Arrangement of Oligomycin-Sensitive Adenosine Triphosphatase in the Mitochondrial Inner Membrane†

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ABSTRACT: Oligomycin-sensitive ATPase has been isolated from beef heart mitochondria by several different approaches including antibody immunoprecipitation. The subunit structure of these preparations has been examined by one- and two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis. All preparations contained 16–18 different polypeptides including the 5 subunits of ATPase, inhibitor protein, dicyclohexocarbodiimide binding proteolipid, and the so-called oligomycin sensitivity conferring protein. The reactivity of isolated F_1ATPase, purified oligomycin-sensitive ATPase, and the membrane-bound complex in intact mitochondria and submitochondrial particles with [^{35}S]diazobenzenesulfonate (DABS) has been compared. The labeling of isolated F_1ATPase by this reagent is distributed equally in the α and β subunits. The γ, δ, and ϵ subunits are also reactive to [^{35}S]DABS. In isolated oligomycin-sensitive ATPase, the α subunit is significantly less reactive to [^{35}S]DABS than the β subunit. Also, the γ and ϵ subunits are partially shielded from labeling by interaction with the membrane sector. The reaction of submitochondrial particles with [^{35}S]DABS resulted in labeling of the β subunit of F_1ATPase, but there was very little reaction with the α, γ, or ϵ subunits. The oligomycin sensitivity conferring protein and at least one membrane sector polypeptide (component 5) were labeled from the matrix side of the membrane. Reaction of intact mitochondria with [^{35}S]DABS resulted in very little labeling of the oligomycin-sensitive ATPase complex. One membrane sector polypeptide, component 12, was, however, labeled from the cytoplasmic side of the inner membrane.

The mitochondrial inner membrane contains several different multipeptide complexes involved in electron transfer and coupled ATP synthesis. When mitochondria or submitochondrial particles are subjected to mechanical disruption by shaking with glass beads (Penefsky, 1967) or to sonic oscillation (Horstman & Racker, 1970; Senior & Brooks, 1970), F_1ATPase is released. This globular, water-soluble protein shows ATPase activity but lacks ATP–Pi exchange activity. It is cold labile and insensitive to inhibitors of ATP synthesis such as oligomycin and DCCD[1] [for reviews, see Senior (1973), Pedersen (1975), and Panet & Sanadi (1976)]. F_1ATPase has been isolated from many different sources and shown to contain five different subunits in an as yet undefined stoichiometry (Tzagoloff & Meagher, 1971; Lambeth & Lardy, 1971; Senior & Brooks, 1971; Knowles & Penefsky, 1972; Abrams & Smith, 1974; Nelson, 1976). Preparations of F_1ATPase from beef heart or rat liver mitochondria often contain small and variable amounts of an inhibitor protein of low molecular weight (Brooks & Senior, 1974; Chan & Barbour, 1976; Cintron & Pedersen, 1979). Recently, F_1ATPases from beef heart (Spitsberg & Haworth, 1977), rat liver (Amzel & Pedersen, 1978), and thermophilic bacterium PS3 (Kagawa, 1978) have been crystallized and X-ray analysis of these crystals should eventually provide a detailed three-dimensional structure for the protein.

After mitochondria or submitochondrial particles have been treated with detergents to disrupt the inner membrane, a complex can be isolated which retains both ATPase and ATP–Pi exchange activity and which is cold stable and inhibited by DCCD and oligomycin. This complex has been prepared in several different ways and variously called F_1–F_0 (Kagawa & Racker, 1966a; Serrano et al., 1976), oligomycin-sensitive ATPase (Tzagoloff et al., 1968; Berden & Voorn-Brouwer, 1978), complex V (Stiggall et al., 1978), or "ATP–Pi exchangease" (Sadler et al., 1974). It contains several polypeptides in addition to F_1 subunits, and these extra polypeptides collectively form the membrane sector or F_0.

According to current theories of energy transduction, the oligomycin-sensitive ATPase acts as a reversible proton pump and the membrane sector contains a channel for transporting protons from one side of the membrane to the other (Mitchell, 1977; Kagawa, 1978). Such a function requires that the complex spans the mitochondrial inner membrane in a particular orientation. Labeling studies using [^{35}S]DABS (Schneider et al., 1972), antibody inhibition studies (Christiansen et al., 1969), and electron microscopic studies (Kagawa & Racker, 1966b) all show that F_1ATPase is present on the matrix face of the inner membrane, but the disposition of the membrane sector and the arrangement of polypeptides within it remain a matter of conjecture.

Here we describe studies in which mitochondria and submitochondrial particles have been reacted with [^{35}S]DABS. The ATP synthetase complex has been isolated by immunoprecipitation, and the labeling of polypeptides from the cytoplasm and matrix sides of the inner membrane has then been examined.

Experimental Procedures

Enzyme Preparations. Oligomycin-sensitive ATPase preparations were isolated according to Tzagoloff et al. (1968), Sadler et al. (1974), Serrano et al. (1976), and Berden &

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; DABS, diazobenzenesulfonate; IgG, immunoglobulin; DCCD, dicyclohexylcarbodiimide; OSCP, oligomycin sensitivity conferring protein; OSATPase, oligomycin-sensitive adenosine triphosphatase.

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Voorn-Brouwer (1978). A preparation of complex V was the kind gift of Dr. Y. Galante. F_1ATPase was isolated according to Senior & Brooks (1970) or according to Beechey et al. (1975). OSCP was prepared by the method of MacLennan & Tzagoloff (1968). Inhibitor protein was the kind gift of Dr. A. E. Senior, University of Rochester.

