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Alkylation of cysteine-containing peptides to mimic palmitoylation

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Abstract: Numerous proteins that are involved in cell signaling and viral replication require post-translational modification by palmitoylation to function properly. The molecular details by which this palmitoyl modification affects protein function remain poorly understood. To facilitate *in vitro* biochemical and structural studies of the role of palmitoylation on protein function, a method was developed for alkylating peptides with saturated C_{16} groups at cysteine residues and demonstrated using peptides derived from the palmitoylated region of Sindbis virus E2 glycoprotein. The synthetic approach takes advantage of disulfide chemistry to specifically modify only the cysteine residues within peptides and covalently links C_{16} groups via disulfide bridges using a new thioalkylating reagent, hexyldexyl-dithiopyridine. The chemistry presented here takes place in solution under mild conditions without the need for protection of the peptide functional groups. A method for purifying these modified peptides is also described. This protocol can be of general use to investigators studying the role of palmitoylation in biological systems.

Abbreviations: CI-MS, chemical ionization mass spectrometry; DMSO, dimethylsulfoxide; DTT, dithiothreitol; FABS, fast atom bombardment mass spectroscopy; Fmoc, N^{α}-flurenylmethyloxycarbonyl; HPLC, high-performance liquid chromatography; MLV, multilamellar vesicle; NMR, nuclear magnetic resonance; PC, L-α-phosphatidylcholine; PDMS, plasma desorption mass spectroscopy; PS, L-a-phosphatidyl-L-serine; TFA, trifluoroacetic acid; TFE, trifluoroethanolamine; TLC, thin-layer chromatography.

Palmitoylation is a process by which proteins are modified post-translationally by the covalent attachment of saturated 16-carbon (palmitoyl) fatty acid groups. These palmitoyl groups are joined by cellular enzymes to targeted cysteine residues via a labile thioester linkage (1). The palmitoylation modification is a reversible event and has been associated with a number of proteins that are involved in signal transduction pathways, such as the members of the *src* family of protein tyrosine kinases (2–7), the G-protein α_s subunit (8, 9), the G-protein receptor kinase GRK6 (10) and members of the Ras family of proteins (11–13). Reversible acylation is considered important in modulating the signal-transducing functions of these proteins by controlling their recruitment to their sites of action at the plasma membrane (3). Various transmembrane glycoproteins of enveloped RNA viruses are also palmitoylated (1, 14–17), but the molecular details by which palmitoyl modification affects the viral life cycle remain speculative. For example, while site-directed mutagenesis studies in alphavirus systems show that envelope protein palmitoylation in alphaviruses is somehow important for proper virion formation, such studies do not provide further insight into a detailed biochemical mechanism for viral assembly (1, 16, 18–20).

In vitro studies of the biochemical and structural effects of palmitoylation require the introduction of palmitoyl groups at cysteine side chains. To date, synthetic methods either do not target palmitoyl groups specifically to cysteines (21, 22) or else rely upon careful protection/deprotection strategies during solid-phase peptide synthesis (23, 24). To assist our biochemical and structural studies of alphavirus assembly in model membrane systems, we developed a simple synthetic approach for cysteine-specific alkylation of peptides with C_{16} groups and demonstrated the technique using peptides derived from the palmitoylated region of the Sindbis virus E2 glycoprotein. To generate these peptide modifications, a new thioalkylating agent, hexyldecyl-dithiopyridine, was developed that specifically tethers saturated C_{16} groups to cysteine residues in peptides via disulfide bridges. The organic synthesis and purification of these alkylated peptides are reported here.

The addition of the C_{16} alkyl groups to peptides as described below is shown to confer a membrane-anchoring capacity in a manner that mimics *in vivo* palmitoylation. Reconstitution of these alkylated peptides into simple model membrane systems allows investigation of protein–lipid interactions and assessment of the biochemical activity of these peptides when anchored to a model membrane surface. In particular, solution NMR structural studies of these alkylated peptides in detergent micelles or bicelles (25–28) are approachable and ultimately more can be learned about the structural role of palmitoylation in diverse biological systems.

