Acyl-CoA Binding Proteins Inhibit the Nonenzymic S-Acylation of Cysteinyl-Containing Peptide Sequences by Long-Chain Acyl-CoAs†

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ABSTRACT: Acyl-CoA binding proteins (ACBPs) from rat and bovine liver were found to inhibit the nonenzymic S-acylation of two representative types of peptides by long-chain acyl-CoAs. As demonstrated previously [Quesnel, S. & Silvius, J. R. (1994) *Biochemistry 33* 13340−13348; Bharadwaj, M., & Bizzozero, O. A. (1995) *J. Neurochem. 65*, 1805−1815], peptides with the sequences myristoyl-GCG, myristoyl-GCV, and IRYCWLRR-NH_2, all representing physiological S-acylation sites in mammalian proteins, become S-acylated at appreciable rates in the presence of long-chain acyl-CoAs and large unilamellar lipid vesicles. Addition of ACBP at physiological molar ratios with respect to long-chain acyl-CoAs strongly inhibits the spontaneous S-acylation reaction, in a manner that can be quantitatively described by assuming that the ACBP sequesters the acyl-CoA with nanomolar affinity in a complex unable to serve as an *S*-acyl donor. From these results, we calculate that at physiological (intracellular) concentrations of ACBP, long-chain acyl-CoAs, and membrane lipids the expected half-times for spontaneous S-acylation of such protein sequences by long-chain acyl-CoAs will lie in the range of several tens of hours. The nonenzymic reaction of protein cysteine residues with long-chain acyl-CoAs is thus unlikely to contribute significantly to the physiological modification of signaling and other proteins that show relatively rapid rates of S-acylation in mammalian cells. However, it cannot be excluded that a nonenzymic reaction with long-chain acyl-CoAs could contribute to the physiological S-acylation of certain membrane proteins if the latter exhibit very slow kinetics of S-acylation *in vivo*.

A variety of permanently and reversibly membrane-associated proteins have been reported to be S-acylated in eukaryotic cells (Schmidt, 1989; Schlesinger et al., 1993; Bouvier et al., 1995; Casey, 1995; Jackson et al., 1995; Milligan et al., 1995; Ross, 1995). To date, the mechanisms of S-acylation of most such proteins remain ill-defined. Enzymic activities have been characterized that mediate the S-acylation of several membrane-associated proteins, including both integral membrane proteins such as the envelope proteins of Semliki Forest virus (Schmidt & Burns, 1989) and reversibly membrane-associating proteins such as N-*ras*, the α-subunits of various heterotrimeric G-proteins, and several *src*-homologous nonreceptor tyrosine kinases (Gutierrez & Magee, 1991; Berthiaume & Resh, 1995; Dunphy et al., 1996; Liu et al., 1996). However, a variety of cellular proteins, including rhodopsin (O'Brien et al., 1987; Moench et al., 1994), the myelin proteins lipophilin (Bizzozero et al., 1987; Ross & Braun, 1988) and P_o (Bharadwaj & Bizzozero, 1995), the α-subunits of certain heterotrimeric G-proteins (Duncan & Gilman, 1996), and pulmonary surfactant protein SP-C (Qunbar & Possmayer, 1994), have been found to undergo spontaneous S-acylation *in vitro* in the presence of long-chain acyl-CoAs.[1] Simple cysteinyl-containing (lipo)peptides have also been shown to undergo spontaneous S-acylation in model systems when incubated with long-chain acyl-CoAs or other *S*-acyl donors (Quesnel & Silvius, 1994; Bharadwaj & Bizzozero, 1995; Jackson et al., 1995).

Whether spontaneous S-acylation of proteins can proceed efficiently under physiological conditions, in which the available concentrations of *S*-acyl donors may be considerably lower than those typically used for *in vitro* assays, is an unresolved question. Long-chain acyl-CoAs represent a potentially important pool of *S*-acyl donors for protein autoacylation *in vivo*. However, within the cytoplasm, long-chain acyl-CoAs bind reversibly and with relatively high affinity to acyl-CoA binding proteins (ACBPs) (Mikkelsen & Knudsen, 1987; Knudsen et al., 1989, 1994; Rasmussen et al., 1990; Rosendal et al., 1993). ACBP has been estimated to be present in significant molar excess over total cytoplasmic long-chain acyl-CoAs in a variety of different eukaryotic cell types examined (Knudsen et al., 1989; Rasmussen et al., 1993). As a result, ACBP is expected to reduce significantly the concentrations of free long-chain acyl-CoAs, both in the cytoplasm and in the cytoplasmic

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[1] Abbreviations: ACBP, acyl-CoA binding protein; CoA, coenzyme A; DOPS, 1,2-dioleoylphosphatidylserine; DTPA, diethylenetriaminepentaacetic acid; DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid sodium salt; MOPS, 3-(*N*-morpholino)propanesulfonic acid sodium salt; N-BioPE, *N*-(biotinylamidocaproyl) phosphatidylethanolamine; N-RhoPE, *N*-(lissamine rhodamine sulfonyl) phosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; TLC, thin-layer chromatography. Peptide sequences are indicated using the standard one-letter amino acid code and the additional abbreviations myr for an N-terminal myristoyl group and -CaBim for an *S*-bimanylcysteamido group.

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surfaces of cellular membranes, and consequently to attenuate processes that are mediated by free (soluble or membrane-bound) long-chain acyl-CoAs. In agreement with this suggestion, *in vitro* assays have demonstrated that ACBP effectively antagonizes the inhibitory effects of long-chain acyl-CoAs on the activities of proteins such as acetyl-CoA carboxylase and the mitochondrial adenine nucleotide translocase (Rasmussen et al., 1993).

