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ON THE IDENTIFICATION OF THE UNCOUPLER BINDING PROTEIN OF BOVINE MITOCHONDRIAL OLIGOMYCIN SENSITIVE ATPase

George A. Blondin

Institute for Enzyme Research
Madison, WI 53706

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SUMMARY: The 55,000 dalton polypeptide component of the membrane sector of the mitochondrial oligomycin sensitive ATPase has been purified by recycling chromatography on BioGel P-100. The amino acid composition of the purified polypeptide differs significantly from that of the α-subunit of F_1 with which it shares a similar apparent molecular weight. However, the amino acid composition of the former is identical to that of the Factor B polypeptide, which is known to occur in oligomeric forms. Evidence is presented which suggests that the mitochondrial uncoupler binding proteins and the various oligomeric forms of the Factor B polypeptide, including the 55,000 dalton species described in the present report, are identical.

Extraction of the mitochondrial oligomycin sensitive ATPase (OS-ATPase)* with 3.5 M NaBr is generally believed to remove the F_1 subunits in soluble form and to leave behind the membrane sector (Fo) subunits as an insoluble residue (1). Nevertheless, Capaldi (2) reported that the NaBr residue from bovine mitochondrial OS-ATPase contained a significant amount of a component with a molecular weight (55,000 daltons) similar to that of the α-subunit of F_1. Since this component was not solubilized by repeated washing of the NaBr pellet with 8 M guanidine hydrochloride (2), it was thought to be a different polypeptide from that of similar molecular weight in F_1, which is readily solubilized by this reagent. While an altered conformational state of a portion of the F_1 α-subunits could explain these findings, an equally compelling position could take the following form; namely, that the Fo derived 55,000 dalton polypeptide represents an oligomeric form of a smaller Fo subunit. Such a view is compatible with the variable occurrence of the Fo derived 55,000 dalton polypeptide [see, for example, Berden and Voorn-Brouwer (3)], and with the documented oligomeric forms of specific Fo subunits (4, 5). This communication deals with the purification and properties of the Fo derived 55,000 dalton subunit and our working hypothesis is evaluated by a comparison of these properties with those reported for other Fo subunits and with those of the α-subunit of F_1.

*ABBREVIATIONS USED: OS-ATPase, oligomycin sensitive ATPase; Fo, membrane sector of the oligomycin sensitive ATPase; NPA, 2-azido-4-nitrophenol; DTT, dithiothreitol; OSCP, oligomycin sensitivity conferring protein.

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METHODS

Preparations: The preparation of bovine heart mitochondria and submitochondrial particles has been described elsewhere (6, 7). The OS-ATPase complex was prepared by the method of Berden and Voorn Brouwer (3). Fo was prepared from the OS-ATPase complex by extraction with 3.5 M NaBr according to the procedure of Tzagoloff et al. (1). Tritiated 2-azido-4-nitrophenol was synthesized as described by Hanstein et al. (8).

Procedures: Protein concentration was determined by the method of Lowry et al. (9) with 0.2% deoxycholate in the assay. The method developed by Swank and Munkres (10) for SDS-urea gel electrophoresis in crosslinked polyacrylamide gels was employed as previously described (11). However, gel concentrations of 8% were used in the present study. The gel permeation method of Hummel and Dreyer (12) was used for the estimation of adenine nucleotide, phosphate and 2-azido-4-nitrophenol binding by purified samples of the 55,000 dalton polypeptide. The gel (BioGel P6) bed dimensions were 1.7 x 13 cm and the equilibrating solution contained 5.0 mM Tris acetate (pH 7.0), 5.0 mM potassium acetate (pH 7.0), 2.0 mM DTT and concentrations of ligand and inhibitor as indicated in the legend of Table 1. Samples of the 55,000 dalton polypeptide (2.5 mg prot.) were sedimented and solubilized in 1 ml of the appropriate radiolabeled ligand - equilibrating solution containing, in addition, 5.0 mg lysolecithin and 1.0 µl of glacial acetic acid. Samples thus prepared were applied to column and eluted with fresh equilibrating solution. Fractions of 1.0 ml each were collected and assayed for radiolabel content. Samples for amino acid analysis were prepared as previously described (13).

