Cysteine-Containing Peptide Sequences Exhibit Facile Uncatalyzed Transacylation and Acyl-CoA-dependent Acylation at the Lipid Bilayer Interface†

Susan Quesnel and John R. Silvius*

Department of Biochemistry, McGill University, Montréal, Québec, Canada H3G 1Y6

Received June 28, 1994; Revised Manuscript Received August 24, 1994⊗

ABSTRACT: A variety of simple cysteine-containing lipopeptides, with sequences modeled on those found in naturally occurring S-acylated proteins, undergo spontaneous S-acylation in phospholipid vesicles at physiological pH when either long-chain acyl-CoAs or other S-acylated peptides are added as acyl donors. Fluorescent or radiolabeled lipopeptides with the sequence myristoyl-GCX- (X = G, L, R, T, or V), a motif found to undergo S-acylation in several intracellular regulatory proteins, and the prenylated peptide -SCRC(farnesyl)-OMe, modeled on the carboxyl terminus of $p21^{H\text{-}ras}$, were all found to be suitable acyl acceptors for such uncatalyzed S-acyl transfer reactions at physiological pH. Acylation of these cysteinyl-containing lipopeptides to high stoichiometry was observed, on time scales ranging from a few hours to a few tens of minutes, in vesicles containing relatively low concentrations (≤ 1 mol %) and only a modest molar excess (2.5:1) of the acyl donor species. No evidence was obtained for acyl transfer to peptide serine or threonine hydroxyl groups under the same conditions. These observations may have significant implications both for the design of *in vitro* studies of the S-acylation of membrane-associated proteins and for our understanding of the mechanisms of S-acylation of these species *in vivo*.

A number of membrane-associated proteins have been found to be modified by the attachment of long-chain fatty acids in thioester linkage to one or more protein cysteinyl residues [for reviews, see Sefton and Buss (1987), Schultz *et al.* (1988), Schmidt (1989), James and Olson (1990), and Schlesinger *et al.* (1993)]. Such modifications were first demonstrated in cytoplasmic juxtamembrane segments of various integral membrane proteins (Rose *et al.*, 1984; Kaufmann *et al.*, 1984; Koch & Hammerling, 1986; Jing & Trowbridge, 1987; Schmidt *et al.*, 1988; Ovchinnikov *et al.*, 1988; O'Dowd *et al.*, 1989; Bizzozero *et al.*, 1990; Curstedt *et al.*, 1990; Weims & Stoffel, 1992; Papac *et al.*, 1992; Crise & Rose, 1992; Fujimoto *et al.*, 1993; Kennedy & Limbird, 1993). S-Acylation has more recently been demonstrated as well in a variety of proteins that exhibit reversible membrane association (Hancock *et al.*, 1989; Skene & Virag, 1989; Fujiyama *et al.*, 1991; Adamson *et al.*, 1992; Degtyarev *et al.*, 1993; Linder *et al.*, 1993; Liu *et al.*, 1993; Paige *et al.*, 1993; Parenti *et al.*, 1993; Veit *et al.*, 1993; Shenoy-Scaria *et al.*, 1993; Wedegaertner *et al.*, 1993; Mumby *et al.*, 1994; Shi *et al.*, 1994), enhancing the affinity and/or the specificity of binding of these proteins to intracellular membranes. The frequent occurrence of such proteins in various intracellular signaling pathways lends added interest to an understanding of the mechanism and the functional consequences of this type of protein modification.

To date, knowledge of the mechanisms of S-acylation of both permanently and reversibly membrane-associated proteins is very limited. Certain integral membrane proteins, notably rhodopsin (O'Brien *et al.*, 1987) and the major proteolipid of myelin (Bizzozero *et al.*, 1987a,b; Ross & Braun, 1988), have been shown to be S-acylated *in vitro* by long-chain fatty acyl-CoAs[1] by a mechanism that may require no other protein mediator. While *in vitro* S-acylation of certain other transmembrane proteins has been demonstrated (Adam *et al.*, 1984, 1988; Berger & Schmidt, 1984; Riendeau & Guertin, 1986; Mack *et al.*, 1987; Hartel-Schenck *et al.*, 1992), little is known about the mechanisms and the possible mediators of these reactions. Still less information is available on the mechanisms of S-acylation of proteins that reversibly associate with membranes, including myristoylated proteins such as $pp56^{lck}$, $pp59^{fyn}$, and the α-subunits of heterotrimeric G_o and G_i family proteins (Linder *et al.*, 1993; Paige *et al.*, 1993; Parenti *et al.*, 1993; Shenoy-Scaria *et al.*, 1993), isoprenylated proteins such as $p21^{H\text{-}ras}$ and $p21^{rhoB}$ (Hancock *et al.*, 1989; Adamson *et al.*, 1992), and proteins that lack other known lipid modifications, such as neuromodulin/GAP-43 (Skene & Virag, 1989; Liu *et al.*, 1993) and the α-subunits of heterotrimeric G_s and G_q family proteins (Degtyarev *et al.*, 1993; Linder *et al.*, 1993; Parenti *et al.*, 1993; Wedegaertner *et al.*, 1993; Veit *et al.*, 1994). A putative membrane-associated S-acylating activity for $p21^{N\text{-}ras}$ has been reported in mouse fibroblasts (Gutierrez & Magee, 1991), and a soluble thioesterase active on palmitoylated $p21^{H\text{-}ras}$ has recently been purified from bovine brain

† This work was supported by a grant from the Medical Research Council of Canada to J.S., who gratefully acknowledges further support in the form of an M. R. C. of Canada Scientist award.

⊗ Abstract published in *Advance ACS Abstracts,* October 15, 1994.

[1] Abbreviations: acyl-CoA, acyl-coenzyme A; (12-DABS)-18 PC, 1-palmitoyl-2-((12-(4-((4-(dimethylamino)phenyl)azo)phenyl)sulfonyl)-methylamino)stearoyl-PC; DCCD, dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; DMF, dimethylformamide; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; DTPA, diethylenetriaminepentaacetic acid; DTT, dithiothreitol; Fmoc-, 9-fluorenylmethoxycarbonyl-; HEPES, N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) sodium salt; HOBT, 1-hydroxybenzotriazole; Mes, 2-(*N*-morpholino)ethanesulfonic acid sodium salt; MOPS, 3-(*N*-morpholino)propanesulfonic acid sodium salt; PC, phosphatidylcholine; POPC (-PE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (-ethanolamine); TLC, thin-layer chromatography. Peptides and lipopeptides are abbreviated using the standard one-letter amino acid code, with side-chain substituents indicated in parentheses following the substituted amino acid, plus the following additional abbrevations: Ac-, acetyl; BimTA-, *S*-bimanylthioacetyl-; -(BimCA), -2-(*S*-bimanylthio)ethylamide; and myr-, *N*-myristoyl-.

0006-2960/94/0433-13340$04.50/0

(Camp & Hofmann, 1993). To date no acylating activities for other proteins that associate reversibly with membranes have been described in any detail.

In the present study we have examined the possibility that peptide cysteinyl residues suitably positioned at the membrane interface may inherently constitute efficient substrates for uncatalyzed[2] S-acylation by other long-chain acyl thioesters present in the same membrane. Interestingly, we find that efficient acylation of membrane-anchored cysteinyl-containing peptides can occur using not only long-chain acyl-CoAs but also other S-acylated peptides as acyl donors. These results underscore some important potential concerns for *in vitro* reconstitutions of membrane protein S-acylation, and they suggest the interesting possibility that some membrane-associated proteins may undergo S-acylation *in vivo* by nonenzymatic transfer of acyl groups from other S-acylated proteins.

