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Acylation of Cellular Proteins with Endogenously Synthesized Fatty Acids†

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ABSTRACT: A number of cellular proteins contain covalently bound fatty acids. Previous studies have identified myristic acid and palmitic acid covalently linked to protein, the former usually attached to proteins by an amide linkage and the latter by ester or thio ester linkages. While in a few instances specific proteins have been isolated from cells and their fatty acid composition has been determined, the most frequent approach to the identification of protein-linked fatty acids is to biosynthetically label proteins with fatty acids added to intact cells. This procedure introduces possible bias in that only a selected fraction of proteins may be labeled, and it is not known whether the radioactive fatty acid linked to the protein is identical with that which is attached to the protein when the fatty acid is derived from endogenous sources. We have examined the distribution of protein-bound fatty acid following labeling with [^{3}H]acetate, a general precursor of all fatty acids, using BC_3H1 cells (a mouse muscle cell line) and A431 cells (a human epidermoid carcinoma). Myristate, palmitate, and stearate account for essentially all of the fatty acids linked to protein following labeling with [^{3}H]acetate, but at least 30% of the protein-bound palmitate in these cells was present in amide linkage. In BC_3H1 cells, exogenous palmitate becomes covalently bound to protein such that less than 10% of the fatty acid is present in amide linkage. These data are compatible with multiple protein acylating activities specific for acceptor protein fatty acid chain length and linkage. The enzyme(s) that link(s) palmitate to protein by amide linkage preferentially use(s) fatty acid generated by fatty acid synthetase in BC_3H1 cells, but apparently has (have) equal access to exogenously and endogenously derived palmitate in A431 cells.

Covalent modification of proteins with fatty acid has been described for a number of specific cellular and viral proteins, including the vesicular stomatitis virus G protein (Schmidt & Schlesinger, 1979), calcineurin B (Aitken et al., 1982), the transferrin receptor (Omary & Trowbridge, 1981), pp60 src (Sefton et al., 1982), the catalytic subunit of cAMP-dependent protein kinase (Carr et al., 1982), and cytochrome b_5 reductase (Ozols et al., 1984). The linkage of fatty acid to acyl proteins

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can be divided into two broad categories—ester or amide—on the basis of the sensitivity of the lipid–protein linkage to hydroxylamine (Magee et al., 1984; Olson et al., 1985). Acylation of a particular protein appears to be specific for linkage and chain length, but different cell lines exhibit different degrees of *overall* linkage specificity with respect to acyl chain. For instance, acyl proteins with fatty acid bound covalently to their amino-terminal glycine residues appear to be highly specific for myristate, an obligatory amide linkages, yet the fraction of protein myristate as amide in different cell types varies from 65% to greater than 90% (Olson et al., 1985). The function of fatty acylation is unknown, but some observations suggest that it is important for acyl protein function at membranes (Buss et al., 1985; Pellman et al., 1985).

The majority of acyl proteins have been detected by labeling cells in culture with radioactive fatty acids. The relative distribution of fatty acids on acyl proteins with respect to chain length and linkage cannot be determined by this technique, since the relative size of the fatty acyl donor pools are unknown. Moreover, exogenous and endogenous fatty acids need not have equal access to these pools. In the present study, we examine the relative amounts of the various cellular acyl proteins in BC_3H1 and A431 cultured cell lines by labeling cultures with [^{3}H]acetate. We document the relative contribution of the various fatty acids to acyl protein with respect to chain length and linkage.

The value of this approach is indicated by the observation that only a small fraction of the protein-bound palmitate is in the form of amide when palmitate is used to label the protein-bound fatty acids in BC_3H1 cells but a very large fraction of the protein-bound palmitate is present in amide linkage when acetate is used as a label.

Materials and Methods

Cell Culture and Labeling Conditions. BC_3H1 muscle cell line (Schubert et al., 1974) and the A431 human epidermal carcinoma cells (Stoscheck & Carpenter, 1983) were grown as described previously (Olson et al., 1983; Rothenberg et al., 1983). The cells were labeled with ^{3}H-labeled fatty acids (Olson et al., 1985) for 4 h in the presence of delipidated serum (Chan & Knowles, 1976) as described previously.

