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THE BIOGENESIS OF RAT LIVER MITOCHONDRIAL ATPase

SUBUNIT COMPOSITION OF THE NORMAL ATPase COMPLEX AND OF THE DEFICIENT COMPLEX FORMED WHEN MITOCHONDRIAL PROTEIN SYNTHESIS IS BLOCKED

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Summary

1. An ATPase complex containing 12 subunits was isolated from rat liver mitochondria.

2. In vivo inhibition of mitochondrial protein synthesis by the chloramphenicol analogue thiamphenicol leads to the formation of an oligomycin-insensitive membrane-bound ATPase complex in mitochondria of regenerating rat liver.

3. This oligomycin-insensitive, membrane-bound ATPase was isolated by the same procedure as the ATPase complex from regenerating livers of untreated animals.

4. SDS-polyacrylamide gel electrophoresis of in vivo labelled ATPase complexes from control and from thiamphenicol-treated rats reveals that three subunits out of the 12 are not synthesized or assembled when the mitochondrial translation activity is blocked.

5. From the subunits synthesized and assembled when mitochondrial protein synthesis is impaired, at least one may be part of the membrane sector (F_o) of the ATPase complex (subunit 5).

6. The oligomycin sensitivity-conferring protein seems absent in the ATPase complex formed in the presence of thiamphenicol.

Abbreviations: F_1, coupling factor I (soluble oligomycin-insensitive ATPase); F_o, coupling factor o (the membrane part of oligomycin-sensitive ATPase); F_{c2}, coupling factor c_2 (part of F_o); OSCP, oligomycin sensitivity-conferring protein (part of F_o); Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DCCD, *N,N'*-dicyclohexylcarbodiimide; SDS, sodium dodecyl sulfate.

Introduction

It is well-established that the biogenesis of some enzymes located in the inner mitochondrial membrane requires the cooperation of two protein-synthesizing systems. One is operating in the cytoplasm, the other in the mitochondria (for a review see ref. 1). For the yeast *Saccharomyces cerevisiae* and the mould *Neurospora crassa* it has already been possible to identify the cytoplasmically and mitochondrially made subunits of the enzymes concerned. It appeared, then, that both in yeast [2] and *Neurospora* [3] three subunits out of seven of cytochrome *c* oxidase and one subunit out of six of the cytochrome bc_1 complex [4,5] are synthesized on mitochondrial ribosomes. In yeast four subunits of the mitochondrial ATPase complex are also synthesized in the mitochondria [6]. These four subunits form, together with OSCP, the F_o part of the enzyme. When the formation of F_o in yeast is prevented by specific inhibition of mitochondrial protein synthesis by chloramphenicol the assembly of the cytoplasmically made subunits of the enzyme complex into the inner mitochondrial membrane does not occur [7]. In *N. crassa* two subunits of the ATPase complex are of mitochondrial origin [8]. It is not yet known if the lack of these two polypeptides is accompanied by impaired assembly and functional disability of the enzyme.

For animal systems much less is known about the identity of products of mitochondrial protein synthesis. However, inhibition of mitochondrial protein synthesis in isolated rat heart cells in culture [9] and in the fast proliferating small intestinal epithelium of rat in vivo [10] leads to the formation of an oligomycin-insensitive ATPase. This indicates that also in mammals one or more subunits not belonging to the F_1 part of the ATPase complex are made by or under strong control of the mitochondrial protein-synthesizing machinery. In contrast with the comparable situation in yeast [7], the oligomycin-insensitive ATPase activity in the epithelial cells remains bound to the inner mitochondrial membrane [10]. This suggest that at least part of the ATPase membrane sector (F_o) to which F_1 is bound is synthesized under these conditions. This membrane-bound character of the oligomycin-insensitive ATPase, as formed during inhibition of mitochondrial protein synthesis, was stressed by Wagner and Rafael [11]. These authors, working with liver mitochondria from mice, which received chloramphenicol in the diet, further showed that the oligomycin-insensitive ATPase does not participate in oxidative phosphorylation. We obtained identical results in this respect with rat small intestinal epithelial mitochondria [12].

We are now studying the bioenergetic and biogenetic aspects of this phenomenon in more detail. The present paper describes our comparative studies of the ATPases isolated from regenerating rat liver of normal and of thiamphenicol-treated rats. In the latter mitochondrial protein synthesis is blocked.

Methods and Materials

Partial hepatectomy. Male Wister rats weighing 180—220 g were partially hepatectomized according to the method of Higgins and Anderson [13]. The anaesthetic we used was a solution containing 0.02% phentanyl and 1% flu-

anisone (Hypnorm®), the dose being 0.5 ml/kg body weight.

Antibiotic and mode of administration. Thiamphenicol glycinate (Urfamycine®) was generously supplied by Zambon via Inpharzam Amsterdam, The Netherlands. Animals were treated as described previously [12]. They received 250 mg thiamphenicol/kg body weight per day or otherwise as indicated in the text.

Preparation of mitochondria, mitoplasts and Lubrol particles. Mitochondria were isolated acoording to Bustamente et al. [14]. The homogenization medium (H-medium, pH 7.4) contained 220 mM mannitol, 70 mM sucrose and 2 mM Hepes. Mitoplasts were prepared as described by Chan et al. [15]. Lubrol particles were prepared from mitochondria or mitoplasts by extraction with Lubrol WX [15].

