Semisynthetic Proteins: Model Systems for the Study of the Insertion of Hydrophobic Peptides into Preformed Lipid Bilayers†

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ABSTRACT: Models of protein translocation and secretion will not be complete without details of the mechanism of lipid bilayer insertion. The study of spontaneous hydrophobic peptide interactions with model membrane systems has been hindered by their very low solubility in aqueous solutions. A novel protocol has been developed that enables the site-specific (N-terminus) attachment of hydrophobic peptides to a water-soluble carrier protein [bovine pancreatic trypsin inhibitor (BPTI)] using a heterobifunctional crosslinker (SPDP). In this initial study H-$(Ala)_{20}$-Tyr-Cys-$CONH_2$ and H-$(Ala)_{10}$-Tyr-Cys-$CONH_2$ were selected as hydrophobic peptides, since alanine is the simplest α-helix-forming amino acid, and the peptides as α-helices are just long enough to span the lipid bilayer and monolayer, respectively. The carrier protein was treated with σ-methylisourea, which resulted in the guanidination of the four lysine ε-amine groups. The chemical modification of BPTI to give G-BPTI allowed the attachment of SPDP specifically to the free N-terminal α-amine group. The peptides were synthesized with a C-terminal cysteine moiety, allowing the site-specific cross-linking of the peptides to the N-terminus. In order to prevent peptide aggregation, the synthetic peptides were cleaved from the preparative resin in detergent and cross-linked to G-BPTI. After cross-linking, the detergent was removed from the mixture by gel filtration employing propionic and formic acids in the mobile phase. The detergent-free, peptide−G-BPTI conjugates were subsequently purified by reversed-phase HPLC. The interaction parameters of the two semisynthetic proteins with large unilamellar vesicles were determined by ultracentrifugation of the equilibrated vesicle−protein mixtures. For comparison, the same semisynthetic proteins were reconstituted into lipid vesicles using an octyl glucoside dilution technique. The incorporation and reconstitution data proved to be quite similar. The results indicated that $(Ala)_{20}$−G-BPTI interacted with LUV to form a stable complex and behaved as a membrane protein in reconstituted bilayer systems. $(Ala)_{10}$−G-BPTI, however, remained in the aqueous phase in both bilayer systems. The thermodynamic interaction data are compared to the theoretical values of total free energy changes calculated for the incorporation of model hydrophobic α-helices. In addition, the solubility and stability of the hydrophobic peptides, both in the aqueous phase and membrane-bound, were studied by cleaving the disulfide bond linking the peptides to G-BPTI using dithiothreitol. Molecular sieve chromatography was used to evaluate the state of self-association of the semisynthetic proteins in aqueous solutions.

Two aspects of the problem of membrane biogenesis that have been the focus of much research are membrane protein targeting and membrane protein translocation [for reviews, see Randall and Hardy (1989), Wickner (1988), and Gierasch (1989)]. Two important questions in these areas are (i) how are individual membrane proteins directed to their proper target membrane, and (ii) how are membrane proteins, often with rather large water-soluble domains, inserted into the nonpolar membrane to achieve the proper topology? The process of protein export can be broken down into three stages: entry, translocation, and release (Randall & Hardy, 1989). The most controversial stage has been the mechanism of translocation. In the past, the prevailing thought has been that the translocation step occurred through protein pores or tunnels. Recently, examples of spontaneous partitioning of proteins into model lipid bilayers (Dumont & Richards, 1984; Rietveld & de Kruijff, 1984; Maduke & Roise, 1993), and the failure to arrest membrane biogenesis in yeast in gene deletion experiments (Hann & Walter, 1991), suggest that parallel pathways of polypeptide insertion may exist and that one of these pathways may be spontaneous insertion into the membrane bilayer. Central to these studies is the problem of the mechanism of interaction of signal sequences with membrane systems to initiate translocation.

One of the most striking features of signal sequences is their lack of sequence homology, with the possible exception of the site of cleavage by signal peptidase. Nevertheless, most signal sequences can be divided into two groups: (1) the amphipathic group, rich in basic and hydroxylated residues that form amphipathic helices and (2) the hydrophobic group (Tamm, 1991). The first group mainly targets proteins to the mitochondria, chloroplasts, and peroxisomes, whereas members of the second group target proteins to the endoplasmic reticulum and bacterial membranes. Analyses of signal sequences from both prokaryotes and eukaryotes has identified three discernible regions: the n-, h-, and c-regions (von Heijne, 1985). The most distinctive part of the leader sequence is the h-region. The length of this highly

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hydrophobic segment runs between 10 and 15 residues, with no sequence homology evident among various leader sequences. The function of this region, proposed to be the partitioning into and spanning of the membrane bilayer (Bedouelle & Hofnung, 1981), has been shown to depend directly on length and hydrophobicity in many different systems (Bankaitis et al., 1984; Chou & Kendall, 1990).

Despite hypotheses that argue for a direct role of the membrane bilayer in protein translocation, experimental evidence has been scarce. The ease in producing arrays of mutant polypeptides by *in vitro* mutagenesis has caused most researchers to focus on structure−function relationships in membrane proteins. The role of the lipid bilayer in protein translocation has largely been ignored, because *in vitro* experimental techniques that can probe the roles of membrane lipids are not applicable to studies that are not well-defined (e.g., *in vitro* translation systems in the presence of microsomal fractions), and, *in vivo*, mutagenic techniques are largely applicable to proteins (directly); those that do affect lipid species (indirectly) most often can be suppressed by other lipids.

Ideally, if the role of the h-region in protein insertion is the subject of research, the logical choice for probes is a series of model peptides differing incrementally in both length and degree of hydrophobicity. However, such peptides are highly insoluble in aqueous solutions, and their tendency to aggregate irreversibly severely limits their study. Generally, physical−chemical studies of protein−lipid interactions have focused on the use of small proteins or model peptides solubilized in membrane-mimetic solvent systems (Rizo et al., 1993) or reconstituted in defined lipid systems (Silvius, 1992). Relatively little work has been done on the insertion of hydrophobic peptides into preformed bilayers. Due to the much greater aqueous solubility of isolated amphipathic signal sequences, most of the biophysical research concerning signal sequence−membrane interactions has focused on this class of peptides. Studies involving the hydrophobic class have almost exclusively been undertaken in artificially reconstituted systems or have employed the addition of charged residues upstream from the signal sequence to increase water solubility (Briggs & Gierasch, 1984). A novel approach to this problem, modeled after type I integral membrane proteins that are anchored to the bilayer by a single transmembrane segment, is to couple the hydrophobic peptides to a water-soluble carrier protein (Moll et al., 1991). Examples of integral membrane proteins of this type are microsomal cytochrome b_5 (Takagaki et al., 1983; Rezpecki et al., 1986) and vesicular stomatitis virus G protein (Patzer et al., 1979). In the case of cytochrome b_5, the amphipathic structure can be divided into the hydrophilic catalytically active domain (11 000 kDa), which binds the heme group, and the hydrophobic membrane-binding domain (5000 kDa) which serves as the membrane anchor. The latter domain consists of ~35 residues at the C-terminus, whose overall hydrophobicity is comparable to signal peptides (Bendzko et al., 1982). This protein, readily dispersible in aqueous phases, inserts spontaneously into preformed unilamellar vesicles of phosphatidylcholine as indicated by the enhancement of the fluorescence of tryptophan residues located in the hydrophobic segment (Dufourcq et al., 1975).

In this paper, we describe a relatively simple and convenient method for the generation of semisynthetic proteins,

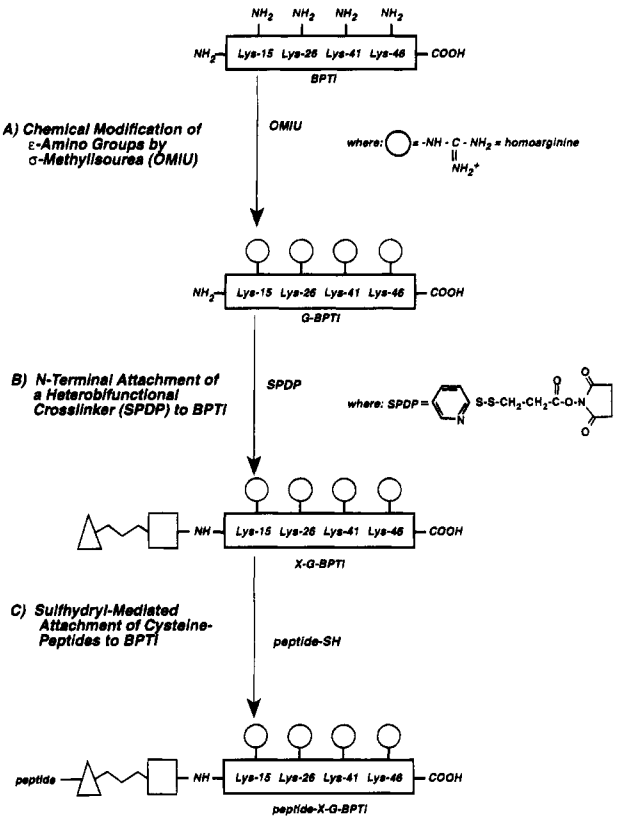

FIGURE 1: General scheme for the production of semisynthetic proteins. The structures of the various intermediates and products are shown schematically.

which are analogs of type I integral membrane proteins. This novel protocol for the covalent attachment of the hydrophobic peptides specifically to the N-terminus of bovine pancreatic trypsin inhibitor (BPTI)[1] requires blocking by guanidination the four lysine groups of the carrier (Lys^{15}, Lys^{26}, Lys^{41}, and Lys^{46}) to produce G-BPTI, and reaction of the lone free α-amine group at the N-terminus with the amine-specific moiety of a heterobifunctional cross-linker, *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP). The C-terminal cysteine of the peptide is then coupled to the sulfhydryl-reactive moiety of SPDP. Reversed-phase HPLC is employed to isolate $(Ala)_n$−G-BPTI conjugates. The end product, G-BPTI with the $(Ala)_n$ peptide covalently and site-specifically attached, is termed a semisynthetic protein. The general chemical structure of such a semisynthetic protein can be written as

$$NH_2\text{-peptide-S-S-CH}_2\text{-CH}_2\text{-CO-NH-(G-BPTI)-COOH}$$

The scheme for the production of semisynthetic proteins that we have employed is shown in Figure 1.