In this study, we have examined the effects of two mammalian ACBPs on the spontaneous transfer of *S*-acyl groups from long-chain acyl-CoAs to synthetic cysteinyl-containing (lipo)peptides that correspond to physiological sites of S-acylation in cellular proteins. Peptides were used as *S*-acyl acceptors in these experiments because, in contrast to the parent proteins, they can be studied in completely defined synthetic systems that are assured to be free of extrinsic *S*-acyltransferase activities. The sequences myristoyl-GCG and myrGCV represent a major site of S-acylation in the human and murine forms, respectively, of the nonreceptor tyrosine kinase $p56^{lck}$ (Shenoy-Scaria et al., 1993; Koegl et al., 1994; Resh, 1994; Yurchak & Sefton, 1995), and peptides incorporating this motif are readily S-acylated *in vitro* by a nonenzymic reaction when incubated with long-chain acyl-CoAs and lipid vesicles (Quesnel & Silvius, 1994). The synthetic octapeptide IRYCWLRR-NH_2, representing the physiological site of S-acylation of the P_o glycoprotein of peripheral myelin (Bizzozero et al., 1994), has likewise been shown to undergo efficient spontaneous S-acylation *in vitro* in the presence of acyl-CoAs (Bharadwaj & Bizzozero, 1995). In the present study, we demonstrate that in the presence of physiological concentrations of ACBP, long-chain acyl-CoAs, and lipid membranes the rates of spontaneous S-acylation of such peptides by acyl-CoAs are far slower than the rates of *in vivo* S-acylation reported for newly biosynthesized proteins or for signaling proteins such as N-*ras* or $G_{s\alpha}$. However, our results do not entirely rule out the possibility that certain membrane proteins, which appear to exhibit very slow kinetics of S-acylation *in vivo*, might become S-acylated physiologically by spontaneous reaction with long-chain *S*-acyl-CoAs.

MATERIALS AND METHODS

Materials. Palmitoyl-, oleoyl-, and stearoyl-CoA and monobromobimane were obtained from Sigma (St. Louis, MO). [9,10-^{3}H]palmitoyl-CoA was synthesized from [9,-10-^{3}H]palmitic acid (DuPont Canada, 40−80 Ci/mmol, accurately diluted with unlabeled palmitate to 1.0 or 0.1 mCi/μmol), using the procedure of Bishop and Hajra (1980). Thin-layer chromatography of the labeled acyl-CoA in 50:30:20 (v:v:v) 1-butanol/acetic acid/water followed by autoradiography revealed a single major band comigrating with authentic palmitoyl-CoA (>95% of total radioactivity by scintillation counting) and minor contamination with free fatty acid. Acyl-CoA stocks were stored at −80 °C in 5 mM potassium phosphate at pH 6.0 and transfered at relatively high (normally millimolar) concentrations and using glass vessels to minimize the loss of acyl-CoA through adsorption. The peptide IRYCWLRR-NH_2 was prepared by Fmoc-based solid phase synthesis on Rink amide MBHA resin (Novabiochem), using as side chain-protecting groups 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Arg), *tert*-butyl (Tyr), trityl (Cys), and *tert*-butoxycarbonyl (Trp). The completed, N-terminally deblocked peptide was cleaved from the resin using 82.5:5:5:2.5 trifluoroacetic acid/water/phenol/dimethyl sulfide/ethanedithiol (5 h at 25 °C), dried, precipitated three times from methanol with ether, and finally purified by HPLC on a C18/C2 reverse phase column (SuperPac Prep-S, Pharmacia, Point-Claire, Québec), eluting with a gradient of 0 to 50% acetonitrile in water. A portion of the purified peptide was labeled with a small excess of monobromobimane in 2:1 methanol/0.1 M HEPES at pH 7.4 and precipitated four times from methanol with diethyl ether, yielding a single fluorescent spot by thin-layer chromatography in 45:30:10:10 1-butanol/pyridine/acetic acid/water.

Rat and recombinant bovine liver ACBP were purified as described (Mandrup et al., 1991; Knudsen et al., 1989); stock solutions prepared in sterile 50 mM MOPS at pH 7.4 were standardized by absorbance at 280 nm (using extinction coefficients of 15.22 mM^{-1} cm^{-1} for rat ACBP and 16.48 mM^{-1} cm^{-1} for bovine ACBP), stored at 4 °C, and normally used within 2 weeks of preparation.

Methods. S-Acylation of myrGCG-CaBim with unlabeled acyl-CoAs in the presence of lipid vesicles was assayed as described previously (Quesnel & Silvius, 1994) with minor modifications. Briefly, large unilamellar lipid vesicles (POPC/POPE/DOPS, 4:4:2 molar proportions), prepared by extrusion through 0.1 μm pore size polycarbonate filters (MacDonald et al., 1991), were preincubated (under argon and in the dark) for 15 min with cysteinyl-containing peptides at final concentrations of 100 mM NaCl, 50 mM HEPES, 1 mM $MgCl_2$, 1 mM DTT, 50 μM DTPA, 2 mM lipid, and 20 μM peptide at pH 7.3. Acyl-CoA (preincubated where relevant with ACBP for 15 min at 37 °C) was then added to 20 μM, and the samples were incubated at 37 °C under argon and with exclusion of light. At varying times, 100 μL samples were withdrawn and the extent of peptide S-acylation was determined by TLC separation and fluorescence quantitation of the unacylated and S-acylated peptide as described previously (Quesnel & Silvius, 1994).

Incubation of myrGCG- or myrGCV-CaBim with [^{3}H]-palmitoyl-CoA (specific activity of 100 μCi/μmol) was carried out as above, but using final concentrations of 40 μM myristoyl-peptide, 10 μM acyl-CoA, and 2 mM lipid vesicles. Samples were extracted and dried as above, mixed with 1 nmol of unlabeled S-palmitoylated peptide, and applied to silica gel G plates (Whatman, PE Sil G), which were developed with 94:6:0.2 (v:v:v) CH_2Cl_2/methanol/acetic acid. The S-acylated peptide band was visualized on the dried plates by fluorescence, scraped into scintillation vials, and incubated for 24 h with 15 mL of Cytoscint (ICN Canada, St. Laurent, Québec) before counting.

S-Acylation of IRYCWLRR-NH_2 with [^{3}H]palmitoyl-CoA was assayed by a modification of the method of Bharadwaj and Bizzozero (1995). Briefly, large unilamellar lipid vesicles (40:40:20 POPC/POPE/DOPS, 5 mM) were incubated with 100 μM peptide and 20 μM [^{3}H]palmitoyl-CoA (specific activity of 1 mCi/μmol) in 50 mM MOPS, 1 mM DTT, and 50 μM DTPA at pH 7.4 nd 37 °C under argon. After incubation for the indicated times, 20 μL samples were withdrawn and rapidly chilled, mixed with 1 mL of methanol containing 1% acetic acid, and dried under a stream of nitrogen. The dried samples were applied to silica gel G plates (Whatman, PE Sil G) which were developed in 45:25:8:8 1-butanol/pyridine/acetic acid/water. The developed plates were imaged by autoradiography after spraying with En^{3}Hance (DuPont Canada, Mississauga,

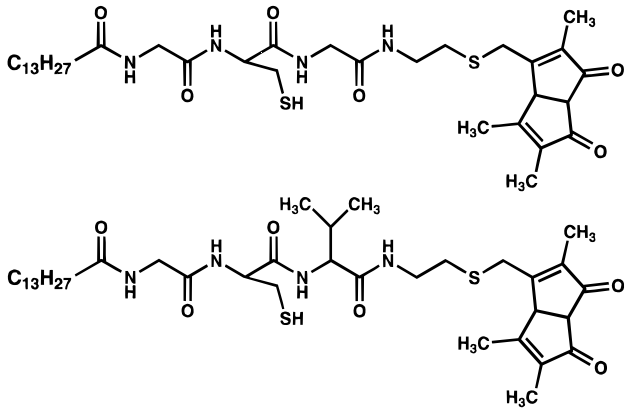

FIGURE 1: Structures of myrGCG- and myrGCV-CaBim.