Purification of the 55,000 Dalton Polypeptide: Fo protein was solubilized by incubation overnight at 38°C in the presence of 4% SDS, 8.0 M urea, 40 mM DTT and 0.1 M sodium phosphate (pH 8.3). The protein concentration was adjusted to 20 mg/ml, and up to 20 ml of the latter solution was introduced onto a BioGel P-100 column (5 x 90 cm) which had been equilibrated with 2% SDS, 4.0 M urea, 0.5 mM DTT and 5.0 mM sodium phosphate (pH 8.3). The column (thermostated and maintained at 40°C) was then developed with equilibrating solvent at a flow rate of 1.5 ml/min, and the first major component which was seen to emerge (LKB Uvicord II, 280 nm) was allowed to recycle a total of two additional times in order to achieve complete separation from the 29,000 dalton polypeptide which migrated just distal to the 55,000 dalton species. The 55,000 dalton polypeptide was harvested free of SDS, urea, DTT and phosphate from the appropriately combined fractions according to a previously described procedure (14).

RESULTS

The polypeptide composition of the Fo sector of the OS-ATPase is shown in Fig. 1 (Trace a). In agreement with other densitometric traces of this sector of the OS-ATPase (2), the 55,000 dalton polypeptide (component 2) is a dominant species along with OSCP (component 9) and components 7 and 8, which migrate as 29,000 and 22,000 dalton polypeptides, respectively. The migration of the trace components 3, 4 and 6 is in agreement with their identification as F_1 α, β and γ subunits, respectively. Trace b of Fig. 1 is a densitometric trace of the purified 55,000 dalton polypeptide, which was obtained by recycling chromatography of the entire Fo sector on BioGel P-100 as described in the Methods section. The preparation shows only minor impurities corresponding to 43,000 and 29,000 dalton polypeptides.

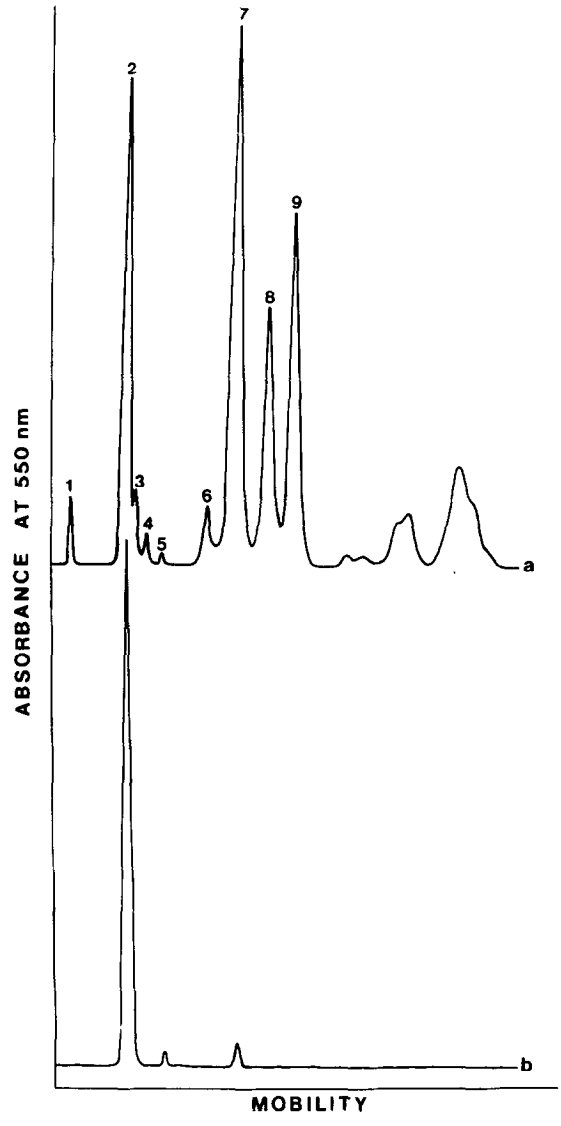

Figure 1. Densitometric traces of the oligomycin sensitive ATPase Fo sector (trace a) and the purified Fo derived 55,000 dalton polypeptide run on 8% polyacrylamide gels.

