

Myristoylation of Proteins in Platelets Occurs Predominantly through Thioester Linkages*

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We have demonstrated by several lines of evidence that in platelets myristate is linked to proteins predominantly via thioester bonds as is palmitate, and the covalent binding of the two long chain saturated fatty acids to proteins involves the same mechanisms. The first piece of evidence to support the thioester linkage between myristate and proteins is that [³H]myristate could be removed from proteins via alkaline methanolysis, which disrupts ester bonds but not amide bonds. The second piece of evidence is that unlabeled palmitate, which can form only thioester bonds in physiologic concentrations, competitively inhibits the formation of alkaline methanolysis-sensitive covalent bonds between [³H]myristate and proteins. Third, by SDS-polyacrylamide gel electrophoresis and fluorography, the patterns of labeled proteins from [³H]myristate- and [³H]palmitate-labeled platelets are identical. Fourth, [³H]myristate-labeled proteins, like [³H]palmitate-labeled proteins, both release their fatty acid moieties when exposed to hydroxylamine at neutral pH, which disrupts thioester but not hydroxyester bonds. These findings indicate that although the covalent binding of palmitate to proteins was found to occur at a faster rate than that of myristate, protein S fatty acid acylation that occurs posttranslationally is not specific for palmitate.

A large number of proteins have been shown to undergo covalent modification by the attachment of long chain saturated fatty acids. Fatty acid acylation confers an extra hydrophobic moiety to proteins, which may facilitate the binding of such proteins to hydrophobic membrane domains and promote hydrophobic protein-protein interactions (for review, see Refs. 1-4). A fatty acid may be linked to a protein through an amide bond via an N-terminal glycine moiety cotranslationally or through a thioester bond via an internal (or C-terminal) amino acid posttranslationally. Present evidence indicates that the fatty acid involved in the first type of modification (amide bond) is myristate, and it is generally accepted that palmitate is the ester-linked fatty acid moiety. The enzyme responsible for the covalent binding of myristate

to proteins, *N*-myristoyl transferase, has been purified from *Saccharomyces cerevisiae* and the gene cloned. This enzyme was found to exhibit a strict substrate specificity toward myristoyl-CoA as the fatty acid donor and N-terminal glycine as the amino acid acceptor (5-10). Much less is known about the biochemical mechanism of posttranslational acylation of proteins with fatty acids through thioester linkages. Thus far, no palmitoyl transferase activity has been purified, and no gene related to this activity has been cloned. In addition, the enzymology of this type of fatty acid acylation is poorly understood. A great number of metabolic labeling experiments in which radiolabeled palmitate became bound to individual proteins via hydroxylamine-sensitive thioester linkages have provided support for the concept that palmitate is the fatty acid involved in protein modification through thioester linkages. However, the results of several studies suggest, but do not conclusively demonstrate, that myristate may also become bound to proteins via thioester bonds. In certain cell-free systems using homogenates rather than intact cells, acylation through thioester bonds has failed to show high specificity with regard to the chain length in the range of C-14 to C-18 (11). Three studies involving the use of intact cells in culture in which the covalent binding of different chain length fatty acids to proteins was analyzed provide conflicting data concerning chain length specificity for thioester-bound fatty acids. In human keratinocytes and squamous carcinoma cell lines (12), as well as in BC3H1 muscle cells (13, 14), little, if any, protein-bound myristate was found in ester linkage, while in 3T3 mouse fibroblasts and PC12 rat pheochromocytoma cells (13), a minor but significant portion of myristate was linked to protein via ester, probably thioester, bonds. These studies are somewhat difficult to interpret, however, because in cells such as these with active protein synthesis, both amide and thioester linkages are constantly being formed, and radiolabeled myristate incorporation into protein could, therefore, be via amide as well as thioester bonds. Also, fatty acids can become covalently bound to proteins as the ester-linked fatty acid moieties of glycosylated phosphatidylinositol membrane anchors (15). This protein modification by fatty acid is a very early posttranslational event, is associated with active protein synthesis (16), and further confounds the interpretation of studies involving cells synthesizing new proteins. Nonetheless, the study involving cell homogenates (11) and the three involving intact cells (12-14) raise the possibility that myristate (14:0) can become covalently bound to proteins by thioester as well as amide linkages. To address this question definitively, we used human platelets, a system with little or no protein synthesis.