MATERIALS AND METHODS

Materials

Amino acids and protected derivatives were purchased from Novabiochem (La Jolla, CA) or Sigma Biochemicals (St. Louis, MO). Other peptide-synthetic reagents were obtained from Novabiochem or from Aldrich Chemical Co. (Minneapolis, MN). Acyl-CoAs, fatty acids, and monobromobimane were obtained from Sigma; phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL) except for dioleoyl phosphatidylserine (DOPS), which was synthesized as described previously (Silvius & Gagné, 1984). All other common chemicals and buffers were of reagent grade or better. Dimethylformamide (analytical grade) was stored over activated molecular sieve 4A for 2 weeks at 4 °C before use; all other solvents were redistilled.

Synthesis of Acylated Peptides. *S*-Bimanylcysteamine·HCl was synthesized by reacting 1 equiv of monobromobimane with 0.97 equiv each of cysteamine·HCl and DIEA in methanol (5 mL per mmol of bromobimane) for 30 min at 25 °C. The reaction mixture was concentrated under nitrogen and dried down overnight under high vacuum.

The acylated peptides myrGCX-(BimCA), where X = glycine, leucine, threonine, and valine, were synthesized as follows. Fmoc-amino acid, HOBT, and DCCD (1, 2, and 1.05 molar equiv, respectively) were reacted together (1 h, 0 °C) in dry DMF (2 mL per mmol of Fmoc-amino acid) and then combined with 1 equiv each of *S*-bimanylcysteamine·HCl and DIEA; for coupling to Fmoc-threonine the preincubation with HOBT and DCCD was omitted. After stirring for 2 h at 0 °C, the mixture was diluted with 5 vol of ethyl acetate, filtered, and concentrated under nitrogen. The residue was partitioned between chloroform and 1:1 methanol/0.1 M HCl in 1 M aqueous KCl, and the chloroform layer was concentrated and dried overnight *in vacuo*. The dried residue, containing a single major fluorescent band in each case, was deprotected with 4:1 (v/v) DMF/piperidine (45 min, 0 °C) and then dried *in vacuo* and coupled to *N*-Fmoc-(*S*-*tert*-butylmercapto)cysteine, using HOBT/DCCD coupling as described above but omitting DIEA. The recovered products were purified by preparative TLC on silica gel 60 plates (solvent 95:5 chloroform/methanol). The major fluorescent band obtained in each case was eluted from the scraped gel with methanol, dried *in vacuo*, deprotected with DMF/piperidine as above, and then added to a mixture of 1.2 equiv of *N*-myristoylglycine (Lapidot *et al.*, 1967), 2 equiv of HOBT, and 1.5 equiv of DCCD which had been preincubated for 2 h at 25 °C in 1:1 CH_2Cl_2/DMF (5 μL per μmol of myristoylglycine). After 1−3 h, TLC showed coupling to be essentially complete, and the products were extracted by chloroform/methanol/aqueous phase partitioning as described above. The residue from concentration of the chloroform phase was incubated (4 h, 65 °C, under argon) with (per μmol of product) 1 mg of DTT, 2 μL of β-mercaptoethanol, and 4 μL each of DMF and 1 M aqueous MOPS, pH 7.4, and then re-extracted as above and finally purified by preparative TLC (solvent 90:10:0.5 chloroform/methanol/acetic acid). The product was eluted from the moistened gel with methanol and partitioned between chloroform and 1:1 methanol/1 M aq KCl, at 0 °C and under argon. The chloroform phase was dried, and the products were stored under argon at −80 °C in methanol or dry CH_2Cl_2, normally in the presence of 1 molar equiv of DTT. The structures and purities of the lipopeptide products were confirmed by ^{1}H NMR and by thin-layer chromatography on silica gel 60 in both the solvent system used for purification and a second, distinct developing solvent (98:2 ethyl acetate/methanol for neutral species, 65:25:2:2 chloroform/methanol/ammonium hydroxide/water for cationic lipopeptides).

The acylated peptide myrGSG-(BimCA) was prepared by the above protocol, using Fmoc-serine in place of Fmoc-(*S*-*tert*-butylmercapto)cysteine (omitting preincubation of the Fmoc-serine with HOBT/DCCD) and omitting the DTT/β-mercaptoethanol reduction step. The peptide myrGCR-(BimCA) was also prepared by the above protocol, but intermediate peptide products were isolated by diethyl ether precipitation rather than by solvent partitioning, and the final product was purified by TLC in 50:20:10:10:5 (v/v/v/v/v) chloroform/acetone/methanol/acetic acid/water. AcGCG-(BimCA) was prepared as described above for myrGCG-(BimCA), using *N*-acetylglycine in place of *N*-myristoylglycine in the final coupling reaction and omitting solvent-partitioning steps from the purification protocol. The prenylated peptide BimTA-SCRC(farnesyl)-OMe was prepared using methods described previously (Silvius & l'Heureux, 1994). [^{3}H]myrGCG-OMe was synthesized as described above for myrGCG-(BimCA), using glycine methyl ester and [^{3}H]myristoylglycine (specific activity 2 μCi/nmol), respectively, in the initial and final condensation steps.

S-Palmitoyl and -oleoyl peptides were synthesized by reaction with a 1.2−5-fold excess of the appropriate acyl chloride in 9:1 (v/v) CH_2Cl_2/pyridine (50 μL/μmol of peptide) for 2 h at 25 °C under argon. The S-acylated products were purified by preparative TLC as described above for the parent peptides. Disulfide-linked conjugates (myrGCX-[BimCA])$_2$ were prepared by treating the corresponding cysteinyl peptides with 0.5 equiv of I_2 in methanol for 30 min at room temperature and then purifying as for the parent peptides.[3]

[2] We here use the term "uncatalyzed" to denote a process of S-acylation of cysteinyl residues that is not catalyzed by any special domain or combination of amino acids within the same or another (poly)peptide chain. This usage is technically incorrect, since it ignores the fact that the lipid bilayer itself acts "catalytically" by concentrating and partially orienting the reacting species. We nonetheless favor this term as conveying more fully the sense of the above definition than do alternative terms such as "nonenzymatic."

Methods

General. Large unilamellar lipid vesicles were prepared from lipid mixtures, dried down under high vacuum from chloroform, by hand extrusion through 0.1-μm pore-size polycarbonate (Nucleopore) membranes (MacDonald *et al.*, 1991). Vesicle suspensions (20−40 mM lipid) were prepared in deoxygenated 150 mM NaCl, 10 mM HEPES, and 0.2−1.0 mM DTPA, pH 7.0, and kept on ice under argon until ready for use. All salt and buffer solutions used were autoclaved before use; the pH of buffer solutions was rechecked but not adjusted after autoclaving. Stock solutions of acyl-CoA and DTT were prepared in sterile 20 mM phosphate, pH 6.0, or sterile water, respectively, and stored at −80 or −20 °C, respectively.

Assays of Vesicle Insertion, Intervesicle Transfer, and Flip-Flop of Lipopeptides. The kinetics of insertion of bimane-labeled lipopeptides into lipid vesicles were monitored by fluorescence enhancement as described previously (Silvius & l'Heureux, 1994), injecting vesicles (0.5 mM) into buffer containing the appropriate lipid probe ($\leq$1 μM) at 37 °C. Interbilayer exchange and transbilayer flip-flop of fluorescent lipopeptides was monitored by observing the time-dependent dequenching of fluorescence as the lipopeptides transferred from large unilamellar or multilamellar vesicles, labeled during preparation with 0.4 mol % lipopeptide and 1 mol % of the nonexchangeable quencher (12-DABS)-18-PC, to a large excess of unlabeled lipid vesicles added to initiate the transfer measurement. In this system, the initial rate of fluorescence dequenching reflects the kinetics of interbilayer transfer of the lipopeptide, while the kinetics and the amplitude of dequenching at longer times provide information about the rate of lipopeptide transbilayer flip-flop [for further details, see Silvius and l'Heureux, (1994)].

