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RESOLUTION OF THE MITOCHONDRIAL N,N'-DICYCLOHEXYLCARBODIIMIDE BINDING PROTEOLIPID FRACTION INTO THREE SIMILAR SIZED PROTEINS

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SUMMARY: Three chloroform-methanol soluble proteins have been resolved by reverse phase HPLC of a mitochondrial proteolipid fraction from Sephadex LH-60 containing predominantly hydrophobic proteins of less than 10,000 daltons. While all three of the purified proteins comigrate during SDS-urea gel electrophoresis, amino acid analyses of the purified components reveal major differences by which each can be easily distinguished. The three proteins occur in yields of 1.58, 0.28, and 0.21 nmoles per mg protein respectively for proteins α, β, and γ. Only hydrophobic protein α is isolatable with N,N'-dicyclohexylcarbodiimide covalently bound, whereas hydrophobic proteins β and γ bind significant levels of ADP and inorganic phosphate.

Mitochondrial membrane sector proteins which are soluble in chloroform-methanol and whose molecular weights fall in the range of 6,000 to 10,000 daltons have recently become the focal point of several key functional activities associated with the energy transducing machinery of the inner mitochondrial membrane. Interest in this group of hydrophobic proteins began almost 10 years ago following the demonstration by Cattell et al. (1) that a potent inhibitor of oxidative phosphorylation, N,N'-dicyclohexylcarbodiimide (DCCD)*, was covalently associated with a 10,000 dalton chloroform-methanol soluble protein isolated from bovine mitochondria. However, more recent studies suggest that, in addition, protonophoric activity (2,3), K^+/H^+ exchange (4), uncoupler binding (5), oligomycin (3,6) and trialkyltin sensitivity (7), and Pi and ADP binding (8,9), are all associated with a 6,000 to 10,000 dalton chloroform-methanol soluble protein or class of proteins. Unfortunately, it is not known at this time whether these various activities are accountable in terms of a single protein or multiple hydrophobic protein species of similar size. Here

*ABBREVIATIONS USED: DCCD, N,N'-dicyclohexylcarbodiimide; HP, hydrophobic protein; ETP, electron transfer particle.

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we present evidence that there are in fact three separate mitochondrial chloroform-methanol soluble proteins of similar size, only one of which is labelled with [^{14}C]-DCCD while at least two are uncoupler sensitive binders of ADP and Pi.

METHODS

PREPARATIONS: The preparation of bovine mitochondria and submitochondrial particles (ETP) has been described elsewhere (10,11). Crude hydrophobic protein was prepared by the method of Cattell et al. (1) with the modifications introduced by Fillingame (12). Radiolabelling of submitochondrial particles was accomplished according to the procedure described by Fillingame (12). The concentration of [^{14}C]-DCCD (ICN Pharmaceuticals, Inc., 7150 cpm/nmole) during the incubation was set at 0.87 nmoles per mg ETP protein.

PROCEDURES: Protein concentration was determined by the method of Lowry et al. with 0.2% deoxycholate in the assay (13). Lipid phosphorus was determined by the method of Rouser and Fleischer (14). The method developed by Swank and Munkres (15) for SDS-urea gel electrophoresis in highly crosslinked polyacrylamide gels was employed for the analysis of hydrophobic protein samples. Sample preparation, buffer solutions, gel composition, and staining and destaining procedures employed were identical to those recommended by Downer et al. (16). Samples for amino acid analysis (0.1 mg per sample) were hydrolyzed in the presence of 1.0 ml of constant boiling HCl for 24, 48, and 72 hours at 110. Analyses were performed in duplicate with a Durrum D-500 amino acid analyzer.

CHROMATOGRAPHY: Sephadex LH-60 was equilibrated in a solvent system which was 60 mM in potassium acetate, pH 7.0, and was composed of 45.75% chloroform, 45.75% methanol, 7.5% water, and 1.0% benzene. After forming a 3 cm bed of Sephadex LH-20 equilibrated in the same solvent system, the slurry of LH-60 was poured into the column to give a bed of 100 cm x 2.8 cm. After application of samples in 10 ml or less of solvent, the column was eluted isocratically with the equilibrating solvent system using gravity flow rates in the range of 0.4 to 0.7 ml per min. The effluent was continuously monitored at 254 nm with an LKB Uvicord II. Reverse phase HPLC was carried out with Whatman Partisil-10 ODS contained in a 9 mm by 50 cm stainless steel column. Solvent delivery was performed with a Perkin-Elmer model 601 system. Sample injections were made through a Waters model U6-K sample injector with a 2.0 ml sample loop. Effluent was continuously monitored at 275 nm with a Perkin-Elmer model LC-55 spectrophotometer. Chromatographic fractions were concentrated at 0-5 C as described by Fillingame (12) prior to use or rechromatography. It is important that the solutions are kept cold during concentration as otherwise considerable losses of HPγ occur by transfer of the latter to the upper aqueous methanol phase. Other conditions are contained in the legend to Figure 1.