We have previously shown that in blood platelets palmitoylation of proteins via thioester linkages is an active process (17, 18). In the present study, we investigated the covalent attachment of myristate to platelet proteins. The aims of the present study were: 1) to determine whether myristate be-

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comes linked to platelet proteins; 2) if so, to determine the type of linkage(s); and 3) if the linkage is a thioester, to compare it qualitatively and quantitatively with palmitoylation of platelet proteins through thioester linkages. We found that myristate is bound to platelet proteins predominantly through thioester linkages, that this binding is effectively prevented by excess palmitate, and that myristate binds to the same set of proteins as palmitate.

EXPERIMENTAL PROCEDURES

Materials—[9,10-³H]Myristic acid (39.8 Ci/mmol), [9,10-³H]palmitic acid (38.0 Ci/mmol), and Formula 989 scintillation solution were purchased from Du Pont-New England Nuclear. [³⁵S]Methionine (1000 Ci/mmol) was obtained from Izinta (Budapest, Hungary). Unlabeled fatty acids (myristate, palmitate, and stearate) and fatty acid methyl esters (methyl myristate, methyl palmitate, and methyl stearate) were products of Nu-Check Prep (Elysian, MN). Hydroxylamine, human thrombin (3000 units/mg), apyrase (grade VII), and fatty acid-free bovine serum albumin were obtained from Sigma. *M*₁ marker proteins were from Bio-Rad. Prostaglandin E₁ was purchased from Advanced Magnetics (Cambridge, MA). ¹⁴C-Methylated *M*₁ marker proteins and autoradiographic image enhancer (Amplify) were from Amersham Corp. High performance liquid chromatography (HPLC)¹ grade solvents were used for delipidation of proteins, alkaline methanolysis, and extraction of fatty acids. Methanolic HCl (3 M) was the product of Supelco (Bellefonte, PA).

Preparation of [³H]Myristate- and [³H]Palmitate-labeled Platelet Suspensions—Isolation of platelets for the present studies performed as we described earlier (17) was followed with minor modifications. Platelet-rich plasma was obtained by centrifugation (120 × *g*, 15 min) of human blood anticoagulated with acid-citrate dextrose containing 0.18 μM prostaglandin E₁. Platelets were then pelleted from platelet-rich plasma (1300 × *g*, 15 min) and resuspended in solution A (140 mM NaCl, 2.5 mM KCl, 0.1 mM MgCl₂, 10 mM NaHCO₃, 0.5 mM NaH₂PO₄, 1 mg/ml glucose, 10 mM HEPES, pH 7.4) containing 3.6 mg/ml fatty acid-free bovine serum albumin, 1 unit/ml apyrase, 0.3 μM prostaglandin E₁, and 300 μCi/ml [³H]myristate (7.5 μM) with or without 30 μM unlabeled palmitate or 300 μCi/ml [³H]palmitate (7.9 μM). The platelet count of the platelet-rich plasma was between 300 and 330 × 10⁶/ml. Labeled and unlabeled fatty acids were dried completely under nitrogen and then solubilized in aqueous medium by vortexing for 60 s in the above buffer. After incubation of platelet suspensions for 1 or 4 h with various combinations of labeled and unlabeled fatty acids, total platelet-associated radioactivity was determined. Platelets were then pelleted by centrifugation (1100 × *g*, 15 min) and resuspended in the same volume of solution A containing 1 unit/ml apyrase and 0.3 μM prostaglandin E₁. Platelets were again centrifuged (1100 × *g*, 15 min) and resuspended in solution A containing 1 unit/ml apyrase. Aliquots were removed for the determination of platelet count and radioactivity incorporated into platelets. The entire procedure was carried out at 37 °C. During centrifugation, the temperature in the centrifuge was maintained between 32 and 37 °C. As determined in separate experiments, platelets radiolabeled for 1 or 4 h responded effectively to 0.1 unit/ml thrombin when tested for aggregation in a platelet aggregometer (Bio Data, Horsham, PA).