Ontario), and the visualized radioactive bands were moistened, scraped into 15 mL of Cytoscint, and quantitated by scintillation counting after incubation for 24 h at room temperature.

Binding of IRYC(bimanyl)WLRR-NH_2 to lipid vesicles (POPC/POPE/DOPS/N-BioPE/N-RhoPE, 40:40:20:2:0.2 molar proportions) was assayed by incubating labeled peptide (2 nmol) with vesicles (100−500 nmol) for 15 min in 100 μL of 50 mM MOPS and 50 μM DTPA at pH 7.4 and room temperature. Avidin (10 μg) was added, and after further incubation for 20 min, the samples were centrifuged (20 min, 13600*g*, 37 °C) to pellet the vesicles. Each supernatant (50 μL) was then mixed with 3 mL of methanol, and the concentration of unsedimented bimanyl-peptide and N-RhoPE-containing vesicles was determined by fluorescence using a Perkin-Elmer LS-50 spectrofluorimeter (excitation and emission settings of 390 and 468 nm, respectively, for bimanyl-peptide and of 525 and 596 nm, respectively, for N-RhoPE).

RESULTS

S-Acylation of myrGCG- and myrGCV-CaBim. Using a fluorescence-based assay, we have previously shown that myristoylated and prenylated peptides containing a free cysteinyl residue readily undergo spontaneous S-acylation at physiological pH when incubated with long-chain acyl-CoAs in the presence of lipid vesicles (Quesnel & Silvius, 1994). Using [^{3}H]palmitoyl-CoA as the *S*-acyl donor and the myristoylated peptides myrGCG-CaBim and myrGCV-CaBim (Figure 1) as acceptors, formation of S-[^{3}H]palmitoylated lipopeptide under these conditions was readily demonstrated by TLC separation and scintillation counting of the products as described in Materials and Methods. In preliminary experiments, we established that coincubation of myrGCG-CaBim (40 μM), large unilamellar lipid vesicles (POPC/POPE/DOPS, 40:40:20 molar proportions, 2 mM), and [^{3}H]palmitoyl-CoA (10 μM) led to the appearance of a radiolabeled band that comigrated exactly with authentic S-palmitoylated peptide upon TLC analysis and which was not formed in control incubations without myristoyl-peptide (Figure 2A). Incorporation of radioactivity into the S-palmitoylated peptide band was linear with time for at least 2 h of incubation at 37 °C (data not shown) and was therefore routinely determined from the extent of reaction after 2 h of incubation at this temperature.

Inclusion of increasing amounts of either rat or bovine ACBP in the above reaction mixtures led to a progressive

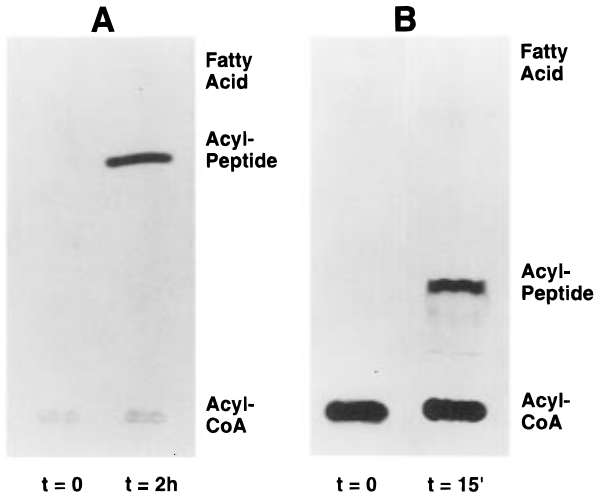

FIGURE 2: Autoradiograms of products of reaction of [^{3}H]palmitoyl-CoA with cysteinyl peptides in the presence of 40:40:20 (molar proportions) POPC/POPE/DOPS vesicles. (A) [^{3}H]Palmitoyl-CoA (specific activity of 100 μCi/μmol), myrGCG-CaBim, and vesicles (10 μM, 40 μM, and 2 mM, respectively) were incubated for 2 h at 37 °C, and the reaction products were extracted and analyzed by thin-layer chromatography (developing solvent, 94:6:0.2 CH_2Cl_2/methanol/acetic acid) and autoradiography as described in Materials and Methods. (B) [^{3}H]Palmitoyl-CoA (specific activity of 1 mCi/μmol), IRYCWLRR-NH_2, and vesicles (20 μM, 100 μM, and 5 mM, respectively) were incubated for 15 min at 37 °C, and the reaction products were analyzed by thin-layer chromatography (developing solvent, 45:25:8:8 1-butanol/pyridine/acetic acid/water) and autoradiography as described in Materials and Methods. The lipid-extraction procedure used to prepare the samples for panel A gave low recovery of [^{3}H]palmitoyl-CoA but essentially quantitative recovery of *S*-acyl peptide, free fatty acids, and acyl-DTT thioesters, as shown by control experiments. The autoradiogram in panel A thus shows only a faint band of [^{3}H]palmitoyl-CoA, while the sample in panel B, which was prepared without solvent extraction, shows a prominent [^{3}H]acyl-CoA band.

inhibition of the spontaneous S-[^{3}H]palmitoylation of liposome-associated myrGCG-CaBim (Figure 3). An almost identical pattern of inhibition was observed for S-acylation of myrGCV-CaBim under the same conditions (not shown). While ACBP and [^{3}H]palmitoyl-CoA were routinely preincubated together for 15 min in these experiments, much shorter times of preincubation (2−5 min) were sufficient to produce results similar to those presented here. The pattern of ACBP inhibition was unaltered when the buffer concentration was increased to 200 mM HEPES (not shown), ruling out the trivial possibility that the effect of ACBP addition could be due to perturbation of the pH of the incubation mixtures. Likewise, ACBP was shown not to promote breakdown of palmitoyl-CoA in control incubations.

