AMINO ACID INCORPORATION BY ISOLATED RAT LIVER MITOCHONDRIA INTO TWO PROTEIN COMPONENTS OF MITOCHONDRIAL ATPase COMPLEX

Lack of incorporation into dicyclohexylcarbodiimide binding proteolipid

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1. Introduction

The role of products of mitochondrial protein synthesis in the biogenesis of the mitochondrial ATPase complex of yeast and fungi is well documented. In *Saccharomyces cerevisiae* four [1] and in *Neurospora crassa* two [2] protein subunits of the complex are synthesized intramitochondrially. Special attention has been paid to the elucidation of the site of the synthesis of DCCD binding proteolipid of the mitochondrial ATPase complex. In yeast [3] this component is synthesized within mitochondria and in fungi [4,5] outside the organelles. Less information exists on the participation of mitochondrial translation products in the formation of mitochondrial ATPase complex and on the site of the synthesis of DCCD binding proteolipid of the complex in animal cells. The intramitochondrial formation of three protein components of mitochondrial ATPase complex in oocytes [6] and in regenerating rat liver [7] has been indicated by selective in vivo inhibition of cytoplasmic or mitochondrial protein synthesis. Incorporation of labeled amino acids by isolated rat [8] and mouse [9] liver mitochondria into DCCD binding proteolipid has been described and it was concluded that the DCCD binding component of rat [8] and mouse [9] liver mitochondrial ATPase complex represents a product of mitochondrial protein synthesis. On the other hand, selective in vivo inhibition of mitochondrial protein synthesis in regenerating rat liver did not affect significantly amino acid incorporation into a component supposed to be identical with the DCCD binding proteolipid of mitochondrial ATPase complex [7].

We found that isolated rat liver mitochondria incorporated [^{35}S]methionine into two protein components of the mitochondrial ATPase complex. One of these components was soluble in chloroform:methanol (2:1) mixture but its electrophoretic mobility was different from that of the DCCD binding proteolipid.

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; F_1, mitochondrial ATPase (coupling factor 1); PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate

2. Materials and methods

Incorporation of [^{35}S]methionine by isolated [10] mitochondria proceeded at 30°C in a sterile medium containing mitochondria (3–5 mg/ml), 50 mM bicine, 90 mM KCl, 1 mM EGTA, 5 mM KH_2PO_4, 1 mM cycloheximide, 0.1% bovine serum albumine, 15 mM $MgCl_2$, 1 mM ATP, 5 mM phosphoenolpyruvate, 25 μg/ml pyruvate kinase, 50 μg/ml unlabeled amino acid mixture [11] without methionine, 50 μCi/ml [^{35}S]methionine (>1000 Ci/mmol), final pH 7.8. After 20 min incubation with shaking, 10 mM unlabeled methionine was added to the mixture. Following additional 5 min incubation the labeled mitochondria were centrifuged and 3 times washed with 0.3 M mannitol, 1 mM EDTA, 0.1% chloramphenicol, 10 mM unlabeled methionine, (pH 7.4).

Rat liver mitochondria labeled with [^{14}C]amino acids in vivo were isolated from 200 g animals injected with 1.5 mCi [^{14}C]leucine (125 Ci/mol), 1.5 mCi [^{14}C]isoleucine (105 Ci/mol), 1 mCi [^{14}C]phenylalanine (225 Ci/mol) in two equal doses 36 and 16 h before sacrifice.

Labeling of mitochondria with [^{14}C]DCCD (32 Ci/mol) was performed by 16 h incubation at 4°C with the inhibitor (0.5–10 nmol/mg protein) in a mixture containing 0.25 M sucrose, 1 mM EDTA, 10 mM Tris–HCl (pH 7.5) and mitochondria (3 mg/ml). The labeled mitochondria were centrifuged and washed 2 times with the incubation medium without DCCD.

Chloroform:methanol (2:1) extraction was performed as detailed in [12]. The labeled immunoprecipitated ATPase complex was coextracted with 10-fold excess of unlabeled mitochondria.

Antisera against purified [13] chloroform released [14] F_1 were obtained using the following schedule. The rabbits were injected into footpad with 0.1, 0.5 and 1 mg F_1 which was dissolved in 0.5 ml 0.8% NaCl, 0.002% KCl, 0.002% KH_2PO_4 and 0.0917% K_2HPO_4, and emulsified with 0.5 ml complete Freund's adjuvant. Intervals between respective doses were 3 weeks. Sera were collected 8 days after the last injection.