Assays of Lipopeptide Acylation. All steps described below were carried in dim light or, where possible, with complete exclusion of light; incubations and extractions were carried out under argon. Lipid vesicles prepared as described above were added to N_2-purged NaCl/DTPA/DTT (final concentrations given below), and myristoylated peptide was then injected in methanol (<1% by volume) at room temperature. After 5 min, concentrated stocks of HEPES buffer, $MgCl_2$, and acyl-CoA were added in rapid succession. The final composition of all reaction mixtures, except where otherwise noted, was 2−5 mM phospholipid incorporating 0.4 mol % lipopeptide and 1 mol % acyl-CoA in 150 mM NaCl, 50 mM HEPES, 1 mM each phosphate, DTT and $MgCl_2$, and 50 μM DTPA. Reaction time courses were normally initiated by adding acyl-CoA at 37 °C or, when multiple incubations were to be initiated simultaneously, by rapidly warming reaction mixtures from 0 to 37 °C within a few minutes after adding acyl-CoA at the lower temperature. After incubation under argon at 37 °C, 200-μL aliquots of

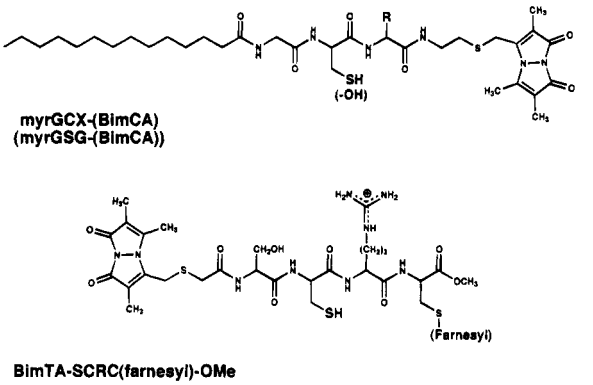

FIGURE 1: Structures of the fluorescent lipopeptides examined in this study.

reaction mixtures were transferred to screw-capped tubes, rapidly frozen in a dry ice/ethanol bath, and mixed without prior thawing with 1.5 mL each of chloroform, methanol, and 0.5 M KCl/0.02 M Mes, pH 5.0. After shaking and phase separation, the chloroform phase was withdrawn, dried under nitrogen, and streaked on TLC plates (silica gel 60, 5 × 10 cm), which were developed in 90:10:0.5 chloroform/methanol/acetic acid (for uncharged lipopeptides) or 50:20:10:10:5 chloroform/acetone/methanol/acetic acid/water (for cationic lipopeptides). Fluorescent bands were visualized with minimal UV illumination and then scraped into screw-capped tubes and suspended by bath sonication in 3.5 mL of methanol. The tubes were vortexed and centrifuged (clinical centrifuge, 5 min), and 3 mL of the methanol extract was taken for fluorescence determination using a Perkin-Elmer LS-5 spectrofluorimeter (excitation and emission wavelengths 390 and 468 nm; slit settings 15/20 nm). Control experiments indicated that corresponding S-acylated and -unacylated lipopeptides gave very similar fluorescence per unit molar amount under these conditions. The hydroxylamine sensitivity of putative S-acylated lipopeptides was tested by incubation for 2 h at 25 °C in 2:1:0.5 (v/v/v) chloroform/methanol/0.2 M aqueous hydroxylamine, pH 7.0.

Interpeptide transfer of S-acyl groups was measured essentially as described above, replacing acyl-CoAs with S-acylpeptides. Water-insoluble *N*-myristoyl-S-acylpeptides were incorporated into lipid vesicles during preparation, and in this case reactions were initiated by injecting the acceptor lipopeptide into the reaction mixture. Acylation of [^{3}H]-myrGCG-OMe was assayed using incubation, extraction, and TLC conditions similar to those described above (adding 0.5 μCi of lipopeptide/incubation mixture), visualizing the TLC-separated products by autoradiography and quantitating them by counting the scraped bands in Cytoscint (ICN).

RESULTS

In Figure 1 are shown the general structures of the fluorescent acylated and isoprenylated peptides (collectively refered to hereafter as "lipopeptides") that were examined in this study. Lipopeptides containing a fluorescent bimane group were used for most assays because they permitted ready assay not only of the acylation/deacylation reactions but also of the bilayer insertion, interbilayer transfer, and transbilayer flip-flop of the lipopeptides (see Materials and Methods). Knowledge of the kinetics of these latter processes considerably facilitated the interpretation of some of the experiments described below. As well, using fluorimetric

[3] In 90:10:0.5 (v/v/v) chloroform/methanol/acetic acid the R_f values for the neutral cysteinyl lipopeptides and their derivatives were as follows (acyl = palmitoyl or oleoyl): myrGCG-(BimCA), 0.50 (-SH), 0.73 (-S-acyl), 0.42 (-SS-); myrGCL-(BimCA), 0.66 (-SH), 0.89 (-S-acyl), 0.55 (-SS-); myrGCT-(BimCA), 0.50 (-SH), 0.71 (-S-acyl), 0.42 (-SS-); and myrGCV-(BimCA), 0.64 (-SH), 0.84 (-S-acyl), 0.56 (-SS-). In 50:20:10:10:5 chloroform/acetone/methanol/acetic acid/water the R_f values for the cationic cysteinyl lipopeptides and their derivatives were as follows: myrGCR-(BimCA), 0.43 (-SH), 0.65 (-S-acyl), 0.32 (-SS-); and BimTA-SCRC(farnesyl)-OMe, 0.42 (-SH), 0.61 (-S-acyl), 0.34 (-SS-).

measurements as described previously (Silvius & l'Heureux, 1994), we established that all of these lipopeptides were almost entirely bound to phospholipid vesicles at the lipid concentrations used in the acylation assays (2−30 mM). Importantly, as discussed later, control experiments with the radiolabeled lipopeptide myrGCG-OMe gave results similar to those obtained using bimane-labeled lipopeptides.

When lipopeptides of the general sequence myrGCX-(BimCA), or the prenylated peptide BimTA-SCRC(farnesyl)-OMe, were incubated with large unilamellar lipid vesicles in the presence (but not in the absence) of long-chain acyl-CoAs, time-dependent formation of a new fluorescent product, with an increased mobility on TLC, was observed. In all cases both the chromatographic migration and the hydroxylamine sensitivity of the new product was identical to that of an authentic sample of the S-acylated compound prepared chemically (see Materials and Methods). The serine-containing lipopeptide myrGSG-(BimCA) showed no detectable acylation even after incubation for 8 h in lipid vesicles in the presence or absence of 2.5 mol % oleoyl-CoA. Significantly, neither myr-GCT-(BimCA) nor BimTA-SCRC(farnesyl)-OMe showed detectable formation of an S,O-diacyl product (as might be expected if S-to-O acyl migration occurred) after even prolonged incubations with oleoyl-CoA in lipid vesicles, although the diacyl derivatives were readily prepared using excess oleoyl chloride in organic solvents (see Materials and Methods).

In the absence of reducing agents (and in the presence or absence of acyl-CoA), the cysteine-containing lipopeptides showed significant acyl-CoA-independent formation of a second product, which in each case was identified as the disulfide-linked lipopeptide dimer based on its comigration on TLC with an I_2-oxidized sample of the parent species and on its reconversion to the original lipopeptide upon incubation with DTT/β-mercaptoethanol. DTT (1 mM) was therefore routinely added to reaction mixtures to minimize formation of the oxidized product. However, it was demonstrated in control experiments that DTT was not essential to the S-acylation reaction (not shown).

