Adenosine Triphosphatase from Rat Liver Mitochondria

I. PURIFICATION, HOMOGENEITY, AND PHYSICAL PROPERTIES*

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SUMMARY

1. The adenosine triphosphatase of rat liver mitochondria is tightly bound to the inner membrane and is solubilized in very poor yield (less than 0.5%) by sonic oscillation procedures alone. Solubilization of the enzyme in good yield (30 to 55%) is obtained by sonic oscillation provided the membranes are first washed exhaustively in low ionic strength buffer and incubated for 10 hours in the presence of ATP and ethylene glycol. Purification of the solubilized enzyme by sequential chromatography on DEAE-cellulose and Sephadex G-200 consistently yields preparations exhibiting a specific activity of approximately 22 μmoles of ATP hydrolyzed per min per mg.

2. The purified enzyme is homogeneous as assessed by gel filtration, sedimentation in sucrose gradients, and sedimentation in the analytical ultracentrifuge. The molecular weight of the ATPase is 384,000 based on determinations of its sedimentation coefficient (s20,w = 12.15), diffusion coefficient (D20,w = 2.96 × 10⁻⁶ cm² sec⁻¹), and partial specific volume (ρ = 0.740 cm³ g⁻¹).

3. Electrophoresis in two dissociating solvent systems indicates that the enzyme contains three classes of subunits. The molecular weights of these three subunits determined by their electrophoretic mobilities in the sodium dodecyl sulfate-mercaptoethanol system are: subunit A, 62,500; subunit B, 57,000; and subunit C, 36,000.

4. The amino acid composition of the purified ATPase from rat liver mitochondria is strikingly similar to amino acid compositions reported for ATPases from such diverse sources as bovine heart mitochondria, spinach chloroplasts, and bacteria. The similarity in chemical and physical properties among these enzymes is discussed in terms of their possibly analogous structural and functional roles in these membrane systems.

Mitochondrial adenosine triphosphatases (ATP phosphohydrolase, EC 3.6.1.3) have been isolated from bovine heart (1).

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retains acceptor control of respiration have presented strong evidence that the ADP-ATP exchange is in fact catalyzed by mitochondrial ATPase in intact membrane systems (24).

It is our goal to elucidate the structural properties and catalytic mechanisms which are important for the participation of mitochondrial membrane systems described above. We have chosen the rat liver mitochondrial membrane system for this study because the physiological and biochemical properties of the mitochondria and component membranes (24, 25) have been extensively studied, and purified components are available. As an initial step in this study, we report here the purification to homogeneity and some physical properties of the membrane ATPase of rat liver mitochondria.

**EXPERIMENTAL PROCEDURE**

**Materials**

Sephadex G-200 was purchased from Pharmacia and prepared for use according to their instruction manual. DEAE-cellulose (Cellex-D), Bio-Rad, 0.7 meq per g) was prepared for use as described by Peterson and Sober (26). SDS and urea were obtained from Sigma and Mallinkrodt Chemical Works, respectively, and used without further purification. ATP and DPNH were obtained from P-L Biochemicals. Phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase were obtained from Sigma. All other chemicals were of reagent grade purity.

The following proteins were used as standards for gel filtration and gel electrophoresis: bovine serum albumin from Pentex; bovine liver catalase, rabbit muscle pyruvate kinase, bovine heart lactic dehydrogenase, rabbit muscle pyruvate kinase, bovine heart lactic dehydrogenase, rabbit muscle glyceraldehyde 3-phosphate dehydrogenase, jack bean urease, and horse heart cytochrome c from Sigma; myoglobin from Mann; and chymotrypsinogen from Worthington Biochemicals. Hemoglobin was prepared from freshly drawn human blood by the method of Ingram (27).

**Methods**

**Isolation of Mitochondria**—Rat liver mitochondria were isolated as described by Schneider and Hogeboom (28) and stored as frozen sediments until use.

**ATP Assay**—ATPase activity was measured spectrophotometrically at 340 nm by coupling the production of ADP to the oxidation of DPNH via the pyruvate kinase and lactate dehydrogenase reactions essentially as described by Pullman et al. (1). The mixture contained in a final volume of 1 ml at pH 7.5, 65 mM Tris·Cl, 300 mM of sucrose, 4.75 mM of MgCl₂, 4.0 mM of ATP, 0.40 mM of DPNH, 0.60 mM of phosphoenolpyruvate, 5.0 mM of KCN, 1 unit of lactic dehydrogenase, and 1 unit of pyruvate kinase. One unit of ATPase activity is defined as the amount of enzyme which hydrolyzes 1 pmole of ATP per min under these conditions.