The ATPase complex was solubilized and immunoprecipitated from mitochondria by direct precipitation with an immunoglobulin fraction [15] from anti-F_1 sera according to [16].

SDS–PAGE was performed [17] in gel slabs with a linear 12–20% acrylamide gradient. The samples were dissolved and depolymerized before electrophoresis by either 2 min heating at 90°C or by 30 min incubation at 50°C with 2% SDS, 4% 2-mercaptoethanol, 20% glycerol, 10 mM Tris–HCl (pH 7.0). Both treatments yielded identical results. Gels were stained for 16 h [18] and destained [19] for 24 h at 20°C. Two-dimensional separation of proteins was according to [20]. Radioactivity distribution in gels was monitored by fluorography [21].

Protein was estimated according to [22].

3. Results and discussion

ATPase complex immunoprecipitated from rat liver mitochondria consisted of ~12 protein components separable by SDS–PAGE. These components were detected in the gel by both staining and determining radioactivity distribution after electrophoresis of the complex immunoprecipitated from mitochondria labeled with [^{14}C]amino acids in vivo (fig.1). Protein constituents of the immunoprecipitated ATPase complex ranged from mol. wt 93 500–8000. The protein composition of the immunoprecipitated ATPase complex was practically identical with that of the oligomycin-sensitive ATPase complex prepared according to [20].

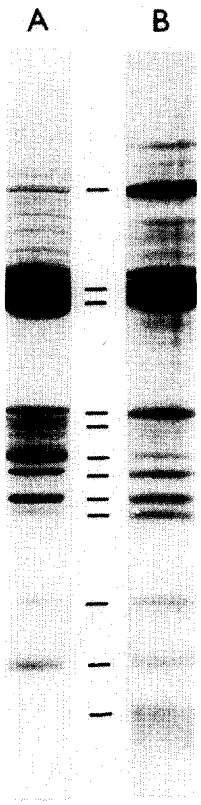

Fig.1. Protein composition of ATPase complex immunoprecipitated from rat liver mitochondria. The complex was precipitated from unlabeled mitochondria (A) or from mitochondria labeled in vivo with [^{14}C]amino acids (B). After electrophoresis the proteins were stained (A) or detected by fluorography (B).

ATPase complex immunoprecipitated from mitochondria labeled with [^{35}S]methionine in vitro contained two discrete labeled components with mol. wt 21 500 and 9000, respectively (fig.2). Incorporation of [^{35}S]methionine by isolated mitochondria into these components was inhibited by chloramphenicol. Thus, under the conditions used, 21 500 and 9000 mol. wt components of ATPase complex were formed and integrated into the complex by isolated rat liver mitochondria. This can be compared with a recent report [7] that 25 000, 22 000 and 9000 mol. wt components of rat liver mitochondrial ATPase complex are synthesized in mitochondria of regenerating rat liver in vivo. We could detect an in vitro labeled product with mol. wt 25 000 in the

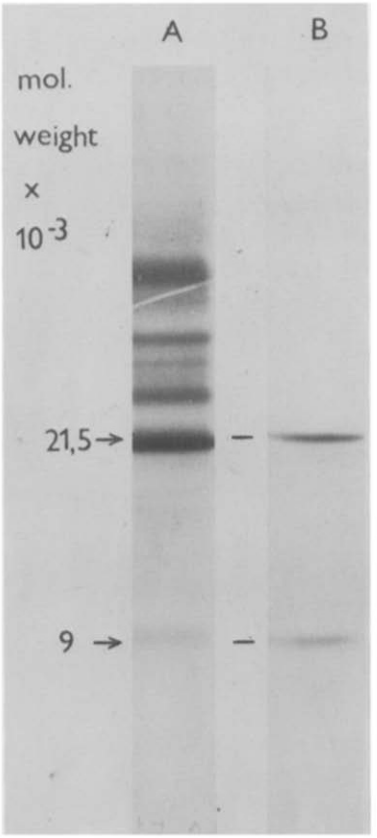

Fig.2. Products of in vitro mitochondrial protein synthesis in whole mitochondria and in mitochondrial ATPase complex. [^{35}S]Methionine labeled products were detected by fluorography after electrophoresis of (A) whole in vitro labeled mitochondria and (B) ATPase complex immunoprecipitated therefrom.

