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# Covalent Modification of Proteins by Arachidonate and Eicosapentaenoate in Platelets*

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**The posttranslational modification of proteins by fatty acids has been shown to involve long chain-saturated fatty acids, predominantly palmitate. In the present study, we demonstrated by metabolic labeling of human platelets with [$^3$H]arachidonate and [$^3$H]eicosapentaenoate that these polyunsaturated fatty acids can also become covalently linked to proteins. The extent of binding of arachidonate to proteins was somewhat less than that of palmitate. Arachidonate binding to platelet proteins was not significantly influenced by the inhibition of cyclooxygenase and lipoxygenase. This finding and the high performance liquid chromatography analysis of radiolabeled products removed from proteins by selective cleavage techniques established that arachidonate, and not its metabolic products, was the protein-linked radiolabeled moiety in [$^3$H] arachidonate-labeled platelets. A 7.5-fold higher concentration of unlabeled palmitate competed to a small extent with [$^3$H]arachidonate for protein labeling. Both arachidonate and eicosapentaenoate were bound to proteins almost exclusively through ester linkages. It was further demonstrated that 61 and 66% of total protein-linked arachidonate and eicosapentaenoate, respectively, were bound via thioester bonds. In contrast, 91% of the binding of palmitate to proteins occurred via thioester linkages. As demonstrated by SDS-polyacrylamide gel electrophoresis and fluorography, the patterns of palmitoylated and arachidonoylated proteins were similar but not identical, with selected proteins only palmitoylated or only arachidonoylated. [$^3$H]Eicosapentaenate labeled the same set of proteins as [$^3$H]arachidonate. The fluorographic pattern of $^3$H-arachidonoylated proteins was not changed by cyclooxygenase and lipoxygenase inhibitors. The binding of a polyunsaturated fatty acid to a protein in place of a saturated fatty acid could significantly influence the hydrophobic interactions of the protein and, thereby, have important functional implications.**

Covalent attachment of a fatty acid to an amino acid residue results in the addition of a hydrophobic moiety to the protein. This may facilitate the interaction of the protein with hydrophobic membrane domains, promote hydrophobic protein-protein interactions, or target proteins to specific membrane receptors (for reviews, see Refs. 1–4). Fatty acids can be linked to an amino acid residue directly or indirectly as a component of a phosphatidylinositol moiety attached to a COOH-terminal amino acid through an intervening glycan structure (5, 6). Direct linkage of fatty acids to proteins can occur both cotranslationally and posttranslationally. The former type of modification involves an $NH_2$-terminal amino acid residue to which the fatty acid is bound through an amide bond. The enzyme which is responsible for this type of modification, *N*-myristoyl transferase, has been purified, and the gene for the protein has been cloned. This protein was found to exhibit strict substrate specificity toward myristoyl-CoA as a fatty acid donor and $NH_2$-terminal glycine as fatty acid acceptor (7–12). The biochemical mechanism of posttranslational fatty acid acylation of proteins has not been studied to the same extent as cotranslational *N*-myristoylation. The enzyme(s) responsible for addition of thioester-linked fatty acids have not been isolated, and the amino acid sequence requirement around the cysteine residue which is involved in the linkage is not known. From a number of studies in which cells were incubated with radiolabeled palmitate, and proteins were covalently bound to this fatty acid, it was concluded that palmitate is the fatty acid involved in this type of modification. However, data from a few reports have indicated that fatty acid acylation through thioester linkages has a more relaxed fatty acid specificity than cotranslational fatty acid acylation. Using microsomal membrane fractions to study protein acylation with a viral polypeptide as a fatty acid acceptor, it has been shown that myristate, stearate, and oleate could replace palmitate as the thioester-linked fatty acid (13). In cultures of epidermoid carcinoma cells, mouse fibrosarcoma cells, and rat pheochromocytoma cells, myristate and stearate were also bound to proteins by ester, probably thioester linkages, although the extent of binding was significantly less than that of palmitate (14, 15). More recently, we have shown that in human platelets, in which *de novo* protein synthesis is negligible and, therefore, no cotranslational fatty acid acylation takes place, palmitoylation is an active process (16, 17), and that myristate also becomes bound to platelet proteins via thioester linkages (18). Taken together, these results establish that saturated long chain fatty acids other than palmitate can be utilized to posttranslationally acylate proteins. The involvement of polyunsaturated fatty acids in the posttranslational fatty acid acylation of proteins has not been studied, although the covalent binding of arachidonate metabolites, eicosanoids, to proteins has also been detected in microsomal cell fractions (19–21) and in intact platelets (22). In the present study, it was demonstrated in human platelets by metabolic labeling with [$^3$H]arachidonate and [$^3$H]eicosapentaenate that these polyunsaturated fatty acids could become

