Acylation of Proteins by Myristic Acid in Isolated Mitochondria*

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Isolated and highly purified mitochondria from rat liver were incubated with [1-14C]myristate, solubilized in boiling sodium dodecyl sulfate, and analyzed by polyacrylamide gel electrophoresis and autoradiography. Six to eight protein bands were found to be radioactively labeled. If the mitochondria were heated for 5 min at 95 °C prior to incubation with this fatty acid, no labeling was observed. By preexposing the mitochondria to unlabeled fatty acids of varying chain lengths, the extent of labeling by [1-14C]myristate was reduced in a chain length-dependent manner, exhibiting maximal inhibition at lauric acid. Reversibility of the labeling was demonstrated by chasing the incorporated radioactivity with unlabeled fatty acids of varying chain length, resulting in a maximal displacement of the tracer again by lauric acid. Fractionation of the labeled mitochondria into mitochondrial matrix and inner mitochondrial membrane components before or after labeling showed that the modified proteins are located inside the inner mitochondrial membrane. In both cases, the pattern of labeling was different from the one observed with intact mitochondria. The labeled bands in the gel were sensitive to alkaline methanol or hydroxylamine treatment. The radioactivity recovered after this treatment co-migrated with myristic acid on thin layer chromatography plates. The chain length specificity and the rapid reversibility of the observed acylation argue for a new type of reaction, different from the acylation observed in whole cells. The possible involvement of the acylated proteins in the regulation of oxidative phosphorylation is discussed.

Fatty acids are known to be regulators of several mitochondrial functions such as ATPase, respiratory enzymes, or carrier-mediated transport across the inner membrane (1, 2). In particular, in liver in vivo they allow an adaptation of the degree of coupling of oxidative phosphorylation such that the production of ATP can run at optimal efficiency under given metabolic constraints imposed by different nutritional regimes (3). Recently, the covalent modification of proteins by fatty acids, occurring either co-translationally or post-translationally has gained wide attention in the context of regulatory functions (for review see Ref. 4 and references therein). These observations have prompted us to investigate whether acylation of proteins can also occur in isolated and purified mitochondria in order to find a possible mechanistic basis for the regulation of oxidative phosphorylation by fatty acids in these organelles. This paper describes the properties of this covalent modification of mitochondrial proteins, which can indeed be observed, and discusses possible implications of this process in the regulation of oxidative phosphorylation. A preliminary report of our work has been published elsewhere (5).

MATERIALS AND METHODS

Isolation and Fractionation of Mitochondria—Liver mitochondria were prepared from 250-g male rats according to the procedure of Johnson and Lardy (6), except that during one wash, defatted bovine serum albumin (1 mg/ml) was included in the medium in order to remove a possible contamination by free fatty acids. For the preparation of mitoplasts, isolated mitochondria were further treated with 0.2% digitonin (7) with the modifications described in procedure XIII A1 in Ref. 8, in order to remove the outer mitochondrial membrane as well as contaminating lysosomes, peroxisomes, and microsomes. Glutamate dehydrogenase (9), arylsulfatase (10), glucose-6-phosphatase (11), and catalase (12) activity were taken as markers for the mitochondrial matrix and lysosomal, endoplasmic reticulum, and peroxisomal contaminations, respectively. Contamination by lysosomes was reduced about 10-fold, by endoplasmic reticulum about 5-fold, and by peroxisomes about 3- to 5-fold. This was calculated from the measured specific activities of the corresponding marker enzymes. Sub mitochondrial particles and matrix protein fraction were prepared as follows. Mitochondria were suspended in 0.5 ml/g liver of a medium containing 2 mM EDTA, pH 8.5. The suspension was sonicated twice for 30 s at 0 °C using a tip sonifier set at about 70 watt (MSE Ultrasonic desintegrator Mk2), followed by centrifugation for 10 min at 10,000 × g. Subsequently, the supernatant was centrifuged for 10 min at 200,000 × g in a Kontron ultracentrifuge rotor (TPT 70.38), the resulting pellet resuspended in a medium containing 250 mM mannitol, 70 mM sucrose, and 10 mM Tris-HCl, pH 7.5, and pelleted as above to give submitochondrial particles. The supernatant of the first high speed centrifugation was used as the matrix protein fraction. When radioactively labeled mitochondria were fractionated, a slightly different procedure was used. After incubation with fatty acid, the mitochondria (1.2 ml, 2.2 mg/ml protein) were cooled to 4 °C and pellet for 3 min at 8,000 × g. The pellet was suspended in 1.2 ml of 2 mM K-EDTA, pH 8.5. The suspension was sonicated four times 15 s at 0 °C using a tip sonifier and subsequently centrifuged in the ultracentrifuge as described above. The supernatant was taken as the matrix protein fraction and the pellet as the inner membrane fraction and analyzed for the presence of acylated proteins as detailed below.