Experimental Procedures

Materials

E2/12 (Ac-KARRECLTPYAL-NH_2) and E2/33 (Ac-KARRECLTPYALAPNAVIPTSLALLCCVRSANA-NH_2) peptides were synthesized at the Purdue University Peptide Synthesis Facility using either Fmoc (for E2/12) or tBoc (for E2/33) chemistry, and were further purified using reverse-phase chromatography. Peptide sequences were confirmed by plasma desorption mass spectroscopy (PDMS) analysis using a Bioion 20R plasma desorption mass spectrometer. Observed $[MH]^+$ for E2/12 was 1462.7 (1462.8 calcd for mass-averaged $C_{64}H_{109}N_{20}O_{17}S$); obsd $[MH]^+$ for E2/33 was 3528.8 (3529.3 calcd for mass-averaged $C_{153}H_{261}N_{46}O_{43}S_3$). Dithiodipyridine (trade name Aldrithiol) and 99% trifluoroacetic acid (TFA) were purchased from Aldrich Chemical Company, and hexyldecyl mercaptan was purchased from TCI America. Formic acid (98–100%) and spectroscopic grade acetonitrile were purchased from VWR Scientific. Dithiothreitol (DTT), trifluoroethanolamine (TFE) and L-α-phosphatidyl-L-serine (PS) were purchased from Sigma Chemical Company and L-α-phosphatidylcholine (PC) was purchased from Avanti Polar Lipids. Silica gel 60 (230–400 mesh) was purchased from E. Merck.

Synthesis of hexyldecyl-dithiopyridine (1)

As outlined in step 1 of Fig. 1, dithiodipyridine (1.7 g, 7.7 mmol) dissolved in 12.5 mL of 0.1 M Tris (pH 8)/*n*-propanol (1 : 1) was added to hexyldecyl mercaptan (0.2 g, 0.77 mmol) in 10 mL of *n*-propanol, and stirred at room temperature for 1.5 h. The reaction mixture was extracted with ethyl acetate (2 × 30 mL) and the organic phase was further dried with Na_2SO_4. Following filtration through Whatman filter paper and concentration via rotary evaporation, the reaction mixture was loaded onto a silica gel 60 column and then eluted with a hexanes/ethyl acetate (16 : 1) mobile phase. A sample of each column fraction was run on a silica TLC plate using a hexanes/ethyl acetate (16 : 1) mobile phase. Exposure of the plate to UV light and to I_2 vapor identified a group of fractions containing a single new compound with an R_f of 0.9. This R_f value is distinct

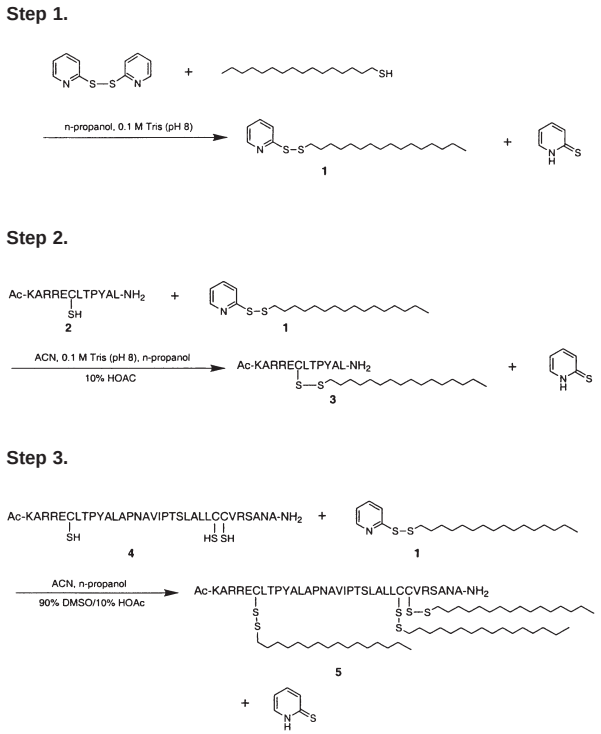

Figure 1. The synthetic approach for preparation of alkylated E2 peptides. Step 1, the activation of hexyldecyl mercaptan; step 2, alkylation of E2 12-mer peptide; step 3, alkylation of E2 33-mer peptide.

from those of the unreacted starting reagents, and thus the new compound was a candidate for **1**. All such fractions were pooled and the total sample volume was reduced by rotary evaporation; the sample was then flash-frozen in liquid nitrogen and placed under vacuum overnight to remove residual solvent, yielding a white powder (0.28 g, 100% yield based upon the weight of hexyldecyl mercaptan). This material was analyzed by ^{1}H NMR using a Varian VXR500 spectrometer and by chemical ionization mass spectrometry (CI-MS) with a Finnigan GCQ (ion trap) mass spectrometer. ^{1}H NMR [500 MHz,$CDCl_3$] δ 8.59 (d, J = 5.3 Hz, 1H), 8.00 (m, 1H), 7.96 (m, 1H), 7.35 (dd, J = 6.3, 6.3 Hz, 1H), 1.2 (m, 28H); CI-MS $[MH]^+$ calcd for $C_{21}H_{38}NS_2$ is 368, obsd $[MH]^+$ is 368. The TLC, NMR and mass spectrometry analysis confirmed the identity of this compound as being **1**.