The dependence of the inhibition of peptide S-acylation on the ACBP concentration shown in Figure 3 cannot be well described by a simple rectangular-hyperbolic equation. However, as described in the Appendix, the inhibition curves can be very well fit by modeling the equilibria of palmitoyl-CoA between aqueous, vesicle-bound, and ACBP-complexed forms, assuming that the ACBP-bound form is inactive in the S-acylation reaction. Using the data of Peitzsch and McLaughlin (1993) to estimate the vesicle/water partition coefficient of palmitoyl-CoA (K_p') as roughly 1.45×10^5 M^{-1}, the curves describing the inhibition of S-palmitoylation of myrGCG-CaBim by either rat or bovine liver ACBP are

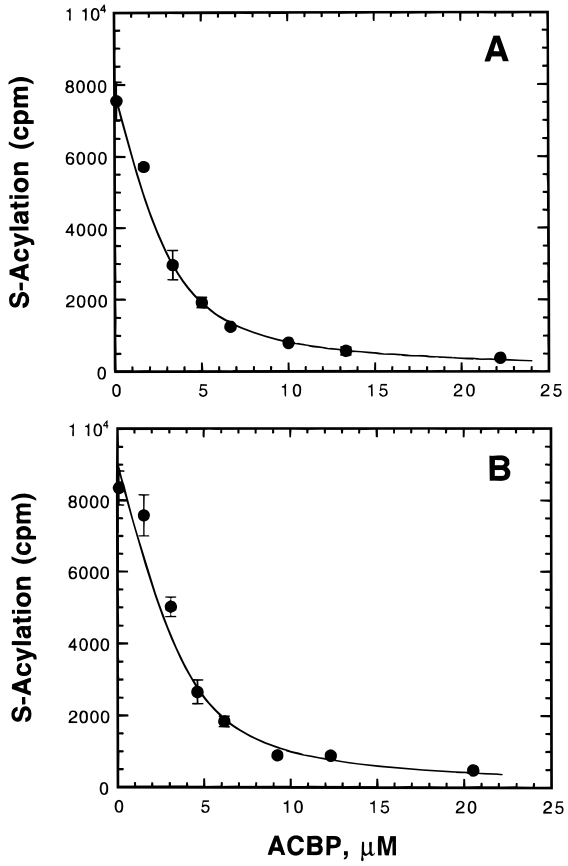

FIGURE 3: Inhibition of S-acylation of myrGCG-CaBim in [^{3}H]-palmitoyl-CoA-containing lipid vesicles by rat liver ACBP (A) or bovine liver ACBP (B). [^{3}H]Palmitoyl-CoA, myrGCG-CaBim, and lipid vesicles (10 μM, 40 μm, and 2 mM, respectively) were incubated at 37 °C, and the rate of S-acylation was determined as described in Materials and Methods. Data points represent the average (±SEM) for a total of nine samples at each ACBP concentration, collected in three separate experiments. The curve shown in panel A was fitted to the data using the model described in the Appendix with the parameter values K_d(ACBP·palmitoyl-CoA) = 5.8 nM and $K_p' = 1.45 \times 10^5$ M^{-1} for palmitoyl-CoA partitioning between the aqueous phase and the vesicle surfaces. The curve shown in panel B was similarly fit assuming $K_d = 5.9$ nM and the same value for K_p'. As discussed in the text, in order to fit the data satisfactorily, it was also necessary to assume that the actual final concentration of palmitoyl-CoA in the reaction mixtures was 4.2 μM (A) or 4.4 μM (B), while the nominal input was 10 μM.

well fit using values of 5.8×10^{-9} M (rat) or 5.9×10^{-9} M (bovine) for the dissociation constant (K_d) for the palmitoyl-CoA−ACBP complex (solid curves, Figure 3). Given that the estimated value of K_d is strongly affected by the value assumed for K_p', these values are in good agreement with those recently estimated for binding of palmitoyl-CoA to these ACBPs (8.9 ± 5.3 nM for rat ACBP and 2.1 ± 1.9 nM for bovine ACBP) on the basis of microcalorimetric measurements made in a medium with an ionic strength similar to that used here (J. K. Knudsen et al., unpublished results).

One surprising feature of the data shown in Figure 3 is the fact that the steeply descending initial phase of each inhibition curve extrapolates to the *x*-axis at a concentration of ACBP significantly less than the nominal concentration of added acyl-CoA, in contrast to the result expected given the previously observed 1:1 stoichiometry of acyl-CoA−ACBP binding (Rasmussen et al., 1989; Knudsen et al., 1990; Kragelund et al., 1993). The simplest explanation for this result would be that the actual concentration of [^{3}H]-palmitoyl-CoA in the reaction mixtures was substantially lower (by roughly 2-fold) than the nominal concentration added. In control experiments, we were unable to demonstrate that either errors in the specific activity of the [^{3}H]-palmitoyl-CoA, hydrolytic breakdown of the acyl-CoA or adsorptive loss of the acyl-CoA on surfaces during transfer, storage, and incubation could reduce to this degree the actual concentration of palmitoyl-CoA in the above reactions. Nonetheless, in analyzing the ACBP inhibition curves shown in Figure 3 (and in Figure 6, discussed later), we have included the actual concentration of [^{3}H]palmitoyl-CoA as an adjustable parameter, which may (for unknown reasons) be markedly lower than the nominal input value. This approach has the virtue that it leads to a *minimum* estimate of the degree to which ACBP will inhibit spontaneous S-acylation of cysteinyl residues at any given concentration of acyl-CoA and ACBP.

Table 1: Rat ACBP-Mediated Inhibition of S-Acylation of myrGCG-CaBim by Different S-Acyl-CoAs[a]

Acyl-CoA (20 μM)	percent inhibition of S-acylation by 30 μM ACBP
palmitoyl	94.5 ± 6.1
oleoyl	90.1 ± 2.2
stearoyl	95.6 ± 0.5

[a] Reaction mixtures containing lipid vesicles (2 mM), acyl-CoA (20 μM), and myrGCG-CaBim (20 μM) were incubated in 100 mM NaCl, 50 mM HEPES, 1 mM $MgCl_2$, 1 mM DTT, and 50 μM DTPA at pH 7.3 for 2 h at 37 °C with or without the prior addition of rat ACBP (30 μM). After incubation for various times, samples were withdrawn and the extent of S-acylation of myrGCG-CaBim was determined as described in Materials and Methods. Data presented represent the derived rates of S-acylation in the presence vs the absence of ACBP, determined from two independent sets of samples in each of two experiments.