Incubation with Radioactively Labeled Myristic Acid—[1-14C]Myristic acid (58.0 mCi/mmol) was obtained from Du Pont-New England Nuclear as an ethanolic solution. The ethanol was evaporated from a suitable amount of the solution under a stream of nitrogen gas and redisolved in 10 μl of 1.3-butanol, and 300 μl of the incubation medium was added. This medium contained 60 mM KCl, 7.5 mM potassium phosphate, pH 7.4, 40 mM triethanolamine-HCl neutralized with KOH to pH 7.4, 15 mM potassium succinate, 2 mM potassium glutamate, 2 mM potassium malate, 1 mM K-ATP, 1 mM MgCl2, and 0.65 μg/ml rotenone. The mixture was placed in a shaking water bath (130 rpm) thermostated at 37 °C. The reaction was started by the addition of mitochondria, submitochondrial particles, matrix proteins, or defatted bovine serum albumin (at a final concentration of 2.2 mg/ml), depending on the experiment. After 10 min, the reaction was stopped by adding 150 μl of concentrated sample buffer...
containing 30 mM sodium phosphate, pH 7.0, 7.5% (w/v) SDS, 36% (v/v) glycerol, 10 mM diethiothreitol, and 0.5 mg/ml bromphenol blue, followed by incubation for 3 min at 95 °C. SDS-PAGE was performed on slab gels according to Laemmli (13) as modified (14) using a 10% (w/v) polyacrylamide gel. 100-150 pg of protein were applied per lane. Parallel lanes of the gels were stained for protein electrophoresis either in 1 ml containing several days to x-ray film at -75 °C. (w/v) polyacrylamide gel. 100-150 pg of protein were applied per lane. SDS-PAGE was performed followed by incubation for 3 min at 95 °C. The lanes were dried, and the remaining radioactivity was visualized by autoradiography. The chemical identity of the radioactivity was determined following a similar procedure as described by Schlesinger et al. (15). Slices corresponding to labeled bands were cut from unfixed gels, homogenized, and incubated for 24 h in 2 ml of a medium containing 1 M hydroxylamine, pH 9.9. The hydrolysis was carried out on a shaker at room temperature. The resulting suspension was centrifuged for 10 min at 10,400 × g, and the supernatant containing the hydrolysate was extracted three times with 2 ml chloroform/methanol (2:1, v/v). The pooled organic solvent extract was evaporated under a stream of nitrogen gas, and the residue was analyzed on thin chromatography plates (C8-silanized silicagel, Sigma) according to Heusser (16), using methanol/dioxan/chloroform/50 mM glycine, pH 3.0, in water (4:3:2:3) as the mobile phase. The standards were 1-14C-labeled myristic and palmitic acid. The dried thin layer plates were impregnated with Enhance spray (Du Pont-New England Nuclear) and exposed to x-ray film.

RESULTS

Acylation in Isolated Rat Liver Mitochondria—Acylation by myristic acid was studied in two different preparations: 1) mitochondria isolated from rat liver homogenates by fractional centrifugation in a medium containing mannitol plus sucrose and 2) mitoplasts obtained by further purification of this mitochondrial fraction with a digitonin treatment in order to minimize the contamination of the mitochondria by lysosomes, peroxisomes, and microsomes. This latter preparation is also known to be essentially free of outer mitochondrial membrane. Both preparations were incubated in the presence of [1-14C]myristate. In order to prevent metabolism of the added fatty acids, β-oxidation was inhibited with rotenone (17) which blocks oxidation of NADH at site I of the respiratory chain. Simultaneously, malate and glutamate were added in order to achieve a maximal reduction of the nicotinamide nucleotide pool. The respiratory chain was supplied with reducing equivalents past the rotenone block by addition of succinate as an oxidizable substrate. As judged from the liberation of 14CO2 from [1-14C]myristate, β-oxidation could be suppressed by about 98% with respect to the control without rotenone. In contrast to β-oxidation, the activation of the fatty acids to CoA esters does not depend on NAD+ but only on the availability of ATP produced by oxidative phosphorylation, which was unimpaired under these chosen experimental conditions in mitochondria as well as in mitoplasts.