**Determination of Stokes Radius by Gel Filtration**—The method used was that of Ackers (29). Protein samples (100 μl, 1.5 to 2.5 mg per ml) in Buffer 1 (200 mM potassium phosphate, 5.0 mM EDTA, pH 7.5) were layered on a Sephadex G-200 column (0.7 × 50 cm) equilibrated with the same buffer. Elution was carried out at room temperature (23-25°) at a flow rate of 2.0 ml per hour maintained by a pump. Sixty fractions containing 0.4 ml were routinely collected. Elution position was determined by measurement of protein, and where appropriate, by measurement of enzymatic activity. Dextran blue (Pharmacia) and 2,4-dinitrophenol were used as exclusion and inclusion volume markers, respectively. Values of the distribution coefficients (Ve - Vd) / Vd, determined for the proteins of known Stokes radius (a) were used to calculate a value for the mean pore radius (r) of the column from the molecular sieve equation of Ackers (29). The unknown Stokes radius of mitochondrial ATPase was then determined from the same equation by means of the mean pore radius and the distribution coefficient measured for the enzyme.

**Polyacrylamide Gel Electrophoresis**—Electrophoresis was carried out vertically in gel columns (0.5 × 7.5 cm) immersed in buffer maintained at 15-20°. Gel electrophoresis in the SDS-mercaptoethanol system of Shapiro, Vinuela, and Maizel (30) was carried out exactly as described by Weber and Osborn (31). The protein samples (2 to 4 mg per ml) were incubated at 40° for 2 hours in 1% SDS, 1% mercaptoethanol, 10 mM sodium phosphate, pH 7.0, dialyzed for 16 hours at room temperature against 0.1% SDS, 0.1% mercaptoethanol, 10 mM sodium phosphate, pH 7.0, and subjected to electrophoresis on 10% polyacrylamide gels containing 0.1% SDS. The gels were fixed and stained for 3 hours in a freshly prepared solution of 0.25% Coomassie brilliant blue in 40.4% methanol (v/v), 9.2% acetic acid (v/v). They were then destained by soaking in several 30-ml volumes of 7.5% acetic acid (v/v), 5% methanol (v/v) at room temperature for 48 hours followed by incubation in several changes of the same solvent at 55° until the background stain was reduced to zero.

Gel electrophoresis in phenol-acetic acid-urea was performed essentially as described by Takayama et al. (32). The protein sample (10 μl, 2 to 4 mg per ml) in Buffer 1 containing 5.0 mM dithiothreitol was treated with 4 volumes of phenol and 2 volumes of acetic acid. Aliquots (50 μl) were then layered on 6% gels containing 5.0 mM urea and subjected to electrophoresis at 2 mA per tube. The gels were stained with Amido black in 7% acetic acid.

The stained gels were scanned at 540 nm with a scanning attachment for a Gilford spectrophotometer. Areas under the peaks in the scanner traces were measured by planimetry.

**Other Methods**—Membrane protein was estimated by the biuret reaction in the presence of 0.53% sodium cholate (33). Soluble protein was measured by the procedure of Lowry et al. (34) except that the volume of the reaction was scaled down to 0.65 ml. If sucrose or ATP were present, the protein was precipitated with 5% trichloroacetic acid prior to the determination of protein. Concentrations of heme proteins used as standards were determined from absorbances at 400 nm.

Densities and viscosities used in calculations of 7.5 cm) immersed in 0.1 cm) immersed in D20, ω and D20,ω were taken from the International Critical Tables (35) and the Handbook of Chemistry and Physics (36). The corrections were not large for the conditions of the experiments.

**RESULTS**

Mitochondrial ATPase from rat liver appears to be substantially more tightly bound to the inner mitochondrial membrane than the corresponding enzymes from yeast or bovine heart. In those cases, disruption of the mitochondrial membranes by sonication or by shaking with glass beads in isotonic sucrose-EDTA
solubilized a substantial fraction of the enzyme (1, 2, 4). Application of similar techniques to rat liver mitochondria solubilized less than 0.5% of the enzymatic activity. However, exhaustive washing of the mitochondrial membranes in low ionic strength media followed by sonication gave greatly improved solubilization. This method is described below and the results are summarized in Table I.