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covalently bound to proteins. Furthermore, the extent of their binding was comparable to that of palmitate. This binding was not prevented by lipoxygenase and cyclooxygenase inhibitors and occurred almost exclusively via ester, predominantly thioester, linkages. As demonstrated by SDS-PAGE[1] and fluorography, most of the proteins that are palmitoylated can also be acylated with arachidonate and eicosapentaenoate, although there were proteins which were acylated only by palmitate.

## EXPERIMENTAL PROCEDURES

*Materials*—[5,6,8,9,11,12,14,15-$^3$H]Arachidonate (76 Ci/mmol), [5,6,8,9,11,12,14,15,17,18-$^3$H]eicosapentaenoate (116.8 Ci/mmol), [9,10-$^3$H]palmitate (38.0 Ci/mmol), and scintillation solution F 989 were purchased from Du Pont-New England Nuclear. Arachidonate, palmitate, docosahexaenoate (22:6), arachidonate methyl ester, and docosatetraenoate methyl ester (22:4) were products of Nu-Check Prep (Elysian, MN). Hydroxylamine, apyrase (grade VII), fatty acid free bovine serum albumin and eicosapentaenoate methyl ester were obtained from Sigma. Eicosapentaenoate was a product of Cayman Chemical Co. (Ann Arbor, MI). $M_r$ marker proteins were purchased from Bio-Rad. Prostaglandin $E_1$ ($PGE_1$) was purchased from Seragen (Cambridge, MA). Baicalein, a selective inhibitor of platelet lipoxygenase (23), was the product of Biomol (Plymouth Meeting, PA). The water-soluble indomethacin megluminate was obtained from Chiesi Farmaceutici S. p. A. (Parma, Italy). $^{14}$C-Methylated $M_r$ marker proteins and autoradiographic image enhancer (Amplify) were obtained from Amersham Corp. HPLC grade solvents were used for HPLC, delipidation of proteins, alkaline methanolysis, and extraction of fatty acids. Methanolic HCl (3 M) was the product of Supelco (Bellefonte, PA). Methylation of docosahexaenoate acid was performed according to the method of Lepage and Roy (24).

*Preparation of [$^3$H]Fatty Acid-labeled Platelet Suspensions*—The procedure described earlier for labeling with [$^3$H]palmitate was followed with minor modifications (16). Platelet-rich plasma was obtained from human blood anticoagulated with acid-citrate-dextrose containing 0.18 $\mu$M $PGE_1$ by centrifugation (120 × *g*, 15 min). Platelets pelleted from platelet-rich plasma at 1300 × *g* (15 min) were resuspended in solution A (140 mM NaCl, 2.5 mM KCl, 0.1 mM $MgCl_2$, 10 mM $NaHCO_3$, 0.5 mM $NaH_2PO_4$, 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, and 0.3 $\mu$M $PGE_1$. [$^3$H]Arachidonate, [$^3$H]eicosapentaenoate, or [$^3$H]palmitate in ethanol was dried completely under nitrogen, then solubilized by vigorous mixing for 60 s in the above solution, and added to an equal volume of platelet suspension. After the incubation for 1 h, total 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 $\mu$M $PGE_1$. Platelets were again centrifuged (1100 × *g*, 15 min) and resuspended in solution A containing 1 unit/ml apyrase and incubated for an additional 30 min. Finally, aliquots were removed for the determination of platelet count and radioactivity incorporated in the platelets. The entire procedure was carried out at 37 °C. During centrifugation, the temperature in the centrifuge was maintained between 32 and 37 °C. The radioactivity in the final suspension was 300 $\mu$Ci/ml which corresponded to mass concentrations of 4 $\mu$M for arachidonate, 2.6 $\mu$M for eicosapentaenoate, and 7.9 $\mu$M for palmitate, which are in the physiological range for nonesterified fatty acids bound to albumin (25). The platelet count was 300–330 × $10^6$/ml. In certain experiments with [$^3$H]arachidonate and [$^3$H]palmitate, the incubation mixture also contained 30 $\mu$M unlabeled palmitate. In the experiments with cyclooxygenase and lipoxygenase inhibitors, the platelet suspension and the washing solutions contained 10 $\mu$M baicalein and 30 $\mu$M indomethacin. In separate experiments, it was shown that under these conditions both the cyclooxygenase and lipoxygenase pathways of eicosanoid production were completely inhibited.

