Stability of Fatty Acyl-Coenzyme A Thioester Ligands of Hepatocyte Nuclear Factor-4α and Peroxisome Proliferator-Activated Receptor-α

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ABSTRACT: Although long-chain fatty acyl-coenzyme A (LCFA-CoA) thioesters are specific high-affinity ligands for hepatocyte nuclear factor-4α (HNF-4α) and peroxisome proliferator-activated receptor-α (PPARα), X-ray crystals of the respective purified recombinant ligand-binding domains (LBD) do not contain LCFA-CoA, but instead exhibit bound LCFA or have lost all ligands during the purification process, respectively. As shown herein: (i) The acyl chain composition of LCFA bound to recombinant HNF-4α reflected that of the bacterial LCFA-CoA pool, rather than the bacterial LCFA pool. (ii) Bacteria used to produce the respective HNF-4α and PPARα contained nearly 100-fold less LCFA-CoA than LCFA. (iii) Under conditions used to crystallize LBD (at least 3 wk at room temperature in aqueous buffer), 16:1-CoA was very unstable in buffer alone. (iv) In the presence of the respective nuclear receptor (i.e., HNF-4α and PPARα), LBD 70–75% of 16:1-CoA was degraded after 1 d at room temperature in the crystallization buffer, whereas as much as 94–97% of 16:1-CoA was degraded by 3 wk. (v) Cytoplasmic LCFA-CoA binding proteins such as acyl-CoA binding protein, sterol carrier protein-2, and liver-FA binding protein slowed the process of 16:1-CoA degradation proportional to their respective affinities for this ligand. Taken together, these data for the first time indicated that the absence of LCFA-CoA in the crystallized HNF-4α and PPARα was due to the paucity of LCFA-CoA in bacteria as well as to the instability of LCFA-CoA in aqueous buffers and the conditions used for LBD crystallization. Furthermore, instead of protecting bound LCFA-CoA from autohydrolysis like several cytoplasmic LCFA-CoA binding proteins, these nuclear receptors facilitated LCFA-CoA degradation.

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Abbreviations: 16:1-CoA, palmitoleoyl-coenzyme A; 17:0-CoA, *n*-heptadecanoyl-coenzyme A; aa, amino acid; ACBP, acyl CoA-binding protein; CoA, coenzyme A; HNF-4α (aa 1–455), full-length hepatocyte nuclear factor 4α; HNF-4α-E (aa 132–370), N- and C-terminal truncation mutant of HNF-4α comprising aa 132–410 (i.e., ligand-binding domain E, but missing the negative regulatory domain F and the DNA-binding domain); HNF-4α-E-F (aa 132–455), N-terminal truncation mutant of HNF-4α comprising aa 132–455 (i.e., ligand-binding domain E and negative-regulatory domain F, but missing the DNA-binding domain); HNF-4α-E-0.5F (aa 132–410), N- and C-terminal truncation mutant of HNF-4α comprising aa 132–410 (i.e., ligand-binding domain E, but missing half of the negative-regulatory domain F and all of the DNA-binding domain); LBD, ligand-binding domain; LCFA, long-chain fatty acid; LCFA-CoA, long-chain fatty acyl CoA; L-FABP, liver fatty acid-binding protein; MPD, 2-methyl-2,4-pentanediol; PPARα, peroxisome proliferator-activated receptor-α, RARα, retinoic acid receptor-α; RXRα, retinoid X receptor α; SCP-2, sterol carrier protein-2.

Two members of the nuclear receptor superfamily, hepatocyte nuclear factor-4α (HNF-4α) and peroxisome proliferator-activated receptor-α (PPARα), bind coenzyme A (CoA) thioesters of long-chain FA (LCFA, C_{14}–C_{22}) in *in vitro* binding assays (1–7). Although early radioligand competition binding assays showed these proteins exhibiting only weak affinities (i.e., μM K_i) for LCFA-CoA (1,6), subsequent direct fluorescence binding assays demonstrated that both HNF-4α (2–4) and PPARα (7) exhibited high affinities (i.e., low nM K_d) for LCFA-CoA. Furthermore, in the case of HNF-4α this high affinity for LCFA-CoA was dependent on the presence of an intact F domain C-terminal to the ligand-binding domain (LBD). Deletion of the F domain abolished LCFA-CoA binding and enhanced that of LCFA (4). The molecular basis for the discrepancy reported in affinities obtained between radioligand competition and fluorescence binding assays is based on the fact that radioligand competition assays are known to significantly underestimate the affinities of ligand-binding proteins for LCFA-CoA and LCFA (8,9). For example, cytoplasmic LCFA-CoA-binding proteins such as acyl-CoA-binding protein (ACBP), sterol carrier protein-2 (SCP-2), and liver FA-binding protein (L-FABP) exhibit M K_d in radioligand binding assays (reviewed in Refs. 8 and 10), whereas those obtained with fluorescence and titration calorimetry binding assays display a 100- to 1000-fold stronger affinity (10–13).

Although PPARα is widely recognized as a ligand-inducible nuclear receptor that is activated only when specific ligands (e.g., peroxisome proliferators, LCFA, nonhydrolyzable LCFA-CoA, etc.) are present in cells (reviewed in Refs. 5, 7, 14, and 15), the transcriptional activity of HNF-4α in the liver, intestine, or pancreas as a function of specific ligands has been highly debated. For instance, some authors considered HNF-4α to be a constitutively active and ligand-independent nuclear receptor, even though its modular structure includes the LBD (16). In contrast, other groups demonstrated that various LCFA (C_{14}–C_{22}) were able to modulate the transcriptional activity of HNF-4α by reporter gene assays in transfected COS-7 cells in culture (1,3). In addition, overexpression of fatty acyl CoA synthase enhanced the ability of LCFA to modulate the transcriptional activity of HNF-4α, whereas overexpression of fatty acyl CoA hydrolase inhibited the ability of LCFA to modulate the transcriptional activity of HNF-4α (1). Furthermore, it was shown that purified full-length HNF-4α and truncation mutants

with an intact LBD and C-terminal F domain had an affinity for the CoA thioesters of LCFA several orders of magnitude higher than for the corresponding free LCFA *in vitro* (1–4).

