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Occurrence of acylated proteins in the membrane of the bacterium *Micrococcus luteus*

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The membrane of the Gram-positive bacterium *Micrococcus luteus* was shown to contain at least four proteins which can be acylated by palmitate. Experiments were carried out by incubating the cells in the presence of [^{14}C]- or [^{3}H]palmitic acid and by studying their distribution between membrane lipids and proteins. One of the acylated proteins of molecular weight around 38 kDa was clearly identified to be acylated by palmitate using a multi-step procedure. This included culture of the bacterium in the presence of [^{14}C]palmitic acid, unlabeled acetate and unlabeled amino acids, followed by preparative polyacrylamide gel electrophoresis of membrane proteins after a careful delipidation step and, for given proteins, electroelution, acid hydrolysis and analysis of the fatty acids by gas chromatography. An overall analysis of the four acylated proteins extracted from bacteria cultured in the absence of exogeneous fatty acids reveals the presence, in situ, of palmitate as the main fatty acid, and of myristic, stearic and arachidonic acids.

Introduction

It is becoming apparent that covalent modification of membrane proteins by fatty acids is more common than previously expected [1–3]. Incubation of eukaryotic cell lines with radiolabeled fatty acids reveals the presence of numerous acylated membrane proteins [4,5]. Chemically and functionally well described membrane proteins have been shown to be acylated by long chain fatty acids [6–8]. More recently, a new group of ectoproteins has been described which contain covalently linked phosphatidylinositol [3].

Although the number of reports of these phenomena has increased rapidly over the last three years, the biological and structural significance of protein acylation still remains unclear. Other questions such as the acylation mechanism and fatty acid specificity also require elucidation.

From another point of view, the property of certain membrane proteins to be acylated is of potential value for the investigation of lipid–protein and protein–protein interactions in membranes. Such proteins could be metabolically acylated by exogenous labeled fatty acids which could then serve as probes for fluorescence, electron spin resonance or photochemical studies. Thus, the glycoprotein of vesicular stomatitis virus has been labeled with 16-(9-anthroyloxy)palmitic acid [9] and with a photo-activatable fatty acid [10].

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Recently, in order to study membrane structure in greater detail, we have introduced a new photochemical approach which uses anthracene as a photo-activatable group, after it has been attached to a fatty acid. 9-(2-Anthryl)monanoic acid has been synthesized [11], and incorporated metabolically into the various membrane lipids of the bacterium *Micrococcus luteus* [12]. A simple and versatile photo-crosslinking method has been used for the determination of the lateral distribution of lipids in model and natural membranes [13,14].

It seemed appealing to further extend this approach to the study of lipid–protein interactions via metabolic incorporation of anthracene fatty acid into acylated proteins. As a first step in this direction, we attempted to detect the presence of acylated proteins in the membrane of *M. luteus*. The data to be presented strongly suggest that the membrane of this bacterium contains at least four acylated membrane proteins.

Materials and Methods

1. Chemicals

[9,10(n)-^{3}H]- and [1-^{14}C]palmitic acids were obtained from Commissariat à l'Energie Atomique (Saclay, France). Salts and chemicals used were of analytical grade.

2. Cell growth

Micrococcus luteus (ATCC 4698-4) was grown at 30°C with shaking in a peptone-containing medium, and then harvested at the end of the exponential phase of growth as described elsewhere [15]. Bacteria were pelleted by centrifugation.

For growth in the presence of radiolabeled fatty acids, a solution containing 0.1 mCi of [^{14}C]palmitate (specific radioactivity 54 mCi/mmol or 18 mCi/mmol) or 0.2 mCi of [^{3}H]palmitate (specific radioactivity 50 Ci/mmol) in 0.2 ml of dimethylformamide was gradually added to the peptone medium (20 ml) [15], containing delipidized bovine serum albumin (1 mg/ml, Corporated NBC) under gentle stirring. Homogeneous dispersion of the fatty acid was achieved by two short periods of sonication (30 s each) using a bath type sonicator. Control experiments indicated that the amount of dimethylformamide used did not influence the growth of bacteria.