Preparation of NaBr particles. Mitoplasts suspended at a protein concentration of 8 mg/ml in a medium containing 250 mM sucrose and 10 mM Tris/sulfate (pH 7.5), were sonicated for four 15-s periods at 0°C with a Branson B12 sonifier (microtip, step 3). Unbroken mitoplasts were spun down at 20 000 × *g* for 10 min. The supernatant was centrifuged at 100 000 × *g* for 30 min. The particles were extracted with 3.5 M NaBr as described by Tzagoloff et al. [16]. The resulting NaBr particles were washed once with a solution containing 250 mM sucrose, 1 mM EDTA and 10 mM Tris/sulfate (pH 7.5), collected by centrifugation at 100 000 × *g* for 30 min, resuspended in a small volume of the same medium and stored in this solution at −80°C.

Isolation of beef heart F_1 ATPase. Beef heart F_1 ATPase was isolated according to Drahota and Houstek [17]. The collected peak fractions obtained after the final gel filtration were made 50% (w/v) in glycerol and stored at −80°C without appreciable loss of activity.

Association of beef heart F_1 ATPase with NaBr particles. NaBr particles were diluted in 250 mM sucrose, 1 mM EDTA and 10 mM Tris/sulfate (pH 7.5) and incubated with 1 μg beef heart F_1 in a final volume of 1 ml. After 30 min incubation at room temperature samples of the mixture were assayed for ATPase activity and inhibition by oligomycin.

Purification of ATPase complex from rat liver mitochondria. Oligomycin-sensitive ATPase was solubilized from Lubrol WX-insoluble fractions as described by Soper and Pederson [18] with the following modifications. After extraction of the mitoplasts with Lubrol WX and subsequent centrifugation the pellet was washed once with PA buffer containing 300 mM K_2HPO_4, 2 mM ATP, 10% (w/v) ethylene glycol, 5 mM EDTA and 0.5 mM dithiotheitol (pH 7.9) and suspended in this buffer at a protein concentration of 2 mg/ml. To this suspension Triton X-100 was added from a 10% (w/v) stock solution to a concentration of 0.4%. The mixture was incubated at 0°C for 15 min and centrifuged at 200 000 × *g* for 90 min. The resulting supernatant was dialyzed at 0°C for 2 h against a buffer of the following composition: 30 mM K_2HPO_4, 2 mM ATP, 1% (w/v) ethylene glycol, 5 mM EDTA and 0.5 mM dithiothreitol (pH 7.9). The dialyzed extract was layered on 8—18% glycerol gradients containing 2% (w/v) ethanol, 2 mM ATP, 0.5 mM dithiothreitol, 0.04% Triton X-100, 5 mM EDTA and 20 mM Tris-sulfate (pH 7.5) and centrifuged at 0°C for 16 h at 22 000 rev./min in a Beckman SW41 rotor. Usually 12—14 fractions were collected and screened for ATPase activity.

In vivo labelling. 1.5 mCi L-[4,5-^{3}H]leucine (specific radioactivity 1 Ci/mmol) in 2 ml saline or 0.5 mCi L-[^{35}S]methionine (specific radioactivity 0.11 Ci/mmol) in 0.2 ml saline were injected intraperitoneally into rats. After 10 h the animals were killed and the isolation procedure for ATPase was started.

SDS gel electrophoresis. Fractions obtained after glycerol centrifugation were precipitated with 10% trichloroacetic acid and the precipitates washed with aceton. Precipitates were dissolved in 0.1 N NaOH. Then one third volume of dissociation solution containing 20 mM NaH_2PO_4, 8% (w/v) SDS, 5% (v/v) glycerol and 5% (v/v) mercaptoethanol (pH 7) was added. The samples were heated at 100°C for 2 min and neutralized with 1 N HCl. Electrophoresis was carried out in the presence of 0.1% (w/v) SDS with 15% (w/v) polyacrylamide slab gels or disc gels and 3% stacking gels, using the buffer system as described by Laemmli [19]. Calibration proteins were serum albumin (68 000), pyruvate kinase (57 000), ovalbumin (43 000), lactate dehydrogenase (36 000), carbonic anhydrase (29 000), myoglobin (17 200) and cytochrome *c* (11 700). The gels were stained with 0.25% (w/v) Coomassie Brilliant Blue R250 in 50% (v/v) methanol, 7.5% (v/v) acetic acid for 1 h at 55°C and destained in 25% methanol and 10% acetic acid.

For the estimation of radioactivity the disc gels were fixed in 10% trichloroacetic acid for 30 min and frozen. The length of the gels was measured. The gels were then cut into pieces of 1 mm with a Mickle gel slicer at 0°C. The slices were weighed to enable us to correct for variations in the thickness of the slices. The slices were digested overnight in 0.5 ml soluene at 40°C and counted in a liquid scintillation counter operated in a ^{3}H and ^{35}S double-label device.

Enzyme assays. Cytochrome *c* oxidase was assayed spectrophotometrically at room temperature [20]. Prior to determination the enzyme was activated at 0°C in 1% digitonine (final protein concentration 10 mg/ml) for 15 min.