Mitochondria were isolated from beef hearts according to Smith (1967) except that phosphate was substituted for Tris when preparations were to be labeled by DABS and EDTA (20 mM) was added. Only fresh mitochondria were used in labeling experiments. Submitochondrial particles were obtained by sonication of a mitochondrial suspension (20 mg/mL) in SPMS buffer (0.25 M sucrose, 10 mM phosphate, 1 mM $MgCl_2$, and 1 mM succinate, pH 7.8) 3 times for 15 s at full setting in an MSE sonicator. The suspension was centrifuged at 10000*g* for 10 min in a Sorvall SS 34 and the pellet discarded. The supernatant was centrifuged at 78000*g* for 30 min, and the pellet was suspended in SPMS buffer. The yield of submitochondrial particles by this mild disruption procedure was 5–15% of starting mitochondria. Antibodies against F_1ATPase were prepared as described by Ludwig & Capaldi (1979).

Labeling with [^{35}S]DABS. [^{35}S]Diazobenzenesulfonate (5–9 Ci/mmol) was prepared from [^{35}S]sulfanilic acid (Amersham/Searle) as described by Tinberg et al. (1974). Isolated F_1ATPase and purified OSATPase were diluted in 20 mM sodium phosphate, pH 7.8, or in 3% $NaDodSO_4$ and 20 mM sodium phosphate, pH 7.8, and reacted with [^{35}S]DABS at room temperature for 30 min. The reaction was stopped by addition of an equal volume of 10 mM histidine. The mixture was then dialyzed repeatedly against 1% $NaDodSO_4$, 20 mM Tris-HCl, and 5 mM histidine, pH 7.8.

Mitochondrial membranes were labeled at 10 mg/mL in a buffer of 0.25 M sucrose and 10 mM sodium phosphate, pH 7.8, containing 40 μM DABS for 20 min at room temperature. The reaction was stopped by addition of an equal volume of buffer containing 0.25 M sucrose, 20 mM Tris-HCl, and 10 mM histidine, pH 7.8. Membranes were washed by centrifugation in the quenching buffer and then stored at −70 °C in small aliquots suspended to 20 mg/mL in the same buffer.

Membrane Solubilization. Membranes were incubated at 1 mg/mL in 1% Triton X-100, 0.1 M KCl, 0.05% sodium azide, 2% methanol, 0.2 mM EDTA, 0.2 mM ATP, and 50 mM Tris-HCl, pH 7.4, for 40 min at 0 °C, and then this solution was centrifuged at 78000*g* for 45 min to remove unsolubilized material. F_1ATPase antiserum was added, and immunoprecipitation was allowed to occur overnight at 0 °C. The pellet was collected by centrifugation at 10000*g* for 20 min and washed twice in distilled water before being solubilized in 5% $NaDodSO_4$, 4 M urea, and 2.5% β-mercaptoethanol by heating at 100 °C for 2 min.

Labeling of OSATPase with [^{14}C]DCCD. OSATPase (10 mg/mL) was labeled with 30 μM [^{14}C]DCCD (50 mCi/mmol) in 250 mM sucrose and 10 mM Tris-HCl, pH 7.5, at 4 °C for 5 h. The complex was precipitated with 50% ammonium sulfate and collected by centrifugation at 20000*g* for 15 min. The pellet was resuspended in sucrose–histidine buffer and precipitated a second time with ammonium sulfate. The protein was finally dissolved in 5% $NaDodSO_4$, 4 M urea, and 2.5% β-mercaptoethanol and incubated at 37 °C for 30 min for gel electrophoresis.

Gel Techniques. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed by the procedure of Swank & Munkres (1971) or Weber & Osborn (1969). Gels were stained and destained as described by Downer et al. (1976). Radioactive gels were sliced in 1-mm thick slices with a Mickle gel slicer. These slices were dissolved in 1 mL of 15% H_2O_2 at 80 °C overnight. Seven milliliters of a solution of Omnifluor (NEN), 2.66 g/L in toluene–Triton X-100 (2:1), was added to each vial, and the radioactivity was measured in a Packard liquid scintillation counter.

Other Methods. Protein was estimated as described by Lowry et al. (1951). ATPase activity was measured according to Serrano et al. (1976). ATP and Triton X-100 were obtained from Sigma.

Results

Polypeptide Composition of OSATPase. Conditions for maximal resolution of the subunits of OSATPase were explored as a preliminary to labeling experiments and in order to fully characterize the immunoprecipitated enzyme. Several different preparations of OSATPase have been described, all of which contain at least 10 different component polypeptides when analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis using the Weber & Osborn (1969) gel system (Stekhoven, 1972; Capaldi, 1973; Sadler et al., 1974; Serrano et al., 1976; Stiggall et al., 1978; Berden & Voorn-Brouwer, 1978). Five of the components are the subunits of F_1ATPase, one is OSCP, and the remainder are concluded to be a part of the membrane sector or F_0 portion of the complex (Capaldi, 1973; Serrano et al., 1976; Berden & Voorn-Brouwer, 1978).

We have examined several different preparations of OSATPase made with bile salts [according to Tzagoloff et al. (1968) and Serrano et al. (1976)], lysolecithin (Sadler et al., 1974), or Triton X-100 (Berden & Voorn-Brouwer, 1978) and confirm that the polypeptide compositions of different preparations are qualitatively similar in Weber–Osborn-type gels. As shown in Figure 1, OSATPase prepared according to Berden & Voorn-Brouwer (1978) was resolved into 10 major bands in this gel system. Berden & Voorn-Brouwer (1978) have examined their preparation of OSATPase by using gel electrophoresis in the Swank & Munkres (1971) buffer system. In agreement with these workers, we see several bands which were not resolved in the Weber–Osborn-type gels (Figure 1).