*Extraction of Noncovalently Bound Lipids*—Washed radiolabeled platelets (1 ml) were precipitated and extracted with 3.5 volumes of cold acetone (vortexing for 60 s) and then kept on ice for 30 min, at which time the precipitate was collected by centrifugation (4500 ×*g*, 4 °C, 10 min). Acetone-precipitated proteins from 1 ml of platelet suspension were first 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 protein 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 methanol. After each extraction step, the samples were left at room temperature for 10 min before centrifugation. The final protein residue contained no radioactivity removable by lipid extracting solvents, and it was dried completely under nitrogen. Selected protein samples were dissolved in SDS-PAGE sample buffer (without $\beta$-mercaptoethanol) by boiling for 5 min and then shaking overnight. Their protein content and radioactivity were determined. Other samples were used for the release of fatty acids and identification and quantitation of the fatty acids bound to proteins.

*Release of Protein-bound Fatty Acids by Alkaline and Acid Methanolysis and Identification of Released Fatty Acids by HPLC*—The dried delipidated protein preparations were first subjected to alkaline methanolysis to disrupt *O*-ester and *S*-ester linkages and subsequently subjected to acid methanolysis to disrupt amide linkages (16). Fatty acids released by alkaline or acid methanolysis were extracted with hexane, then dried completely under nitrogen and redissolved in methanol. Aliquots were removed for determination of extracted radioactivity before HPLC analysis. The nonextractable radioactivity was also measured. To the remainder of the samples, 2.4 $\mu$g of unlabeled fatty acid (arachidonate or eicosapentaenoate), and unlabeled fatty acid methyl esters (methyl arachidonate and methyl 22:4 *n*-6 or methyl eicosapentaenoate and methyl 22:6 *n*-3) were added as standards. Separation of fatty acids and fatty acid methyl esters 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 a 40–90% (v/v) acetonitrile, 17 mM phosphoric acid gradient for 20 min and then with 90% acetonitrile, 17 mM phosphoric acid for 35 min at a flow rate of 1 ml/min. Fractions of 1 ml were collected. 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.

*Release of Protein-bound Fatty Acids by Neutral Hydroxylamine Treatment*—Delipidated platelet proteins from 0.1 ml of platelet suspension were resuspended in 0.5 ml of $H_20$ with a 10-s burst of sonication. Equal volumes of 2 M hydroxylamine, pH 6.95, were added and the mixture was incubated for 12 h at room temperature with constant shaking. It was then extracted with 3 × 1 ml chloroform/methanol (2:1). The organic phases were combined and dried completely under nitrogen. The dried material was resuspended in 0.2 ml of acetonitrile and 0.1 ml of water. Then 4.5 ml of F989 was added, and the radioactivity was determined. Alternatively, delipidated proteins dissolved in 50 $\mu$l of SDS sample buffer were also treated with 1 M hydroxylamine (pH 6.95) and extracted as described above. The amount of radioactivity released by neutral hydroxylamine was essentially identical using both methods. Treatment with 1 M Tris-HCl, pH 6.95, did not result in any release of radioactive material from the proteins.

*SDS-PAGE, Hydroxylamine Treatment, and Fluorography of Polyacrylamide Gels*—SDS-PAGE of reduced (with 50 mg/ml $\beta$-mercaptoethanol) or nonreduced platelet proteins in a 5–20% gradient gel, as well as fixing, staining, and destaining of gels, and 1 M hydroxylamine treatment of fixed gels have all been described earlier (16). Destained gels were soaked in Amplify solution supplemented with 0.5% glycerol for 1 h, briefly rinsed with distilled $H_20$, and dried at 60 °C. The dried gels were exposed to Kodak X-OMAT AR film (Rochester, NY) at −70 °C for 4 weeks.

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

## RESULTS

*Uptake and Covalent Binding to Proteins of [$^3$H]Fatty Acids*—A substantial amount of added [$^3$H]arachidonate (26.5% of the total added radioactivity) was incorporated into platelets during the 1-h incubation period (Table I). The amount of dpm incorporated into cells incubated with [$^3$H]arachidonate was approximately 2.5- and 3-fold higher than in [$^3$H]eicosapentaenoate and [$^3$H]palmitate-labeled platelets, respectively. The different amounts of incorporated radioac-

[1] The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; $PGE_1$, prostaglandin $E_1$; dpm, disintegrations/minute.