The amino acid composition of the purified Fo derived 55,000 dalton polypeptide is shown in Table I. In addition, the amino acid composition reported for Factor B (15), a documented oligomeric component of Fo (5, 22), and for the α-subunit of F_1 (16, 17) are also shown. Finally, the amino acid composition of the adenine nucleotide carrier protein (18) is shown since the latter may be related to the 29,000 dalton polypeptide of Fo and since the carrier protein has been reported to be capable of forming stable oligo-

Table I. Amino acid composition of subunits of mitochondrial oligomycin sensitive ATPase

Amino acid	Polypeptide				
	This Study	$F_1\alpha$[a]	$F_1\alpha$[b]	Factor B[c]	Adenine nucleotide carrier[d]
Aspartate	9.56	8.33	8.33	9.94	8.22
Threonine	5.06	4.96	5.28	4.97	4.61
Serine	6.41	7.34	6.10	6.06	5.15
Glutamate	10.68	11.50	11.97	10.90	8.08
Proline	4.81	3.57	3.45	5.25	3.35
Glycine	9.27	11.30	9.56	9.33	10.71
Alanine	9.21	9.92	9.69	8.72	11.20
Cysteic acid[e]	1.54	0.59	0.59	1.57	1.71
Valine	6.56	7.93	7.27	6.66	7.43
Methionine	2.26	1.58	1.94	1.85	2.27
Isoleucine	5.49	6.94	6.88	5.40	5.04
Leucine	10.09	8.92	9.22	9.71	7.94
Tyrosine	2.63	1.78	2.94	2.64	4.01
Phenylalanine	3.66	2.38	2.74	3.50	6.32
Histidine	2.42	1.19	1.02	2.56	0.94
Lysine	6.03	6.34	6.25	6.81	7.63
Arginine	4.19	5.35	6.70	4.04	5.60
Leu/Iso	1.84	1.28	1.34	1.80	-
Glx/Pro	2.22	3.22	3.47	2.08	-
Met/Cys	1.47	2.68	3.34	1.47	-

[a] Data of Knowles and Penefsky (17).

[b] Data of Brooks and Senior (16) with the incorporation of cysteic acid value of Knowles and Penefsky (17).

[c] Data of Lam et al. (15).

[d] Data of Aquila et al. (18).

[e] Determined after performic acid oxidation.

meric (dimer) species (19), a feature which could account for its migration in the region of the Fo-derived 55,000 dalton polypeptide. An analysis of the amino acid composition of the polypeptides described in Table I reveals the following: 1) the amino acid composition of the Fo derived 55,000 dalton subunit is identical to that of Factor B and shows several major differences in composition to that of the adenine nucleotide carrier protein, especially with respect to the contents of proline, tyrosine, phenylalanine and histidine residues; and 2) the composition of the Fo derived 55,000 dalton subunit

Table II. Binding properties of the Fo derived 55,000 dalton polypeptide

Additions	nmoles Ligand/mg Protein		
	ATP	Pi	NPA
None	25.9	4.93	31.3
Pentachlorophenol	0.2	0	-

The concentration of ATP-C^{14} and of Pi^{32} was set at 100 μM and the concentration of tritiated NPA (2-azido-4-nitrophenol) at 50 μM. The concentration of pentachlorophenol was set at 20 μM.

is similar to that of the α-subunit of F_1, nevertheless, at least three major differences are apparent as shown in Table I, i.e., the ratios of glutamate to proline, leucine to isoleucine and methionine to cysteic acid. On the basis of these differences and in view of the extremely close compositional correspondence between the Fo derived 55,000 dalton subunit and Factor B, the co-identity of these two polypeptides appears reasonable.