Synthesis and purification of alkylated E2 peptides (3 and 5)

In step 2 of Fig 1, 3 μmol (1.1 mg) of **1** in 750 μL of 0.1 M Tris buffer (pH 8)/*n*-propanol/acetonitrile (1 : 1 : 1) was stirred for 20 min and added to 0.3 μmol (0.5 mg) of **2** in 375 μL of 10% acetic acid. The reaction mixture was then stirred for 12 h at room temperature. Following centrifugation to remove insoluble material, the clarified supernatant was loaded onto an analytical reverse-phase C_{18} column (Pharmacia). Using a 0–100% gradient in Solvent B developed over 30 min (Solvent A: 95% H_2O, 5% acetonitrile, 0.1% TFA; Solvent B: 95% acetonitrile, 5% H_2O, 0.1% TFA) with a flow rate of 1 mL/min (detection at 280 nm), a new compound was found to elute at 56% B; this compound was analyzed by fast-atom bombardment mass spectrometry (FABS) using a Kratos MS-50 instrument.

Samples of both this compound and of unmodified **2** that contained a 10-fold molar excess of DTT were analyzed by HPLC. Twenty-five micrograms of each sample in 100 μL of potassium phosphate buffer (20 mM potassium phosphate, 1 mM NaN_3, pH 7.4) were loaded onto a reverse-phase C_4 analytical column (Vydac) and eluted using the gradient described above (detection at 214 nm); the profiles from these chromatograms were then compared. Another sample of the new compound was prepared containing a 10-fold molar excess of DTT; 25 μg of this sample in 100 μL of potassium phosphate buffer was loaded onto the column and eluted with the same gradient. The profile from this chromatographic run was examined for similarities to the elution profile of **2**. The mass spectrometry and HPLC analysis confirmed the identity of this new compound as being **3**, which was obtained at 47% yield based upon the weight of **2**.

The E2/33 peptide (**4**) was alkylated using similar protocols as those used for **2**. In step 3 of Fig 1, 1.6 μmol (0.6 mg) of **1** in 380 μL of dimethyl sulfoxide (DMSO)/*n*-propanol/acetonitrile (1 : 1 : 1) was stirred for 20 min and added to 57 nmol (0.2 mg) of **4** in 200 μL of 90% DMSO/10% acetic acid. The reaction mixture was then purged with nitrogen gas and stirred under nitrogen for 24 h at room temperature. After centrifugation to remove insoluble material, the reaction mixture was loaded onto a reverse-phase C_4 preparatory column (Vydac) and eluted at a flow rate of 3.5 mL/min (Solvent A: 75% H_2O, 5% acetonitrile, 20% formic acid; Solvent B: 75% acetonitrile, 5% H_2O, 20% formic acid) using the following gradient: 0% B, 0–3 min; 30–60% B, 3–26.5 min; and finally 60–100% B, 26.5–50.5 min (detection at 280 nm). A peak that eluted at 98% B was collected and lyophilized, yielding a white powder (25% yield based upon the weight of **4**); an analysis of this material by PDMS confirmed the presence of triply alkylated E2/33 [5]. The PDMS analysis also indicated the presence of singly and doubly alkylated peptide in the recovered material.

Characterization of the membrane-targeting properties of the alkyl modification

A stock of 95% PC/5% PS multilamellear vesicles (MLVs) was prepared by dissolving in a round-bottomed flask 0.25 mg of PS and 4.75 mg PC in chloroform and drying the solution to a thin film under a stream of nitrogen. Samples were placed under a vacuum overnight to remove residual chloroform, rehydrated with 250 µL of potassium phosphate buffer and freeze–thawed with vortexing several times to yield MLVs. The liposomes were then centrifuged at 10 000 **g** for 10 min and the supernatant containing the smaller vesicles was removed. The pellet was gently resuspended and the sample was centrifuged and resuspended twice more to isolate the larger vesicles. The liposome suspension containing predominantly large MLVs was resuspended to 20 mg/mL in potassium phosphate buffer.