Cells grown as described above (0.1 mCi [^{14}C]palmitate; 54 mCi/mmol) were pelleted and washed twice with 50 mM Tris buffer (pH 7.4), twice with the same buffer containing 0.2% (w/v) of the detergent Tyloxapol T 8761 (Sigma, St Louis, USA) and then twice with Tris buffer. Radioactivity associated with each washing solution was counted by scintillation (Intertechnique, France), and was found to decrease from $2 \cdot 10^6$ cpm in the first wash to a constant value of 10^4 cpm after the fourth wash. In these experiments and in the following, radioactivity counting was determined to within ± 10%.

For kinetic studies, fatty acid (5 mg unlabeled palmitic acid and 0.35 mCi [^{14}C]palmitic acid) and fatty acid-free bovine serum albumin (100 mg) were added to the culture medium (100 ml) during the exponential phase of growth of the bacteria for various lengths of time.

3. Membrane preparation and delipidation

Bacteria were suspended in Tris buffer (pH 7.4) (10 ml) in the presence of lysozyme (0.1 mg/ml, Sigma), deoxyribonuclease (1 μg/ml, Sigma) and *p*-chloromercuriphenylsulfonate (0.1 mM, Sigma) and incubated for 30 min at 37°C. Protoplast formation was monitored by the decrease in optical density at 650 nm. The suspension was centrifuged (2000 × *g*, 5 min) in order to remove the residual intact bacteria, then ultracentrifuged (70 000 × *g*, 1 h) to pellet the membrane fraction. Membranes were resuspended in water (4 ml) to which was added methanol (10 ml) and chloroform (5 ml). After vortexing, the mixture was kept overnight at 4°C. Proteins were then pelleted by centrifugation (2000 × *g*, 10 min) and further delipidized as follows: two additional extractions with chloroform/methanol (1 : 2, v/v) two extractions with chloroform/methanol (1 : 2, v/v) containing 0.1% $NaDodSO_4$ (sodium dodecyl sulfate) and to further extractions with chloroform/methanol (1 : 2, v/v). At each extraction step, radioactivity associated with the organic phase was counted and found to decrease from $2 \cdot 10^6$ cpm in the first extract to a constant value of 10^4 cpm after the 7th extraction step. The two first organic extracts were combined to give about 2

mg of lipids. Delipidized proteins were lyophilized to give about 10 mg of dry material (both lipids and proteins were extracted from 20 ml of bacterial culture at the end of the exponential phase of growth, optical density at 250 nm ≈ 12–14).

4. Protein gel electrophoresis and fluorography

Lyophilized delipidized proteins (around 50 μg) were solubilized in a migration buffer heated at 60 °C for 10 min, and subjected to electrophoresis on a polyacrylamide gel (10%) containing 0.1% $NaDodSO_4$ [16].

After migration (16 h at 20 mA), gels were stained with Coomassie blue. They were then autoradiographed for one week after a fluorographic treatment [17].

5. Isolation and analysis of acylated proteins

40 mg of delipidized proteins were subjected to preparative polyacrylamide gel electrophoresis. After staining and fluorography of the gels, the desired bands were cut out of the gel and dialyzed in the presence of 20 mM ammonium acetate buffer (pH 7.4), 0.1% $NaDodSO_4$. For each slice, proteins were elecroeluted (100 V) overnight at room temperature [17] and then lyophilized. Electroelution yield was more than 90%. Residual $NaDodSO_4$ was removed by precipitating the proteins twice in cold acetone (−80 °C; 2 h).

6. Lipid, fatty acid and amino acid analysis

Lipids from a total lipid extract were chromatographed on silica-gel plates (Merck G-60 0.1 mm, chloroform/methanol/acetic acid/water (65 : 25 : 10 : 4, v/v)). Radioactivity associated with the various lipids was counted directly on the chromatogram with radioscanner (Berthold, Germany).