To label cells with [^{3}H]acetate, the cells were first incubated overnight in medium containing delipidated serum in order to induce fatty acid synthetase (Bloch & Vance, 1977) and thereby maximize the conversion of acetate to fatty acids. BC_3H1 cells were plated on collagen-coated 150 mm diameter Falcon petri dishes. When the cells were almost confluent, the medium was changed to 20 mL of DME[1]–20% delipidated fetal calf serum supplemented with L-glutamine (0.1 mg/mL), 5 mM pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin. Following the overnight incubation, the medium was replaced with 15 mL of identical medium containing 1 mCi of [^{3}H]acetate (4.7 Ci/mmol). After 4 h the cells were rinsed 3 times with cold DME–1% fetal calf serum and removed by scraping with 50 mL of calcium- and magnesium-free Hank's solution containing 15 mM iodoacetamide. The cells were collected by centrifugation and extracted with 500 μL of NaCl/P_i containing 0.5% Triton X-100, 0.05% Na$DodSO_4$, 0.5% bovine serum albumin, 1 mM phenylmethanesulfonyl fluoride, 10 μg/mL soybean trypsin inhibitor, 10 μg/mL aprotinin, and 0.5 mM iodoacetamide, at 4 °C for 10 min. Particulate material, including nuclei, was removed by centrifugation at 8000*g* for 5 min. The protocol for labeling A431 cells was identical except that collagen coating of the dish was not required to prevent loss of cells from the dish during medium changes.

Analysis of Protein-Bound Fatty Acids. Delipidated cell protein was prepared by slight modification of the protocol previously described (Olson et al., 1985). The cell proteins were delipidated by exhaustive extraction of the 0.5-mL cell extract with 6 times 15 mL of chloroform–methanol (2:1 v/v). The protein pellet was resuspended with sonication in 0.5 mL of 0.1% Na$DodSO_4$ in NaCl/P_i on the third and fifth chloroform–methanol extractions. Further treatment of this protein with chloroform–methanol–concentrated HCl (67:33:1 v/v/v) only extracted small quantities of additional radioactivity averaging 6% of the label in different preparations labeled either with acetate or with fatty acids. The delipidated protein was then suspended in 200 μL of distilled H_2O. Twenty microliters of the extract was diluted with 80 μL of distilled H_2O and 100 μL of 2 M hydroxylamine either at pH 7.0 or at pH 10.0. After incubation for 4 h at 23 °C, the reaction was extracted 3 times with an equal volume of chloroform–methanol (2:1 v/v). The combined organic phase containing fatty acids released from protein with hydroxylamine (esters and thio esters) was evaporated under a stream of nitrogen, and the fatty acid composition was determined by reverse-phase HPLC after transmethylation to form the fatty acid methyl esters (Olson et al., 1985). The remaining aqueous phase was treated with 17% concentrated HCl in methanol (v/v yielding a final concentration of HCl of 2 N) for 24 h at 95 °C in sealed tubes under nitrogen to release amide-linked fatty acids from protein as the corresponding methyl esters. These were extracted into petroleum ether, and the chain length distribution was determined as described (Olson et al., 1985). Standards of myristate, palmitate, and stearate methyl esters were included with each HPLC analysis. The protein from one 150 mm diameter dish labeled with 1 mCi of [^{3}H]acetate contained on the average 160 000 dpm of fatty acid covalently linked to A431 cellular protein and 100 000 dpm to BC_3H1 cellular protein.

The identification of the fatty acids was carried out on a reverse-phase μBondapak C_{18} column (Waters). Samples were redissolved in methanol, and the column was eluted with 80% acetonitrile at the rate of 1 mL/min for the first 25 min and 2 mL/min for the remainder of the elution. The elution of standards was monitored at 214 nm.

Isolation of Fatty Acyl Amino Acids and Peptides. Delipidated protein from one 150 mm diameter dish labeled with 1 mCi of the appropriate fatty acid for 4 h was suspended in 500 μL of 20 mM glycine, pH 10.2, and dispersed by treatment for 1 h at 30 °C in a bath sonicator. The solution was acidified to pH 2.0 with 20 μL of 1 N HCl and extracted 3 times with an equal volume of petroleum ether. After the final extraction, solvent was removed under a stream of nitrogen, and the aqueous solution was adjusted to pH 10.2 with 130 μL of 200 mM glycine, pH 10.2. The sample was digested with alkaline Pronase for 48 h (1 mg of enzyme was added each 24 h). Three-hundred twenty-five microliters of 500 mM KP_i, pH 7.2, was then added to bring the pH to 7.2, and digestion was continued with Pronase E for an additional 48 h (1 mg of enzyme added each 24 h). One drop of toluene was added during Pronase digestion to retard bacterial growth.

The digested sample was extracted 3 times with 1 volume of chloroform–methanol (2:1 v/v), and the combined organic

[1] Abbreviations: DME, Dulbecco's minimal essential medium; NaCl/P_i, phosphate-buffered saline (10 mM phosphate), pH 7.2; Na$DodSO_4$, sodium dodecyl sulfate; HPLC, high-pressure liquid chromatography.

