Mitochondrial ATP Synthasome

CRISTAE-ENRICHED MEMBRANES AND A MULTIWELL DETERGENT SCREENING ASSAY YIELD DISPERSED SINGLE COMPLEXES CONTAINING THE ATP SYNTHASE AND CARRIERS FOR Pᵢ AND ADP/ATP

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The terminal step of ATP synthesis in intact mitochondria is catalyzed by the ATP synthase (FᵢFₒ) that works in close synchrony with the Pᵢ and ADP/ATP carriers. Each carrier consists of only a single polypeptide chain in dimeric form, while the ATP synthase is highly complex consisting in animals of 17 known subunit types and more than 30 total subunits. Although structures at high resolution have been obtained for the water-soluble Fₒ part of the ATP synthase consisting of only five subunit types, such structures have not been obtained for either the complete ATP synthase or the Pᵢ and ADP/ATP carriers. Here, we report that all three proteins are localized in highly purified cristae-like vesicles obtained by extensive subfractionation of the mitochondrial inner membrane. Moreover, using a multiwell detergent screening assay, 4 nonionic detergents out of 80 tested were found to disperse these cristae-like vesicles into single soluble complexes or "ATP synthasomes" that contain the ATP synthase in association with the Pᵢ and ADP/ATP carriers. These studies offer new mechanistic insights into the terminal steps of oxidative phosphorylation in mitochondria and set the stage for future structural efforts designed to visualize in atomic detail the entire complex involved. They also provide evidence that the cristae are a subcompartment of the inner membrane.

As mitochondria comprise the site of most ATP production in animal cells by a process known as oxidative phosphorylation, there has been intense interest in understanding its mechanism (1–3). This remarkably complex process requires that four major events take place: electron transport, generation of a proton gradient, transport of Pᵢ and ADP, and finally coupling the proton gradient to ATP synthesis, a process catalyzed by the ATP synthase complex (FᵢFₒ).1 The latter two events are closely synchronized as each ATP molecule that is made on the Fₒ unit of the ATP synthase inside the mitochondria exits this organelle as new Pᵢ, and ADP molecules enter simultaneously on separate transporters, referred to here as PIC and ANC, respectively. As the ATP synthase has long been known to be associated with inner membrane regions or extensions called "cristae" (4, 5), it is here that PIC and ANC are also most likely localized.

A major impediment to fully understanding the terminal events of mitochondrial oxidative phosphorylation is the absence of atomic resolution structures for the complete ATP synthase, PIC, and ANC. In this regard, it seems likely that within the mitochondria, as for other complicated biological systems, supercomplexes exist. One or more may involve the electron transport chain complexes and another an ATP synthase-PIC-ANC complex. Significantly, biochemical evidence for respiratory chain supercomplexes in both mitochondria and bacteria has been obtained recently (6, 7), as has highly suggestive evidence for an association of the ATP synthase, PIC, and ANC (8, 9). Considering recent structural achievements in obtaining high resolution data on the 70 S ribosome-RNA complex from two different laboratories (10, 11), it is not unrealistic to assume that similar achievements are likely to be forthcoming for other "supercomplexes" including those located in the mitochondrial inner membrane (6, 7). Here, however, the problem is compounded as the first barrier that must be overcome is not that of obtaining two- or three-dimensional crystals. Rather it is to identify an appropriate detergent that will maintain the complex or supercomplex of interest intact, active, and in soluble dispersal form (12). This difficulty likely contributes substantially to the fact that of the 19,551 structures currently reported in the Research Collaboratory for Structural Biology data base, less than 30 are membrane proteins. Also, in examining several reports where remarkable success has been achieved (13–16), it is clear that no single detergent is appropriate for all membrane proteins. Rather, an exhaustive search must be conducted to identify the appropriate detergent(s) for each (12).