For fatty acid analysis, total lipids, total delipidized proteins or isolated proteins were suspended in 2 ml of a 6 M HCl solution in a sealed vial, and heated at 110 °C overnight. Released fatty acids were extracted three times with diethyl ether, and then counted for radioactivity. The extracted fatty acids were methylated with diazomethane and analyzed by gas chromatography. Cold fatty acids were identified on a Girdel 630 apparatus equipped with a 3% OV1 silicone glass column, 1 m long. The ^{14}C-labeled fatty acids were analyzed on a Varian gas chromatograph equipped with an Intertechnique radioactivity detector. They were also analyzed by thin-layer chromatography on silica gel plates (Merck G60, petroleum ether/diethyl ether (8 : 2, v/v)).

Amino acids obtained after hydrolysis of radiolabeled proteins were subjected to chromatography on cellulose thin-layer plates (Merck, butanol/pyridine/water (6 : 4 : 3, v/v)), and the radioactive amino acids were detected after scanning the chromatogram with a Berthold radioscanner.

7. Identification of the acyl linkage

For the identification of the acyl linkage, proteins acylated with [^3H]palmitate were treated with a freshly prepared solution of hydroxylamine (1 M, pH 8) at 20 °C, for 30 min with stirring. Lipids were extracted with a methanol/diethyl ether (1 : 1, v/v) mixture and hydroxamate derivatives were identified by thin-layer chromatography on silicic acid (toluene/methanol/acetic acid (8 : 2 : 0.1, v/v)). Residual proteins were hydrolysed under alkaline conditions (methanol/benzene (7 : 3, v/v), 5% potassium hydroxide, 50 °C, 4 h). Finally, proteins were hydrolyzed with a 6 M HCl solution as previously described. At each step, the fatty acids were extracted and counted for radioactivity.

For identification of proteins specifically acylated via amide linkages, delipidized proteins were treated with 1 M hydrochloric acid in a chloroform/methanol (1 : 1, v/v) mixture and then refluxed at 57 °C for 2 h.

Results

1. [^{14}C]Palmitate incorporation into membrane lipids and proteins of M. luteus

When the growth medium was supplemented with [^{14}C]palmitic acid, the bacterium was found to incorporate the exogenous fatty acid at a high rate into its lipids (phosphatidylglycerol, dimannosyldiacylglycerol, phosphatidylinositol and cardiolipin). The rate of incorporation of [^{14}C]palmitate into the lipids of *M. luteus* was then followed for various incubation times at the beginning of the exponential phase of growth of the bacteria. After 10 min incubation, [^{14}C]palmi-

tate was found in bacteria, partly in the free acid form (47% of the total radioactivity) and was already incorporated into the various lipids, mainly into phosphatidylglycerol (44%). After 30 min, only a slight increase in incorporation into phospholipids and glycolipids at the expense of the neutral lipids was observed and this became more pronounced after an overnight (16 h) incubation time (dimannosyldiacylglycerol 26%; phosphatidylglycerol and cardiolipin 59%; phosphatidylinositol 6%) (data not shown).