In our studies of the structure of electron transfer complexes, we have found that two-dimensional gels in which enzyme is electrophoresed in the Weber & Osborn (1969) gel system in the first dimension and in the Swank & Munkres (1971) gel system in the second dimension often resolve components not seen in either one-dimensional system (Capaldi et al., 1977). The slab gel in Figure 1 shows a two-dimensional gel of the Berden & Voorn-Brouwer (1978) preparation of OSATPase. Eighteen different polypeptides were seen in this system when large amounts of protein were applied to the gels (i.e., 350–500 μg). OSATPase isolated in bile salt as described by Serrano et al. (1976) (shown in Figure 2), enzyme isolated with lysolecithin as the solubilizing detergent (Sadler et al., 1974) (result not shown), and complex V prepared according to Stiggall et al. (1978) (results not shown) were examined by two-dimensional gel electrophoresis and found to contain all of the components seen in the Berden & Voorn-Brouwer (1978) preparation.

Small amounts of F_1ATPase, the inhibitor protein, and OSCP were coelectrophoresed with the total complex firstly in one-dimensional gels and where necessary in two-dimensional gels (e.g., for the inhibitor protein) in order to identify the various bands or spots on the gels. These identifications are shown in Figure 1 and are listed in Table I. The optimal separation of OSCP (band 6) and band 7 (a component of unknown function) was obtained on 20% gels (and Swank–Munkres gel conditions) as shown in Figure 3. The proteolipid

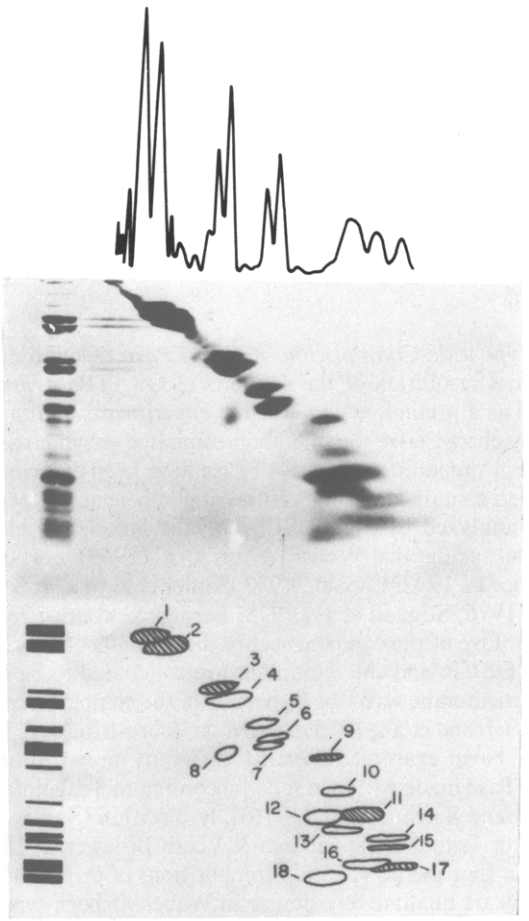

FIGURE 1: Two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis of OSATPase prepared according to Berden & Voorn-Brouwer (1978). 350 μg of OSATPase was electrophoresed on Weber & Osborn (1969) type gels [13% acrylamide and 0.433% bis(acrylamide)] in a tube gel for the first-dimension separation. Duplicate samples were run; one was stained and is shown in the upper gel trace, and the second was cemented onto a slab gel 5-mm thick made with 15% acrylamide 0.5% bis(acrylamide) and electrophoresed in the Swank & Munkres (1971) buffer system (second dimension). A standard of 180 μg of OSATPase was run on the left-hand side of the gel. Polypeptides are numbered in the order they migrated in the second dimension. Shaded spots 1, 2, 3, 9, and 17 are the five subunits of F_1ATPase; 11 is the inhibitor protein (see text).

protein or DCCD binding protein was identified after reacting OSATPase with [^{14}C]DCCD (Figure 4). The major peak of radioactivity in DCCD-labeled OSATPase was band 16 (apparent M_r 8500), and there were other much smaller peaks of radioactivity in the positions of bands 2, 4, and 10 on gels (with apparent molecular weights of 44 000, 30 000, and 18 000, respectively). These could indicate aggregates of the DCCD binding protein not dissociated under our conditions of gel electrophoresis [see also Tzagoloff & Akai (1972) and Graf & Sebald (1978)]. Alternatively, other components of OSATPase may have buried carboxyls which can react with DCCD, albeit with a much lower affinity for the reagent than the proteolipid protein [see, for example, Pougeois et al. (1979)].

Ten bands on gels were not identified with previously purified components of OSATPase. One of these, band 4, is probably the so-called uncoupler binding protein which has been covalently labeled by a radioactive derivative of 2-azido-4-nitrophenol (Hanstein, 1976). Other bands may be coupling factor B (Lam & Yang, 1969; Lam et al., 1969) and F_6 (Kanner et al., 1976), which has been implicated in binding F_1ATPase to the membrane.

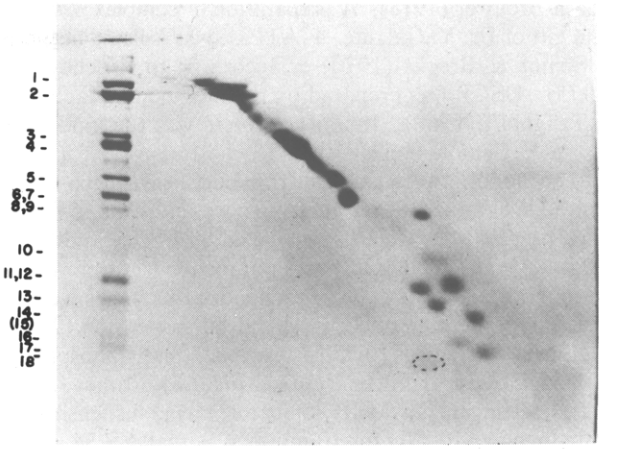

FIGURE 2: Two-dimensional NaDodSO$_4$–polyacrylamide gel of OSATPase prepared according to Serrano et al. (1976). 360 μg of protein was used for the two-dimensional analysis; 180 μg was used as a standard on the slab. The gel conditions were the same as those described in the legend to Figure 1. The dotted line shows the position of component 18 seen on the slab but not evident in the photograph.