With the above thoughts in mind, the objectives of the work reported here were to obtain a highly enriched cristae-like vesicular fraction containing the ATP synthase in association with PIC and ANC and to identify detergents most appropriate

1 The abbreviations used are: FₒFᵢ, ATP synthase; PIC, phosphate carrier; ANC, adenine nucleotide carrier; ATP synthasome, ATP synthase-PIC-ANC complex; TEM, transmission electron microscopy; SEM, scanning electron microscopy; CMC, critical micelle concentration; Cymal-5, cyclohexyl-pentyl-β-maltoside; Hega-11, undecanoyl-N-hydroxyethylgluamide; PBS, phosphate-buffered saline; IMF, inner mitochondrial membrane fraction; Tricine, N-[2-hydroxyethyl]glycine; D-maltoside; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; Chaps, 3-[3-cholamidopropyl]dimethylammonio]-2-hydroxy-1-propanesulfonic acid.
for solubilizing this membrane associated supercomplex in an active dispersed form so that future structural studies could be conducted.

EXPERIMENTAL PROCEDURES

Materials

Rats (Harlan Sprague-Dawley® white males) were obtained from Charles River Breeding Laboratories, reagents for electron microscopy from Pella, digitonin from Calbiochem, and Lubrol WX from General Biochemicals (Pella). Digitonin was suspended first in 16 ml of Buffer A (300 mM potassium Pi, 4 mM ATP, 10% ethylene glycol, 5 mM EDTA, and 0.5 mM dithiothreitol, pH 7.9), frozen in dry ice and acetone, and stored overnight at −20 °C. The IMF was then slowly thawed, made up to 65 ml with Buffer B (300 mM potassium Pi, 50 mM EDTA, pH 7.9), frozen in dry ice and acetone, and stored overnight at −20 °C. The IMF was then slowly thawed, made up to 65 ml with Buffer B (300 mM potassium Pi, 50 mM EDTA, pH 7.9), and washed for 15 min by stirring in an ice-cold 100-ml beaker. Centrifugation was then carried out for 30 min in a Ti 70.1 rotor at 50,000 rpm in a Beckman LE 80K ultracentrifuge. The combined pellets in the multiple tubes were suspended in 65 ml of Buffer C (300 mM potassium Pi, 50 mM EDTA, 1 mM ATP, pH 7.9) for 15 min and centrifuged again for 1 h at 50,000 rpm. The

ATPase and Respiration Assays—ATPase activity (±0.6 µg of oligomycin/mg of protein) and respiration (oxygen consumption) were monitored as described previously (17, 18) in the presence, respectively, of 3.0 mM ATP and 7.8 mM succinate. SDS-PAGE and Western analysis were carried out by the method of Laemmli (19), and Western analysis and N-terminal sequence analysis were carried out exactly as described previously (20).

Cristae-like Inner Membrane Vesicles—Each preparation commenced by preparing from four rats an inner mitochondrial membrane fraction (IMF) (18), after which a previously described procedure (21) was modified to prepare the cristae-like membranes. Specifically, the IMF was suspended first in 16 ml of Buffer A (300 mM potassium Pi, 4 mM ATP, 10% ethylene glycol, 5 mM EDTA, and 0.5 mM dithiothreitol, pH 7.9), frozen in dry ice and acetone, and stored overnight at −20 °C. The IMF was then slowly thawed, made up to 65 ml with Buffer B (300 mM potassium Pi, 50 mM EDTA, pH 7.9), and washed for 15 min by stirring in an ice-cold 100-ml beaker. Centrifugation was then carried out for 30 min in a Ti 70.1 rotor at 50,000 rpm in a Beckman LE 80K ultracentrifuge. The combined pellets in the multiple tubes were suspended in 65 ml of Buffer C (300 mM potassium Pi, 50 mM EDTA, 1 mM ATP, pH 7.9) for 15 min and centrifuged again for 1 h at 50,000 rpm. The
combined pellets were now suspended in 32 ml of Buffer C and centrifuged for 10 min at 6,000 rpm in a SS-24 rotor in a Sorvall RC-2B centrifuge. After saving the supernatants, the combined pellets were suspended in 16 ml of the same buffer and centrifuged as before in the Sorvall SS-24 rotor. The supernatants were saved again, and once more the pellets were suspended in 16 ml of the same buffer and centrifuged. Then pellets from the two previous steps were discarded, while the supernatants were combined and, after 15 min, subjected to centrifugation for 30 min at 50,000 rpm in the Beckman ultracentrifuge as described above. The pellets were suspended in 32 ml of Buffer C and centrifuged for 20 min at 10,000 rpm in the SS-24 rotor in a Sorvall RC-2B centrifuge. After saving the supernatants, the tubes were tapped gently to dislodge the membrane pellets from underlying glycogen pellets. Then the membrane pellets were rinsed out of the tubes, suspended in 16 ml of Buffer C, and centrifuged as before at 10,000 rpm. The pellets were discarded, while the saved supernatants were pooled. Ethylene glycol was then added to the pooled supernatants to give a final concentration of 10%, and after 30 min this fraction was diluted to 65 ml with Buffer A and centrifuged at 50,000 rpm in a Beckman ultracentrifuge. Aliquots were then removed from the top and subjected to ATPase assays in a multiwell plate reader. Absorbance readings in row A minus those in row B were used to determine the degree of solubility of the cristae-like membranes. Finally, a 1-μl aliquot was removed from each well in row A to assay for ATPase activity ± oligomycin.

