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De novo synthesis and desaturation of fatty acids at the mitochondrial acyl-carrier protein, a subunit of NADH:ubiquinone oxidoreductase in *Neurospora crassa*

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We have cultivated the *cel* mutant of *Neurospora crassa* defective in cytosolic fatty acid synthesis with [2-^{14}C]malonate and found radioactivity covalently attached to the mitochondrial acyl-carrier protein (ACP), a subunit of the respiratory chain NADH:ubiquinone oxidoreductase. We purified the ACP by reverse-phase HPLC: the bound acyl groups were trans-esterified to methylesters and analyzed by gas chromatography. The saturated C_6 to C_{18} fatty acids and oleic acid were detected. De novo synthesis and desaturation of fatty acids at the ACP subunit of NADH:ubiquinone oxidoreductase and use of the products of this mitochondrial synthetic pathway for cardiolipin synthesis is discussed.

NADH:ubiquinone oxidoreductase; Complex I; Mitochondrial fatty acid synthesis; Cardiolipin

1. INTRODUCTION

Fatty acid synthesis is catalysed by two different types of fatty acid synthetases (FAS) with diverse molecular structure using the same ubiquitous mechanism [1,2]. In both FAS types, the growing fatty acid is linked via a thioester bond to phosphopantetheine. In FAS I this group is bound to the ACP domain within the multifunctional protein, whereas in FAS II it is bound to a discrete, small acyl-carrier protein (ACP). Some years ago such a 'prokaryotic' ACP with the phosphopantetheine was discovered in mitochondria of *Neurospora crassa* [3], and some evidence that the ACP participates in de novo fatty acid synthesis independent of fatty acid synthesis in the cytosol was provided [4]. The product(s) of this mitochondrial synthetic pathway, however, remained unclear. Recently the mitochondrial ACP was also found in bovine heart [5], and it was shown that both *N. crassa* and bovine ACP are tightly associated with the respiratory chain NADH:ubiquinone oxidoreductase, also known as complex I [5,6]. The sequences of bovine and *N. crassa* mitochondrial ACP were determined and a close relationship to bacterial ACP was demonstrated [5,6]. The question which remains to be answered is what is the role of this mitochondrial ACP?

We report here that in *N. crassa* the mitochondrial ACP participates not only in de novo synthesis of long-chain saturated fatty acids but also in fatty acid desaturation. For the entire synthetic pathway the ACP remains associated with respiratory complex I. We discuss the possibility that the mitochondrial ACP donates fatty acids to form key intermediates for cardiolipin synthesis.

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2. MATERIALS AND METHODS

The *cel* mutant of *N. crassa* (University of Kansas, FGSC stock 2947) was grown at 25°C in 0.1 l minimal medium plus 2% sucrose, 0.04% Tween 80 and 0.05 mg [2-^{14}C]malonate (51 Ci/mol, NEN). An inoculum of 10^{10} conidia provided exponential growth for 16 h and a wet cell yield of 0.8 g. Procedures for isolation of mitochondria from labelled hyphae, sucrose gradient centrifugation of Triton X-100-solubilized mitochondria, immunoprecipitation and enzymatic analysis of complex I are described elsewhere [6].

All steps of ACP purification, except reverse-phase HPLC, were carried out at 4°C. Complex I (30 mg protein) purified from 600 g (wet mass) *N. crassa* [6] was dissolved in 1 ml 50 mM Tris-HCl, pH 7.5, 0.1% Triton X-100 and 0.7 M NaI, and then centrifuged on a 12 ml gradient of 0–10% sucrose in 0.8 M NaJ, 50 mM Tris-HCl, pH 7.5, 0.1% Triton X-100 and 0.2 mM phenylmethylsulfonyl fluoride ($PhMeSO_2F$) at 36,000 rpm for 18 h. The top 4 ml of the gradient, containing the ACP, was dialyzed against 50 mM Tris-HCl, pH 7.5, and 0.2 mM $PhMeSO_2F$ for 2 h. The solution was loaded onto a DEAE-Sepharose-Cl 6B column (0.6 × 10 cm; Pharmacia) equilibrated in 50 mM Tris-HCl, pH 7.5. A 60 ml gradient of 0–0.6 M NaCl in 50 mM Tris-HCl, pH 7.5, at a flow rate of 20 ml/h was used for elution. Fractions containing the ACP were dialyzed as above and loaded onto a Mono Q HR 5/5 column (Pharmacia) equilibrated with 50 mM Tris-HCl, pH 7.5. A gradient of 0–1 M NaCl in this buffer at a flow rate of 0.5 ml/min was used for elution (Fig. 2). The ACP fractions were concentrated 10-fold by ultrafiltration (Diaflo PM 10, Amicon) and subjected to reverse-phase HPLC (C_4-HPLC column, 300 Å pore size, 5 μm particles, 250 × 4 mm, Macherey und Nagel, Düren, Germany) equilibrated in H_2O with 0.1% trifluoroacetic acid.