[1] Abbreviations: BPTI, Bovine pancreatic trypsin inhibitor; G-BPTI, guanidinated-BPTI; DABS, (dimethylamino)azobenzenesulfonyl chloride; DCM, dichloromethane; DIPEA, diisopropylethylamine; DMF, dimethyl formamide; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; LUV, large unilamellar vesicles; MTBE, methyl *tert*-butyl ether; $NaBH_4$, sodium borohydride; OMIU, *O*-methylisourea; $(Ala)_n$-G-BPTI, semisynthetic protein; SUV, small unilamellar vesicles; SDS, sodium dodecyl sulfate; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; TFA, trifluoroacetic acid; TMS-Cl, trimethylchlorosilane; X-G-BPTI, cross-linker-BPTI.

The peptide $(Ala)_{20}$ was selected initially, since alanine is the simplest helix-forming amino acid, and the length of $(Ala)_{20}$ is just long enough in an α-helical conformation to span the bilayer. Biophysical studies carried out on poly(L-alanine) have shown that it exists as a right-handed α-helix in the solid state (Elliott & Malcolm, 1959), in nonpolar solvents (Downie et al., 1957), and in aqueous solution as a block copolymer (Ihara et al., 1982). Since one objective of this research is to distinguish the differences in membrane insertion of signal sequences (~12 residues) and transmembrane segments (~25 residues), a peptide containing 10 alanine residues was also used as a comparison, with a length as an α-helix long enough to span one monolayer of the membrane. It is interesting to note that the N-terminal signal sequence of yeast cytochrome *c* oxidase contains a stretch of 10 alanine residues in the h-region (Kaput et al., 1982).

Bovine pancreatic trypsin inhibitor, selected as the carrier protein, is a basic polypeptide consisting of 58 residues (6512 kDa). Due to its size and stability, the structure of BPTI has been extensively examined by X-ray crystallography and neutron diffraction (Wlodawer et al., 1984), two-dimensional NMR (Wüthrich et al., 1982), energy minimization (Levitt, 1981), molecular dynamics (Levitt & Sharon, 1987), and circular dichroism (Kosen et al., 1983). Protein folding studies using BPTI as a model have been carried out by a number of workers (Creighton, 1978; Kim & Baldwin, 1990; Weissman & Kim, 1991). Because it is a highly charged protein (16 charges at neutral pH; net charge = +6), the semisynthetic proteins prepared from it are expected to be water soluble as is cytochrome b_5. The steric arrangement of the three intramolecular disulfide bridges are presumably responsible for the extreme stability of BPTI over a wide range of pH and temperature; no loss of activity occurs when exposed to 2.5% trichloroacetic acid at 80 °C or when boiled in dilute acid (Kraut et al., 1930; Green & Work, 1953). It is not digested by an array of proteolytic enzymes, except by thermolysin at temperatures above 60 °C (Wang & Kassell, 1970). Functional stability is also maintained after treatment with several chemical denaturants, including sodium dodecyl sulfate (SDS), implying that there is very limited solvent accessibility to anything but the surface of the molecule (Broze & Miletich, 1987). These inherent properties are critical to the process of producing the semisynthetic proteins, since the chemical modification and protein purification techniques are generally quite harsh. In this paper we also report studies of the state of aggregation of these semisynthetic proteins in the aqueous phase, and the thermodynamics of their interaction with phospholipid bilayers. A preliminary report of the interaction studies has appeared elsewhere (Moll & Thompson, 1992).

EXPERIMENTAL PROCEDURES

Materials. BPTI was obtained from Mobay Chemical Corp. (FBA Pharmaceuticals). Folin and Ciolalteus phenol reagent, σ-methylisourea (sulfate salt), diisopropylethylamine, trimethylchlorosilane, L-alanine, dithiothreitol, sodium dodecyl sulfate, sodium borohydride, 5,5′-dithiobis(2-nitrobenzoic acid), and β-mercaptoethanol were obtained from Sigma. Sephadex CM-25 and Sephadex G-50 (super fine) were obtained from Pharmacia. *N*-Succinimidyl 3-(2-pyridyldithio)-propionate, acetonitrile (HPLC grade), fluorescamine, dimethyl formamide (anhydrous), acetic acid, and trifluoroacetic acid were obtained from Pierce Chemical Corp. Calcium oxide, Fmoc-Cl, propionic acid, methyl *tert*-butyl ether, ethanedithiol, and ethyl acetate were obtained from Aldrich. Hexane, chloroform, dichloromethane, and ethyl ether were obtained from Mallinckrodt. L-[2,3-^{3}H]Alanine (41 Ci/mmol) was obtained from Amersham. Silica gel plates were obtained from Analtech. Formic acid, toluene, *n*-propanol, and methanol (HPLC grade) were obtained from Fisher Scientific. Acridine orange was obtained from Eastman Organic Chemicals. Dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) were obtained from Avanti Polar Lipids. Octyl glucoside was obtained from Pfanstiehl Laboratories.

Guanidination of BPTI. The method of Chervenka and Wilcox (1956) was used with modifications. A solution of σ-methylisourea (OMIU) was prepared by dissolving 10 g of the OMIU·sulfate in 20 mL of dH_2O on ice. The OMIU·sulfate was converted to free base by the slow addition of 20 g of barium hydroxide. The solution, diluted to 100 mL ($[OMIU]_F$ = 0.58 M), was centrifuged at 4 °C for 30 min at 10000*g* to remove the white precipitate (barium sulfate). The supernatant was kept on ice and adjusted to pH 11.0−11.3 with the addition of 1 M HCl. Lyophilized BPTI (200 mg/31 μmol) was added to the solution and incubated at 4 °C with stirring for 7 days. The solution was centrifuged for 30 min at 10000*g* to get rid of a small amount of precipitate and then dialyzed (2×) against 3 L of 0.5 M NaCl, (2×) against 3 L of 0.2 M NaCl, (2×) against 3 L of dH_2O, and finally against 50 mM glycine (pH 10.5)/100 mM NaCl. Guanidinated BPTI (G-BPTI) was loaded onto a Sephadex CM-25 column (0.4 × 20 cm) equilibrated in 50 mM glycine (pH 10.5)/100 mM NaCl. The protein was eluted with a linear gradient from 0.1 to 0.7 M NaCl (200 mL). Protein fractions were monitored at 280 nm, and the major protein peak eluting at 0.4 M NaCl was pooled, dialyzed exhaustively against distilled H_2O, lyophilized, and stored at −20 °C.

Amino Acid Analysis of BPTI and G-BPTI. Amino acid analysis was performed by the Protein and Nucleic Acid Research Facility at the University of Virginia Health Sciences Center. After complete hydrolysis, the amino acids were derivatized with (dimethylamino)azobenzenesulfonyl chloride (DABS) using a modification of the procedure of Knecht and Chang (1986). DABS-derivatized amino acids were separated by reversed-phase chromatography on an analytical Metasil ODS column using a 30 min nonlinear gradient between buffer A (16 mM citrate in 4% DMF) and buffer B (16 mM citrate in 4% DMF and 70% acetonitrile). Ionic conditions were altered from standard protocols to ensure the separation of homoarginine peaks from arginine, proline, and alanine peaks.

Attachment of SPDP to Guanidinated BPTI. In a typical preparation, 25 mg of G-BPTI was dissolved in 1.8 mL of 100 mM phosphate buffer (pH 7.6). Four milligrams of SPDP was dissolved in 400 μL of DMF. Three hundred microliters of SPDP in DMF was added dropwise while vortexing to allow maximum uniform dispersion before hydrolysis. Although SPDP is completely soluble in DMF, the addition to an aqueous solution resulted in the formation of an emulsion, which tended to aggregate or coalesce over time. The reaction mixture was incubated for 1.5 h at room temperature with constant mixing. The small amount of precipitate generated during the reaction was removed by centrifugation at 15000*g* for 10 min. The solution was

loaded onto a Sephadex G-50 (super fine) column (120 × 1.5 cm) equilibrated with 100 mM phosphate buffer (pH 7.6). X-G-BPTI fractions, monitored at 280 nm, were pooled, lyophilized, and stored at −20 °C.

Extent of Cross-Linking Reaction. One milligram of BPTI with the cross-linker attached (X-G-BPTI) was dissolved in 1 mL of 100 mM phosphate buffer (pH 7.6) and placed into a 1-cm cuvette. An initial UV spectrum was recorded between 220−375 nm on a Shimadzu (UV-260) UV−visible recording spectrophotometer to determine protein concentration by absorbance at 276 nm and to generate a blank baseline for pyridine-2-thione analysis monitored by absorbance at 343 nm. Ten microliters of DTT (0.5 M) or 10 μL of β-mercaptoethanol (1 M) was added directly to the cuvette and stirred. The mixture was allowed to react for 10 min at room temperature. A duplicate UV spectrum was recorded and used to determine the amount of pyridine-2-thione by using a molar extinction coefficient at 343 nm of 8000 M^{-1} cm^{-1}.

Peptide Synthesis. $(Ala)_n$ peptides were synthesized at Tufts University by Fmoc methodology on a MilliGen Excell 9400 peptide synthesizer using a PAL (peptide amide linker) (0.1 mmol) and BOP/HOBt activation/coupling procedures. Coupling employed a 5-fold excess of Fmoc-amino acid with a single 2-h coupling time. The cysteine moiety was protected by a trityl group, and tyrosine was protected by a tBu group. Deprotection was in 30% piperidine in DMF. The last two couplings employed tritiated alanine. The completed peptide-resins were washed with DCM/MeOH, lyophilized, and shipped to the University of Virginia for the cleavage reactions. The Fmoc-[^{3}H]alanine was synthesized following a standard protocol with slight modification (Bolin et al., 1989).

Cleavage from the Resin of $(Ala)_n$ Peptides in SDS with TFA. Peptide-resin (0.1−0.4 g) was placed into a glass vial equipped with a polyseal cap. A micro-stir-bar was added, and the vial was put on ice. The cleavage mixture (0.25 mL of EDT/0.25 mL of dH_2O/9.50 mL of TFA/250 mg of SDS) was cooled in a 25-mL beaker on ice. The cleavage mixture was added to the peptide-resin on ice, blanketed with N_2, and incubated for 5 min. It was allowed to come to room temperature with gentle stirring for 1.5 h. The mixture was then slowly vacuum-filtered (Whatman #2 medium porosity) into 30 mL of cold MTBE. TFA (2 × 0.5 mL) was added back to the vial and filtered. The filtrate was incubated at 4 °C for 10−12 h and then transferred to Teflon FEP (fluorinated ethylene propylene) tubes and centrifuged at 2000*g* for 10 min at 4 °C. The pellet was resuspended and centrifuged four times with 25-mL MTBE aliquots and finally dried for 6−12 h *in vacuo* over KOH. The dry pellet was dissolved in 10 mL 30% acetic acid and centrifuged for 10 min at 2000*g*. The solubilized SDS−peptide mixture was lyophilized, redissolved in distilled H_2O, relyophilized, and stored under N_2 at −60 °C.

