Identification and Quantitation of the Fatty Acids Composing the CoA Ester Pool of Bovine Retina, Heart, and Liver*

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Several proteins found in retinal photoreceptor cells (guanylate cyclase activating protein, protein kinase A, recoverin, and transducin) are N-terminally modified with the fatty acids 12:0, 14:0, 14:1-n-9, and 14:2-n-6, whereas similar proteins in other tissues contain only 14:0. It has been hypothesized that the acyl-CoA pool of the retina contains amounts of 12:0, 14:1-n-9, and 14:2-n-6 elevated over 14:0, in comparison to other tissues, and this accounts for the specificity of N-terminal fatty acylation. To test this hypothesis, we performed fatty acid analysis on total acyl-CoAs purified from bovine retina (light-adapted), heart, and liver. We also examined the N- and S-linked fatty acid composition of the total protein pools from these tissues. Acyl-CoAs were prepared from heart, liver, and retina and separated by high performance liquid chromatography (HPLC). Identities of peaks were based on HPLC of standard 12:0, 14:0, 14:1-n-9, and 14:2-n-6 CoAs. Total protein was subjected to base hydrolysis followed by acidic methanolysis to release S- and N-linked fatty acids, respectively, and fatty acid phenacyl esters were prepared for HPLC analysis. Retina had levels of 12:0 (2.7 ± 2.1%), 14:1-n-9 (2.9 ± 2.2%), and 14:2-n-6 (1.6 ± 0.7%) CoAs below that of 14:0 CoA (7.0 ± 1.8%). Likewise, heart levels of 14:2-n-6 CoA (3.7 ± 0.1%) were near and 12:0 (2.6 ± 0.6%) and 14:1-n-9 (0.7 ± 0.3%) CoAs were below that of 14:0 CoA (3.8 ± 1.0%). Liver had levels of 12:0 (16.1 ± 5.7%) and 14:2-n-6 (8.1 ± 1.2%) CoAs above and 14:1-n-9 CoA (1.2 ± 0.6%) below that of 14:0 CoA (5.9 ± 0.8%). Fatty acid analysis of total protein showed that all tissues contained S-linked 16:0, 18:0, and 18:1-n-9. Retina proteins contained N-linked 14:0, 14:1-n-9, and 14:2-n-6, whereas heart and liver had only 14:0. Our findings do not support the hypothesis that the CoA ester pool of the retina is enriched with 12:0, 14:1-n-9, and 14:2-n-6 over 14:0, in comparison to other tissues. This suggests that alternative models must be considered for the regulation of N-terminal fatty acylation of proteins in photoreceptor cells.

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Coenzyme A (CoA) is the major fatty acid carrier molecule in plant and animal cells. Fatty acids are bound to CoA through a thioester linkage, the formation of which is carried out in mammalian cells by long chain acyl-CoA synthetases (1, 2). Fatty acylated CoAs (acyl-CoAs) play many diverse functional roles in cells (for reviews, see Refs 1 and 3–5). In this investigation, acyl-CoAs served as substrates for enzymes that acylate proteins. Several proteins found in retinal photoreceptor cells (guanylate cyclase-activating protein, protein kinase A, recoverin, and transducin) are N-terminally acylated with 12:0, 14:0, 14:1-n-9, and 14:2-n-6, whereas similar proteins in other tissues contain only 14:0 (6). In the case of guanylate cyclase-activating protein, recoverin, and transducin, the percentages of 12:0, 14:1-n-9, and 14:2-n-6 present are near or greater than that of 14:0 (6). Consequently, it appears modification with 12:0, 14:1-n-9, and 14:2-n-6 is a retina-specific phenomenon (6) with potential functional significance as described for recoverin (7). N-terminal acylation of proteins is carried out by N-terminal myristoyltransferase (NMT) for (for reviews, see Refs 8–9). Human and yeast NMTs are known to have a high substrate affinity for 14:0 CoA (2, 20, 21). Although 14:2-n-6 CoA has not been tested, 12:0 and 14:1-n-9 CoAs are utilized by the human NMT at a 3.1- and 1.9-fold lower catalytic efficiency, respectively (Vmax/Km), than 14:0-CoA (20). A recent examination of myristoyltransferase activity for yeast NMT toward 12:0, 14:1-n-9, and 14:2-n-6 CoAs, compared with 14:0 CoA (22), showed equal activity for 14:1-n-9 CoA and significantly lower activity for 12:0 and 14:2-n-6. Overall, the studies for NMTs show that 12:0, 14:1-n-9, and 14:2-n-6 CoAs are not superior substrates compared with 14:0 CoA. Consequently, to compete for the NMT, levels of available 12:0 and 14:2-n-6 CoAs must be higher and 14:1-n-9 at least equal to that of 14:0 CoA.

