

High-Yield Synthesis and Purification of an α -Helical Transmembrane Domain

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Polypeptides corresponding to hydrophobic transmembrane α -helices, such as residues 69–101 of glycoprotein A, are notoriously difficult to prepare in quantities sufficient for biophysical experiments. Simple synthetic and purification approaches reported here have been developed by combining a few modifications to standard procedures, without resorting to elevated temperatures, expensive activation strategies, or complex hydrophobic solvent mixtures. The cost of screening projects, preparing labeled peptides, and examining sequence variations is thereby significantly reduced. The quality of the peptide synthesized by this small-scale 9-fluorenylmethoxycarbonyl (Fmoc) strategy is comparable to that of the peptide synthesized by an experienced resource facility using a large-scale *tert*-butyloxycarbonyl strategy. Using reverse-phase HPLC, the desired peptide was separated from the primary side product (a Leu or Ile deletion) and quantitatively recovered at greater than 98% purity. Baseline resolution was achieved using a water:acetonitrile gradient to elute the peptides from a cyanopropyl column at ambient temperature. Combining these approaches readily yields 10 to 20 mg of pure transmembrane peptide from a small-scale Fmoc synthesis. The approaches are readily transferable to transmembrane sequences not previously synthesized and do not require setting up a specialized facility. The time and start-up expense required to launch new studies are thereby reduced expanding the range and detail with which questions in membrane protein biophysics can be explored. © 2001 Academic Press

Key Words: hydrophobic peptides; α -helix; solid-phase peptide synthesis; SPPS; Fmoc; reverse-phase HPLC.

Examination of genome sequences for stretches of predominately hydrophobic amino acids 15–23 residues in length suggests that as much as 30% of open reading frames encode proteins that may cross membranes using hydrophobic helices (1, 2). Despite the abundance of membrane proteins and the important roles they play in many cellular processes, investigation of membrane protein folding and assembly lags far behind studies of soluble protein folding. Use of the tools of molecular biology to express predicted transmembrane domains, either directly or as chimeric fusion proteins (3), has a number of potential limitations. The gene products may be toxic to the host, may form insoluble aggregates, or may be rapidly degraded thereby preventing accumulation of the polypeptide. Thus, facile chemical synthesis emerges as a desirable approach.

Development of reliable synthetic approaches utilizing a *tert*-butyl (Tboc)³ protection strategy has provided a source of crude material. The cost of a Tboc synthesis, however, limits the number of transmembrane peptide sequence variations that can be studied. In addition, the necessary use of hydrofluoric acid to cleave the peptides from the resin limits the approach to specialized facilities. Although a small-scale 9-fluorenylmethoxycarbonyl (Fmoc) strategy can reduce the cost and eliminate the use of hydrofluoric acid, synthesis of transmembrane peptides using an Fmoc strategy is not yet routine (4–6). Common features of natural transmembrane sequences still present significant synthetic chal-

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³ Abbreviations used: DIPEA, *N,N*-diisopropylethylamine; Fmoc, 9-fluorenylmethoxycarbonyl; GpA, glycoprotein A; HBTU, *O*-benzotriazol-1-yl-*N,N,N',N'*-tetramethyluroniumhexafluorophosphate; HOBt, hydroxybenzotriazole; HPLC, high-pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption time of flight mass spectrometry; PEG-PS, polyethyleneglycol-polystyrene; Tboc, *tert*-butyloxycarbonyl; TFA, trifluoroacetic acid.

lenges, such as the length of polypeptide necessary to span a lipid bilayer, the high frequency of β -branched amino acids, and the presence of cysteines.