Table I: Fatty Acid Composition of Acyl Proteins from [^{3}H]Acetate-Labeled Cells[a]

	protein-bound fatty acid (fraction of total)							
	myristate		palmitate		stearate		total	
cell line	ester	amide	ester	amide	ester	amide	ester	amide
BC_3H1	0.01	0.11	0.54	0.22	0.08	0.04	0.63	0.37
A431	0.03	0.14	0.47	0.27	0.06	0.03	0.56	0.44

[a] Cells were labeled for 4 h with [^{3}H]acetate and the fatty acids linked to cellular proteins determined as described under Materials and Methods. For each fatty acid, the fraction released by hydroxylamine at pH 7.0 is designated as ester linked and the remainder as amide linked. Hydroxylamine at pH 10 did not release additional fatty acid from the protein. The mean (±) SD) fraction of palmitate in amide linkage in BC_3H1 cellular protein was 0.29 (±0.06), $n = 4$. The results are means of two or more determinations, which did not vary by more than 10% of the reported value.

Table II: Fatty Acid Composition of Acyl Protein from Fatty Acid Labeled Cells[a]

	myristate-labeled cells						palmitate-labeled cells					
	myristate		palmitate		stearate		myristate		palmitate		stearate	
cell line	ester	amide	ester	amide	ester	amide	ester	amide	ester	amide	ester	amide
BC_3H1	0.09	0.78	0.13					0.01	0.76	0.07	0.15	0.01
A431	0.15	0.33	0.34	0.17					0.55	0.25	0.10	0.07

[a] Cells were labeled as described under Materials and Methods and the composition and linkage of the fatty acid to protein determined. The fraction of the radioactivity on the protein not present as fatty acid has been characterized as amino acids derived from the oxidation of the fatty acid. The percentage of total radioactivity covalently associated with protein present as fatty acid was 70 and 89% for myristate- and palmitate-labeled BC_3H1 cells, respectively, and 82 and 86% for myristate- and palmitate-labeled A431 cells, respectively. The data are expressed as the fraction of total fatty acid label at the chain length indicated. Data for BC_3H1 cells labeled with myristate were taken from Olson et al. (1985). The mean (± SD) fraction of palmitate in BC_3H1 cellular protein in amide linkage was 0.09 (±0.04), $n = 7$.

phases were back-washed with 0.7 volume of chloroform–methanol–500 mM KP_i, pH 7.2, 1/10/10 (v/v/v).

The aqueous phase was adjusted to pH 2.0 with 420 μL of 1 N HCl and extracted as above, except that the back-wash solution was chloroform–methanol–0.1 N HCl (1/10/10 v/v/v).

In each case the organic phase was evaporated under nitrogen and redissolved in UV-grade methanol, and the radioactive components were analyzed by HPLC using a 3.9-mm × 30-cm Waters μBondapak C_{18} column eluted with a 1%/min linear gradient from 0 to 100% acetonitrile with an aqueous phase of 0.05% trifluoroacetic acid adjusted to pH 7.5 with triethylamine. One-milliliter fractions were collected. Myristoyl amino acids were synthesized by reacting myristoyl chloride with radioactive amino acid as follows. Five microcuries of ^{35}S- or ^{14}C-labeled amino acid was taken to dryness under vacuum. To this was added 70 μL of anhydrous diethyl ether with 2.5 μL of myristoyl chloride. After 2 h at 23 °C, the solvent was evaporated with nitrogen, and the residue was suspended in 20% methanol in H_2O and analyzed by reverse-phase HPLC as above. Residual amino acid elutes with the column front, and radioactivity released on the column represents myristoyl amino acids. In the case of myristoyl lysine, the amino group to which myristate is linked is not known.

Materials. Radioactivity was determined in 3a70 (Research Products International Inc.). Proteolytic enzymes, fatty acid methyl ester standards, myristoyl chloride, and hydroxylamine were obtained from Sigma Chemical Co. Radioactive fatty acids and amino acids and [^{3}H]acetate were purchased from New England Nuclear. All other chemicals were reagent-grade. UV-grade methanol and chloroform were obtained from American Scientific Products.

Electrophoresis. Electrophoresis of 10% $NaDodSO_4$–polyacrylamide gels was performed as described previously (Olson et al., 1984). After staining and destaining gels were prepared with EN^{3}HANCE (New England Nuclear) prior to fluorography. Hydroxylamine treatment of protein following electrophoresis was performed as previously described (Olson et al., 1984), except that treatment was for 6 h instead of 18 h. This shortened treatment period gave comparable release of esterified fatty acid but with less diffusion of protein from the gel.