Table I: Molecular Weights and Identification of the Polypeptides of OSATPase

band no.	mol wt from Weber–Osborn gels	mol wt from Swank–Munkres gels	mol wt from amino acid comp[a]	identification
1	54 000	48 000	60 200	α
2	48 500	44 000	53 300	β
3	31 500	33 000	33 160	γ
4	29 000	30 500		
5	24 500	25 000		
6	21 500	20 800		OSCP
7	22 000	20 500		
8	29 000	20 000		
9	15 000	19 500	16 100	δ
10	13 000	14 700		
11	10 000	12 300		IP
12	15 500	12 300		
13	13 000	11 800		
14	7 000	10 700		
15	7 000	10 200		
16	10 000	8 500	7 627	DCCD
17	7 000	7 700	5 850	ε
18	6 500	6 500		

[a] Calculated from Knowles & Penefsky (1972).

Antibody Precipitation of OSATPase from the Mitochondrial Inner Membrane. For labeling experiments involving high levels of radioactivity, it is convenient to have a simple one-step purification of the enzyme being studied. Therefore, an antibody was raised with which to selectively immunoprecipitate OSATPase from the detergent-solubilized inner membranes. Chloroform-treated F_1ATPase (Beechey et al., 1975) was used as an antigen, and antibodies against this fraction have now been raised in four different rabbits. The purification and full characterization of the ATPase antibody have been described elsewhere (Ludwig & Capaldi, 1979).

A two-dimensional gel of the immunoprecipitate obtained by reacting the F_1ATPase antibody with Triton X-100 solubilized submitochondrial particles is shown in Figure 5. The major bands on the gel are the two subunits of IgG. In addition, all of the bands seen in conventionally isolated preparations of OSATPase are present, with the possible exception of band 4 (M_r 29 000), which runs very close to the small subunit of IgG and is not clearly discernible in the gels.

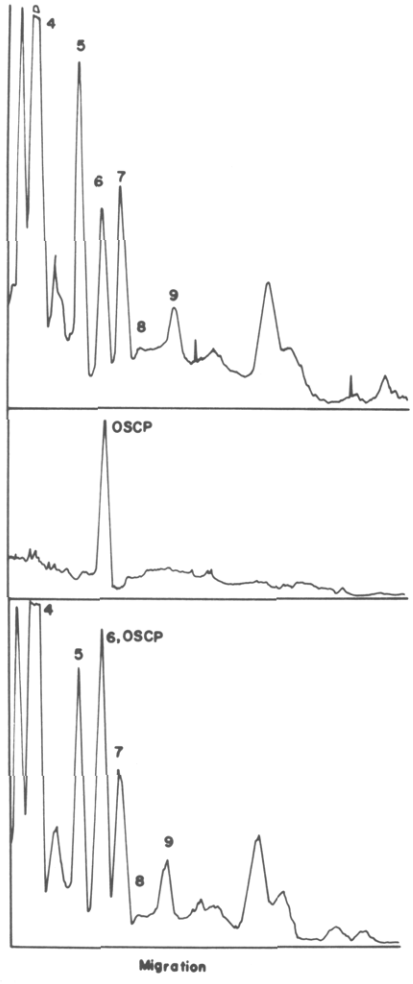

FIGURE 3: OSATPase prepared according to Berden & Voorn-Brouwer (1978) run on 20% polyacrylamide gels [20% acrylamide and 0.67% bis(acrylamide)] under the Swank & Munkres (1971) buffer conditions. Upper trace, 68 μg of OSATPase; middle trace, 7 μg of OSCP; lower trace, 68 μg of OSATPase + 7 μg of OSCP.

OSATPase immunoprecipitated from detergent-solubilized mitochondria was qualitatively similar in polypeptide composition to that shown in Figure 5 except for containing considerably more of the δ subunit of F_1ATPase. This result suggests that the δ subunit is released during preparation of submitochondrial particles (perhaps during sonication), a possibility which warrants further study because of the controversy about the stoichiometry of subunits in F_1ATPase [see Senior (1979)].

Labeling of F_1ATPase and OSATPase with [^{35}S]DABS. Purified F_1ATPase and isolated OSATPase were both reacted with [^{35}S]DABS in order to determine the relative exposure of subunits to the aqueous medium. Samples of each preparation were divided into two equal aliquots, one was diluted with buffer (native enzyme) and the other with 1% $NaDodSO_4$ to effect a denaturation of the protein, and then both were reacted with an equal amount of [^{35}S]DABS. All of the subunits of F_1ATPase were labeled in native F_1ATPase (Figure 6, upper trace) and in OSATPase (Figure 7, upper trace). The reactivity of individual subunits was quantitated by summing the number of counts incorporated after labeling the native enzyme and dividing this by the number of counts incorporated in the $NaDodSO_4$-treated sample. These values, expressed as percentages, are given in Table II. Clearly, the α subunit and, to a smaller extent, the γ and ϵ subunits are less reactive to DABS when F_1ATPase is associated with the membrane sector (in OSATPase) than when it is free in solution.

