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Antimitochondrial Antibodies (AMA) in Primary Biliary Cirrhosis. I. Separation of the PBC Antigen Activity from Mitochondrial ATPase Activity[1]

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Abstract

Antimitochondrial antibodies are found in a variety of autoimmune liver diseases, particularly primary biliary cirrhosis. The antigen against which these antibodies are directed is localized on the inner mitochondrial membrane. Earlier work suggested that this antigen was associated with the mitochondrial ATPase. However, we have succeeded in separating the enzyme activity from the antigenic activity using gel filtration and ion-exchange chromatography. Furthermore, the antigenic activity is not affected by modulators of ATPase enzymatic activity like aurovertin or oligomycin. The antigenic activity is, however, very susceptible to reagents which block thiol groups. The mitochondrial antigen, in contrast to the ATPase enzyme, is found in high amounts in brown fat mitochondria. Identification of this antigen may help to explain why specific antimitochondrial antibodies arise in the sera of patients with primary biliary cirrhosis.

Key Words: Antimitochondrial antibodies (AMA); mitochondrial antigen; mitochondrial ATPase; primary biliary cirrhosis; brown fat mitochondria; autoimmune liver diseases; inner mitochondrial membrane.

Introduction

Separation of the PBC Antigen Activity from F_1-ATPase

Initial investigations of the antimitochondrial antibodies (AMA) in primary biliary cirrhosis (PBC) and other related liver diseases (Berg *et al.*, 1967) localized the antigen to the inner mitochondrial membrane—a finding that

[1]Abbreviations: ATPase, adenosine triphosphatase; PBC, primary biliary cirrhosis; AMA, antimitochondrial antibodies; SMPs, submitochondrial particles; CFT, complement fixation test; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; BAT, brown adipose tissue.

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was confirmed by the electron microscopic studies of Bianchi *et al.* (1976). More recently it has become apparent that there is considerable heterogeneity of antimitochondrial antibodies in liver diseases (Berg and Binder, 1976); however, the majority of sera from patients with PBC or AMA-positive choleostatic chronic active hepatitis ("mixed forms") possess antibodies directed against what is apparently a single common antigen (Sayers *et al.*, 1980; Meek *et al.*, 1980; Berg and Baum, 1980). Although it is quite likely that even sera from "classical" PBC contain antibodies directed at other antigens, the evidence for a single major antigen in these cases is strong, and it is with this antigen that the present communication is concerned.

Using affinity chromatography, Ben-Yoseph *et al.* (1974) substantially purified this antigen. However, its role in the mitochondrion and its identity remained unknown. Furthermore, its mode of preparation, its insolubility (except in the presence of high concentrations of urea), and its apparent association with lipid make it difficult to compare it with the fractions isolated in the present work.

AMA when incubated with mitochondria or submitochondrial particles (SMPs) failed to have any effect on respiration or oxidative phosphorylation (Berg *et al.*, 1969; Sayers, 1977). Although mitochondrial ATPase activity was not inhibited by these sera, it was noted that preparations of chloroform-released ATPase from beef heart mitochondria, which were relatively pure as assessed by the peptide pattern on SDS gel electrophoresis, were very antigenic in the complement fixation test (CFT) (Sayers and Baum, 1976b). Furthermore, when this ATPase preparation was subjected to electrophoresis on 5% polyacrylamide gels, the majority of the antigenicity comigrated with the ATPase band, as demonstrated by subsequent elution from gel slices and testing by CFT against standard PBC sera. This finding led to the proposal that the PBC antigen might be a subunit of mitochondrial F_1-ATPase (Baum *et al.*, 1979). However, it was recognized that a major weakness in this argument was the fact that brown fat mitochondria possessing not only a low ATPase enzyme activity but also low absolute amounts of the enzyme (Cannon and Vogel, 1977) contained high concentrations of the PBC antigen assessed by quantitative CFT. In order therefore to establish what relationship exists between the mitochondrial ATPase and the major antigen reacting with PBC sera, we have extended studies on the nature of this antigen, and report here evidence that it is neither a catalytic nor an essential structural constituent of the detachable sector of the membrane ATPase.