Several measures were employed to confirm that the observed S-acylation of lipopeptides by acyl-CoAs was not catalyzed by enzymic (or other) contaminants in the reaction mixtures. All procedures were carried out using sterilized vessels and sterile technique, and solutions were autoclaved wherever possible. Components that could not be autoclaved (DTT, acyl-CoAs, and lipids) were prepared in sterile media; in addition, as described below, the results of control experiments appear to exclude the possibility that contaminants in any of these materials could be responsible for the observed lipopeptide S-acylation reactions. First, as already noted DTT could be omitted from the reaction medium with only a modest reduction (<30%) in the acylation rate and yield, which was entirely attributable to partial oxidation of the cysteinyl-lipopeptide substrates. Second, as discussed below several different long-chain acyl-CoAs gave very similar extents and kinetics of lipopeptide S-acylation, suggesting that (presumably random) contamination from this source is also unlikely to explain the observed S-acylation reaction. Finally, vesicles composed of either POPC, dioleoyl-PC or dimyristoyl-PC were found to support oleoyl-CoA-dependent acylation of the lipopeptide myrGCG-(BimCA) with similar efficiencies (initial rates 18%/h, 20%/h, and 11%/h, respectively, at pH 7.4, 2 mM lipid, and 20 μM oleoyl-CoA). It thus appears that contamination from the phospholipid preparations (notably phospholipase A_2, which is used in the synthesis of mixed-acyl-PCs, although it should be efficiently removed during lipid purification) is also unlikely to explain the observed lipopeptide S-acylation reactions.

In Figure 2A is shown a representative time course of reaction of the acylated peptide myrGCG-(BimCA) (1 mol %) with oleoyl-CoA (2.5 mol %) in 40:40:20 (molar proportions) POPC/POPE/DOPS large unilamellar vesicles. As shown in the accompanying semilog transformation (Figure 2B), the time course exhibits pseudo-first-order (exponential) kinetics until over one-half of the peptide has been acylated, at which point acyl-CoA depletion is becoming significant. Apparent rate constants for acyl-CoA-dependent lipopeptide acylation were thus determined from the initial slopes of semilog plots like those shown in Figure 2B, normally including in the analysis only time points for which the reaction was <25% complete. However, in all cases longer incubations gave much higher stoichiometries of lipopeptide S-acylation (>75% using a 2.5:1 acyl-CoA/lipopeptide ratio).

In Figure 2C is shown the pH dependence of the S-acylation of myrGCG-(BimCA) (0.4 mol %) by oleoyl-CoA (1 mol %) incorporated into 40:40:20 POPC/POPE/DOPS large unilamellar vesicles. A similar, but slightly steeper, pH dependence was observed using large unilamellar POPC vesicles under the same conditions (not shown). The strong pH dependence of the lipopeptide S-acylation rate in this pH range suggests that the ionized (thiolate) form of the lipopeptide cysteinyl residue is the active nucleophile in this reaction. The above results were not substantially altered when magnesium (1 mM) was replaced by 1 mM EDTA in the incubation medium (not shown). In order to mimic the conditions expected at the cytoplasmic face of cellular membranes, all further experiments described below employed large unilamellar POPC/POPE/DOPS (40:40:20 molar proportions) vesicles at 37 °C in 150 mM NaCl (or KCl, with equivalent results in all cases), 50 mM HEPES, 1 mM phosphate, 1 mM $MgCl_2$, 1 mM DTT, and 50 μM DTPA, pH 7.4. Under these standard experimental conditions, the rate of spontaneous hydrolysis of the S-acylated peptides was <5% per hour (data not shown).

When fixed amounts of myrGCG-(BimCA) and palmitoyl- or oleoyl-CoA are incubated with increasing concentrations of lipid vesicles (4−30 mM), the initial rate of lipopeptide acylation decreases steadily. In this range of lipid concentrations, both species should be almost completely partitioned into the vesicle bilayer membranes [a conclusion based on fluorescence measurements of lipopeptide-vesicle binding (Silvius & l'Heureux, 1994) and on extrapolation from the data of Peitzsch and McLaughlin (1993) for acyl-CoA binding to lipid vesicles], and their effective *bilayer* concentrations should thus be inversely proportional to the concentration of added vesicles. As shown in Figure 2D, the rate of S-acylation of myrGCG-(BimCA) by acyl-CoAs is in fact linearly proportional to the *bilayer* concentration of acyl-CoA up to at least 1.25 mol % of the latter component.

In Table 1 are listed the initial rates measured for acylation of myr-GCG-(BimCA) in 40:40:20 POPC/POPE/DOPS vesicles by various acyl-CoAs, added to 1 mol % with respect to the vesicle phospholipids. Very similar rates of reaction

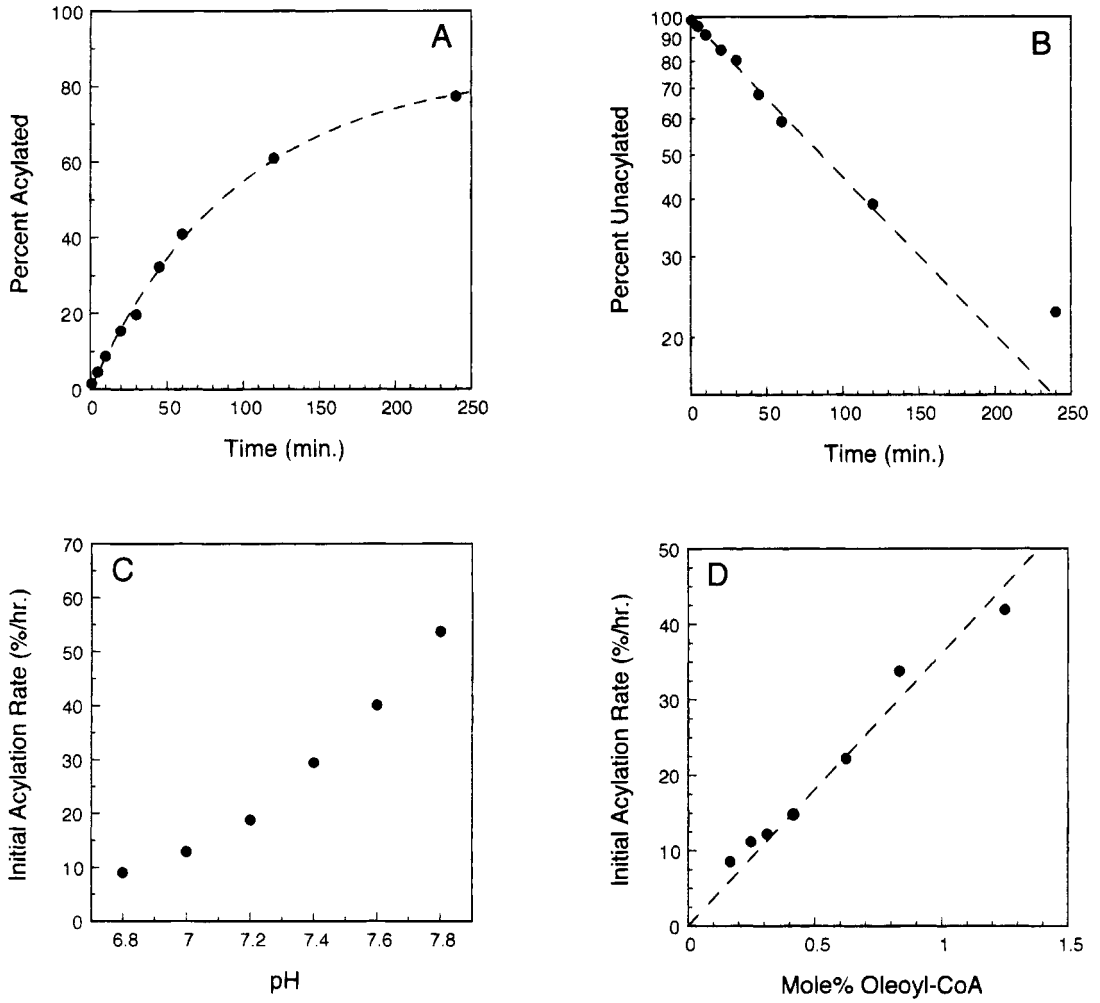

FIGURE 2: (A) Time course of acylation of myrCGC-(BimCA) (1 mol % with respect to vesicle lipids) by oleoyl-CoA (2.5 mol %) in 40:40:20 (molar proportions) POPC/POPE/DOPS large unilamellar vesicles at 37 °C, pH 7.4. Other experimental conditions were as described under Materials and Methods. (B) Semilog transformation of the data in panel A (note that the *y*-axis now represents the percentage of *unacylated* peptide). (C) pH dependence of the rate of acylation of myrGCG-(BimCA) (0.4 mol % with respect to vesicle lipids) by oleoyl-CoA (1 mol %) in POPC/POPE/DOPS (40:40:20) large unilamellar vesicles. Other experimental conditions were as described under Materials and Methods. (D) Variation of the rate of acylation (pH = 7.4) of myrGCG-(BimCA) in the presence of varying surface concentrations of oleoyl-CoA in 40:40:20 POPC/POPE/DOPS large unilamellar vesicles. Lipopeptide and oleoyl-CoA, in a fixed molar ratio of 1:2.5, were added to varying concentrations of lipid vesicles to give the indicated mole percentages of acyl-CoA with respect to vesicle lipids.