Extraction of Noncovalently Bound Lipids—Precipitates from 1 ml of washed radiolabeled platelets were generated by addition of 3.5 volumes of cold acetone (vortexing for 60 s) to the platelet suspension. The precipitates were kept on ice for 30 min, at which time they were collected by centrifugation (4500 × *g*, 0 °C, 10 min). The precipitates from the 1-ml platelet suspension were then extracted with 6 ml of chloroform/methanol (2:1). The mixture was vortexed for 60 s and then incubated at room temperature for 30 min. The precipitate, pelleted by centrifugation (4500 × *g*, room temperature, 15 min), was extracted twice more with 6 ml of the same chloroform/methanol (2:1) solvent, then twice with 6 ml of chloroform/methanol/water (1:1:0.3), and finally with 6 ml of methanol. After each extraction step, the samples were left at room temperature for 10 min and centrifuged as described. The final protein residue contained no radioactivity extractable by lipid-extracting solvents, and it was dried completely under nitrogen. Certain samples were dissolved in SDS-PAGE sample buffer (without β-mercaptoethanol) by boiling for 5 min and then shaking overnight. The protein content and radioactiv-

ity of the samples were determined. Other samples were used for identification and quantitation of fatty acids bound to proteins by HPLC.

Release of Protein-bound Fatty Acid and Identification of Released Fatty Acids by HPLC—The dried delipidated protein preparations were first subjected to alkaline methanolysis to disrupt ester linkages. In this process, the delipidated protein was treated with 2 ml of 0.2 M KOH in methanol for 30 min at 37 °C and then centrifuged (4500 × *g*, room temperature, 10 min). The supernatant was removed, acidified with 2 M HCl, and the fatty acid methyl esters generated by this procedure were extracted three times with 1.5 ml of hexane each time. Approximately 95% of the radioactivity released by methanolic KOH was recovered in the combined hexane extract. This extract was then dried completely under nitrogen and redissolved in methanol. Aliquots were removed for determination of extracted radioactivity before HPLC analysis. At this point, 150 μg of each unlabeled fatty acid (myristate, palmitate, stearate) and corresponding unlabeled fatty acid methyl esters were added to the samples as standards. Separation of the three fatty acids and three fatty acid methyl esters as six separate peaks was achieved by reverse-phase HPLC using a Microsorb C18 column (4.6 mm × 30 cm) (Rainin, Woburn, MA). The fatty acids were eluted with 94% acetonitrile, 17 mM phosphoric acid (v/v) for 24 min and then with 97% acetonitrile, 17 mM phosphoric acid (v/v) for 12 min at a flow rate of 1 ml/min. Fractions of 1 ml were collected and counted in 4 ml of scintillation fluid. The absorbance profile of eluted compounds was monitored at 205 nm, and retention times of radioactive alkaline methanolysis products were determined by coelution with unlabeled fatty acid and fatty acid methyl ester standards.

After the removal of alkaline methanol, the protein pellet was washed with 2 ml of methanol and then subjected to acid methanolysis to release any amide-bound fatty acid. The samples were treated with 2 ml of 3 M methanolic HCl at 110 °C for 48 h under nitrogen. The reaction solutions were extracted three times with 1.5 ml of hexane, and the combined hexane fractions were processed for HPLC as described above for alkaline methanolysis products. The radioactivity that was not released by alkaline or acid methanolysis was also determined.