Materials and Methods

Submitochondrial particles were prepared from beef heart mitochondria according to the method of Hansen and Smith (1964).

Mitochondrial ATPase was isolated by the chloroform method of Beechey *et al.* (1975).

The ATPase enzyme activity was estimated by a procedure described by Pullmann *et al.* (1960), and the *antigenic activity* by a microtiter complement fixation test (CFT) of Roitt and Doniach (1969). Fractions were tested for complement fixing activity with high-titer PBC marker sera obtained from either the Medizinische Klinik, Tübingen, or from Dr. S. Hadjiyannis, Hippokration Hospital, Athens. Data are presented as reciprocal CFT titers. An assessment of specific antigenic activity in the CFT was performed by titrating descending protein concentrations of a fraction against a standard by high titer PBC serum.

SDC gel electrophoresis was carried out in 10% gels according to the method of Weber and Osborne (1969). BSA, trypsin, trypsin inhibitor (ovomucoid), α-amylase, and cytochrome *c* were used as molecular-weight standards. Protein concentrations were determined either by the Lowry method (Lowry *et al.*, 1951) or by the dye-binding method of Bradford (1976).

Treatment of SMPs with Reagents Affecting Enzymatic Activity

Aurovertin. SMPs were incubated with aurovertin at a concentration of 60 nmol aurovertin/mg of protein. Incubation was carried out for 10 min at room temperature. The SMPs were then diluted in a large volume of buffer and pelleted at 100,000 *g* for 30 min. The pellet was resuspended, washed twice in buffer, then compared to control SMPs which had undergone the same procedures apart from the addition of the inhibitors. ATPase and antigenic activities of control and treated SMPs were then estimated.

Oligomycin. SMPs were incubated with oligomycin at a concentration of 3 μg oligomycin/mg of protein. Re-isolation, washing, and testing for antigenicity were performed as described above.

Isolation of ATPase from Mercurial-Treated SMPs

SMPs were incubated with mersalyl acid at 1 and 0.1 μmol of mersalyl/mg protein respectively. After incubation for 20 min the SMPs were washed and re-isolated. The chloroform treatment was applied to the SMPs from these and control incubations. The fractions obtained were tested for ATPase and antigenic activities.

Further Purification of ATPase Prepared by the Chloroform Extraction Method

Ion-Exchange Chromatography. The ATPase fraction obtained from SMPs using the chloroform procedure was further purified by the ion-

exchange chromatography method of Tuena de Gomez-Puyou and Gomez-Puyou (1977). The extracted enzyme was dialyzed overnight against a large volume of 2 mM ATP, 2 mM EDTA, and 0.15 M sucrose, pH 7.3. Then about 13 ml of this solution (0.5 mg/ml) was applied to a column of A-H Sepharose (1 × 15 cm). The solution containing ATPase was applied at a rate of approximately 0.5 ml/min. After the chloroform-released ATPase had been absorbed onto the column, buffer was passed through until no protein was detected in the eluate. After this a stepwise gradient was constructed with 0.1, 0.3, and 1 M KCl introduced onto the column respectively when no more protein could be detected in the eluate. The various protein peaks were dialyzed overnight against 20 mM Tris-SO_4 buffer, pH 7.4, then concentrated on an Amicon filter with a molecular weight cutoff of 5000. All protein peaks were tested for ATPase and antigenic activity.

Gel Filtration. Chloroform-released ATPase was purified according to a procedure described by Houstek *et al.* (1977). A 6-ml portion of the enzyme (4 mg/ml) was applied to a Sepharose 6B column (1.5 × 80 cm). All peaks obtained were concentrated on an Amicon filter with a molecular weight cutoff of 5000 and were tested for ATPase activity. The same fractions were dialyzed against CFT buffer and were tested for antigenic activity in CFT.

Brown Fat SMPs

Brown fat mitochondria were prepared from newborn rabbit tissue based on the method of Cannon and Lindberg (1979); SMPs prepared as previously described were subjected to the chloroform treatment. The extract obtained was tested for both antigenic and ATPase activity and further separated by ion-exchange chromatography and gel filtration respectively. Fractions obtained from the column were tested for antigenicity.