Washing of Membranes—Frozen mitochondria (approximately 2.5 g of mitochondrial protein) were thawed and suspended by means of a glass homogenizer with a Teflon pestle in a volume of 3.0 mM Tris-Cl, 50 mM EDTA, pH 7.5, sufficient to give a protein concentration of 50 mg per ml. The suspension was sonicated at maximum intensity with the large probe of a Bronwill Biosonic sonicator for 2 min at 0°. The mitochondrial membrane fragments were then sedimented at 150,000 x g for 45 min at 0°. The sedimented membranes were resuspended in a volume of 3.0 mM Tris-Cl, 50.0 mM EDTA, pH 7.5, sufficient to give a protein concentration of 20 mg per ml. The membranes were then washed five times in this buffer by centrifugation at 150,000 x g for 45 min followed by resuspension to the initial volume. The temperature was maintained near 0° throughout the five washes. During this procedure, 40% to 50% of the membrane protein was released into the supernatant.

Incubation and Solubilization—The washed membranes were resuspended in the original volume of 3.0 mM Tris-Cl, 50.0 mM EDTA, pH 7.5, and then adjusted to 10% ethylene glycol and 4.0 mM ATP. The suspension was incubated at room temperature (23-25°) for 16 hours. After this incubation, the membranes were sedimented by centrifugation at 150,000 x g for 45 min at room temperature and resuspended to the starting volume with 250 mM sucrose, 3.0 mM Tris-Cl, 5.0 mM EDTA, pH 7.5. During this incubation and wash, an additional 20% of the original membrane protein was released into the supernatant. Mitochondrial ATPase was then dissociated from the membrane by sonication at maximum intensity using the large probe of a Bronwill Biosonic sonicator for 30 min in three 10-min intervals. During sonication the suspension was immersed in a 22° water bath. The temperature of the suspension at the end of each 10-min interval was approximately 30°. After sonication, the membranes were removed by sedimentation at 150,000 x g for 1 1/2 hours at room temperature. It was important to carry out this centrifugation immediately since a slow reassociation of the enzyme with the membrane seemed to take place. Because of the cold lability of the soluble enzyme, all subsequent operations were carried out at room temperature.

Chromatography on DEAE-cellulose—Immediately after centrifugation, the clear yellow supernatant was adsorbed to a column of DEAE-cellulose (8 x 2 cm) which had been equilibrated with 20 mM potassium phosphate, 5.0 mM EDTA, pH 7.5. If the soluble supernatant was not immediately adsorbed to this column, the solution became turbid and activity was lost. The enzyme was eluted from the column with a linear gradient of potassium phosphate (pH 7.5, 50 mM to 250 mM). The buffers also contained 5.0 mM EDTA. The flow rate was 2 ml per min and 5-ml fractions were collected. The enzymatic activity eluted as a single peak at a phosphate concentration of about 180 mM.

The active fractions from the DEAE-cellulose column were pooled and concentrated to a volume of about 5 ml by means of an Amicon Diaflo apparatus with a PM-10 filter. A 10 to 15% loss of activity accompanied this procedure, but other procedures for concentration gave even poorer yields.

Chromatography on Sephadex G-200—The pooled and concentrated DEAE enzyme was layered on a column of Sephadex G-200 (2.1 x 90 cm) equilibrated with 200 mM potassium phosphate, 5.0 mM EDTA, pH 7.5 (Buffer 1). The enzymatic activity emerged in a single zone shortly after the void volume. The active fractions were combined and concentrated as described above to a protein concentration of about 4 mg per ml. The specific activity of the preparation at this point was usually about 22 units per mg.

Stability and Storage of Enzyme—The purified enzyme is stable at room temperature at a concentration 0.5 mg per ml in Buffer 1 for up to 24 hours. Greater than 90% of the enzymatic activity is lost in 1 hour at 0°. Activity is also lost rapidly at room temperature in the absence of phosphate. Occasionally in gel filtration and zone sedimentation experiments described below, dissociation amounting to 5 to 10% of the total protein was observed. This could be prevented by inclusion of 4.0 mM ATP in Buffer 1. Inclusion of ATP in the buffer had no effect on the physical properties of the enzyme.