After incubation of mitochondria with [1-14C]myristate, the reaction was stopped by boiling in SDS and subsequently analyzed by SDS-PAGE and autoradiography. Fig. 1 depicts the results of incubations with the crude mitochondrial preparation and with mitoplasts. Both the pattern of the acylated bands and the protein pattern are similar in these two different preparations. This demonstrates that acylation of mitochondrial proteins indeed occurs in highly purified mitochondria and that contaminations by acylated proteins from other subcellular fractions can be neglected in our experiments.

1 The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

Fig. 1. Acylation of mitochondria. Mitochondria were isolated and incubated for 10 min with 75 mM [1-14C]myristate as described under "Materials and Methods." Radioactivity incorporated into mitochondrial proteins was visualized by subjecting the destained proteins to SDS-PAGE and subsequent autoradiography. Parallel lanes were stained for protein with Coomassie Blue (P) or processed for autoradiography (A) as follows: the gel was soaked for 30 min in 1 M salicylate, pH 5, dried, and exposed for several days to x-ray film at -75 °C.

Identification of the Radioactivity Incorporated into Proteins—Lanes from unfixed, unstained gels were incubated immediately after electrophoresis either in 1 M Tris, pH 9.5, 1 M hydroxylamine, pH 9.9, or 1 M KOH in 20% (v/v) methanol in water and hydrolyzed for 2 h at room temperature. The lanes were dried, and the remaining radioactivity was visualized by autoradiography. The chemical identity of the radioactivity was determined following a similar procedure as described by Schlesinger et al. (15). Slices corresponding to labeled bands were cut from unfixed gels, homogenized, and incubated for 24 h in 2 ml of a medium containing 1 M hydroxylamine, pH 9.9. The hydrolysis was carried out on a shaker at room temperature. The resulting suspension was centrifuged for 10 min at 10,400 × g, and the supernatant containing the hydrolysate was extracted three times with 2 ml chloroform/methanol (2:1, v/v). The pooled organic solvent extract was evaporated under a stream of nitrogen gas, and the residue was analyzed on thin chromatography plates (C8-silanized silicagel, Sigma) according to Heusser (16), using methanol/dioxan/chloroform/50 mM glycine, pH 3.0, in water (4:3:2:3) as the mobile phase. The standards were 1-14C-labeled myristic and palmitic acid. The dried thin layer plates were impregnated with Enhance spray (Du Pont-New England Nuclear) and exposed to x-ray film.

There are at least six protein bands which are labeled. Starting at the bottom of the gel, these acylated proteins have the following relative molecular masses as determined from the co-migration of added marker proteins with known molecular weights: α (43 kDa), β (47 kDa), γ (50 kDa), δ (56 kDa), ε (69 kDa), and a weak band sometimes visible ξ (140 kDa). Occasionally, we also observed a diffuse pattern of radioactivity in the range of 29 to 40 kDa. Furthermore, the β-band may actually consist of two adjacent bands with very similar molecular weights. In control experiments, it was shown that treating of mitochondria for 5 min at 95 °C prior to incubation with [1-14C]myristate abolished labeling completely.

Slices corresponding to the band that incorporated most radioactivity, the δ band were excised and the radioactivity determined by liquid scintillation counting. The amount of the label incorporated corresponded to about 40 pmol/mg mitochondrial protein. This estimate assumes that there is no hydrolysis of label during the preparation of the samples and analysis with SDS-PAGE.

In the experiment in Fig. 2, the time course of acylation and deacylation was analyzed. For this end, the mitochondria
were incubated with [1-14C]myristate, and samples were taken at the times indicated in the figure. Significant labeling occurs within the first minute, but the time course for the different bands varies. Maximal labeling is reached after 2–10 min, depending on the band. After 15 min of incubation, excess unlabeled myristate was added, which resulted in a significant decrease of the labeling after 15 s of all bands except the 8-band. This experiment demonstrates that acylation of mitochondrial proteins is reversible and that acylation and deacylation are relatively rapid processes.

In order to study the localization of these acylated proteins and the proteins that mediate acylation, subfractions of the acylated mitochondrial preparation were isolated, namely sonified inside-out submitochondrial particles, representing mainly the inner membrane portion of the mitochondria and the supernatant of this fraction, containing matrix proteins. The left panel of Fig. 3 depicts the results of this experiment. It should be noted that during fractionation, some of the labeled bands were lost. All of the bands that persist during fractionation are located in the matrix. The absence of radioactive bands in submitochondrial particles suggests absence of mitochondrial contamination in this fraction. The pattern obtained with intact mitochondria (IM) is shown for comparison. In the experiment shown in the right panel of Fig. 3, isolated subfractions were incubated with [1-14C]myristate after fractionation. A comparison of the results for intact mitochondria (IM), submitochondrial particles (SM), and matrix proteins (MA) shows that not all of the proteins are labeled to the same extent when only a subfraction of the mitochondria is incubated with myristate and that mitochondria must be intact in order to produce the whole spectrum of acylated proteins. When recombined subfractions were incubated with [1-14C]myristate, no labeling could be observed (not shown). This suggests either that the proteins involved in acylation are very labile or that deacylation becomes more prominent after fractionation.