BINDING STUDIES: The Pi and ADP binding activity of isolated proteins was measured according to the method of Kadenbach and Hadvary (17) with modifications introduced by Kessler (9). The protein (.1 mg) was dissolved in 1.0 ml of chloroform:n-butanol:methanol (50/150/25) and 0.1 ml of an aqueous solution containing 25 mM [^{32}P]-Pi at pH 7.0 was added. After vortexing the contents, 1.0 ml of an identical aqueous solution containing in addition 2.0 M sucrose was added. The contents were then vortexed for 90 seconds and clean phase separation was achieved by centrifugation. An aliquot (0.5 ml) of the upper organic phase was then counted in a liquid scintillation system. For the study of ADP binding, the aqueous solutions contained 2.5 mM [^{14}C]-ADP.

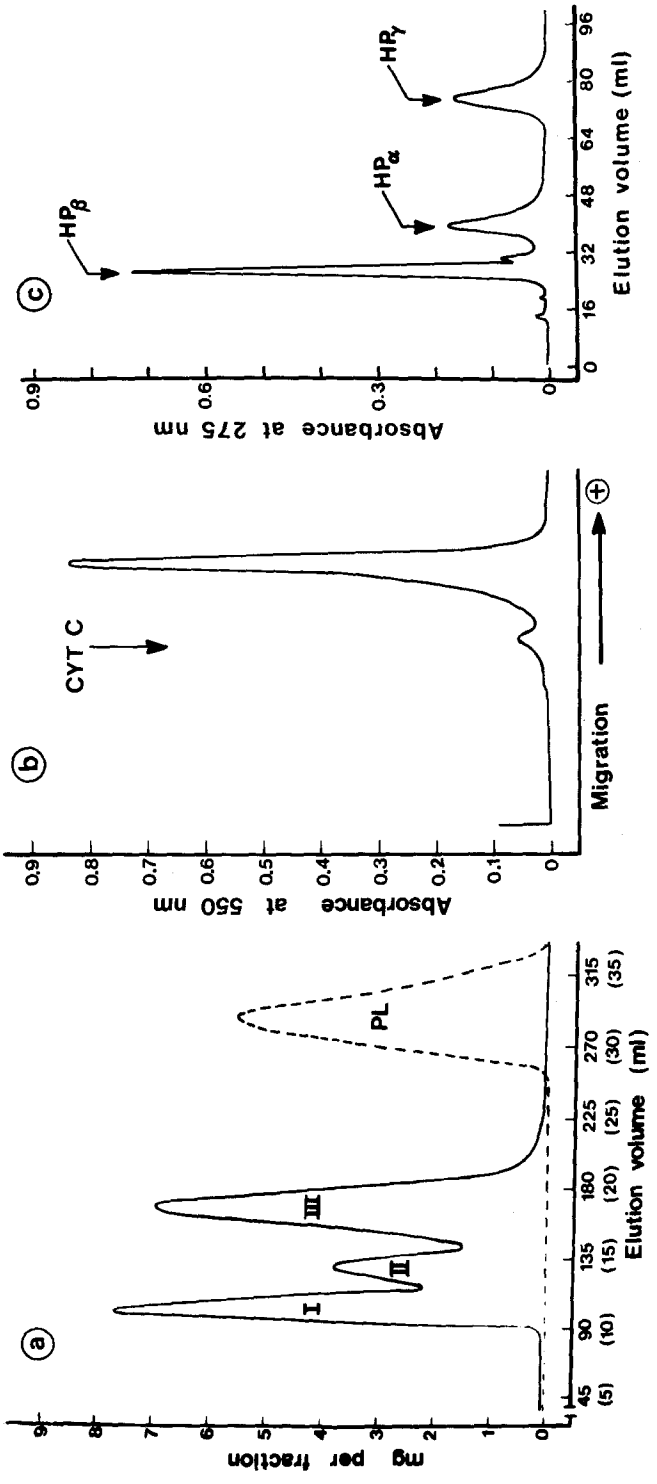

Figure 1. (a) Chromatography of unmodified bovine mitochondrial proteolipid on Sephadex LH-60. The values in parentheses are fraction numbers. The solid line represents protein and the dashed line phospholipid (PL). An identical pattern was obtained from [14C]-DCCD modified proteolipid in which 94% of the applied radioactivity emerged associated with Zone III protein. (b) SDS-urea gel electrophoresis of Zone III protein derived from Sephadex LH-60 chromatography. (c) HPLC of Zone III protein on Whatman partisil 10 ODS. Isocratic elution was carried out with 0.24 M potassium acetate, pH 6.5, in methanol:Chloroform (2:1) containing 8% water and 1% benzene. The flow rate was 2.0 ml per min at 2000 psi.

Ethanolic solutions of uncoupler or DCCD were added to protein samples prior to evaporation to dryness and addition of chloroform:butanol:methanol. Experiments containing DCCD were preincubated for 2 hours at 0-5 C.

RESULTS

The 40 to 70 mg of twice ether precipitated crude mitochondrial proteolipid which is obtainable from 1.0 gm of submitochondrial particle protein was first submitted to exclusion chromatography on Sephadex LH-60 in order to obtain a less than 10,000 dalton fraction. In a typical experiment as described in Figure 1a, the chromatography of 49.4 mg of proteolipid resulted in the recovery