Twenty microliters of this MLV stock solution were added to 150 µL of 0.25 mg/mL **3** in potassium phosphate buffer (35 : 1 molar ratio of lipid/peptide) and incubated at room temperature for 45 min. Following this incubation, the mixture was centrifuged at 10 000 **g** for 10 min and the supernatant was transferred to a new microfuge tube; the pellet was resuspended with 20 µL of TFE, followed by the addition of 150 µL of potassium phosphate buffer. An identical experiment with a mixture containing liposomes and **2** (30 : 1 molar ratio of lipid/peptide) was performed in parallel, as well as a control reaction containing **3** but no liposomes. The fractions from each experiment were loaded onto a reverse-phase C_4 analytical column (Vydac) and **2** or **3** were eluted with a gradient of 0% B, 0–3 min; 0–100% B, 3–33 min (Solvent A: 95% H_2O, 5% acetonitrile, 0.1% TFA; Solvent B: 95% acetonitrile, 5% H_2O, 0.1% TFA) at a flow rate of 1 mL/min (detection at 214 nm). Subsequent inspection of the resulting chromatogram profiles readily determined whether **2** or **3** display affinity for phospholipid membranes.

Results and Discussion

Synthesis, purification and characterization of alkylated peptide compounds

The aim of this study was to covalently link saturated C_{16} alkyl groups via disulfide linkages to cysteines in peptides and thus mimic the palmitoylated state of a protein. A simple synthesis scheme (Fig. 1) was developed to achieve this aim. The scheme features the use of a new thioalkylating reagent, hexyldecyl-dithiopyridine [1], that can transfer saturated C_{16} groups to peptides with the desired cysteine specificity. The purified reaction products from step 1 of Fig. 1 were initially analyzed by TLC as described above to verify the successful synthesis of **1**. The TLC analysis identified a new compound with an R_f value of 0.9, which is distinct from the R_f values of the starting reagents. This new compound is UV-positive, indicating the presence of a conjugated system within the compound. Furthermore, the ^{1}H NMR spectrum of this compound is consistent with that expected for **1**, displaying the anticipated peaks in the aromatic and aliphatic regions of the spectrum (see Experimental Procedures). Finally, CI-MS analysis confirmed that the new compound has a molecular mass corresponding to **1**. Observed $[MH]^+$ = 368 (368 calcd for $C_{21}H_{38}NS_2$). Together, the TLC, NMR and mass spectrometry data confirmed the generation of **1**. When stored in nitrogen gas at –20°C and under desiccating conditions, this compound retains excellent thioalkylation activity towards cysteine-containing peptides (*vide infra*) for many months.

Using **1**, E2/12 (**2**) was alkylated as described in step 2 of Fig. 1. The reaction products from step 2 were eluted from a C_{18} reverse-phase column using a conventional water/acetonitrile/0.1% TFA solvent system (see Experimental Procedures). A peak that eluted at 56% B was collected and analyzed by FABS, this analysis confirmed that the collected peak contained a compound having a molecular mass corresponding to the alkylated product **3** (Fig. 2A). This

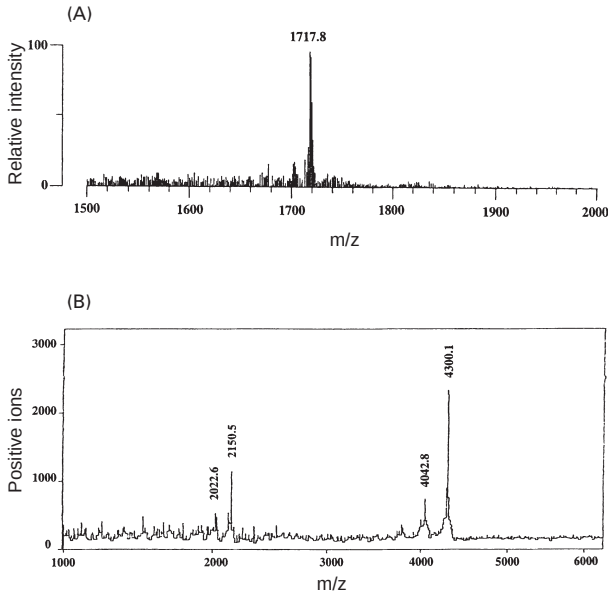

Figure 2. (A) FABS analysis of the product from the reaction containing **2** as the starting material. (B) PDMS analysis of the product from the reaction containing **4** as the starting material.

compound was obtained at 47% yield based upon the weight of **2**. Observed $[MH]^+$ = 1717.8 (1719.3 calcd for mass-averaged $C_{80}H_{141}N_{20}O_{17}S_2$). The observed molecular mass of this purified reaction product agrees to within 0.09% of the calculated molecular mass of **3**, thus confirming successful synthesis and purification of the singly alkylated peptide **3**.