Despite the above data indicating that both PPARα and HNF-4α bind LCFA-CoA with high affinity, the crystal structures of the LBD of PPARα (17) and HNF-4α (18,19) do not contain endogenously bound LCFA-CoA. Instead, these truncated proteins contain either no endogenously bound ligand, as for the PPARα LBD (17), or, in the case of the HNF-4α LBD, contain constitutively bound LCFA that do not influence the open vs. closed conformational state of the HNF-4α LBD. However, LCFA-CoA is known to alter PPARα conformation, as demonstrated by circular dichroism (7), and a nonhydrolyzable LCFA-CoA analog is known to alter PPAR LBD conformation, as evidenced by sensitivity to protease digestion and the ability to bind to co-activators (7,20). Likewise, LCFA-CoA and nonhydrolyzable LCFA-CoA analogs (but not LCFA) alter the conformation of full-length HNF-4α as well the HNF-4α LBD containing the C-terminal F domain (2,4,21). Such ligand-induced conformational changes are a hallmark of ligand-activated nuclear receptors (reviewed in Refs. 2 and 22–25).

Although the molecular basis for the above discrepancies is not yet known, at least three possibilities may be considered: First, the level of endogenous LCFA-CoA may be very small as compared with that of LCFA in the bacteria used to produce the recombinant truncated PPARα and HNF-4α constructs. Second, the FFA detected within the binding site of HNF-4α LBD by X-ray crystallography may arise from residual LCFA formed by degradation of CoA thioesters during the crystallization process. Third, truncation of the C-terminal F domain, especially long in HNF-4α, significantly alters the ligand specificity of the recombinant N- and C-terminal truncation proteins used for crystallography (4). The purpose of the present work was: (i) to examine the relative proportions of LCFA-CoA and LCFA in the recombinant bacteria, (ii) to show the stability of LCFA-CoA in the context of protein crystallization conditions; (iii) to show the effect of nuclear receptors that bind LCFA-CoA, i.e., HNF-4α and PPARα, on LCFA-CoA stability; and (iv) to determine the stability of LCFA-CoA in the context of the cytoplasmic LCFA-CoA-binding proteins ACBP, SCP-2, and L-FABP.

MATERIALS AND METHODS

Materials. Palmitoleoyl-CoA (16:1-CoA) and *n*-heptadecanoyl-CoA (17:0-CoA) were purchased from Sigma Chemical Co. (St. Louis, MO). Protease inhibitor cocktail, ultralow range color markers for SDS-PAGE, gel filtration M.W. markers (range 6,500–66,000 Da), alkaline phosphatase conjugated goat anti-rabbit IgG, alkaline phosphatase conjugated rabbit anti-goat IgG, and 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT) for Western analysis were purchased from Sigma-Aldrich (St. Louis, MO). Protein concentration was determined by the Bradford method using Protein Assay Dye Reagent Concentrate purchased from Bio-Rad Laboratories (Richmond, CA).

Rabbit polyclonal anti-rat HNF-4-LBD antisera were prepared according to Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines and further purified by affinity chromatography on protein-A-sepharose as described earlier (26). Rabbit polyclonal anti-mouse PPARα antibodies were from Affinity BioReagents (Golden, CO). Polyclonal antisera to murine recombinant L-FABP, human recombinant SCP-2 (12), and murine recombinant ACBP (28,29) were prepared as described (29) in the cited papers. The specificity of appropriate dilutions of the purified antibodies was determined as described earlier (30). None of the antisera cross-reacted with proteins other than the one against which the antisera were raised.

Expression and purification of recombinant full-length and deletion mutant forms of rat HNF-4α. The full-length rat HNF-4α [amino acids (aa) 1–455] and the N-terminal deletion mutant HNF-4α-E-F (aa 132–455) recombinant proteins were obtained as described previously (1,2). The cDNA of the rat C-terminal truncation mutant lacking the entire F domain, HNF-4α-E (aa 132–455) was obtained by PCR using the sense 5′-CATGCCATGGGCAGCCATCATCATCATCACAGGTCAAGCTACGAG and antisense 5′-GAAGATCTCTAGGCAGACCCTCCAAG primers. Rat N-terminal His-tagged HNF-4α1 (aa 132–410) recombinant was prepared by PCR using the sense 5′-CATGCCATGGGCAGCCATCATCATCATCACAGGTCAAGCTACGAG and antisense 5′-GAAGATCTCTAGGTGGACATCTGTCC primers. The PCR products were cloned into pET11d plasmid. The recombinants plasmids were expressed in the *Escherichia coli* BL21(DE3)pLyS strain, and the His-tagged proteins were purified by affinity chromatography on nickel nitrilotriacetic resin (Qiagen, Chatsworth, CA) and stored at −70°C. Purity of the recombinant proteins was assessed by SDS-PAGE and Western blotting as described earlier (2–4).

Expression and purification of recombinant PPARα-ΔAB (aa 101–468). The cDNA encoding mouse PPARα with a deletion of the amino-terminal A/B domain (i.e., encoding PPARα aa 101–468) cloned into a $(His)_6$-tagged bacterial expression vector (pET-PPARα-ΔAB) was a gift from Dr. Noa Noy (Cornell University, Ithaca, NY) (31). Recombinant PPARα-ΔAB protein was expressed in BL21(DE3)pLysS strain of *E. coli* and purified by affinity chromatography with cobalt resin (BD Biosciences, Clontech, Palo Alto, CA) as described earlier (7). The purified recombinant PPARα-ΔAB protein eluting from the column was dialyzed against a buffer containing 10 mM Hepes (pH 8.0), 0.1 mM EDTA, 1 mM DTT, 400 mM KCl, and 10% glycerol, and stored in 25% glycerol at −80°C. Protein purity was assessed by SDS-PAGE and Western blotting as described earlier (7,31).

