region between residues 186 and 202 of the subunit, and includes the two dicyclohexylcarbodiimide (DCCD)-reactive glutamic acid residues which have been suggested to be involved in the catalytic activity of the enzyme complex (Table I). It has been shown that the inactivation of the bovine mitochondrial F_1-ATPase by DCCD is due to the modification of a specific glutamic acid residue in the β-subunit [24,6], and that the inactivation could not occur in the presence of Mg^{2+}. The suggested explanation of this observation is that a glutamic acid residue at the active site of the ATP synthase β-subunit binds Mg^{2+}, and Mg^{2+} complexes of adenosine nucleotides [6]. However, it is also possible that Mg^{2+} affects the reactivity of the glutamic acid side chain with DCCD by binding to an allosteric site where it induces a conformation charge.

Characterization of the antipeptides' antibodies

Two rabbits were immunized with each peptide, conjugated through their NH_2-terminal to keyhole limpet haemocyanine (KLH). When monitored by ELISA, the rabbit sera were found to react with the immunogenic carrier KLH (Fig. 1), as expected. To examine the antipeptide antibodies in the sera, therefore, bovine serum albumin (BSA) peptide conjugates were used; in all cases the anti sera did not show any reactivity to BSA (Fig. 1). Both peptide conjugates were found to induce antibody formation to the peptides, as the antisera reacted with the BSA-peptide conjugates as shown in Fig. 1 The titres of the antibodies against each peptide detected in the immune sera reached their maximum at around 1 : 25 000 to 1 : 30 000 after two booster injections (data not shown).

TABLE II

Comparison of the amino acid sequence of the ATP synthase β-subunit from different organisms

Alignment of the amino acid sequence of the human, bovine, and yeast DCCD binding and C-terminal regions of the ATP synthase β-subunit are shown.

	Amino acid sequence	
	DCCD binding region	C-terminal region
	190 200	471 480
Human	G V G E R T R E G N D L Y H E M I	A K A D K L A E E H S S
Bovine	. . . . .	. . . . .
Yeast	. . . . .	R . . E . . . R . A N

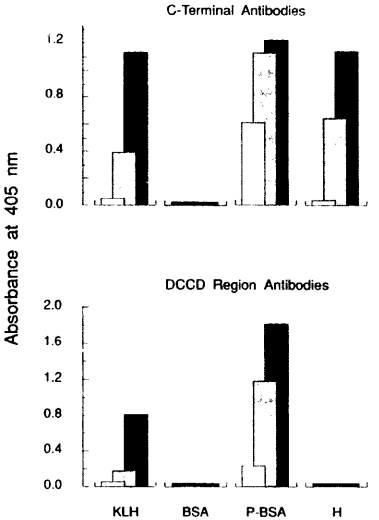

Fig. 1. Characterization of the antipeptide antibodies to the ATP synthase β-subunit by ELISA. The assay was carried out as described in Materials and Methods. Antibodies were incubated with various concentrations of microtitre plate-bound antigen, and binding of the antibody was detected with β-galactosidase-linked donkey anti-rabbit Ig. Antigens used were keyhole limpet haemocyanine (KLH), bovine serum albumin (BSA), peptide coupled to BSA (P-BSA) and human mitochondrial F_1-ATPase (H), at 1 μg (□), 10 μg (▨) and 100 μg (■) per well.

The reactivity of the antipeptide antibodies to the β-subunit of the ATP synthase was examined with an F_1-ATPase preparation from human placental mitochondria. Direct ELISA results, using this F_1-ATPase preparation as an antigen, showed that C-terminal antipeptide antibodies reacted with the enzyme complex, presumably with its β-subunit (Fig. 1). In contrast, the antibodies to the DCCD binding region were found to be not reactive in this experiment.