Results

Effects of Inhibitors

Aurovertin and oligomycin at concentrations which strongly inhibited the ATPase activity of SMPs had no effect on the antigenicity of these particles (Table I), suggesting that the antigenic site was neither associated with the enzymatic site of the ATPase nor the aurovertin or oligomycin binding sites. This is consistent with the finding (Leoutsakos and Baum, unpublished observations) that the chloroform-released ATPase regains oligomycin sensitivity on incubation with ATPase-depleted membranes whether or not either or both fractions are pre-incubated with an excess of PBC sera. On the other hand, mersalyl acid did affect antigenicity in SMPs, and also in

Table I. Effect of Oligomycin and Aurovertin on Enzymatic and Antigenic Activity of SMPs

	ATPase activity (μmol/min/mg protein)	Antigenic activity[a]
SMPs	3.1	1000
Oligomycin-treated SMPs (3 μg oligomycin/mg protein)	0.7	1000
Aurovertin-treated SMPs (60 nmoles aurovertin/mg protein)	1.24	1000

[a]For assessment of antigenic activity 100 μ/ml of SMP protein was tested against a standard high-titer PBC sera in the CFT. Reciprocal CFT titers are given.

Table II. Effect of Mersalyl on the Enzymatic and Antigenic Activity of Chloroform-released ATPase

Mersalyl concentration[a] (μmol/mg protein)	ATPase activity (μmol/min/mg protein)	Antigenic activity (reciprocal CFT titers)[b]
0	3.6	1000
0.1	3.6	−ve
1	0.21	−ve

[a]SMPs were incubated at the concentration shown and the ATPase then isolated and assayed as described in the Materials and Methods section.
[b]100μg/ml each chloroform extract was tested in the CFT against a standard high-titer PBC sera.

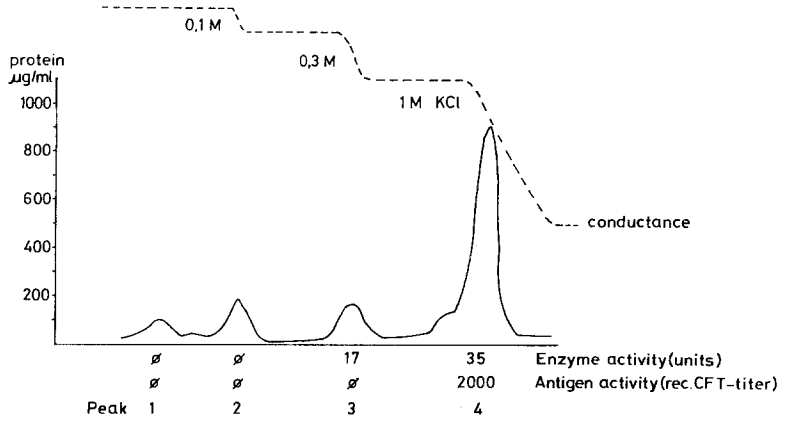

Fig. 1. Further purification of the chloroform-released ATPase by ion-exchange chromatography. The original chloroform extract had an ATPase specific activity of 5 units, and optimal complement fixation (titer 2000) with a standard marker serum could still be obtained at a protein concentration of 75 μg/ml. Peaks 1, 2, and 3 showed no antigenic activity at 75 μg/ml whereas only 25 μg/ml of peak 4 protein gave good fixation.

the chloroform-extracted ATPase (Table II). As can be seen from Table II the antigenicity of the chloroform-released preparation is more susceptible to the mercurial than is the enzymatic activity. At 0.1 μmol mersalyl/mg of protein, the chloroform-extracted ATPase preparation has the same enzymatic activity as the control, while the antigenicity has been abolished.