Results

To investigate the relative amounts and composition of cellular fatty acyl proteins, we labeled cultures of BC_3H1 cells and A431 cells with [^{3}H]acetate. In order to maximize the incorporation of acetate with fatty acids, the cells were first incubated overnight in delipidated serum, a condition that in a number of other cells has been shown to increase the rate of endogenous fatty acid synthesis (Bloch & Vance, 1977). After 4-h incubation with [^{3}H]acetate, the cells were harvested, and delipidated protein was prepared by extraction with chloroform–methanol. The delipidated protein was then analyzed for hydroxylamine-labile (ester or thio ester) and hydroxylamine-stable, acid-labile (amide) fatty acid by treatment with either neutral or alkaline hydroxylamine. In each instance, the released fatty acid hydroxamates were converted to methyl esters, and the composition of the fatty acids was determined by reverse-phase HPLC as described under Materials and Methods. The radioactivity in each fatty acid fraction was normalized to the number of acetate equivalents for calculation of relative molar contribution to total protein-bound fatty acid.

Table I summarizes the results obtained with BC_3H1 cells and with A431 cells labeled with [^{3}H]acetate. In both cell lines, palmitate accounted for approximately 75% of the protein-bound fatty acid, while myristate and stearate contributed about equally to the remaining 25%. Fatty acids of other chain lengths were not detected. In each case, fatty acid amide represents about 40% of total protein-bound fatty acids, but surprisingly, for each cell type myristate only accounts for approximately $^1/_3$ to $^1/_4$ of the total amide-linked fatty acids. In previous experiments (Olson et al., 1985), we had labeled BC_3H1 cells with either myristic acid or palmitic acid and could detect no significant level of exogenous palmitic acid linked by amide linkage to protein. A431 cells had not been examined previously.

In Table II we present the distribution of fatty acids linked to protein in BC_3H1 cells and A431 cells labeled with myristate or palmitate for 4 h. Since BC_3H1 cells had not been plated

on collagen or preincubated overnight in delipidated serum, the previous experiments were repeated under conditions where the labeling with [^{3}H]acetate and ^{3}H-labeled fatty acids and subsequent treatments are strictly comparable, and no significant difference existed between results from two procedures for fraction of total protein as ester and amide. There was a significant increase, however, in the amount of stearate seen linked to protein from palmitate labeling, presumably due to a greater rate of elongation of exogenous fatty acid in these lipid-starved cells.

The most striking difference between the results in Tables I and II is the presence of a large fraction of palmitate amide in the acetate-labeled cells, which can also be seen in A431 cells when labeled with palmitate, but not in BC_3H1 cells. The fraction of protein-bound palmitate radioactivity present in amide linkage in BC_3H1 cells from acetate labeling was significantly different from that obtained with palmitate labeling ($P < 0.005$, unpaired t test). A431 cells are unique in this regard among the cell lines examined to date. We have previously reported that when either BC_3H1 cells, 3T3 cells, or PC12 pheochromocytoma cells are labeled with palmitate, all of the protein-bound fatty acid is present as palmitate linked to protein by ester or thio ester linkage (Olson et al., 1985). Possible implications of these observations for the biosynthesis of fatty acylated proteins will be presented under Discussion. Note that significant fatty acid elongation has taken place in A431 cells, a phenomenon that occurs to a much smaller degree in BC_3H1 cells, even when lipid starved prior to labeling.

In a few specific proteins examined to date, myristate has been detected linked to N-terminal glycine (Aitken et al., 1982; Carr et al., 1982; Ozols et al., 1984; Henderson et al., 1983; Schultz et al., 1985). To examine the generality of this observation, we labeled BC_3H1 cells with [^{3}H]myristic acid and then exhaustively digested delipidated protein with Pronase as described under Materials and Methods. The Pronase digest was first extracted with chloroform–methanol at pH 7.0 and then at pH 2.0. With this protocol, 85% of the protein-bound fatty acid that was myristate amide was extracted into organic solvent. The organic phases were analyzed by reverse-phase HPLC as illustrated in Figure 1. The material in the organic phase extracted at pH 7.0 coincided in elution position with myristoylglycine and differed from a variety of other myristoylated amino acids (Figure 1A). The material extracted at pH 2.0 also contained myristoylglycine, but the major component eluted from the reverse-phase HPLC column did not correspond in elution position to any of the myristoyl amino acids (Figure 2B). In all cases, acid methanolysis followed by reverse-phase HPLC showed that all of the label was present as myristic acid.

We considered the possibility that the material eluting at 65 min in Figure 1B represented myristate acid linked to a peptide(s). We therefore isolated this material by reverse-phase HPLC, removed the solvent with a stream of nitrogen, and redissolved the sample in UV-grade methanol. Aliquots of the material were subjected to partial acid hydrolysis in 0.0125 N HCl at 95 °C under N_2. At appropriate intervals, samples were extracted with chloroform–methanol and analyzed by reverse-phase HPLC. The results are shown in Figure 2. Note that the position of the peaks is slightly different than that in Figure 1, because a different HPLC column had to be used in these experiments. Partial acid hydrolysis converts a significant fraction of the fast-eluting material to material that coelutes with myristoylglycine. Very little free myristic acid is formed under these conditions for short hydrolysis times.