Specificity of Acylation and Deacylation with Respect to the Chain Length of Saturated Fatty Acids—In order to test the chain length specificity of acylation, we incubated mitochon-

![Fig. 3. Localization of the labeled bands within the mitochondria.](image1)

![Fig. 4. Chain length dependence of acylation and deacylation.](image2)
radioactive spot on the autoradiography could be detected. Hence, apparently the sample buffer is able to completely wash out noncovalently bound fatty acids from this protein. If mitochondria were also present during the incubation of bovine serum albumin (MB), we surprisingly found that the albumin was acylated in addition to the mitochondrial bands. This phenomenon is further discussed below.

To further prove that myristate is covalently bound to mitochondrial proteins, we treated the SDS gels of a standard incubation with various agents. The results of this experiment are depicted in Fig. 6. Extraction of the gel with 1 M Tris-HCl buffer did not remove the radioactivity. In contrast, hydrolysis with either 1 M hydroxylamine or methanolic KOH removed a large fraction of the radioactivity (A) without hydrolyzing the proteins on the gels as judged from the Coomassie Blue staining (P).

In order to characterize the products of this hydrolysis, we treated gel slices containing the individual bands α, β, γ, and δ with hydroxylamine and separated the hydrolysates by thin layer chromatography. As shown in Fig. 7, the radioactively labeled products of the hydrolysis co-migrated with myristic acid, suggesting that the unmetabolized myristic acid is liberated from the proteins by the treatment with hydroxylamine. No hydroxamates could be detected after hydrolysis as shown by staining in separate thin layer runs using hydroxylamine. No hydroxamates could be detected after hydrolysis as shown by staining in separate thin layer runs using hydroxylamine and \( \text{NH}_2\text{OH, pH 9.9, as described under "Methods and Materials."} \) The resulting extract was subjected to thin layer chromatography, and the bands on the plate visualized by autoradiography. The figure shows an analysis obtained with extracts from gel regions corresponding to the bands indicated in the figure and the radioactive markers \( [{}^1\text{C}]\)myristic acid and \( [{}^1\text{C}]\)palmitic acid (Std).

DISCUSSION

Thus far, acylation and deacylation of proteins by myristic and palmitic acid have been demonstrated in whole cells. Covalent modification by myristate is thought to specifically occur co-translationally on N-terminal glycine residues, whereas palmitate modification is supposed to form thioester bonds with cysteine residues and probably is a post-translational process (4). Protein fatty acyltransferase (18) has been located in the rough endoplasmic reticulum, and fatty acyltransferase activity has been found in microsomal preparations (19). The substrate for the transferase reaction is probably the coenzyme A ester (18, 20). At least in vitro, the transferase uses fatty acids of differing chain lengths (21). In some cases, protein acylation also occurs nonenzymatically, e.g., in Semliki Forest Virus protein E2 (21) and in the bovine retinal rhodopsin (22). At present, the physiological role of protein acylation remains unclear. A number of possible roles have been suggested such as provision of a hydrophobic anchor to enable membrane binding for otherwise soluble proteins (23), or involvement in protein-sorting (4).

Here we show for the first time that reversible and specific acylation and deacylation of proteins also occurs in isolated mitochondria from rat liver. The question whether this mitochondrial acylation is spontaneous or enzyme-catalyzed cannot be answered conclusively on the basis of our experiments. The observed specificity and also the fact that bovine serum albumin is acylated to some extent in the presence but not in the absence of mitochondria speaks rather in favor of an enzyme-catalyzed acylation. Therefore, our findings suggest the existence of acylase(s) and/or deacylase(s) in the mitochondria. Since mainly proteins of the mitochondrial matrix and at least one protein of submitochondrial particles are acylated, these enzymes are probably localized on the inner side of the inner mitochondrial membrane. This is not
necessarily in contradiction to the observed acylation of added albumin, since this could be explained by the contamination of our mitochondrial preparation with broken mitochondria or simply could be due to a sluggish quenching of protein fatty acid acylase activity after the addition of SDS to our incubations.