Attempts to detect acylated membrane proteins with [^{14}C]palmitate were unsuccesful. Indeed, after 10 and 30 min incubation of bacteria in the presence of [^{14}C]palmitate, a significant amount of radioactivity was found in the lipids ($1.3 \cdot 10^6$ and $2.5 \cdot 10^6$ cpm/mg, respectively). In contrast, the radioactivity associated with proteins was low (10^4 and $3 \cdot 10^4$ cpm/mg, respectively) and presumably corresponded to background radioactivity. Fluorography of the corresponding gel (Fig. 1, lane A) revealed no labeling of proteins (Fig. 1, lane B). After 16 h of incubation, i.e. at the end of the exponential phase of growth of the bacteria, the amount of radioactivity associated with proteins had significantly increased ($1.6 \cdot 10^5$ cpm/mg) as compared to that in the lipids ($8 \cdot 10^6$ cpm/mg). In this case, many labeled bands appeared on the corresponding fluorogram (Fig. 1, lane D) which was almost identical to the protein pattern (Fig. 1, lane C) revealed by Coomassie blue staining. However, the radioactivity associated with proteins may not have been due to direct acylation by [^{14}C]palmitate. It could have been due either to residual adsorbed lipids or to the incorporation of radiolabeled amino acids derived from [^{14}C] acetate. As a control, delipidized membrane proteins were submitted to total acid hydrolysis and the aqueous hydrolysate was extracted with diethyl ether in order to separate fatty acids from amino acids. Only one third of the radioactivity (22000 cpm) was found in the ether phase, the remainder (45000 cpm) being associated with the aqueous phase. Thin-layer chromatography of this amino acid fraction on cellulose plates revealed that lysine, threonine, aspartic and glutamic acids were radiolabeled. All four amino acids are known to be synthesized from acetate units.

Fig. 1. Polyacrylamide gel electrophoresis of *M. luteus* membrane proteins after culture in the presence of [^{14}C]palmitate during 10 min (lanes A and B) or during 16 h (lanes C and D); Coomassie blue staining (lanes A and C) and fluorography (lanes B and D).

2. Isolation and analysis of an acylated protein

In order to reduced non-specific labeling of proteins due to catabolism of [^{14}C]palmitate, the growth culture medium was enriched with unlabeled acetate and with those amino acids which were observed to be labeled, i.e. lysine, threonine, aspartic and glutamic acids. The distribution of radioactivity between amino acids and fatty acids during a 16 h labeling period was then measured after a complete acid hydrolysis of the delipidized membrane proteins. In this case, two thirds of the radioactivity (42000 cpm) was found associated with the ether phase and only one third (23000 cpm) with the aqueous phase. When this set of

proteins was submitted to polyacrylamide gel electrophoresis, the fluorographic pattern did not exactly correspond to the Coomassie blue staining pattern (Fig. 2, lanes A and B). Many, but not all of the proteins were weakly labeled, and one band, of molecular weight around 38 kDa (band c), appeared to be much more radioactive than the others.

The labeled *M. luteus* membrane proteins were then submitted to preparative polyacrylamide gel electrophoresis. Band c was eluted as well as three other bands (a, b and d, Fig. 2, lane B) which were arbitrarily chosen, for control experiments. These proteins were submitted to total acid hydrolysis and radioactivity was counted both in the ether and water phases of the hydrolysates, corresponding to fatty acids and amino acids, respectively. For band c, an ether to water counts ratio of 3:1

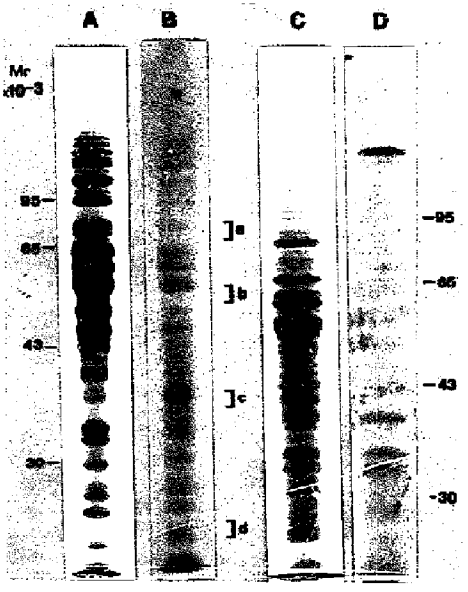

Fig. 2. Polyacrylamide gel electrophoresis of *M. luteus* membrane proteins after culture of the bacteria in the presence of unlabeled acetate, lysine, threonine, glutamic and aspartic acids and [^{14}C]palmitate (lane A and B) or [^{3}H]palmitate (lane C and D). Proteins were revealed after Coomassie blue staining (lane A and C) and fluorography (lane B and D).