TABLE I

*The incorporation of radiolabeled fatty acids into platelets*

Platelets were incubated with 300 μCi/ml of the [$^3$H]fatty acid for 1 h at 37 °C, and the radioactivity incorporated by the cells was determined. The concentration of nonlabeled palmitate, when present, was 30 μM. Inhibitors: platelet lipoxygenase and cyclooxygenase were inhibited by baicalein and indomethacin. Values represent means ± S.E.

| Components in the incubation mixture | No. of experiments | Incorporated radioactivity | |
|---|---|---|---|
| | | dpm/ng protein | % of added radioactivity |
| [$^3$H]Palmitate | 10 | 93.9 ± 3.5 | 9.5 ± 0.3 |
| [$^3$H]Arachidonate | 7 | 259.0 ± 9.5 | 26.5 ± 1.0 |
| [$^3$H]Arachidonate and inhibitors | 6 | 222.3 ± 23.1 | 23.5 ± 2.4 |
| [$^3$H]Arachidonate and palmitate | 3 | 221.9 ± 7.5 | 23.9 ± 0.8 |
| [$^3$H]Eicosapentaenoate | 2 | 118.0 ± 1.2 | 11.8 ± 0.1 |

TABLE II

*Covalent binding of incorporated radiolabeled fatty acids to platelet proteins*

Platelets were incubated with 300 μCi/ml of the [$^3$H]fatty acid for 1 h at 37 °C. Platelet proteins were precipitated, noncovalently bound lipids were extracted, and protein-bound radioactivity was measured. The concentration of non-labeled palmitate, when present, was 30 μM. Inhibitors: platelet lipoxygenase and cyclooxygenase were inhibited by baicalein and indomethacin. Values represent means ± S.E.

| Components in the incubation mixture | No. of experiments | Radioactivity covalently linked to proteins | |
|---|---|---|---|
| | | dpm/ng protein | % of incorporated radioactivity |
| [$^3$H]Palmitate | 8 | 1.91 ± 0.06 | 2.03 ± 0.06 |
| [$^3$H]Arachidonate | 5 | 1.72 ± 0.11 | 0.66 ± 0.04 |
| [$^3$H]Arachidonate and inhibitors | 4 | 1.16 ± 0.13 | 0.52 ± 0.06 |
| [$^3$H]Arachidonate and palmitate | 3 | 1.13 ± 0.10 | 0.51 ± 0.04 |
| [$^3$H]Eicosapentaenoate | 2 | 0.68 ± 0.01 | 0.58 ± 0.01 |

tivity probably reflect differences in the rates of fatty acid uptake. Incubation with cyclooxygenase and lipoxygenase inhibitors slightly decreased the uptake of [$^3$H]arachidonate. A 7.5-fold excess of unlabeled palmitate resulted only in a slight decrease of incorporation of radiolabeled arachidonate, which is consistent with the concept that in platelets two distinct mechanisms are involved in the uptake of saturated and polyunsaturated fatty acids (26).

A relatively small but reproducible portion of radioactivity (1.72 ± 0.11 dpm/ng protein) became covalently linked to platelet proteins in [$^3$H]arachidonate-labeled cells, which is somewhat less than the value (1.91 ± 0.06 dpm/ng protein) obtained with [$^3$H]palmitate (Table II). Since the amount of incorporated radioactivity in [$^3$H]palmitate-labeled platelets is considerably lower than in [$^3$H]arachidonate-labeled cells, the amount of protein-bound radioactivity obtained with [$^3$H] palmitate represents a 3-fold higher percentage of total radioactivity incorporated in the cells than that measured in [$^3$H] arachidonate-labeled cells (2.03 ± 0.06% *versus* 0.66 ± 0.04%). Since the percentage of total incorporated [$^3$H]eicosapentaenoate that was linked to proteins was approximately the same (0.58 ± 0.01%) as the value obtained with [$^3$H]arachidonate, the relatively low protein-bound dpm in [$^3$H]eicosapentaenoate-labeled platelets (0.68 ± 0.01 dpm/ng protein) is apparently due to the relatively low uptake of this fatty acid rather than to the impaired binding of eicosapentaenoate to proteins. The incubation of platelets with baicalein and indomethacin resulting in the combined inhibition of platelet cyclooxygenase and lipoxygenase activities decreased the protein-linked radioactivity in [$^3$H]arachidonate-labeled platelets by 33%. If the slight inhibition of uptake of [$^3$H]arachidonate in these platelets was also taken into account, the decrease was only 21%. The presence of excess (30 μM) unlabeled palmitate resulted in a 23% decrease in the binding of [$^3$H]arachidonate to platelet proteins. The same amount of unlabeled palmitate competed more efficiently with the posttranslational protein binding of [$^3$H]myristate (18) and of [$^3$H]palmitate than with the posttranslational protein binding of [$^3$H]arachidonate in Table II (51% inhibition for [$^3$H]myristate, 61% inhibition for [$^3$H]palmitate, and 23% inhibition for [$^3$H]arachidonate). This was true despite the fact that the experiments involving radiolabeled palmitate and radiolabeled myristate included these fatty acids in higher mass concentrations (7.5 and 7.9 μM, respectively) than experiments involving radiolabeled arachidonate (4 μM).