Regardless of the synthetic approach, the quality of the synthesis and the ease of purification are tightly linked for natural transmembrane sequences. Separation of the desired polypeptide from very similar deletion and truncation products remains challenging because of the limited solubility of the by-products (7) and the tendency of natural transmembrane sequences to form aggregates or oligomers (8). As the number of byproducts increases the number of peaks in a chromatogram increases and the ability to resolve the peaks declines. Extreme measures are sometimes required, such as loading the peptides in high concentrations of trifluoroacetic acid or formic acid, raising the temperature or adding chaotropic agents (9–11). Even though these approaches facilitate dissolving the products and binding the peptides to the column such harsh conditions reduce column lifetimes. The correct peptide is frequently found in several peaks, together with a subset of the by-products, indicating that the transmembrane peptides are binding to the column as oligomers or aggregates. Significant yield is lost in order to obtain acceptable purity. In order to recover the correct peptide in reasonable yield it is necessary to resolve it from all similar sequences in a single chromatographic run. Thus, the ability to dissolve and bind the peptides to the column as monomers and elute them separately depends critically on reducing the number of by-products.

Here we describe a general approach to synthesize and purify long hydrophobic peptides such as the transmembrane domain of human glycoporphin A (see Fig. 1). The 33-residue peptide includes 12 β -branched amino acids and forms stable oligomers in a variety of solvents. A high synthetic yield of several different transmembrane sequences has been achieved without using expensive activation reagents, elevated temperatures, or chaotropic agents. In addition, quantitative recovery of the desired products at >98% purity has been achieved with relative ease, in a single HPLC run without resorting to elevated temperatures or complex hydrophobic solvents. Furthermore, the methods developed with glycoporphin A have been transferable to other transmembrane peptides not previously synthesized, reducing the time and start-up expense required to launch new biophysical studies. In this report we compare the quality and yield of the glycoporphin A transmembrane peptide synthesized by two approaches: a small-scale synthesis in our lab using an Fmoc strategy and a large-scale synthesis by an experienced resource facility using a Tbc strategy.

MATERIALS AND METHODS

Peptide synthesis. A polypeptide corresponding to residues 69–101 of human glycoporphin A (GpA) was synthesized on polyethylene glycol–polystyrene (PEG-PS) resin substituted at 0.16 mmol g mol⁻¹ (Perseptive Biosystems). The Applied Biosystems Model 433A peptide synthesizer protocols were modified in order to compensate for the 12 β -branched amino acids and the length of the GpA transmembrane peptide. Using the subroutines programmed by the manufacturer, the coupling time was increased from ~20 to 35 min by insertion of a 15-min wait module after the initial coupling module. The solvent to resin ratio was increased twofold by using 0.125 mmol resin with the standard 0.250 mmol procedure. Adjustment of the solvent-to-resin ratio was necessary due to the increased swelling of the PEG-PS resin and to ensure access of the growing peptide chain to the reagents. Fmoc and side-chain-protected amino acids (Advanced Chem Tech or NovaBiochem) were used at eightfold molar excess over N termini and were activated *in situ* by the following standard reagents; *O*-benzotriazol-1-yl-*N,N,N',N'*-tetramethyluroniumhexafluorophosphate, hydroxybenzotriazole, and *N,N*-diisopropylethylamine (HBTU/HOBT/DIPEA) (6). Using HBTU/HOBT is considerably cheaper than using HATU/HOAT in the activation strategy. All other reagents were used as recommended in the protocols supplied with the instrument: piperidine, *N*-methylpyrrolidone, dimethylformamide, and dichloromethane (J. T. Baker). As shown in Fig. 1, the synthesizer was programmed from the C terminus to double couple the last eight residues and to triple couple at positions surrounding the two threonines. One-third of the resin peptide was acetylated at the N terminus before cleavage of the peptide from the resin using 30% acetic anhydride/HOBT/DIPEA for 10 min. The remaining two-thirds were reserved for fluorescent labeling as described elsewhere (12). The amidated C terminus resulted from the PAL linker that was preloaded on the resin (Perseptive Biosystems).

