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ANALYSIS OF THE PROTEIN SUBUNIT STRUCTURE OF THE OLIGOMYCIN SENSITVE ATPASE PROTEOLIPID FRACTION BY REVERSE PHASE HIGH PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY: The proteolipid fraction which is obtained from the bovine mitochondrial oligomycin sensitive ATPase complex by extraction with chloroform:methanol is resolvable into 7 components by preparative reverse phase high pressure liquid chromatography. Each of these 7 components is present together with 4 additionally resolved components in the proteolipid fraction which is obtained by extraction of submitochondrial particles with chloroform: methanol. Of the 7 components derived from the oligomycin sensitive ATPase, 4 have been identified with known protein subunits of the membrane sector of this complex, 2 are newly documented subunits of this complex, and 1 remains uncharacterized.

The N,N'-dicyclohexylcarbodiimide (DCCD)* binding proteolipid fraction from bovine heart mitochondria, which appears homogeneous during SDS-urea polyacrylamide gel electrophoresis, has recently been resolved by reverse phase high pressure liquid chromatography (HPLC) into three component proteins of similar size (1). These have been designated HPα (7.0 K daltons), HPβ (7.2 daltons), and HPγ (6.8 K daltons), respectively. HPα was identified as the DCCD binding subunit (1) which is known (2) to be a component of the membrane sector (F_0) of the oligomycin sensitive ATPase (OS-ATPase). HPβ and HPγ were found to be capable of uncoupler sensitive binding of stoichiometric levels of ADP and inorganic phosphate; however, their relationship to the OS-ATPase complex remained unknown (1). Since the latter two subunits i.e., HPβ and HPγ, appeared to be functionally qualified as subunits of the OS-ATPase complex, it was decided to test this hypothesis directly by chloroform: methanol extraction of the complex itself and subsequent resolution of the proteolipid as had been previously carried out with similarly derived proteolipid from submitochondrial particles (ETP). Here, we also document a new HPLC gradient elu-

*ABBREVIATIONS USED: DCCD, N,N'-dicyclohexylcarbodiimide; HPLC, high pressure liquid chromatography; F_0, membrane sector of oligomycin sensitive ATPase; OS-ATPase, oligomycin sensitive ATPase; ETP, electron transfer particle; OSCP, oligomycin sensitivity conferring protein.

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tion sequence which has enabled us to resolve the entire proteolipid fraction from both ETP and the OS-ATPase complex without the preliminary requirement for molecular sizing by gel filtration on Sephadex LH-60 (1). This latter procedure has enabled us to document the finding that 7 out of the 11 component proteins found in proteolipid from ETP are derivable from the OS-ATPase complex.

METHODS

PREPARATIONS: The preparation of bovine heart mitochondria and submitochondrial particles has been described elsewhere (3,4). Proteolipid was prepared from a suspension of ETP at pH 6.5 by the method of Cattell et al. (5) with the modifications introduced by Fillingame (6). The OS-ATPase complex was prepared by the method of Berden and Voorn-Brouwer (7) with the following modification: the solid sodium sulfate was added to the Triton incubation mixture of step 1 at room temperature prior to the addition of the ETP suspension. The entire mixture was then cooled to 5° during the recommended 5 minute incubation period. Proteolipid was prepared from the OS-ATPase complex as described above for ETP except that the pH of the suspension was adjusted to 6.0 prior to extraction with chloroform: methanol.

PROCEDURES: Protein concentration was determined by the method of Lowry et al. with 0.2% deoxycholate in the assay (8). The method developed by Swank and Munkres (9) for SDS-urea gel electrophoresis in highly crosslinked polyacrylamide gels was employed as described previously (1). The following molecular weight markers were used in the gel electrophoresis procedure for the determination of subunit size: ovalbumin (45,000), D-amino acid oxidase (37,000), chymotrypsinogen (25,000), myoglobin (18,500), cytochrome c (12,500), and insulin (5,700).