It can be hypothesized that the retina contains elevated amounts of 12:0, 14:1-n-9, and 14:2-n-6 CoAs over 14:0 CoA compared with other tissues, accounting for the specificity of heterogenous N-terminal acylation (6). We describe here experiments designed to test this hypothesis by analyzing the fatty acid composition of the acyl-CoA pools from bovine heart, liver, and retina. As a complementary study, we determined the 12:0, 14:0, 14:1-n-9, and 14:2-n-6 content of proteins from bovine heart, liver, and retina. Our results show that all three tissues contain CoA derivatives of 12:0, 14:1-n-9, and 14:2-n-6, yet these fatty acids are N-acylated only to retinal proteins. Thus, the heterogeneous N-terminal fatty acylation in retina may reflect unusual synthesis and utilization of 12:0, 14:1-n-9, and 14:2-n-6 CoAs, rather than acyl-CoA pool composition.

1 The abbreviations used are: NMT, N-terminal myristoyltransferase; FAPE, fatty acid phenacyl ester; HPLC, high performance liquid chromatography.
Fatty Acid CoAs in Bovine Retina, Heart, and Liver

EXPERIMENTAL PROCEDURES

Reagents—12:0, 14:0, 16:0, 17:0, 18:0, 18:1 n-9, 14:0, 14:1 n-9, 18:1, 18:2 n-6, 18:3 n-3, and 20:4 n-6 CoAs standards were from Sigma-Aldrich. [3H]14:0, 14:1 n-9, 20:4 n-6, and 14:2 n-6 CoAs were from Radiolabeled Chemicals, Inc. (St. Louis, MO), and Sigma-Aldrich, respectively. 14:1 n-9, and 14:2 n-6 CoAs were synthesized according to the method of Hajra and Bishop (23, 24). Standards [3H]14:0, [3H]16:0, and 22:6 n-3 CoAs were unresolvable and so are assigned as a single peak. B. HPLC profile of the extract made from two retinas. Peak identities were based on comparison to Rf values (Rf = t sample/t standard) and radioactivity profile for HPLC of the extract made from retinas that had been spiked with [3H]14:0 and [3H]16:0 CoAs (100,000 dpm/0.2 nmol each) prior to extraction.

Tissue Preparation—Bovine eyes, heart, and liver were obtained from Mikkelson Beef Inc. (Oklahoma City, OK). Eyes were obtained from Dr. K. Jackson, University of Oklahoma Health Sciences Center). KH2PO4, acetonitrile, CHCl3, hexane, isopropanol, and MeOH were HPLC grade, and 12 N HCl was Optima TM grade (Fisher). Ethanol was HPLC grade, and KOH was semiconductor grade (Sigma-Aldrich). All other chemicals were of reagent grade (Fisher). Ethanol was HPLC grade, and KOH was semiconductor grade (Sigma-Aldrich). All synthesized acyl-CoAs were judged to be 100% pure by HPLC (described below), thin layer chromatography (silica gel 60 plates (EM Science, Philadelphia, PA) using a butanol/HOAc/H2O (50:30:20, v/v/v) solvent system), and liquid chromatography/mass spectrometry (courtesy of Dr. K. Jackson, University of Oklahoma Health Sciences Center). KH3PO4, acetone, CHCl3, hexane, isopropanol, and MeOH were HPLC grade, and 12 N HCl was Optima grade (Fisher). Ethanol was HPLC grade, and KOH was semiconducter grade (Sigma-Aldrich). All other chemicals were of reagent grade (Fisher or Sigma-Aldrich).