The peptide was cleaved and deprotected in 80% trifluoroacetic acid (TFA), including the following scavengers: 4% ethane dithiol, 4% ethylmethyl sulfide, 4% phenol, 4% thioanisole, and 4% water (v/v) (13). The solution was prepared fresh and mixed at a ratio of 2 to 4 ml per 0.1 g resin peptide. The peptides were deprotected and cleaved after 4 h of stirring at room temperature in a 30-ml fritted glass filter covered with parafilm. The yellow-orange solution was filtered and the resin was washed with 2 vol of TFA. The filtrate and wash were combined and concentrated to approximately 2 ml on a rotary evaporator. The crude peptides were precipitated by adding the solution dropwise to 100 ml of ice-cold diethylether. The large flakes were

collected in a bench-top centrifuge at 10,000 rpm for 5 min and washed six times with ice-cold diethylether. After drying overnight at room temperature, the pellet was crushed to a white powder. Crude peptides prepared in this way and stored at -20°C in a desiccator were stable against TFA acetylation and methionine oxidation for at least 2 years. In order to evaluate the quality of the small-scale Fmoc approach, the same peptide sequence was synthesized by Dr. J. Elliot using a large-scale Tbc strategy previously employed for several transmembrane sequences (William M. Keck Resource Facility, Yale University).

Peptide purification. Peptides were purified by reverse-phase high-performance liquid chromatography (HPLC) using a water/acetonitrile gradient. Both the aqueous phase (A) and the acetonitrile phase (B) included 0.3% TFA. A semipreparative Zorbax cyanopropyl column (SB-CN 9.4 \times 250 mm, 80 Å, 5 μm) was preequilibrated in 70%A/30%B at a flow rate of 3 ml min^{-1} . Including a 5-ml injection loop, the total volume was ~ 16 ml.

Many protocols have focused on modifying the mobile phase in order to elute transmembrane peptides from reversed-phase columns (14). We found that a mixture of solvents at the loading step provided an easier and more effective approach. Although both formic acid and trifluoroacetic acid are generally excellent solvents for transmembrane peptides, formic acid was avoided because of the potential for N terminal modification. When loaded in trifluoroacetic acid many transmembrane peptides elute in broad unresolved peaks, indicating that a range of elution conditions is required for desorption. Since transmembrane proteins are totally denatured in strongly acid solvents (15, 16) the broad elution profiles may reflect conformational heterogeneity when the peptides bind to the column. Trifluoroethanol promotes helix formation but generally interferes with peptide binding to many stationary phases. Mixing the two solvents was expected to promote the formation of soluble helices that bind to the stationary phase as folded polypeptides. A large loading volume was used to promote dissociation and increase the likelihood that glycophorin A might bind to the column in monomeric form.

In a typical run 2–8 mg of crude peptide was dissolved in 200 μl trifluoroacetic acid, 200 μl trifluoroethanol, and 400 μl acetonitrile for 10–15 min. The sample was filtered through a Millex-SR 0.5- μm filter just prior to injection. The dead volume of the filter was recovered with a 400- μl acetonitrile rinse that was added to the filtrate. The sample was diluted with the equilibrating solvent to a final volume of 4.5 ml after which 400 μl of water was added in order to reduce the overall organic composition. The sample was loaded into a 5-ml loop, injected immediately onto the column,



FIG. 1. Sequence of the transmembrane domain of glycophorin A. The numbering corresponds to the human sequence while the numbers of dots show single, double, or triple couplings used at each position.

and eluted over 30 min from 30% B to 90% B. The GpA peptide eluted at $\sim 83\%$ B.

Peptide evaluation. HPLC (described above) and amino acid analysis (Myron Crawford, Keck Facility, Yale University) were used to determine the composition, yield, and purity of the peptides. Electrospray mass spectrometry was used to determine the composition of the crude material (Dr. W. McMurray, Keck Facility, Yale University). Matrix-assisted laser desorption time of flight mass spectrometry (MALDI-TOF) was used to verify that the purified peptides corresponded to the predicted molecular weight. MALDI-TOF spectra of HPLC fractions were collected on a Voyager System (PE Biosystems) using equal volumes of the sample and saturated sinapinic acid as the ionizing matrix. Note that during laser desorption the sinapinic acid matrix can form adducts that add satellite peaks to the spectrum at +206 to the relative mass of the original products. The synthetic yield was calculated by comparing the total amino acid mass in the crude material to the theoretical mass of product assuming 99.9% coupling efficiency (34 couplings, including N terminal acetylation). The purified yield was calculated from the mass of lyophilized pure peptide obtained per unit mass of crude material. Synthetic and purification yields are discussed below.