ATPase activity was measured at room temperature in the presence of an ATP-regenerating system [21], either by determining the phosphate liberated [22] from ATP as described previously [12] or by coupling the hydrolysis of ATP to the loss of absorbance of NADH at 340 nm as described by Soper and Pederson [18].

Protein was determined by a modified Lowry method as described by Peterson [23].

Results

Association of beef heart F_1 with NaBr particles

Treatment of rats with thiamphenicol, a specific inhibitor of mitochondrial protein synthesis, leads to the formation of oligomycin-insensitive ATPase in mitochondria from regenerating liver. This ATPase activity is bound to the inner membrane as appears from its association with Lubrol particles (Table 1). Similar results were already obtained in mice by Wagner and Rafael [11]. This association with the membrane fragments suggests that at least part of F_o which connects F_1 to the membrane is still present in the ATPase complex that is formed under conditions of inhibition of mitochondrial protein synthesis. This suggestion is further supported by the experiment of Fig. 1. It has already been

TABLE I

ATPase ACTIVITY AND INHIBITION BY OLIGOMYCIN IN LUBROL PARTICLES ISOLATED FROM REGENERATING LIVER MITOCHONDRIA OF CONTROL AND THIAMPHENICOL-TREATED RATS

ATPase activity is expressed as μmol ATP hydrolyzed/min per mg protein. Results are given as mean values ± S.E. of three experiments. Thiamphenicol treatment was started 4—6 h after partial hepatectomy and was for 64 h.

	ATPase	% inhibition by oligomycin (2 μg/ml)
Control animals	0.72 ± 0.10	82 ± 3.0
Thiamphenicol-treated animals	0.69 ± 0.11	57 ± 2.8

shown by Drahota and Houstek [17] that beef heart F_1 hybridizes quite well with F_1-depleted submitochondrial particles (NaBr particles). In the experiment of Fig. 1 a fixed amount of beef heart F_1 is incubated with increasing amounts of NaBr particles isolated from regenerating liver mitochondria of normal and thiamphenicol-treated rats. After 30 min of incubation the ATPase activity and the inhibition by oligomycin was measured. Addition of more than about 125 μg of both preparations of NaBr particles/μg F_1 does not increase the percentage of inhibition by oligomycin. However in NaBr particles from control animals this level of inhibition is about 90%, whereas in the NaBr particles from thiamphenicol-treated animals not more than 60% of the reconstituted ATPase activity can be inhibited by oligomycin (Fig. 1). This corresponds with percentages of inhibition by oligomycin of 82% and 57% in Lubrol particles of control and thiamphenicol-treated animals, respectively (Table I). It should be noticed here that the NaBr particles of the treated animals still contain an amount of functional F_o derived from the normal mitochondrial membranes already present at the onset of the in vivo inhibition with thiamphenicol. The protein con-

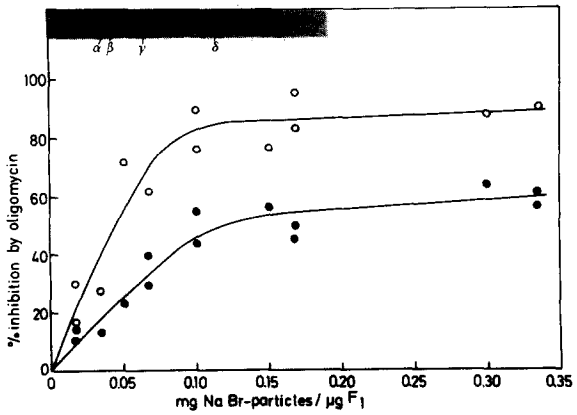


Fig. 1. Inhibition by oligomycin of ATPase activity of beef heart F_1 bound to NaBr particles, isolated from mitochondria of regenerating liver of control rats (○———○) and thiamphenicol-treated rats (●———●). The combined results are given from experiments performed with three different preparations of NaBr particles. ATPase activity of NaBr particles was always less than 0.004 μmol/mg protein per min. ATPase activity of beef heart F_1 was 10 μmol/mg protein per min. The band pattern of F_1 in a SDS-polyacrylamide gel is shown in the inset. The ε subunit is not visible.

tent of regenerating liver as well as the amount of total mitochondrial protein increase about two fold during the period of the thiamphenicol treatment of 64 h. The specific activity of cytochrome *c* oxidase, the synthesis of which is also dependent in part on the mitochondrial translation system [24] is 47% lower in the mitochondria of the thiamphenicol-treated rats than in the mitochondria of control animals (data not shown). Therefore, it can be assumed that the amount of normal F_o in the mitochondria of the treated animals is also about 40—50% lower than in the control preparations, at least if the turnover of cytochrome *c* oxidase and the membrane part of the ATPase complex is the same. Mitochondrial protein turnover, however, is not an important factor in this respect during the relative short period of treatment [25]. The experiment shown in Fig. 1 shows that the added F_1 ATPase is bound both to the normal F_o and to the deficient newly synthesized F_o, which are both present in NaBr particles of the thiamphenicol-treated animals. For, if F_1 ATPase were only bound to the normal F_o one would expect to obtain 90% inhibition of the ATPase activity by oligomycin after reconstitution with particles from the treated animals. This 90% inhibition should be reached in that case at a ratio of NaBr particles to F_1 of about 250, i.e. twice as high as for the control particles.