CHROMATOGRAPHY: Preparative reverse phase HPLC was carried out as previously described (1) with Whatman Partisil-10-ODS-2 contained in a 9 mm by 50 cm stainless steel column. The linear gradient time was set at 45 minutes and the composition of the solvent system was as follows: solvent B contained 0.32 M potassium acetate, pH 6.5, in methanol:water (75:25), and solvent A contained 0.32 M potassium acetate, pH 6.5, and 8% water in chloroform:methanol (2:1). After injection of the sample, the first 24 ml of effluent was collected at a flow rate of 2.0 ml per min., while the remainder of the sequence was carried out at a flow rate of 3.0 ml per min.

SAMPLE PREPARATION: Proteolipid samples were prepared for HPLC by dissolving the twice ether precipitated proteolipid in chloroform:methanol (2:1) followed by evaporation at 25°C of the constant stirring solution under a stream of nitrogen until the volume had been reduced to approximately one fourth the original. This procedure produces a solution with a density of 1.05 equivalent to a chloroform: methanol composition of approximately 1:2. Further removal of chloroform generally causes excessive loss of proteolipid by precipitation. Sufficient solvent is added initially in order to give a final protein concentration of approximately 3 mg/ml for OS-ATPase proteolipid and 5 mg/ml for ETP proteolipid. The final solution is centrifuged at 2000 rpm for 10 minutes in order to remove insolubles. The fractions derived from HPLC were assayed for protein directly after removal of organics by evaporation. However, prior to gel electrophoresis of the collected components, it was found necessary to remove potassium acetate. Although this was most easily accomplished by evaporation of the organics and washing of the resulting precipitate with H_2O, certain of the components failed to precipitate. A more reproducible, albeit tedious, procedure (6) involved adjusting the final chloroform:

methanol:H_2O concentration of each component to 8:4:3. (A 20 ml system space volume was used to calculate the solvent composition from the indicated gradient value.) After vortexing the resulting biphasic solution, the phases were separated by centri fugation at 2000 rpm for 10 minutes at 5°C and the upper methanol:water:potassium acetate phase aspirated and discarded. The protein remaining in the resulting lower phase was then in a suitable form for gel electrophoresis after removal of organics.

RESULTS

The protein subunit composition of our OS-ATPase preparation on SDS-urea polyacrylamide gels is shown in Figure 1. The band pattern is almost identical to that of other published traces of this complex (7,10,11) except for the greater resolution at the lower molecular weight end which is typical of Swank and Munkres gels. The five subunits of F_1 are identifiable as α through ε, band 3 is OSCP, band 5 is probably the F_1 inhibitor subunit, and the unnumbered band at the very top of the gel is almost assuredly a contaminant as suggested by Capaldi (10).

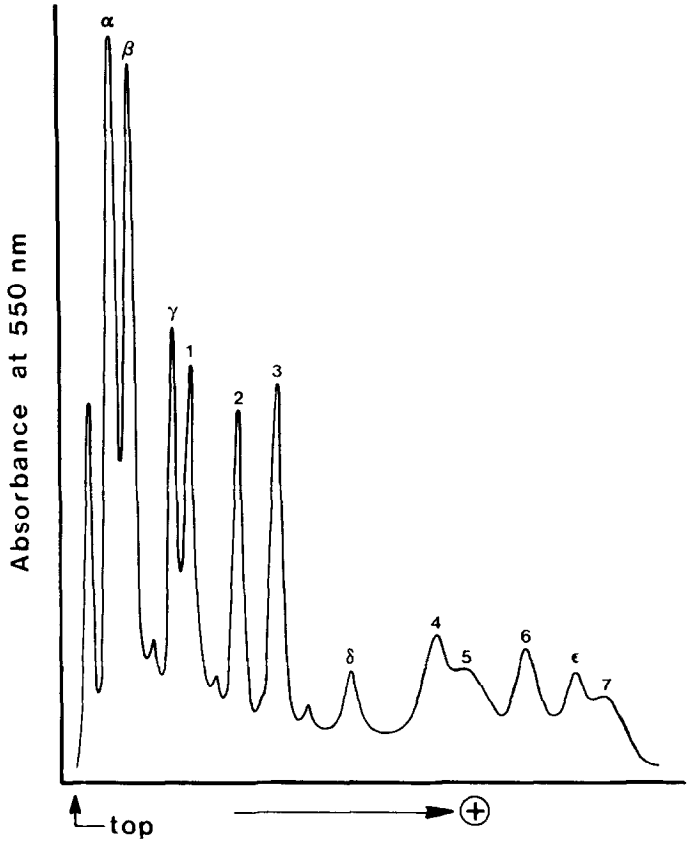

Figure 1. Densitometric trace of the OS-ATPase complex run on SDS-urea polyacrylamide gel.