RESULTS

Peptide synthesis. Simple modifications to standard protocols have allowed a 33-residue peptide corresponding to the transmembrane domain of human glycophorin A (Fig. 1) to be efficiently synthesized and purified. The amino acid content of the crude material was determined by extended hydrolysis in hydrochloric acid (M. Crawford, W. M. Keck Facility): 72% (Fmoc) and 65% (Tbc). Assuming 99.9% coupling efficiency the theoretical maximum yields were 442 and 1820 mg for the 0.125 mmol synthesis (Fmoc) and the 0.5 mmol synthesis (Tbc), respectively. The actual yields were 339 mg (Fmoc) and 1190 mg (Tbc), which correspond to crude synthetic yields of 77 and 66%, respectively. The amino acid correlation coefficient for each of the synthetic strategies was 0.919 (R^2) (using all amino acids except tryptophan, data not shown).

The crude peptides were evaluated by electrospray

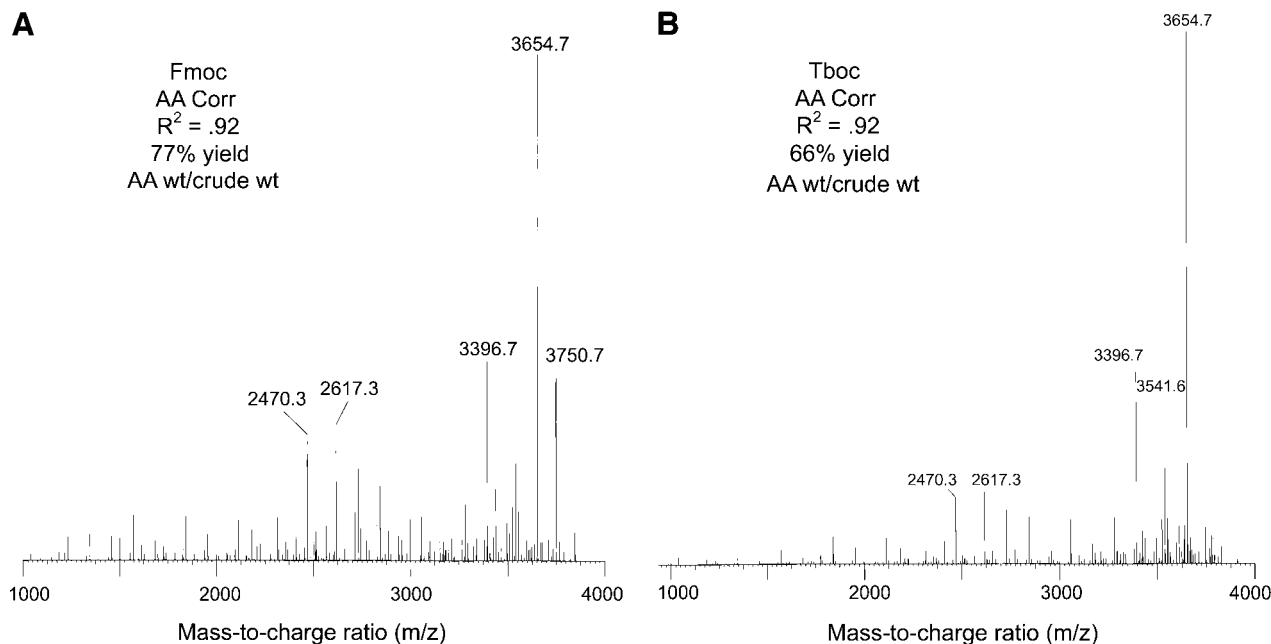


FIG. 2. Electrospray mass spectra of the crude synthetic products. Fmoc synthesis (A). Tbc synthesis (B).