*Type of Linkages and Identity of Fatty Acids Involved in Covalent Bindings to Proteins*—As reported earlier (16), radiolabeled palmitate was bound to proteins almost exclusively via ester linkages. Similarly, more than 90% of the radioactivity bound to proteins in [$^3$H]arachidonate and [$^3$H]eicosapentaenoate-labeled cells could be released from proteins by alkaline methanolysis, which disrupts all ester bonds (0-ester and S-ester) (Table III). After alkaline methanolysis, for all three labeled fatty acids the release of radioactivity by acid methanolysis (*i.e.* fatty acids bound via amide linkages) as well as the nonreleasable radioactivity that remained with the protein after such treatment were negligible. It was shown by HPLC analysis that in [$^3$H]arachidonate and [$^3$H]eicosapentaenoate-labeled cells, 95 and 92% of the radioactivity bound to protein through ester linkage was [$^3$H]arachidonate and [$^3$H]eicosapentaenoate, respectively (Fig. 1). This clearly indicates that predominantly arachidonate and eicosapentaenoate were bound to proteins and not their metabolic products. Neither the inhibition of cyclooxygenase and lipoxygenase nor the addition of cold palmitate influenced this situation, since the contribution of [$^3$H]arachidonate to the total of esterified protein-linked radioactivity was still greater than 90% under these conditions (data not shown). Using neutral hydroxylamine treatment which selectively cleaves thioester but not 0-ester bonds (27–29), nearly 100% of [$^3$H] palmitate bound to protein via ester linkage was identified as thioester linked fatty acid (Table III). The major portion of radiolabeled arachidonate and eicosapentaenoate was also bound to protein through thioester linkages. However, a minor but significant portion was involved in other type(s) of ester, probably 0-ester, linkages. The relative proportion of thioester-linked [$^3$H]arachidonate within the total protein-bound pool was slightly decreased (61 *versus* 53%) when [$^3$H]arachidonate labeling of platelets was performed in the presence of 30 μM unlabeled palmitate.

*Detection of [$^3$H]Fatty Acid-labeled Proteins by SDS-PAGE and Fluorography*—Studies were performed to determine if the two tritiated polyunsaturated fatty acids used in the experiments became bound to the same set of proteins as were labeled with [$^3$H]palmitate. The fluorographic patterns of radiolabeled proteins from platelets separately incubated with [$^3$H]arachidonate and with [$^3$H]palmitate were similar but not identical (Fig. 2, *lanes P* and *A*). Even using only one-dimensional electrophoresis, on the reduced gel, at least four palmitoylated bands (129, 47, and 37, and 17 kDa) were identified (see *arrowheads* on *lane P*, reduced gel) which had no corresponding band in lane A with arachidonoylated proteins. On the nonreduced gel, two such bands were also detected (see *arrowheads*, nonreduced gel, *lane P*). The absence of a band

TABLE III

*Release of radiolabeled fatty acids covalently linked to platelet proteins by selective cleavage techniques*

Parallel samples of lipid extracted proteins from platelets labeled with [$^3$H]fatty acids were subjected to neutral hydroxylamine treatment to release thioester linked fatty acids or to alkaline methanolysis to release all ester linked fatty acids. The radioactivity liberated was determined by scintillation counting. Protein samples treated with alkaline methanolysis were subsequently subjected to acid methanolysis to release amide linked fatty acids. The radioactivity released, as well as the radioactivity remaining with the protein precipitate (nonreleasable pool), were determined. The percentage of radioactivity liberated by alkaline and acid methanolysis plus the nonreleasable pool sum to 100% in each case. Results are means of at least two separate experiments, the differences of which were less than 4% (hydroxylamine treatment and alkaline methanolysis) or 2% (acid methanolysis and nonreleasable dpm).