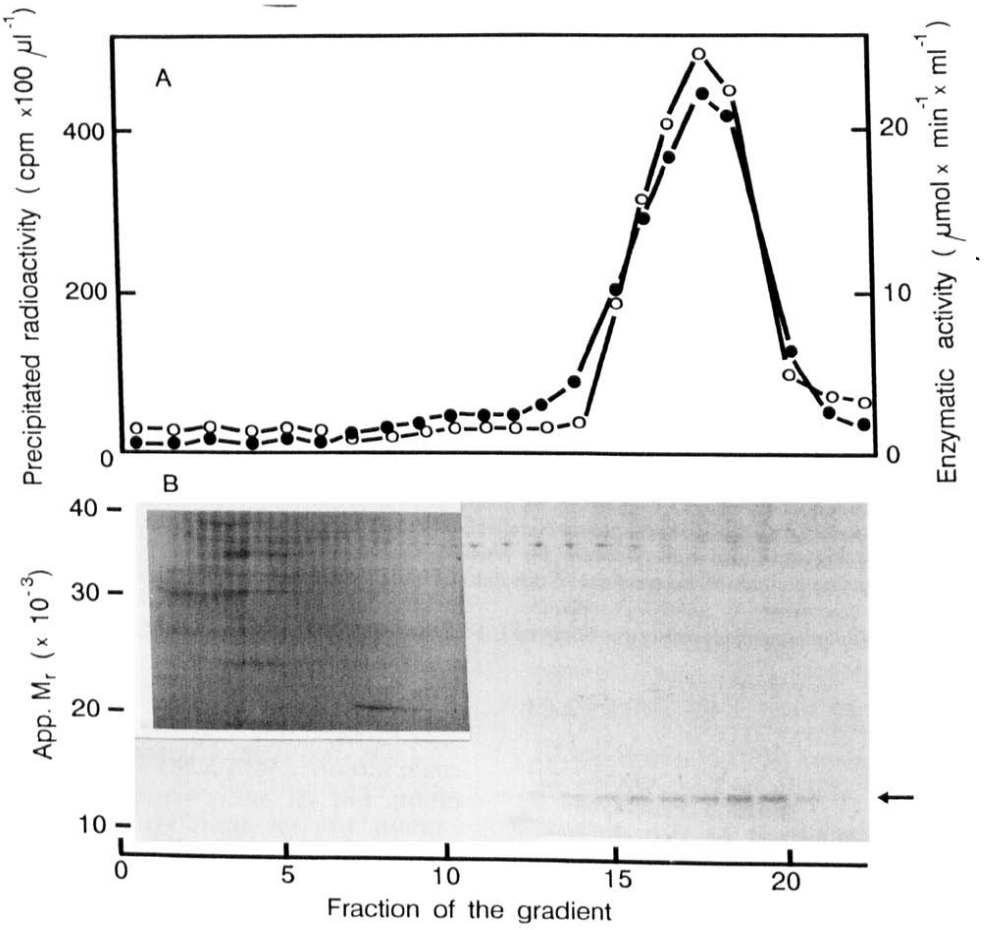

Fig. 1. In vivo labelling by [2-^{14}C]malonate of the acyl groups at the ACP subunit of complex I. (A) Distribution of immunoprecipitated radioactive complex I protein (●) and NADH-ferricyanide reductase activity after sucrose gradient centrifugation of detergent-solubilized mitochondria (○). (B) Radioactively labelled ACP subunit (arrow) of complex I visualized by SDS-PAGE and autoradiography of the immunoprecipitated complex I protein. The inset at the left side shows the pattern of radioactively labelled polypeptides in the total mitochondrial protein. Besides the ACP a number of other polypeptides are labelled but they sedimented less fast through the gradient than complex I.