Using **1**, E2/33 (**4**) was alkylated as described in step 3 of Fig. 1. While **2** dissolves readily in 10% acetic acid prior to reaction with **1**, **4** is more difficult to solubilize (20 nonpolar residues among the last 28 of this peptide) and solubilization requires 90% DMSO/10% acetic acid prior to reaction with **1**. Furthermore, triply alkylated E2/33 (**5**) could not be eluted from a C_4 reverse-phase column using a conventional water/acetonitrile/0.1% TFA solvent system, the coupling of three saturated C_{16} groups to an already hydrophobic peptide resulted in irreversible adsorption of the compound to the column. To overcome this difficulty, a solvent system based upon the methods of Heukeshoven & Dernick (29) was developed. This solvent system contains 20% formic acid (see Experimental Procedures) and was successfully employed to elute the highly nonpolar compound **5**.

HPLC purification of the reaction mixture from step 3 using the formic acid/acetonitrile/water solvent system results in a peak that elutes at 98% B from the C_4 reverse-phase column. Analysis of this peak using PDMS verified that the peak contained the alkylated product **5** (Fig. 2B). Observed $[MH]^+$ was 4300.1 (4297.8 calcd for mass-averaged $C_{201}H_{357}N_{46}O_{43}S_6$). This observed molecular mass agrees to within 0.05% of the calculated molecular mass **5**, thus confirming successful synthesis. The mass spectrum also indicates that singly and doubly alkylated **4** had copurified with **5**; given the extreme hydrophobicity of these alkylated peptides, the two partially alkylated peptide species had probably aggregated with the triply alkylated **5** on the column, resulting in the observed coelution.

To further characterize the attachment of C_{16} groups using the designed synthesis scheme, both **2** and **3** were analyzed by reverse-phase HPLC (Fig. 3A–C). Twenty-five micrograms of **2** with a 10-fold molar excess of the disulfide-reducing agent DTT were loaded onto an analytical C_4 reverse-phase column and eluted using a 0–100% acetonitrile gradient (see Experimental Procedures); **2** elutes at ≈ 30% B (Fig. 3A). An identical amount of **3** was also analyzed and this compound typically elutes at ≈ 57% B (Fig. 3B). The increased hydrophobicity of this compound, as indicated by the longer elution time during the chromatographic run, is consistent with the addition of the hydrophobic C_{16} group to peptide **2**. Finally, compound **3** was incubated for 2 h with