| Fatty acids in the incubation mixture | % of total protein-linked radioactivity | | | | Total protein-linked radioactivity dpm/μg protein |
|---|---|---|---|---|---|
| | Released by | | | Nonreleasable | |
| | Neutral hydroxylamine | Methanolysis | | | |
| | | Alkaline | Acid | | |
| [$^3$H]Palmitate | 91 | 94 | 3 | 3 | 1980 |
| [$^3$H]Arachidonate | 61 | 93 | 3 | 4 | 1605 |
| [$^3$H]Arachidonate and inhibitors | 56 | 93 | 3 | 4 | 1146 |
| [$^3$H]Arachidonate and palmitate | 53 | 91 | 4 | 5 | 1062 |
| [$^3$H]Eicosapentaenoate | 66 | 90 | 5 | 5 | 575 |

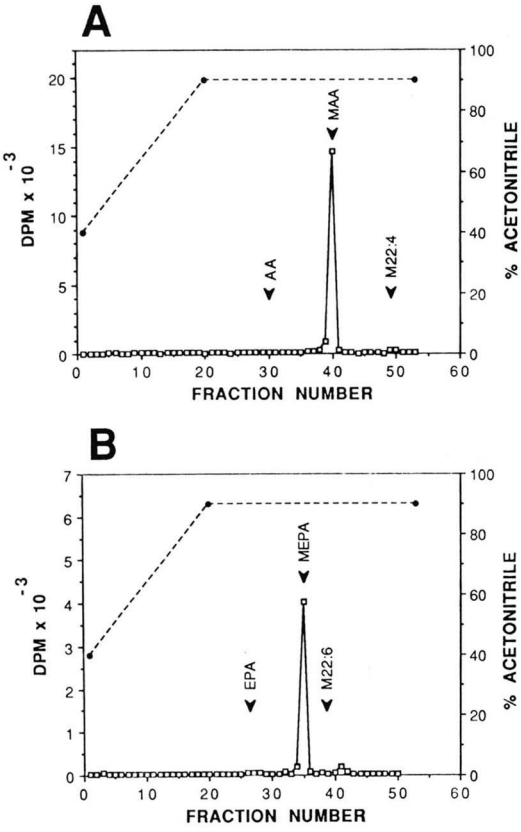


FIG. 1. **Identification and quantitation of radiolabeled fatty acids released by alkaline methanolysis from platelet proteins labeled with [$^3$H]arachidonate (*A*) or [$^3$H]eicosapentaenoate (*B*).** Using reverse-phase HPLC, elution positions of arachidonate (*AA*), methyl arachidonate (*MAA*), and methyl 22:4 (*M22:4*) as well as those of eicosapentaenoate (*EPA*), methyl eicosapentaenoate (*MEPA*), and methyl 22:6 (*M22:6*) were determined by monitoring absorbance at 205 nm. Both elution profiles represent the mean values of duplicate experiments with essentially identical results.