$NaBH_4$ Reduction of Peptide Disulfide Bonds. Immediately prior to conjugation with X-G-BPTI, 10 mg of the SDS−peptide was weighed into a 13 × 100 mm test tube. A fresh solution of $NaBH_4$ was made (0.5 M in 3.0 M NaOH and 1 mM EDTA). The $NaBH_4$ mixture was cooled in an ice bath, and 2.0 mL was slowly added to the peptide. The mixture was blanketed with N_2 and incubated for 1 h. The reaction was ended by slowly adding 0.5 M HCl in a ventilated hood to lower the pH of the solution down to 3. Care was taken to avoid excess foaming. Aliquots were analyzed for free sulfhydryl group content (Ellmann, 1959).

Conjugation of Peptides to X-G-BPTI. Lyophilized X-G-BPTI (20 mg) was dissolved in 4 mL of 100 mM sodium phosphate buffer at pH 7.8. Small amounts of precipitate were removed by centrifugation at 10000*g* for 10 min. A 2-fold excess of peptide in SDS (freshly reduced) was added, blanketed with N_2, and incubated at room temperature. Aliquots were taken at specified intervals and monitored for pyridine-2-thione at 343 nm. The reaction was stopped by freezing the mixture in liquid N_2 and lyophilizing.

Detergent Removal by Gel Filtration with Propionic/Formic Acid. Gel filtration of peptide−protein conjugate mixtures was performed on a Sephadex G-25 SF column (30 × 0.7 cm) equilibrated with 2:1:2 (v/v) propionic acid/formic acid/H_2O. Lyophilized peptide−protein conjugates were dissolved in the running buffer and loaded onto the column. Fractions (0.2 mL) were collected and assayed for protein at 280 nm and for tritium radioactivity. The radioactive peak eluting in the void volume was diluted with H_2O and lyophilized. SDS was assayed in the fractions by the acridine orange method of Sokoloff and Frigon (1981), which can detect as little as 0.001% SDS. Aliquots of the samples (20 μL) were added to 13 × 100 mm polypropylene tubes equipped with snap-on caps. Acridine orange reagent (1% in 0.5 M sodium sulfate) was added (100 μL) to the sample and mixed. Toluene (3.0 mL) was added and vortexed vigorously. The samples were centrifuged for 2 min at 2000*g*. The organic layer was monitored at 499 nm.

Reversed-Phase HPLC of Peptide−G-BPTI. Detergent-free peptide−protein mixtures were loaded onto an analytical Zorbax ODS reversed-phase column equilibrated in buffer A (0.1% v/v TFA in H_2O). The flow rate was maintained at 1.0 mL/min, and the elution of peptide−protein conjugates was monitored at 220 nm using a Gilson HM variable-wavelength UV detector. The peptide−proteins were eluted using a step gradient of 0−40% buffer B (0.1% TFA v/v in 2:2:1 v/v acetonitrile/*n*-propanol/H_2O) for 5 min, holding for 5 min, 40−50% for 30 min, and 50−100% in 5 min. Peaks were assayed for radioactivity.

Molecular Sieve Chromatography. Sephadex G-50 superfine was equilibrated in a column (120 × 0.38 cm) with 50 mM ammonium bicarbonate (pH 7.6)/50 mM NaCl. For the control elution, 2.5 mg of BPTI was dissolved in 200 μL of Blue Dextran (2 mg/mL) and 100 μL of DNP-lysine (2 mg/ml). The column was eluted with buffer at a flow rate of 0.3 mL/min with 1-mL fractions. For the semisynthetic proteins, the same conditions were used with loading volumes of 250 μL (360−435 nmol). The fractions were monitored by measuring absorbance at 280 nm, and aliquots were assayed by liquid scintillation counting. Initial samples of the semisynthetic proteins were made by dissolving lyophilized materials directly in the elution buffer. Excess sample was stored in buffer at 4 °C for one week and reeluted.

Preparation of Liposomes. Lipids or lipid mixtures were lyophilized from chloroform using a rotary evaporator at 40 °C and then dried for 4−12 h under vacuum. The round-bottom flask containing the lipid film was swirled gently in a water bath at 40 °C for 5 min, and buffer equilibrated at the same temperature was added. The mixture was rotated in the 40 °C bath for another 15 min and vortexed to form a crude suspension of multilamellar vesicles (MLV). In order

to ensure uniform solvent distribution among the vesicle population and to eliminate small vesicles, the suspension was carried through five freeze−thaw cycles by submerging the mixture in liquid nitrogen and then placing it in a water bath at 50 °C (Mayer et al., 1985).

LUV dispersions were prepared by extrusion (Hope et al., 1985; Mayer et al., 1986; Nayar et al., 1989) using a high-pressure extrusion apparatus obtained from Lipex Biomembranes, Inc., equipped with two polycarbonate filters in series (Nucleopore; 0.1-μm pore size). The device was equilibrated at temperatures above the phase transition temperatures of the lipids by immersion in a water bath. At least 10 sequential passes through the extruder were required in order to achieve a uniform vesicle population as determined by photon correlation spectroscopy (Nicomp Model HN5-90 equipped with a Model 170 autocorrelator). The size distribution was typically found to have average mean diameters of 80−110 nm. LUV dispersions were stored under N_2 at 30 °C.

Semisynthetic Protein−LUV Binding Assay. Semisynthetic protein samples (1 mM) were incubated with DMPC and DMPC/DPPC LUV (5 mM) in 50 mM Tris-HCl and 10 mM NaCl at pH 7.4 for 24 h in a 37 °C water bath. The mixtures were transferred to polyallomer tubes (150 μL) and centrifuged for 1 h at 30 psi (135000g) in a Beckman Airfuge. Aliquots of the supernatant were assayed for protein and lipid as described below.

Phospholipid Determination. The method of Anderson and Davis (1982) was used with minor modifications. Aliquots (10−20 μL) of the supernatant were pipetted into 100 × 12 glass Pyrex test tubes and dried down with N_2. For the standard curve, aliquots containing 20−400 nmol of a 2 mM Na_2HPO_4 stock solution were used. Concentrated sulfuric acid (100 μL) was added to the dried mixtures, and the samples were digested for 10 min on a heating block (160 °C). After cooling, 50 μL of a 6% (v/v) solution of H_2O_2 was added and mixed. The samples were returned to the heating block for another 40 min. To the cooled tubes was added 2 mL of H_2O followed by vortexing. Stock solutions of ammonium molybdate (1.25 g/100 mL) and ascorbic acid (4.93 g/100 mL) were made and stored on ice. Immediately before use, the solutions were mixed (1:1 v/v) on ice, and 800 μL was added to the samples while vortexing. Glass marbles were placed on the tubes, which were then placed in a boiling water bath for 7−10 min. The mixtures were allowed to cool, and the absorbance of the samples was measured at 797 nm. Linearity was observed between the range 3−200 nmol phosphate.

Protein Determination. Supernatant samples (25−100 μL) were transferred to scintillation vials containing 10 mL of EcoLite (+) (ICN Biochemicals) scintillant solution. Samples were assayed for radioactivity ([^{3}H]alanine) in a Packard Tri-Carb Model 460CD liquid scintillation counter.

Reconstitution. A 60-fold molar excess of octylglucoside was added to semisynthetic proteins (200−400 nmol) in 50 mM Tris-HCl/10 mM NaCl (pH 7.4) at a final concentration of 30 mM (CMC_{OG} = 25 mM). A 10-fold molar excess of lipid (DMPC or DMPC/DPPC, 50:50 mol %) was added to the mixture and equilibrated under N_2 at 25 °C for 5 h. The mixture was added dropwise at a rate of 10 mL/h into dilute buffer while vortexing, leading to a final octyl glucoside concentration of 10 mM, thus assuring that all the phospholipid originally in the mixed protein−lipid−detergent micelles was converted into vesicles (Jackson & Litman, 1985). The reconstituted vesicles were dialyzed against 2 × 500 mL buffer at 4 °C for 4 h. Aliquots of the samples were then centrifuged as in the spontaneous insertion procedure and the supernatant assayed for protein and lipid as described.

Cleavage of $(Ala)_{20}$−G-BPTI by Dithiothreitol. For the water-soluble form, a 1 mg/mL solution of $(Ala)_{20}$−G-BPTI (24 μCi/mol) in 100 mM Tris-HCl (pH 7.4)/50 mM NaCl was placed into a 1.5-mL polypropylene tube. Dithiothreitol was added at final concentration of 1−5 mM, and the mixture was incubated at 30 °C in a water bath for 1 h. The sample was centrifuged for 15 min in an Eppendorf microcentrifuge (8800g). The supernatant was transferred to an Amicon MPS-1 filtration unit equipped with YM5 (14-mm) filters (MWCO = 5000 Da) and centrifuged at 2000g for 45 min. The supernatant was diluted with 100 mM Tris-HCl (pH 7.4)/50 mM NaCl to give a final volume of 0.5 mL. The dilute solution was assayed for protein by the Lowry method and for radioactivity by adding aliquots to 8 mL of scintillation cocktail (Lowry et al., 1951).

For the membrane-bound form, $(Ala)_{20}$−G-BPTI was incubated with DMPC LUV as described in the spontaneous insertion procedure. After the incubation period, dithiothreitol was added and the mixture incubated for an additional hour. Samples were transferred to polyallomer tubes (150 μL) and centrifuged for 1 h at 30 psi (135000g) in a Beckman Airfuge. The supernatant was filtered as described above to get rid of the reducing agent, which interferes with the Lowry assay, and assayed for protein and radioactive peptide.

RESULTS

Cleavage of Resin-Bound Peptides in the Presence of SDS. The synthetic peptides, attached to the resin in lyophilized form at room temperature [NH_2-$(Ala)_{10}$-Tyr-Cys-$CONH_2$ and NH_2-$(Ala)_{20}$-Tyr-Cys-$CONH_2$, designated $(Ala)_{10}$ and $(Ala)_{20}$], were cleaved without aggregation from the resin in the presence of SDS. Yields were generally 65−75%, as determined by radioactive specific activities and peptide determination. The $(Ala)_{10}$ and $(Ala)_{20}$ peptides behaved differently during the solubilization procedures. When acetic acid was added to the $(Ala)_{20}$−SDS−peptide mixture, very little aggregation and no foam were evident, even after extensive mixing and incubation. The $(Ala)_{10}$−SDS mixture, however, tended to have a higher turbidity accompanied by substantial foam production. The probable reason for this difference in behavior is that $(Ala)_{20}$ is more hydrophobic than its shorter counterpart and thus more readily interacts with SDS to form a stable micelle complex in solution. Studies that examined the solubility of $(Ala)_{10}$ when cleaved from the resin in the absence of SDS supported this explanation.