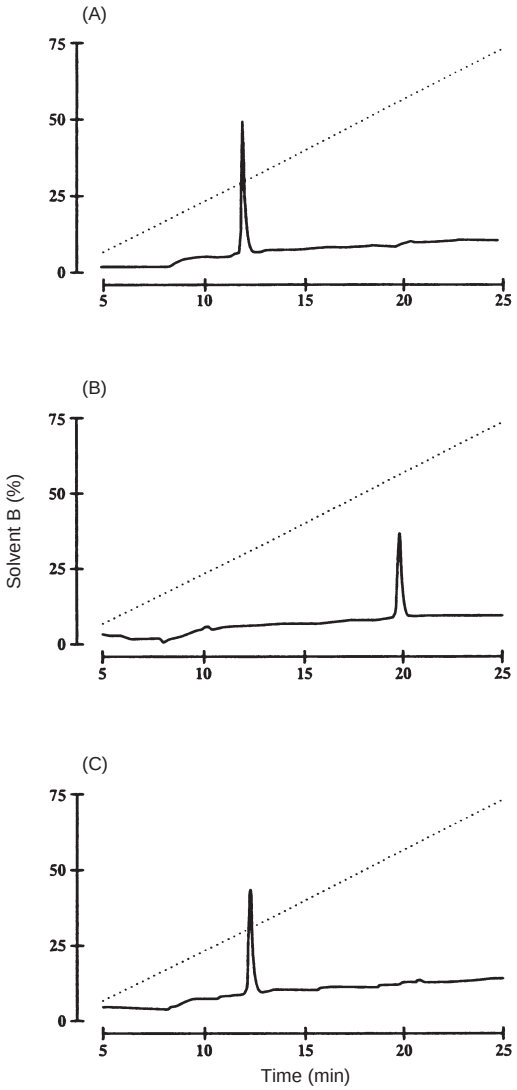

Figure 3. Reverse-phase HPLC analysis of (A) 25 μg of **2**; (B) 25 μg of the compound that was isolated from the reaction in step 2 of Fig. 1; (C) same sample as in (B), but with a 10-fold molar excess of DTT added to reduce any disulfide bonds.

10-fold molar excess of DTT to reduce the putative disulfide bond and 25 μg of this sample was subsequently analyzed by HPLC as described above (Fig. 3C). The resulting peak appears on the chromatogram at the identical position of the peak that corresponds to **2** (Fig. 3A). This observation is consistent with the idea that the added DTT reduces the disulfide bond in the product **3** and regenerates the original reactant **2**. This result further confirms that the synthesis scheme that has been developed alkylates peptides successfully through a disulfide bond as designed.

Membrane-targeting function of the alkyl modification

The anticipated result of alkylating cysteine-containing peptides as described above is that such a modification will

confer a membrane-anchoring capacity to these peptides. This membrane-anchoring capacity was demonstrated using a centrifugation binding assay. Compound **3** was added to a preparation of 95% PC/5% PS multilamellear liposomes in a 35 : 1 molar ratio of lipid/peptide. A similar mixture containing unmodified compound **2** with these vesicles (30 : 1 molar ratio of lipid/peptide) was also prepared, as was as a 'control reaction' containing an identical amount of **3**, but no liposomes (see Experimental Procedures). Following 45 min incubation, the mixtures of membranes and peptide were centrifuged; the resulting pellet and supernatant fractions from these mixtures were each analyzed by reverse-phase HPLC (Fig. 4A–C).

Affinity for the membranes used in this study is indicated by the presence of peptide in the pellet fractions. Unmodified peptide **2** does not have affinity for these vesicles, as this compound is found primarily in the supernatant fraction (Fig. 4A). Conversely, modified peptide **3** is found primarily in the pellet fraction (Fig. 4B), indicating that this compound binds preferentially to the vesicles under the conditions employed. The appearance of this compound in the pellet fraction is the result of the presence of a membrane surface (and not an artifact of centrifugation), as the experiment with the control reaction containing **3** but no membranes show that **3** remains primarily in the supernatant when membranes are absent from the solution (Fig. 4C). Since the sole difference between compounds **2** and **3** is the alkyl group in **3**, this alkyl group must be responsible for targeting **3** to the phospholipid vesicles. Thus, under the conditions tested the alkylation performs its expected function of targeting peptides to a membrane surface, thus mimicking the putative function of the palmitoyl modification in proteins.

Stability of thioalkylated peptides

The disulfide bond is labile in the presence of reagents that promote thiol–disulfide exchange reactions (30, 31). Clearly, reducing agents (such as glutathione or DTT) that are commonly employed in biochemical assays should be used