**Multiwell Detergent Screening Assay**—The screen was conducted using 96-well microtiter plates equipped with a multiwell plate reader. In each plate four different detergents at 12 different concentrations ranging from 0 to 2× CMC (in mM) were tested at 4 °C. For example, detergent “X” and ~1 mg of cristae-like membranes in a total volume of 100 μl were placed in wells in row A at increasing concentrations of detergent. Row B was the same as row A except the cristae-like membranes were not included. After 12 h the absorbance in all wells was measured at 405 nm within 5 s in a multiwell plate reader. Absorbance readings in row A minus those in row B were used to determine the degree of solubility of the cristae-like membranes. Finally, a 1-μl aliquot was removed from each well in row A to assay for ATPase activity ± oligomycin.

**Sedimentation Analysis in Sucrose**—The membrane solution to be sedimented contained the following ingredients in a final volume of 5 ml: 10 mg cristae-like membranes, 0.5% detergent as indicated, 1 mM ATP, 25 mM EDTA, 0.5 mM dithiothreitol, 5% ethylene glycol, and 50 mM Tricine, pH 7.9. This solution was layered onto a bed of 25 ml 25% sucrose containing the same components except the detergent concentration was 0.25%. This solution was then subjected to centrifugation for 10 h at 25,000 rpm in a SW 28 rotor at 4 °C in the Beckman LE 80K ultracentrifuge. Aliquots were then removed from the top and subjected to ATPase assays, SDS-PAGE, and, where indicated, to Western blot analysis for PIC and ANC.

**Protein Determinations**—For determining membrane protein the biuret procedure (22) was used. Other protein determinations were made using either the method of Lowry et al. (23) or the Coomassie dye binding procedure (Pierce). In all cases the standard was bovine albumin.

**RESULTS**

**Purification of an Oligomycin-sensitive ATPase-enriched Subfraction of the Mitochondrial Inner Membrane**—The first step employed the widely used digitonin/Lubrol WX method (18) to obtain a highly purified mitochondrial inner membrane fraction (Fig. 1A). This fraction exhibits heterogeneity both in vesicle size (60–400-nm diameter) and in content of ATP synthase complexes (projecting in part as “lollipop-like” structures) with some vesicles being saturated and others completely nude (18). For this reason, in studies reported here, we subjected the purified inner membrane fraction to an extensive subfractionation approach (see “Methods”) in which steps involving lower centrifugal forces were used first to remove larger inner membrane fragments while retaining in the supernatant the smaller fragments. By monitoring ATPase specific activity inhibited by oligomycin, a potent ATP synthase inhibitor, it became immediately