The reactivity of the antipeptide antibodies to the β-subunit was verified by Western immunoblotting against the human F_1-ATPase. Only one band, corresponding to the ATP synthase β-subunit (51 kDa), was observed for both antibodies (Fig. 2). Similar Western immunoblotting experiments were carried out using the total human mitochondrial protein and octyl glucoside extract of the human mitochondrial membrane. In both cases, one band corresponding to a 51 kDa protein could be consistently demonstrated (Fig. 2) with the two antipeptide antibodies. No significant cross-reactivity to other mitochondrial proteins could be observed, except for the antibodies to the DCCD binding region when tested against the total mitochondrial proteins.

Species specificity of antipeptide antibodies

As shown in Table II, the human ATP synthase β-subunit shares some homology in their amino acid sequence, both at the carboxy terminal and at the residues 186 to 202 region, with that of bovine and yeast. It is of interest to determine the species speci-

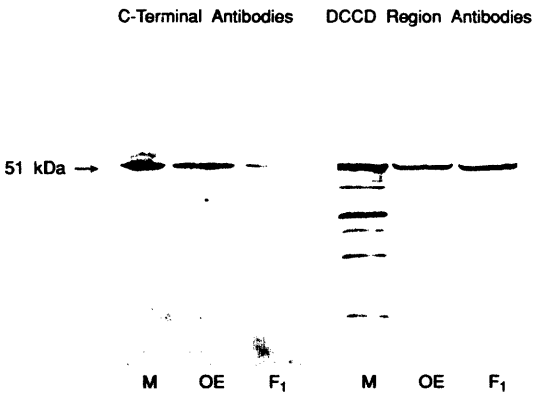

Fig. 2. The reactivity of antipeptide antibodies to the human F_1-ATPase β-subunit in Western immunoblotting. Total human mitochondrial proteins [M], octylglucoside extract of human mitochondrial membrane [OE], and a human F_1-ATPase preparation (chloroform extract) [F_1] were separated by electrophoresis in 12% SDS-polyacrylamide gel, electrophoretically transferred onto nitrocellulose filter, and immunoblotted with antipeptide antibodies. In all cases, a band corresponding to a 51 kDa protein could be consistently demonstrated with the two antipeptide antibodies.

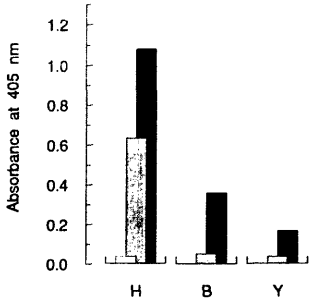

Fig. 3. Species specificity of antipeptide antibodies by ELISA. The assay was carried out as described in Fig. 2. Antibodies to the C-terminal region was examined against human F_1-ATPase (H), bovine F_1-ATPase (B), and yeast F_1-ATPase (Y) as antigens, bound to the microtitre plates. The concentration of antigens were 1 μg (□), 10 μg (▨) and 100 μg (■) per well.

ficity of the antipeptide antibodies, as the pattern of the cross-reactivity might provide information with regard to the epitopes recognized by the antibodies. For this purpose, the reactivity of the antipeptide antibodies was examined by ELISA. Since the antibodies to the DCCD binding region are not reactive under ELISA, only the antibodies to the C-terminal region were tested for this purpose. These antibodies were found to cross-react with the bovine F_1-ATPase, but at a significantly less intensity compared to their reactivity to the human enzyme (Fig. 3). The antibodies reacted only weakly with the yeast F_1-ATPase. Western immunoblotting experiment against human, bovine and yeast mitochondria confirmed the ELISA results (data not shown). The above observation could be perhaps explained on the basis of the homology of the amino acid sequence of the human, bovine and yeast ATP synthase β-subunit at the C-terminus. The human ATP synthase β-subunit [5] shows a complete amino acid homology in the DCCD binding region with that of bovine [12] and yeast [30]. Similarly, complete sequence homology was observed at the C-terminal region between the human and bovine ATP synthase β-subunit, except for one serine residue which is absent in bovine. The homology of the human and the yeast C-terminal ATP synthase β-subunit, however, is low.