The mitochondrial acylation shows a spectrum of specificities shifted to fatty acids of shorter chain lengths than usually seen in whole cell acylation. Assuming that the acylation of the mitochondrial proteins also occurs via the coenzyme A esters rather than via the free fatty acid, the specificity of the activation of fatty acids and notably also the transport of the acyl moieties across the inner membrane (25) may blur the true specificity of acylation and deacylation of mitochondrial proteins. However, the fact that the acylation of mitochondrial proteins is readily reversible on a short time scale and shows the highest apparent affinity at C12 suggests that here we deal with a new type of acylation which cannot be compared to the acylation reported for whole cells. This is further supported by our observation that acylation by myristate and palmitate yields the same pattern of labeling.

The nature of the acylated proteins is not known at the present time. That they are simply CoA esters covalently bound to the proteins in whole cells should consider this possibility. Clearly, we deal with a new type of acylation which cannot be compared to the acylation reported for whole cells. This is further supported by our observation that acylation by myristate and palmitate yields the same pattern of labeling.

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REFERENCES
1. Wojtczak, L. (1976) J. Bioenerg. Biomembr. 8, 293–311
2. Batayneh, N., Kopacz, S. J., and Lee, C. P. (1986) Arch. Biochem. Biophys. 250, 476–487
3. Soboll, S., and Stucki, J. W. (1985) Biochim. Biophys. Acta 807, 245–254
4. Sefton, B. M., and Buss, J. E. (1987) J. Cell Biol. 107, 1449–1453
5. Stucki, J. W., and Sigel, E. (1988) Experientia 44, A81
6. Johnson, D., and Lardy, H. A. (1987) Methods Enzymol. 10, 94–98
7. Schnaitmann, C., Erwin, V. G., and Greenawalt, J. W. (1961) J. Cell Biol. 32, 719–735
8. Peterson, P. L., Greenawalt, J. W., Reynafarje, B., Hullien, J., Decker, G. L., Soper, J. W., and Bustamente, E. (1978) Methods Cell Biol. 20, 411–481
9. Schmidt, E. (1979) in Methoden der enzymatischen Analyse (Bergmeyer, H. U., ed) pp. 607–613, Verlag Chemie, Weinheim
10. Baum, H., Dogson, K. S., and Spencer, B. (1959) Clin. Chim. Acta 4, 453–455
11. Baginski, E. S., Faas, P. P., and Zak, B. (1970) in Methoden der enzymatischen Analyse (Bergmeyer, H. U., ed) pp. 839–841, Verlag Chemie, Weinheim
12. Aebi, H. (1970) in Methoden der enzymatischen Analyse (Bergmeyer, H. U., ed) pp. 636–641, Verlag Chemie, Weinheim
13. Laemmli, U. K. (1970) Nature 227, 680–685
14. Douglas, M., and Butow, R. A. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 1083–1086
15. Schlesinger, M. J., Magee, A. I., and Schmidt, M. F. G. (1980) J. Biol. Chem. 255, 10021–10024
16. Heusser, D. (1968) J. Chromatogr. 33, 62–69
17. Lättipam, P. M., Karki, T. T., Hiltunen, J. K., and Hassinen, I. E. (1986) Biochim. Biophys. Acta 875, 293–300
18. Berger, M., and Schmidt, M. F. G. (1985) FEBS Lett. 187, 289–294
19. Berger, M., and Schmidt, M. F. G. (1986) J. Biol. Chem. 261, 14912–14918
20. Riendeau, D., and Guertin, D. (1986) J. Biol. Chem. 261, 976–981
21. Berger, M., and Schmidt, M. F. G. (1984) J. Biol. Chem. 259, 7245–7252
22. O'Brien, P. J., St. Jules, R. S., Reddy, T. S., Bazan, N. G., and Zatz, M. (1987) J. Biol. Chem. 262, 5210–5215
23. Magee, A. I., and Schlesinger, M. J. (1982) Biochim. Biophys. Acta 694, 279–289
24. Schmidt, M. F. G. (1983) Curr. Top. Microbiol. Immunol., 102, 101–129
25. Bremer, J. (1983) Physiol. Rev. 63, 1420–1440
26. Wall, L., Rodriguez, A., and Meighen, E. (1986) J. Biol. Chem. 261, 15981–15988
27. Stanley, K. K., and Tubbs, P. K. (1975) Biochem. J. 150, 77–88
28. Fleming, P. J., and Hajra, A. K. (1973) Biochem. Biophys. Res. Commun. 55, 743–751
29. Sunegi, B., and Srere, P. A. (1984) J. Biol. Chem. 259, 8748–8752
30. Srere, P. A. (1987) Annu. Rev. Biochem. 56, 89–124