Table 1: Kinetics of Acylation of Myristoyl-GCG-(BimCA) by Various Acyl-CoAs in Large Unilamellar 40:40:20 POPC/POPE/DOPS Vesicles

acyl-CoA	initial rate of acylation (%/h)[a]
hexanoyl	<0.4
palmitoyl	20.6 ± 2.1
stearoyl	20.1 ± 2.7
oleoyl	20.2 ± 0.7
linoleoyl	19.1 ± 3.0

[a] Acylation rates were determined at 37 °C and pH 7.4 as described under Materials and Methods, at lipid and acyl-CoA concentrations of 5 mM and 50 μM, respectively. Values shown represent the mean (± the averaged half-range) of duplicate determinations in two independent experiments.

are observed using different saturated and unsaturated C_{16}- and C_{18}-acyl-CoAs. A much (at least 50-fold) slower acylation reaction is observed using hexanoyl-CoA, which in contrast to the long-chain acyl-CoAs should partition very weakly into the vesicles at the concentrations used here [see Peitzsch and McLaughlin (1993)]. This result strongly suggests that the observed acyl-transfer reaction occurs between *vesicle-bound* acyl-CoA and lipopeptide molecules.

In agreement with this conclusion, under similar reaction conditions (using 2.5 mol % palmitoyl- or oleoyl-CoA in POPC/POPE/DOPS vesicles), the measured rate of S-acylation of the N-acetylated peptide AcGCG-(BimCA) was only 0.05−0.06% per hour, a value almost 10^3-fold lower than that measured for the corresponding N-myristoylated peptide under the same conditions (Figure 2A). Somewhat faster S-acylation of the N-acetylated peptide (1.0−1.2%/hr.) could be observed using micellar palmitoyl- or oleoyl-CoA dispersions (100 μM) in the absence of both phospholipid and DTT. However, under these conditions as well, the initial rates of S-acylation measured for the lipopeptides myrGCG-(BimCA) and BimTA-SCRC(farnesyl)-OMe were at least 100-fold greater (data not shown).

In view of the very similar behavior of different long-chain acyl-CoAs in the above lipopeptide-acylation reactions, most of the experiments presented in this report were carried out using oleoyl-CoA (or S-oleoyl peptides), which could be added to reaction mixtures at 0 °C (a technical advantage in certain types of experiments) without concern for possible phase transitions that might occur for long-chain saturated acyl-CoAs or S-acylpeptides at low temperature. However,

Table 2: Kinetics of S-Acylation of Various Cysteine-Containing Lipopeptides by Oleoyl-CoA and S-Acylated Peptides in Large Unilamellar 40:40:20 POPC/POPE/DOPS Vesicles

acceptor lipopeptide	initial rate of acylation[a] (%/h) by oleoyl-CoA	myr-GC(ol)G-OMe	Ac-LC(ol)R-OMe
myr-GCG-(BimCA)	22.0 ± 2.8	17.2 ± 1.3	55.4 ± 3.4
myr-GCL-(BimCA)	57.2 ± 6.6	37.4 ± 1.6	116.9 ± 24.3
myr-GCR-(BimCA)	17.8 ± 1.1[b]	18.7 ± 3.0	24.5 ± 0.2[b]
myr-GCT-(BimCA)	26.6 ± 3.6	12.7 ± 4.7	61.6 ± 5.6
myr-GCV-(BimCA)	51.7 ± 10.3	22.0 ± 1.5	110.9 ± 34.6
BimTA-SCRC(farnesyl)-OMe	8.6 ± 0.6[b]	9.0 ± 1.2	15.2 ± 0.4[b]
[^{3}H]myrGCG-OMe	9.4 ± 0.2	10.0 ± 0.4	35.5 ± 2.9

[a] Acylation rates were determined in 37 °C, pH 7.4; acceptor lipopeptides and donor species were added to 0.4 and 1.0 mol %, respectively, with respect to vesicle lipids. Other experimental details were as described under Materials and Methods. Values shown represent the mean of duplicate determinations (± the averaged half-range) in two independent experiments. [b] Acylation rates listed for these donor/acceptor pairs have been corrected, as discussed in the text for acyl-CoA-dependent acylation reactions, for the artifactual rate enhancement expected when both donor and acceptor species are concentrated in the vesicles' surface monolayers.

all key results were confirmed using the corresponding S-palmitoyl species as well.

In Table 2 we compare the initial rates of acylation observed when different peptides of the sequence myrCGX-(BimCA), as well as the prenylated peptide (BimTA)-SCRC-(farnesyl)-OMe, were incubated with large unilamellar POPC/POPE/DOPS vesicles containing 1 mol % oleoyl-CoA. These lipopeptides were constructed to represent known S-acylated sequences found in various nonreceptor tyrosine kinases (myrGCV- = murine pp56^{lck} and pp59^{fyn}; myrGCG- = human pp56^{lck}), heterotrimeric G-protein α-subunits (myrGCT- = α_o and $\alpha_{i_1-i_3}$; myrGCR- = α_z; GCL- = α_s, a sequence not however myristoylated in the native protein) and p21$^{H\text{-}ras}$ (-SCRC[farnesyl]-OMe, with the lysine residue found in the human and murine proteins replaced by arginine to facilitate synthesis of an authentic S-acylated lipopeptide standard).

Separate fluorescence experiments (described under Materials and Methods; results not shown) demonstrated that the uncharged lipopeptides examined in the experiments of Table 2 exhibit very rapid bilayer insertion, intervesicle transfer, and transbilayer flip-flop on the time scale of the acylation experiments. In contrast, the arginine-containing lipopeptides also exhibited very rapid bilayer insertion and intervesicle transfer but showed negligible transbilayer flip-flop on the assay time scale. The cationic (but not the neutral) lipopeptides examined here, like the highly charged acyl-CoAs, are thus likely to be largely confined to the surface monolayers of the lipid vesicles under the conditions of the acylation assays. Since the rate of lipopeptide S-acylation is linearly proportional to the local concentration of acyl-CoA (Figure 2D), it can be predicted that the rates of acyl-CoA-dependent acylation measured for the arginine-containing lipopeptides will be artifactually enhanced relative to those for the neutral peptides by the factor ($[L_{total}]/[L_{surface}]$), representing the ratio of total to surface-exposed vesicle lipids.[4] The acylation rates listed for the arginyl-containing lipopeptides in Table 1 have been corrected for this effect, using the assay of Nordlund *et al.* (1981) to estimate the factor just noted.

It can be seen from the data in Table 2 that the amino acid sequence of a membrane-anchored cysteinyl-containing peptide has a significant, albeit limited effect on the kinetics of its acylation by oleoyl-CoA. Interestingly, the cationic (arginyl-containing) peptides do not show faster (corrected) rates of acylation by the anionic oleoyl-CoA than do the neutral peptides. In the family of peptides myrGCX-(BimCA) the presence of a hydrophobic amino acid (valine or leucine) adjacent to the cysteinyl residue appears to enhance the rate of acylation significantly, perhaps by further enhancing the positioning of the cysteinyl group close to the lipid−water interface.