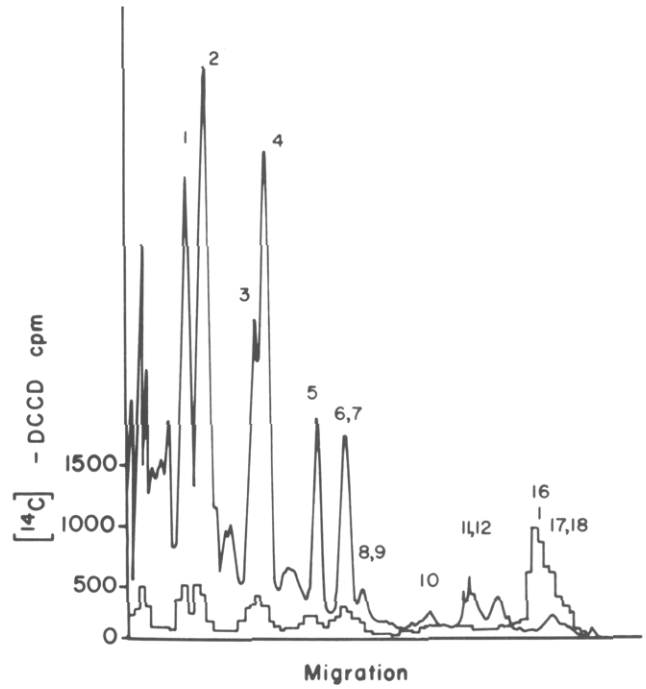

FIGURE 4: Reaction of OSATPase with [^{14}C]DCCD. Labeled protein was run on a 15% gel (1:30 cross-linker). The figure shows the trace of Coomassie blue labeling in the gel as well as the distribution of counts as a bar graph.

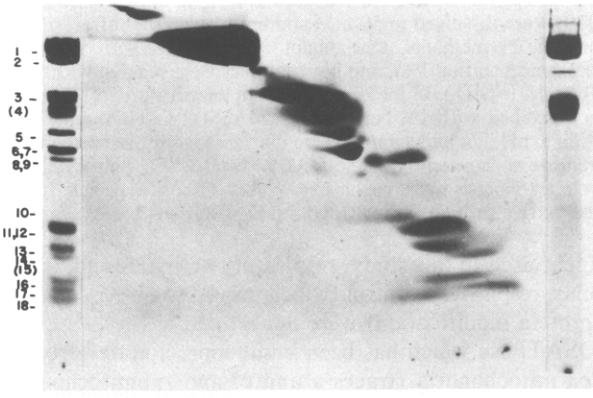

FIGURE 5: Two-dimensional $NaDodSO_4$–polyacrylamide gel electrophoresis of OSATPase immunoprecipitated from submitochondrial particles by an antibody raised against purified F_1ATPase. The standard on the left is immunoprecipitated enzyme; the standard on the right is purified IgG. Gel conditions were the same as those used in Figure 1.

Other polypeptides labeled in OSATPase included components 4, 5, 6, and 7. There was also a broad band of radioactivity in the region of the gel containing components 11–18, but none of these components were heavily labeled by the water-soluble protein-modifying reagent.

Labeling of OSATPase in the Mitochondrial Inner Membrane. [^{35}S]DABS was also used to label OSATPase while in the mitochondrial inner membrane. For these experiments, freshly isolated mitochondria were used as a source of membranes with the cytoplasmic surface available for labeling and submitochondrial particles were used as a preparation with the matrix side of the membrane outermost. The effect of DABS on the functional activity of the mitochondrial inner membrane has been examined in connection with our studies on the orientation of complexes II and III and cytochrome *c* oxidase, and comprehensive data are published in Merli et al. (1979). Briefly, these studies have shown that at 4 nmol of DABS per mg of protein, the level of protein-modifying

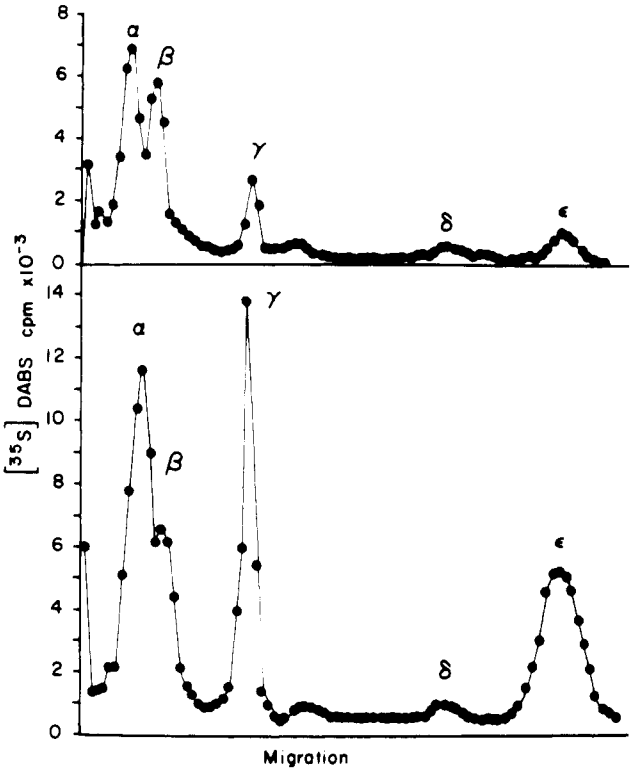

FIGURE 6: Labeling of F_1ATPase with [^{35}S]DABS. Two aliquots (each 300 μg) of F_1ATPase prepared according to Senior & Brooks (1970) were dissolved in 50 mM sodium phosphate buffer, pH 7.8, containing 2% methanol. One aliquot was dissociated with $NaDodSO_4$ (final concentration 1%), and both samples were reacted with 15 μL of 0.4 mM [^{35}S]DABS for 20 min at room temperature. The reaction was quenched with 1% $NaDodSO_4$, 50 mM Tris-HCl, and 5 mM histidine, pH 7.6, and samples were dialyzed against the same buffer to remove noncovalently bound DABS. $NaDodSO_4$–polyacrylamide gel electrophoresis was done on 13% Weber–Osborn-type gels. Upper trace, native protein; lower trace, $NaDodSO_4$-treated enzyme.