Expression and purification of recombinant ACBP, SCP-2, and L-FABP. Murine recombinant L-FABP (27), human recombinant SCP-2 (12), and murine recombinant ACBP (28,29) were purified as described in the cited papers. Protein purity was also determined by SDS-PAGE, silver staining, and Western blotting as described therein.

Analysis of LCFA-CoA content and distribution in purified recombinant HNF-4α and in E. coli cells expressing HNF-4α. After a known amount of 17:0-CoA internal standard was added

to each sample, total LCFA-CoA were extracted from purified full-length HNF-4α (aa 1–455), HNF-4α truncation mutants (i.e., HNF-4α-E, aa 132–370, and HNF-4α-E-F, aa 132–455), and cell homogenate of *E. coli* expressing full-length HNF-4α, by a solid-phase extraction procedure (32). The LCFA-CoA were then converted to fluorescent etheno CoA esters and resolved by HPLC with fluorescence detection (33). Comparison with the known standard LCFA-CoA in this procedure allowed determination of the total mass of LCFA-CoA/mg protein, mass of the individual LCFA-CoA species/mg protein, and relative percentage distribution of individual LCFA-CoA species basically as described earlier (34).

Analysis of free LCFA content of purified recombinant HNF-4α and E. coli expressing HNF-4. Purified full-length HNF-4α and bacterial cell homogenate were analyzed for total FFA using a Waco NEFA C test kit (Waco Chemicals USA, Inc., Richmond, VA) following the procedures provided by the manufacturer. A reagent blank, specimen blank, and standard curve with a known amount of FA were run as suggested.

Determination of HNF-4α protein concentration in E. coli expressing HNF-4α. E. coli cells expressing HNF-4α were lysed by sonication in the presence of protease inhibitor cocktail used for purification of His-tagged proteins from Sigma. The unbroken cells and debris were removed by centrifugation at 50,000 × *g* for 10 min at 4°C. The supernatant was used to determine: (i) total protein by the BCA Protein Assay kit from Pierce (Rockford, IL) and (ii) HNF-4α protein by SDS-PAGE and Western blot analysis as described previously (2,26). Quantitative estimation of HNF-4α protein in Western blots was performed by densitometric analysis after image acquisition using a single-chip CCD (charge-coupled device) video camera and a computer workstation (IS-500 system; Alpha Innotech, San Leandro, CA). Image files were analyzed (mean 8-bit gray-scale density) using NIH Image (available by anonymous FTP). To obtain the HNF-4α protein concentration in the bacterial lysate supernatant samples, the HNF-4α pixel density in Western blots of bacterial lysate supernatant samples was compared with that of known amounts of pure recombinant His-tagged HNF-4α protein run as standards on the same blot.

Stability of LCFA-CoA under conditions used to crystallize N- and C-terminal truncation mutants of HNF-4α: Effect of ACBP. Equal concentrations of the three most prevalent cytosolic acyl-CoA binding proteins (ACBP, SCP-2, L-FABP), full-length HNF-4α (aa 1–455), HNF-4α-E-F (aa 132–455), HNF-4α-E-F (aa 132–410), and HNF-4α-E (aa 132–370) were individually incubated with 16:1-CoA (1:1 molar ratio of protein to 16:1-CoA) at room temperature in a buffer system previously described for HNF-4α-E (aa 132–382) crystallization [0.1 M sodium citrate, pH 8.0, 0.7 M ammonium acetate, 16% 2-methyl-2,4-pentanediol (MPD), and 10 mM DTT] (18). 16:1-CoA in buffer only was used as a blank for autohydrolysis in the absence of protein under the same conditions. After 1 d, 1 wk, and 3 wk, aliquots of the mixtures were taken and a known amount of 17:0-CoA internal standard was added to each sample as well as to a fresh aliquot of 16:1-CoA in buffer without protein and without incubation. The amount of remaining 16:1-CoA was determined by extracting the 16:1-CoA using a solid-phase extraction procedure (32), converting the 16:1-CoA to fluorescent etheno CoA esters (33), and analyzing by HPLC with a fluorescence detector (33) as described earlier (34).

Stability of LCFA-CoA in the crystallization buffer used in PPARα LBD X-ray studies. PPARα-ΔAB or ACBP, both at 0.5 μM, were incubated with equimolar 16:1-CoA at room temperature in each of the following buffers previously used for PPARα LBD protein crystallization: (i) buffer 1 (5.5–9.6% PEG 35K, 50 mM di-ammonium hydrogen citrate, pH 4.9, 50 mM 1,3-bis-tris-propane (BTP), pH 7.0, 10% MPD (35); (ii) buffer 2 (10 mM Tris, pH 8.0, 75 mM sodium chloride, 5% glycerol, 0.5 mM tris-2-carboxyethyl)-phosphine hydrochloride, 1.6 M sodium formate, 50 mM Hepes, pH 7.5) (17). 16:l-CoA alone without added protein was used in each buffer for comparison. After 1 d, 1 wk, and 3 wk, aliquots of the mixtures were analyzed for remaining 16:1-CoA as compared with fresh 16:1-CoA controls and fresh 17:0-CoA that was added to each sample as an internal control. To determine the amount of each fatty acyl-CoA remaining, the fatty acyl-CoA were extracted by solid-phase extraction (33), converted to fluorescent etheno CoA esters, and analyzed by HPLC (33).

RESULTS

LCFA-CoA content of purified recombinant HNF-4α proteins. In vitro ligand-binding assays have clearly shown that recombinant full-length HNF-4α exhibits affinity for LCFA-CoA several orders of magnitude higher than LCFA in solution (1–4). Basically similar data are obtained with N-terminal truncation mutants containing the complete E and F domains (1–4). However, heretofore there have been no reports examining the presence of endogenously bound LCFA-CoA in purified recombinant full-length HNF-4α or in N-terminal truncation mutants of HNF-4α containing the complete E and F domains. Therefore, the content of LCFA-CoA in recombinant full-length HNF-4α (aa 1–455), as well as in its truncation mutants HNF-4α-EF (aa 132–455) and HNF-4α-E (aa 132–370), in solution was determined as described in the Materials and Methods section. However, no LCFA-CoA were detected in either the full-length HNF-4α (aa 1–455) or its truncation mutants containing the complete ligand-binding E and negative-regulatory F domains, i.e., HNF-4α-E-F (aa 132–455). Interestingly, the N-terminal and C-terminal truncation mutant HNF-4α-E (aa 132–370) also did not contain any detectable endogenously bound LCFA-CoA. The latter observation confirmed earlier data from X-ray crystals of such N- and C-terminal HNF-4α truncation mutants that contained bound LCFA, but not LCFA-CoA (18,19).