For long term storage, the enzyme was lyophilized from Buffer 1 and stored at -20°. In this form, the enzyme has been stored for up to 6 months without loss of activity or change in physical properties. All experiments reported here were done on enzyme stored in this manner and used immediately after being redissolved.

Gel Filtration Analysis: Stokes Radius and Diffusion Coefficient—A typical Sephadex G-200 column chromatographic profile of purified mitochondrial ATPase is presented in Fig. 1. The enzyme emerges as a single peak of protein and activity near the void volume. The specific activity throughout the peak is constant. This behavior is consistent with the presence of a single protein species homogeneous with respect to Stokes radius.

Ackers (29) has shown that the movement of macromolecules in a Sephadex G-200 gel matrix is best described as a restricted diffusion process. As predicted by this model, the elution position of a number of well characterized proteins is more closely related to their Stokes radii than to their molecular weights. Gel filtration analysis of a protein of unknown Stokes radius on a column of Sephadex G-200 whose pore radius has been calculated from the elution position of proteins of known Stokes radius allows the determination of the unknown Stokes radius. Data from such an experiment is presented in Table II. The Stokes

### Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
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<tbody>
<tr>
<td>Mitochondria</td>
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<tr>
<td>Sonicated membranes</td>
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<td>88</td>
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<tr>
<td>Washed membranes</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>Incubated membranes</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>Soluble extract</td>
<td>8.4 (4.2-12.3)</td>
<td>43 (30-55)</td>
</tr>
<tr>
<td>DEAE-enzyme</td>
<td>20.5</td>
<td>27</td>
</tr>
<tr>
<td>Sephadex G-200 enzyme</td>
<td>22.0</td>
<td>24</td>
</tr>
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</table>
radius of the purified enzyme determined by this technique is 72.2 Å ± 4.5 Å. The Stokes radius is related to the free diffusion coefficient by the Stokes-Einstein relationship (37). The free diffusion coefficient calculated in this way is $2.96 \times 10^{-7}$ cm² sec⁻¹.

**Sedimentation Velocity Analysis**—Analysis of the purified enzyme by zone sedimentation in sucrose gradients also results in a single coincident peak of protein and enzymatic activity (Fig. 2). Comparison of the sedimentation rate with that of catalase sedimented under identical conditions indicates a value of $s_{20,w} = 12.3 ± 0.1$.

Sedimentation of the enzyme in the analytical ultracentrifuge with the schlieren optical system yields a single sharp symmetrical boundary pattern (Fig. 3). Experiments at concentrations ranging from 4.0 to 1.0 mg per ml indicated that the sedimentation coefficient was not concentration-dependent in this concentration range. Averaging these data, a value of $s_{20,w} = 12.15 ± 0.15$ was obtained.

These two types of sedimentation experiments indicate the presence of a single protein species homogeneous with respect to sedimentation coefficient.

**Electrophoretic Analysis**—Electrophoretic analysis of the purified enzyme in dissociating solvent systems was undertaken both to provide further evidence for the homogeneity of the

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**TABLE II**

Determination of Stokes radius by Sephadex G-200 chromatography

<table>
<thead>
<tr>
<th>Protein</th>
<th>Distribution coefficient</th>
<th>Stokes radius (Å)</th>
<th>Pore radius (Å)</th>
</tr>
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<tr>
<td>Bovine serum albumin</td>
<td>0.375</td>
<td>30.1</td>
<td>178</td>
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<tr>
<td>Catalase</td>
<td>0.204</td>
<td>52.2</td>
<td>173</td>
</tr>
<tr>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
<td>0.279</td>
<td>43.0</td>
<td>170</td>
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<tr>
<td>Glutamate dehydrogenase</td>
<td>0.065</td>
<td>64.0</td>
<td>155</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>0.491</td>
<td>30.8</td>
<td>200</td>
</tr>
<tr>
<td>Urease</td>
<td>0.000</td>
<td>68.0</td>
<td>154</td>
</tr>
<tr>
<td>Mitochondrial ATPase</td>
<td>0.090</td>
<td>(72.2 ± 4.5)</td>
<td>(174 ± 10.7)</td>
</tr>
</tbody>
</table>

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Fig. 1. Gel filtration analysis of mitochondrial ATPase on Sephadex G-200. Mitochondrial ATPase (50 µl, 6.6 mg per ml) in Buffer 1 containing 4.0 mM ATP was layered on a column of Sephadex G-200 (0.7 X 50 cm) equilibrated with the same buffer. The enzyme was eluted at a flow rate of 6.0 ml per hour at room temperature (23-25°C). Fractions of 0.5 ml were collected. Protein (X--X) and ATPase activity (O--O) were determined as described under “Methods.” Arrows indicate the elution positions of dextran blue and 2,4-dinitrophenol.