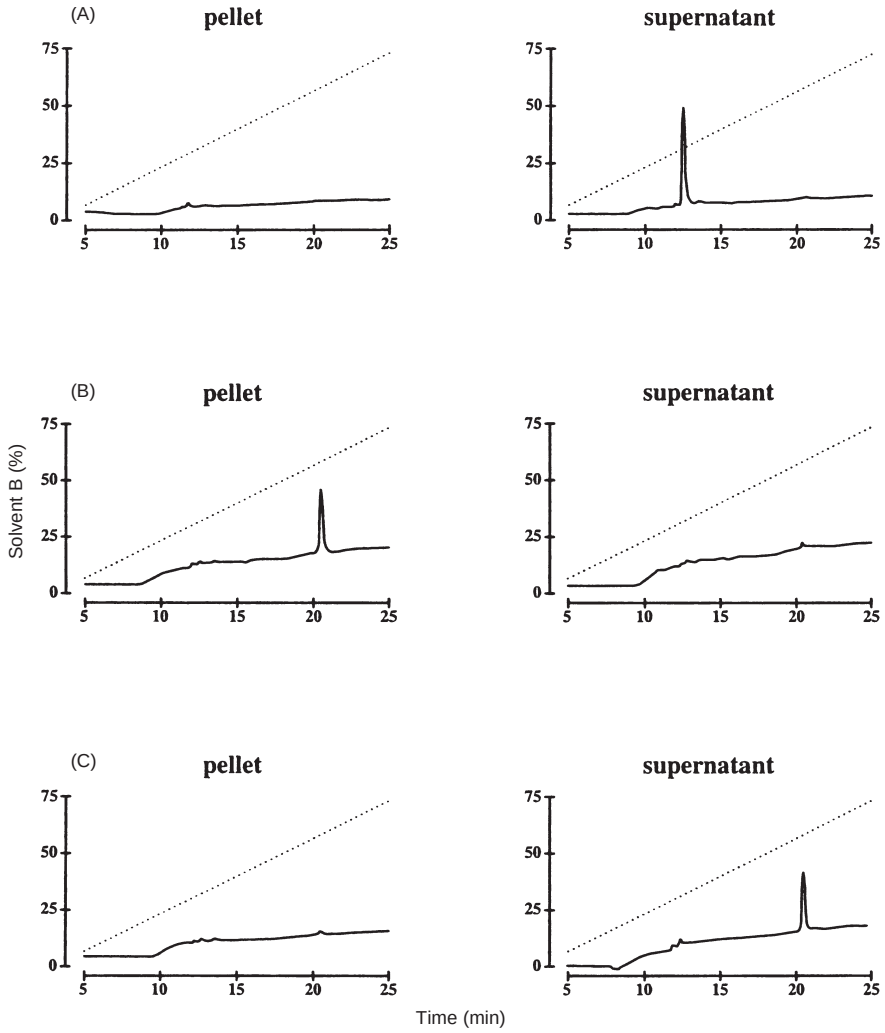

Figure 4. Reverse-phase HPLC analysis of (A) pellet and supernatant fractions of the mixture containing phospholipid membranes and **2**; (B) pellet and supernatant fractions of the mixture containing phospholipid membranes and **3**; (C) pellet and supernatant fractions of the 'control reaction' containing **3** without phospholipid membranes.

with caution in any studies that involve thioalkylated peptides, as the disulfide bond in these modified peptides can be sensitive to such reagents. Conversely, the disulfide bond is expected to be quite stable in the absence of free thiols, which can catalyze the formation of mixed disulfide products; in the absence of thiol, the modified peptides are not expected to be prone to disulfide rearrangement. In accord with this expectation, when **3** or **5** were reconstituted into lipid bilayers in thiol-free phosphate buffer (pH 7.2–7.4), stored at 4°C and then analyzed by analytical reverse-phase HPLC 15 days (for **3**) or 8 days (for **5**) after peptide reconstitution, the resulting chromatograms indicated the presence of **3** or **5** but provided no evidence of the formation of new rearrangement products (data not shown). Thus, under the conditions tested the modified peptides are stable within the explored time frame. The observed disulfide stability is consistent with findings from a previous report of the stability of an intrachain disulfide bridge in a nine-residue neuropeptide Y antagonist. In this study, dimeric peptide species were generated readily via mixed disulfide linkages in the presence of DTT, but in the absence of free thiol no formation of mixed disulfide products occured [32].

Discussion

The synthetic approach that has been chosen to mimic protein palmitoylation has a number of attractive features. First, the synthesis and purification method is robust, having been demonstrated with both water-soluble (**2**) and water-insoluble (**4**) peptides. Also, the chemistry is straightforward, having been performed in solution under mild conditions and in few steps. Importantly, **1** specifically modifies the SH group on cysteine and will not react with OH or NH_2 groups, so the synthesis method described above does not require strategies to prevent unwanted reactions with side chains of other amino acids such as lysine, serine, threonine or tyrosine. This manner of alkylation provides a 'membrane anchor' at the cysteine side chain in a way that mimics *in vivo* palmitoylation. The procedure should prove advantageous in modifying cysteines in other synthetic, fully deprotected peptides, as well as peptides that are produced from enzymatic digests of natural proteins. This method can be used to produce a new class of peptide compounds that can be reconstituted into model membrane systems (such as detergent micelles or bicelles) for subsequent characterization of biochemical activity or for macromolecular structure studies using NMR spectroscopy. From such studies in simple model systems, more can be learned about the influence of the palmitoyl modification upon protein function, leading to a deeper understanding of the effects of protein palmitoylation upon molecular events in living cells.

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