The firm binding of F_1 to the deficient F_o offers the possibility to isolate the oligomycin-insensitive ATPase complex in an intact form and to compare its subunit composition with a normal oligomycin-sensitive ATPase complex, obtained by the same isolation procedure.

Isolation of the ATPase complex

The enzyme was solubilized from Lubrol particles with Triton X-100, essentiallly as described by Soper and Pederson [18], followed by glycerol gradient centrifugation of the solubilized enzyme. The results of a typical experiment are summarized in Table II. After addition of Triton X-100 to the Lubrol particles the total activity declines about two-fold. About 70% of all ATPase activity is in a solubilized or dispersed form in the sense that it can not be spun down upon centrifugation at 200 000 × *g* for 90 min. 80% inhibition of the ATPase activity in the Triton X-100 extract was reached at 1 μg oligomycin/ml corresponding to 25 μg/mg protein.

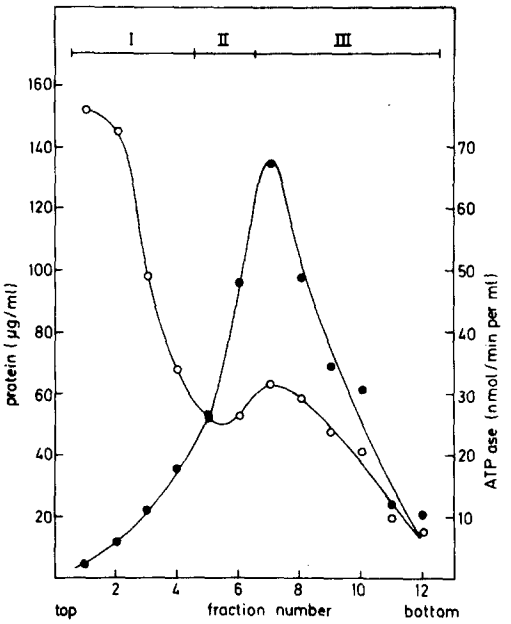
The Triton X-100 extract was layered on 8—18% glycerol gradients and subjected to centrifugation. The ATPase activity separated from most other proteins (Fig. 2), as was shown before for a similar preparation of yeast rutamycin-

TABLE II

SPECIFIC ATPase ACTIVITY AND INHIBITION BY OLIGOMYCIN IN TRITON X-100-SOLUBLE AND INSOLUBLE FRACTIONS DERIVED FROM LUBROL PARTICLES

ATPase activity is expressed as μmol ATP hydrolyzed/min per mg protein.

Fraction	Total amount of protein (mg)	Specific activity	% inhibition by oligomycin
Lubrol particles	8.5	0.64	97
Triton X-100-soluble fraction	5.4	0.34	80
Triton X-100-insoluble fraction	3.9	0.08	85



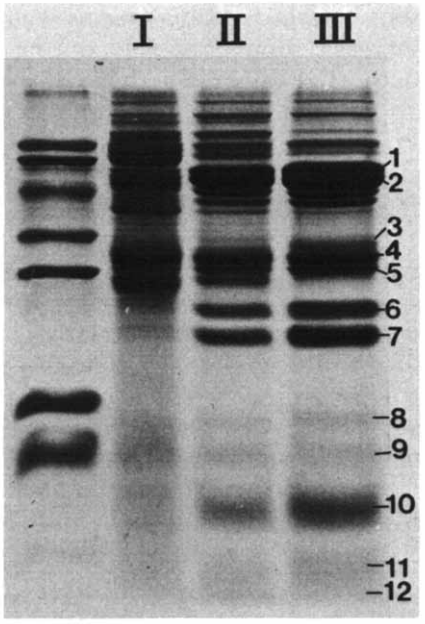


Fig. 2. Glycerol gradient centrifugation of Triton X-100 extract of Lubrol particles. ○———○, protein; ●———●, ATPase activity. For experimental details see Methods and Materials.

Fig. 3. SDS-polyacrylamide slab gel electrophoresis of fractions I—III. The slots from left to right represent a mixture of standard proteins (4 μg each), 60 μg fraction I, 40 μg fraction II and 60 μg fraction III, respectively.

sensitive ATPase [26]. The specific activity of the peak fraction was up to three-fold the activity of the crude Triton X-100 extract. A decline of oligomycin sensitivity to 40% inhibition at 1 μg oligomycin/ml was observed in the fractions containing ATPase activity. Usually the fractions were pooled as indicated in Fig. 2 and further designated fraction I, II and III. The results were similar if the complex was isolated from thiamphenicol-treated animals: the centrifugation profiles, the yields and specific enzyme activities were strictly comparable, while the inhibition by oligomycin was 20—30% lower in the various fractions.