Further Purification of the ATPase by Ion-Exchange Chromatography

The chloroform-released preparation could be resolved into several peaks by ion-exchange chromatography (Fig. 1). It can be seen from Fig. 1

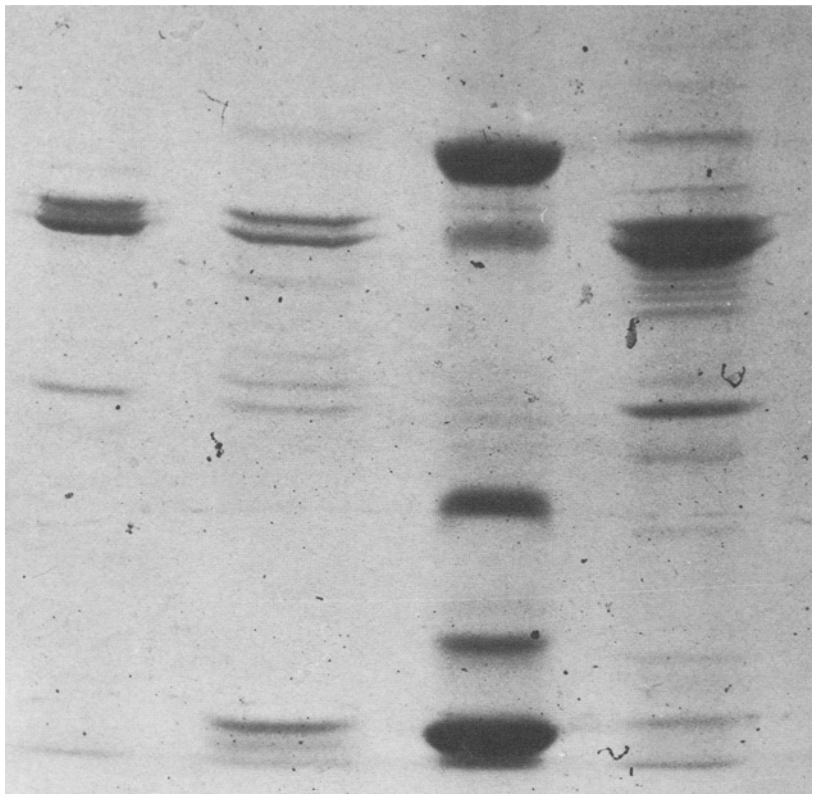

Fig. 2. SDS polyacrylamide electrophoresis of fractions obtained from ion-exchange chromatography of the chloroform ATPase. Fractions were run on the gel at 30 μg protein. (1) Fraction eluted by 1 M KCl (peak 4); (2) fraction eluted by 0.3 M KCl (peak 3); (3) molecular-weight-marker bovine serum albumin (64,000), α-amylase (56,000), trypsin inhibitor protein (28,000), trypsin (23,800), and cytochrome *C* (13,000); (4) original chloroform-released ATPase.

that in peak 3 (eluted by 0.3 M KCl) some ATPase activity is detectable. However, there is no antigenicity. The major protein peak contains both enzymatic and antigenic activity.

SDS polyacrylamide gel electrophoresis (Fig. 2) was performed in order to compare the constituent proteins of the original chloroform ATPase preparation with peaks 3 and 4. A total of 30 μg of protein was applied to the gel in each case.

The original extract contains bands which could tentatively be assigned to the major subunits of the ATPase molecule, but precise assignment of the minor subunits is rendered difficult by the various contaminants present in the preparation. Peak 3 also shows contaminants, although it is no longer antigenic. On the other hand, the highly antigenic peak 4 appears to show definite bands for α, β, and λ and seems to be a relatively pure preparation, although some protein is apparent above and between the α and β bands. The increase in specific activity of ATPase following column chromatography is a common finding, probably related to removal of the inhibitor protein.

Purification by Gel Filtration

The original chloroform-released ATPase, which had a specific activity of 4.8 units, could be resolved into a major and minor protein peak by gel