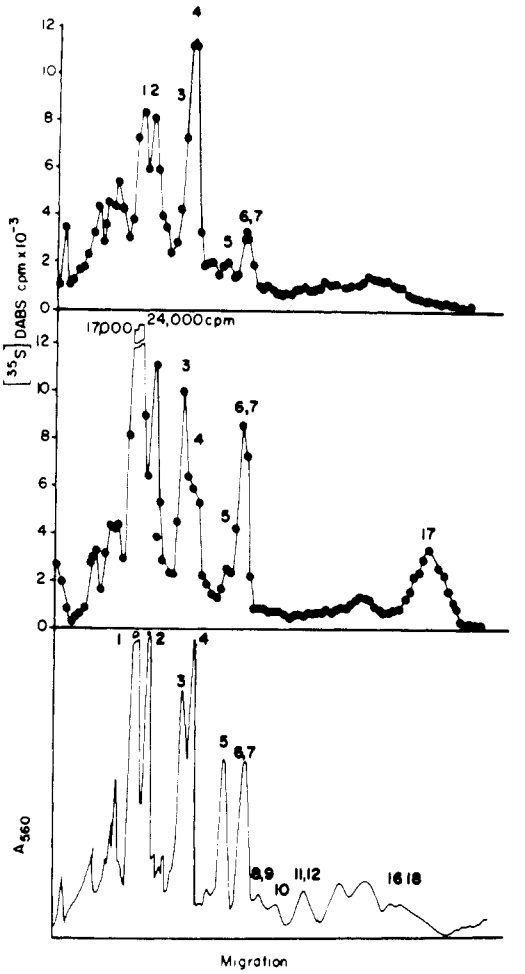

FIGURE 7: Labeling of OSATPase with [^{35}S]DABS. OSATPase prepared according to Berden & Voorn-Brouwer (1978) dissolved in 0.2% Triton X-100, 0.1 M sodium sulfate, and 50 mM sodium phosphate, pH 7.8, containing 2% methanol was labeled with [^{35}S]DABS in the native state (upper trace) and after $NaDodSO_4$ treatment (middle trace) as described in the legend to Figure 6. Gel electrophoresis was conducted in Swank–Munkres-type gels (15% acrylamide, 1:30 cross-linker). The lower trace shows the Coomassie blue staining profile of the $NaDodSO_4$-treated sample.

reagent used in this study, respiratory activities and, in particular, respiratory control (which proved to be very sensitive to protein modification) were not altered.

OSATPase which has been immunoprecipitated from labeled mitochondria (traces a and c) and submitochondrial particles (traces b and d) is shown in Figure 8. The data presented are from a typical experiment in which 5 mg of mitochondria and 2.5 mg of submitochondrial particles were reacted with [^{35}S]DABS. These preparations were solubilized by Triton X-100, and then F_1ATPase antibody was added to precipitate down all of the ATPase. The immunoprecipitates were run on gels, and these gels were first stained and destained (traces c and d of Figure 8) and then sliced and counted (traces a and b of Figure 8). Clearly, the extent of labeling of OSATPase is much greater in submitochondrial particles than in mitochondria (the gels in Figure 8 contain about the same amount of protein as judged by the staining profiles). This is in contrast to the findings for cytochrome *c* oxidase where the majority of the labeling was from the cytoplasmic side of the membrane (Ludwig et al., 1979) or for complex III in which the labeling from the outer and inner surfaces of the mitochondrial inner membrane was approximately equal in amount (Bell et al., 1979). (Experiments on cytochrome *c* oxidase and complex III were done with the same batches of [^{35}S]DABS-labeled membranes that were used for the studies reported here.)

The labeling of OSATPase in mitochondria is in three bands, two of which, bands 2 and 11/12, are components of the protein complex; the third peak, M_r 40 000, is a high

Table II: Reactivities of the Subunits of F_1ATPase to [^{35}S]DABS[a]

prepn	subunits				
	α	β	γ	δ	ε
F_1ATPase	50	100	20	50	20
OSATPase	20	100	10		10

[a] Values given are the number of counts in native enzyme divided by the number of counts in the $NaDodSO_4$-treated enzyme expressed as a percentage and corrected so that the reactivity of the β subunit is arbitrarily set at 100%.

labeled impurity seen as a very small peak in the Coomassie blue stained profile.

The extent of labeling of band 2, the β subunit of F_1ATPase, was 5–13% (five experiments) of that obtained by reaction of [^{35}S]DABS with submitochondrial particles. As our mitochondrial preparations were only around 90–95% intact [as measured by NADH oxidase activity; see Merli et al. (1979)], this small amount of labeling of the β subunit is probably with inside-out vesicles or in leaky mitochondria.

The labeling of band 11/12 is likewise small in comparison with the extent of labeling of this band in submitochondrial particles. However, if we take the labeling of band 2 as a