**Table 1**

| Detergent (type)          | Solubility (% CMC) | Retention of ATPase activity (%) | Retention of inhibitor sensitivity (%) | Candidate for structural studies |
|---------------------------|--------------------|----------------------------------|----------------------------------------|----------------------------------|
| CHAPS (Z)                 | 100 (1.6)          | +                                | +                                      | Promising                        |
| Cymal-5 (N)               | 100 (1.6)          | +                                | +                                      | Promising                        |
| C-Hega-9 (N)              | 100 (1.1)          | +                                | +                                      | Promising                        |
| n-Nonyl-β-n-glucopyranoside (N) | 100 (1.5)      | +                                | +                                      | Promising                        |
| Hega-9 (N)                | 100 (1.1)          | +                                | +                                      | Promising                        |
| Hega-10 (N)               | 100 (1.8)          | +                                | +                                      | Promising                        |
| Hega-11 (N)               | 100 (1.9)          | +                                | +                                      | Promising                        |
| n-Decyl-N,N-dimethylglycine (Z) | 100 (1.2)      | +                                | +                                      | Promising                        |
| n-Dodecyl-N,N-dimethylglycine (Z) | 100 (1.3)      | +                                | +                                      | Promising                        |
| n-Undecyl-β-n-maltopyranoside (N) | 100 (1.9)     | +                                | +                                      | Promising                        |
| n-Dodecyl-β-n-maltopyranoside (N) | 100 (1.5)     | +                                | +                                      | Promising                        |
| n-Tridecyl-β-n-maltopyranoside (N) | 100 (1.8)    | +                                | +                                      | Promising                        |
| Mega-9 (N)                | 100 (1.1)          | +                                | +                                      | Promising                        |
| n-Octyl-β-n-thiomaltopyranoside (N) | 100 (1.7)   | +                                | +                                      | Promising                        |
| n-Nonyl-β-n-thiomaltopyranoside (N) | 100 (1.4)    | +                                | +                                      | Promising                        |
| n-Decyl-β-n-thiomaltopyranoside (N) | 100 (1.6)    | +                                | +                                      | Promising                        |
| Triton X-100 (N)          | 100 (54)           | +                                | +                                      | Promising                        |
apparent that this enzyme is greatly enriched in the combined supernatants. When these were subjected to a final step involving a high centrifugal force, the resultant membrane fraction (Fig. 1A) was found to have a very high specific ATPase activity (14.8 ± 0.32 μmol of ATP hydrolyzed per min/mg of protein), 5–6-fold higher than the mean value of 2.76 ± 0.61 obtained for the starting inner membrane fraction. Moreover, this activity is inhibited 90–95% by oligomycin. In experiments not reported here, the capacity of this inner membrane subfraction to respire was barely detectable with most of the activity (~30 nanomols of oxygen/min/mg of protein) recovered in the larger membrane fragments that were discarded.

**Characterization of the ATPase-enriched Inner Membrane Subfraction by SDS-PAGE, N-terminal Sequence Analysis, Western Analysis, and Electron Microscopy**—Further characterization of the ATPase-enriched inner membrane fraction by SDS-PAGE (Fig. 1B) revealed 17 peptide components, 15 attributable to the ATP synthase and 1 each to PIC and ANC. All were verified either by N-terminal sequence or Western analysis and where indicated by both methods. The only undetectable ATP synthase components were its two regulatory proteins IF1, an inhibitor of ATP hydrolysis (24), and Factor B, an activator of ATP synthesis (25). As both are known to be loosely associated with the ATP synthase complex, they were most likely depleted during preparation of the membranes.

Following the above studies, the purified cristae-like membrane fraction containing the ATP synthase, PIC, and ANC was subjected to both transmission and scanning electron microscopy (TEM and SEM, respectively). Micrographs obtained by TEM of samples negatively stained with uranyl acetate (Fig. 1C) show vesicles with an average diameter of about 120 nm that are densely packed with ATP synthase molecules. These are distinctly evident from the typical “lollipop” morphological features of those F1 headpieces projecting from the periphery. The micrograph obtained by SEM (Fig. 1D) of samples fixed with glutaraldehyde and stained with uranyl acetate, depict a more in depth “top” view of the F1 headpieces projecting from the membrane surface.

These studies provided evidence that we had isolated a cristae-like subfraction of the mitochondrial inner membrane and that this subfraction contains in addition to the ATP synthase also PIC and ANC.

**Identification of Four Detergents That Readily Solubilize the Cristae-like Membranes Containing the ATP Synthase, PIC, and ANC while Retaining ATPase Activity Sensitive to Oligomycin**—To reduce the task of identifying detergents meeting these criteria, we first set up a multiwell screening assay using a 96-well microtiter plate (Fig. 2A), which could be divided into five different categories, Types I–V (Fig. 2B). Of these, only Type I detergents that solubilize cristae-like membranes with the least effect on ATPase activity, and also preserve oligomycin sensitivity, were selected as “very promising” for future experiments. The four detergents identified were Cymal-5, n-decyl-β-D-thiomaltopyranoside, Hega-11, and n-tridecyl-β-D-maltopyranoside, all of which are nonionic and exhibit similar volumes and surface areas (Fig. 2C).