Composition of the ATPase complex

The subunit structure as judged from the band pattern after SDS gel electrophoresis of fractions II and III (Fig. 3) resembles the subunit structure of beef heart oligomycin-sensitive ATPase as isolated by Berden and Voorn-Brouwer [27] and by Serrano et al. [28]. Besides some minor bands of molecular weight higher than 50 000 and two or three bands at about 40 000 molecular weight the preparation of fraction III contains 12 bands which are numbered from 1 to 12, beginning with the band of the highest molecular weight. Analogous to the preparations from beef heart [27,28] and rat liver F_1 ATPase (for a review see Ref. 29) band 1 and 2 with molecular weight of 53 000 and 49 000, respectively, are considered the α and β subunit of F_1 ATPase. The identity of band 3, which is lacking in fraction II is not clear. No band of this molecular weight

was detected in the preparation of Serrano et al., while in the preparation of Berden and Voorn-Brouwer a similar band is ascribed to an impurity. Band 4 with molecular weight 34 000 could be ascribed to the γ subunit F_1. The adenine nucleotide translocator [27,28] and the uncoupler binding protein [27] can be mentioned as candidates for component 5, the molecular weight of which is 32 000. Band 6 with molecular weight 25 000 is also clearly present in the beef heart preparation [27,28] and may belong to F_0. Presumably band 7 represents OSCP. The reported weight of 22 500 [30] is in good agreement with the molecular weight of 22 000 of this band.

The oligomycin-sensitive ATPase complex of beef heart contains a number of proteins with a molecular weight close to or lower than 14 000. These are the δ subunit of F_1 with a molecular weight of 12 500 [29], F_{c2} [31] of which molecular weights of 8 000 [32] and 10 000 [27] are reported, the DCCD binding protein with a molecular weight of 9 000—14 000 [27,33], the F_1 inhibitor, also isolated from rat liver mitochondria as a protein with a molecular weight of 9 500 [34] and the ϵ subunit of F_1 ATPase with a molecular weight of about 7 500 [29]. The preparations II and III contain five bands in this range of molecular weights. These are numbered 8 (14 000), 9 (12 000), 10 (9 000), 11 (6 800) and 12 (6 200). Most likely band 9 contains the δ subunit and band 11 or 12 contains the ϵ subunit. It is difficult to say at this moment which of the bands 8—10 and 11 or 12 contain the other subunits mentioned above.

We conclude that the final preparations II and III contain most likely all the subunits from functional oligomycin-sensitive ATPase preparations described by others [27,28].

Comparison of the subunit composition of the ATPase complex isolated from control animals and from thiamphenicol-treated animals

In order to compare the subunit composition of the ATPase complex isolated from control animals and from thiamphenicol-treated animals the following type of experiment was performed with partially hepatectomized rats. One rat received a dose thiamphenicol of 125 mg/kg body weight on the third day after partical hepatectomy. A second dose was given 8 h later together with 1.5 mCi [^{3}H]leucine. A second rat was given only 1.5 mCi [^{3}H]leucine at the same

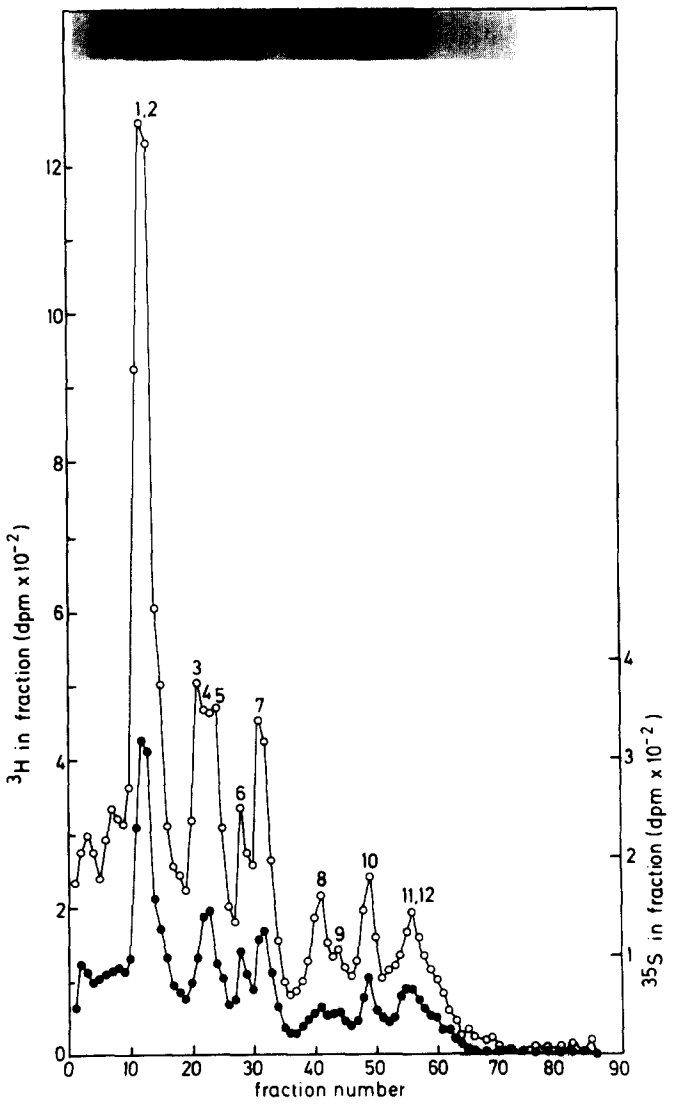
TABLE III

^{3}H RADIOACTIVITY INCORPORATED INTO PROTEIN OF MITOCHONDRIA AND FRACTION III ISOLATED FROM REGENERATING RAT LIVER OF CONTROL AND THIAMPHENICOL-TREATED RATS

Mitochondria and fraction III were isolated after mixing the ^{3}H-labelled liver homogenate with a ^{35}S-labelled liver homogenate from a control rat, as indicated in Methods and Materials and in the text.