To assess whether ACBP also inhibits the S-acylation of myrGCG-CaBim by other long-chain acyl-CoAs, we incubated the cysteinyl-lipopeptide with nonradioactive palmitoyl-, oleoyl-, or stearoyl-CoA in lipid vesicles (with or without ACBP) and then quantitated the production of S-acylated lipopeptide by fluorescence as described in Materials and Methods. As reported previously (Quesnel & Silvius, 1994), no detectable S-acyl-peptide is formed (<1% of input peptide) when myrGCG-CaBim is incubated in POPC/POPE/DOPS (40:40:20) vesicles for up to 6 h at 37 °C in the absence of long-chain acyl-CoA. By contrast, a substantial fraction of the cysteinyl-lipopeptide becomes S-acylated after incubation for 1−2 h when 1 mol % acyl-CoA is added to the vesicles. As shown in Table 1, ACBP in a 1.5:1 molar ratio with respect to acyl-CoA causes a similar inhibition of lipopeptide S-acylation when either palmitoyl-, oleoyl-, or stearoyl-CoA is used, indicating that ACBP competes effectively with lipid bilayers for binding of all three long-chain acyl-CoAs.

S-Acylation of IRYCWLRR-NH$_2$. The juxtamembrane octapeptide sequence IRYCWLRR has been shown to represent the physiological site of S-acylation of the peripheral myelin P_o glycoprotein (Bizzozero et al., 1994), and the corresponding octapeptide amide is spontaneously S-acylated *in vitro* by acyl-CoAs in the presence of detergent micelles (Bharadwaj & Bizzozero, 1995). We therefore examined the effects of ACBP on the acylation of this peptide by [^{3}H]-

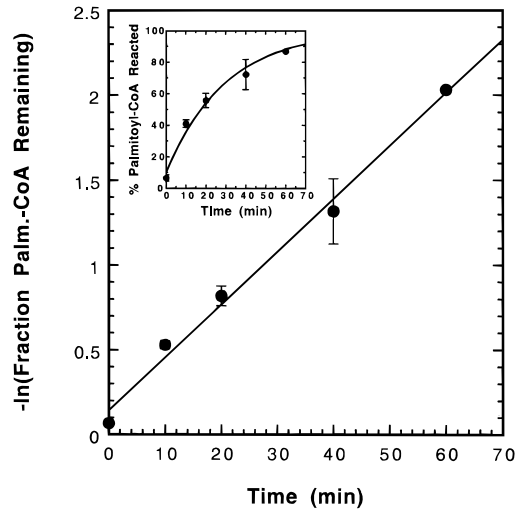

FIGURE 4: Time course of formation of [^{3}H]palmitoylated IRYCWLRR-NH_2 upon incubation of peptide (100 μM), [^{3}H]palmitoyl-CoA (20 μM), and lipid vesicles (5 mM) in 50 mM MOPS, 1 mM DTT, and 50 μM DTPA at pH 7.4. In the main figure, the data are plotted in the appropriate manner to linearize the time course of a pseudo-first-order reaction, while the inset presents the untransformed data. Data shown represent the mean (±standard deviation) determined for three samples at each time point in a representative experiment; the estimate of k' reported in the text was determined from four such experiments. Other experimental details were as described in Materials and Methods.

palmitoyl-CoA in the presence of lipid vesicles (POPC/POPE/DOPS, 40:40:20 molar proportions). To facilitate the interpretation of the results of these experiments, we first characterized the nature of the S-acylation reaction in this system, as described below.

When peptide amide, [^{3}H]palmitoyl-CoA, and lipid vesicles (100 μM, 20 μM, and 5 mM, respectively) were incubated together at 37 °C, formation of radiolabeled S-acyl-peptide could be readily detected, as reported previously by Bharadwaj and Bizzozero (1995). This product and unreacted palmitoyl-CoA were the major radioactive species detected by autoradiography (Figure 2B); no significant formation of the dithiothreitol thioester of [^{3}H]palmitate was detected, in contrast to the results obtained using Triton X-100 micelles in similar incubations [this study (not shown) and Bharadwaj and Bizzozero (1995)]. The time course of reaction under our standard conditions was well-described by a first-order rate equation, as expected for a simple bimolecular reaction when the peptide component is present in large excess (Figure 4). In subsequent experiments, a standard reaction time of 20 min was used, and the effective rate constant for the reaction was estimated assuming pseudo-first-order kinetics as just noted.

From data like those shown in Figure 4, an apparent second-order rate constant for the S-acylation reaction can be estimated, yielding a value of 305 ± 13 min^{-1} M^{-1}, a value similar to that reported by Bharadwaj and Bizzozero (1995) for the analogous reaction in the presence of Triton X-100 micelles. However, under the experimental conditions used in the present study, essentially all (>99%) of the added [^{3}H]palmitoyl-CoA will be bound to the lipid vesicles[2] (Peitzsch & McLaughlin, 1993; Requero et al., 1995). Given this fact, the S-acylation reaction is expected to proceed largely at vesicle surfaces rather than in solution, in which case the reaction rate is more meaningfully expressed as a function of the *surface* concentrations of the reactants:

$$\frac{\text{d[S-acyl-peptide]}}{\text{dt}} = k'x(\text{peptide})_{surf}x(\text{palm-CoA})_{surf}[\text{lipid}]_{eff} \quad (1)$$

where k' is the dimensionless rate constant relating the reaction rate to the surface concentrations (mole fractions x_{surf}) of the two components and $[\text{lipid}]_{eff}$ is the concentration of lipids exposed at the vesicles' outer surfaces. In order to determine the value of k' in the above equation, it is necessary to estimate the surface concentration of vesicle-bound peptide as well as that of vesicle-bound acyl-CoA. This was accomplished as described below.