The ATP Synthase, PIC, and ANC, Localized in Cristae-like Membranes, Sediment as a Single Species in Each of the Four “Type I” Detergents That Disperse Them as Individual ATP Synthase-PIC-ANC Complexes—The cristae-like membrane fraction was solubilized in each of the four selected detergents and sedimented at 50,000 rpm in the Beckman LE 60K ultracentrifuge for 30 min at 4 °C. This resulted in the absence of a membrane pellet verifying the efficacy of the four detergents in completely solubilizing the cristae-like membranes. The clear fractions were then placed on a 25-ml bed of 25% sucrose and centrifuged at 25,000 rpm for 10 h (see “Methods”). In each case, the individual fractions formed a single sharp band at a distance about one-third from the top. These bands were removed and assayed for ATPase activity with and without oligomycin and also subjected to SDS-PAGE and Western analysis using specific antibodies to the ATP synthase β-subunit, PIC, and ANC. Fig. 3A shows that, for three of the four detergents, ≥80% of the ATPase activity characteristic of the solubilized cristae-like membranes put on the gradient is recovered in the one sedimenting band. For the fourth detergent, the recovery of 70% is still quite good. There is also good retention of the capacity of oligomycin to inhibit the recovered ATPase activity in each case.
Significantly, Western analysis presented in Fig. 3B shows that the single sedimenting band also contains in each case both PIC and ANC that are visualized just as clearly as the β-subunit of the ATP synthase. Here, it is important to note from the summary table presented in Fig. 3C that the PIC/β and the ANC/β ratios based on staining intensities remain nearly constant throughout the purification (four experiments), consistent with the presence of a native ATP synthase-PIC-ANC complex. In other data not presented, the SDS-PAGE pattern of the single sedimenting band was in each case nearly identical to that presented earlier in Fig. 1B, lane 2, thus ruling out that one or more of the detergents causes some polypeptides to “fall off” the complex. Finally, when samples were subjected to negative staining and then electron microscopy (see “Methods”), a well dispersed set of single ATP synthase-PIC-ANC complexes with a tripartite structure (head-piece, base-piece, connecting stalk) was observed in all cases (Fig. 3D).

DISCUSSION

One of the greatest challenges in mitochondrial research remains that of obtaining detailed structural information about the terminal steps of oxidative phosphorylation, a complex process involving an ATP synthase to make ATP from P_i and ADP, and two transporters, PIC and ANC, to respectively allow the entrance of these two substrates and the exit of ATP. Studies reported here provide evidence that both the ATP synthase and its required transporters are localized in a cristae-like subfraction of the mitochondrial inner membrane where they form an ATP synthase-PIC-ANC complex. Other work involving an exhaustive screen of 80 different detergents has identified four that solubilize this complex intact and in dispersed form. Thus, these studies have satisfied several important requirements essential for future work that will focus on obtaining detailed structural information about this “super-complex” or “ATP synthasome.”

As the complex that we have isolated in this study contains in addition to the ATP synthase, also PIC and ANC, the base-piece (membrane sector) is expected to be significantly larger than that characteristic of the ATP synthase alone. This appears to be the case as the base piece of the bovine ATP synthase in a recent image reconstruction (26) has a width of only 84 Å, whereas single ATP synthasomes reported here have basepieces of greater than 100 Å. However, further analysis will be necessary taking into consideration detergent and lipid content. As yet, we do not know how “tight” the ATP synthase-PIC-ANC complex is and cannot exclude the possible presence of one or more other essential polypeptides.

Finally, the importance of the work described here deserves comment. First, as it concerns the mechanism of oxidative phosphorylation in mitochondria, these studies indicate that the substrates (P_i and ADP) for ATP synthesis are delivered directly to the ATP synthase and, following ATP synthesis, the product (ATP) is delivered directly to ANC for export to the cytoplasm. Second, as it concerns mitochondrial structure this work provides direct support for the emerging view (27, 28) that the cristae represent a distinct subcompartment of the inner membrane that harbors the terminal proteins of oxidative phosphorylation. Third, these studies provide a method for identifying an appropriate detergent to solubilize any membrane protein and in the case of the ATP synthase-PIC-ANC complex set the stage for structural studies of the complete terminal complex of oxidative phosphorylation in mitochondria.

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