The Pool of Fatty Acids Covalently Bound to Platelet Proteins by Thioester Linkages Can Be Altered by Exogenously Supplied Fatty Acids

Laszlo Muszbek[b], Gizella Haramura[b], Joanne E. Cluette-Brown[a], Elizabeth M. Van Cott[a], and Michael Laposata[a,*]

[a]Department of Pathology, Division of Laboratory Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114-2696, and [b]Department of Clinical Chemistry, University Medical School, Debrecen, Hungary H-4012

ABSTRACT: The goals of this investigation were, first, to develop a chemical strategy to identify and quantitate the mass of fatty acid which is covalently bound to proteins by thioester linkage in unactivated platelets, and, second, to determine whether exogeneously added fatty acids can alter the fatty acid composition of thioester bound fatty acids. Studies with radiolabeled fatty acids cannot identify and quantitate the actual fatty acids bound to proteins because they permit analysis of only the radiolabeled fatty acids added and their metabolites. Therefore, in the absence of metabolic labeling by radiolabeled fatty acids, we isolated the thioester-linked fatty acids from platelet proteins using hydroxylamine at neutral pH to form fatty acid hydroxamates. The hydroxamates were subsequently converted to fatty acid methyl esters by acid methanolysis for quantitation by gas chromatography–mass spectrometry. Using platelet specimens from 14 subjects, 74% of the fatty acid recovered from the unactivated platelet proteins as thioester linked was palmitate. Importantly, however, 22% was stearic acid, and oleate was 4% of the total thioester bound fatty acid. There was minimal variability (2.6-fold at maximum) between the subjects in the amount of the thioester-linked palmitate and thioester-linked stearate. However, there was substantial variability (>100-fold at maximum) between subjects in the amount of thioester-linked oleate. We also demonstrated that incubation of platelets with exogenous fatty acids can alter the profile of fatty acids bound to platelet proteins by thioester linkages. Incubation of platelets with 100 μM palmitate for 3 h increased the amount of thioester-linked palmitate by up to 26%, and incubation of platelets with 100 μM stearate increased the amount of thioester-linked stearate up to 30%. In support of the observation that radiolabeled fatty acids other than palmitate were shown to be capable of binding to platelet proteins by thioester linkage, our results indicate that the fatty acids actually bound to unactivated platelet proteins include a significant amount of stearate, and variable amounts of oleate, as well as palmitate. In addition, the data show that palmitate and stearate can be increased, as a percentage of total protein-bound fatty acid, by incubation with exogenous palmitate and stearate, respectively.

Fatty acids can become covalently bound to proteins by three different types of linkages (reviewed in Refs.1–4). First, fatty acids can be linked to proteins by way of an amide linkage, and the fatty acid found in this type of linkage is almost exclusively myristate (14:0). Second, fatty acids can become bound to proteins by way of thioester linkages. Fatty acids able to bind with this linkage include myristate (5), palmitate (16:0) (the most commonly studied fatty acid in thioester linkage (1–4)), stearate (18:0) (1–4), oleate (18:1) (6), arachidonate (20:4n-6) (7), docosatetraenoate (22:4n-6) (7), eicosapentaenoate (20:5n-3) (7), and docosahexaenoate (22:6n-3) (7). Fatty acids may also be bound covalently to proteins indirectly in glycosylphosphatidylinositol (GPI) anchor proteins (reviewed in Refs. 1–4). In this situation, the fatty acid is associated by an O-ester linkage with a phospholipid moiety which is linked through a variety of sugars to a protein. Although the fatty acid is not directly bound to the protein, in the isolation of total protein from the cells, the fatty acid is recovered. In trypanosomes, the common fatty acid in O-ester linkage in GPI anchor proteins is myristate (8). However, in other organisms the fatty acids in O-ester linkages of GPI anchor proteins are highly varied (3,4).

Nearly all previous studies on fatty acid acylation of proteins identified which fatty acids can become covalently bound to proteins and not the actual fatty acids that are bound to proteins in the native state. In studies with radiolabeled fatty acids, the fatty acids identified as protein-bound will only be those radiolabeled fatty acids added to the cells and their metabolites. If the radiolabeled fatty acids incorporated by the cells do not represent the fatty acids actually bound to protein, then an incorrect conclusion will be drawn concerning the identity of the fatty acids bound to the proteins. This

Lipids 34, S331–S337 (1999).

*To whom correspondence should be addressed at Director of Clinical Laboratories, Room 235—Gray Building, Massachusetts General Hospital, Boston, MA 02114-2696.