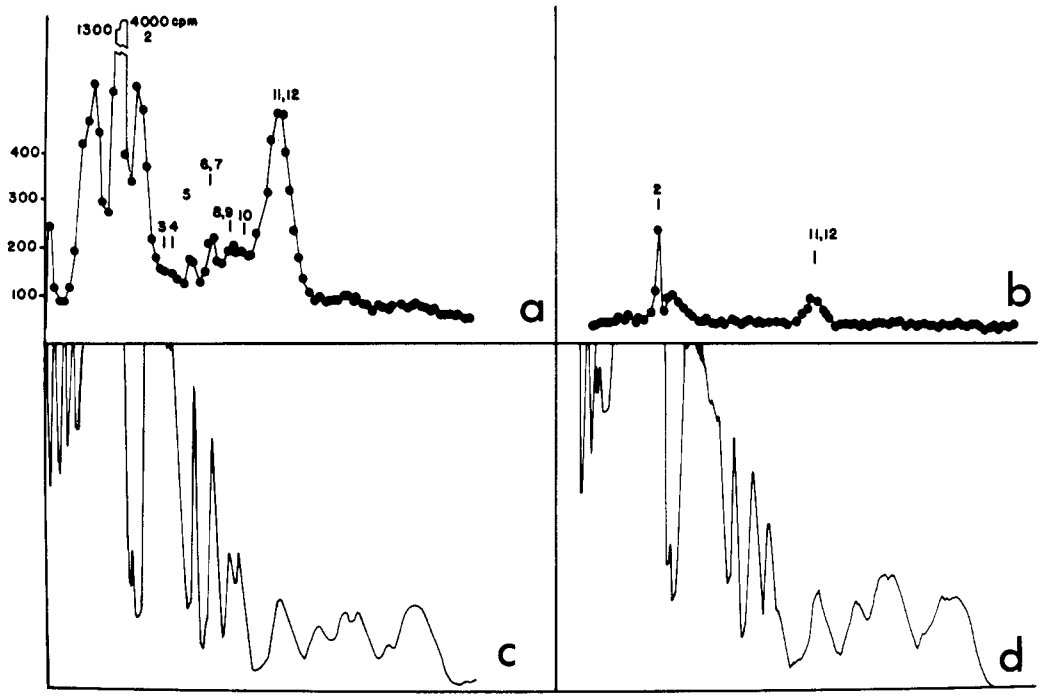

FIGURE 8: [^{35}S]DABS labeling of OSATPase in the membrane. Labeled mitochondria (traces b and d) and labeled submitochondrial particles (traces a and c) were solubilized in Triton X-100 and KCl (for details, see Experimental Procedures) and incubated with optimal amounts of the F_1ATPase antibody. The washed immunoprecipitates were dissociated in 4% $NaDodSO_4$, 8 M urea, 20 mM Tris–phosphate, pH 6.8, and 2% β-mercaptoethanol for 1 h at 37 °C and subjected to electrophoresis in 15% gels by the Swank–Munkres buffer system. The upper traces are radioactive profiles, and the lower traces are Coomassie blue staining profiles.

measure of the leakiness and/or the amount of wrongly oriented membranes, then the labeling of band 11/12 in mitochondria is significant because it is much higher in relation to the labeling of band 2 in this preparation than it is in submitochondrial particles. (The labeling of band 11/12 is 80% of that of band 2 in mitochondria but less than 20% of that in band 2 in submitochondrial particles.) The two components of band 11/12 are the ATPase inhibitor protein and a membrane sector polypeptide of as yet unknown function. The former binds tightly to F_1ATPase and should be localized on the matrix side. This leaves component 12 as the polypeptide exposed on the cytoplasmic side of the membrane.

Both the α and β subunits of F_1ATPase were labeled in submitochondrial particles. It was not possible to quantitate the reactivity of these subunits to DABS in the membrane experiments. However, from trace c of Figure 8, it is clear that the extent of labeling of the α subunit in relation to the β subunit was much less in submitochondrial particles than in isolated OSATPase or F_1ATPase. The γ, δ, and ϵ subunits of F_1ATPase were not visibly labeled in submitochondrial particles. There was labeling of band 11/12 probably in the inhibitor protein. The band containing OSCP (band 6 or 7) was labeled, and the membrane sector component 5 also incorporated a significant number of counts from the matrix side.

Discussion

Purified preparations of OSATPase all show ATP hydrolysis activity, and in addition most show an ATP–Pi exchange activity thought to be a partial reaction of the ATP synthesis reaction [see Senior (1973, 1979), Pedersen (1975), and Panet & Sanadi (1976) for reviews]. Here we have examined the subunit structure of oligomycin-sensitive ATPase made by several different procedures and the composition of enzyme immunoprecipitated from the mitochondrial inner membrane with antibodies made against F_1ATPase. Optimal resolution of the various polypeptides in all of these different preparations required the two-dimensional gel electrophoresis system developed in our recent studies of the mitochondrial electron transport complexes (Capaldi et al., 1977). All of the preparations we examined were very similar and contained five subunits of F_1ATPase with molecular weights of 54 000, 48 500, 31 500, 15 000, and 7000 on Weber–Osborn gels and 48 000, 44 000, 33 000, 19 500, and 7700 on Swank–Munkres gels. The stoichiometry of these five polypeptides in the complex is still an unanswered question. Senior & Brooks (1971) proposed a stoichiometry of 3:3:1:1:1 for the five subunits of beef heart F_1ATPase based upon Coomassie blue labeling. *N*-Ethylmaleimide labeling experiments in contrast indicated a 2:2:2 ratio for the α, γ, and ϵ subunits (Senior, 1975).

Other well-defined polypeptides found in OSATPase include the inhibitor protein and OSCP. The inhibitor protein was present in a large amount in OSATPase made by the method of Berden & Voorn-Brouwer (1978) and in enzyme immunoprecipitated from Triton X-100 solubilized membranes with the F_1ATPase antibody. It was present in a lower amount in preparations made with bile salts such as the procedure of Serrano et al. (1976). OSCP was present in all preparations, migrating with an apparent molecular weight of around 21 000 on both Weber–Osborn and Swank–Munkres gels. In the latter gel system, OSCP ran as the larger molecular weight component of a closely spaced doublet. Eleven polypeptides in addition to those listed above were resolved by our gel procedures. Of these, only the 29 000-dalton component was completely missing in any preparation and this was from enzyme immunoprecipitated from the inner membrane with antibody made against F_1ATPase. Two other components, 15 and 16, were present in variable amounts in different preparations and could be contaminants.