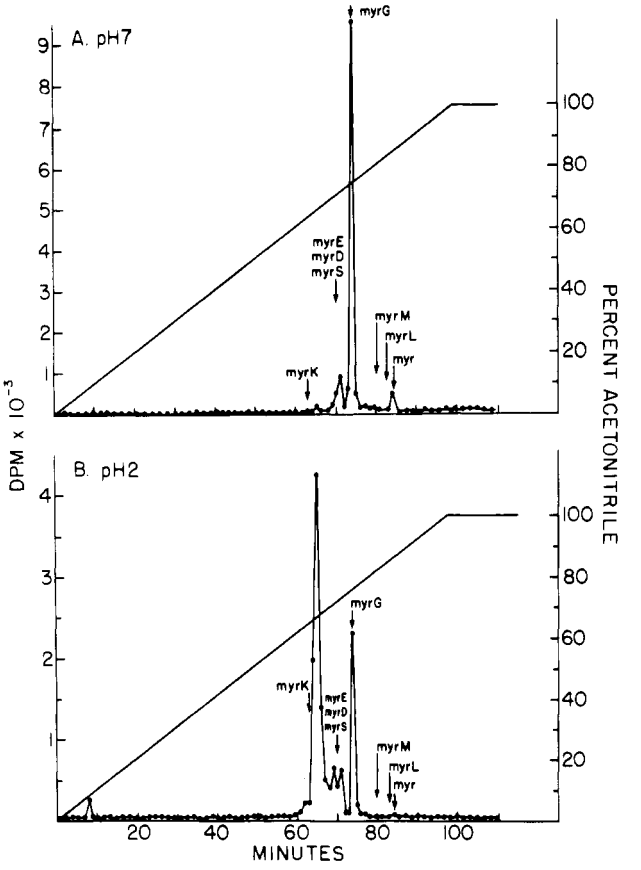

FIGURE 1: Analysis of Pronase digest of [^{3}H]myristate-labeled proteins from BC_3H1 cells. Myristate-labeled proteins were digested with Pronase as described under Materials and Methods, and the digest was extracted with chloroform–methanol, first at pH 7.0 and then at pH 2.0. The radioactive components in the organic phase were separated by reverse-phase HPLC. (Panel A) Radioactivity extracted at pH 7.0; (panel B) radioactivity extracted at pH 2.0. After Pronase digestion, 85% of the protein-bound myristic acid was extracted into solvent. The elution position of myristoyl amino acid standards prepared as described under Materials and Methods is indicated by the arrows. Myristate and myristoyl are abbreviated as myr, while the standard single-letter abbreviations are used for the various amino acids.

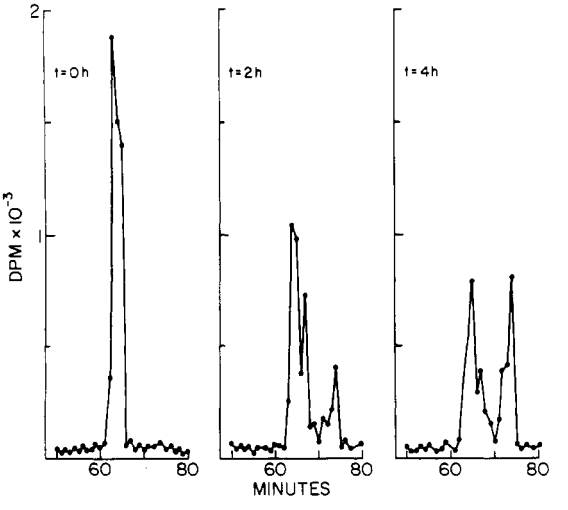

FIGURE 2: Partial acid hydrolysis of myristoyl peptide. The myristate-labeled material eluting at 65 min following reverse-phase HPLC of a Pronase digest, as in panel B in Figure 1, was treated with 0.0125 M HCl under N_2 at 95 °C for the times indicated and then analyzed as in Figure 1. The data were obtained on a different C_{18} μBondapak column than that used in Figure 1, which accounts for the slightly different elution position of radioactivity in this figure. All of the radioactivity in the sample could be accounted for as myristic acid methyl ester after acid methanolysis.