SDS-PAGE, Hydroxylamine Treatment, and Fluorography of Polyacrylamide Gels—The platelet protein pellet recovered by acetone precipitation was washed with another volume of cold acetone, dried, and dissolved in SDS-PAGE sample buffer (half the volume of the final platelet suspension). In certain experiments, radiolabeled platelets not exposed to acetone were also pelleted and dissolved directly in sample buffer. Acetone treatment did not remove any radiolabeled platelet proteins, since with or without acetone there was no difference in the protein or fluorographic pattern following gel electrophoresis. Samples in the solubilizing buffer for SDS-PAGE were either reduced with 50 mg/ml β-mercaptoethanol for 1 h at room temperature or processed without reduction. SDS-PAGE was performed on a 5–20% gradient gel according to the method of Laemmli (19). After electrophoresis, the gels were first fixed in a 50% ethanol, 5% acetic acid solution for 45 min and stained as previously described (18). Selected fixed gels were washed three times for 10 min (each wash) in distilled H₂O and treated for 12 h at room temperature with 1 M hydroxylamine, pH 7.0 (to disrupt thioester linkages), or with 1 M Tris-HCl, pH 7.0 (as a control). Hydroxylamine or Tris was removed by rinsing the gel three times for 10 min (each wash) in distilled H₂O. The gels were then stained and destained. Destained gels were soaked in Amplify solution supplemented with 1% glycerol for 1 h, briefly rinsed with distilled H₂O, and dried at 60 °C. The dried gels were exposed to Kodak X-Omat AR film (Rochester, NY) at –70 °C for 4 days or 4 weeks. As determined in separate experiments, staining and destaining did not decrease the intensity of radiolabeled bands on the fluorograms to any significant extent; treatment with Tris-HCl or hydroxylamine did not result in any change in the protein patterns on the gel, and Tris-HCl had no effect on the fluorograms. Reduced and unreduced samples were run either on separate gels prepared from the same acrylamide gradient or, if they were run on the same gel, the reduced and unreduced samples were separated from each other by at least eight lanes to prevent even minimal contamination of unreduced samples with the β-mercaptoethanol in the wells with reduced samples.

Metabolic Labeling of Platelets with [³⁵S]Methionine and the Determination of Trichloroacetic Acid-precipitable Radioactivity—Labeling of platelets with 300 μCi/ml [³⁵S]methionine was performed exactly as described for ³H-labeled fatty acids. For determination of total protein-bound ³⁵S, 200 μl of platelet suspension was treated for 10

¹ The abbreviations used are: HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

min with 4 ml of 10% boiling trichloroacetic acid, and then the precipitate was washed three times with 10% trichloroacetic acid. The final precipitate was dissolved in 0.1 M NaOH and the protein and radioactivity in the precipitate quantitated.

Other Methods—Protein concentration was measured with the BCA protein assay kit (Pierce Chemical Co.). Radioactivity was counted in a Beckman Instruments LS 6800 scintillation counter.

RESULTS AND DISCUSSION

Uptake of Fatty Acids into Platelets and Covalent Binding to Proteins—Platelets incorporated a significant portion of the total added myristate ($6.20 \pm 0.15\%$, 1.55 nmol of myristic acid/ 10^9 platelets) after 4 h. The fact that unlabeled palmitate competed to a significant extent with the uptake of [3 H]myristate into platelets (Fig. 1) is consistent with the concept that the incorporation of the two fatty acids into platelets involves similar mechanisms. Studies involving the esterification of different fatty acids into platelets (20) have indicated that there are two fatty acid uptake systems in platelets, one for eicosanoid precursor fatty acids and one for all other fatty acids including myristate and palmitate. We observed, however, that the rate of uptake of myristate was approximately 3-fold lower than that of palmitate, suggesting that among the saturated fatty acids, there are preferred fatty acid substrates for uptake into platelets.