Guanidination of BPTI. The four lysine ϵ-amino groups of BPTI are readily guanidinated by reaction with σ-methylisourea. The reaction proceeds to completion within 7 days at 4 °C as evidenced by the homoarginine content of the protein after hydrolysis. Figure 2 is the amino acid analysis of BPTI and guanidinated-BPTI. The extent of guanidination is indicated by the appearance of homoarginine peaks and the disappearance of lysine peaks (arrows). The α-amino group of BPTI was not guanidinated as determined by the extent of reaction with fluorescamine (data not shown), which is specific for free amine groups (either α- or ϵ-amine).

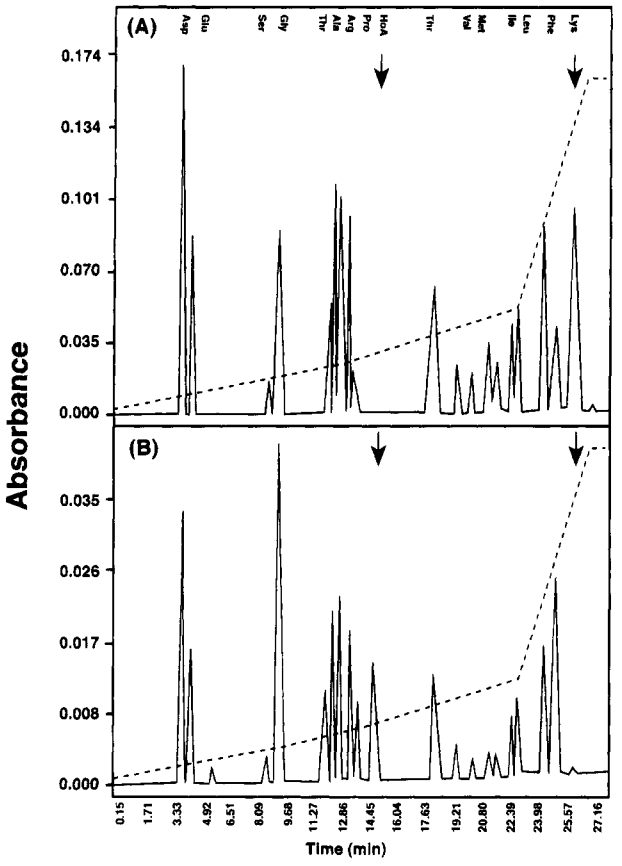

FIGURE 2: Amino Acid analysis of native (A) and guanidinated BPTI (B). Protein (2 μg) was hydrolyzed with HCl vapor at 110 °C for 24 h. After complete hydrolysis, the amino acids were derivatized with DABS. DABS-derivatized amino acids were separated by reversed-phase chromatography on an analytical Metasil ODS column. Amino acids were eluted from the column using a 30-min nonlinear gradient between buffer A (16 mM citrate in 4% DMF) and buffer B (16 mM citrate in 4% DMF and 70% acetonitrile), and the fractions were monitored at 436 nm. Ionic conditions were altered from standard protocols to ensure the separation of homoarginine peaks from arginine, proline, and alanine peaks. HoA refers to homoarginine.

Previous biochemical studies of the inhibitor activity of G-BPTI against trypsin showed very little difference between native BPTI and guanidinated BPTI (Kassell & Chow, 1966). Inhibitory assays using G-BPTI in this study are indistinguishable from those employing unmodified BPTI (data not shown). Studies have indicated no backbone conformational changes are induced by guanidination (Ebina et al., 1989). G-BPTI has been shown to be stable when lyophilized and stored at −20 °C for up to 1 year.

Attachment of SPDP to G-BPTI. Reaction of G-BPTI with the heterobifunctional cross-linker SPDP proceeded rapidly under mild conditions to form a stable intermediate for formation of the semisynthetic proteins. The reaction was essentially complete within 30−40 min. The cross-linker−G-BPTI, designated X−G-BPTI, was easily separated from excess reagent and DMF using a Sephadex G-50 gel filtration column. SPDP was stoichiometrically cross-linked to G-BPTI; yields were generally in the range of 65−75%. X−G-BPTI was stable for up to 6 months when stored lyophilized at −20 °C. The extent of cross-linker incorporation was assayed by monitoring the production of pyridine-2-thione after reaction with DTT or β-mercaptoethanol. Protein concentrations were determined prior to the addition of sulfhydryl reagent due to the strong absorbance below 300 nm of the thiol group. The assay was also used to determine the time course of the reaction (<1 h). Hydrophobic peptides that contain a cysteine group were attached to X−G-BPTI to form a semisynthetic protein by reacting with the 2-pyridyl disulfide moiety. The disulfide bond formed between the peptide and the cross-linker can be selectively cleaved by the addition of the proper concentration of a reductant. For example, the state of aggregation of a membrane-inserted peptide can be studied free of the carrier protein by reacting the membrane-bound form of the semisynthetic protein with a reducing agent. Also, the stability within the lipid bilayer of the inserted peptide can be examined when cleaved from BPTI.

Reduction of Disulfide-Bonded Peptide Dimers. Initial studies were carried out in an attempt to reduce chemically intramolecular disulfide bonds between peptides after cleavage and solubilization. General thiol protecting reagents cannot be included into the cleavage mixture because they are readily destroyed by TFA (Lundt et al., 1978). Dithiodipyridine (2-PDS), DTT, and β-mercaptoethanol successfully reduced the disulfide bonds of the cleaved peptides in aqueous solutions; problems arose when trying to remove the excess thiols afterward. Gel filtration employing SDS in the running buffers was successful in separating thiol reagent from the peptide; however, peptide recoveries were extremely low (5−10%). Dialysis studies produced similar results: thiol was readily dialyzed, but peptides tended to aggregate or adhere to the semipermeable dialysis tubing.

Direct reduction methods were sought that would enable the cleavage of disulfide bonds without the additional removal of reagent or byproduct. The quantitative reduction of disulfide bonds between peptides was carried out in aqueous systems of $NaBH_4$. The selective reduction of several native proteins by $NaBH_4$ has been accomplished, including trypsin (Light & Sinha, 1967), trypsin inhibitor (Kress & Laskowski, 1967), and tryptophanyl-RNA ligase (Kuehl et al., 1976). Reoxidation in the presence of the detergent did not appear to be a problem. EDTA was added to prevent reoxidation by trace amounts of transition metal ions. After incubation with peptide, the excess $NaBH_4$ was decomposed by acidification with HCl. No additional procedures were required to remove any reagents that would interfere with the conjugation reaction. The free sulfhydryl content was easily calculated by measuring the concentration of TNB^{2-} produced upon reaction with DTNB (Ellmann, 1959) and indicated that between 50−65% of the peptides were previously present as disulfide-bonded dimers (Figure 3). The increase in absorbance was slightly higher with the $(Ala)_{10}$ peptide, suggesting perhaps that the disulfide bond between monomers is more solvent exposed than the $(Ala)_{20}$ peptides within the peptide−SDS micelles. The reduced peptide−SDS mixture was then immediately conjugated to X−G-BPTI.

Conjugation of Peptides to X−G-BPTI. The conjugation of peptides to X−G-BPTI in the presence of SDS proceeded much more slowly than model hydrophilic cysteine-containing peptides in the absence of SDS (data not shown), probably due to the increased viscosity of the detergent−protein mixture. As stated previously, BPTI is stable in solutions of SDS. The extent of the reaction was monitored by the increase in absorbance of pyridine-2-thione measured at 343 nm; the reaction is essentially complete after 5 h. The crude mixture of excess X−G-BPTI, BPTI−$(Ala)_n$

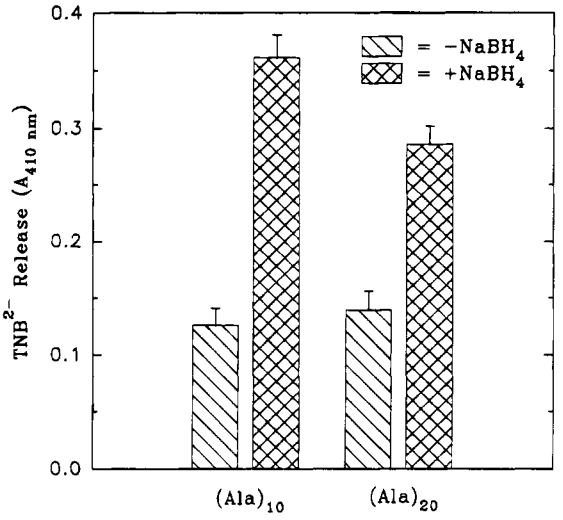

FIGURE 3: $NaBH_4$ reduction of peptide disulfide bonds. Immediately prior to conjugation with X−G-BPTI, 10 mg of peptide was added to a fresh solution of $NaBH_4$ (0.5 M in 3.0 M NaOH and 1 mM EDTA). The mixture was blanketed with N_2 and incubated for 1 h. The reaction was ended by slowly adding 0.5 M HCl in a ventilated hood to lower the pH of the solution down to 3. Aliquots were analyzed for free sulfhydryl group content by the method of Ellmann (1959).

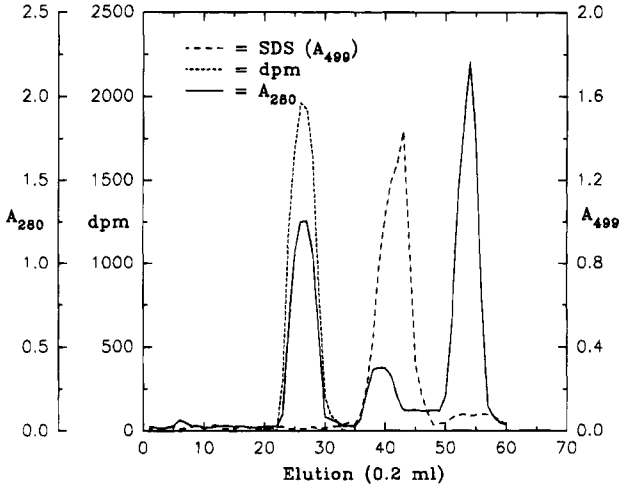

FIGURE 4: SDS removal by gel filtration with propionic/formic acid. Gel filtration of peptide−protein conjugate mixtures was performed on a Sephadex G-25 SF column (30 × 0.7 cm) equilibrated with 2:1:2 (v/v) propionic acid/formic acid/dH_2O. Fractions (0.2 mL) were collected and assayed for protein (A_{280}), SDS (acridine orange-toluene assay), and radioactivity (^3H liquid scintillation). The radioactive peak eluting in the void volume was diluted with dH_2O and lyophilized.