LCFA-CoA and free LCFA content of E. coli expressing the full-length HNF-4α. It is important to consider that not only the affinity for LCFA-CoA vs. LCFA but also the relative availability of the respective ligands in *E. coli* will contribute to the

TABLE 1
FFA and Long-Chain Fatty Acyl-Coenzyme A (LCFA-CoA) Content of *Escherichia coli* Cells Expressing Hepatocyte Nuclear Factor-4α (HNF-4α)[a]

Ligand	Ligand/total protein (nmol /mg)	Ligand/HNF-4α (nmol/mg)	Ligand/HNF-4α (mol/mol)
FFA	4.95 ± 0.62	396 ± 50	20
LCFA-CoA	0.055 ± 0.005	4.36 ± 0.36	0.22
FFA/LCFA-CoA	91:1	91:1	91:1

[a]Data presented are mean ± SE (n = 5).

type of endogenous ligand associated with HNF-4α and its mutants. Therefore, this possibility was examined in *E. coli* overexpressing the full-length HNF-4α. The contents of LCFA, LCFA-CoA, and HNF-4α were determined as described in the Materials and Methods section. As shown in Table 1, bacterial cells expressing the full-length HNF-4α contained 4.95 ± 0.62 nmol LCFA/mg total protein, equivalent to 396 ± 50 nmol LCFA/mg HNF-4α. When expressed on a molar basis, the molar ratio of LCFA/HNF-4α was 20:1. In contrast, *E. coli* contained much less LCFA-CoA, only 54.5 ± 4.5 pmol/mg total bacterial protein (Table 2). When expressed on the basis of HNF-4α content, this was equivalent to 4.36 ± 0.36 nmol LCFA-CoA/mg HNF-4α (Table 1). Thus, the molar ratio of LCFA-CoA/HNF-4α in bacterial extract was only 0.22:1.

LCFA-CoA composition of E. coli cells expressing HNF-4α. The acyl chain length and level of saturation of endogenous LCFA associated with HNF-4α constructs isolated from bacterial extracts differ substantially from that present in bacteria (3,18). For example, even though 16:0 and 18:1 are the most abundant LCFA present in *E. coli*, full-length HNF-4α (Hertz, R., personal communication), HNF-4α-E-F (aa 132–455) (3), or HNF-4α (aa 132–383) (19) contain 50–65% of 16:1, an LCFA barely detectable in bacteria (3,36). This finding cannot be explained simply on the basis of differences in affinities of the HNF-4α constructs (1–4). Instead, high endogenous 16:1 bound to HNF-4α constructs might reflect that in the bacterial LCFA-CoA pool, rather than in the LCFA pool. Hydrolysis of the bound LCFA-CoA during recombinant protein isolation could then account for the high endogenous 16:1 associated with the HNF-4α constructs. To examine this possibility, the acyl chain composition of bacterial LCFA-CoA was determined by HPLC analysis after solid-phase extraction of bacterial LCFA-CoA. The results showed that the most abundant LCFA-CoA was 16:1-CoA (34.9 ± 1.1%), followed by significantly less 16:0-CoA (13.8 ± 0.4%) and 18:1-CoA (25.8 ± 1.0%) (Table 2). Thus, the acyl chain composition of the bacterial LCFA-CoA pool, rather than that of the LCFA pool, reflected the acyl chain distribution of endogenously bound LCFA in recombinant HNF-4α constructs.

TABLE 2
Composition of LCFA-CoA Thioesters of *E. coli* Cells Expressing Recombinant Rat HNF-4α[a]

LCFA-CoA	%	LCFA-CoA	%
16:0	13.8 ± 0.4	18:1	25.8 ± 1.0
16:1	34.9 ± 1.1	20:0	12.1 ± 1.4
18:0	5.8 ± 0.9		

[a]For abbreviations see Table 1.

Stability of LCFA-CoA under protein crystallization conditions. Since X-ray crystallography detects only endogenous LCFA bound in N- and C-terminal truncation mutants of HNF-4α and HNF-4γ (19), it was concluded that only LCFA are endogenous ligands of HNF-4α and HNF-4γ (18,19). However, no data were provided supporting the assumption that LCFA-CoA were stable under the stringent crystallization conditions used therein. To begin to resolve this issue, the stability of 16:1-CoA under crystallization conditions (19) was tested, as described in the Materials and Methods section. In the crystallization buffer at 24°C, 16:1-CoA quickly decomposed. As quickly as 1 d at room temperature, 75% of the 16:1-CoA was degraded (Fig. 1A). Since crystals were typically formed over periods ranging in weeks, the stability was also examined after 1 and 3 wk. Only 13 and 6.6% of the 16:1-CoA remained intact after 1 and 3 wk, respectively (Fig. 1A). Thus, nearly 94% of 16:1-CoA was degraded by 3 wk, the time used for crystallizing the HNF-4α N- and C-terminal F domain truncation mutant (19). Thus, the instability of LCFA-CoA in solution, together with the lengthy isolation procedure required for isolating the recombinant proteins and, even more so, the 3 or more weeks at room temperature required for crystallization of such proteins, likely contributed to the lack of detectable endogenously bound LCFA-CoA therein.