Extraction of Total Acyl-CoAs—The extraction procedure was a modification of the methods of Woldegiorgis et al. (25) and Corkey (26). Frozen tissue was finely powdered by grinding in a mortar chilled by dry ice; the amount per extraction was ~1.5 g of heart, ~1.5 g of liver, and two retinas. Powdered frozen tissue was suspended in 5 ml of isopropanol, 50 mM KH2PO4, pH 7.2 (1:1 v/v) and warmed to 4 °C. For some samples, extraction efficiency was determined by adding 25,000–100,000 dpm (0.05–0.2 nmol) [3H]14:0 CoA or [3H]16:0 CoA. In some retina samples, 29,000 dpm (0.06 nmol) [3H]14:0 CoA and 5 nmol each of 12:0, 14:0, 14:1 n-9, and 14:2 n-6 CoAs were added. The samples were acidified with 100 μl of glacial acetic acid, warmed to 23 °C, and extracted twice with 4 ml of hexane/isopropanol (2:1 v/v) and then twice with 3 ml of hexane. The hexane phases containing nonpolar lipids were discarded. To the aqueous phase was added 200 μl of saturated (NH4)2SO4 and 10 ml MeOH/CHCl3 (2:1 v/v) to form a monophase. After 20 min, the precipitate was removed by centrifugation and washed once with 4 ml of MeOH/CHCl3/H2O (5:2.5:1 v/v/v). Protein pellets were saved for analysis of covalently bound fatty acids. To the pooled supernatant, 4 ml of water was added to form a stable bilayer. The CHCl3 phase containing glycerolipids was removed and combined with subsequent CHCl3 extracts. The aqueous phase was extracted twice more with 3 ml of CHCl3, the pooled CHCl3 phases were back-extracted twice with 1.5 ml of H2O, and the water phases added to the previous aqueous phase. The CHCl3 phases were then discarded. After adding ~10 mg of butylated hydroxy toluene, the aqueous pool was taken to dryness by nitrogen gas evaporation and lyophilization (VirTis lyophilizer, VirTis, Gardiner, NY). The residue, containing acyl-CoAs, was solubilized in 0.5–1.0 ml of 25 mM KH2PO4 (pH 5.3) and passed through a 0.45 μm filter (nylon-66 Microfilterfuge TM Rainin Instruments, Inc., Woburn, MA); this material was used for HPLC analysis.

HPLC Separation of Acyl-CoAs—HPLC was carried out following the modified methods of Woldegiorgis et al. (25) and Corkey (26). Standards or acyl-CoAs extracts from tissues (entire extract injected) were separated using a Waters (Milford, MA) Nova-PakTM C-18 column (60 A, 4 μm, and 3.9 mm x 15 cm inner diameter). Elution (2 ml/min) was done using a linear gradient of 25 mM KH2PO4 (pH 5.3)/acetonitrile started...
Table I

| Tissue | 12:0 CoA | 14:1n-9 CoA | 14:2n-6 CoA | 14:0 CoA |
|---|---|---|---|---|
| Retina | 0.4 | 0.4 | 0.2 | 1.0 |
| Heart | 0.7 | 0.2 | 1.0 | 1.0 |
| Liver | 2.7 | 0.2 | 1.4 | 1.0 |

For release of thioester-linked fatty acids, the protein was saponified in 6 ml of 2% KOH/EtOH (w/v) at 37 °C for 4 h. Following addition of 2 ml of H2O and 300 μl of 12 N HCl, the hydrolysate was extracted three times with 4 ml of hexane. The hexane extracts were combined and used to prepare fatty acid phenacyl esters (FAPEs) by the method of Wood and Lee (28). FAPEs were separated on HPLC using a Supelco (Bellefonte, PA) Supelcosil™ LC-18 column (25 cm × 4.6 mm inner diameter), with elution (2 ml/min) by a linear gradient of acetonitrile/H2O starting at 80/20 (v/v), increased to 92:8 after 45 min, held at 92:8 for 10 min, and returned to 80/20 after 5 min. FAPE elution was monitored by absorbance at 242 nm. After adding 6 ml of EtOH to the aqueous phase to decrease polarity, the protein was pelleted by centrifugation, and the supernatant was discarded. The protein pellet was washed once with 10 ml of EtOH, twice with 10 ml CHCl3/MeOH (2:1 v/v), and three times with 10 ml MeOH. The protein was dried in a Speed Vac concentrator and subjected to acidic methanolysis as described below.

To release amide-linked fatty acids, the proteins were hydrolyzed in 6 ml of 2 N HCl/83% MeOH at 100 °C for 6 h, under nitrogen. The hydrolysate was extracted three times with 4 ml of hexane. Fatty acid methyl ester extracts were dried under nitrogen and saponified in 2 ml of 2% KOH/EtOH at 100 °C for 60 min. After adding 1 ml H2O and 100 μl of 12 N HCl, the hydrolysate was extracted with three times with 2 ml of hexane. FAPEs were prepared and subjected to HPLC as described above.