To estimate the extent of binding of IRYCWLRR-NH_2 to lipid vesicles under the conditions of our S-acylation assays, we employed two different experimental approaches. First, using a centrifugation-based assay as described in Materials and Methods, we measured the binding of the *S*-bimanyl-labeled peptide to lipid vesicles identical to those used in the S-acylation assays. Previous studies (Skerjanc et al., 1987; Silvius & l'Heureux, 1994) have shown that *S*-bimanyl labeling of various amphipathic (lipo)peptides does not substantially alter the affinities of the peptides for lipid bilayers. At the pH, ionic strength, and peptide:lipid ratio used in our S-acylation assay, >90% of IRYC(bimanyl)-WLRR-NH_2 was found to be vesicle-bound at lipid concentrations of ≥1.5 mM, suggesting that a similarly high proportion of the unmodified peptide will be vesicle-bound under the standard conditions of our S-acylation measurements (5 mM lipid). Increasing the ionic strength of the assay buffer to 150 mM (using NaCl) reduced the fraction of vesicle-bound peptide to ca. 50% at 5 mM lipid and in parallel experiments decreased the rate of S-acylation of the unlabeled peptide by roughly 50% at the same lipid concentration (not shown).

To corroborate the above binding results, we measured the rate of *S*-[^{3}H]acyl-peptide formation from unmodified IRYCWLRR-NH_2 upon incubation of the peptide (100 μM) with varying concentrations of lipid vesicles (2.5−20 mM), incorporating a fixed proportion of [^{3}H]palmitoyl-CoA (0.2 mol %). If we assume that the S-acylation reaction proceeds mainly at vesicle surfaces, from eq 1, we predict that in this experiment the rate of formation of *S*-[^{3}H]acyl-peptide will increase with increasing lipid concentration when only a portion of the peptide is vesicle-bound but will plateau when the fraction of bound peptide approaches 100%. By contrast, if the reaction were occurring mainly in solution rather than at vesicle surfaces, the rate of formation of *S*-[^{3}H]acyl-peptide should strongly *decrease* with increasing lipid concentration.[3] As shown in Figure 5, in the experiment just described, the initial rate of formation of [^{3}H]palmitoylated peptide essentially plateaus at lipid concentrations of ≥5 mM, as expected if the reaction occurs predominantly at the vesicle

[2] By extrapolation from the data of Peitzsch and McLaughlin (1993), we can estimate the bilayer/water partition coefficient for palmitoyl-CoA as roughly 1.45×10^5 M^{-1}. Large unilamellar lipid vesicles similar to those employed in this study expose 46 ± 2% of their total phospholipid at their outer surfaces (Shahinian & Silvius, 1995); in a 5 mM suspension of such vesicles, the concentration of lipids exposed to the extravesicular medium is thus ca. 2.3 mM. Using these values, we can calculate that >99% of the palmitoyl-CoA will be membrane-bound under the experimental conditions used to measure S-acylation of IRYCWLRR-NH_2. This conclusion would not be substantially altered if the bilayer/water partition coefficient for palmitoyl-CoA in the present systems differed even by severalfold from the value given above.

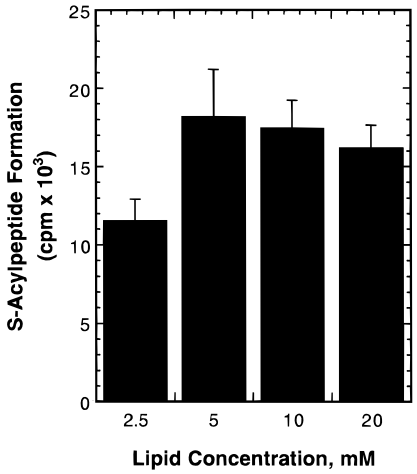

FIGURE 5: Relative efficiency of formation of [^{3}H]palmitoylated IRYCWLRR-NH_2 when the peptide (100 μM) was incubated with varying concentrations of lipid vesicles incorporating a fixed proportion (0.2 mol %) of [^{3}H]palmitoyl-CoA. Data shown represent the mean (±standard deviation) of three samples at each lipid concentration in a representative experiment. A duplicate experiment (not shown) gave a similar pattern of results (notably including a very similar ratio of the extents of *S*-acyl peptide formation at 2.5 and 5 mM lipid) but slightly higher absolute extents of *S*-acyl peptide formation at all lipid concentrations. Other details of experimental methods and interpretation are given in the text.

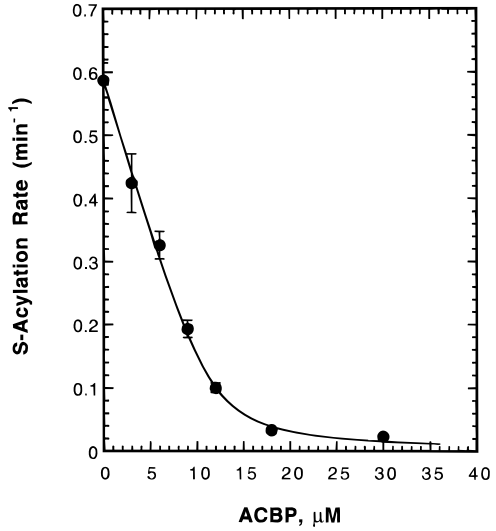

FIGURE 6: Inhibition by rat liver ACBP of S-acylation of IRYCWLRR-NH_2 adsorbed to palmitoyl-CoA-containing lipid vesicles. [^{3}H]Palmitoyl-CoA, myrGCG-CaBim, and lipid vesicles (20 μM, 100 μM, and 5 mM, respectively) were incubated for 20 min at 37 °C, and the rate of S-acylation was determined as described in Materials and Methods. Data points represent the average (±SEM) for a total of nine samples at each ACBP concentration, collected in three separate experiments. The curve shown was fitted to the data using the model described in the Appendix with parameter values K_d(ACBP·palmitoyl-CoA) = 5.8 nM and $K_p' = 3.8 \times 10^4$ M^{-1} for palmitoyl-CoA partitioning between the aqueous phase and the vesicle surfaces, and assuming that the actual final concentration of palmitoyl-CoA in the reaction mixtures was 9.6 μM while the nominal input of this reactant was 20 μM.

surfaces and if (as the above *S*-bimanyl-peptide binding results suggest) virtually all of the peptide is bound to the vesicles at lipid concentrations of ≥5 mM. Applying eq 1 to data like those shown in Figure 4, and assuming that under our reaction conditions >90 and >99% of the peptide and the [^{3}H]palmitoyl-CoA, respectively, are bound to the vesicles [at their outer surface (Boylan & Hamilton, 1992)], we estimate a value for the rate constant k' of 0.76 ± 0.04 min^{-1} (mean of four determinations) for the S-acylation of IRYCWLRR-NH_2 by palmitoyl-CoA at the vesicle surface.