A small portion of incorporated [3 H]myristate became covalently attached to platelet proteins (Table I), as indicated by the fact that it remained associated with the protein residue even after exhaustive lipid extraction or treatment with SDS. This was similar to our earlier finding with [3 H]palmitate (18). The amount of protein-linked myristate in disintegration/min/ng of protein was significantly less than that of palmitate (0.13 ± 0.01 versus 1.93 ± 0.11 at 1 h and 0.34 ± 0.01 versus 4.10 ± 0.32 at 4 h for a 12–15-fold difference). The results suggest that this difference is not likely to be only a result of the lower rate of uptake of [3 H]myristate versus [3 H]palmitate (3-fold difference in uptake versus 12–15-fold difference in fatty acid acylation). The same percentage of incorporated radioactivity was found to be protein bound after 1 and 4 h of incubation of radiolabeled fatty acids with platelets. Therefore, within this interval at least, the ratio of protein bound to total platelet radioactivity did not depend

TABLE I

The covalent attachment of incorporated radiolabeled fatty acids to platelet proteins

Values are mean \pm S.E. from three experiments. The [3 H]myristate concentration was $7.5 \mu\text{M}$; [3 H]palmitate was $7.9 \mu\text{M}$, and unlabeled palmitate was $30 \mu\text{M}$.

Fatty acid incubated with platelets	Time of incubation	Protein-linked radioactivity	
		dpm/ng protein	% of total dpm incorporated by platelets
	h		
[3 H]Myristate	1	0.13 ± 0.01	0.53 ± 0.03
[3 H]Myristate	4	0.34 ± 0.01	0.53 ± 0.02
[3 H]Myristate + palmitate	4	0.09 ± 0.01	0.26 ± 0.03
[3 H]Palmitate	1	1.93 ± 0.11	2.12 ± 0.12
[3 H]Palmitate	4	4.10 ± 0.32	2.05 ± 0.16

on the amount of incorporated ^3H -labeled fatty acid. Taken together, the results indicate that myristate can become covalently bound to proteins, but palmitate is a preferred substrate. The data in Table I show that the addition of unlabeled palmitate reduced the covalent binding of [3 H]myristate to platelet proteins by 3.7-fold, and the percentage of protein-linked [3 H]myristate within the total incorporated radiolabeled myristate pool also decreased by 2-fold. This suggests that palmitate and myristate compete for covalent binding to proteins.

Hydrolysis of Fatty Acids from Proteins and Fatty Acid Identification—From the experiments involving fatty acid hydrolysis from proteins (Table II), we determined, as found in our previous experiments, that more than 90% of the protein-bound radioactivity in [3 H]palmitate-labeled cells was released by alkaline methanolysis. A much smaller amount (2.9%) of total released radioactivity or 49 dpm/ μg platelet protein was nonreleasable. In the present experiments, we observed that 72.8% of the [3 H]myristate (1 h of incubation) was releasable by alkaline methanolysis and, therefore, linked to protein via an ester bond (S- or O-ester). We also found that 26 dpm/ μg platelet protein was nonreleasable in the [3 H]myristate-labeled cells. This absolute value was similar to that observed for palmitate (26 versus 49 dpm/ μg), but since the total amount of protein-bound radioactivity in the [3 H]myristate-labeled cells was much smaller, the nonreleasable fraction is higher by percentage calculations than in [3 H]palmitate-labeled cells. Presumably, a fixed small amount of labeled fatty acid is β -oxidized, and the ^3H is removed from the fatty acid molecule and becomes associated with protein by another mechanism. Acid methanolysis following alkaline methanolysis released only small amounts of additional protein-linked radioactivity. Importantly, our results do not indicate that proteins myristoylated through amide linkages are absent in platelets. Active protein synthesis in megakaryocytes provides the necessary opportunity for such cotranslational fatty acid acylation of proteins. Thus, we conclude that above a small fixed base-line amount of nonreleasable disintegrations/min, [3 H]myristate in platelets is largely bound to protein by ester linkages.