of 22 mg of phospholipid free protein (zone III) which was found to contain predominantly 6500 to 7500 dalton protein judging from its mobility during SDS-urea gel electrophoresis (Figure 1b). Analytical reverse phase HPLC of zone III protein (Figure 1c) resolved the mixture into 3 components emerging at 26 ml, 37 ml, and 75 ml, respectively.

Since it had been determined that the injection of up to 4.0 mg of zone III protein did not seriously degrade the separation properties of the column, several mg of each of the three components were collected by repeated injection of 1.5 ml aliquots equivalent to 3.7 mg of zone III material. A summary of the final yield of each component is shown in Table I, and the amino

TABLE I

YIELD OF 7000 DALTON MITOCHONDRIAL HYDROPHOBIC PROTEINS

Designation	Total mg	nmoles per mg ETP protein	nmoles bound DCCD per mg protein
HPα	11	1.58	26
HPβ	2	0.28	0
HPγ	1.4	0.21	0

Yields are derived from 1.0 gm ETP protein with or without DCCD pretreatments. Minimum molecular weights from amino acid analyses were used to calculate nmoles HP per mg ETP protein.

TABLE II

AMINO ACID ANALYSES OF 7000 DALTON HYDROPHOBIC PROTEINS

AMINO ACID	HPα mol/mol Arginine	HPα Best Integral	HPβ mol/mol Alanine	HPβ Best Integral	HPγ mol/mol Tyrosine	HPγ Best Integral
ASP	2.95 ± .03	3	3.87 ± .05	4	3.04 ± .02	3
THR	3.29 ± .15	3	8.76 ± .20	9	2.27 ± .07	2
SER	4.79 ± .06	5	2.94 ± .09	3	3.86 ± .10	4
GLU	3.11 ± .06	3	4.69 ± .08	5	4.53 ± .12	5
PRO	1.20 ± .05	1	5.92 ± .10	6	4.27 ± .09	4
GLY	10.76 ± .55	11	1.04 ± .02	1	5.69 ± .23	6
ALA	12.14 ± .37	12	1.00	1	5.28 ± .15	5
CYS	.08 ± .06	0	0.03 ± .02	0	0.05 ± .02	0
VAL	4.17 ± .16	4	1.32 ± .02	1	1.87 ± .11	2
MET	2.90 ± .24	3	3.86 ± .16	4	2.71 ± .13	3
ILE	5.48 ± .27	5	2.37 ± .10	2	1.08 ± .05	1
LEU	8.21 ± .11	8	12.04 ± .31	12	5.74 ± .22	6
TYR	1.97 ± .19	2	1.95 ± .02	2	1.00	1
PHE	6.22 ± .06	6	3.05 ± .09	3	5.94 ± .34	6
HIS	0.09 ± .02	0	1.92 ± .04	2	3.02 ± .06	3
LYS	1.76 ± .24	2	5.73 ± 0.13	6	4.77 ± .18	5
ARG	1.00	1	0.82 ± 0.31	1	2.83 ± .03	3
Total residues	69		62		59	
Minimum M.W.	6950		7180		6600	
Daltons	6980 ± 240 (5)		7250 ± 180 (6)		6790 ± 260 (4)	