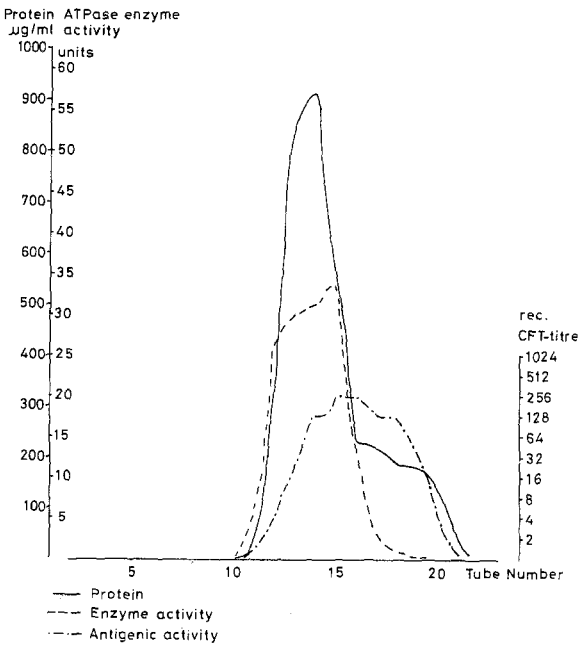

Fig. 3. Dissociation of antigenic and ATPase activity of the chloroform-released ATPase after gel filtration on Sepharose 6B. 6-ml fractions were collected.

filtration (Fig. 3). It can be seen that ATPase activity is confined to the major peak while antigenic activity is present in both major and minor peaks. It was noted that the specific antigenic activity of fractions eluted from the column was lower than that of the original extract so 1 M NaCl was passed down the column. The small amount of protein subsequently eluted had a very high antigenic activity. This suggests either that the PBC antigen nonspecifically binds to Sepharose or that the antigen might dissociate into protomers of very low molecular weight that are not eluted from the column until well after the ATPase.

SDS polyacrylamide gel electrophoresis was carried out using protein

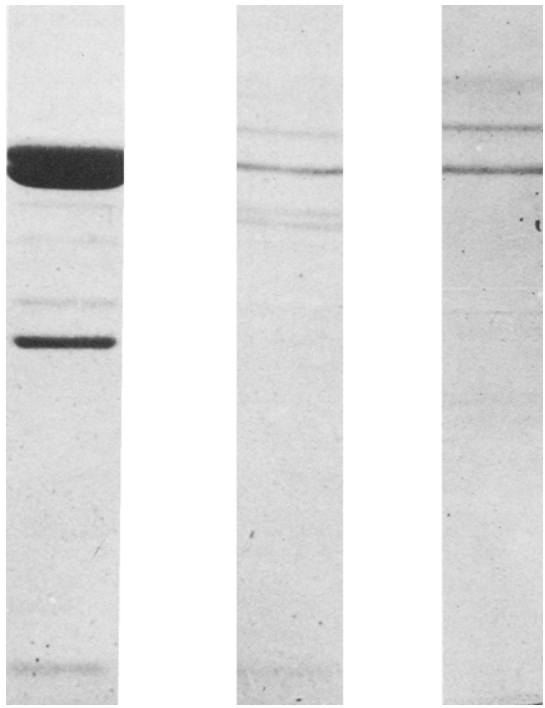

Fig. 4. SDS polyacrylamide gel electrophoresis of fractions obtained from gel filtration of the chloroform-released ATPase on Sepharose 6B. (1) Fraction 13 (high enzyme and antigenic activity); (2) Fraction 17 (low enzyme and high antigenic activity); (3) PBC antigen purified by column chromatography (Sepharose 6B) of a chloroform extract from brown fat SMPs.

fractions from gel filtration which exhibited ATPase activity and antigenicity or just antigenicity (Fig. 4; gels 1 and 2). It can be seen from Fig. 4 that fraction 13 from the major peak shows bands which could be assigned to the α, β, and γ subunits, while fraction 17 does not appear to contain any enzyme constituents. However, there is a band which lies somewhere between the α and the β subunits. This band has also been observed in various other fractions lacking enzyme activity but exhibiting very high antigenic activity (Leoutsakos and Lindenborn-Fortinos, unpublished data).

Antigen from BAT Mitochondria

The chloroform extract from brown fat SMPs possessed very low ATPase activity (0.17 units), although on a protein basis its antigenic activity was four times greater than that of beef heart SMPs. The behavior of the chloroform-extracted antigen on ion-exchange chromatography is shown in Fig. 5. Once again the antigenic activity elutes at high salt concentrations using ion-exchange chromatography. In contrast to the extract from beef heart, most of the protein elutes at lower concentrations. Consequently this method allows the preparation of an antigenic fraction of high specific activity.