mass spectrometry (Dr. W. McMurray, W. M. Keck Facility), as displayed in Fig. 2. In each synthesis the mass of the primary product was 3654.7 Da corresponding closely to the predicted monoisotopic mass of 3655.1 Da. In the Fmoc synthesis (Fig. 2A) the mass of the primary impurities most likely correspond to the following products: a trifluoroacetic acid adduct (3751 Da), Ile and Phe deletions (3397 Da), truncation after Phe (2617 Da), and truncation before Phe (2470 Da). In the Tbc synthesis (Fig. 2B), there is no trifluoroacetic acid adduct, but the same deletion and truncation products are present: Ile deletion (3542 Da), Ile and Phe deletion (3397 Da), truncation after Phe (2617 Da), and truncation before Phe (2470 Da).

Evaluated either by amino acid analysis or by mass spectrometry, the crude yield and composition of the small-scale Fmoc synthesis are comparable to the crude yield and composition of the large-scale Tbc synthesis.

Peptide purification. Semipreparative HPLC chromatograms of the crude peptides prepared by the Fmoc and the Tbc syntheses are shown in Fig. 3. The products are eluted with acetonitrile at room temperature in less than 40 min. In each case, the primary peak is well separated from the other peaks. This unusually high resolution was achieved by using a less hydrophobic column and simple modifications to commonly reported HPLC purification protocols for transmembrane peptides (see Materials and Methods). In order to determine the overall yield, the HPLC fraction corresponding to the main peak was lyophilized and the amino acid mass was determined by extended hydro-

lysis. The purified yields, 12% (Fmoc) and 15% (Tbc), correspond to the mass of pure lyophilized peptide per unit mass of crude material.

Analyzed by MALDI-TOF mass spectrometry (A. Lee, Yale University), the spectrum of the pure peptide

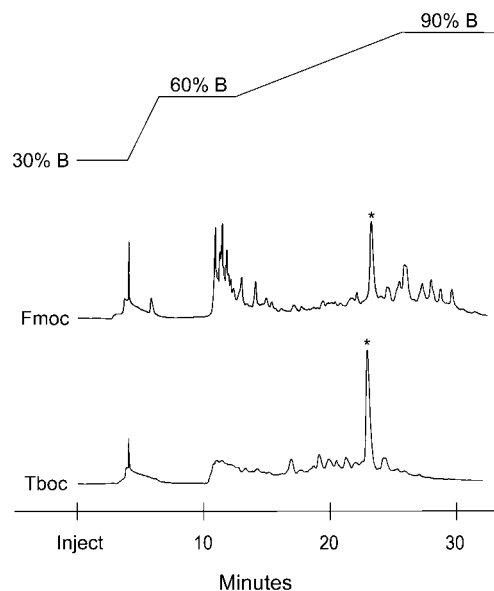


FIG. 3. Semipreparative HPLC purification of glycoporphin A transmembrane peptides synthesized by Fmoc and Tbc strategies. The water/acetonitrile elution gradient described under Materials and Methods is shown above the chromatograms of the peptides synthesized by Fmoc (middle) and Tbc (bottom) strategies. The absorbance was measured at 280 nm. The peak corresponding to the glycoporphin A transmembrane peptide is marked (*).