Fig. 2. Zone sedimentation of mitochondrial ATPase in sucrose gradients. Mitochondrial ATPase (60 µl, 3.3 mg per ml) in Buffer 1 containing 4.0 mM ATP was layered on a linear (5 to 20%, 5 ml) sucrose gradient also buffered with Buffer 1 containing 4.0 mM ATP. Identical gradients were layered with samples (60 µl) containing mitochondrial ATPase (3.8 mg per ml) and catalase (3.3 mg per ml) used as a sedimentation marker ($s_{20,w} = 11.3$ (38, 39)). Sedimentation was for 4 hours at 50,000 rpm in a Spinco SW 50 L rotor. Sample temperature at the end of the run was 24.6°C. Immediately after the run, tubes were punctured from the bottom and 10-drop fractions were collected. Protein concentration and enzymatic activity were determined as described under “Methods.” The sedimentation position of catalase is marked by the arrow.

Fig. 3. Sedimentation velocity analysis of mitochondrial ATPase in the analytical ultracentrifuge. Equal volumes of Buffer 1 and mitochondrial ATPase (3.8 mg per ml) in Buffer 1 were placed in a double sector cell and sedimented at 50,100 rpm in a Spinco model E analytical ultracentrifuge. The temperature was maintained at 22.1°C. Photgraphs were taken at 8-min intervals beginning 10 min after reaching speed. The bar angle setting was 70°. Sedimentation coefficients were determined by measuring the maximum ordinate in the Schlieren patterns with a micro-comparator (Nikon).
enzymatic preparation and to give an indication of its subunit composition. Analysis of the enzyme in the phenol-acetic acid-urea system (32) showed the presence of two sharp protein peaks (Fig. 4). The ratio of staining intensities was consistently 10.0:1.0. Previous treatment of the enzyme with 8.0 M urea or at 62° resulted in the formation of other more slowly moving bands suggesting that aggregation phenomena had occurred. This result indicates that solubilization in phenol-acetic acid-water followed by electrophoresis in 5.0 M urea may not dissociate the protein to its most basic subunits (5) and, therefore, that the two bands observed under standard conditions might be resolved still further under more rigorously dissociating conditions.

Electrophoretic analysis in SDS-mercaptoethanol accomplished this objective. Three protein bands are resolved from the purified mitochondrial ATPase preparation under these conditions (Fig. 5, right). These results indicate that mitochondrial ATPase contains three types of subunits which differ in molecular weight. Comparison of the mobilities of the three ATPase subunits with the mobilities of several standard proteins according to the method of Weber and Osborn (31) indicates molecular weights of 62,500, 57,000, and 36,000 for the A, B, and C subunits, respectively (Fig. 5, left). The standard deviation in each case is approximately ±2000. The ratio of stain intensity in the three bands is 6.5:4.9:1.0. Correcting this weight ratio for the molecular weights of the three subunits yields a molar ratio of 3.7:3.1:1.0. It must be remembered when considering this ratio, however, that staining by Coomassie blue has not been shown to be rigorously proportional to protein concentration.

Weber and Osborn (31) have shown that 40 proteins with well characterized subunit composition are dissociated to their polypeptide chain subunits under the conditions employed here. Of the proteins tested, only myosin and actin failed to dissociate completely in 1% SDS, 1% mercaptoethanol. Addition of 8.0 M urea to the incubation solution succeeded in dissociating these molecules also.

Accordingly, we have tried the following more stringent incubation conditions in an attempt to further dissociate the apparent polypeptide chain subunits described above: (a) inclusion of 8.0 M urea in the incubation solution; (b) previous heating at 100° for 10 min in SDS-mercaptoethanol followed by the standard incubation; (c) increasing the concentration of reagents to 4% SDS and 4% mercaptoethanol; and (d) extraction of the protein with chloroform-methanol (2:1, v/v) or acetone-water (9:1, v/v) before the standard incubation procedure. After each of these procedures, dialysis and electrophoresis as described by Weber and Osborn (31) indicated no change in the electrophoretic pattern. We conclude, therefore, that the A, B, and C subunits described above most probably are single polypeptide chains.