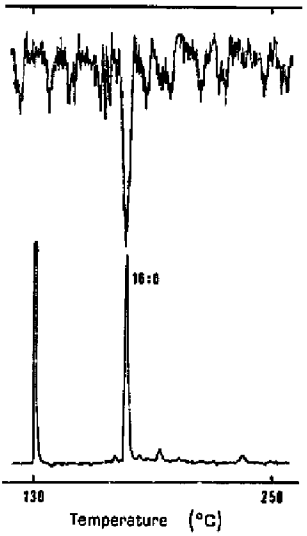

Fig. 3. Gas chromatographic and radioactivity profiles (arbitrary units) of the fatty acid methyl esters obtained after hydrolysis of protein band C (see Fig. 2, lane B).

(62000 cpm/20000 cpm) was found, while no radioactivity could be detected in the ether phase from bands a, b and d. The four ether extracts were also analyzed by thin-layer and gas chromatography. Only the ether extract from band c showed a single radioactive spot on the thin-layer chromatogram at the expected R_f for a long chain fatty acid. As can be seen in Fig. 3, this compound was identified as [^{14}C]palmitate (98%) by gas chromatography coupled with detection of radioactivity. This clearly demonstrates the attachment of palmitate to the 38 kDa acylated protein.

3. [^{3}H]Palmitate incorporation into the membrane proteins of M. luteus

To test for the existence of other acylated proteins, incorporation experiments were carried out with tritiated-palmitate with high specific radioactivity. The incubation time was restricted to 3 h in order to minimize the risk of labeling aminoacids by acetate units which could originate from the β-oxydative degradation of palmitate. As can be seen in Fig. 2, lane D, at least four radiolabeled

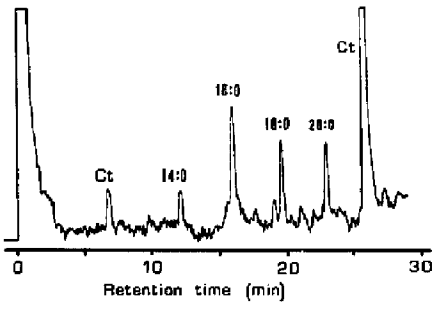

Fig. 4. Gas chromatographic profile of the fatty acid methyl esters obtained after hydrolysis of the four acylated proteins shown in Fig. 2, lane D. Proteins were purified by preparative polyacrylamide gel electrophoresis. They were extracted from bacteria cultured in the absence of exogeneous fatty acids in the growth medium. 14:0, myristic acid; 16:0, palmitic acid; 18:0, stearic acid; 20:0, arachidonic acid; Ct, contaminents from the polyacrylamide gel.

proteins could be identified: a triplet of molecular weight around 32, 35 and 38 kDa, and one band of molecular weight higher than 100 kDa.

In order to ensure that the labeled bands corresponded to palmitoylated proteins, their fatty acid composition was studied. Membrane proteins from a culture in the absence of exogeneous fatty acid were separated by preparative gel electrophoresis. The four bands at the migration positions of the labeled proteins were electroeluted and submitted as a whole to acid hydrolysis. The corresponding ether extract was analyzed by gas chromatography. As shown in Fig. 4, it was found to contain mainly palmitate but also myristate, stearate and arachidonate. This experiment shows that, 'in situ', those proteins are acylated by palmitate and also by other long chain fatty acids.

4. Characterization of the chemical linkages between palmitate and proteins

It has been observed that palmitoylated proteins are usually linked through ester or thioester bonds. From bacteria cultured in the presence of [^{3}H]palmitate, we submitted delipidated proteins to hydroxylamine, then to basic hydrolysis and then to acidic hydrolysis. 18%, 75%, and 7% of the radioactivity were, respectively, released after each treatment indicating that, in *M. luteus*, proteins are palmitoylated mainly through ester linkages.