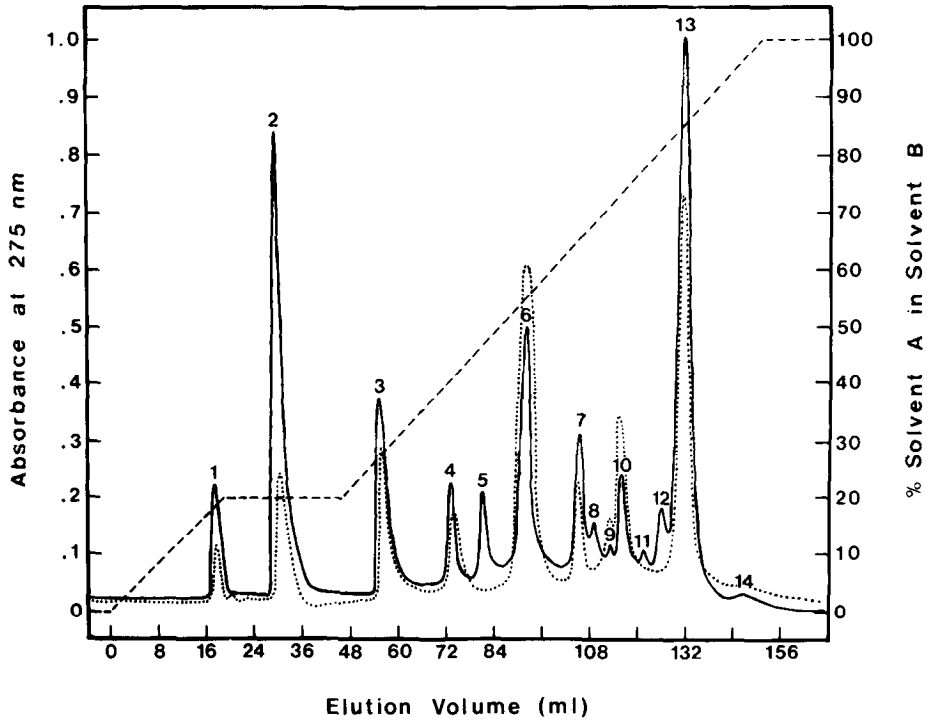

Figure 2. Preparative HPLC analysis of ETP proteolipid (———) and OS-ATPase proteolipid (·····) on Whatman Partisil 10 ODS-2. The solvent gradient is described by the dashed (-----) line. Sample injections of 2.0 ml each were made through a Waters U6-K sample injector. Pressure values were 600 psi at the beginning of each run and approximately 2200 psi at the termination. A 60 ml equilibration with solvent B (see methods) was performed prior to each injection.

The location of the remaining 5 bands (1,2,4,6 and 7) is in good agreement with their assignment to the membrane sector (F_0) of the OS-ATPase. Their molecular weights are 29 K, 21 K, 12 K, 8.8 K, and 7 K, respectively. Bands 1, 2, and 7 are well documented F_0 subunits (12), band 4 is probably identifiable with Factor B (13), and band 6 with F_6 (14), both of which are suspected of being authentic F_0 subunits (12). The important point to be emphasized is that there are no major unexpected bands present.

Extraction of this complex with chloroform:methanol yields 13-15 mg of twice ether precipitated proteolipid per 100 mg complex. Assuming extraction of only F_0 subunits and a molecular weight for F_0 of 92,000 (15), this is equivalent to 70% of the available F_0 protein. It is important to emphasize at this juncture that very little proteolipid was extracted at pH 7.5. For example, in a pH graded