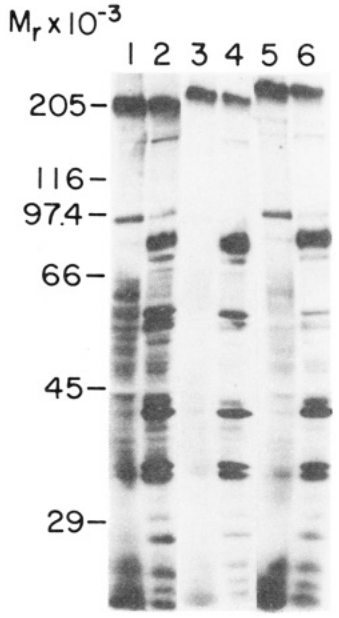

FIGURE 3: Sensitivity of acylated proteins to hydroxylamine. A431 cells were labeled with [^{3}H]myristate or [^{3}H]palmitate, and cell extracts were prepared as described in Olson et al. (1985). After separation of labeled proteins by electrophoresis on 10% polyacrylamide gels, gels were fixed, washed with water, and then treated for 6 h at room temperature either with freshly prepared 1 M hydroxylamine, pH 7.0, or with 1 M Tris, pH 7.0. Gels were then prepared for fluorography. (Lanes 1, 3, and 5) [^{3}H]Palmitate-labeled proteins; (lanes 2, 4, and 6) [^{3}H]myristate-labeled proteins. (Lanes 1 and 2) No treatment; (lanes 3 and 4) hydroxylamine-treated gels; (lanes 5 and 6) Tris-treated gels. Note that a significant fraction of the palmitate-labeled protein is stable to hydroxylamine treatment.

These results are consistent with the notion that the fast-eluting material represents myristic acid linked to a small oligopeptide. Partial acid hydrolysis converts this material to myristoylglycine as well as intermediate forms, which possibly also contain myristoylglycine. Of the total amide-linked myristic acid, 70–80% can be accounted for as myristoylglycine.

A similar analysis was attempted with palmitate-labeled A431 cells. However, none of the solvent-extractable radioactivity following Pronase digestion showed the chromatographic behavior of a fatty acyl amino acid. The solvent-extractable material appeared to be a very complex mixture of fatty acylated hydrophobic peptides that have not been characterized.

The pattern of cellular acyl protein from A431 cells labeled with radioactive fatty acid was examined by $NaDodSO_4$–polyacrylamide gel electrophoresis followed by fluorography. The data in Table II indicate that this is a valid technique in A431 cells since for the labeling periods used greater than 80% of the protein-associated radioactivity is as fatty acid. Lanes 1 and 2 in Figure 3 show the pattern of acylation from [^{3}H]palmitate- and [^{3}H]myristate-labeled A431 cells, respectively. Note that although the overall pattern of acylation differs with the two labels, a number of proteins of the same apparent molecular weight label both with palmitate and with myristate. This is not unexpected, since the data in Table II indicate that 51% of the protein-bound fatty acid from myristate-labeled A431 cells is as palmitate, presumably the result of microsomal elongation of exogenous label and subsequent attachment to protein. This pattern of acyl protein and comigration of palmitate- and myristate-labeled protein is not seen in BC_3H1 cells (Olson et al., 1985), which only yield 13% of protein-bound fatty acid as palmitate following labeling with myristic acid. To examine which of these acyl proteins bound fatty acid in ester linkage, gels were treated either with 1 M hydroxylamine, pH 7.0, or as a control with 1 M Tris, pH 7.0. As can be seen in Figure 2, myristate-labeled proteins appear to be largely hydroxylamine stable, while a large portion of the palmitate proteins are hydroxylamine-labile (ester or thio ester). As predicted from Table II, however, some of the protein-bound fatty acid myristate labeling was hydroxylamine-labile, and some from palmitate labeling was hydroxylamine-stable, a significant portion of which is present in a protein of apparent M_r of 200 000. The fatty acid linked to this protein must be either palmitate or stearate, since no significant metabolic conversion of palmitate to myristate occurs in these cells (Table II). The relative contribution of this acyl protein to total amide-linked acyl protein is impossible to quantitate by this technique, since other acyl proteins present in small amounts would not be detected by this technique. Hydroxylamine treatment of palmitate-labeled BC_3H1 protein separated by electrophoresis releases essentially all of the protein-bound radioactivity (Olson et al., 1985) as would be predicted from the data in Table II.

Discussion

Acylation of a number of specific cellular proteins has been described, encompassing both ester (Omary & Trowbridge, 1981; Magee et al., 1984; Kaufman et al., 1984; Slomiany et al., 1984a) and amide (Aitken et al., 1982; Carr et al., 1982; Ozols et al., 1984; Henderson et al., 1983; Schultz et al., 1985) linkages, but to date there has been no investigation of the relative distribution of acyl proteins with respect to acyl chain and linkage. To do this, we have labeled cells with [^{3}H]acetic acid and have determined relative amounts and acyl chain specificity for ester and amide linkages. Mysitoylglycine has been described to be the amide linkage present in the bovine cardiac muscle catalytic subunit of cAMP-dependent protein kinase (Carr et al., 1982), calcineurin B (Aitken et al., 1982), NADH-cytochrome b_5 reductase (Ozols et al., 1984), and pp60src (Schultz et al., 1985) and p15 Rauscher and Moloney murine leukemia viruses (Henderson et al., 1983). In BC_3H1 cells, we have shown that the majority of the myristoylated proteins have myristate attached to glycine. Thus, the acyl transferase activity responsible for attaching myristate to proteins in amide linkage appears to be specific for N-terminal glycine.