Our laboratory has been systematically examining the adenine nucleotide and phosphate binding capability of isolated OS-ATPase subunits (13) and the availability of the purified Fo derived 55,000 dalton subunit allowed for an examination of its binding properties. The data of Table II show that this polypeptide binds both ATP and Pi and that the binding of both of these ligands is completely uncoupler (pentachlorophenol) sensitive. Furthermore, the Fo derived 55,000 dalton polypeptide binds the tritiated uncoupler 2-azido-4-nitrophenol (NPA) in almost identical stoichiometry to the binding of ATP. Finally the data of Table III show that the bound NPA is capable of photoactively labeling the 55,000 dalton polypeptide in a manner analogous to the photoactive labeling properties of the uncoupler binding protein of Hanstein and Hatefi (20). Appropriate control experiments show that NPA does not photoactively label the lysolecithin micelles and that nonphotoactivated NPA is washed out of the polypeptide-lysolecithin complex in transit through the column.

DISCUSSION

The primary conclusion of the present communication relates to the non-identity of the Fo derived 55,000 dalton polypeptide with the α-subunit of F_1 and with the identity of the former with an oligomeric form of the Factor B polypeptide. The evidence in support of this conclusion derives principally from two observations. In the first place, the 55,000 dalton Fo derived subunit is not solubilized by repeated washing with 8 M urea (2), a reagent

Table III. Photoactive labeling of the Fo derived 55,000 dalton polypeptide with tritiated 2-azido-4-nitrophenol[a]

Sample	Covalently attached 2-azido-4-nitrophenol (nmoles/mg protein)
Photoactivated polypeptide-lysolecithin[b]	16.3
Dark Polypeptide-lysolecithin[b]	0.4
Lysolecithin micelles	0[c]

[a]Photoactive labeling was performed as described by Hanstein and Hatefi (20). Non covalently attached NPA was removed on BioGel P6.

[b]Originally contained 25.9 nmoles bound NPA/mg protein.

[c]Expressed as nmoles/mg lysolecithin. Originally contained 10 nmoles NPA/mg lysolecithin.

which is known to completely solubilize the α-subunit of F_1 (16); and secondly, as reported here, the amino acid composition of the Fo derived 55,000 dalton subunit is identical to that of the Factor B polypeptide and significantly different with respect to the ratios of three separate sets of amino acid residues from the α-subunit of F_1.

Several comments are in order regarding the possible relationship of the uncoupler binding protein and the Factor B polypeptide as suggested by the data presented in Tables II and III. Hanstein and Hatefi described the labeling of specific mitochondrial (20) and OS-ATPase (21) uncoupler binding sites with tritiated 2-azido-4-nitrophenol. The labeling pattern of the resulting polypeptides determined by gel electrophoresis (8) established the following distribution: two peaks corresponding to molecular weights of 56,000 and 31,000 contain approximately 60% of the photoactively incorporated label and two minor peaks were detected corresponding to molecular weights in the range of 12,000 to 14,000 and 43,000. The 31,000 dalton polypeptide was the major labeled component, and subsequent evidence (21) indicated that it corresponded to the 29,000 dalton component of the membrane sector (Fo) of the OS-ATPase (Complex V in their terminology). On the other hand, the monomeric form of Factor B is thought to be in the range of 13,000 to 14,000 daltons (22); and evidence has been presented for oligomeric forms of this polypeptide corresponding to the 29,000 dalton dimer (15), the 47,000 dalton trimer (5) and the 55,000 dalton tetramer (present study). Thus the dalton spectrum of oligomeric Factor B polypeptides corresponds perfectly to the dalton spectrum of the uncoupler binding protein species. In addition, the

uncoupler sensitive Pi and ATP binding of the oligomeric Factor B preparation of the present study and the binding and photoactively dependent covalent attachment of tritiated NPA to the latter mimics exactly the properties of the uncoupler binding protein of Hanstein and Hatefi (20).

Finally the views presented here are consistent with the recent findings of Joshi et al. (22) that Factor B depleted OS-ATPase shows very little stimulation of OS-ATPase activity in the presence of uncoupler, whereas the uncoupler stimulated OS-ATPase activity is increased 3.6 fold when the complex is fortified with exogenous Factor B. The uncoupler sensitivity conferring property of Factor B is thus totally consistent with the view that Factor B is, in fact, the principal uncoupler binding protein and that the latter is capable of migrating on polyacrylamide gels as oligomeric species.

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