Effect of HNF-4α (full-length and truncation mutants) on LCFA-CoA stability as compared with other LCFA-CoA-binding proteins under protein crystallization conditions. Although it has been hypothesized that cytosolic LCFA-CoA-binding proteins may protect LCFA-CoA from being hydrolyzed by intracellular esterases (reviewed in Refs. 8, 27, and 37), nothing is known regarding the effect of either cytosolic or nuclear LCFA-CoA-binding proteins on the autohydrolysis of LCFA-CoA in buffer alone, especially under protein crystallization conditions. To assess whether LCFA-CoA are protected from autodegradation in the buffer by association with a binding protein, the effects of several LCFA-CoA-binding proteins were examined.

First, the effect of intracellular LCFA-CoA-binding proteins such as ACBP, SCP-2, and L-FABP was examined. These cytosolic proteins bind LCFA-CoA with affinities in the order ACBP ≥ SCP-2 > L-FABP (reviewed in Refs. 8, 9, 11, and 12). When these proteins were individually incubated at 1:1 molar ratio with 16:1-CoA under conditions used to crystallize HNF-4α (i.e., crystallization buffer at room temperature), only the

highest-affinity proteins (ACBP, SCP-2) significantly reduced, but did not completely prevent, 16:1-CoA hydrolysis (Fig. 1B). The lower-affinity protein L-FABP was without effect (Fig. 1B). After 1 d in the presence of ACBP, SCP-2, or L-FABP, the majority of LCFA-CoA was degraded: 63, 65, and 80%, respectively (Fig. 1B). Thereafter, at longer incubation times only ACBP significantly protected 16:1-CoA from hydrolysis, albeit 81% of available 16:1-CoA was still hydrolyzed after 3 wk (Fig. 1B).

Second, the effects of the nuclear receptor HNF-4α (full-length) and HNF-4α deletion mutants on LCFA-CoA stability were similarly tested. Although full-length HNF-4α (aa 1–455) exhibits high affinity (i.e., nM K_d) for LCFA-CoA in *in vitro* binding assays (2–4), this did not protect LCFA-CoA from degradation. Instead, full-length HNF-4α (aa 1–455) enhanced hydrolysis as compared with incubation in the buffer alone: nearly 10-fold accelerated conversion of LCFA-CoA to free LCFA within 1 d of incubation and total degradation at longer incubation times (Fig. 1A). Similarly, the N-terminal DNA-binding domain deletion construct, i.e., HNF-4α-E-F (aa 132–455), also exhibits high affinity (i.e., nM K_d) for LCFA-CoA in *in vitro* binding assays (2–4), but it did not protect LCFA-CoA from degradation. Instead, 16:1-CoA hydrolysis was enhanced ninefold within 1 d of incubation, followed by total degradation at longer time points (Fig. 1A). Surprisingly, the N-terminal and partial C-terminal F domain deletion construct, HNF-4α-E-0.5F (aa 132–410), showed some protection against 16:1-CoA hydrolysis during the first day of incubation but not thereafter, since 16:1-CoA was degraded to the same high extent as in the crystallization buffer only at longer incubation times (Fig. 1A). The N-terminal and C-terminal F domain deletion construct, HNF-4α-E (aa 132–370), comprising only the LBD, only weakly binds LCFA-CoA in *in vitro* binding assays (4) and had no or little effect on LCFA-CoA degradation. At 1 d and 1 wk of incubation, 16:1-CoA hydrolysis was the same in the presence of HNF-4α-E (aa 132–370) as for buffer alone. Only at 3 wk incubation did HNF-4α-E (aa 132–370) slightly enhance 16:1-CoA hydrolysis as compared with buffer alone.

Stability of LCFA-CoA in the presence of PPARα vs. ACBP. To determine whether the unusual effect of HNF-4α to enhance LCFA-CoA degradation was a unique feature of this nuclear regulatory protein, the effect of another nuclear receptor that binds LCFA-CoA, i.e., PPARα (7,20), on LCFA-CoA stability was examined. Despite the high affinity (very low nM K_d) PPARα displays for LCFA-CoA, endogenously bound LCFA-CoA have also not been detected in X-ray crystal structures of PPARα LBD (17,35). To begin to address this issue, the possibility that LCFA-CoA are unstable under PPARα crystallization conditions was considered. To test the stability of LCFA-CoA under crystallization conditions, aliquots of 16:1-CoA were incubated in the absence or presence of added LCFA-CoA binding protein (i.e., PPARα or ACBP) in two types of buffers previously used to crystallize recombinant PPARα-LBD protein as described in the Materials and Methods section (17,35).

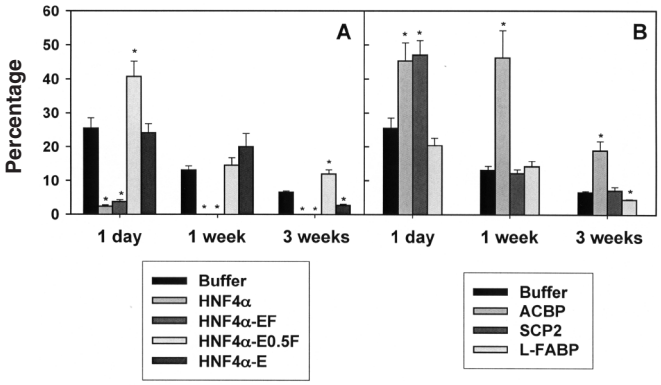

FIG. 1. Long-chain fatty acyl-coenzyme A (LCFA-CoA) stability under hepatocyte nuclear factor 4α (HNF-4α) crystallization conditions. 16:1-CoA was incubated in HNF-4α crystallization buffer (as described in the Materials and Methods section) in the absence or presence of proteins known to have affinity for LCFA-CoA, in a molar ratio of 1:1, for various time periods (1 d to 3 wk). (A) Percentage of 16:1-CoA recovered after incubation with full-length HNF-4α or truncated forms of HNF-4α, such as HNF-4α-E-F [amino acids (aa) 132–455; ligand-binding domain (LBD) E and negative-regulatory domain F, but missing the DNA-binding domain], HNF-4α-E-0.5F (aa 132–410; LBD E, but missing half of the negative-regulatory domain F and all of the DNA-binding domain), and HNF-4α-E (aa 132–370; LBD E, but missing the negative regulatory domain F and the DNA-binding domain). (B) Percentage 16:1-CoA recovered after incubation with acyl CoA-binding protein (ACBP), sterol carrier protein-2 (SCP-2), or liver FA-binding protein (L-FABP).