We describe here a new linkage of palmitate to cellular protein—that of palmitate amide. In A431 cells, palmitate amide can be detected chemically by labeling cells with either radioactive palmitate or acetate, and [^{3}H]palmitate-labeled cellular proteins with this palmitate amide linkage can be discerned on $NaDodSO_4$–polyacrylamide gels after treatment with hydroxylamine. In BC_3H1 cells, however, this linkage can only be detected chemically by labeling with acetate. This implies that, in these cells, endogenously synthesized palmitate is the donor for attaching palmitate to protein in amide linkage. Moreover, in animal cells, newly synthesized palmitate is released from the fatty acid synthesis by hydrolysis, i.e., as the free fatty acid (Bloch & Vance, 1977). Thus, either the palmitate amide acyl transferase receives its fatty acyl donor via a specific interaction (direct or indirect) with the fatty acid synthetase complex, or a compartment for palmitate amide acylation exists that kinetically has preferential access to biosynthetic palmitate. In A431 cells, this restriction cannot be detected, perhaps because a different step in the biosynthesis of acyl protein becomes rate limiting, perhaps due to the existence of a different palmitate acyltransferase or the presence of a specific acyl donor transporter.

Olson et al. (1984) have shown that the α and β subunits of the acetylcholine receptor in BC_3H1 cells contain covalently

bound fatty acid from palmitate labeling, visible on NaDodSO$_4$–polyacrylamide gels only after immunoprecipitation and prolonged fluorographic development and that this fatty acid is bound in an amide linkage. The low levels of palmitate radioactivity incorporated into such a relatively abundant acyl protein in these cells could be explained by the preference of endogenously synthesized palmitate for palmitate amide linkage to proteins, as discussed above. It should be noted that the chain length of the fatty acid linked to the acetylcholine receptor was not determined. This channeling of acetate into palmitate amides would specifically decrease the specific activity of palmitate in such linkages with respect to palmitate esters. Cellular acyl proteins containing palmitate ester linkages label well with exogenous radioactive fatty acid, and several groups have shown that acyl-CoA can act as the acyl donor to acyl proteins with ester linkages (Berger & Schmidt, 1984; Slomiany et al., 1984b; Adam et al., 1984; Ross & Brown, 1985). The acyl donor for linakge of fatty acids to proteins by amide bonds is as yet unknown.

Olson et al. (1984) also determined that cerulenin, a known inhibitor of fatty acid biosynthesis (Omura, 1976), inhibits surface acetylcholine receptor expression in BC$_3$H1 cells and propose that acylation is necessary for subunit assembly, a process known to be the regulated step for surface expression and required for subunit stability (Merlie et al., 1983; Merlie, 1984). While the suggestion was made that cerulenin may inhibit the fatty acid acylation of the acetylcholine receptor, the data presented in this paper make it equally likely that cerulenin may be inhibiting acylation of the receptor by inhibiting fatty acid synthesis, thus preventing the formation of the physiological donor of the fatty acid for the receptor. Note, however, that cerulenin has been shown to block acylation of Sindbis and vesicular stomatitis viral glycoproteins (Schlesinger & Malfer, 1982) and the in vitro acylation of myelin proteolipid protein (Ross & Braun, 1985), acyl proteins that have fatty acid bound in thio ester or ester linkages (Magee et al., 1984; Agrawal et al., 1982).

The residue to which palmitate is bound in amide linkage in protein is yet unknown. For the acetylcholine receptor, Olson et al. (1984) have pointed out the likelihood that the fatty acid is attached to the ϵ-amino group of a lysine residue, since the amino-terminal group of the receptor is known not to be blocked (Conti-Tronconi et al., 1982).

It is interesting to note that saturated acyl chains of 14–18 carbons in length accounted for all of the detected endogenously synthesized fatty acid covalently bound to cellular protein. Although previous studies using radioactive fatty acids to label cellular proteins have implied this result (Olson et al., 1985), this need not have been the case, since (a) labeling with a specific fatty acid biases detection of protein acylation toward those acyl proteins containing either that particular fatty acid or a metabolite thereof and (b) Berger & Schmidt (1984) have shown that little if any acyl chain specificity exists within their microsomal acyl transferase system for acylation of Semliki Forest virus E1 protein in a hydroxylamine-sensitive linkage (ester or thio ester). In this light, it is remarkable that myristate comprises only 1.6 and 5.4% of fatty acid esterified to protein while it represents 12 and 17% of the total protein-bound fatty acid in BC$_3$H1 and A431 cells, respectively. Moreover, in both cell types, myristate accounted for 30% of the fatty acid bound to protein in amide linkage. Clearly, the enzymes involved in transferring fatty acids to protein in ester and amide linkages exhibit marked differences in specificities for acyl donor chain length.