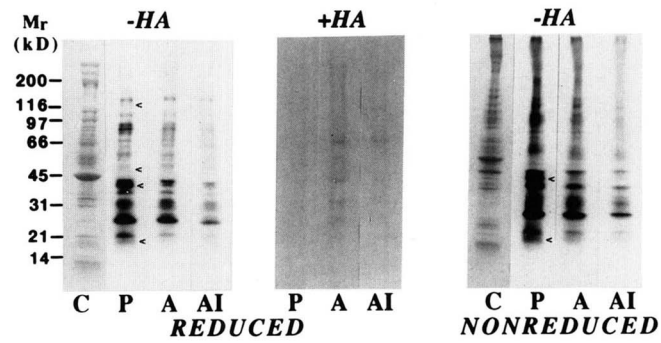


FIG. 2. **$^3$H-Arachidonoylated and $^3$H-palmitoylated proteins in platelets.** Washed platelets were labeled with [$^3$H]fatty acid. Platelet proteins were then precipitated, extracted, and dissolved in sample buffer. Reduced and nonreduced samples (40 μg protein/well) were analyzed by SDS-PAGE after which the radiolabeled proteins were detected by fluorography. SDS-PAGE of reduced samples was performed in duplicate on two parallel gels, and one of the fixed gels was subjected to neutral hydroxylamine (*+HA*) treatment. *Lanes C*, Coomassie-stained gels of platelet proteins; *lanes P*, [$^3$H]palmitate-labeled platelets; *lanes A*, platelets incubated with [$^3$H]arachidonate; *lanes AI*, [$^3$H]arachidonate-labeled platelets incubated with baicalein and indomethacin to inhibit cyclooxygenase and lipoxygenase. *Horizontal lines* on the *left side* of *first gel* represent the migration distance of Mr marker proteins. *Arrowheads* on the *P lane* identify bands which can be detected only on the fluorogram of $^3$H-palmitoylated proteins.

in the arachidonoylated lane which corresponds to the highly intense [$^3$H]palmitoylated band at 37 kDa is especially noticeable on the nonreduced gel. These polypeptides either cannot be arachidonoylated or the extent of their arachidonoylation is too low to appear on the fluorogram. It was also found that [$^3$H]eicosapentaenoate labeled the same set of proteins as [$^3$H]arachidonate (Fig. 3). As expected from the results of neutral hydroxylamine treatment of radiolabeled proteins in solution, hydroxylamine treatment of the gels completely eliminated the radiolabeled bands from the fluorogram of $^3$H-palmitoylated proteins (hydroxylamine-treated gel, *lane P*), while some of the arachidonoylated proteins, among them a 66-kDa polypeptide, retained its radiolabel (hydroxylamine-treated gel, *lane A*). No change in the fluorographic pattern of arachidonoylated proteins could be detected when platelet cyclooxygenase and lipoxygenase were both inhibited (*lanes AI versus lanes A*), although the lanes representing samples with inhibitors were somewhat less intense than those without inhibitors.

## DISCUSSION

The primary purpose of this study was to determine if two polyunsaturated fatty acids which can be converted to biolog-

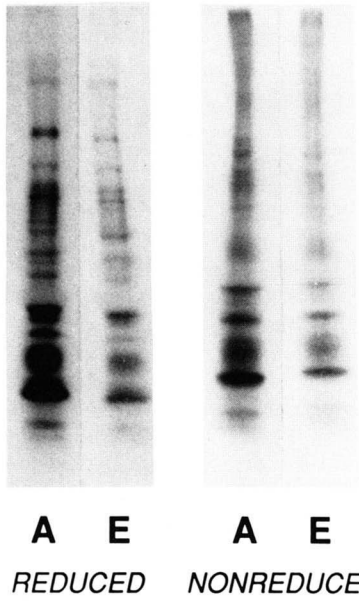


FIG. 3. **Fluorograms of platelet proteins labeled with [$^3$H] arachidonate and [$^3$H]eicosapentaenoate.** Reduced and unreduced protein samples (50 $\mu$g protein/well) of platelets labeled with [$^3$H]arachidonate (*A*) and [$^3$H]eicosapentaenoate (*E*) were analyzed by SDS-PAGE and fluorography.

ically active metabolites, arachidonate and eicosapentaenoate, could be utilized for posttranslational fatty acid acylation of proteins. To perform this study using nucleated cells with active protein synthesis would impose a number of difficulties regarding interpretation of data on the posttranslational incorporation of radiolabeled fatty acids into the cells. In nucleated cells with active protein synthesis, fatty acids can be $\beta$-oxidized, and the radiolabel may appear in amino acids utilized for protein synthesis, or in fatty acids used for cotranslational acylation of proteins. Therefore, platelets, which are highly important in hemostasis, provide an excellent tool for specifically studying posttranslational fatty acid acylation of proteins.

The covalent binding of [$^3$H]arachidonate or [$^3$H]eicosapentaenoate to platelet proteins was proven by several lines of evidence in this study. First, in platelets radiolabeled with these fatty acids, the radiolabel was recovered with protein after exhaustive lipid extraction to remove noncovalently associated lipids. Second, the radiolabeled fatty acid could not be separated from the proteins by SDS-PAGE. Third, the platelet-bound radioactivity was cleaved from the protein using techniques which selectively disrupt certain types of covalent bonds, and the released radioactivity was identified as the respective fatty acids by HPLC. It remains to be determined if the binding of [$^3$H]arachidonate and [$^3$H]eicosapentaenoic to proteins is a platelet-specific process or, more likely, a generalized phenomenon.