Fraction	Control animals		Thiamphenicol-treated animals	
	^{3}H radioactivity (dpm/mg protein)	^{3}H/^{35}S	^{3}H radioactivity (dpm/mg protein)	^{3}H/^{35}S
Mitochondria	$222 \cdot 10^3$	3.42	$217 \cdot 10^3$	3.35
Fraction III	$250 \cdot 10^3$	4.03	$175 \cdot 10^3$	2.79

time while the third rat received 0.5 mCi [^{35}S]methionine. The rats were killed 10 h after the administration of the labelled amino acid and the livers were excised and homogenized. Equal portions of the ^{35}S-labelled homogenate were mixed with the two ^{3}H-labelled homogenates and ATPase was isolated from the mixed homogenates. The incorporation of the radioactivity into protein of the mitochondria and the final fraction III is given in Table III. It should be noticed that the specific ^{3}H radioactivity of fraction III from the treated animals and the ^{3}H:^{35}S ratio is only about 30% lower than in the corresponding fractions of the control experiment. On the other hand the ^{3}H:^{35}S ratio in the two preparations of mitochondria is practically equal. The somewhat lower amount of radioactivity in the whole preparation III from the thiamphenicol-treated rats can be ascribed to the specific inhibition of the incorporation into three



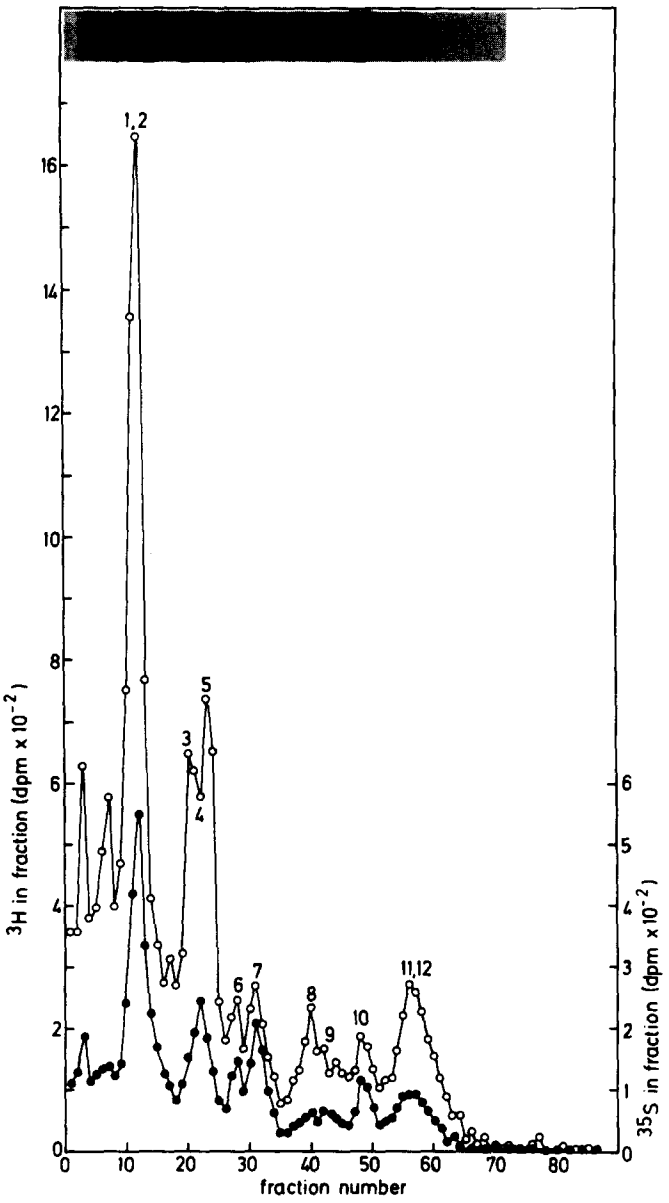


Fig. 4. SDS-polyacrylamide disc gel electrophoresis of fraction III. Fraction III was obtained after in vivo labelling of rats with [^{3}H]leucine or [^{35}S] methionine as described in Methods and Materials and in the text. Radioactivity in the gel was determined as described in Methods and Materials. (A) Fraction III isolated from a mixture of a ^{3}H-labelled liver homogenate and one-half of a ^{35}S-labelled liver homogenate both from partially hepatectomized rats (control). (B) Fraction III isolated from a mixture of a ^{3}H-labelled liver homogenate from a partially hepatectomized, thiamphenicol-treated rat and the other half of a ^{35}S-labelled liver homogenate from a partially hepatectomized control rat. o———o, ^{3}H; •———•, ^{35}S. An SDS (slab) gel of fraction III after coloring with Coomassie Brilliant Blue is shown in the inset.