The complexity of the fatty acid acylation pattern of cellular proteins suggests that a number of distinct protein fatty acyl transferases may exist in cells. These activities exhibit specificity toward their acyl protein substrates, since only specific proteins become acylated, and toward acyl chain length and linkage as demonstrated by hydroxylamine-sensitivity data and analysis of acyl amino acids generated by proteolytic digestion of acyl proteins.

Fatty acid:protein acyl transferases may recognize specific amino acid sequences in proteins, but any given acylating enzyme may use as a substrate a variety of proteins containing that sequence. An examination of the sequence of four proteins known to contain myristic acid linked to N-terminal glycine residues reveals no obvious homology other than the N-terminal glycine residue. The precise signals involved in protein acylation await identification and characterization of these acyl transferases in cell-free systems with defined acyl acceptors as substrates.

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Registry No. Palmitic acid, 57-10-3; myristic acid, 544-63-8; stearic acid, 57-11-4; glycine, 56-40-6.

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Stability and Substructure of Cardiac Myosin Subfragment 1 and Isolation and Properties of Its Heavy-Chain Subunit†

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ABSTRACT: The substructure and the thermal stability of the subunit interactions of bovine cardiac myosin subfragment 1 (SF1) have been examined. The results are in agreement with previous reports that the cardiac protein is cleaved in a very similar manner [Flink, I. L., & Morkin, E. (1982) *Biophys. J. 37*, 34; Korner, M., Thiem, N. V., Cardinaud, R., & Lacombe, G. (1983) *Biochemistry 22*, 5843-5847] but at a much faster rate [Applegate, D., Azarcon, A., & Reisler, E. (1984) *Biochemistry 23*, 6626-6630] than the skeletal protein. Additionally, it is found that the long-lived, steady-state intermediates formed by these proteins with MgATP at high ionic strength differ in their susceptibilities to tryptic attack especially at the 27K/50K junction of the associated heavy chains, suggesting a different conformation for these intermediates of the cardiac and skeletal SF1's. The thermal stability of the subunit interactions under conditions approaching the physiological state was examined by thermal ion-exchange chromatography of cardiac SF1 at 39.5 °C in the presence of MgATP. This results in the separation of part of the protein as the isolated heavy chain which is found to exhibit high levels of ATPase activity in the absence and presence of actin. Tryptic digestion of cardiac SF1 prior to thermal ion-exchange chromatography produces greater dissociation, with the heavy chain in this case being isolated as a complex of 27K, 50K, and 18-20K fragments. This tryptic heavy chain exhibits similar levels of ATPase activities as its parent tryptic SF1, showing the same V_{max} for the actin-activated MgATPase as the undigested cardiac SF1 and heavy chain but a larger K_m for actin.

It is now well established that the contractile properties of cardiac and skeletal muscles differ in accordance with the physiological requirements of these two types of muscles. The slower speed of contraction of cardiac muscle is apparently related to the lower rate of actin-activated MgATPase of its constituent myosin compared to that for the skeletal tissue (Taylor & Weeds, 1976). At the molecular level, it has been shown that despite the similarities in size and subunit structure of these two types of myosin, the chemical structures (primary structure) of the heavy and light polypeptide chains of cardiac myosin differ from their counterparts in skeletal myosin (Hoh et al., 1979; Flink et al., 1979; Chizzonite et al., 1982; Weeds, 1975; Leger & Elzinga, 1977). Since the higher orders of structure necessary for the expression of function are directly related to the primary structure, it is not unreasonable to suggest that the kinetic differences are directly attributable to differences in the structures of these two myosins. The ways in which the conformation of the cardiac protein differs from that of the skeletal protein have not as yet been fully characterized, but it is likely that if these differences do exist, they would be found in the myosin subfragment 1 (SF1)[1] regions where the sites for ATPase and actin binding are located.

The SF1 isolated by chymotryptic digestion of skeletal myosin is comprised of a heavy chain of 95K and a light chain

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[1] Abbreviations: DEAE, diethylaminoethyl; SF1, SF1(A1), and SF1(A2), myosin subfragment 1 and the A1- and A2-containing isoenzymes, respectively; A1 and A2, two alkali light chains; T1 and T2, two regions located at about 75 and 27 kilodaltons, respectively, from the amino terminus of the heavy chain and which are vulnerable to tryptic attack; HC, heavy chain of SF1; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

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