E-mail: MLaposata@Partners.org

Abbreviations: GC–MS, gas chromatography—mass spectrometry; GPI, glycosylphosphatidylinositol; FAME, fatty acid methyl ester; PGE_1, prostaglandin E_1.

is a particularly important question with regard to thioester linked fatty acids because so many different fatty acids have been shown to be capable of binding to proteins in thioester linkage. If 16:0 is added to platelets, with covalent binding of the fatty acid and its metabolites to proteins, the fatty acids detectable will include 16:0, 18:0, and 18:1. This is a very limited number of fatty acids relative to the number of naturally occurring fatty acids. If 14:0 is added to platelets, the fatty acids detectable will include primarily 14:0 and 16:0. If 20:4n-6 is added to the cells, 20:4n-6 and 22:4n-6 may be detected, and if 20:5n-3 is added to the cells, 20:5n-3 and 22:6n-3 are detectable.

There are two major limitations to identifying and quantitating the fatty acids bound to cellular proteins by thioester-linkage in cells in the native state. First, the small mass of thioester-linked fatty acid presents a major limitation to identifying and quantitating the fatty acids. A second obstacle is the isolation of thioester-linked fatty acids away from O-ester-linked fatty acids in GPI anchor proteins. A common means to liberate thioester-linked fatty acids from proteins is alkaline methanolysis, which removes both thioester-linked and O-ester-linked fatty acids (1–4). Thus, this method cannot be used to selectively evaluate the thioester-linked fatty acid pool. To selectively remove thioester-linked fatty acids from proteins and allow O-ester-linked fatty acids to remain bound, it is necessary to use hydroxylamine at neutral pH. Hydroxylamine at neutral pH will specifically liberate thioester-linked fatty acids from protein (9), but it does not lead to formation of fatty acid methyl esters (FAME) which are detectable and quantifiable by gas chromatography (GC). Instead fatty acid hydroxamates are formed. Thus, the efficient chemical conversion of a very small mass of fatty acid hydroxamates to FAME is necessary for detection and quantitation of the fatty acids bound to platelet proteins in thioester linkage. We performed this analysis in the present studies, creating fatty acid hydroxamates and then FAME from the thioester-linked fatty acids bound to platelet proteins. We determined that the fatty acids actually covalently bound to platelet proteins by thioester linkage include stearate with highly variable amounts of oleate, as well as palmitate. Most importantly, however, we showed that the percentages of palmitate and stearate thioester linked to platelet proteins can be altered by incubation of the platelets with palmitate or stearate. In model membranes an increase of two carbons in fatty acid chain length greatly increases the time required for flip-flop and greatly decreases (by approximately 10-fold) the rate of dissociation of the fatty acids from the bilayer (10). This raises the possibility that proteins with the same primary amino acid sequence may be bound to membranes with different affinities because of differences in thioester-bound fatty acids which influence membrane binding.

MATERIALS AND METHODS

Isolation of the washed platelet suspension. Blood (6 mL) was collected from fasting subjects in acid citrate dextrose. Then 0.28 mM prostaglandin E_1 (PGE_1) was added to the blood, and the sample was centrifuged at $137 \times g$ for 15 min at 37°C. The platelet-rich plasma (PRP) was aspirated from a total of eight samples, pooled, and subsequently centrifuged at $1230 \times g$ for 15 min at 37°C. The supernatant from the centrifugation was aspirated, and the platelet pellet was resuspended in 10 ml of washing solution [36.0 mg fatty acid free bovine serum albumin (BSA) in 10 mL solution A] (10 mM HEPES, 0.14 M NaCl, 2.5 mM KCl, 0.1 mM $MgCl_2$, 0.1% glucose, 0.5 mM NaH_2P0_4, 10 mM $NaHC0_3$, pH 7.4), containing 0.28 mM PGE_1 supplemented with 50 µL 200 U/mL apyrase.). The resuspended platelet pellet was then centrifuged at $1000 \times g$ for 15 min at 37°C. The supernatant from this centrifugation was aspirated, and the platelet pellet was washed an additional two times using the washing solution without bovine serum albumin.

Incubation of platelets with fatty acids. In some experiments, the platelets were incubated for 3 h at 37°C at this point with either 100 µM palmitate or 100 µM stearate in the albumin-containing washing solution described above. An aliquot was removed for a platelet count. The final pellet was resuspended in 0.5 ml SDS buffer (2.0% SDS, 62.5 mM Tris-HCl, pH 6.8). The platelet suspension in SDS was heated in boiling water for 5 min.