The presence of rat ACBP strongly reduces the rate of spontaneous S-acylation of IRYCWLRR-NH_2, by vesicle-associated [^{3}H]palmitoyl-CoA, as shown in Figure 6. As was found above using myrGCG-CaBim as the acceptor peptide, the inhibition of S-acylation by ACBP can be well described by assuming that ACBP sequesters the palmitoyl-CoA with nanomolar affinity in a complex unable to participate in the spontaneous S-acylation reaction (see the Appendix). In order to fit the data in Figure 6 using the value for K_d(ACBP·palmitoyl-CoA) estimated from the myrGCG-CaBim S-acylation experiments described above (5.8 nM), it was necessary to use a value for K_p', the water/vesicle partition coefficient for [^{3}H]palmitoyl-CoA, that was roughly 4-fold smaller than in the experiments using myrGCG-CaBim as the *S*-acyl acceptor. A reduced value for K_p' is plausible in the reactions using IRYCWLRR-NH_2 as the acyl acceptor, as the lower ionic stength and the absence of divalent cations in these reaction mixtures will augment charge repulsions between the anionic vesicle surfaces and palmitoyl-CoA molecules.

DISCUSSION

The results presented in this study demonstrate that, at physiological concentrations of long-chain acyl-CoAs and of bilayer-forming phospholipids, ACBP can sharply reduce the rates of spontaneous S-acylation of two very different types of (lipo)peptide substrates at the bilayer surface. The experimental confirmation that long-chain acyl-CoAs bound to ACBP cannot participate in spontaneous *S*-acyl transfer reactions supports previous speculation (Quesnel & Silvius, 1994; Reverey et al., 1996) that in the presence of physiological levels of ACBP nonenzymic S-acylation of proteins by long-chain acyl-CoAs will be strongly suppressed.

Our findings with the model substrates myrGCG- and myrGCV-CaBim, containing the N-terminal myrGC- S-acylation motif found in a variety of G-protein α-subunits and nonreceptor tyrosine kinases, can be well described using a simple model in which ACBP sequesters long-chain acyl-CoAs with nanomolar affinity in a complex that cannot transfer *S*-acyl groups spontaneously to cysteinyl residues. Spontaneous S-acylation of the octapeptide IRYCWLRR-NH_2, representing the juxtamembrane S-acylation site of the P_o glycoprotein of peripheral myelin, is inhibited by ACBP in a quantitatively similar maner. These S-acylation reactions proceed largely at vesicle surfaces under our experimental conditions [this study and Quesnel and Silvius (1994)]. Our results are thus consistent with prior demonstrations showing

[3] In the experiment presented in Figure 4, the surface concentration of [^{3}H]palmitoyl-CoA [x(palm-CoA)$_{surf}$] will be essentially constant (since virtually all of the palmitoyl-CoA is vesicle-bound at millimolar lipid concentrations), and the product x(peptide)$_{surf}$[lipid]$_{eff}$ will first increase with increasing lipid concentration and then plateau as the proportion of peptide bound to vesicles approaches 100%. From these considerations, we obtain the results predicted in the text in the case where the S-acylation reaction proceeds mainly at the surfaces of vesicles. In the same experiment, the concentration of *free* peptide in the aqueous phase will decrease, and that of palmitoyl-CoA in the aqueous phase will remain essentially constant, as the lipid concentration rises. From these latter considerations, we predict that the rate of S-acylation would strongly *decrease* with increasing vesicle concentration if the reaction proceeded mainly in the aqueous phase.

that ACBP can extract a substantial fraction of long-chain acyl-CoAs from artificial and natural membranes (Rasmussen et al., 1990, 1993; Rosendal et al., 1993; Fyrst et al., 1995).

The physiological molar ratio of ACBP to acyl-CoA in the cytoplasm has been estimated to lie in the range of 1.25:1 to ca. 5:1 in different mammalian systems (Knudsen et al., 1989; Rasmussen et al., 1993). Assuming a cytosolic ACBP concentration of 30 μM (Rasmussen et al., 1994) and given that cellular membranes expose lipids to the cytoplasmic compartment at an effective concentration in the low millimolar range (Griffiths et al., 1989), we can estimate from our present results and the equations described in the Appendix that the level of long-chain acyl-CoAs in the cytoplasmic leaflet of cellular membranes will be reduced by roughly 8−10-fold by cytoplasmic ACBP at a 1.5:1 molar ratio of ACBP to acyl-CoA and by up to 20-fold at a 4:1 ratio of these components. This simple calculation assumes that the major long-chain acyl-CoAs present in the cytoplasm resemble palmitoyl-CoA in their relative affinities of binding to lipid bilayers vs ACBP. Our finding that rat ACBP inhibits with similar efficiency the S-acylation of vesicle-associated myrGCG-CaBim by either palmitoyl-, oleoyl-, or stearoyl-CoA suggests that this is the case. In this case, the molar percentage of long-chain acyl-CoAs partitioned into the cytoplasmic faces of intracellular membranes is predicted to lie in the range of 0.02−0.1 mol %, while the concentration free in the cytoplasm is predicted to lie in the range of 10^{-9}−10^{-8} M (see the Appendix). These values are much lower than those utilized in most *in vitro* studies of spontaneous S-acylation of proteins, where the effective concentration of acyl-CoAs in membranes or detergent micelles typically equals or exceeds 0.5−1 mol % (O'Brien et al., 1987; Bizzozero et al., 1987; Quesnel & Silvius, 1994; Bharadwaj & Bizzozero, 1995; Duncan & Gilman, 1996).