The chloroform extract from brown fat SMPs was also passed down a Sepharose 6B column and the fractions were tested for antigenic activity (Fig. 6). Antigenic activity was found between the two major protein peaks.

SDS electrophoresis was performed with this antigenic fraction and the

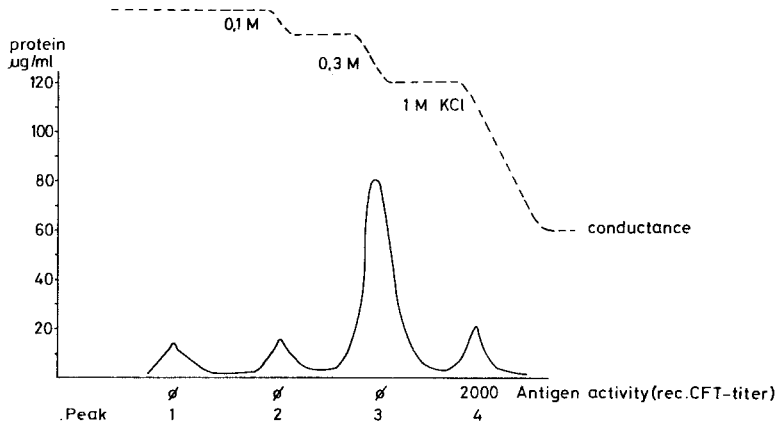

Fig. 5. Purification of the PBC antigen by ion-exchange chromatography of a chloroform extract from brown fat submitochondrial particles. The chloroform extract possessed no detectable ATPase activity but gave good complement fixation with a standard marker serum at 25 µg protein/ml. Peaks 1, 2, and 3 showed no antigenic activity at this concentration whereas only 5 µg/ml of peak 4 protein gave good fixation.

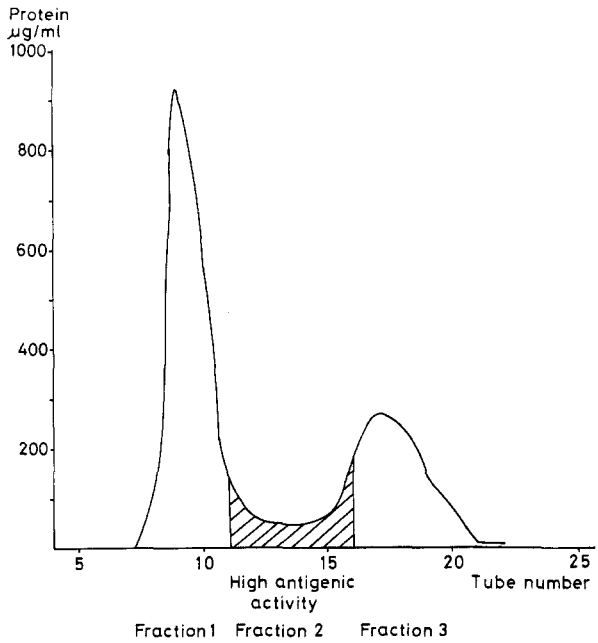

Fig. 6. Purification of the PBC antigen by gel chromatography of a chloroform extract from brown fat submitochondrial particles. 6-ml fractions were collected, and the antigenic activity in each was titrated. Tubes 11–16 still gave optimal complement fixation at 5 μg protein/ml whereas all other tubes were negative at 25 μg/ml.

chloroform-released ATPase from bovine heart SMPs for comparison (Fig. 4; gel 3). Once again bands were observed either above or between the α and β subunits.