Removal of noncovalently bound fatty acids. To precipitate the platelet proteins and remove the lipids noncovalently associated with the proteins, the denatured platelet solution was mixed with 15 mL of $CHCl_3$/MEOH (2:1, vol/vol). The mixture was vortexed and incubated for 30 min at room temperature. The sample was vortexed again for 1 min and centrifuged at $4,468 \times g$ at room temperature for 15 min. The supernatant was aspirated and discarded. At that point, 15 mL of $CHCl_3$/MEOH (2:1, vol/vol) was added to the pellet with vortexing for 1 min. The mixture was incubated for 5 min at room temperature, after which the sample was centrifuged at $4,468 \times g$ at room temperature for 15 min. The supernatant was aspirated and discarded. The extraction sequence described above was repeated using 15 mL of $CHCl_3$/MEOH (2:1), $CHCl_3$/MEOH (1:2), $CHCl_3$/MEOH/H_2O (1:1:0.3) and MEOH. The final precipitate was dissolved in 1.0 mL guanidine HCl (6M) containing 20 mM TRIS-HCl and 0.02% Na_2 EDTA. An aliquot was removed for protein determination.

Release of protein-bound fatty acids and extraction of released fatty acid hydroxamates. To release the thioester-linked fatty acids from the platelet proteins by neutral hydroxylamine treatment and extract fatty acid hydroxamates, the protein precipitate from the platelets was divided into two aliquots. To one aliquot of platelets was added hydroxylamine and 4 nmol of 17:0 acyl-CoA as the thioester-linked internal standard. This was denoted as the "sample." To the second aliquot of platelet protein precipitate, 4 nmol of 17:0 acyl-CoA without hydroxylamine was added. This was denoted as the "sample blank." In place of hydroxylamine, 1 mL of 2 M TRIS-HCl was added. There were two additional blanks which contained no platelets. One blank included 1 mL of hydroxylamine with 1.0 mL of 6 M quanidine HCl to account

for any contamination introduced by hydroxylamine (the "hydroxylamine blank"). The final blank (the "TRIS blank") included 1 mL of TRIS buffer with 1.0 mL of 6 M guanidine HCl to account for any contaminating fatty acids introduced into the "sample blank" which was dissolved in 2 M TRIS-HCl rather than hydroxylamine. All tubes were vortexed gently and incubated for 18 h at room temperature. To the sample and the three blanks was added 2 mL of $CHCl_3$/ethyl acetate (1:1, vol/vol). The four tubes were then vortexed for 1 min and centrifuged at $4,468 \times g$ for 15 min at room temperature. This resulted in the formation of two phases. The infranatant was removed and saved, and 2 mL of $CHCl_3$/ethyl acetate (1:1, vol/vol) was added to the remaining supernatant. The sample was vortexed for 1 min and then centrifuged at $4,468 \times g$ for 15 min at room temperature. The infranatant was again collected. This process was repeated one additional time, such that for each tube, three infranatants were collected and dried completely under nitrogen. The combined dried infranatants from each tube were resuspended in 0.2 mL $CHCl_3$/ethyl acetate (1:1, vol/vol) and vortexed vigorously.

Conversion of fatty acid hydroxamates into FAME by acid methanolysis. The tube was centrifuged at $1,115 \times g$ for 5 min at room temperature. The 0.2 mL supernatant was transferred to a hydrolysis tube. The original centrifuge tube was then rinsed with another 0.2 mL of $CHCl_3$/ethyl acetate (1:1, vol/vol), vortexed, and centrifuged again at $1,115 \times g$ for 5 min at room temperature. The 0.2 mL rinse was added to the hydrolysis tube. The combined contents of the hydrolysis tube were dried completely under nitrogen. At that point, 1 mL of 3 M methanolic HCl was added to the hydrolysis tube which was then closed and gently agitated. The tube was then incubated at 100°C for 24 h. After cooling to room temperature, the contents of the tube were transferred to a vial for extraction three times with 1.0 mL hexane. In each 1.0 mL-extraction with hexane, the tube was vortexed vigorously. The upper hexane phase was aspirated each time and the three extracts were pooled. To these extracts was added 4 nmol of methyl 15:0 as a second internal standard to account for evaporation losses. Each specimen was then dried completely under nitrogen and resuspended in 10 μL hexane, and 1 μL of the 10 μL was then injected into a Hewlett-Packard 5890 series II model gas chromatograph-mass spectrometer (GC–MS) with a Hewlett-Packard 5971 mass spectrometer (Hewlett-Packard, Wilmington, DE). The column was a Supelcowax 10 capillary column (Supelco, Bellefonte, PA). The injector temperature was 260°C with a detector temperature of 280°C. The flow rate of the carrier gas was 0.7 ml per minute, and the carrier gas was helium. To elute the FAME from column, the GC was programmed to initiate elution at 150°C for 2 min. At that point the temperature was increased 10°C per minute to 200°C where it was held for 4 min. At that time, the temperature was increased at 5°C per minute to 240°C and held for 3 min. Finally, the temperature was ramped at 10°C per minute to 270°C where it was held for 6 min. This temperature program eluted all of the FAME. For each of the analyses, single ion monitoring using ions 55, 67, 69, 74, 79, and 91 was performed. Total ion scans were also run at 50–500 AMU. The fatty acids in thioester linkage were quantitated by determining (the pmol fatty acid in the sample) minus (the pmol fatty acid in the hydroxylamine blank) minus (the pmol fatty acid in the sample blank) minus (the pmol fatty acid in the Tris blank)/mg platelet protein. The 15:0 methyl ester blank was included to account for changes in FAME concentration as a result of evaporation, but because the recovery of the 15:0 methyl ester was found to be greater than 95% in all cases, it had a neglible impact on the calculations. The 17:0 fatty acyl-CoA, therefore, which contains a fatty acid thioester linked to CoA, was the internal standard used to account for essentially all losses in the procedure.