Solid-phase extraction was then used to determine the proportion of intact vs. hydrolyzed 16:1-CoA, as described in the Materials and Methods section.

In the absence of added LCFA-CoA binding protein (i.e., PPARα or ACBP), LCFA-CoA was very unstable in the incubation buffers and conditions (room temperature) used to crystallize PPARα LBD. Within 1 d of incubation, 70–75% of the 16:1-CoA was degraded (Figs. 2A,B). By 1 wk, 85–88% of the 16:1-CoA was degraded, and by 3 wk 86–97% was hydrolyzed (Fig. 2B). These data suggest that by the end of 3–6 wk, typical conditions used to crystallize the PPARα LBD protein, almost no LCFA-CoA would remain intact. However, it must be considered that protein-bound ligands are typically thought to be more stable in solution than free ligands. Therefore, the aforementioned experiments with the two crystallization buffers were repeated in the presence of LCFA-CoA binding proteins (i.e., PPARα or ACBP).

Recently, it was shown that PPAR is a nuclear receptor that binds LCFA-CoA (15) with very high affinity (low nM K_d) (7). However, the data indicate that in the presence of the LCFA-CoA binding protein PPARα, 16:1-CoA was not protected from degradation (Fig. 2). Instead, depending on the buffer used and the time period examined, PPAR enhanced 16:1-CoA degradation by 2- to 10-fold (Fig. 2). In any case, by 3 wk of incubation all of the 16:1-CoA was degraded in the presence of PPARα, regardless of the buffer used.

Although ACBP is primarily a cytosolic protein, low

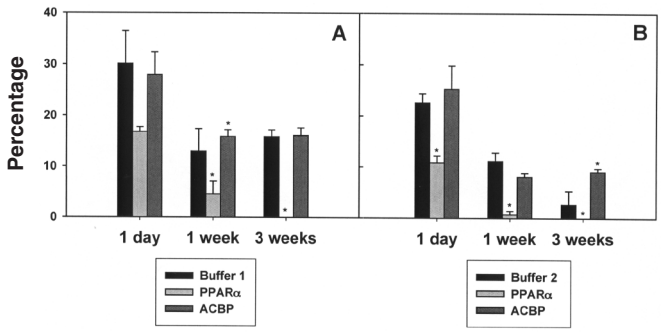

FIG. 2. LCFA-CoA stability under peroxisome proliferator-activated receptor-α (PPARα) crystallization conditions. 16:1-CoA was incubated in two different buffers that were previously used to crystallize the PPARα LBD (as described in the Materials and Methods section) in the absence or presence of PPARα and ACBP. (A) Buffer 1, as described in the Materials and Methods section. (B) Buffer 2, as described in the Materials and Methods section. For other abbreviations see Figure 1.

amounts of ACBP are detected in nuclei, where it interacts with nuclear receptors to influence transcriptional activity (26). Similar to PPARα, ACBP exhibits very high affinity (very low nM K_d) for LCFA-CoA (8,11,38). In contrast to PPARα, however, ACBP did not accelerate LCFA-CoA degradation but, depending on the buffer used, actually protected 16:1-CoA from hydrolysis. In buffer 2 (Fig. 2B), but not buffer 1 (Fig. 2A), ACBP protected 16:1-CoA from degradation at long time points, i.e., 3 wk, but not at short time points. As shown above, ACBP was even more protective of 16:1-CoA degradation in another buffer (i.e., 0.75 M ammonium phosphate, pH 5.0, and 10 mM DTT) used to crystallize HNF-4α truncation mutants (Fig. 1B).

DISCUSSION

Despite the importance of nuclear receptors such as HNF-4α and PPARα in glucose and FA metabolism, relatively little is known regarding the nature of the endogenous ligands, and in particular LCFA-CoA associated with these proteins.

A wide body of evidence is consistent with LCFA-CoA as putative endogenous, physiologically significant ligands of these nuclear receptors: (i) *In vitro* ligand-binding assays performed with the respective full-length proteins (or their N-terminal truncation constructs containing intact C-terminal F domains) clearly demonstrate that these nuclear receptors bind LCFA-CoA in aqueous buffers with equal (PPARα) (7,20) or considerably higher (HNF-4α) (2–4) affinities than exhibited for LCFA. (ii) The nM K_d obtained for LCFA-CoA binding by direct fluorescence binding assays (2–4,7) are in the same range as the concentration of LCFA-CoA in the nucleus of living cells (34). (iii) LCFA-CoA, but not LCFA, alter the conformation of HNF-4α (1–4,21) and PPARα (7,20). However, under other *in vitro* conditions only slight (39) or no (16) effects on conformation of HNF-4α were observed. (iv) Normal LCFA-CoA and nonhydrolyzable LCFA-CoA modulate the transactivation of HNF-4α in living cells (3,4,38). (v) Several investigators used purified recombinant HNF-4α to show that LCFA-CoA, but not LCFA, regulate HNF-4α homodimer formation and DNA binding (gel mobility shift) *in vitro* (1,40). This action is analogous to LCFA-CoA regulating the binding of Fad-R (an *E. coli* DNA-binding protein) to its cognate DNA response element *in vitro* (41). However, the finding that LCFA-CoA regulates HNF-4α homodimer formation and DNA binding may depend on the exact conditions used for this *in vitro* assay (16,39). (vi) Recombinant purified PPARα has been used to show that LCFA-CoA or nonhydrolyzable LCFA-CoA analog binding increases PPARα co-activator recruitment (7). (vii) Manipulation of cellular LCFA-CoA levels affects HNF-4α transcriptional activity consistent with LCFA-CoA being endogenous HNF-4α ligands: Fatty acyl-CoA synthase overexpression enhances HNF-4α-mediated transactivation, whereas overexpression of fatty acyl-CoA hydrolase inhibits HNF-4α-mediated transactivation in living cells (1). Also, treatment with the fatty acyl-CoA synthase inhibitor triacsin C prevents suppression of HNF-4α transcriptional activity by inhibitory proligands (3).