5. Fatty acid analysis of membrane proteins

Membrane proteins were also analyzed directly for their total fatty acid composition in comparison to that of phospholipids. From a normal bacterial culture grown in the absence of exogeneous fatty acids, membrane proteins were extracted, delipidized and divided into two batches. The first part was submitted to acid hydrolysis. Fatty acids were extracted and analyzed by gas chromatography as described in Materials and Methods. From the results shown in Table I, the fatty acid distribution of delipidized proteins was different from that of lipids with an increase in the ratio of saturated and unsaturated over methyl-branched fatty acids.

The second batch of delipidized membrane proteins was tested for proteins specifically acylated through amide linkages. Delipidized proteins were submitted to an acidic treatment before total acid hydrolysis. Control experiments showed that this treatment induced an hydrolysis of ester bonds in phospholipids. It is likely that such a treatment would also hydrolyze thioester and ester bonds in proteins. In this situation, large differences were found in the fatty acid composition of lipids and proteins. While the former mainly contained (60%) the iso- and ante-iso methyl-branched myristic

TABLE I

FATTY ACID COMPOSITION (%) OF MEMBRANE LIPIDS AND ACYLATED PROTEINS FROM *M. LUTEUS*

All these values were determined to within 5% in relative value.

Fatty acid	Fatty acid composition (%)		
	Total lipids	Delipidized proteins	Acid-washed proteins
C14:0	6	6	9
C14:1	1	–	–
C15r	53	40	16
C16:0	18	28	27
C16:1	5	8	5
C17r	7	5	1
C18:0	3	6	11
C18:1	5	4	10
C20:0	2	3	15
C20:1	–	–	6
Branched fatty acids	60	45	17
Saturated and unsaturated fatty acids	40	55	83

and palmitic acids, the latter contained predominantly (80%) the normal myristic, palmitic, stearic, oleic and arachidonic acids. This suggests the presence of proteins acylated via amide linkages to long chain saturated fatty acids.

Discussion

A clear cut identification of acylated proteins is very difficult to achieve. The only unambiguous proof for the presence of acylated proteins rests on the identification of a covalent linkage between an amino acid and a lipid. This has been achieved for some proteins such as myelin lipophilin [6], *E. coli* proteolipid [7] and calcineurin [8]. In each case, the polypeptide sequence as well as the fatty acyl chain was known and the covalent linkage between the amino acid and the lipid was identified as an amide, or an ester (or a thioester) bond.

The usual approach for identification of acylated proteins by incorporation of an exogenous radiolabeled fatty acid is meaningful provided that the radioactivity resides exclusively in the fatty acids covalently attached to the protein. Fatty acid analysis following membrane protein delipidation also relies on the assumption that the proteins have been totally delipidized. If acylated proteins still contain adsorbed lipids, they will probably the released during protein hydrolysis and the fatty acid content and composition of the presumed acylated proteins will be erroneous. In fact, delipidation of membrane proteins is difficult to achieve and control. Most workers consider that repeated chloroform/methanol extraction leads to total protein delipidation. Others consider that adsorbed lipids are totally released from proteins during polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$. None of these assumptions can be accepted as valid generally.

In the present work, chloroform/methanol extraction appeared to be insufficient to properly delipidate the proteins from the membrane of *M. luteus*. Organic extraction in the presence of $NaDodSO_4$ was found to increase the release of free fatty acids and lipids. Nevertheless, after such a working procedure, the occurrence of residual lipids adsorbed to proteins in significant amount could be ruled out since after 30 min of incubation, lipids were strongly labeled whereas proteins were not.