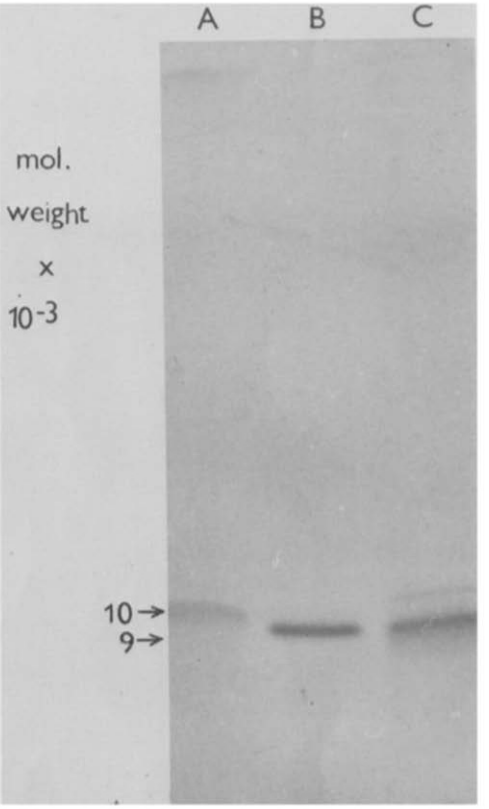

Fig.3. Chloroform:methanol soluble components of mitochondrial ATPase complex labeled with DCCD and amino acids. Chloroform:methanol extracts of ATPase complex immunoprecipitated from mitochondria labeled with (A) [^{14}C]DCCD, (B) [^{35}S]methionine in vitro and (C) [^{14}C]amino acids in vivo were electrophoresed and fluorographed.

immunoprecipitated ATPase complex when the precipitation reaction lasted longer as a consequence of the use of immunoglobulin fraction from less potent antisera. This fact along with the low labeling of the 25 000 mol. wt product recovered in the immunoprecipitate under the conditions mentioned probably indicates a disposition of this product to associate and to coprecipitate non-specifically with the complex.

The 9000 mol. wt component of the ATPase complex was extractable with chloroform:methanol (2:1) mixture as the single in vitro labeled product from both ATPase complex and whole mitochondria (fig.3). Relation between this proteolipid component of the ATPase complex and DCCD binding proteolipid was next examined. Independently of the concentration of [^{14}C]DCCD used for the labeling of rat liver mitochondria the majority of the radioactivity bound to the immunoprecipitated ATPase complex was recovered in two components with mol. wt 16 500 and 10 000, respectively (fig.4). In chloroform:methanol extracts of both the [^{14}C]DCCD-labeled mitochondria and the ATPase complex only the 10 000 mol. wt labeled component was found. As detected by two-dimensional separation of [^{14}C]DCCD-labeled mitochondria the DCCD binding components with mol. wt 16 500 and 10 000 exhibited identical isoelectric point (pI 7.5) (fig.4). Accordingly, the DCCD binding component with mol. wt 16 500 may represent an aggregated form of the 10 000 mol. wt DCCD binding proteolipid. The tendency of DCCD binding proteolipid of beef heart mitochondria to migrate as an aggregate in SDS–PAGE [24] along with the iden-