Amino Acid Composition—The results of amino acid analyses on acid hydrolysates of the enzyme are shown in Table III, column 1. No amino sugars or other ninhydrin-positive compounds were detected in the hydrolysates. Separate analyses for chloroform-methanol extractable lipid phosphates were negative. The analyses could have clearly detected 2.5% by weight phospholipid (assuming a lipid molecular weight of 700). The apparent partial specific volume calculated from the amino acid composition as described by Cohn and Edsall (42) is 0.740 cm³ per g.

For comparison, the amino acid compositions of three other membrane ATPases are presented in Table III, columns 2, 3, and 4. The data were taken from References 18 and 19 and normalized to 30,000 g of protein. The four enzymes from unrelated sources have surprisingly similar amino acid compositions.

Estimated Molecular Weight—With the Svedberg equation, the diffusion coefficient estimated from gel filtration (D_m, w = 2.96 × 10⁻⁹ cm² sec⁻¹), the sedimentation coefficient extrapolated to zero concentration (s_m, w = 12.15), and the partial specific volume calculated from the amino acid composition (P = 0.740 cm³ per g) can be combined to give a reliable molecular weight estimate. The molecular weight calculated from these data is 384,000. The apparent uncertainty in this value due to the
DISCUSSION

The experiments reported here describe the purification of mitochondrial ATPase from rat liver to homogeneity and, in addition, provide information concerning its subunit composition, molecular weight, and relationship to other highly purified ATPase preparations. Gel filtration studies and sedimentation velocity analysis by means of two different techniques revealed no indications of heterogeneity in the preparation. The results of disc gel electrophoresis carried out on several preparations in dissociating and partially dissociating systems were also consistent with the presence of a homogeneous enzyme consisting of multiple subunits.

The estimated molecular weight of 384,000 calculated from sedimentation velocity, gel filtration, and amino acid analysis data is in the same range as the molecular weights reported for the ATPases from S. faecalis, spinach chloroplasts, and bovine heart mitochondria whose amino acid compositions (Table III) and physiological functions are similar. The correlation with the reported molecular weight of 386,000 for the S. faecalis enzyme (19) is closer than with the values of 325,000 or 358,000 reported for the chloroplast enzyme (18) or the value of 284,000 reported for the bovine heart mitochondrial enzyme (3). Differences in aggregation state under the conditions of the molecular weight determinations may account for the different molecular weights observed (cf. Reference 18).

In addition to the close similarity in molecular weights of the enzymes isolated from rat liver and S. faecalis, a very recent communication from the laboratory of Abrams (43) has documented the importance of a 37,000 molecular weight protein in the binding of the enzyme to the membrane. This protein is released by the washing procedure used for release of the membrane ATPase (17). In early preparations of the S. faecalis enzyme, this protein apparently appeared as a third subunit (44) on electrophoresis in urea but was lost in the more rigorous purification procedures used subsequently (19). Since that washing procedure is similar to the one used here, it is attractive to speculate that the 36,000 molecular weight subunit observed here may have an analogous function.

Three subunits of mitochondrial ATPase have been demonstrated here under a variety of stringently depolymerizing conditions. In light of the extensive study by Weber and Osborn (31) on the depolymerizing properties of SDS-mercaptoethanol solutions and the physical studies of Tanford's group (45, 46) which put these empirical observations on quasi-theoretical ground, it seems very probable that these three subunits are in fact fully dissociated polypeptide chains of molecular weight 62,500, 57,000, and 36,000. On the other hand, rigorous proof of this point requires chemical studies on the separated subunits. The observation of these three bands in similar ratios in electrophoretic studies of whole inner mitochondrial membranes suggests that a similar ATPase complex may exist in the intact membrane (47).