TABLE I

COMPOSITIONAL SUMMARY OF ETP + OS-ATPASE PROTEOLIPID

Component No.	Designation	ETP		OS-ATPase		
		mg	mg/gm ETP	mg	mg/gm ATPase	mole/mole ATPase
1	mixture	0.43	2.76	0.19	1.9	--
2	mixture	3.56	22.85	1.05	10.5	--
3	9 K	0.89	5.71	0.74	7.4	0.39
4	HPγ	0.28	1.80	0.27	2.7	0.18
5	unknown	0.41	2.63	--	--	--
6	HPβ	0.36	2.31	0.50	5.0	0.34
7	11.5 K	0.43	2.76	0.34	3.4	0.14
8	unknown	0.18	1.16	--	--	--
9	unknown	0.10	0.64	0.15	1.5	--
10	HPα	1.47	9.44	2.02	20.2	1.36
11	unknown	0.08	0.51	--	--	--
12	unknown	0.31	1.99	--	--	--
13	29 K	7.40	47.51	5.40	54.0	0.86
Total recovered		15.9		10.66		
Total applied		19.8		13.9		
% Recovery		80%		77%		

Each compositional analysis was derived from pooled material collected from two HPLC analyses of 2 ml aliquots each of ETP and OS-ATPase proteolipid, respectively. The component numbers are referable to the peak identifying numbers of Figure 2. A molecular weight of 470,000 for the OS-ATPase complex was used for the calculation of mole subunit per mole ATPase.

series of extractions the yield of OS-ATPase proteolipid was 2.26%, 3.45%, 7.68%, and 14.2% at pH 7.5, 7.0, 6.5, and 6.0 respectively. Previous unsuccessful attempts by other investigators to demonstrate the general extractability of F_o proteins other than the DCCD binding subunit (e.g., see ref. 7) is almost assuredly related to the inefficiency of extraction at pH 7.5.

The resolution of the OS-ATPase proteolipid by reverse phase HPLC is shown in Figure 2 (dotted line) as is the resolution of proteolipid derived from ETP (solid line). On gross inspection, it is apparent that (1) all of the resolved subunits of F_o proteolipid are present in and therefore easily obtainable from ETP proteolipid, and (2), only 9 of the 13 ETP proteolipid protein subunits are derivable from

F_0. Components 4, 6, and 10 have been identified as HPγ, HPβ, and HPα respectively by HPLC analysis of authentic materials and components 3, 7, and 13 have been assigned molecular weights of 8.8 K, 11.5 K, and 29 K respectively on the basis of SDS-urea gel electrophoresis of collected samples. Components 5, 8, 9, 12, and 11 have thus far not been characterized, component 14 appears to be non protein and components 1 and 2 are non-homogeneous mixtures of component 3 and 7 plus component 4 and/or 6. A fuller account of each of the resolved components shown in Figure 2 is given in Table I.

DISCUSSION

It is apparent from the data of Figure 2 and Table I that significant amounts of HPβ and HPγ are associated with the OS-ATPase complex and, in fact, appear to be concentrated in this complex by a factor of approximately 2 when compared with the levels of these components found in ETP. While the calculated ratio (mole per mole ATPase complex) of these two subunits is admittedly less than unity (0.34 and 0.18 for HPβ and HPγ, respectively) it must be pointed out that both the 9 K (F_6, component 3) and 11.5 K (Factor B, component 7) F_0 subunits fall in this very same range (0.39 and 0.14, respectively). These sub-stoichiometric values are undoubtedly expressions of protein losses in any one or more of three major areas. In the first place, approximately 30% of the theoretical amount of F_0 is unextractable under our conditions. Secondly, because only material directly under each emerging peak was collected, we sustained losses of approximately 20% during HPLC; and thirdly, an undetermined amount of HPβ and/or HPγ emerges early in the HPLC gradient sequence in peaks 1 and 2 of Figure 2. As indicated in the results section, these two bands are inhomogeneous and contain, in addition, major amounts of the 9 K and 11.5 K subunits which emerge in pure form in peaks 3 and 7 respectively. The phenomenon probably represents chasing by the higher chloroform concentration of the injection vehicle which causes partial premature emergence of selected components. In view of these considerations, we feel that the present data supports the conclusion that HPβ and HPγ are authentic subunits of the OS-ATPase complex. We are, however, mindful of the fact that the most compelling evidence in support

of this conclusion would derive from reconstitution experiments. While this has not yet been accomplished even as regards the 29 K and the DCCD binding (HPα) subunits, the present technology, which has enabled us to separate all of the apparent subunits of F_o, should soon permit such an evaluation.

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