Another possibility of misinterpreting fluorographic patterns occurs when exogenous fatty acids have been catabolized into acetate units which are then reincorporated into aminoacids and hence into proteins. This pathway is ignored in most publications even though it has been observed when control experiments were carried out [18]. This source of artifact should be checked routinely when using exogeneous radiolabeled fatty acids. Indeed, when a fatty acid is incubated with any cell system for a short period of time (10–30 min), the extent of lipid catabolism probably remains too low to significantly interfere with protein labeling and acylated proteins can be clearly identified. This is the situation in various viruses for which the presence of acylated proteins is clearly established [19–21]. On the contrary, for most experiments reported so far for eukaryotic cells and bacteria, incubation of the cells in the presence of radiolabeled fatty acids was performed for several hours and, after isolation of membrane proteins, many labeled proteins could be observed [4,5,18]. In certain cases, protein acylation was ascertained by hydroxylamine treatment of the gel, which selectively removed the radiolabeled fatty acid [4].

In *M. luteus* membrane proteins which had been carefully delipidized, two third of the total radioactivity was found to be associated with proline, threonine, glutamic and aspartic acids, a group of amino acids which are known to originate from acetate. This can explain why practically all membrane proteins were found to be radiolabeled. An approach including culture of the bacterium in the presence of [^{14}C]palmitate, unlabeled acetate and amino acids, preparative polyacrylamide gel electrophoresis of membrane proteins, electroelution of given proteins, acid hydrolysis and analysis of their fatty acids by gas chromatography allowed us to demonstrate unambiguously that protein band c was an acylated protein of molecular weight around 38 kDa. The function of this protein is unknown.

The fluorographic pattern of membrane proteins after incubation of the bacteria in the presence of [^{3}H]palmitate revealed the presence of at least four acylated bands, among which the protein corresponding to band c was prominent. On the other hand, an analysis of the corresponding

protein extract shows that palmitoylated proteins are acylated mainly via ester bonds. This is in agreement with results obtained for other cell systems [4,5]. Taking into account the conditions of incubation of the cells in the presence of this fatty acid, one cannot conclude that 'in situ', these proteins should be substituted only with palmitate. In other words, one cannot rule out the possibility that the bacterial membrane contains other types of acylated proteins which cannot be detected with the help of radiolabeled palmitate. In this respect, palmitate was found as the main fatty acid released from the four bands isolated from a culture in the absence of exogeneous fatty acid. This suggests that some of these proteins are probably palmitoylated 'in situ'. Nevertheless, myristate, stearate and arachidonate were also found in these proteins. Furthermore, various long chain fatty acids were also released from a total protein extract from the same bacteria (Table I). In both cases, the fatty acid composition was quite different from that of the host phospholipids with a clear cut tendency toward saturated fatty acids. This tendency was markedly increased for the acid-treated proteins which can be considered as being mainly acylated via amide bonds. This suggests that assembly of the acylated proteins might involve a selected fatty acid pool. This has already been observed in the case of other proteins acylated via amide bonds [21]. The nature of the fatty acid is a key point in the understanding of acylated protein anchorage and function.

Reports taking into account the distinction between acylated proteins formed after addition of an exogenous fatty acid and naturally occurring acylated proteins in which the lipid part corresponds to the molecule as it is 'in situ', in the cell are not abundant. Brain lipophilin [6], and gastric mucus glycoprotein [22] contain palmitate, stearate and oleate as the main fatty acid components. However, myristate is found at the N-terminus of some proteins [23] as well as in the pp-60 protein from Rous sarcoma virus [21]. Unfortunately, in none of these studies have the protein bound fatty acids been compared with membrane lipid fatty acids.

There are not many studies related to acylated proteins in bacterial systems [24,25]. However, it appears that bacteria contain a few such proteins [24]. If *Escherichia coli* lipoprotein is a major constituent of this bacterial membrane, the presence of other acylated proteins can be revealed only with fatty acids of very high specific radioactivity [26]; this suggests that such proteins are not abundant. In *M. luteus*, acylated proteins were revealed only after a long time of exposure of the gel to fluorography. The reason for such a discrepancy between prokaryotic and eukaryotic systems is still unknown.

At the present time, we are synthesizing a tritiated derivative of 9-(2-anthryl)nonanoic acid in order to be able to detect metabolic incorporation of this compound into acylated proteins.

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