RESULTS

Figure 1 shows the electron-impact tracing of the GC–MS peaks which had the expected retention times for methyl palmitate and methyl stearate. The mass spectra of authentic standards of methyl palmitate and methyl stearate are shown for comparison with the fatty acid peaks eluting at the appropriate times for methyl palmitate and methyl stearate. The major ion peaks characteristic of FAME and ion peaks unique to methyl palmitate and methyl stearate were found in the methyl palmitate and methyl stearate peaks, respectively. The ions unique to methyl palmitate and methyl stearate were the molecular ions of 270 (M^+) for methyl palmitate and 298 (M^+) for methyl stearate.

Because the amount of fatty acid covalently bound to proteins by thioester linkage was expectedly low, it was important to establish the reproducibility of the measurement for thioester-linked fatty acid. To assess the precision of the analytical method, we performed five analyses on one sample of pooled platelets. For the quantitation of thioester-bound palmitate, the mean value for the sample tested was 796 pmol/mg with a standard deviation of 62 pmol/mg. The coefficient of variation was calculated to be 7.8%. A similar result was obtained for the quantitation of thioester-linked stearate. The mean value was 267 pmol/mg, and the standard deviation was 18 pmol/mg, resulting in a coefficient of variation of 7.0%. Thus, the measurement showed excellent reproducibility, and, therefore, major differences in values between subjects most likely reflect biological variability rather than analytical variability in the measurement.

The fundamental question addressed in these studies was the identity and quantitation of the mass of fatty acids covalently bound to platelet proteins by thioester linkage. Table 1 shows the results of this quantitation from 14 normal healthy subjects in pmol fatty acid/mg platelet protein, and as percentage of total fatty acid recovered. As shown in the table, the three fatty acids detected were 16:0, 18:0, and 18:1. Although palmitate was the predominant fatty acid, there was a significant amount of stearate which was thioester-linked to platelet proteins. In some subjects, oleate was also recovered, but it represented on average, less than 5% of total fatty acid. Particularly noteworthy, stearate represents a significant