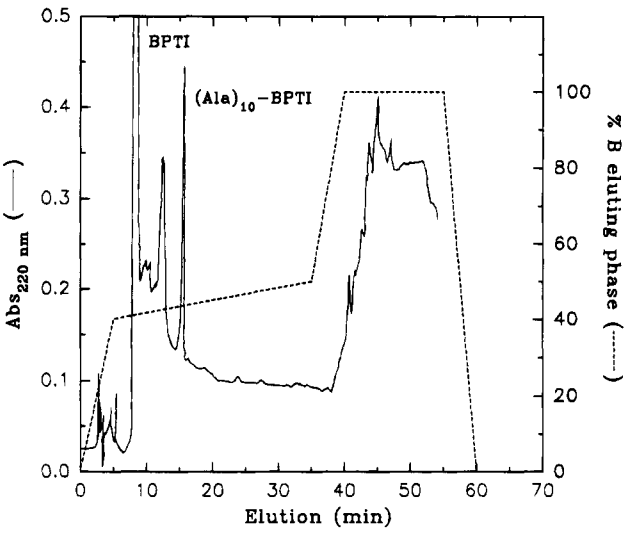

FIGURE 5: Reversed-phase HPLC of $(Ala)_n$−G-BPTI. Detergent-free samples were loaded onto an analytical Zorbax ODS reversed-phase column equilibrated in buffer A (0.1% v/v TFA in dH_2O). The flow rate was maintained at 1.0 mL/min, and the elution of peptide−protein conjugates was monitored at A_{220} (AUFS = 0.5) using a Gilson HM variable-wavelength UV detector. The peptide−proteins were eluted using a step gradient of 0−40% buffer B (0.1% TFA v/v in 2:2:1 v/v acetonitrile/*n*-propanol/dH_2O) for 5 min, holding for 5 min, 40−50% for 30 min, and 50−100% in 5 min.

conjugates, and free peptide−SDS micelles were stored under N_2 at −20 °C to prevent reduction of the disulfide bond.

Removal of SDS by Propionic/Formic Acid Gel Filtration. Before the conjugated species could be purified using RP-HPLC procedures, it was necessary to remove SDS from the mixture. Although several methods are available that quantitatively remove SDS from small peptides (Hanaoka et al., 1979; Henderson et al., 1979; Hager & Burgess, 1980), the conditions are not suitable for native proteins. A method for removing SDS from intact proteins has been described by Amons and Schrier (1981), which is based on the disruption of SDS micelles and SDS−protein complexes by strong acids. It has been used to successfully separate SDS from BSA, catalase, ovalbumin, myoglobin, lysozyme, cytochrome *c*, and collagen (Amons & Schrier, 1981).

Sample fractions were assayed for protein, SDS, and radioactivity (Figure 4). SDS was assayed by the acridine orange/toluene assay (Sokoloff & Frigon, 1981). Peptide−G-BPTI conjugates, along with unreacted X−G-BPTI, eluted in the void volume. SDS monomers were eluted between 6.2 and 9.6 mL. Pyridine-2-thione eluted within the include volume. However, unreacted peptide was not eluted from the column, apparently binding strongly to the column matrix. The same result was obtained when attempting to purify reduced peptides after DTT treatment on gel filtration columns equilibrated with SDS buffer. As a result, column matrices were used once and discarded.

Reversed-Phase HPLC Purification of Peptide−BPTI Conjugates. The final purification of peptide−G-BPTI conjugates was accomplished using RP-HPLC on an ODS column (Figure 5). Usually, only one radioactive peak (43−45% B) was evident after assaying the eluting fractions. A very shallow gradient was required to separate peptide−G-BPTI conjugates from the excess BPTI species (large fraction eluting at ~10 min). Unreacted X−G-BPTI eluted early from the column during the initial linear gradient (30−35% B). When samples were allowed to incubate for more than 24 h, however, the main peptide−G-BPTI disappeared with the appearance of two very small peaks. The peptides were apparently hydrolyzed during prolonged exposure to TFA. This resulted in the elution of dimer and monomer at extremely low yields. As with other chromatographic results, the free peptide species were most likely irreversibly bound to the column. Column guards were employed throughout these studies to protect the column from clogging with hydrophobic contaminants. Radioactive peaks were pooled and lyophilized. The sample was resuspended in ammonium bicarbonate and lyophilized to eliminate trace amounts of TFA. The final purified product was stored under N_2 at −60 °C.

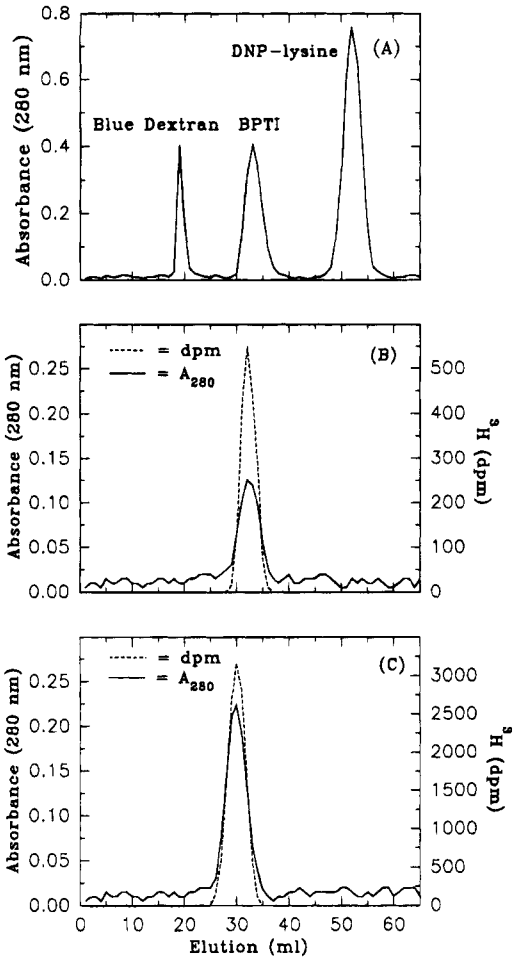

FIGURE 6: Molecular sieve chromatography of $(Ala)_n$–G-BPTI semisynthetic proteins. Proteins were eluted on a Sephadex G-50 SF (120 × 0.38 cm) column equilibrated with 50 mM ammonium bicarbonate (pH 7.6)/50 mM NaCl. (A) Control elution chromatogram of Blue Dextran (void volume), native BPTI, and DNP-lysine (include volume). (B) $(Ala)_{10}$–G-BPTI and (C) $(Ala)_{20}$–G-BPTI were loaded onto the same column employing the same running buffer and flow rate (0.3 mL/min) as the control. Fractions (1.0 mL) were monitored for protein by measurement of absorbance at 280 nm and assayed for conjugated peptide by ^3H liquid scintillation.

State of Aggregation of the Semisynthetic Proteins in Aqueous Buffer. The state of aggregation was assayed by molecular sieve chromatography in aqueous buffers using gel filtration media with a nominal separation range of 500–8000 Da (Sephadex G-50 superfine). As markers for the control elution chromatogram, Blue Dextran (av MW = 2 000 000) and *N*-ε-(2,4-dinitrophenyl)-L-lysine (DNP-Lys; MW = 312) were used to define the void volume and included volume, respectively. BPTI (MW = 6512) was used to distinguish the elution pattern of the semisynthetic proteins from the native protein.

The control elution chromatogram using the markers described is displayed in Figure 6. $(Ala)_{10}$–G-BPTI and $(Ala)_{20}$–G-BPTI were loaded onto the column employing the same buffer system and flow rate. $(Ala)_{10}$–G-BPTI eluted one fraction (1.0 mL) ahead whereas $(Ala)_{20}$–G-BPTI eluted three fractions (3.0 mL) ahead of BPTI. The elution peaks of the two semisynthetic proteins correspond well with their monomeric molecular weights [$(Ala)_{10}$–G-BPTI = 7593; $(Ala)_{20}$–G-BPTI = 8304]. In addition, no higher-order aggregates were detected. No radioactivity was measured in the void volume where aggregates of dimers and larger would elute. Although lyophilized samples dissolved in buffer and chromatographed immediately were completely recovered in the eluate, such was not the case if aqueous aliquots were stored at 4 °C for 1 week. After storage $(Ala)_{20}$–G-BPTI recovery was decreased by 53%, whereas $(Ala)_{10}$–G-BPTI recovery was decreased by only 6% (data not shown). These results suggest that time-dependent aggregation of the semisynthetic proteins did occur under storage conditions. The fact that the aggregates did not elute from the column in the void volume suggests the possibility that they were irreversibly bound to the column matrix or were too large to enter the column. The semisynthetic proteins were therefore stored in the lyophilized form and dissolved in buffer immediately prior to subsequent experiments. No aggregation with this procedure was detected.

Table 1: Spontaneous Insertion of $(Ala)_n$–G-BPTI Proteins into Phospholipid Vesicles[a]

	DMPC		DMPC/DPPC	
	$lipid_{sup}$(%)	$protein_{sup}$(%)	$lipid_{sup}$(%)	$protein_{sup}$(%)
control	4.8 ± 1.4		5.6 ± 1.8	
G-BPTI	3.9 ± 1.1	93.9 ± 4.2	4.1 ± 2.1	92.2 ± 6.1
BPTI-MI3	4.1 ± 2.3	89.1 ± 5.3	3.8 ± 2.8	93.3 ± 3.9
BPTI-$(Ala)_{10}$	6.1 ± 2.0	96.2 ± 2.0	4.5 ± 2.2	97.6 ± 2.7
BPTI-$(Ala)_{20}$	3.7 ± 2.5	66.3 ± 4.2	4.0 ± 1.9	74.4 ± 2.5

[a] Semisynthetic protein samples (1 mM) were incubated with DMPC and DMPC/DPPC LUV (5 mM) in 50 mM Tris-HCl (pH 7.4)/10 mM NaCl for 24 h at 37 °C. The protein–LUV mixtures were centrifuged for 1 h at 135000*g* in a Beckman Airfuge. Aliquots of the supernatant were assayed for protein and lipid as described under Experimental Procedures.