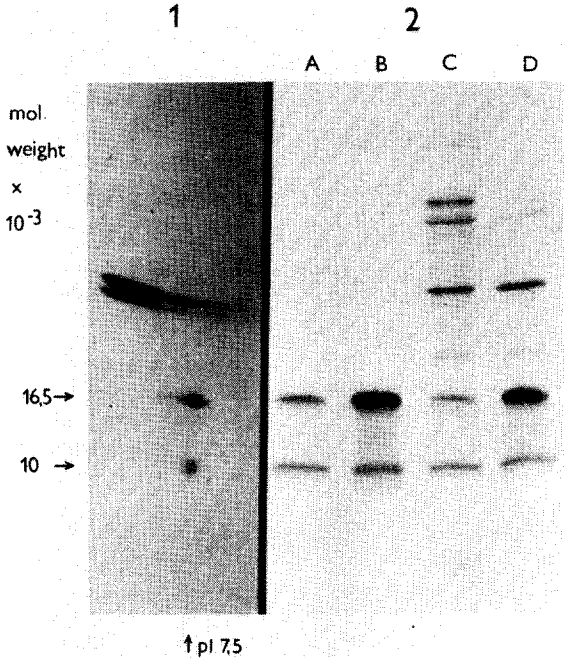

Fig.4. Electrophoretic properties of DCCD binding components of mitochondrial ATPase complex. (1) Fluorogram of the gel slab with [^{14}C]DCCD labeled mitochondria separated in two dimensions. Mitochondria were labeled in the presence of 0.75 nmol [^{14}C]DCCD/mg protein and separated in two dimensions with the omission of 2-mercaptoethanol. The radioactivity was recovered only in the demonstrated part of the slab. (2) Fluorogram of the electrophoresed whole mitochondria labeled in the presence of (D) 0.5 nmol [^{14}C]DCCD/mg protein, (C) 10 nmol [^{14}C]DCCD/mg protein, and of electrophoresed ATPase complex immunoprecipitated from mitochondria labeled in the presence of (B) 0.5 nmol [^{14}C]-DCCD/mg protein, (A) 10 nmol [^{14}C]DCCD/mg protein.

tical electrophoretic properties of DCCD binding components of beef heart and rat liver mitochondrial ATPase complex (not shown) favour this proposal.

In the ATPase complex immunoprecipitated from [^{14}C]DCCD-labeled mitochondria no DCCD binding component with the electrophoretic mobility corresponding to the 9000 mol. wt proteolipid labeled with [^{35}S]methionine in vitro was detected (see fig.3,4). This observation is at variance with data pointing to the synthesis of DCCD binding proteolipid in isolated rat [8] and mouse [9] liver mitochondria. Such discrepancy can be most simply explained by the different conditions of the preparation of mitochondrial proteolipid(s) and by the higher resolving power of the fluorographic detection of radioactivity distribution in electrophoreograms used in this study.

It could be argued that the 10 000 mol. wt DCCD binding proteolipid represented in fact a DCCD modified form of the 9000 mol. wt component labeled in vitro with [^{35}S]methionine which would have an altered electrophoretic mobility upon DCCD binding. However, no labeled 10 000 mol. wt component was detected in chloroform:methanol extract of mitochondria labeled in vitro with [^{35}S]methionine and subsequently incubated with unlabeled DCCD (fig.5).

It can be concluded that:

1. Two protein components of rat liver mitochondrial

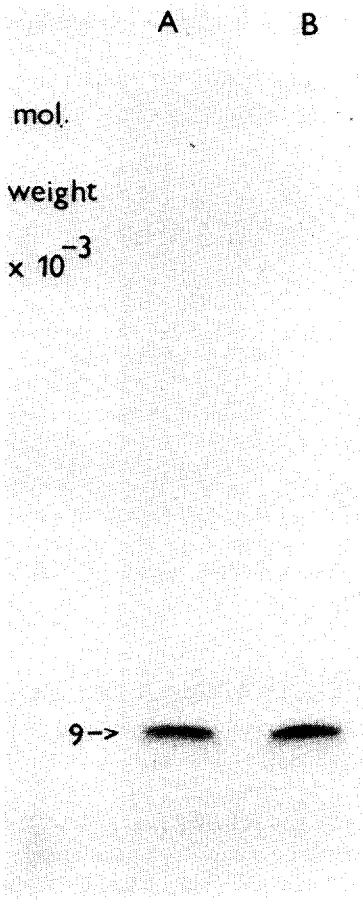

Fig.5. Lack of the effect of DCCD on electrophoretic properties of chloroform:methanol soluble component labeled with [^{35}S]methionine in vitro. Mitochondria were labeled with [^{35}S]methionine in vitro and suspended (2 mg protein/ml) in 0.25 M sucrose, 1 mM EDTA, 10 mM Tris–HCl (pH 7.5) without (A) or with (B) unlabeled DCCD (250 nmol/mg protein). After 2 h incubation at 4°C the mitochondria were pelleted and extracted with chloroform:methanol. The extracts were electrophoresed and fluorographed.

ATPase complex are synthesized and integrated into the complex by isolated mitochondria;

2. The in vitro synthesized component that is soluble in chloroform:methanol differs from the DCCD binding proteolipid in DCCD binding ability, electrophoretic mobility and probably in the intracellular site of synthesis.

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