The weight ratios of protein subunits determined from staining intensities in SDS-mercaptoethanol and phenol-acetic acid-urea gel electrophoresis experiments were 6.5:4.9:1.0 and 10.1:1.0, respectively. Comparison of these two ratios suggests that the A and B subunits observed in SDS-mercaptoethanol gel electrophoresis both contribute to the slower moving band observed in phenol-acetic acid-urea gel electrophoresis. This behavior can be ascribed either to coincidental comigration or, more likely, to incomplete depolymerization of the parent molecule under the conditions of phenol-acetic acid-urea gel electrophoresis. Correction of the 10.1:1.0 weight ratio for molecular weight gives a molar ratio of (A + B)/C = 6.2:1.0. This ratio is in fair agreement with the molar ratio of A:B:C = 3.7:3.1:1.0 from SDS-mercaptoethanol gel electrophoresis. Since staining of protein by Amido black has been shown to be proportional to protein concentration in the three investigations (48-50), the data suggest a tentative formulation of the subunit composition as ABC. The molecular weight of 394,000 obtained by summing these subunits is in good agreement with the molecular weight determined from hydrodynamic studies.

Gel electrophoretic results similar in some respects to those described here have been reported for three different types of preparations of the bovine heart mitochondrial ATPase (5, 13). MacLennan, Spudis, and Tzagoloff (13) reported that a preparation made according to Pullman et al. (1) contained two subunits separable by phenol-acetic acid-urea having mobilities similar to those described here. However, a high salt extractable preparation containing coupling factor 1 activity gave only one

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>Rat liver mitochondrial ATPase</th>
<th>Bovine heart mitochondrial ATPase</th>
<th>Spinach chloroplast ATPase</th>
<th>S. faecalis ATPase</th>
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<tr>
<td>Cysteine</td>
<td>0.7</td>
<td>1.1</td>
<td>1.1</td>
<td>0.9</td>
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<tr>
<td>Aspartic acid</td>
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<td>16.0</td>
<td>17.0</td>
<td>12.8</td>
</tr>
</tbody>
</table>

standard deviation in the determinations of $D_{20, w}$ and $e_{20, w}$ is approximately 8%.

The experiments reported here describe the purification of mitochondrial ATPase from rat liver to homogeneity and, in addition, provide information concerning its subunit composition, molecular weight, and relationship to other highly purified ATPase preparations. Gel filtration studies and sedimentation velocity analysis by means of two different techniques revealed no indications of heterogeneity in the preparation. The results of disc gel electrophoresis carried out on several preparations in dissociating and partially dissociating systems were also consistent with the presence of a homogeneous enzyme consisting of multiple subunits.

The estimated molecular weight of 384,000 calculated from sedimentation velocity, gel filtration, and amino acid analysis data is in the same range as the molecular weights reported for the ATPases from S. faecalis, spinach chloroplasts, and bovine heart mitochondria whose amino acid compositions (Table III) and physiological functions are similar. The correlation with the reported molecular weight of 386,000 for the S. faecalis enzyme (19) is closer than with the values of 325,000 or 358,000 reported for the chloroplast enzyme (18) or the value of 284,000 reported for the bovine heart mitochondrial enzyme (3). Differences in aggregation state under the conditions of the molecular weight determinations may account for the different molecular weights observed (cf. Reference 18).

In addition to the close similarity in molecular weights of the enzymes isolated from rat liver and S. faecalis, a very recent communication from the laboratory of Abrams (43) has documented the importance of a 37,000 molecular weight protein in the binding of the enzyme to the membrane. This protein is released by the washing procedure used for release of the membrane ATPase (17). In early preparations of the S. faecalis enzyme, this protein apparently appeared as a third subunit (44) on electrophoresis in urea but was lost in the more rigorous purification procedures used subsequently (19). Since that washing procedure is similar to the one used here, it is attractive to speculate that the 36,000 molecular weight subunit observed here may have an analogous function.

Three subunits of mitochondrial ATPase have been demonstrated here under a variety of stringently depolymerizing conditions. In light of the extensive study by Weber and Osborn (31) on the depolymerizing properties of SDS-mercaptoethanol solutions and the physical studies of Tanford's group (45, 46) which put these empirical observations on quasi-theoretical ground, it seems very probable that these three subunits are in fact fully dissociated polypeptide chains of molecular weight 62,500, 57,000, and 36,000. On the other hand, rigorous proof of this point requires chemical studies on the separated subunits. The observation of these three bands in similar ratios in electrophoretic studies of whole inner mitochondrial membranes suggests that a similar ATPase complex may exist in the intact membrane (47).