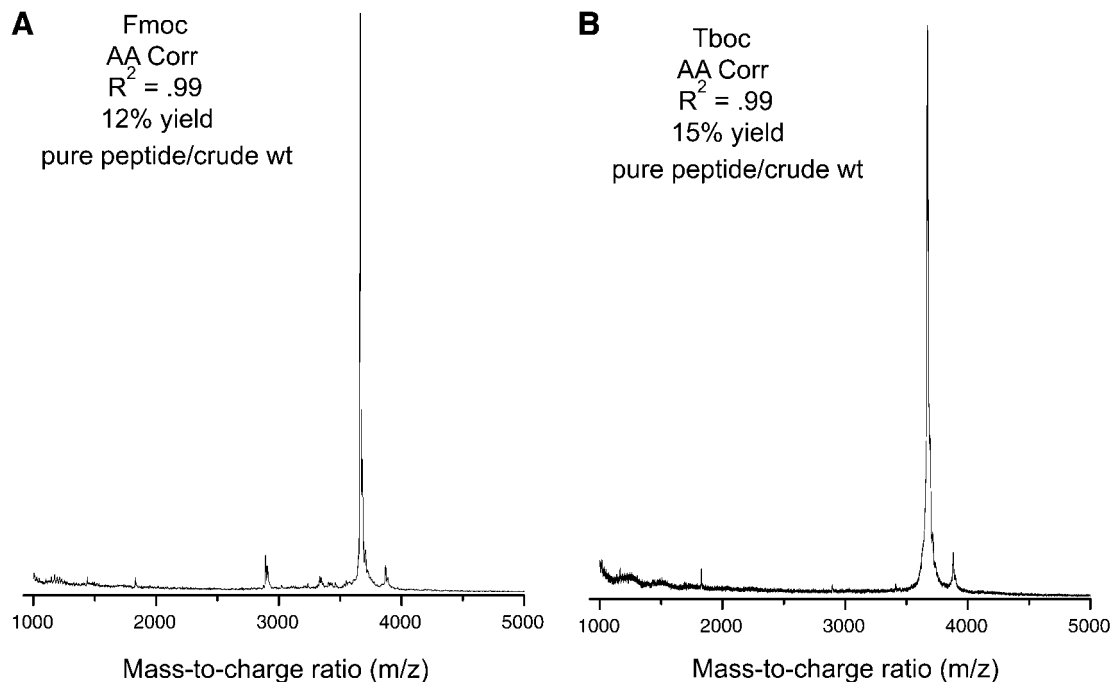


FIG. 4. MALDI-TOF mass spectra of pure GpA transmembrane peptide synthesized by Fmoc (A) and Tbc (B) strategies. The satellite peaks at +206 Da are sinnapinic acid adducts formed during ionization.

is shown in Fig. 4. The satellite peaks at higher molecular weight correspond to sinnapinic acid adducts (+206 Da) and are a consequence of the laser desorption method rather than impurities in the sample. The amino acid correlation was also consistent with a single peptide ($R^2 = 0.998$, all amino acids except tryptophan, data not shown). Integration of the HPLC chromatogram of the lyophilized peptide indicated that the sample purity is at least 98% (data not shown). Taking into account the amino acid content of the crude material and the integrated peaks of the chromatograms the pure peptides were recovered at >80% yield using a simple water/acetonitrile gradient at ambient temperature.

DISCUSSION

The transmembrane α -helix corresponding to residues 69–101 of glycoporphin A has been efficiently synthesized and purified. The charged residues flanking the hydrophobic residues were included to preserve potentially important interactions with lipid head groups (see Fig. 1). Inclusion of polar or charged amino acids facilitates purification by increasing the solubility of the products especially when they are included at the C terminus where coupling efficiency is very high. The most problematic residues, the β -branched threonines at positions 74 and 87, were triple coupled, and the N terminal eight residues were double coupled. These extra couplings were intended to compensate for

the reduced efficiency of coupling due to steric hindrance and the increasing peptide length, respectively. An alternative strategy using HATU instead of HBTU was not used because of the significantly increased cost of HATU and the need to increase the temperature (10). These few modifications to standard procedures eliminated the need for elevated temperatures, expensive activation strategies, or complex hydrophobic solvent mixtures.

Both the Fmoc and the Tbc approaches have yielded the desired peptide as the primary product and reveal the same sections of the polypeptide sequence that are difficult to synthesize (see Fig. 2). Note that the peak at 3542 Da, which is 113 Da less than the desired peptide, may comprise a collection of products from deletion of any of the eight isoleucines or four leucines. From the relative intensity of the 3542 Da peak in Fig. 2A the Fmoc strategy may reduce the number of 113 Da deletion products, although there are significant losses due to TFA adduct formation. The Fmoc approach can be implemented in most laboratories because cleavage/deprotection by TFA instead of HF circumvents the need for specialized facilities. The smaller synthetic scales available with the Fmoc approach reduces the time and cost of screening projects, preparing labeled peptides, or examining sequence variations.