Normalization of HPα to arginine was made in accordance with the analysis of Graf and Sebald (19). The values quoted here are averaged from duplicate experiments with and without DCCD modification. The normalization of HPβ to alanine was made because of the large errors consistently associated with the arginine values from HPβ. The values for HPβ represent the average of 6 analyses and for HPγ of 4 analyses. The absence of cysteine was not confirmed by performic acid oxidation and tryptophane was not determined. All of the values quoted here were derived from 48 hr. hydrolyses which gave the most consistent results. Values for minimum M.W. are based on summation of best integrals and dalton values are based on migration during SDS-urea gel electrophoresis with values in parentheses representing the number of determinations.

acid composition of each of the three components is shown in Table II. In addition, the results of an experiment in which all three components were isolated by the same procedures from a preparation which was preincubated with [^{14}C]-DCCD is described in column 4 of Table I.

TABLE III

EFFECT OF UNCOUPLER AND DCCD ON THE BINDING Pi AND ADP

BY 7000 DALTON MITOCHONDRIAL HYDROPHOBIC PROTEINS

Protein	nmoles ligand per mg protein					
	no additions		+ uncoupler		+ DCCD	
	ADP	Pi	ADP	Pi	ADP	Pi
HPα	2	<0	--	--	--	--
HPβ	141	345	0	0	68	0
HPγ	22	62.5	0	0	29.3	89.4

The uncoupler used in this study was pentachlorophenol which was present in the organic phase at a concentration of 2×10^{-5} M. Assays conducted in the presence of DCCD contained 0.1 μmole of unlabelled inhibitor. Other conditions are described in the section on methods.

The results of experiments designed to determine the ADP and Pi binding capability of each component are described in Table III. While the binding values obtained in the presence of HPα are uniformly negative in this assay system, it is important to mention that the determination of protein concentration in both the organic and aqueous phases of the assay failed to reveal the presence of HPα. However, careful examination of the interface revealed the presence of a film of protein precipitate of HPα in this assay system. A monophasic assay system based on a methodology developed by Hummel and Dreyer (18) is presently being explored for the analysis of the binding capability of HPα.

DISCUSSION

It is apparent that the HPα component of the present report is identical to the DCCD binding protein of bovine mitochondria described by Graf and Sebald (19). The amino acid analysis is almost identical, and the yield (1.1%) and specific activity of bound DCCD (26 nmoles per mg protein) are in very close agreement with the values reported by Graf and Sebald (0.6% and 39 nmoles per mg protein respectively). The higher specific activity re-

ported by these authors could easily be referable to the higher level of ligand (2-3 nmoles versus 0.87 nmoles per mg protein) utilized by Graf and Sebald. On the other hand, the higher absolute yield of HPα in the present report may be referable to the fact that the Graf and Sebald procedure is encumbered by losses of from 40 to 50% of the protein during CM-cellulose chromatography, whereas we have observed near quantitative recovery of both protein and radioactivity during both of our chromatographic procedures.

While the three proteins described in the present report are easily distinguishable chromatographically, their distinguishability on the basis of amino acid analysis is equally as compelling. HPα is dominated by the presence of glycine and alanine while HPβ is practically devoid of these two amino acid residues and is in turn dominated by the presence of threonine and leucine. HPγ on the other hand has equal amounts of glycine, leucine, and phenylalanine.

The Pi and ADP binding capabilities of HPβ and HPγ are remarkable in the context of previously reported values for Pi binding to an isolated mitochondrial proteolipid which were in the range of 5 to 6 nmoles per mg protein (8). In the present report for example, it can be seen that the binding of ADP by HPβ is exactly stoichiometric (theory predicts 139 nmoles per mg protein for a 1:1 complex), whereas that of Pi is superstoichiometric. The complete inhibition of Pi and ADP binding by 2×10^{-5} M uncoupler is also of special interest in view of the recent report by Katre and Wilson (5) describing the photoaffinity labelling by uncoupler of a small molecular weight proteolipid from mitochondria.

Although DCCD is not isolatable in bound form with HPβ or HPγ, its postulated interaction with HPβ (20) is based on the inhibitory activity of DCCD as described in Table III. In addition, since the N-acyl urea bond formed between DCCD and an acidic protein residue is a potentially labile bond, the possibility does exist that the former ligand does in fact react with HPβ *in situ* but is displaced by some other ligand as dicyclohexylurea

either in situ or during the isolation. In agreement with this interpretation, we have observed that HPβ isolated from DCCD treated submitochondrial particles is inactive in the Pi binding assay (21).

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