In the light of the results presented thus far, it was of interest to determine whether S-acylated peptides as well as acyl-CoAs could serve as donors for acylation of cysteinyl peptides anchored at the lipid/water interface. In a first set of experiments, POPC/POPE/DOPS (40/40/20) vesicles were prepared containing 1 mol % of either myrGC(oleoyl)G-(BimCA) or myrGC(oleoyl)R-(BimCA) as the acyl donor; myrGCR-(BimCA) or myrGCG-(BimCA), respectively, was then added as the acyl acceptor, and the time course of S-acyl transfer was monitored as described under Materials and Methods. Efficient S-transacylation was observed in both these systems, as demonstrated both by the conversion of a portion of the acceptor lipopeptide to its S-acylated form (Figure 3) and by a parallel de-S-acylation of a portion of the donor lipopeptide (not shown). Acyl transfer between myrGCG-(BimCA) and other peptides was also observed (data not shown), although in some cases the S-acylated "reactant" and "product" peptides were only marginally resolved by TLC, hindering precise kinetic analysis. To overcome this limitation, we compared the rates of S-acylation of various bimane-labeled lipopeptides in POPC/POPE/DOPS vesicles incorporating as acyl donors 1 mol % of the unlabeled S-acylated peptides myrGC(oleoyl)G-OMe or Ac-LC(oleoyl)R-OMe. As shown in Table 2, transacylation is observed at substantial rates in all cases. Very similar results were observed using the corresponding S-palmitoyl peptides as acyl donors (not shown). The reaction rates measured using the lipopeptide Ac-LC-(oleoyl)R-OMe as the acyl donor are particularly noteworthy, ranging up to 110−120%/h using myrGCL-(BimCA) or myrGCV-(BimCA) as the acyl acceptor.

In a final set of experiments, we examined the S-acylation of the radiolabeled lipopeptide [^{3}H]myr-GCG-OMe using as the acyl donors oleoyl-CoA, myr-GC(oleoyl)G-OMe and Ac-

[4] This conclusion can be demonstrated as follows. Consider the vesicle preparation to consist of two lipid "compartments": the external monolayer of each vesicle and any internal monolayers (including those of internal lamellae, if present). If the effective rate constant for acylation of the lipopeptide molecules in a given compartment is linearly proportional to the local mole fraction (x) of acyl-CoA, with a constant of proportionality k_{ac}, the measured overall rate constant will be $k_{ac}[f(\text{lipopeptide})_{int}x(\text{acyl-CoA})_{int} + f(\text{lipopeptide})_{ext}x(\text{acyl-CoA})_{ext}]$, where f(lipopeptide) is the fraction of the lipopeptide in either compartment. If a given lipopeptide is present at equal mole fractions in both compartments, for a given mole fraction of *total* acyl-CoA with respect to *total* lipids [$x(\text{acyl-CoA})_{total}$], the measured rate constant reduces to [$k_{ac}x(\text{acyl-CoA})_{total}$], independent of the distribution of acyl-CoA between the compartments. By contrast, if the lipopeptide and the acyl-CoA are both entirely confined to the surface-monolayer compartment, the measured rate constant will be $k_{ac}x(\text{acyl-CoA})_{ext} = k_{ac}x(\text{acyl-CoA})_{total}f_{ext}$, where $f_{ext} = [\text{lipid}]_{ext}/[\text{lipid}]_{total}$ is the fraction of total vesicle lipids that is exposed at the vesicles' outer surfaces.

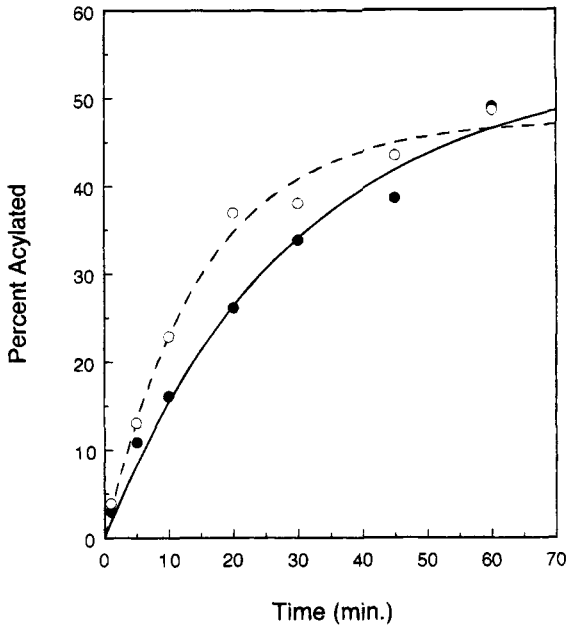

FIGURE 3: Time courses of transacylation (pH 7.4) of myrGCR-(BimCA) by myrGC(oleoyl)G-(BimCA) (open circles) and of myrGCG-(BimCA) by myrGC(oleoyl)R-(BimCA) (closed circles) in 40:40:20 POPC/POPE/DOPS large unilamellar vesicles. Other experimental details were as described under Materials and Methods.

LC(oleoyl)R-OMe. As shown in Table 2, the observed rates of acylation of the radiolabeled peptide fall within the range measured for the various bimane-labeled lipopeptides examined, suggesting that the presence of the bimanyl group *per se* is not a major factor in the acylation reactions demonstrated here.

DISCUSSION

The results presented in this study have significant implications for several aspects of the study of S-acylation of membrane-associated proteins, certainly *in vitro* and possibly *in vivo* as well. First, it seems clear that acyl-CoAs can serve as efficient acyl donors to cysteine residues within interfacially disposed peptide sequences, without requiring any protein-mediated catalytic or autocatalytic activity. This conclusion supports previous reports of the *in vitro* S-acylation of certain membrane proteins by processes described as "nonenzymatic" or "autocatalytic" (Bizzozero *et al.*, 1987b; O'Brien *et al.*, 1987; Ross & Braun, 1988; Hartel-Schenk & Agre, 1992), and it suggests that such proteins need little if any specific sequence information, beyond that required to ensure a suitable interfacial disposition and steric accessibility of the "target" cysteinyl residue(s), in order to exhibit such autoacylating activity.

It is difficult to assess whether membrane-associated proteins may be directly acylated *in vivo* by long-chain acyl-CoAs in the manner we have observed for cysteine-containing lipopeptides *in vitro*[5]. Two critical questions in this regard concern the concentrations of "free" long-chain acyl-CoAs that are found in living cells and the possible affinities with which particular membrane proteins bind these compounds. While the *total* concentration of long-chain acyl-CoAs in the cytoplasm of mammalian cells is of the order of tens of micromolar, most of this appears to be bound to cytosolic acyl-CoA-binding proteins (Rasmussen *et al.*, 1993, 1994), and the concentration of "free" long-chain acyl-CoAs in the cytoplasm may lie in the picomolar range.[6] Extrapolating from the partitioning data of Peitzsch and McLaughlin (1993), we can estimate that under these conditions the level of long-chain acyl-CoAs partitioned into the lipid bilayers of cellular membranes would be of the order of $10^{-4}-10^{-3}$ mol % with respect to the endogenous membrane lipids. Our data suggest that this level of membrane bilayer-associated acyl-CoA would support rather inefficient uncatalyzed S-acylation of membrane proteins unless the latter exhibit strongly preferential binding of such amphiphiles in competition with membrane lipids [for discussions of this question, see Brotherus *et al.* (1981) and Marsh (1985)] and/or exceptionally high reactivity of one or more cysteine residues positioned at the membrane interface. It would be of considerable interest to determine whether significant "nonenzymatic" S-acylation of proteins such as rhodopsin (O'Brien *et al.*, 1987) and myelin proteolipid protein (Bizzozero *et al.*, 1987b; Ross & Braun, 1988) can be detected *in vitro* in the presence of cytoplasmic acyl-CoA-binding proteins (Rasmussen *et al.*, 1993, 1994) to maintain physiologically low concentrations of free and membrane-associated acyl-CoAs. A similar question may be posed for putative enzymatic S-acylating activities (Berger & Schmidt, 1984; Adam *et al.*, 1984, 1988; Riendeau & Guertin, 1986; Moench *et al.*, 1994) as well as other membrane-associated processes (cf. Glick & Rothman, 1987; Pfanner *et al.*, 1989) that have been reconstituted *in vitro* using acyl-CoAs (or their precursors) at free and membrane-bound concentrations that may be considerably higher than physiological values.