Catalytic Hydrogenation of FAPEs—FAPE peaks from fatty acids released during base hydrolysis and acidic methanolysis were collected, 20 nmol of 17:0 FAPE was added as carrier, and the total FAPEs were extracted with hexane. The FAPEs were saponified with 2% KOH/ EtOH as described above. Free fatty acids were solubilized in 2 ml of EtOH/hexane (2:1) and bubbled with hydrogen (20 min) in the presence of ~ 10 mg of PtO2 (Matheson, Norwood, OH). Spent PtO2 was removed by centrifugation. Fatty acids were phenacylated and separated on HPLC as described above, with the modification of a 30-min elution.
after subjecting to mild alkaline hydrolysis.

extract after mixing with standards.

\[ ^{3}H \]16:0 CoA was 12.5 

CoAs prior to extraction.

mated (12:0, 14:0, and 16:0 CoAs, 2 nmol each) to be 250

ence standard (\([^{3}H] \) 16:0 CoA) in the extractions.

separation. While retaining complete separation from the other

14:1

standards, 18:2

were discernible. Shown in Fig. 4

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values were calculated rel-

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those calculated for standard acyl-CoAs. Peaks near the

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were adjusted to a theoretical 100% yield, based on

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Peaks near the appropriate RF positions for all the acyl-CoA standards were discernible. The HPLC profile for the extract after mixing with the standards is shown in Fig. 5B. Co-elution with the acyl-CoA standards was seen for the peaks found in the extract, lending support to their identity assignments. There were also several prominent peaks in the trace that did not match the RF value or co-elute with any standard, making identity assignment impossible.

A HPLC profile for liver acyl-CoA extracts after base hydrolysis is shown in Fig. 5C. Complete disappearance was seen for all the acyl-CoA candidate peaks, as well as those that were unidentifiable, supporting the idea that they are thioester-linked lipids.

The average percentage of each identifiable acyl-CoA species in the extracts (n = 2) is shown in Fig. 3B. The most abundant was 18:2n-6/20:4n-6/22:6n-6 CoA (35.3 ± 4.0%), followed by 16:0 (20.1 ± 0.9%), 12:0 (16.1 ± 5.7%), 18:3n-3 (9.3 ± 1.6%), 14:2n-6 (8.1 ± 1.2%), 14:0 (5.9 ± 0.8%), 18:1n-9 (2.4 ± 0.1%), 18:0 (1.8 ± 1.3%), and 14:1n-9 (1.2 ± 0.6%) CoAs. The levels of 12:0 and 14:2n-6 CoAs were greater than that of 14:0 CoA, and the level of 14:1n-9 CoA was lower than that of 14:0 CoA (Table I). Total acyl-CoA yield was estimated to be 5.3 ± 1.4 nmol/g of wet weight.

Fatty Acid CoAs in Bovine Retina, Heart, and Liver

FIG. 6. Base hydrolysis of total protein from bovine retina, heart, and liver. HPLC profiles of FAPEs for fatty acids released from retina, heart, and liver total protein by base hydrolysis. Control represents contaminant fatty acids in reagents. Identities of all peaks were based on retention times for standard FAPEs (profile not shown).

FIG. 7. Acidic methanolysis of total protein from bovine retina, heart, and liver. HPLC profiles of FAPEs for fatty acids released from retina, heart, and liver total protein by acidic methanolysis. Control represents contaminant fatty acids in reagents. Identities of all peaks were based on retention times for standard FAPEs (profile not shown).
FAPEs (profile not shown). Identities of all were based on retention times for standard profiles, respectively. Retinol total protein, after being subjected to hydrogenation, FAPEs for 14:1 retina total protein by acidic methanolysis.

Acids 12:0, 14:1 n-9, and 14:2 n-6 were not found in any of the base hydrolysates.

The HPLC profiles of the FAPEs released by acidic methanolysis of retina total protein, after being subjected to hydrogenation. Control (C) represents contaminant fatty acids in reagents. 17:0 peak is from 17:0 (20 nmol) added as carrier during the procedure. The y axes for profiles A, B, and C are shown at a reduction of × 2.0, × 1.6, and × 2.1, respectively. Identities of all were based on retention times for standard FAPEs (profile not shown).

min, the identities of which are unknown. The short chain fatty acids 12:0, 14:1–n-9, and 14:2–n-6 were not found in any of the base hydrolysates.