A comparison of the polypeptide composition of OSATPase from beef heart (this study) with the complex isolated from

Table III: Polypeptide Composition by Molecular Weight of OSATPase Isolated from Different Sources

	beef heart (this study)	*N. crassa* (Sebald, 1977)	yeast (Ryrie & Gallagher, 1979)	chloroplast (Pick & Racker, 1979)	PS_3 (Sone et al., 1975	*E. coli* (Foster & Fillingame, 1979)
F_1 ATPase						
α	54 000[a]	59 000	52 000	59 000	56 000	55 000
β	48 000[a]	56 000	48 000	55 000	53 000	50 000
γ	33 000	36 000	31 000	37 000	32 000	37 000
δ	19 500	15 000	14 500	17 500	13 500	20 000
ϵ	7 700	12 000	10 700	13 500	11 000	12 000
inhibitor protein	12 300	[b]	7 000			
OSCP	20 800	22 000	23 000			
membrane sector	25 000	21 000	28 500	17 500	19 000	24 000
	20 500	19 000	24 500	15 500	13 500	19 000[c]
	20 000	16 000	21 500	13 500	5 400	8 400
	14 700	8 000	16 700	7 500		
	12 300		12 700			
	11 800		9 000			
	8 500					
	6 500					

[a] Molecular weight from Weber–Osborn gels as opposed to Swank–Munkres gels used for other components. [b] Inhibitor does not precipitate with the purified enzyme. [c] Recent studies have led Kagawa and colleagues to suggest that the 19 000-dalton component is an impurity of preparations (Sone et al., 1978).

procaryotes (Sone et al., 1975; Foster & Fillingame, 1979), yeast (Ryrie & Gallagher, 1979), *Neurospora crassa* (Sebald, 1977), and chloroplasts (Pick & Racker, 1979) shows the added complexity of the mammalian enzyme (Table III). This could of course reflect the fact that none of the procedures used to date isolate OSATPase from beef heart (or rat liver) mitochondria in a pure form. Alternatively, there are several indications that mitochondrial OSATPase is more complex than the enzyme from chloroplasts or procaryotes. For example, there is a distinct inhibitor protein for the F_1ATPase in mitochondria while in chloroplasts and procaryotes the ϵ subunit appears to serve this function (Nelson et al., 1972; Smith & Sternweis, 1977). Also, mitochondrial ATPase contains OSCP, a component not identified so far in OSATPase from chloroplasts or procaryotes. It is not unreasonable then that the membrane sector may contain additional components.

The gross organization of the mitochondrial OSATPase complex has been determined by electron microscopic studies (Soper et al., 1979). The F_1ATPase portion is seen as a globular protein of 90-Å diameter extending from the matrix side of the mitochondrial inner membrane. The membrane sector is considered to be an intrinsic part of the membrane continuum. In some electron micrographs a stalk is observed which is considered to contain OSCP (MacLennan & Asai, 1968) and possibly F_6 (Knowles et al., 1971; Kanner et al., 1976) as both of these polypeptides have been shown to be involved in binding of F_1ATPase to the membrane sector.

The labeling studies reported here are our first effort toward providing a more detailed picture of the structure of OSATPase. They confirm that most of the complex including the F_1ATPase portion is on the matrix side of the membrane. The very poor labeling of OSATPase in mitochondria suggests that very little of this complex is exposed on the cytoplasmic side of the inner membrane.

Considering the F_1ATPase protein first, the α and β subunits were found to label very differently in different preparations of the enzyme. In purified F_1ATPase, the α subunit incorporated about one-half of the number of counts as the β subunit; in OSATPase it incorporated only about one-fifth of the number of counts and in submitochondrial particles about one-tenth of the number of counts as the β subunit. There are two possible explanations for these results. It could be that binding of F_1ATPase to the membrane sector alters the conformation of the enzyme so that the α subunit becomes more buried within the globular unit itself. Alternatively and more likely, the binding of F_1ATPase to the membrane sector could result in the shielding of the α subunit. In this connection, Leimgruber et al. (1978) have reported that the α subunit of F_1ATPase from *Staphylococcus aureus* is cleaved by chymotrypsin when the enzyme is free in solution but is protected from the action of the protease when F_1ATPase is bound to the membrane. These workers have concluded that the α subunit is involved directly in membrane attachment in this organism.

The labeling of both the γ and ϵ subunits is also lower in OSATPase and with the membrane-bound complex than it is in isolated F_1ATPase. It appears therefore that these subunits are partly shielded from water by binding to the membrane sector.

In contrast to the F_1ATPase portion, the membrane sector of OSATPase (not including the 29 500-dalton polypeptide) was poorly labeled by [^{35}S]DABS both in the isolated enzyme and in the inner membrane preparations. One component at least was labeled from the matrix side (component 5) and another (component 11) was labeled from the cytoplasmic side. The membrane sector therefore spans the inner membrane but probably with the major portion of the protein within the bilayer.

DABS is a rather specific protein-modifying reagent, reacting only with Lys, His, Tyr, and Cys residues. It is possible then that a significant portion of the membrane sector is exposed but without these particular amino acids present. For this reason we have begun experiments with *N*-(4-azido-2-nitrophenyl)-2-aminoethyl[^{35}S]sulfonate (NAP-taurine), a water-soluble, lipid-insoluble, protein-modifying reagent in which the reactive group is a photoactivated nitrene. The studies of Matheson et al. (1977) have shown that NAP-taurine is less specific with respect to which amino acids are modified. Our preliminary studies show a labeling pattern for isolated OSATPase with NAP-taurine which is very similar to that with DABS: that is, with very little labeling of membrane sector polypeptides (Prochaska et al., 1980). This is added evidence that the membrane sector is mainly bound within the bilayer. Finally, Montecucco et al. (1979) have recently described experiments using arylazidophospholipids

to identify directly those polypeptides intercalated into the lipid bilayer for membranous OSATPase. They found very heavy labeling of the membrane sector polypeptides and in particular the extremely hydrophobic DCCD binding protein (Graf & Sebald, 1978) but no labeling of the F_1ATPase portion of the complex.

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