Our present observations underscore the need for stringent criteria to assess the evidence for enzymatic mediation of protein S-acylation reactions that are reconstituted *in vitro* using long-chain acyl-CoAs or their precursors. For a given protein the exposure of cysteinyl residues at the membrane

[5] A high degree of selectivity in the spectrum of fatty acyl chains incorporated into a given S-acylated protein (compared, for example, to the diversity of acyl chains found in the cytoplasmic pool of long-chain acyl-CoAs) might of course suggest that the acylation process was enzymatically mediated. Unfortunately, this issue has been addressed in any detail for only a few S-acylated proteins, and in the majority of these cases [with the possible exception of rhodopsin (Papac *et al.*, 1992; compare, however, O'Brien *et al.*, 1987)] very significant heterogeneity has been found in the S-acyl chains incorporated (Berger & Schmidt, 1984; Bizzozero *et al.*, 1986; Adam *et al.*, 1988; Fujimoto *et al.*, 1993; Hallak *et al.*, 1994; and references therein). Given these findings, and our very limited knowledge of the relative proportions and availabilities of different long-chain acyl-CoAs in the cytoplasm of most cell types (Oldrich *et al.*, 1981; Prasad *et al.*, 1987; Woldgegiorgis *et al.*, 1988), it appears that for most systems the available protein acyl-composition data can offer little to clarify the mechanism(s) of the S-acylation process.

[6] Rasmussen *et al.* (1994) have recently suggested that the dissociation constants (K_d) for binding of long-chain acyl-CoAs to cytosolic acyl-CoA binding proteins (ACBPs), and hence the concentrations of free cytoplasmic long-chain acyl-CoAs, may lie in the femtomolar range under physiological conditions. While it seems clear from the various data presented by these authors (Rasmussen *et al.*, 1993, 1994) that the K_d values for ACBP binding of long-chain acyl-CoAs lie in the subnanomolar range, it is less clear that a K_d value in the midfemtomolar range, as estimated for the ACBP/palmitoyl-CoA complex, could be determined with any precision by the titration-calorimetric approach described. Extrapolating from the K_d values reported by these authors for the binding of shorter-chain acyl-CoAs to ACBP, we could estimate an average K_d value of the order of 10^{-10} M for binding of long-chain acyl-CoAs and a likely concentration of free cytoplasmic long-chain acyl-CoAs of the same order.

interface, and hence the potential susceptibility of the protein to uncatalyzed S-acylation, may vary depending on a number of factors, including the protein's orientation, conformation, dynamics, and (for a nonresident membrane-associating protein) extent of membrane binding. In some assay systems these factors may vary considerably under different conditions, or between different membrane fractions, such that an uncatalyzed S-acylation reaction could mimic many properties of an enzymatically mediated process.

While acyl-CoAs are often described (correctly) as "high-energy" acyl donors, as O'Brien *et al.* (1987) have noted, thioester linkages in S-acylated proteins are of comparable energy and should be considered in a similar light. Given this fact and the intrinsically high nucleophilicity of free sulfhydryl groups, our observation of significant exchange of S-acyl groups between cysteinyl-containing peptide sequences at the membrane bilayer interface can be readily rationalized. It is nonetheless rather striking to observe how comparatively rapid this process can be (giving half-times of transfer of a few tens of minutes to a few hours) in a simple model system entirely lacking any catalytic activity (beyond the simple concentrative and orienting effects of the membrane surface) or any evident complementarity between the donor and acceptor species.

The observations just noted suggest the interesting possibility that some membrane-associated proteins, which are particularly readily S-acylated on interfacial cysteine residues (by nonenzymatic, catalytic or autocatalytic reactions), may subsequently donate acyl groups to other membrane-associated proteins by a mechanism that requires neither specific catalytic sequences within the donor protein nor, potentially, specific recognition between the acyl-donating and -accepting sequences. In some cases such S-acyl transfer might proceed simply through random collisions and be relatively nonselective (within the constraints imposed by steric interactions between the participating proteins) and possibly purely adventitious from a functional standpoint. It is interesting to contemplate to what degree this possibility could apply to some of the number of membrane proteins that are S-acylated on cytoplasmic sequences immediately adjoining transmembrane α-helixes, a modification whose functional significance has yet to be elucidated and which has in fact been questioned for certain systems (Kotwal & Ghosh, 1984; Rose *et al.*, 1984; Jing & Trowbridge, 1987).

Greater selectivity and faster rates of interprotein S-acyl transfer could be achieved, using a "noncatalytic" mechanism like that proposed above, in cases where putative acyl-donor and -acceptor proteins form even a transient complex. Given the relatively high rates of interpeptide S-acyl transfer that we observe in our model systems, where such reactions are expected to be purely collision-mediated, it seems plausible that such complex formation could promote rapid (seconds to minutes) and selective interprotein S-acyl transfer simply by effecting a prolonged apposition and, at least to some extent, a favorable mutual orientation of the two proteins. Again, this proposed mechanism would inherently require neither a catalytic sequence or domain in the donor protein nor specific recognition between the donor and acceptor sequences themselves, so long as these sequences were mutually accessible within the donor–acceptor complex. In principle, an S-acyl transfer process of this type could even proceed in a favored direction (i.e., entail a significant net free energy change). For example, a cysteine residue that was strongly constrained to lie very near the membrane interface even when unacylated (e.g., by close juxtaposition to a transmembrane α-helix or to a second acyl- or isoprenyl-modified amino acid) might serve as a favorable acceptor of S-acyl groups from an S-acylated sequence that acquired significant conformational and/or positional freedom upon loss of its S-acyl "tether." The possibilities presented above, while obviously speculative, may be useful to consider in searching for the frequently elusive mediators of membrane protein S-acylation. They also offer a potential explanation for the seeming absence of clear consensus motifs for S-acylated sequences in many membrane-associating and membrane-integrated proteins.

REFERENCES

Adam, M., Rodriguez, A., Turbide, C., Larrick, J., Meighen, E. A., & Johnstone, R. M. (1984) *J. Biol. Chem. 259*, 15460–15463.

Adam, M. Turbide, C., & Johnstone, R. M. (1988) *Arch. Biochem. Biophys. 264*, 553–563.

Adamson, P., Marshall, C. J., Hall, A., & Tilbrook, P. A. (1992) *J. Biol. Chem. 267*, 20033–20038.

Berger, M., & Schmidt, M. F. G. (1984) *J. Biol. Chem. 259*, 7245–7252.

Bizzozero, O. A., McGarry, J. F., & Lees, M. B. (1986) *J. Neurochem. 47*, 772–778.

Bizzozero, O. A., McGarry, J. F., & Lees, M. B. (1987a) *J. Biol. Chem. 262*, 2138–2145.

Bizzozero, O. A., McGarry, J. F., & Lees, M. J. (1987b) *J. Biol. Chem. 262*, 13550–13557.

Bizzozero, O. A., Good, L. K., & Evans, J. E. (1990) *Biochem. Biophys. Res. Commun. 170*, 375–382.