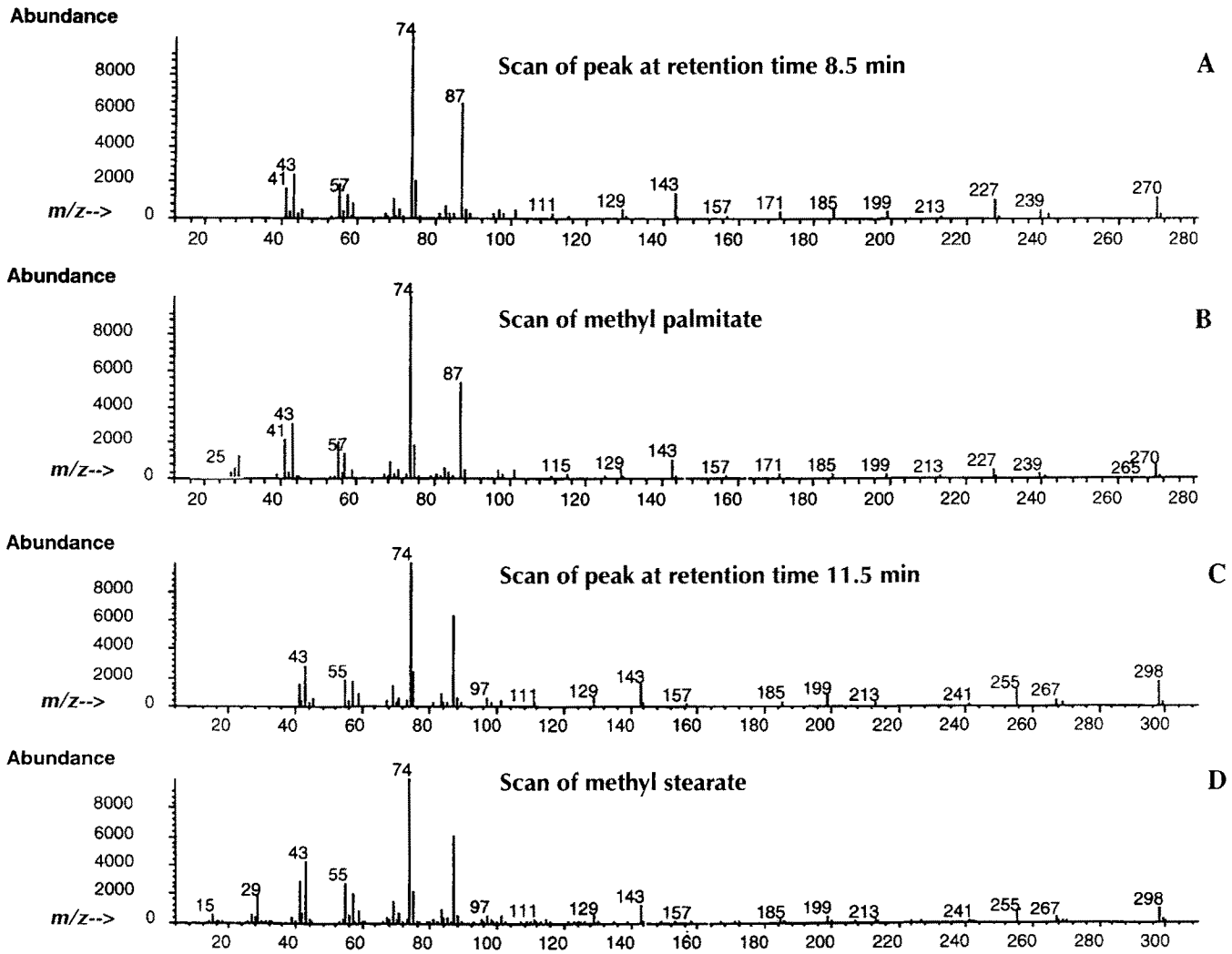

FIG. 1. Gas chromatography—mass spectrometry scans (GC–MS). Electron impact scan from GC–MS of (**A**) peak with retention time of methyl palmitate (8.5 min); (**B**) methyl palmitate standard; (**C**) peak with retention time of methyl stearate; (**D**) methyl stearate standard.

quantity of the fatty acid which is thioester-linked to protein. In studies with radiolabeled fatty acids using radiolabeled palmitate, where stearate generation is dependent on an elongase enzyme activity which converts palmitate to stearate, only trace amounts of stearate, far less than 22% of thioester-linked fatty acid, were found to be protein bound.

Table 1 also shows that there was a relatively modest amount of biological variability in the percentage of thioester-linked palmitate, and even less variability in stearate among the individual subjects. The amount of protein-bound oleate in several of the subjects (numbers 1, 3, 4, 10, and 11) was notably higher than in the others, while some subjects had undetectable levels of oleate. Although it was not possible to determine the basis for this disparity, this finding raised the possibility that the ingestion of dietary fat with a high amount of oleate, such as olive oil, may be responsible for this variability between individuals in fatty acid composition. We therefore asked whether the fatty acid composition bound to platelet proteins by thioester linkage could be modified by exogenously provided fatty acids.

To address this question, platelets were incubated for 3 h with 100 μM palmitate or 100 μM stearate to determine whether incubation with these fatty acids could alter the fatty acid composition of the thioester-linked fatty acids. Figure 2 shows the increase in thioester-linked palmitate after incubation with palmitate, and Figure 3 shows the increase in thioester-linked stearate after incubation with stearate. In each of the three samples, platelets incubated with palmitate showed an increase in the amount of covalently bound palmitate. It should also be noted, although not shown in the figure, that subjects 2 and 3 showed significant increases in the amount of protein-bound stearic acid after incubation with palmitate. This is not unexpected since platelets are able to elongate palmitate to stearate (11). Subject 1 did not show an increase in protein-bound stearate after incubation with palmitate, possibly because the platelets were less effective at elongating palmitate to stearate. The increase in stearate in subjects 2 and 3 after incubation with palmitate was, in absolute amount, less than the increase in palmitate. The increases in thioester-linked stearate after incubation of the