To confirm that amide linkages would survive alkaline hydrolysis under the experimental conditions used in our studies with platelets, we performed parallel experiments with a mouse fibrosarcoma cell line (HSDM₁C₁) with active protein synthesis and, thereby, active formation of amide bonds between proteins and fatty acids. The labeling conditions for the fibrosarcoma cells and the platelets were identical. We found that 30–40% of protein-linked [3 H]myristate in fibrosarcoma cells was resistant to alkaline methanolysis but that

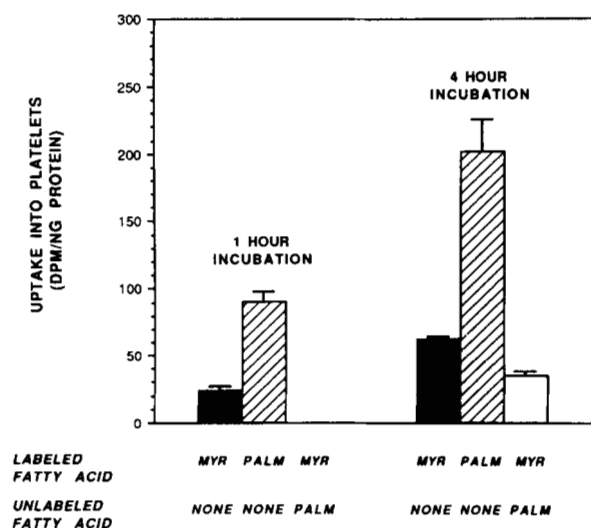


FIG. 1. Incorporation of [3 H]myristate (MYR) and [3 H]palmitate (PALM) into platelets. Platelets were incubated with $300 \mu\text{Ci/ml}$ ($7.5 \mu\text{M}$) [3 H]palmitate, $300 \mu\text{Ci/ml}$ ($7.5 \mu\text{M}$) [3 H]myristate, or $300 \mu\text{Ci/ml}$ ($7.5 \mu\text{M}$) [3 H]myristate + $30 \mu\text{M}$ palmitate for 1 or 4 h, and the total radioactivity incorporated into platelets was determined. The values represent the mean \pm S.E. of three experiments.

TABLE II

Analysis of fatty acids covalently linked to proteins in [^3H]myristate- and [^3H]palmitate-labeled platelets

Values represent means of duplicate experiments, the results of which did not differ from each other by more than 10%. FA, fatty acid; ND, not determined.

Fatty acid	Incubation time	Released by methanolysis		Nonreleasable	Total
		Alkaline	Acid		
	<i>h</i>	<i>dpm/μg platelet protein</i>			
[³ H]Myristate ^a	1	83 (72.8%)	5 (4.4%)	26 (22.8%)	114 (100%)
Myristate ^b		71 (62.3%)			
Palmitate ^b		12 (10.5%)	ND		
Stearate ^b		0			
[³ H]Palmitate ^a	1	1602 (93.6%)	61 (3.5%)	49 (2.9%)	1712 (100%)
Myristate ^b		16 (0.9%)			
Palmitate ^b		1538 (89.8%)	ND		
Stearate ^b		48 (2.9%)			
[³ H]Myristate ^a	4	193 (67.7%)	30 (10.5%)	62 (21.8%)	285 (100%)
Myristate ^b		164 (57.5%)	30 (10.5%)		
Palmitate ^b		29 (10.2%)	0		
Stearate ^b		0	0		
[³ H]Myristate + palmitate ^a	4	27 (35.5%)	10 (13.2%)	39 (51.3%)	76 (100%)
Myristate ^b		21 (27.6%)	10 (13.2%)		
Palmitate ^b		6 (7.9%)	0		
Stearate ^b		0	0		

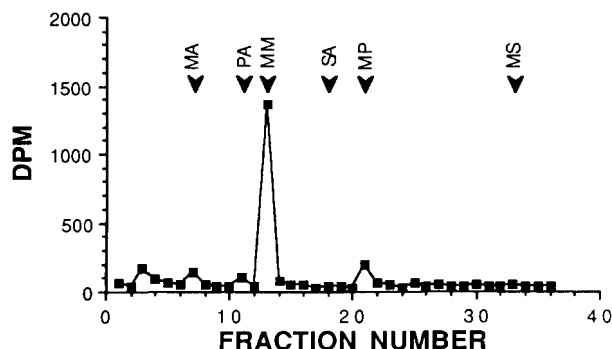
^a, labeling FA; (alkaline + acid + nonreleasable) sums to 100%.^b, released FA; (myristate + palmitate + stearate) sums to percentage of labeling FA.