Brotherus, J. R., Griffith, O. H., Brotherus, M. O., Jost, P. C., Silvius, J. R., & Hokin, L. E. (1981) *Biochemistry 20*, 5261–5267.

Camp, L. A., & Hofmann, S. L. (1993) *J. Biol Chem. 268*, 22566–22574.

Crise, B., & Rose, J. K. (1992) *J. Biol. Chem. 267*, 13593–13597.

Curstedt, T., Johansson, J., Persson, P., Eklund, A., Robertson, B., Löwenadler, B., & Jörnvall, H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2985–2989.

Degtyarev, M. Y., Spiegel, A. M., & Jones, T. L. Z. (1993) *Biochemistry 32*, 8057–8061.

Fujimoto, T., Stroud, E., Whatley, R. E., Prescott, S. M., Muzsbek, L., Laposata, M., & McEver, R. P. (1993) *J. Biol. Chem. 268*, 11394–11400.

Fujiyama, A., Tsunasawa, S., Tamano, F., & Sakiyama, F. (1991) *J. Biol. Chem. 266*, 17296–17931.

Glick, B. S., & Rothman, J. E. (1987) *Nature 326*, 309–312.

Guttierez, L., & Magee, A I. (1991) *Biochim. Biophys. Acta 1078*, 147–154.

Hallack, H., Muszbek, L., Laposata, M., Belmonte, E., Brass, L. F., & Manning, D. R. (1994) *J. Biol. Chem. 269*, 4713–4716.

Hancock, J. F., Magee, A. I., Childs, J. E., & Marshall, C. J. (1989) *Cell 57*, 1167–1177.

Hartel-Schenk, S., & Agre, P. (1992) *J. Biol. Chem. 267*, 5569–5574.

James, G., & Olson, E. N . (1990) *Biochemistry 29*, 2623–2634.

Jing, S. Q., & Trowbridge, I. S. (1987) *EMBO J. 6*, 327–331.

Kaufmann, J. F., Krangel, M. S., & Strominger, J. L. (1984) *J. Biol. Chem. 259*, 7230–7238.

Kennedy, M. E., & Limbird, L. E. (1993) *J. Biol. Chem. 268*, 8003–8011.

Koch, N., & Hammerling, G. J. (1986) *J. Biol. Chem. 261*, 3434−3440.
Kotwal, G. J., & Ghosh, H. P. (1984) *J. Biol. Chem. 259*, 4699−4701.
Lapidot, Y., Rappoport, S., & Wolman, Y. (1967) *J. Lipid Res. 8*, 142−145.
Linder, M. E., Middleton, P., Hepler, J. R., Taussig, R., Gilman, A. G., & Mumby, S. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 3675−3679.
Liu, Y., Fisher, D. A., & Storm, D. R. (1993) *Biochemistry 32*, 10714−10719.
MacDonald, R. C., MacDonald, R. I., Menco, B. Ph. M., Takeshita, K., Subbarao, N. K., & Hu, L. (1991) *Biochim. Biophys. Acta 1061*, 297−303.
Marsh, D. (1985) in *Progress in Lipid−Protein Interactions* (Watts, A., & de Pont, J. J. H. H. M., Eds.) Vol. 1, Chapter 4, Elsevier, Amsterdam.
Mack, D., Berger, M., Schmidt, M. F. G., & Kruppa, J. (1987) *J. Biol. Chem. 262*, 4297−4302.
Moench, S. J., Terry, C. E., & Dewey, T. G. (1994) *Biochemistry 33*, 5783−5790.
Mumby, S. M., Kleuss, C., & Gilman, A. G. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 2800−2804.
Nordlund, J. R., Schmidt, C. F., Dicken, S. N., & Thompson, T. E. (1981) *Biochemistry 20*, 3237−3241.
O'Brien, P. J., St. Jules, R. S., Reddy, T. S., Bazan, N. G., & Zatz, M. (1987) *J. Biol. Chem. 262*, 5210−5215.
O'Dowd, B. F., Hnatowich, M., Caron, M. G., Lefkowitz, R. J., & Bouvier, M. (1989) *J. Biol. Chem. 264*, 7564−7569.
Oldrich, A., Dietl, B., & Lynen, F. (1981) *Anal. Biochem. 113*, 386−397.
Ovchinnikov, Y. A., Abdulaev, N. G., & Bogachuk, A. S. (1988) *FEBS Lett. 230*, 1−5.
Paige, L. A., Nadler, M. J. S., Harrison, M. L., Cassady, J. M., & Geahlen, R. L. (1993) *J. Biol. Chem. 268*, 8669−8674.
Papac, D. I., Thornburg, K. R., Büllesbach, E. E., Crouch, R. K., & Knapp, D. R. (1992) *J. Biol. Chem. 267*, 16889−16894.
Parenti, M., Viganó, M. A., Newman, C. M. H., Milligan, G., & Magee, A L. (1993) *Biochem. J. 291*, 349−353.
Peitszch, R. M., & McLaughlin, S. (1993) *Biochemistry 32*, 10436−10443.
Pfanner, N., Orci, L., Glick, B. S., Amherdt, M., Arden, S. R., Malhotra, V., & Rothman, J. E. (1989) *Cell 59*, 95−102.
Prasad, M. R., Sauter, J., & Lands, W. E. M. (1987) *Anal. Biochem. 162*, 202−212.
Rasmussen, J. T., Rosendal, J., & Knudsen, J. (1993) *Biochem. J. 292*, 907−913.
Rasmussen, J. T., Faergeman, N. J., Kristiansen, K., & Knudsen, J. (1994) *Biochem. J. 299*, 165−170.
Riendeau, D., & Guertin, D. (1986) *J. Biol. Chem. 261*, 976−981.
Rose, J. K., Adams, G. A., & Gallione, C. J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2050−2054.
Ross, N. W., & Braun, P. E. (1988) *J. Neurosci. Res. 21*, 35−44.
Schlesinger, M. J., Veit, M., & Schmidt, M. F. G. (1993) in *Lipid Modifications of Proteins* (Schlesinger, M. J., Ed.), pp 1−19, CRC Press, Boca Raton, FL.
Schmidt, M. F. G. (1989) *Biochim. Biophys. Acta 988*, 411−426.
Schmidt, M., Schmidt, M. F. G., & Rott, R. (1988) *J. Biol. Chem. 263*, 18635−18639.
Schultz, A. M., Henderson, L. E., & Oroszlan, S. (1988) *Annu. Rev. Cell Biol. 4*, 611−647.
Sefton, B. M., & Buss, J. E. (1987) *J. Cell Biol. 104*, 1449−1453.
Shenoy-Scaria, A. M., Gauen, L. K. T., Kwong, J., Shaw, A. S., & Lublin, D. M. (1993) *Mol. Cell. Biol. 13*, 6385−6392.
Shi, Y., Veit, B., & Baekkeskov, S. (1994) *J. Cell. Biol. 124*, 927−934.
Silvius, J. R., & Gagné, J. (1984) *Biochemistry 23*, 3232−3240.
Silvius, J. R., & l'Heureux, F. (1994) *Biochemistry 33*, 3014−3022.
Skene, J. H. P., & Virag, I. (1989) *J. Cell Biol. 108*, 613−625.
Viet, M., Nürnberg, B., Spicher, K., Harteneck, C., Ponimaskin, E., Schultz, G., & Schmidt, M. F. G. (1994) *FEBS Lett. 339*, 160−164.
Wedegaertner, P. B., Chu, D. H., Wilson, P. T., Levis, M. J., & Bourne, H. R. (1993) *J. Biol. Chem. 268*, 25001−25008.
Weims, T., & Stoffel, W. (1992) *Biochemistry 31*, 12289−12296.
Woldegiorgis, G., Spennetta, T., Corkey, B. E., Williamson, J. R., & Shrago, E. (1988) *Anal. Biochem. 150*, 8−12.