TABLE 1
Quantitation of Fatty Acids Covalently Bound to Platelet Proteins Through Thioester Linkage[a]

	pmol fatty acid/mg protein					
Subject	16:0	(%)	18:0	(%)	18:1	(%)
1	444	(67)	149	(23)	67	(10)
2	453	(77)	135	(23)	n.d.	
3	355	(69)	128	(25)	33	(6)
4	489	(67)	185	(25)	54	(7)
5	731	(78)	192	(21)	11	(1)
6	928	(82)	210	(18)	n.d.	
7	564	(79)	141	(20)	6	(1)
8	860	(78)	232	(21)	7	(1)
9	709	(74)	250	(26)	n.d.	
10	678	(67)	218	(22)	116	(11)
11	779	(63)	277	(22)	178	(14)
12	651	(78)	185	(22)	n.d.	
13	669	(78)	191	(22)	n.d.	
14	565	(77)	173	(23)	n.d.	
MEAN + SEM	634 ± 44	(74 ± 2)	190 ± 12	(22 ± 1)	34 ± 14	(4 ± 1)

[a]n.d., none detected.

platelets with 100 μM stearate ranged from 10 to 30% for the three subjects. There was a decrease or no change in the amount of thioester-linked palmitate when the platelets were incubated with stearate. This was expected since incubation of platelets with stearate is unlikely to generate palmitate by retroconversion in the platelet. In some of the GC–MS tracings, arachidonate was identified as a thioester-linked fatty acid. However, the amount detected was too low to quantitate in these studies with unactivated platelets. In previous studies with radiolabeled arachidonate, we showed that arachidonate can become covalently bound to proteins by way of thioester linkages (7). These results raise the possibility that arachidonate becomes covalently bound to proteins only when the platelets are activated. In support of this hypothesis, arachidonate is liberated in substantial quantity from phospholipids upon cell activation, and the free arachidonate concentration in the platelet increases significantly at that time.

DISCUSSION

The elaborate methods used in our studies, to identify and quantitate the fatty acids thioesterified to platelet proteins, were required to remove the O-ester-linked fatty acids associated with the GPI anchor proteins from the thioester-bound fatty acids. Amide-linked fatty acids do not represent a potential confounding variable because platelets have little, if any, capacity for protein synthesis, and amide-linked fatty acids are added cotranslationally. In addition, alkaline hydrolysis will remove and methylate the O-ester bound fatty acids from the GPI-linked proteins as well as thioester-bound fatty acids, but not liberate the amide-linked fatty acids (12). In addition to identifying the relative amounts of fatty acids thioester-linked to platelet proteins, these data provide a quantitation in pmol fatty acid/mg platelet protein of the actual amount of fatty acid covalently bound to platelet proteins in thioester linkages. This number is not obtainable in studies

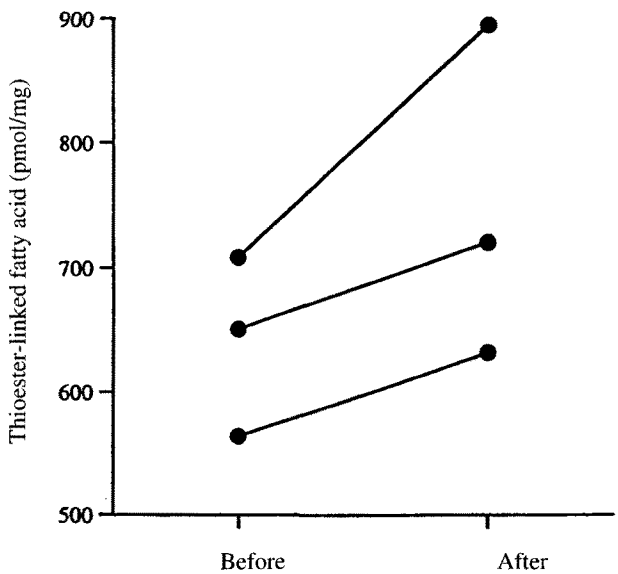

FIG. 2. Effect of palmitate (16:0) incubation on platelet protein fatty acid composition. Change in mass of thioester-linked palmitate after incubation of platelets from three subjects *in vitro* with 100 μM palmitate for 3 h at 37°C.