Our examination of bovine heart, liver, and retina for 12:0, 14:0, 14:1–n-9, and 14:2–n-6 CoA content was essential in explaining how heterogeneous N-terminal fatty acylation arises. In heart and retina, 12:0, 14:0, 14:1–n-9, and 14:2–n-6 CoAs were sometimes difficult to distinguish from background; therefore, the reported percentages should be taken as an upper limit for their presence. Although our data are relative percentages, there was no obvious indication in heart and retina that 12:0, 14:1–n-9, and 14:2–n-6 CoAs exceeded 14:0 CoA by a substantial amount. On the other hand, liver acyl-CoA levels were all significantly above background levels, showing amounts of 12:0 and 14:2–n-6 CoAs slightly higher than that of 14:0 CoA, whereas 14:1–n-9 CoA was somewhat lower. As in heart and retina, there was no dramatic enrichment of 12:0, 14:1–n-9, and 14:2–n-6 CoAs over 14:0 CoA in liver.

Based on the catalytic efficiency (Vmax/Km) data for the human NMT (20), there should be at least 2.6- and 3.1-fold higher amounts of 12:0 and 14:1–n-9 CoAs, respectively, relative to 14:0 CoA for equal utilization. Activity (Vmax) and dissociation constant (Kd) data for the yeast NMT toward 14:0 and 14:2–n-6 CoAs (22) suggest that, minimally, 18.9- and 5.0-fold higher amounts, respectively, than 14:0 CoA would be required for equal incorporation. Although the amounts of 12:0, 14:1–n-9, and 14:2–n-6 CoAs in heart are consistent with the acylation of heart proteins with only 14:0, the levels of these CoAs in the retina are definitely not high enough to lead to the heterogeneous acylation pattern seen for photoreceptor proteins. Likewise, the relative amounts of 12:0 and 14:2–n-6 CoAs in liver suggest that these fatty acids should be found in liver proteins in addition to 14:0 (104 and 7–28% of 14:0, respectively), but this is not the case (35). Consequently, it is very unlikely heterogeneous N-terminal fatty acylation is determined by differences in the acyl-CoA composition of the tissues.

Our results suggesting the presence of 12:0, 14:1–n-9, and 14:2–n-6 CoAs in heart and liver led us to question whether there might exist heterogeneously acylated proteins in these tissues that have yet to be identified. We performed fatty acid analysis on the total protein precipitates from heart, liver, and retina. Base hydrolysates released considerale 16:0 from all of the proteins, which is consistent with the thioester modification of proteins by 16:0 (15). We also observed the release of 14:0, 18:0, 18:1–n-9, and 18:2–n-6, which can also be S-linked to proteins (36, 37). Although the representative palmitoylated proteins in heart and liver are uncertain, the major modified protein in retina is likely the photoreceptor protein rhodopsin (38). Acidic methanolysis of heart and liver total protein showed release of 14:0 but not of 12:0, 14:1–n-9, and 14:2–n-6.

Retina total protein released 14:0, 14:1–n-9, and 14:2–n-6, consistent with the presence of these fatty acids in photoreceptor...
proteins. Thus, whereas all three tissues may contain 14:1-9 and 14:2-6 CoAs, only retinal proteins contained these two fatty acids in an amide linkage.

In light of our results, alternative models must be considered for regulation of N-terminal fatty acylation in photoreceptors. Previously, we showed that the retina synthesizes 14:1-9 and 14:2-6 by retroconversion of 18:1-9 and 18:2-6 or 20:4-6, respectively (39). We hypothesized that retina peroxisomes might be more active in producing 14:1-9 and 14:2-6. It is also possible that the retina acyl-CoA synthetase is more efficient at generating the required 18:1-9 and 14:1-9 respectively (39). We hypothesized that retina peroxisomes contain NMT isoforms (42–44), and bovine brain and spleen NMTs are cytosolic proteins (48–51), the retina NMT could be compartmentalized where it is more accessible to 14:1-9 and 14:2-6 CoAs than 14:0 CoA. Our previous studies showed that 14:1-9 and 14:2-6 CoAs are not generated by peroxisomes.

CONTROL OF NMT ACTIVITY

We conclude that alternative models must be considered for controlling NMT activity at work in photoreceptors.

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