For elution a 14 ml gradient of 0–99% acetonitrile in 0.1% trifluoroacetic acid, at a flow rate of 0.5 ml/min was used (Fig. 2).

The procedure for gas chromatography is described elsewhere [7]. The ACP solution was concentrated 10-fold by vaporization, 10 µl portions were dried in 1 ml glass vials under a stream of nitrogen, and 5 µl 0.2 M trimethylsulfoniumhydroxide in methanol (Macherey und Nagel) were added to convert the acyl groups derived from the ACP into the methyl ester form. Portions of 1 µl were applied to a HP 5890 gas chromatograph (Hewlett Packard, Avondale, USA) equipped with a flame ionization detector, a split/splitless inlet, a 20 m × 0.25 mm × 0.32 µm film fused-silica capillary column (SE-54, cross-linked 5% phenylmethyl silicone). Hydrogen, with a column head pressure of 50 kPa and a split ratio of 20:1, was used as carrier gas. The temperature of the injector was 250°C and that of the flame ionization detector 300°C. The gas flow was 35 cm/s.

3. RESULTS

A culture of the *cel* mutant of *N. crassa*, grown in the presence of [2-^{14}C]malonate, was harvested in the exponential growth phase after 90% of the radioactivity had been taken up from the medium. After sucrose gradient centrifugation of Triton X-100-solubilized mitochondria, the immunoprecipitated radioactively labelled complex I protein was identified by SDS-PAGE and autoradiography (Fig. 1). An antiserum against the 22 kDa subunit of complex I which does not cross-react with the ACP subunit was used. The experiment showed that in vivo incorporation of [2-^{14}C]malonate leads to labelling of the ACP subunit of complex I.

Complex I was isolated from *N. crassa* wild-type cells [6] and treated with the chaotropic agent, NaJ, to detach the peripheral subunits, among them the ACP [8]. Upon sucrose gradient centrifugation in the presence of Triton X-100, these subunits remained in the top third of the gradient, while the membrane part of the complex sedimented to two thirds of the way through the gradient. The ACP was purified by two anion-exchange chromatographic steps in detergent-free buffer, the latter step performed as FPLC (Fig. 2). To remove any non-covalently bound lipid, the purified ACP was subjected to reverse-phase HPLC in acetonitrile/water/trifluoroacetic acid. The ACP eluted as a single peak at 48% acetonitrile (chromatogram not shown).

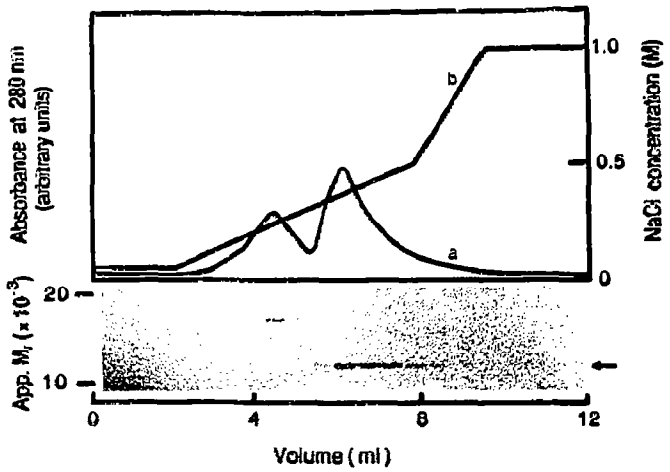

Fig. 2. Purification of the ACP by anion-exchange FPLC. Profile of the absorbance at 280 nm (a), NaCl concentration (b), and SDS-PAGE of the eluted proteins. The ACP is marked with an arrow.

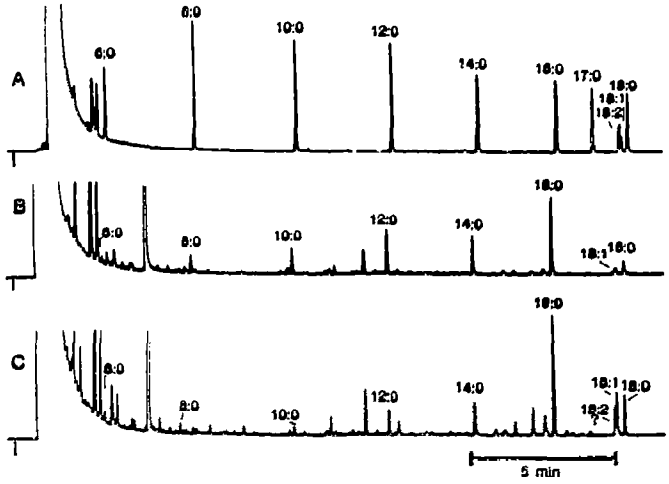

Fig. 3. Capillary gas chromatography of fatty acid methyl esters. (A) Standards, (B,C) two different probes *trans*-esterified from the ACP.