components of the ATPase complex (see Fig. 4), whereas also a somewhat decreased rate of assembly of the other subunits of the ATPase complex into the inner mitochondrial membranes of the thiamphenicol-treated rats may contribute to the slightly declined incorporation into fraction III. In any case it

can be concluded that most of the oligomycin-insensitive ATPase complexes which are formed under conditions of inhibition of mitochondrial protein synthesis, can be isolated by the same isolation procedure as the normal oligomycin-sensitive ATPase complexes of the control animals. The distribution of radioactivity of fraction III from the control and the thiamphenicol-treated animals in a SDS-polyacrylamide disc gel is shown in Fig. 4. Most of the bands numbered 1—12 can be distinguished on the basis of the distribution of ^{3}H and ^{35}S radio-activity in the gel. Bands 1 and 2 (α and β subunits) are not separated in the radioactivity profile. The other components except 11 and 12 are clearly visible as separate peaks. It is evident that relatively less [^{3}H]leucine is incorporated into the polypeptides of band 6, 7 and 10 of Fig. 4B as compared to Fig. 4A. A quantitative elaboration of the data is given in Table IV. Similar results were obtained with fraction II (see Fig. 2). It is evident from these experiments that inhibition of mitochondrial protein synthesis only influences the labelling of polypeptides present in the bands 6, 7 and 10 and has little effect on the synthesis of the components numbered 1—5, 8, 9, 11 and 12.

By the same technique we have also determined the $^{3}H/^{35}S$ ratio in the diffurent components of the Triton X-100-insoluble fraction derived from the Lubrol particles. This fraction contains nearly all cytochrome aa_3 present in the Lubrol particles. At least one component of the Triton X-100-insoluble fraction from the thiamphenicol-treated rats has a relatively low $^{3}H/^{35}S$ ratio. This component with a molecular weight of about 24 000 could be a subunit of cytochrome aa_3, the synthesis of which is dependent on mitochondrial protein synthesis [24]. The residual amount of ATPase in the Triton X-100-insoluble fraction is too low to account for this component. The assemblage of the cytochrome bc_1 complex into the inner mitochondrial membrane is also dependent

TABLE IV

IN VIVO [^{3}H]LEUCINE AND [^{35}S]METHIONINE INCORPORATION INTO THE DIFFERENT SUBUNITS OF THE ATPase COMPLEX ISOLATED FROM REGENERATING LIVER MITOCHONDRIA OF CONTROL RATS AND THIAMPHENICOL-TREATED RATS

ATPase (fraction III) was fractionated in the different subunits on SDS-polyacrylamide disc gels. Radioactivity in the gels was determined as described in Methods and Materials. Column a: $^{3}H/^{35}S$ ratio in the peak fractions of the subunits of the ATPase complex (Fig. 4A) isolated from a mixture of a ^{3}H-labelled liver homogenate and one-half of a ^{35}S-labelled liver homogenate both from partially hepatectomized rats (control). Column b: $^{3}H/^{35}S$ ratio in the peak fractions of the subunits of the ATPase complex (Fig. 4B) isolated from a mixture of a ^{3}H-labelled liver homogenate from a partially hepatectomized thiamphenicol-treated rat and the other half of a ^{35}S-labelled liver homogenate from a partially hepatectomized rat. Column c; b/a × 100%. Column d: as column c, data from another experiment.

Subunit number	a	b	c	d
1,2	3.94	3.00	76	89
3	5.15	4.23	82	81
4	3.22	2.36	73	66
5	5.16	4.96	96	101
6	3.20	1.68	53	37
7	3.92	1.28	33	40
8	4.50	3.67	82	76
9	3,38	2.48	73	71
10	3.09	1.64	53	46
11,12	3.03	3.02	100	90

on mitochondrial protein synthesis. However, no components with a low $^3H/^{35}S$ ratio could be detected in fraction I (see Fig. 2) from the thiamphenicol-treated animals although this fraction contains most of the cytochrome *b* and c_1 present in the Lubrol particles. It is unlikely that the low ^3H incorporation in the components 6, 7 and 10 of fractions II and III from thiamphenicol-treated animals is caused by loss of one or more of these components during the isolation procedure to other fractions. Neither fraction I of the glycerol gradient, nor the Triton X-100-isoluble material from thiamphenicol-treated rats contain components of molecular weights, in the range of those of the lacking subunits, with a relatively high $^3H/^{35}S$ ratio. The experimental data reflect, therefore, the real subunit composition of the ATPase complex as it is formed during inhibition of mitochondrial protein synthesis.

Discussion

The aim of this study was to compare the subunit structure of the oligomycin-insensitive ATPase, as it is formed during in vivo inhibition by thiamphenicol of mitochondrial protein synthesis, with the subunit structure of the oligomycin-sensitive ATPase from control rats. The purification for the ATPase complex from rat liver mitochondria described in this paper was suitable for this aim. In the first place it emerged from SDS-polyacrylamide gel electrophoresis that the purified enzyme (fraction III, Fig. 2) from control rats has a subunit composition comparable with the beef heart oligomycin-sensitive ATPase purified by Serrano et al. [28] and by Berden and Voorn-Brouwer [27]. It has been demonstrated that these preparations, when integrated in phospholipid vesicles show ATP-P_i exchange [28] and ATP and Mg^{2+}-dependent energization of these vesicles [27].