In contrast to these findings, X-ray crystallographic analysis does not detect the presence of LCFA-CoA as endogenously bound ligands in HNF-4α and PPARα. Unfortunately, the X-ray crystallographic studies are limited by the inability to crystallize the full-length HNF-4α and PPARα. Thus, all available data were obtained with truncation mutants missing the DNA-binding domain and all or most of the C-terminal F domain. For example, X-ray analysis of crystalline recombinant LBD of both PPARα (17,35) and HNF-4α (18,19) does not contain any endogenously bound LCFA-CoA. In the case of PPARα, it is not yet completely clear whether the absence is due to the fact that the truncated nuclear receptors used for X-ray crystallography do not reflect the properties of the full-length PPARα or whether other factors contribute to the loss of LCFA-CoA. With regard to crystals of HNF-4α-E (i.e., the LBD) missing both the N-terminal DNA-binding domain and most of the C-terminal F negative regulatory domain, these crystals contain bound LCFA but not LCFA-CoA (18,19). *In vitro* binding assays performed in solution show that the truncated HNF-4α-E only binds LCFA with high affinity, but not LCFA-CoA (4), opposite to what was observed with the full-length HNF-4α or constructs containing the intact C-terminal F negative regulatory domain (2–4). In summary, the X-ray crystallographic findings with truncated proteins in the absence of water may not necessarily accurately reflect the endogenously bound ligand distribution of the respective full-length proteins in solution. As shown in the present investigation, several additional properties of LCFA-CoA and the nuclear receptors themselves also contribute to the inability to detect endogenously bound LCFA-CoA in these proteins.

First, the acyl chain distribution of the endogenously bound LCFA (3,18,19) reflected that of the bacterial LCFA-CoA pool (Table 2) rather than that of bacterial LCFA (3,18,19). Analysis of the LCFA associated with a variety of HNF-4α constructs revealed that 16:1 accounted for 50–65% of the total acyl chains of wild type HNF-4α (3,18,19). Although bacterial LCFA are enriched in 16:0 and 18:1, the level of 16:1 is barely

detectable in bacterial LCFA (3,18,19). In contrast, within the bacterial LCFA-CoA pool the 16:1-CoA was the single most prevalent LCFA-CoA (present data).

Second, the relative proportions of LCFA-CoA and LCFA in the recombinant bacteria heavily favored the availability of LCFA over LCFA-CoA for binding to the recombinant nuclear receptor proteins and their truncation mutants. The bacteria contained nearly 100-fold more LCFA than LCFA-CoA. Furthermore, the total concentration of LCFA-CoA was so low as to be sufficient for binding to only 20% of the available HNF-4α. Thus, even if the endogenous LCFA-CoA was not degraded during isolation of the respective recombinant proteins or by the nuclear receptors themselves, the vast majority of available nuclear receptor ligand-binding sites would be expected to be occupied by LCFA rather than LCFA-CoA.

Third, LCFA-CoA were unstable in the aqueous buffers used to isolate HNF-4α and PPARα or to crystallize the respective N- and C-terminal truncation mutants of HNF-4α and PPARα. Nearly 75–80% of LCFA-CoA was degraded by 1 d incubation in buffer alone. By 3 wk or longer, the incubation typically required to obtain crystalline protein, more than 97% of LCFA-CoA was degraded. Thus, the intrinsic instability of LCFA-CoA thioesters in aqueous buffers alone can significantly contribute to the inability to detect endogenously bound LCFA-CoA in the full-length nuclear receptors in solution or in crystals of truncation mutant proteins.

Fourth, although it is commonly assumed that bound ligands are more stable than in solution, this was the case for cytoplasmic LCFA-CoA binding proteins, but not the nuclear LCFA-CoA binding proteins. For example, the cytoplasmic LCFA-CoA-binding proteins ACBP, SCP-2, and L-FABP bound and protected LCFA-CoA from degradation directly in proportion to their relative affinities for this ligand. ACBP exhibits not only the highest affinity for LCFA-CoA in this group (reviewed in Refs. 8, 11, and 38), but was the most protective of LCFA-CoA hydrolysis. In fact, freshly isolated recombinant ACBP contains some detectable endogenously bound LCFA-CoA (42). In contrast, neither HNF-4α nor PPARα protected LCFA-CoA from degradation, but instead significantly accelerated LCFA-CoA hydrolysis by nearly 10- and 2-fold, respectively, within 1 d of incubation. Longer incubation periods reduced LCFA-CoA levels to almost nothing. Therefore, it is not surprising that despite the high affinities of full-length HNF-4α (aa 1–455) for LCFA-CoA, lipid extraction of full-length HNF-4α (aa 1–455) or HNF-4α truncation mutants and subsequent analysis did not detect the presence of endogenously bound LCFA-CoA (3,4,18,19). Instead, endogenously bound LCFA was detected even though LCFA is a much lower-affinity ligand for the respective proteins.

Together, the latter findings suggest that this enzymatic activity of nuclear receptors could operate to shut down LCFA-CoA-induced transcription activation/inhibition by LCFA-CoA processing such that it would not further affect the transcription activation function. Most models of molecular mechanisms underlining the ligand-induced activation function of nuclear receptors do not explain how the ligand effect is diminished and eliminated after the fact of modulation. Several possibilities could be considered: (i) The ligand is chemically modified (esterified, hydrolyzed, etc.) so that its binding in the LBD is reduced. In this case, the enzymatic activity required to modify the bound ligand could either be the nuclear receptor itself (as shown herein for HNF-4α and PPARα) or an entirely different protein proximal to it; (ii) complex formation of nuclear receptor molecules with co-activators/co-repressors may induce further conformational changes of the nuclear receptor, resulting in reduced affinity for the ligand and even its release from the binding site. Our data demonstrated that, like HNF-4α, PPARα hydrolyzed LCFA-CoA to free LCFA, and the effect of the catalytic activity of these nuclear receptors on the ligand-induced transcriptional activation function deserves further study. Taken together, these findings may help to explain the absence of endogenously bound LCFA-CoA, the presence of endogenously bound LCFA, and the acyl composition of the endogenously bound LCFA detected in a variety of recombinant HNF-4α proteins (3,19).