The weight ratios of protein subunits determined from staining intensities in SDS-mercaptoethanol and phenol-acetic acid-urea gel electrophoresis experiments were 6.5:4.9:1.0 and 10.1:1.0, respectively. Comparison of these two ratios suggests that the A and B subunits observed in SDS-mercaptoethanol gel electrophoresis both contribute to the slower moving band observed in phenol-acetic acid-urea gel electrophoresis. This behavior can be ascribed either to coincidental comigration or, more likely, to incomplete depolymerization of the parent molecule under the conditions of phenol-acetic acid-urea gel electrophoresis. Correction of the 10.1:1.0 weight ratio for molecular weight gives a molar ratio of (A + B)/C = 6.2:1.0. This ratio is in fair agreement with the molar ratio of A:B:C = 3.7:3.1:1.0 from SDS-mercaptoethanol gel electrophoresis. Since staining of protein by Amido black has been shown to be proportional to protein concentration in the three investigations (48-50), the data suggest a tentative formulation of the subunit composition as ABC. The molecular weight of 394,000 obtained by summing these subunits is in good agreement with the molecular weight determined from hydrodynamic studies.

Gel electrophoretic results similar in some respects to those described here have been reported for three different types of preparations of the bovine heart mitochondrial ATPase (5, 13). MacLennan, Spudis, and Tzagoloff (13) reported that a preparation made according to Pullman et al. (1) contained two subunits separable by phenol-acetic acid-urea having mobilities similar to those described here. However, a high salt extractable preparation containing coupling factor 1 activity gave only one
band corresponding to the major band observed in our studies (13). Senior and Brooks (5) reported that bovine heart mito-
chondrial ATPase made by means of a new purification
procedure contained four separable protein bands under the
conditions of both phenol-acetic acid-urea and SDS-mercapto-
ethanol gel electrophoresis. The two major bands observed in
phenol-acetic acid-urea gel electrophoresis were similar in mo-
tility to those observed here. They observed one major species
(mol wt 55,000) and 3 minor species (mol wt 25,000, 12,000,
and 8,000) in SDS-mercaptoethanol gel electrophoresis. Since
the resolution of bands in 5% gels used in the experiments of
Senior and Brooks (5) is poorer than in 10% gels used here, it is
possible that the major band observed by these investigators
contains two species differing slightly in molecular weight similar
to those observed here.

It is noteworthy that changes in the solubilization and purifi-
cation procedures yield apparently homogeneous enzymes with
somewhat different subunit compositions. In all cases, however,
the major species resolved electrophoretically under depoly-
merizing conditions is similar in mobility to the A and B subunits
described here. It seems likely to us that different solubilization
procedures might lead to solubilization of the catalytic subunit of
mitochondrial ATPase in association with different protein
species from the surrounding membrane. Complete charac-
terization of each of these preparations should yield critical
information concerning the identity of the protein species inter-
acting with the mitochondrial ATPase catalytic subunit in the
membrane.

An interesting aspect of the studies of membrane ATPases
reported to date has been the extensive physical similarity among
the enzymes isolated from different membrane systems. As
documented in Table III, the amino acid compositions of the
enzymes from mammalian mitochondria, spinach chloroplasts,
and bacteria show extensive homology. The molecular weights
of the intact homogeneous enzymes are all in the range from
250,000 to 350,000 daltons. The subunit compositions are
complex but similar with various combinations of subunits of
30,000 to 60,000 daltons indicated in each case (18, 19). In
addition to these structural similarities, several properties
suggesting similarities in catalytic mechanisms have been ob-
erved. These properties include: (a) inhibition of the mem-
brane-bound enzymes by N \(^{\prime}\)N\(^{\prime}\)-dicyclohexylcarbodiimide (51,
52); (b) activation of the membrane-bound enzymes by uncou-
plers (15, 53); (c) activation of the soluble enzymes by heat

treatment (1, 19, 54); and (d) inactivation of the soluble enzymes
by incubation at low temperature (1, 55).

These similarities are remarkable since the sources of the
enzymes are phylogenetically unrelated and the processes they
participate in are different. Thus, mitochondrial ATPase is
primarily involved in oxidative phosphorylation, chloroplast
ATPase in photosynthetic phosphorylation (15), and S. faecalis
ATPase in ATP-driven translocation of cations, phosphate, and
some amino acids (52). The most attractive explanation of
these findings, in our view, is the hypothesis that each of these
processes represents one manifestation of a central mechanism
of energy coupling and transduction that remains unknown and
upon which other energy-linked membrane processes depend.

Physical and chemical studies of the separated subunits of
mitochondrial ATPase from rat liver are currently in progress
in our laboratory.

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