In the second place, it became evident that the oligomycin-insensitive ATPase present in the Lubrol particles of thiamphenicol-treated rats can be isolated by the same procedure as the normal oligomycin-sensitive ATPase. This evidence derived from the fact that during the inhibition of mitochondrial protein synthesis in the thiamphenicol-treated rats the total in vivo amino acid incorporation into fraction III was only slightly lower than the incorporation in fraction III from the control rats (Table III). It should be noticed that at least 80% of the total radioactivity in fraction III from control as well as thiamphenicol-treated animals is associated with the components 1—12. These components, except component 3, are considered to belong to the ATPase complex.

Less than 30% inhibition of [^3H]leucine incorporation into the components belonging to bands 1—5, 8, 9, 11 and 12 of fraction III was observed, when rats were treated in vivo with thiamphenicol, a specific inhibitor of mitochondrial protein synthesis (Fig. 4; Table IV). This indicates that these components are synthesized in the cytoplasm. Among these components the five subunits of F_1 ATPase numbered 1, 2, 4, 8 or 9 and 11 or 12 may be recognized. As pointed out already, band 3 most likely represents an impurity. So in addition to the five subunits of F_1 three other components (5, 8 or 9, 11 or 12), associated with the ATPase complex, are of cytoplasmic origin. These are not only synthesized, but also assembled into the ATPase complex formed under conditions

of inhibition of mitochondrial protein synthesis in a similar way as under control conditions. Component 5 may belong to F_0 because a similar band in beef heart oligomycin-sensitive ATPase complex could not be extracted by 6 M urea [27].

The three bands numbered 6, 7 and 10 show a low [^{3}H]leucine incorporation during the thiamphenicol treatment. This indicates that either the synthesis or assembly of these components is inhibited under conditions of inhibition of mitochondrial protein synthesis. Component 7 represents OSCP, as already pointed out in Results. Hence it is understandable that the ATPase complex formed under conditions of mitochondrial protein synthesis becomes insensitive to inhibition by oligomycin [9] and does not participate in oxidative phosphorylation [11,12], because the indispensability of OSCP for the right functioning of the enzyme is well-established [35]. It is also somewhat surprising, however, that the deficient ATPase complex formed during thiamphenicol treatment is OSCP deficient, because this ATPase is still membrane bound. So we are forced to conclude that the availability of OSCP is not the sole prerequisite for binding of the F_1 sector of the enzyme complex to F_0. This conclusion is supported by the observation of Russel et al. [30] and Vădineanu et al. [36], that F_{c2} alone is able to bind F_1 to F_0, although OSCP seems to strengthen this binding [36]. The results shown in Fig. 1 suggest that beef heart F_1 binds with the same affinity to the normal F_0 as the deficient F_0 present in the preparations of NaBr particles from thiamphenicol-treated rats.

Also the synthesis of component 6, with a molecular weight of 26 000, appears to be blocked in thiamphenicol-treated rats. A similar subunit is present in beef heart oligomycin-sensitive ATPase and probably belongs to F_0 [27]. The third band in which the [^{3}H]leucine incorporation is lower during inhibition of mitochondrial protein synthesis is band 10 with a molecular weight of 9 000. It has recently been shown that isolated rat liver mitochondria are able to synthesize a hydrophobic polypeptide which is able to bind DCCD. The molecular weight of this protein is also 9 000 [37]. It is known that ATPase complexes of different sources [33,38,40] contain a polypeptide, possibly functioning as the proton channel, which specifically and covalently binds DCCD. In yeast, this DCCD binding protein is synthesized in the mitochondria [38] and is coded for the mitochondrial DNA [41], whereas in *N. crassa* this subunit is made by the cytoplasmic ribosomes and coded for by a nuclear gene [42]. It is of great interest to identify this DCCD binding subunit in the ATPase complex of the rat, to isolate this compound and to compare its properties with the DCCD binding protein synthesized by rat mitochondria in vitro. Preliminary experiments suggest that ^{14}C-labelled DCCD, added to fraction III or to a crude Triton X-100 extract of Lubrol particles, is associated with band 12, with a molecular weight of 6 000—7 000 and with band 8 which has a molecular weight of 14 000 and possibly contains the dimers of the DCCD binding protein [27]. Band 10, however, was not labelled in these experiments.

The results together show beyond doubt that active mitochondrial protein synthesis is indispensable for the synthesis of the components 6, 7 and 10 of the rat liver ATPase complex. This being established, there are still a number of possible explanations from a mechanistic point of view. The most straightforward is that all three subunits are synthesized on the mitochondrial ribosomes

and coded for by the mitochondrial DNA. It cannot be excluded, however, that one or more of the polypeptides are synthesized in the cytoplasm but that they need other mitochondrially synthesized proteins for their assembly into the ATPase complex or that their synthesis is strongly controlled and regulated by products of mitochondrial synthesis. It is, perhaps, even likely that one of the latter mechanisms holds for OSCP, which is synthesized in the cytoplasm at least in yeast [43]. Further research is necessary to identify one or more of the components 6, 7 and 10 as mitochondrial products.

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