Peptide purification. Purification of transmembrane peptides by reverse-phase HPLC is especially challenging when the purification requires separation

of hydrophobic sequences that differ by only one or two hydrophobic amino acid deletions. One strategy to reduce deletion products is to truncate the peptides that did not couple by adding an acetylation step. This does not necessarily solve the problem, however, because the N termini that were inaccessible to extension may remain inaccessible to acetylation. If the N termini then become accessible to extension at a later step the result is a deletion product. Separating deletion products from the desired product can be very difficult for natural transmembrane sequences, such as glycoporphin A, that form stable oligomers in detergents and organic solvents.

Due to their limited solubility transmembrane peptides have often been dissolved with high concentrations of trifluoroacetic or formic acid and loaded directly onto a column equilibrated with a significantly aqueous solvent. Elution of the desired product often requires that solvents such as isopropanol, butanol, or trifluoroethanol be added to acetonitrile in order to increase the hydrophobicity of the organic phase (10, 14). Even with these modifications, the desired product is frequently eluted in broad overlapping peaks. Alternatively, the desired product is present in more than one peak along with a subset of the impurities. The presence of multiple peaks indicates that aggregation, oligomerization, or different peptide conformations each gives rise to adsorbed peptides that require different elution conditions. As crude material accumulates on the column both the reproducibility of the chromatograms and the column lifetime are reduced. In some cases it is necessary to raise the temperature in order to achieve the desired purification (10, 11).

In the approach described here the peptides are initially dissolved in strongly organic solvents trifluoroacetic acid and trifluoroethanol but are then diluted 10-fold with a more aqueous solvent just before loading onto the column. Dilution of the sample promotes dissociation while simultaneously lowering the organic solvent content in order to promote adsorption of the peptides to the column. Loading a 5-ml sample onto a ~10-ml column at a nearly matched solvent composition may also increase utilization of the column capacity thereby reducing the number of runs required to purify milligram quantities of material (9). Since glycoporphin A is completely denatured in trifluoroacetic acid (15), reduction of the trifluoroacetic acid content to ~0.4% may reduce the potential for aggregation or irreversible adsorption of the peptides to the solid phase. The desired GpA peptide elutes in a single peak suggesting that the peptides are indeed binding to the column as monomers in a unique conformation (see Fig. 3). The relatively mild conditions significantly extend the lifetime of the column, which was used hundreds of times without any loss of column performance or need for regeneration. The small-scale Fmoc synthe-

ses typically yield ~120 mg of crude material for a 0.050 mmol synthesis and ~350 mg of crude material for a 0.125 mmol synthesis. Thus the Fmoc approach can readily yield 10–20 mg of pure transmembrane peptide, an amount sufficient for many studies using biophysical approaches.

CONCLUSIONS

Simple modifications to standard procedures have allowed the glycoporphin A transmembrane peptide to be efficiently synthesized and purified. The success of the approach suggests that the linkage of synthetic and purification yield is based upon problems of solubility. During synthesis, the challenge of maintaining accessibility of the growing peptide chain may be met by using the more polar PEG-PS resin with a lower substitution and higher solvent volume. Selection of the sequence to include several charged or polar groups, especially at the C terminus, enhances the solubility of both the desired product and any by-products. The potential loss in synthetic yield due to the length of the peptide is compensated by the higher purification yield resulting from high-resolution HPLC chromatography. Adsorption of monomeric peptides to the solid phase may be achieved by dissolving the crude peptides in a good solvent and subsequently diluting them while lowering the acidic organic solvent content. Since the more aqueous loading solvent allows peptides to adsorb to less hydrophobic columns, elution is possible at ambient temperature with a simple water/acetonitrile gradient. A number of other transmembrane domains have also been made using these methods, suggesting that the approaches are readily transferable to new projects.

Synthetic peptides corresponding to natural transmembrane α -helices can be efficiently prepared without resorting to expensive activation reagents, elevated temperatures, complex hydrophobic solvents, or specialized facilities. The reduced cost of the small-scale Fmoc synthesis expands the range and detail with which questions in membrane protein biophysics can be explored.

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