Discussion

It seems evident from the results presented here that the mitochondrial ATPase enzymatic activity can be dissociated from the PBC antigenic activity. Aurovertin and oligomycin appear to have no effect on the antigenic activity of submitochondrial particles. Mersalyl acid affects both antigenicity and enzyme activity. However, these effects can be discriminated at low mersalyl concentrations. Ion-exchange chromatography and gel filtration allow the separation of enzymatic from antigenic activity. Also the extract from brown fat mitochondria possesses a low enzymatic but high antigenic activity. However, it is known that only three subunits, α, β, and γ, are

required for TF_1-ATPase activity (Kagawa *et al.*, 1979). Nonetheless, SDS gel electrophoresis of the fractions obtained from the described separation procedure suggests that none of the F_1-ATPase subunits play a role in the PBC antigen. This conclusion is confirmed by the fact that the F_1-ATPase prepared according to the method of Knowles and Penefsky (1972) is not antigenic, although it clearly showed all five subunits of the F_1-ATPase after polyacrylamide gel electrophoresis (Leoutsakos *et al.*, 1979).

Since the antigen co-purifies with the ATPase (it is only removed in the last step of the Knowles and Penefsky preparation; Leoutsakos and Baum, unpublished observations), one possibility is that the antigen has very similar physicochemical properties to the F_1-ATPase. Consequently procedures which lead to the removal of the F_1-ATPase like chaotropic agents or chloroform treatment are also capable of removing the PBC antigen. Discrimination is further complicated by the fact that the antigen elutes from either ion-exchange or gel chromatography columns together with the ATPase. Furthermore, a probable component (or aggregate form) of the antigen migrates on polyacrylamide gels between the α and β subunits of the ATPase. Other bands were observed in the polyacrylamide gel electrophoresis of purified antigen preparations from beef heart and brown fat SMPs, but this was the only band which appeared in all highly antigenic fractions. It is incidentally noteworthy that, in our experience, the chloroform-released ATPase (antigenic) always manifests a "fuzziness" between α and β bands on SDS polyacrylamide gel electrophoresis, whereas the separation between these bands is clear in the case of the Knowles and Penefsky preparation.

Attempts have been made to estimate the molecular weight of the PBC antigen on Sephadex G150, but it always appeared in the void volume. Presumably strong dissociating conditions are required to break up the complexes formed by the antigen.

Physicochemical similarity might not be the sole reason for co-purification of the antigen with the ATPase. It is possible that there is a specific association between the two species, since the antigen can be eluted from the major ATPase band of 5% polyacrylamide disc gels (Baum *et al.*, 1979). Whether this association is due to protein–protein interactions or has some functional significance remains unclear. It is of interest to note that the antigen seems to bind nonspecifically to Sepharose columns. Since column chromatography is often used in the preparation and purification of F_1-ATPase it is possible that the antigen is at first associated but is removed during the subsequent purification procedure. A pure F_1-ATPase preparation generously provided by Dr. D. A. Harris and purified without use of column chromatography methods contained both high PBC antigenic activity and high levels of reactive sulfhydryl groups.

A possible identity between the antigen and coupling factor B has

already been considered (Leoutsakos *et al.*, 1979) and is being further investigated. However, a difficulty encountered in this and all related studies with this antigen is that it appears to have only a very limited number of antigenic determinants, so that it exhibits "prozone" effects on purification, and its quantitative antigenicity is apparently dependent on its state of aggregation.

The sensitivity of the antigen to mercurials is of interest not only by analogy to the mercurial sensitivity of factor B but also because specific thiol groups appear to play an important role in inner membrane processes. For example, various substrates are known to affect the number of exposed titratable thiol groups (Hatase *et al.*, 1977; Harris *et al.*, 1979), which may help to explain earlier data which showed an effect of substrates and hormones on antigenic activity (Berg, unpublished observations).

The antibodies of this specificity occur in the sera of over 90% of patients with PBC and persist throughout the course of the disease. They are known to play no role in the pathogenesis of the disease (Doniach and Walker, 1969) and do not appear in the large immune complexes found in the sera of many of these patients (Wands *et al.*, 1978). However, primitive eukaryotes such as yeast, fungi, and parasites possess mitochondria, some of which we have already shown to react with PBC sera (Leoutsakos and Sayers, unpublished data). Further, certain bacteria contain many components in common with those of mammalian mitochondria (Sayers and Baum, 1976a). It is therefore possible that antimitochondrial antibodies arise due to an immunological cross reaction. Thus identification and characterization of this antigen may provide a clue to the etiology of PBC and other related liver diseases.

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