Interaction of Semisynthetic Proteins with LUV. The interaction of the semisynthetic proteins with bilayer membranes was studied by determining their partition coefficients between bilayer and aqueous phase using Airfuge ultracentrifugation. Large unilamellar vesicles were used since their physical properties and overall characteristics are closer to those of biological membrane bilayers than are the properties of SUV (Sheetz & Chan, 1972; Chrzeszczyk et al., 1977; Parente & Lentz, 1984; Wimley & Thompson, 1990). Phospholipid dispersions prepared from DMPC and from a 1:1 molar mixture of DMPC/DPPC were used. Semisynthetic proteins (1 mM) were incubated with LUV (5 mM) for 24 h at 37 °C to achieve equilibrium. Equilibration was determined by standard titration of semisynthetic protein aliquots with variable concentration lipid and measuring bound peptide at various time intervals (data not shown). The systems were then centrifuged in an Airfuge. Aliquots of the resulting supernatant were assayed for free protein by measuring the radioactivity of the aqueous sample. The amount of unpelleted LUV (probably in the form of heterogenous SUV) in the supernatant was determined by measuring the amount of inorganic phosphate present using a modified Bartlett assay (Anderson & Davis, 1982).

The water-vesicle partition coefficients for $(Ala)_{10}$–G-BPTI and $(Ala)_{20}$–G-BPTI in LUV of DMPC and DMPC/DPPC (50:50 mol %) at 37 °C are given in Table 1. As a control for the amount of LUV pelleted in the centrifuge experiments, samples containing only LUV were assayed for the content of lipid present in the supernatant. Typically, around 5% of the lipid content was found in the supernatant

Table 2: Reconstitution of $(Ala)_n$−G-BPTI Proteins[a]

	DMPC		DMPC/DPPC	
	$lipid_{sup}$(%)	$protein_{sup}$(%)	$lipid_{sup}$(%)	$protein_{sup}$(%)
control	7.2 ± 2.3		6.9 ± 2.9	
G-BPTI	8.0 ± 2.6	95.4 ± 3.6	7.6 ± 3.2	91.2 ± 5.2
BPTI-MI3	6.6 ± 3.0	92.1 ± 5.2	5.6 ± 2.8	90.9 ± 3.4
BPTI-$(Ala)_{10}$	8.2 ± 3.1	92.3 ± 3.2	7.4 ± 2.6	96.2 ± 3.0
BPTI-$(Ala)_{20}$	6.9 ± 2.8	60.1 ± 4.4	6.5 ± 2.2	62.7 ± 3.3

[a] Semisynthetic proteins (200−400 nmol) were reconstituted in a 60-fold molar excess of octyl glucoside and a 10-fold molar excess of lipid (DMPC or DMPC/DPPC, 50:50). The mixture was diluted to give a final octyl glucoside concentration of 10 mM and dialyzed against 2 × 500 mL of 50 mM Tris-HCl/10 mM NaCl (pH 7.4). Protein−vesicles were centrifuged and analyzed as in the spontaneous insertion assays.

using these procedures, probably in the form of SUV. As a control for nonspecific binding, BPTI and G-BPTI conjugated to a nonhydrophobic model peptide (MI3 sequence: QVVISVNPYKPLGC) were used in the assay; neither bound to LUV to any significant degree. Finally, semisynthetic proteins in the absence of LUV remained in the supernatant.

The binding data shown in Table 1 suggest that $(Ala)_{10}$−G-BPTI did not spontaneously interact with either DMPC or DMPC/DPPC LUV in detectable amounts. $(Ala)_{20}$−G-BPTI bound to the DMPC LUV with a partition coefficient $K_p = (4.38 \pm 0.18) \times 10^3$, and to DMPC/DPPC LUV with $K_p = (3.06 \pm 0.08) \times 10^3$. At 37 °C, the experimental values of K_p for $(Ala)_{20}$−G-BPTI partitioning into lipid vesicles yielded values of ΔG_t equal to −5.21 kcal/mol (DMPC) and −4.99 kcal/mol (DMPC/DPPC). Within experimental error, there did not appear to be any difference in the extent of interaction with $(Ala)_{20}$−G-BPTI into pure DMPC vesicles compared to the mixed-phospholipid vesicles.

Reconstitution Studies. Semisynthetic protein-reconstitution studies into DMPC and DMPC/DPPC vesicles were performed using the octyl glucoside dilution technique (Jackson & Litman, 1985). The reconstituted vesicles were assayed for protein incorporation in the same way as in the spontaneous interaction experiments. The results are shown in Table 2. Surprisingly, the incorporation data are quite similar to the interaction experiments. G-BPTI and the control MI3−G-BPTI conjugate did not incorporate into the reconstituted vesicles. Similarly, the $(Ala)_{10}$−G-BPTI protein did not appreciably form a stable complex with the DMPC/DPPC vesicles, although there appeared to be a small amount of incorporation into DMPC vesicles. $(Ala)_{20}$−G-BPTI reconstituted into both DMPC and DMPC/DPPC vesicles to a similar extent, which was slightly higher than the spontaneous incorporation results. It is quite possible that the semisynthetic proteins were entrapped within the vesicles during formation. This, however, cannot account for more than a few percent of the amount bound based on the internal volume fraction of the reconstituted vesicles. Electrostatic interactions between semisynthetic protein molecules may also play a role in the increased incorporation into the reconstituted vesicles.

Cleavage of Peptide from Carrier Protein. The conjugated peptide was cleaved from G-BPTI by the reduction of the disulfide bond linkage using dithiothreitol. Reduction was carried out on the semisynthetic protein in aqueous solution in order to observe the aqueous phase behavior of the cleaved hydrophobic peptide. Reduction was also performed on

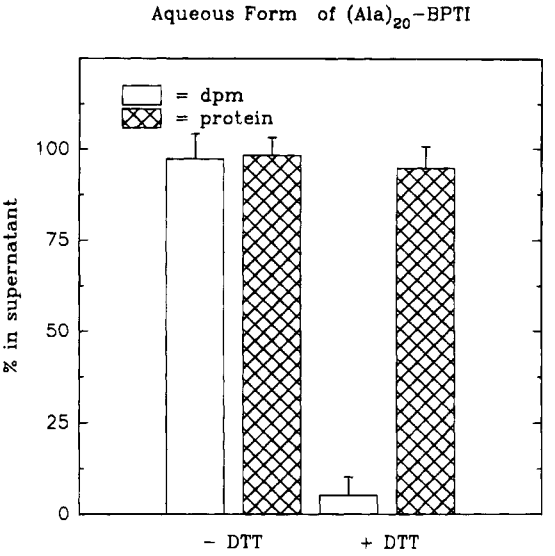

FIGURE 7: Cleavage of $(Ala)_{20}$−G-BPTI in aqueous solution by DTT. $(Ala)_{20}$−G-BPTI [1 mg/mL in 100 mM Tris-HCl/50 mM NaCl (pH 7.4)] was incubated with DTT (1−5 mM) in a 30 °C water bath for 1 h. The mixture was centrifuged at 8800*g* for 15 min. The supernatant was transferred to a MPS-1 filtration unit equipped with YM5 filters (MWCO = 5000 Da) and centrifuged for 45 min at 2000*g*. The supernatant was diluted (V_{Tot} = 0.5 mL) and assayed for protein and radioactivity as described under Experimental Procedures.

semisynthetic protein/LUV complexes to generate the membrane-bound peptide free of G-BPTI. The data are shown in Figure 7. In the absence of LUV, the reduction of the disulfide bond resulted in the aggregation of free peptides, which were pelleted by centrifugation. About 80% of the cleaved peptide was present in the aggregate, as indicated by the loss of radioactivity in the supernatant. Unsuccessful attempts were made to dissolve the aggregate in several solvents. The majority of G-BPTI was present in the supernatant as determined by protein determination after ultrafiltration and dilution to lower the concentration of DTT to levels compatible with the Lowry assay. The cleavage of membrane-bound $(Ala)_{20}$−G-BPTI was carried out in a similar way. The results are shown in Figure 8. The amount of $(Ala)_{20}$−G-BPTI remaining in the supernatant was the same as in the spontaneous insertion experiments. After treatment with the reducing agent and centrifugation, an increase in G-BPTI was evident in the supernatant, and yet no increase in radioactivity was observed. The membrane-bound peptide was apparently stable in the bilayer without G-BPTI and pelleted with the vesicles. It is clear that the disulfide bond linkage between the peptide and G-BPTI was easily cleaved using 1 mM dithiothreitol.

DISCUSSION

The model peptides employed in the preparation of semisynthetic proteins consisted of only three amino acids: alanine (for studying peptide insertion into lipid bilayers), cysteine (for conjugation to BPTI), and tyrosine (for quantitative peptide concentration determination). In this initial study, H-$(Ala)_{20}$−Tyr-Cys-$CONH_2$ and H-$(Ala)_{10}$−Tyr-Cys-$CONH_2$ were synthesized, since $(Ala)_{20}$ is just long enough to span a bilayer (~30 Å), assuming it adopts an α-helical conformation. Alanine is the simplest helix-forming amino acid and has been shown to exist as a right-handed α-helix in several states (Elliot & Malcolm, 1959; Downie et al.,

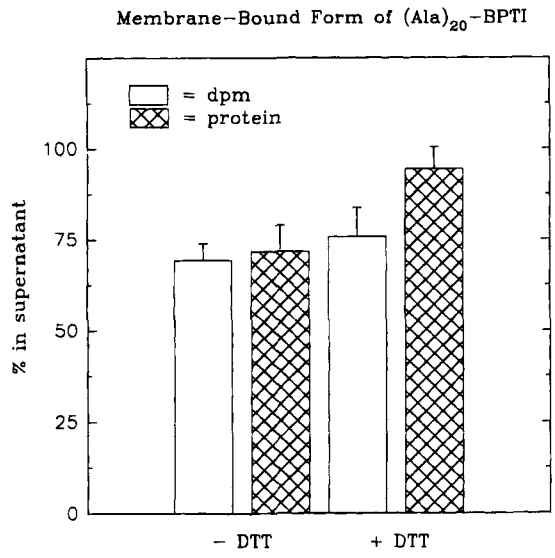

FIGURE 8: Cleavage of membrane-bound $(Ala)_{20}$-G-BPTI by DTT. $(Ala)_{20}$-G-BPTI was incubated with DMPC LUVs as described in the spontaneous insertion assays. After the incubation, DTT (1-5 mM) was added, and the sample was allowed to react for an additional hour. Samples were centrifuged for 1 h at 135000*g*. The supernatant was filtered as in the aqueous-phase studies to get rid of reductant, and the samples were assayed for protein and radioactivity.