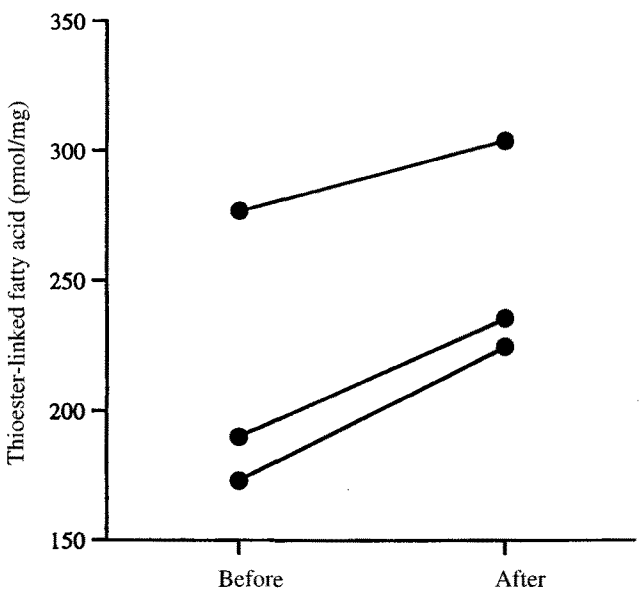

FIG. 3. Effect of stearate (18:0) incubation on platelet protein fatty acid composition. Change in mass of thioester-linked stearate after incubation of platelets from three subjects *in vitro* with 100 μM stearate for 3 h at 37°C.

with radiolabeled fatty acids because upon incorporation into the cell, the specific activity of the radiolabeled fatty acid is decreased to an undeterminable value by dilution with an unknown amount of unlabeled precursor fatty acid already in the cell. The amount of unlabeled precursor fatty acid is unknown because the precursor fatty acid pool for thioester-linked fatty acids in the cell has not been identified. The data also permit an estimation of the stoichiometry between fatty

acids and platelet proteins. Assuming an average molecular weight of 65,000 daltons for a platelet protein, with the data from our studies showing 858 pmol of thioester-linked (palmitate + stearate + oleate)/mg platelet protein, on average 0.056 molecules of fatty acid are thioester-linked to protein if a single fatty acid is bound to a single protein. Therefore, if a single fatty acid becomes bound to a single protein molecule, approximately one out of every 20 protein molecules in the platelet would be fatty acid-acylated with a thioester linkage. It is quite possible, however, that at any given time, certain proteins are acylated with more than one fatty acid. Thus, one out of 20 protein molecules acylated with a thioester-linked fatty acid represents a maximal estimate.

The results of our studies indicate that the fatty acids thioester-linked to platelet proteins in the unactivated state include stearate and oleate as well as palmitate. The studies also demonstrate that exogenously presented fatty acids can augment the mass of thioester-linked fatty acid. There is evidence that differences in fatty acid chain length and unsaturation affect the ability of fatty acids to promote protein binding to membranes (13–15). The significance of stearate as a fatty acid bound in thioester linkage to protein is that the additional two carbons on the saturated chain relative to palmitate may enhance its ability to promote protein binding to membranes. In studies evaluating the dissociation of fatty acids from phospholipid bilayers, the rate constants (Koff $(s)^{-1}$) for fatty acid desorption from bilayers were 39.0 for myristate, 8.2 for palmitate, and 0.5 for stearate (10). Thus, there is a 16.4-fold difference in the strength of the association for the phospholipid bilayer between palmitate and stearate. Accordingly, a change from palmitate to stearate or palmitate to oleate may significantly impact the function of a protein without any alteration in the primary amino acid sequence of the protein molecule.

There are at least three studies which support our finding that stearate is not a trace fatty acid among thioester-linked fatty acids. In a study involving a single platelet protein, P-selectin was isolated from human platelets, and by GC–MS it was shown that stearate as well as palmitate was thioester-linked to the isolated P-selectin molecule (16). In work by Zeng *et al.* (17), it was demonstrated that asialoglycoprotein receptor subunits contained both thioester-linked palmitate and stearate. The relative amounts of palmitate and stearate covalently bound in thioester linkage to the asialoglycoprotein receptor could not be determined, but the data suggest that stearate is unlikely to be a trace fatty acid. Although these results were obtained with individual proteins, they are consistent with the results of this investigation which identified and quantitated the fatty acids bound to all platelet proteins. In a third study, Berthiaume and Resh (18) reported on the biochemical characterization of a palmitoyl acyltransferase activity that palmitoylates myristylated proteins. They evaluated the fatty acyl-CoA specificity of the palmitoyl acyltransferase enzyme preparation. The investigators found, as expected, that unlabeled palmitate was the most effective fatty acid at inhibiting the acylation of radiolabeled palmitate to their substrate. Importantly, however, they showed that stearate was also effective in inhibiting the acylation of radiolabeled palmitate to their substrate, indicating that stearate is an acceptable substrate for palmitoyl acyltransferase. Taken together, our results indicate that stearate, as well as palmitate, is covalently bound in thioester linkage to platelet proteins and that exogenously presented fatty acids can alter the fatty acid composition of the thioester-linked fatty acid pool.