Using the above estimates and the effective rate constants (k') estimated for spontaneous S-acylation of membrane surface-bound mGCG-CaBim and IRYCWLRR-NH_2 [this study and Quesnel and Silvius (1994)], we can estimate the expected half-times of spontaneous S-acylation of these peptides at physiological bilayer concentrations of long-chain acyl-CoA [$x(\text{acyl-CoA})_{surf}$ = 0.0002−0.001] using the following equation:

$$t_{1/2} = \ln(2)/[k' \cdot x(\text{acyl-CoA})_{surf}] \qquad (2)$$

For the P_o glycoprotein-derived peptide, a half-time of ca. 15−75 h is thereby estimated for spontaneous S-acylation at physiological concentrations of membrane lipid, long-chain acyl-CoAs, and ACBP, while for myrGCG-CaBim, the expected half-time under these conditions would be on the order of 30−150 h. Similarly long half-times can be predicted from the data of Duncan and Gilman (1996) for the spontaneous S-acylation of α-subunits of G_{i1} and other heterotrimeric proteins under physiological conditions.[4] These half-times are far longer than the half-times (minutes to several tens of minutes) observed for palmitoylation of regulatory proteins such as $G_{s\alpha}$, N-*ras*, and GAP-43 in intact cells (Magee et al., 1987; Skene & Virág, 1989; Degtyarev et al., 1993; Mumby et al., 1994; Wedegaertner & Bourne, 1994) and than the time scales estimated for S-acylation of newly biosynthesized membrane proteins in their transit through the endoplasmic reticulum and Golgi apparatus (Schmidt & Schlesinger, 1980; Dunphy et al., 1981; Bonatti et al., 1989). It is thus highly unlikely that spontaneous reaction with long-chain acyl-CoAs could contribute significantly to the S-acylation of such proteins under physiological conditions.

While *in vivo* incorporation of *S*-acyl groups into proteins like the above appears to be much too rapid to be mediated by spontaneous reaction with acyl-CoAs, certain membrane-associated proteins show much slower rates of incorporation of radiolabeled *S*-acyl groups in intact cells (St. Jules & O'Brien, 1984; Staufenbiel, 1988). Unfortunately, from the results reported to date, it is unclear whether proteins of this latter class exhibit an intrinsically slow rate of S-acylation or merely slow turnover of *S*-acyl groups and a high steady state level of S-acylation. Further study will be needed to clarify whether nonenzymic acylation by long-chain acyl-CoAs could contribute significantly to the physiological S-acylation of proteins of this type.

APPENDIX

The binding equilibrium for association of a long-chain acyl-CoA with ACBP in the aqueous phase can be described as a simple 1:1 binding event with a dissociation constant K_d (Knudsen et al., 1989; Rasmussen et al., 1990):

$$\text{acyl-CoA}_{aq} + \text{ACBP} \rightleftharpoons \text{ACBP}\cdot\text{acyl-CoA} \qquad (3)$$

$$K_d = \frac{[\text{acyl-CoA}]_{aq}[\text{ACBP}]}{[\text{ACBP}\cdot\text{acyl-CoA}]} \qquad (4)$$

At low ratios of acyl-CoA to lipid, the association of long-chain acyl-CoA with lipid bilayers can be described as a simple partitioning equilibrium, governed by a dimensionless partition coefficient K_p relating the mole fractions (x) of acyl-CoA in the aqueous and bilayer compartments (Peitszch & McLaughlin, 1993):

$$K_p = x(\text{acyl-CoA})_{bilayer}/x(\text{acyl-CoA})_{aq} \qquad (5)$$

For low solute concentrations, the partition coefficient can be expressed alternatively as $K_p' = K_p/(55.5\ \text{M})$, which for low ratios of acyl-CoA to lipid reduces to the form

$$K_p' = \frac{[\text{acyl-CoA}]_{bilayer}/[\text{lipid}]_{ext}}{[\text{acyl-CoA}]_{aq}} \qquad (6)$$

where $[\text{lipid}]_{ext}$ is the concentration of lipid exposed at the

[4] From the data of Duncan and Gilman (1996), the initial rate of nonenzymic S-acylation of $G_{i\alpha1}$ in CHAPS micelles (15 mM) in the presence of 20 μM palmitoyl-CoA (a physiological concentration) is expected to be roughly 0.8%/min at pH 7.4. In control experiments using 20 μM palmitoyl-CoA, we found that the rate of nonenzymic S-acylation of myrGCG-CaBim was roughly 1.4-fold greater when assayed in 15 mM CHAPS than when measured in the presence of lipid vesicles (2 mM) in place of detergent. We can calculate from the equations described in the Appendix that in the experiments using vesicles the addition of ACBP at a 1.5:1 ACBP:acyl-CoA molar ratio will reduce the bilayer concentration of acyl-CoA by roughly 8-fold. Applying these results together, and noting that Duncan and Gilman found that the initial rate of nonenzymic S-acylation of $G_{i\alpha1}$ varies linearly with the acyl-CoA concentration in this concentration range, we predict that at physiological levels of long-chain acyl-CoAs, ACBP, and membrane lipids (20 μM, 30 μM, and 2 mM, respectively) the rate of spontaneous S-acylation of myrGCG-edNBD will be on the order of 0.07%/min and the half-time for spontaneous S-acylation on the order of 15−20 h. The half-time predicted for this process will be even longer at higher effective (membrane) lipid concentrations.

vesicle's outer surfaces [the surface to which the acyl-CoA has access (Boylan & Hamilton, 1992)] and $[\text{acyl-CoA}]_{\text{bilayer}}$ and $[\text{acyl-CoA}]_{\text{aq}}$ represent the concentrations of vesicle-associated and free acyl-CoA, respectively.

For any fixed values of $[\text{lipid}]_{\text{ext}}$ and $[\text{acyl-CoA}]_{\text{total}}$, using the constraints

$$[\text{ACBP}]_{\text{total}} = [\text{ACBP}] + [\text{ACBP·acyl-CoA}] \quad (7)$$

and

$$[\text{acyl-CoA}]_{\text{total}} = [\text{acyl-CoA}]_{\text{aq}} + [\text{acyl-CoA}]_{\text{bilayer}} + [\text{ACBP·acyl-CoA}] \quad (8)$$

the above equations can be solved as a function of $[\text{ACBP}]_{\text{total}}$, using K_p' and K_d as known (or adjustable) parameters. To estimate the bilayer/water partition coefficient $K_{p'}$ for palmitoyl-CoA, we extrapolated from the data of Peitzsch and McLaughlin (1993) for myristoyl-CoA, assuming that the value of K_p varies in the same manner with acyl chain length for acyl-CoAs as reported for free fatty acids in the cited study, to obtain a value of 1.45×10^5 M^{-1} under conditions where electrostatic repulsion betwen the lipid surface and acyl-CoA molecules can be neglected.

Combining the above equations with estimates for K_p' and K_d, we can also calculate the soluble and membrane concentrations of free long-chain acyl-CoAs expected at any given concentrations of membrane lipid, long-chain acyl-CoA, and ACBP.

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