Immunoprecipitation of human mitochondrial ATP synthase

The antipeptide antibodies have been used to immunoprecipitate the human ATP synthase complex in a solid phase system [see Material and Methods for experimental details]. When the immunoprecipitated complex obtained with the antibodies to the C-terminal was analysed by electrophoresis on a 12% SDS-polyacrylamide gel, 14 protein bands were observed (Fig. 4). At least five of these were observed also when a normal non-immune human Ig fraction was used instead of the antibodies, indicating that these proteins were non-specifically associated to the Sepharose 4B beads used in the immunoprecipitation, despite of the pre-absorption of the antisera with the beads prior to immunoprecipitation. Two major bands of 53 and 51 kDa could be identified as the α- and the β-subunits of the F_1 sector of the enzyme complex. The other two subunits of the F_1 sector, γ and δ, appeared as bands with the mobility of 31 kDa and a 14 kDa protein, respectively. Three other bands corresponding to 29, 24 and 18 kDa proteins were observed. One of these bands (24 kDa) was tentatively identified as that of the mitochondrially synthesized subunit 6 of the F_o-sector. The other two, are presumably the equivalent of the P25 and P18 subunits of the yeast ATP synthase [1,4], which have been also reported to be present in the bovine heart complex [25]. The electrophoretic system employed did not resolve the ϵ-subunit of the F_1-sector and subunits 8 and 9 of the F_o-sector at the bottom of the gel. The other polypeptides which were observed on the gel were probably aggregation or breakdown products of the α and β-subunit, as described previously [1].

The antibodies to the DCCD binding region did not appear to immunoprecipitate the ATP synthase complex. SDS-polyacrylamide gel electrophoresis analysis

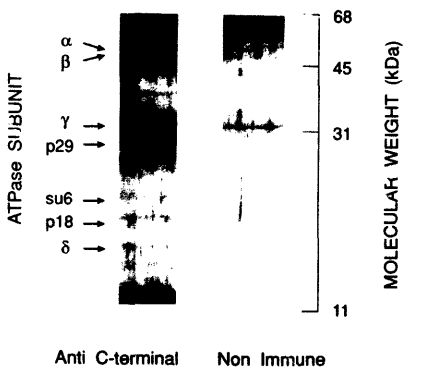

Fig. 4. Immunoprecipitation of human mitochondrial ATP synthase. Human mitochondria were isolated as in Materials and Methods, and membrane proteins were solubilized from the mitochondria with octyl glucopyranoside (5%). Protein antigens were then immunoprecipitated from the detergent extract by using the antipeptide antibodies to the carboxy terminal of the human ATP synthase β-subunit [Anti C-terminal] and a normal non-immune serum [Non Immune] in a solid phase system. Immunoprecipitated proteins were separated by electrophoresis on 12% SDS-polyacrylamide gels, and were visualized by silver staining.

of the fraction obtained with these antibodies by immunoprecipitation did not even show the β-subunit (data not shown). Interestingly, however, the Western immunoblotting results clearly showed that these antibodies recognized the β-subunit of the ATP synthase (Fig. 2), and not only of human but also of a range of other organisms, including yeast (data not shown). It appears, that the epitopes recognized by these antibodies are hidden in the interior of the F_1-ATPase, and become exposed under the membrane solubilization and electrophoretic conditions employed in the Western immunoblotting experiment.

Reactivity of the antipeptide antibodies in immunohistochemical experiment

One of the potential use of the antibodies described in the present study is in the immunohistochemical examination of the ATP synthase in the skeletal muscle and other tissue materials, in patients with various mitochondrial respiratory chain disorders. The ability of the antipeptide antibodies to the C-terminal region to react with the ATP synthase under histochemical conditions, therefore, has been examined. Mouse liver, muscle and stomach sections were used for this purpose.