ACKNOWLEDGMENTS

This work was supported by grant #DK-37454 from the National Institutes of Health, grant #T 025760 from the Hungarian Research Fund, and grant #P30 DK-40561 from the Clinical Nutrition Research Center at the Massachusetts General Hospital.

REFERENCES

1. Casey, P.J. (1995) Protein Lipidation in Cell Signaling, *Science 268*, 221–225.
2. Milligan, G., Parenti, M., and Magee, A.I. (1995) The Dynamic Role of Palmitoylation in Signal Transduction, *Trends Biochem. Sci. 20*, 181–187.
3. Low, M.G. (1989) Glycosyl-Phosphatidylinositol: A Versatile Anchor for Cell Surface Proteins, *FASEB J. 3*, 1600–1608.
4. Low, M.G., and Saltiel, A.R. (1988) Structural and Functional Roles of Glycosyl-Phosphatidylinositol in Membranes, *Science 239*, 268–275.
5. Muszbek, L., and Laposata, M. (1993) Myristoylation of Proteins in Platelets Occurs Predominantly Through Thioester Linkages, *J. Biol. Chem. 268*, 8251–8255.
6. Muszbek, L., and Laposata, M. (1989) Covalent Modification of Platelet Proteins by Palmitate, *Blood 74*, 1339–1347.
7. Muszbek, L., and Laposata, M. (1993) Covalent Modification of Proteins by Arachidonate and Eicosapentaenoate in Platelets, *J. Biol. Chem. 268*, 18243–18248.
8. Masterson, W.J., Raper, J., Doering, T.L., Hart, G.W., and Englund, P.T. (1990) Fatty Acid Remodeling: A Novel Reaction Sequence in the Biosynthesis of Trypanosome Glycosyl Phosphatidylinositol Membrane Anchors, *Cell 62*, 73–80.
9. Kaufman, J.F., Krangel, M.S., and Strominger, I.L. (1984) Cysteines in the Transmembrane Region of Major Histocompatibility Complex Antigens are Fatty Acylated *via* Thioester Bonds, *J. Biol. Chem. 259*, 7230–7238.
10. Hennes, A.R., Awai, K., Hammerstrand, K., and Duboff, G.S. (1966) Carbon-14 in Carboxyl Carbon of Fatty Acids Formed by Platelets from Normal and Diabetic Subjects, *Nature 210*, 839–841
11. Wilcox, C.A., Hu, J.S., and Olson, N.E. (1987) Acylation of Proteins by Myristic Acid Occurs Cotranslationally, *Science 238*, 1275–1278
12. Buss, J.E., Solski, P.A., Schaeffer, J.P., MacDonald, M.J., and Der, C.J. (1989) Activation of the Cellular Proto-Oncogene Product p21 Ras by Addition of a Myristylation Signal, *Science 243*, 1600–1603.
13. Wedegaertner, P.B., Chu, D.H., Wilson, P.T., Levis, M.J., and Bourne, H.R. (1993) Palmitoylation is Required for Signaling Functions and Membrane Attachment of Gqα and Gsα, *J. Biol. Chem. 268*, 25001–25008.
14. O'Dowd, B.F., Hnatowich, M., Caron, M.G., Lefkowitz, R.J., and Bouvier, M. (1989) Palmitoylation of the Human $ß_2$-Adrenergic Receptor, *J. Biol. Chem. 264*, 7564–7569.
15. Zhang, F., Kamp, F., and Hamilton, J.A. (1996) Dissociation of Long and Very Long Chain Fatty Acids from Phospholipid Bilayers, *Biochemistry 35*, 16055–16060.

16. Fujimoto, T., Stroud, E., Whatley, R.E., Prescott, S.M., Muszbek, L., Laposata, M., and McEver, R.P. (1993) P-Selectin is Acylated with Palmitic Acid and Stearic Acid at Cysteine 766 Through a Thioester Linkage, *J. Biol. Chem. 268*, 11394–11400.
17. Zeng, F.Y., Kaphalia, B.S., Ansari, G.A.S., and Weigel, P.H. (1995) Fatty Acylation of the Rat Asialoglycoprotein Receptor, *J. Biol. Chem. 270*, 21382–21387.
18. Berthiaume, L., and Resh, M.D. (1995) Biochemical Characterization of a Palmitoyl Acyltransferase Activity that Palmitoylates Myristoylated Proteins, *J. Biol. Chem. 270*, 22399–22405.