Finally, although the exact identity and structure of the esterase sites in HNF-4α and PPARα remain to be determined, comparisons with known long-chain acyl-CoA hydrolases/thioesterases may provide some insights. Long-chain acyl-CoA hydrolases/thioesterases are enzymes that catalyze the hydrolysis of fatty acyl-CoA to the corresponding FFA and CoA. Long-chain acyl-CoA hydrolases from rat liver microsomes (43,44), from rat liver and brain cytosol (45,46), and from rat liver mitochondria and peroxisomes (47,48) have been purified and characterized, demonstrating a wide variety of structures and mechanisms of catalysis. The long-chain acyl-CoA hydrolases from liver and brain cytosol are serine or cysteine-dependent esterases with a catalytic triad consisting of a nucleophile (serine or cysteine), an acidic group (aspartic or glutamic acid), and a histidine (43,46). Other forms of acyl-CoA hydrolases like those found in mitochondria and peroxisomes have an active site serine motif (Gly-X-Ser-X-Gly) common to carboxyl esterases and lipases in general (47,48). Although the existing X-ray crystal structures do not provide structural data on the F domain in HNF-4α (19,49) or in HNF-4γ (coded by a completely different gene and sharing only 37% sequence homology in the F domain with that of HNF-4α) (18), structural and sequence comparison with other long-chain acyl-CoA hydrolases/thioesterases suggests several possibilities comprising a potential hydrolytic/esterasic site. With regard to the catalytic motif of typical esterases containing a Gly-X-Ser-X-Gly motif, analysis of the full-length HNF-4α aa sequence indicated that there was no Gly-X-Ser-X-Gly motif within the LBD E (aa 132–370), the negative regulatory domain F (aa 371–455), or in any other parts of the protein. However, several residues of Ser, Cys, Glu/Gln, Asp/Asn, and His were present within the LBD E (aa 132–370) and the negative regulatory domain F (aa 371–455), suggesting that the acyl-CoA hydrolytic activity of HNF-4α may be explained by a coordinated action of Ser/Cys, Glu/Gln (or Asp/Asn), and His residues forming a 3-D triad positioned around the acyl-CoA thioester linkage. These aa can be separated by a variable number of aa, as long as the active

head groups are in the proper orientation to form a triad. The data presented herein demonstrated that only full-length HNF-4α (aa 1–455) and the truncation mutant containing the entire F domain (aa 132–455) exhibited acyl-CoA hydrolysis. In contrast, the truncation mutants containing a small part of the F domain (i.e., aa 132–410) or no F domain at all (i.e., aa 132–370) had no hydrolytic activity. Examination of the X-ray crystal structure of a truncated HNF-4α comprising the LBD (aa 132–382) (19,49) suggests that Cys246 and Glu184 are close to the carboxylic end of the FFA within the FA binding site. Only one histidine residue (His218) is present in the LBD (aa 132–382), but this residue is not close to the bound FA or FA carboxylate. Instead, the His involved in the catalytic activity could be provided by a histidine-rich sequence within the F domain (i.e., aa 371–455 containing His375, His377, His378, His381, His383, His388, and His402). The fact that the HNF-4α truncation mutant consisting of aa 132–410 did not exhibit acyl-CoA hydrolysis indicated that at least one of the three amino acids of the catalytic triad was missing. The HNF-4α F domain contains six serine residues (Ser427, Ser430, Ser432, Ser434, Ser436, and Ser452) and two Glu residues (Glu422 and Glu435) in the region between aa 411 to 455. These considerations would suggest that one or more of these Ser and/or His residues in the F domain, together with a Glu residue in the LBD E, could form a 3-D catalytic triad that accounts for the esterasic activity of HNF-4α.

Analysis of the PPARα aa sequence and structure (17) also suggests several possible esterasic motifs: (i) Analysis of the aa sequence of the LBD of PPARα reveals a potential SerX-HisXAsp motif (i.e., aa 414–418). However, as for the HNF-4α LBD, the crystal structure shows that these amino acids are not in the proper orientation/proximity to form an active esterase triad. (ii) Another potential esterase motif comprising Cys, Glu, His, and Asp is found at aa 191–194 of the PPARα (within the DNA-binding domain). However, since this region has not been crystallized, it is impossible to determine the orientation of the amino acids. Further, the presence of this region within the DNA-binding domain would suggest that it is not responsible for the acyl-CoA hydrolysis noted with the PPARα. (iii) Another potential esterase motif that may account for PPARα hydrolytic activity is composed of Gly, Ser, and Gly with one to two aa between them (50). Although a similar glycine- and serine-rich motif is present in the A/B domain of PPARα, comprising aa 42–50 (GlyXXSerSerGlySerXGly), this region is outside the area used for the crystallization studies and the recombinant protein used for the experiments described herein. Therefore, it is unlikely that this region was responsible for the acyl-CoA hydrolysis demonstrated in the Results section. (iv) Finally, a conserved motif of Cys, His, and Cys (formed by orientation rather than sequence order) has been suggested to serve as the catalytic site for thiolases (51). Examination of the X-ray crystal structure of PPARα reveals the presence of such a motif comprised of C278 folding in to interact with H274 and C275. The orientation of this motif and its location within the ligand-binding site suggests that this may represent an active thiolytic site. In summary, although the exact structure and location of the esterase sites in HNF-4α and PPARα are not known, examination of the respective aa sequences and available structures of truncation mutants of these proteins suggests several possible motifs consistent with those of known fatty acyl-CoA hydrolase/esterase enzymes.

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