The acyl groups attached to the ACP were *trans*-esterified to fatty acid methyl esters which were analyzed by capillary gas chromatography [7]. The saturated C_6 to C_{18} fatty acids and unsaturated oleic acid were regularly detected in all chromatograms (Fig. 3). As a control a mixture of standard and ACP-derived fatty acid methyl esters were also analyzed, yielding increased peak heights but no broadening or splitting of peaks (data not shown). There was also a minor peak at the position of linoleic acid but the height of this peak varied from experiment to experiment like the heights of other peaks not regularly found. 3-Hydroxytetradecanoate, reported to be the predominant acyl group at the ACP [4], was not detected.

4. DISCUSSION

Synthesis of saturated fatty acids up to stearic acid in fungi and animals has so far been believed to occur only in the cytosol at the FAS type I multi-enzyme complex [1,2]. Further elongation and unsaturation is carried out by accessory enzymes at the endoplasmic reticulum. Our finding that saturated fatty acids up to stearic acid and unsaturated oleic acid are found at the mitochondrial ACP provides strong evidence that a 'prokaryotic' FAS II type complex operates in the mitochondrion of fungi. This assumption can probably also be extended to mammals. Electrospray mass spectrometry of the ACP subunit of bovine complex I before and after incubation at alkaline pH under reducing conditions gave a difference of 302 mass units [5], suggesting stearic acid as the candidate for the (predominant) fatty acid. In view of the transport of fatty acids by the carnitin translocase system from the cytosol into the mitochondria, the question of what the mitochondrial fatty acid synthetic pathway should be used for arises. Mitochondria are not autonomous in their membrane lipid production and import most of their phospholipids from the endoplasmic reticulum [9]. But cardiolipin is made only in the inner mitochondrial membrane by a cardiolipin synthetase of bacterial origin [10].

The phospholipid synthesizing enzymes of most bacteria work with ACP-bound fatty acids and not with CoA-bound fatty acids [11]. Also the growing fatty acids in bacteria are desaturated while covalently attached to ACP [12]. From the similarity between the bacterial phospholipid synthesizing system and the fatty acid synthesis occurring at the mitochondrial ACP we conclude that the role of mitochondrial fatty acid synthesis is to provide the acyl-products used for the synthesis of cardiolipin.

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REFERENCES

[1] McCarthy, A.D. and Hardie, D.G. (1984) Trends Biochem. Sci. 9, 60–63.
[2] Ohlrogge, J.B. (1987) in: The Biochemistry of Plants, vol. 9. (Stumpf, P.K., ed.) pp. 137–157, Academic Press, Orlando.
[3] Chuman, L. and Brody, St. (1989) Eur. J. Biochem. 184, 643–649.
[4] Mikolajczyk, S. and Brody, St. (1990) Eur. J. Biochem. 187, 431–437.
[5] Runswick, M.J., Fearley, I.M., Skehel, J.M. and Walker, J.E. (1991) FEBS Lett. 286, 121–124.
[6] Sackmann, U., Zensen, R., Röhlen, D., Jahnke, U. and Weiss, H. (1991) Eur. J. Biochem. 200, 463–469.
[7] Müller, K.D., Husmann, H. and Nalik, H.P. (1990) Z. Bakteriol. 274, 174–182.
[8] Weiss, H., Friedrich, T., Hofhaus, G. and Preis, D. (1991) Eur. J. Biochem. 197, 563–576.
[9] Daum, G. (1985) Biochim. Biophys. Acta 822, 1–42.
[10] Schlamme, M. and Hostetler, K.Y. (1991) J. Biol. Chem. 266, 22398–22403.
[11] Cooper, C.L. and Lueking, D.R. (1984) J. Lipid Res. 25, 1222–1232.
[12] Garwin, J.L., Klages, A.L. and Cronan Jr., J.E. (1980) J. Biol. Chem. 255, 3263–3265.