Lecomte *et al.* (22) have also studied protein labeling in platelets incubated with [$^3$H]arachidonate. However, they found only minimal labeling of proteins in resting platelets which was significantly increased during activation by thrombin. Based on experiments with cyclooxygenase, lipoxygenase, and thromboxane synthetase inhibitors, they concluded that cyclooxygenase and lipoxygenase products, and not arachidonate, were the moieties covalently bound to proteins, although the protein-linked moieties presumed to be eicosanoids were not isolated and identified. The differences between their findings and ours may be partly explained by different experimental conditions for fatty acid labeling of the proteins. In their experiments, the radioactivity used for labeling was significantly less; the labeling period was much shorter, and the platelet count was higher. As a result, the process of arachidonoylation in resting platelets could have been below the limit of detection. It is to be noted that under the conditions used in their experiments, palmitoylation of platelet proteins also could not be detected. It has been shown, however, in our previous studies (16, 17) and in the work of others (30, 31) that posttranslational modification of platelet proteins with palmitate is an active process. In the present study, the combined inhibition of lipoxygenase and cyclooxygenase did not prevent radiolabeling of proteins by [$^3$H] arachidonate, and the radioactivity liberated from proteins by alkaline methanolysis was quantitatively recovered as arachidonate. These findings indicate that the fatty acid and not its eicosanoid metabolites was bound to platelet proteins.

The majority of protein-linked arachidonate and eicosapentaenoate, and nearly all protein-bound palmitate in our experiments, was bound through thioester linkages. Most of the palmitoylated proteins were also arachidonoylated, but several were only palmitoylated. This might indicate that there are two protein-*S*-fatty acyltransferases, only one of which utilizes polyunsaturated fatty acids. Alternatively, there could be substrate proteins to which polyunsaturated fatty acids cannot bind because unlike saturated fatty acids, they are not straight chain molecules. If the protein-linked [$^3$H]fatty acid binding in our study is expressed as dpm/$\mu$g protein, the binding of [$^3$H]palmitate to proteins was only 11% higher than that of [$^3$H]arachidonate, but when expressed as percent of total incorporated [$^3$H]fatty acid, the amount of protein-linked palmitate was three times greater than the amount of protein-linked arachidonate. Although arachidonate is incorporated into platelets much more rapidly and to a greater extent than palmitate, a much higher percentage of arachidonate becomes esterified in phospholipids (26). This may indicate that relatively more palmitoyl-CoA than arachidonoyl-CoA is available for protein acylation and explain our observed results. Unlabeled palmitate in relatively high concentration competed with radiolabeled arachidonate for protein binding to a lesser extent than expected on the basis of the above finding, and on the basis of its competition with the straight chain radiolabeled fatty acids [$^3$H]palmitate and [$^3$H]myristate. One possible explanation for this observation is, as mentioned previously, that there may be two protein-*S*-fatty acyltransferases, only one of which utilizes polyunsaturated fatty acids. It may also be that in the concentration ranges used in our studies, the two fatty acids do not compete effectively for binding to proteins.

The ester-bound arachidonate and eicosapentaenoate which are not thioester-linked probably represent fatty acids esterified to proteins through *O*-ester linkages. This may involve hydroxyl groups of serine or threonine residues. Alternatively, [$^3$H]fatty acid may be incorporated into the phosphatidylinositol moiety of a protein-linked glycophosphatidylinositol anchor.

Protein-linked arachidonate represents a hitherto unrecognized cellular arachidonate pool, the compartmentalization of which depends on the compartmentalization of the proteins to which it is linked. The release of arachidonate from proteins by a putative protein-*S*-fatty acylesterase, if the conditions were suitable, may even result in eicosanoid production. It is to be emphasized that the mass concentration of fatty acids used in these experiments resembles their physiological concentrations in the plasma as nonesterified fatty acids bound to albumin. This suggests that binding of these fatty acids to platelet proteins is a continuous process under normal con-

ditions. This also raises the possibility that the composition of the fatty acid pool linked to platelet proteins depends on the composition of one or more pools of fatty acids in the plasma, such as the albumin-bound pool of nonesterified fatty acids. If this is true, changes in the concentration of certain plasma fatty acids may alter the composition of fatty acids bound to proteins, which then could have structural and functional implications. The replacement of a protein-linked palmitate, for instance, with arachidonate, a fatty acid of different hydrophobicity and conformation, may significantly influence its relationship with membrane lipids or hydrophobic domains of other proteins.

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