1957; Ihara et al., 1982; Marqusee et al., 1989). $(Ala)_{10}$ was chosen because its length would be just long enough to span one monolayer of the bilayer. Peptides were made radioactive by incorporating Fmoc-[^{3}H]alanine into the peptide synthesis. Thus, the partitioning of the peptides into model lipid bilayers could be assayed quantitatively by liquid scintillation. In addition, the peptides were synthesized as the C-terminal blocked peptide amide. Peptide insertion of the semisynthetic proteins into model membranes could then be studied without the interference of a charged carboxylate group.

In standard protocols, the peptides were cleaved from the resin, solubilized, and purified using conventional methods. Problems arose with peptide sequences that were mainly hydrophobic in nature; cleaved peptides tended to form aggregates that were not easily solubilized by organic solvents or detergents. Hydrophobic peptides that were successfully solubilized were nevertheless difficult to purify, as they tended to bind irreversibly to most types of HPLC columns. Tomich and co-workers, when synthesizing membrane-spanning sequences of the rat brain sodium channel using Boc SPPS, incorporated SDS into their HF cleavage protocol to prevent the irreversible aggregation that occurred in the absence of detergent (Tomich et al., 1988). Yields were generally in excess of 80% of the final product, compared to 5-10% without SDS. The presence of detergent during the cleavage reaction resulted in the preferential formation of SDS-peptide complexes rather than irreversible peptide-peptide aggregates. The peptides were purified, and SDS was removed, by a novel RP-HPLC protocol that included octyl glucoside in both the aqueous and organic buffers; column temperatures >55 °C were also required. Several other hydrophobic peptides were also successfully cleaved and purified using this procedure. In general, SDS was added to the cleavage of peptides whose overall hydrophobicity (using hydropathy profiles) was positive for more than half of the sequence (Tomich et al., 1988).

Using this general strategy, we have developed a protocol that resulted in the formation of SDS-peptide complexes upon cleavage from the resin. SDS was included in the TFA/EDT cleavage mixture, and the crude SDS/peptide mixture was stored as the lyophilized powder. Instead of purifying the peptide and then conjugating to X-G-BPTI, the crude SDS-peptide mixture was directly added to X-G-BPTI, and the conjugated (and solubilized) form was then purified. SDS was removed from the mixture prior to purification using a propionic/formic acid-based gel filtration procedure. The modified protocol prevented contamination of the reversed-phase columns by SDS or octyl glucoside, which irreversibly altered both the binding characteristics and elution profiles. Since the complete $(Ala)_n$ peptides included a radioactive label, the complete peptide-protein conjugates were monitored from the column eluate using liquid scintillation measurements.

BPTI is a potent inhibitor of serine proteases; the K_D value for the BPTI-trypsin complex is about 6×10^{-14} M (Vincent & Lazdunski, 1972), reflecting a high affinity of the inhibitor for the enzyme. Although most of the molecular surface is comprised of polar amino acids, there are numerous nonpolar side chains in the region of the trypsin binding site. Pro^{13}, Cys^{14}-Cys^{38}, Lys^{15}, Ala^{16}, and Ile^{18} are partially accessible in the free inhibitor and become shielded upon formation of an enzyme-inhibitor complex. Hydrophobic interactions apparently contribute to the high binding energy (Fritz et al., 1969). G-BPTI has many features that assist in the characterization of the peptide-protein conjugates. The high net charge helps solubilize the hydrophobic peptides in aqueous solutions. The rigid conformation and extreme stability allows the rather harsh chemical modification and SDS removal procedures to be performed and resists overall conformational changes brought about by the hydrophobic nature of the peptides. The hydrophobic peptides were conjugated to G-BPTI at the N-terminus, which is located at the base of the protein molecule. The physical separation between the peptide conjugation site and the trypsin binding site permits the assay of inhibitory activity, whether in solution with peptide attached, or anchored to a lipid bilayer by the insertion of the hydrophobic peptide. Hence, the peptide-BPTI conjugates can be examined for functional and conformational stability both in aqueous solutions and membrane-bound.

Chemical modification by guanidination using σ-methylisourea is one of the most highly selective derivatization techniques available. Despite its relative instability in aqueous systems, σ-methylisourea is remarkably specific for the reaction with ϵ-amino groups of lysyl residues but not the N-terminal α-amino groups of proteins (Hughes et al., 1949; Chervenka & Wilcox, 1956; Klee & Richards, 1957; Hettinger & Harbury, 1964; Kassell & Chow, 1966). Due to the high pH requirement, the protein must be stable in highly alkaline solutions. Although the mechanism is unknown, the pH dependence implies that at least one of the reacting species must be protonated. The conversion of lysine residues to homoarginine does not alter the net charge of proteins in the neutral or acidic pH range (pK_a of guanidino group = 12.5; pK_a of ϵ-amino group = 10.0) and does not introduce a chemical group which is foreign to biological systems. The biophysical characterization of guanidinated BPTI by two-dimensional NMR showed no change in the backbone conformation of the modified form

as compared to the native inhibitor (Ebina et al., 1989).

The method of choice for the coupling of peptides to the carrier protein consisted of cross-linking the two moieties using a heterobifunctional cross-linking reagent. When selecting a specific cross-linker, several physical properties were considered, including homo- versus heterobifunctionality, reaction specificity, hydrophobicity, cross-linkable distance, and cleavability. By blocking the four lysyl ϵ-amino groups in BPTI by guanidination, the only free amino group was at the N-terminus. Using an amino-specific reagent at one end of a heterobifunctional cross-linker resulted in the site-specific attachment of the cross-linker to the N-terminus. Syntheses of peptides with a single cysteine group located at the C-terminus then allowed the attachment of peptides directly to the N-terminus using sulfhydryl-specific reagents at the other end of the cross-linker. The result was a semisynthetic protein containing the proper orientation of N- and C-termini.

The cross-linker employed in the current studies is a commercially available heterobifunctional cross-linker, SPDP. Ebina et al. (1989) have successfully attached synthetic peptides to BPTI by such methods in order to study how short peptides can induce the production of antibodies that react with sequences in the intact folded protein. In the experiments involving the conjugation of antigenic peptides to BPTI, several tested cross-linkers were found to induce aggregation upon incorporation of the peptide. Two widely used cross-linkers, *m*-maleimidobenzoic acid *N*-hydroxysuccinimide (MMBS) and N-(γ-maleimidobutyryloxy)succinimide (GMBS), were found to be inadequate for their 2D NMR studies due to extensive aggregation problems; admittedly, high protein concentrations were required for sharp and well-resolved resonances. In contrast to MMBS and GMBS, peptide−protein conjugates employing SPDP as the cross-linker showed no evidence of aggregation, even at high protein concentrations. Hence, SPDP was a logical first choice as a coupling reagent. The reaction occurs rapidly (10−20 min), is temperature-independent (4−25 °C), and was efficient under a wide range of concentrations (50 μM−9 mM) (Vanin & Ji, 1981). SPDP was stoichiometrically cross-linked to G-BPTI. Excess reagent and organic solvent was removed by gel filtration to yield the purified product, cross-linker-G-BPTI (X−G-BPTI). By reacting X−G-BPTI with a thiol reagent, the number of thiopyridyl groups introduced into BPTI could be determined spectrophotometrically by evaluating the quantity of pyridine-2-thione groups released. The other end of SPDP contains a thiol-directed reagent, 2-pyridyl disulfide. The reaction with a free cysteine group on a peptide or protein involves a direct displacement of thiopyridine, releasing pyridine-2-thione, and producing a new disulfide bond between the cross-linker and the protein. The release of pyridine-2-thione offered a convenient means of monitoring the extent of the reaction; the molar extinction coefficient of the chromophore is quite high (ϵ_{343nm} = 8000 M^{-1} cm^{-1}).

The peptide−G-BPTI conjugation was the final step in the preparation of semisynthetic proteins. The peptide−SDS mixture, which was freshly reduced by $NaBH_4$, was directly added to an aqueous solution of X−G-BPTI. Conjugation of peptides to G-BPTI occurred at neutral pH under anaerobic conditions. The extent of conjugation was monitored by measuring the release of pyridine-2-thione at A_{343}. Due to the high concentration of SDS, the reaction times were substantially increased (from 1 to 24 h). The removal of detergent was accomplished using gel filtration with propionic/formic acid included in the eluting solvent system. After the removal of SDS, the peptide−BPTI conjugates were purified from unreacted X−G-BPTI and deletion peptide−BPTI conjugates. Reversed-phase HPLC has become an integral step in most protein or peptide purifications due to its exceptional resolving power and was used as the final purification step. The semisynthetic proteins thus produced proved to be stable when stored lyophilized and under N_2.

The interaction of the semisynthetic proteins with phospholipid bilayers was examined by determining their water−vesicle partition coefficients using ultracentrifugation. As a comparison, the incorporation of the semisynthetic proteins into vesicles was studied using a well-established reconstitution procedure.

In order to assess the binding of the semisynthetic proteins to preformed lipid vesicles, it is necessary to know the state of aggregation of these proteins in the aqueous phase. Our result showed that neither $(Ala)_{10}$−G-BPTI nor $(Ala)_{20}$−G-BPTI aggregated to any appreciable degree in aqueous solution. This is in contrast to some other type I membrane-interactive proteins. For example, cytochrome b_5, consisting of a hydrophilic catalytic domain (bearing the heme moiety) and an independent hydrophobic domain (single transmembrane segment of ~40 amino acids) which anchors the protein to the membrane, has been shown to exist in distinct states of self-association in aqueous solutions (Spatz & Strittmatter, 1971; Calabro et al., 1976; Leto & Holloway, 1979). Glycophorin A, another example of protein anchored to the membrane by a single transmembrane segment, is also water-soluble, largely due to the presence of 60% by weight carbohydrate. It is known to readily self-associate to form aggregates consisting of 10−20 monomers (Springer et al., 1966). The reason for the lack of aggregation of the semisynthetic proteins in the aqueous phase is most probably the high surface charge density of G-BPTI. It seems unlikely that the linked peptide caused the G-BPTI to adopt a conformation that shielded the hydrophobic peptide from the aqueous environment, since BPTI exhibits unusually high conformational stability, as noted earlier.