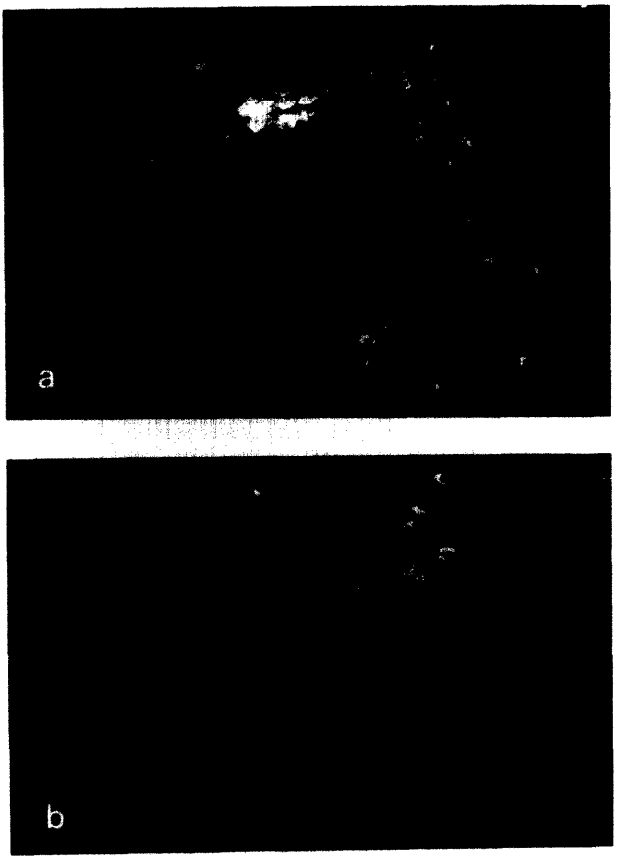

Fig. 5. Reactivity of the antipeptide antibodies in immunohistochemical experiment. Results shown are for the mouse stomach. (a) The immunofluorescence pattern observed with the antimitochondrial autoantibodies in the serum of a patient with primary biliary cirrhosis. (b) The immunofluorescence pattern observed for the C-terminal antipeptide antibodies.

In all cases the antibodies were found to react positively by immunofluorescence. The immunofluorescence pattern was similar to that observed when antimitochondrial auto-antibodies from a patient with primary biliary cirrhosis were used, as shown in Fig. 5 for a section from a mouse stomach. Primary biliary cirrhosis is an autoimmune disease affecting primarily the intrahepatic biliary ductular, and characterized by the finding of auto-antibodies to mitochondrial antigens in serum of patients [26,27]. The autoantigens have recently been identified as the E2 components of the mitochondrial 2-oxoacid dehydrogenase complexes, in particular the E2 of pyruvate dehydrogenase complex I [28,29].

Concluding remarks

The production of the antipeptide antibodies to the human ATP synthase subunit represents one of our research activities, aimed to elucidate the processes involved in the assembly of the mitochondrial energy transducing membrane in humans. These antibodies would be useful for the investigation of the structure and function of the human ATP synthase complex in normal and disease situations. As an example, the antipeptide antibodies to the C-terminal region of ATP synthase β-subunit have been used in a study to investigate the association of the autoantigens of primary biliary cirrhosis, an autoimmune disease characterized by the presence in sera of patients of autoantibodies to mitochondrial inner membrane proteins, with the ATP synthase. The important issue of whether any of the PBC reactive polypeptides are associated with the mitochondrial ATP synthase has been thus resolved [31].

The antipeptide antibodies against the C-terminal region of the ATP synthase have been also used now in the histochemical examination of this enzyme complex in the skeletal muscle of patients with mitochondrial disorders, in particular those with chronic progressive external ophthalmoplegia (CPEO) [32]. This particular type of mitochondrial disorder has been shown to be associated with the presence of partially deleted mtDNA, which coexists in tissues in a heteroplasmic manner with the normal mtDNA population.

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