FIG. 2. Identification and quantitative analysis of radiolabeled fatty acids released from proteins of [^3H]myristate-labeled platelets by alkaline methanolysis. Elution positions of myristate (MA), palmitate (PA), stearate (SA), methyl myristate (MM), methyl palmitate (MP), and methyl stearate (MS) (arrowheads) were determined by monitoring absorbance at 205 nm. This chromatogram is representative of two essentially identical chromatograms.

this radioactivity could be released by acid methanolysis.² This result confirms that any [^3H]myristate bound to platelet proteins through amide linkages, if present, could have survived alkaline methanolysis as expected and supports the conclusion that only trace amounts of [^3H]myristate, if any, were linked to platelet proteins via an amide linkage.

The identification of radiolabeled fatty acids released by alkaline and acid methanolysis was carried out by reverse-phase HPLC using unlabeled fatty acid and fatty acid methyl ester standards (Fig. 2). As previously reported (18), in [^3H]palmitate-labeled platelets, the protein-bound radiolabeled fatty acid was almost exclusively thioester-linked palmitate. In [^3H]myristate-labeled cells, we found 85% of protein-linked ^3H -labeled fatty acid released by alkaline methanolysis to be [^3H]myristate (Table II). The remaining 15% was recovered as [^3H]palmitate formed by chain elongation (21) of the [^3H]myristate prior to its attachment to proteins. The competition of unlabeled palmitate using physiologic concentrations of

nonesterified palmitate in the plasma ($30\ \mu\text{M}$)³ with [^3H]myristate for protein binding revealed a number of important observations. While there was a 2–3-fold change in the non-releasable (62 versus 39 dpm/ μg) and amide-linked (30 versus 10 dpm/ μg) pools, in the presence of unlabeled palmitate, there was an 8-fold decrease in the ester-linked [^3H]myristate pool (164 versus 21 dpm/ μg). These data indicate that in platelets, palmitate competes with myristate for binding to proteins through thioester linkages.

SDS-PAGE and Fluorography—The results of our fluorographic experiments shown in Fig. 3 indicate that as in [^3H]palmitate-labeled cells, nearly all of the [^3H]myristate covalently linked to platelet proteins was released by neutral hydroxylamine treatment. This indicates that the bulk of the protein-bound [^3H]myristate is linked through *S*-ester linkages rather than *O*-esters. Hydroxylamine at neutral or slightly acidic pH disrupts thioester linkages but, as verified with model compounds (22, 23) and with the glycosyl phosphatidylinositol membrane anchor (15), at this pH, hydroxylamine does not cleave the hydroxyester linkage.

Finally, we compared the SDS-PAGE patterns of platelet proteins labeled with [^3H]palmitate with ^3H -labeled proteins labeled with myristate. Due to less uptake and less intense binding to proteins of [^3H]myristate-labeled samples relative to [^3H]palmitate-labeled samples, as noted earlier, lanes with [^3H]myristate samples required much longer exposure than those with [^3H]palmitate to obtain comparable intensity (Fig. 3). A thorough comparison of fluorograms from both reduced and unreduced protein samples of homogenates from [^3H]myristate and [^3H]palmitate-labeled platelets showed identical fluorographic patterns after SDS-PAGE.

It has been previously shown that the extent of protein synthesis in the platelet is insignificant when compared with the amount of protein synthesis in nucleated cells, with much of the protein synthesis in these cells involving major cytoskeletal proteins (24, 25). The amount of acid-precipitable ^{35}S radioactivity in platelets metabolically labeled with [^{35}S]methionine, which reflects radioactivity incorporated into platelet proteins, was less than 2% of the amount obtained with

² L. Muszbek and M. Laposata, unpublished observations.³ Hallaq, Y., Becker, T. C., Manno, C. S., and Laposata, M. (1993) *Lipids*, in press.