The thermodynamic data obtained for the $(Ala)_{20}$−G-BPTI protein bound to vesicles can be compared to the theoretical values of total free energy changes calculated for the incorporation of model hydrophobic α-helices (Jähnig, 1983). The total free energy change upon incorporation of a peptide into a lipid bilayer can be described (Engelman & Steitz, 1981; Jähnig, 1983) as

$$\Delta G_t = \Delta G_{fob} + \Delta G_{fil} + \Delta G_{con} + \Delta G_{imm} + \Delta G_{lip} \quad (1)$$

where

ΔG_{fob} = free energy gained from the hydrophobic effect

ΔG_{fil} = free energy gained from hydrogen bonding/hydrophilic effects

ΔG_{con} = free energy due to conformational changes (e.g., α-helix formation)

ΔG_{imm} = free energy change due to peptide immobilization effects

ΔG_{lip} = free energy change due to lipid perturbation

The free energy gained from the hydrophobic effect, ΔG_{fob}, can be calculated from the change of the peptide/water interfacial area and the free energy change per unit area using $\Delta G_{fob} = C_s A$ (Richards, 1977). C_s is the solvation parameter. The interfacial area of a peptide, A, is the area accessible to water molecules. Water-accessible surface areas used in the calculation were obtained from Gly-X-Gly peptides as models for the extended standard state (Chothia, 1976) and defined relative to a stochastic standard state based on an ensemble mean accessibility of ϕ, ψ, and χ dihedral angles taken from X-ray diffraction data of 23 proteins (Rose et al., 1985). The value of the water-accessible surface area for alanine is 118.1 Å^2. An alanine 20-mer then has a total surface area of 2362 Å^2. Assuming a solvation parameter free energy per area of $C_s = -16$ cal/mole·Å^2 (Eisenberg & McLachlan, 1986) gives a free energy change upon peptide incorporation of $\Delta G_{fob} = -37.8$ kcal/mol.

The loss in free energy due to peptide immobilization in the membrane, ΔG_{imm}, results from a loss of one rotational and two translational degrees of freedom and has been estimated to be equal to +16 kcal/mol (Janin & Chothia, 1978; Jähnig, 1983). The contribution from lipid perturbation, ΔG_{lip}, has been proposed to be equal to +2 kcal/mol (Jähnig, 1983). The ΔG_{fil} and ΔG_{con} contributions due to hydrogen bonding and conformational changes can be considered negligible by assuming the free energy change upon insertion is the same for an α-helical peptide as for a random coil peptide; that is, the free energy loss due to a conformational change is balanced by the free energy increase due to the hydrophobic effect (Engelman & Steitz, 1981).

The total free energy upon insertion of the peptide into the lipid bilayer is then given as

$$\Delta G_t = \Delta G_{fob} + \Delta G_{imm} + \Delta G_{lip}$$
$$= -19.8 \text{ kcal/mol}$$

The largest contribution to the total negative free energy of transfer is a result of the hydrophobic effect. Although ΔG_{fob} depends linearly on peptide length when considering single amino acid peptides, ΔG_{imm} is independent of peptide length. Figure 9 is a plot of ΔG_t vs peptide length for alanine peptides. $\Delta G_t = 0$ when the number of residues is equal to 9.5. ΔG_t for $(Ala)_{10}$−G-BPTI is expected to be very close to the minimum length for spontaneous insertion into a lipid bilayer. This conclusion is consistent with the observation that $(Ala)_{10}$−G-BPTI did not partition or reconstitute into the bilayers as shown in Tables 1 and 2. However, the values of ΔG_t attained for $(Ala)_{20}$−G-BPTI, which did bind to the bilayers (−5.21 and −4.99 kcal/mol for DPPC and DMPC/DPPC bilayers, respectively), are substantially smaller than the value of −20 kcal/mol indicated in Figure 9. There are several possible reasons for this difference. First is the fact that the C-terminal residues of the $(Ala)_{20}$−G-BPTI molecule are Tyr and Cys, and there is also a linker region, -S-CH_2-CH_2-CO-NH-, connecting the peptide to G-BPTI. Tyrosine is a bulky, moderately polar amino acid that tends to remain in the interfacial areas (Jacobs & White, 1989). Although cysteine is considered to be moderately nonpolar, the disulfide bond between cysteine residues is less so. The second reason may be that the G-BPTI interacts with the

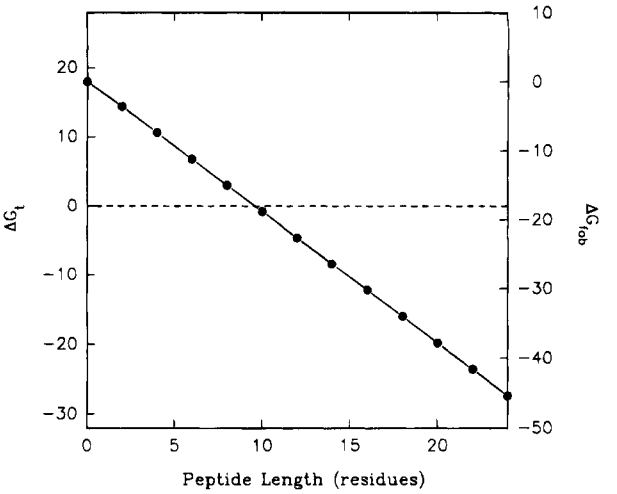

FIGURE 9: Theoretical total free energy of peptide insertion vs peptide length of alanine polymers. The total free energy of peptide insertion (ΔG_t) is estimated by eq 1. ΔG_{fob} is estimated using a value of $C_s = -16$ cal/mol·Å and a water-accessible surface area of 118.1 Å^2 per alanine residue.

bilayer surface in an energetically unfavorable fashion. A third cause for the lower value of ΔG_t may be the electrostatic repulsion of $(Ala)_{20}$−G-BPTI molecules on the vesicle surface. Even though the C-terminus of the peptide is blocked, the presence of several charged residues on the surface of G-BPTI might effect the degree of interaction with the hydrocarbon region. A fourth cause may be that the peptide interacts with the G-BPTI moiety in such a way as to reduce the effective length of the $(Ala)_{20}$ residues.

These results can be compared to other experimental data obtained from studies of peptide and protein incorporation into bilayers. Cytochrome b_5, whose transmembrane segment is considerably larger than $(Ala)_{20}$, spontaneously associates with egg phosphatidylcholine vesicles with a binding energy of $\Delta G_t = -11$ kcal/mol (Leto & Holloway, 1979). However, the specific conformation and orientation of the transmembrane segment is not known with certainty. Melittin (26 amino acids) interacts with DMPC vesicles with a binding energy of $\Delta G_t = -9$ kcal/mol; the presence of a charged residue within the hydrophobic segment accounts for the decreased value (Vogel, 1981). These experimental values are lower than calculated theoretical values for ΔG_t. Although water-soluble, the pentapeptide pentagastrin has been shown to spontaneously bind to liposomes containing only zwitterionic phosphatidylcholines; the value of ΔG_t was not reported (Surewicz & Epand, 1984).

Under the experimental conditions used in this study, the number of $(Ala)_{20}$−G-BPTI molecules associated with a single LUV is about 250. This is roughly 10% of the maximum number of semisynthetic molecules that can be accommodated on the surface of an LUV of diameter 1000 Å (as determined by quasielastic light scattering) assuming $(Ala)_{20}$−G-BPTI to have the dimensions of BPTI (Huber et al., 1971).

Although the critical length for peptide insertion in a bilayer has yet to be elucidated, the experimental data obtained so far, along with the theoretical calculations of the free energy required for spontaneous insertion as a function of length, tend to support recent *in vivo* and statistical studies examining the role of signal sequences in protein translocation. In a systematic study of the hydro-

phobic core region of the *Escherichia coli* alkaline phosphatase signal peptide, the wild-type sequence was replaced with homoploymeric units varying in length and hydrophobicity (Chou & Kendall, 1990). The hydrophobic core of the wild-type signal sequence, IALALPLF, was replaced using cassette mutagenesis to create mutant core regions containing, among others, $(Ala)_{10}$, $(Ala)_{15}$, $(Ala)_{20}$, and $(Ala)_{25}$ homopolymer units. Transport and processing of the enzyme was followed in the mutants. In the wild-type the phosphatase is cleaved and released as the mature enzyme into the periplasm. Chou and Kendall found that the $(Ala)_{10}$-mutant was incapable of effecting periplasmic localization, whereas activity appeared in the $(Ala)_{15}$-mutant and was further increased with the $(Ala)_{20}$- and $(Ala)_{25}$-mutants. Similarly, the total extent of processing was examined using pulse−chase studies and showed that only 8% of the $(Ala)_{10}$-mutant enzyme had been processed after 20 min, compared to 96% for the wild-type and 43% for the $(Ala)_{20}$-mutant. Further experiments replacing alanine with leucine showed that the length requirements for transport and processing were decreased as a result of the increased hydrophobicity, suggesting a subtle interrelationship between the net hydrophobicity and length of the core. Further evidence in support of this hypothesis was provided by statistical studies by von Heijne (1985), who found that the most hydrophobic signal sequences analyzed were also the shortest. Statistical studies were also undertaken by Bedouelle and Hofnung (1981), who analyzed the functional export characteristics of several signal sequences along with known mutations. Their survey suggested the existence of a threshold hydrophobic axis length for signal peptides, below which export is abolished. Their analysis predicted that 12 amino acids (corresponding to ~18 Å when in an α-helix confirmation) is the minimum length that could support export. It is interesting to note that with $(Ala)_{10}$−G-BPTI, which did not associate with or reconstitute in lipid bilayers, the hydrophobic peptide is short of this minimum length.

The studies of the interaction of two semisynthetic proteins, $(Ala)_{10}$−G-BPTI and $(Ala)_{20}$−G-BPTI, with phospholipid bilayers reported in this paper demonstrate the feasibility and general utility of this approach for the examination of the interaction of water-soluble, hydrophobic peptides with preformed bilayers. Studies of the interactions with bilayers of a variety of peptides, comprised of the same or several different amino acids, are currently underway using the semisynthetic protein approach. In these studies attention is focussed on the topology of the peptide in the lipid bilayer. The fact that the disulfide bond linking the peptide to G-BPTI proved to be readily reducible in the interactant complex by water-soluble dithiothreitol offers the possibility of generating bilayers containing hydrophobic peptides all with the same transbilayer orientation; a result that is rarely obtained by reconstitution. Since the orientation of all peptides in the bilayer is the same, the effects of specific amino acids and their position in the peptide on peptide−peptide interactions in the bilayer can be examined.

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