FIG. 3. ^3H -Myristoylated and ^3H -Palmitoylated proteins in platelets. Platelets were washed and labeled with ^3H -labeled fatty acid; platelet proteins were precipitated and extracted with acetone, then analyzed with SDS-PAGE and fluorography as described under "Experimental Procedures." C, Coomassie Blue staining; M and P, fluorogram of proteins from [^3H]myristate- (4-week exposure) and [^3H]palmitate- (4-day exposure) labeled platelets, respectively; -HA and +HA, gels incubated in the absence or presence of 1 M neutral hydroxylamine. To each well, 63 μg of platelet protein was applied. Arrows represent the migration distance of standard proteins of the following molecular mass, from top to bottom: 200, 116, 97.4, 66.2, 45, 31, 21.5, and 14.4 kDa.

mononuclear peripheral blood cells. The value obtained using ^{35}S -labeled platelets was 0.99 ± 0.03 dpm/ng protein (1-h incubation, mean \pm S.E., $n = 3$). The results of this control study support the conclusions that little, if any, cotranslational binding of myristate to proteins occurs in platelets via amide linkages and that myristate is linked to platelet proteins posttranslationally via thioester bonds.

We have proven by several lines of evidence in this study that myristate can indeed be linked to proteins via thioester bonds in a cell system where there is little or no protein synthesis and, therefore, as our data support, no active formation of amide bonds between myristate and cell proteins. The posttranslational binding of myristate and palmitate to proteins in platelets apparently involves identical mechanisms, and the two fatty acids become bound to the same set of substrate proteins. These findings strongly support the view that, as far as chain length of saturated fatty acid is concerned, fatty acid acylation of proteins through thioester linkage is of somewhat relaxed specificity. Therefore, instead of the generally used term, palmitoylation, protein S fatty acylation would be a more accurate term to describe this process.

We observed that modification of platelet proteins with [^3H]myristate occurred at a lower rate than with [^3H]palmitate. There are several possible explanations for this observation. Proteins S fatty acyl transferase(s) may have a lower affinity for myristoyl-CoA than palmitoyl-CoA. A slower rate of myristoyl-CoA synthesis or a higher rate of deacylation of thioester-linked myristate by a protein-fatty acylase could also be responsible for this difference. In the plasma, the nonesterified palmitate concentration is much higher than the nonesterified myristate concentration. We have found in our laboratory that the nonesterified palmitate concentration in the plasma is 30.5 ± 16.5 μM , and the nonesterified myristate concentration is 2.3 ± 1.5 μM (mean \pm S.D., $n = 11$ different nonfasting individuals).³ Because of the relative

abundance of the two fatty acids in the plasma and the preferential uptake of palmitate over myristate into the platelets, we suggest that *in vivo* palmitate is much more likely to be bound to proteins by thioester linkages than myristate, although both fatty acids can be thioester linked to proteins.

There may be a much greater complexity in the process of fatty acid acylation via thioester bonds than is presently understood. It has been shown that this type of posttranslational modification involves at least two distinct classes of target proteins for fatty acid binding. One class includes the proteins localized to the inner surface of the plasma membrane, which are resistant to extraction procedures that usually release peripheral membrane proteins (26–29). The other set of proteins includes transmembrane proteins or glycoproteins in which the fatty acid is linked to a cysteine residue located just below the hydrophobic transmembrane domain in the cytoplasmic region (18, 30–32). It has been suggested that fatty acids might be thioester linked to proteins by different fatty acyl transferases located in different subcellular compartments. Indeed, plasma membrane, endoplasmic reticulum, Golgi apparatus, and cytoplasm have all been suggested as sites where posttranslational fatty acid acylation of proteins can take place (1–4). Our data raise the possibility that different fatty acyl transferases are responsible for the protein binding of myristate and palmitate via thioester bonds. The hydrophobicity of the fatty acid moiety is clearly a critical factor in the functional modification of certain proteins. Since hydrophobicity is greatly affected by differences in carbon chain length, the issue of fatty acid specificity for protein modifications, as addressed in this study, may have major functional implications.

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