Synthesis and Biophysical Characterization of a Multidomain Peptide from a *Saccharomyces cerevisiae* G Protein-coupled Receptor*

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We attached peptides corresponding to the seventh transmembrane domain (TMD7) of the α-mating factor receptor (Ste2p) of *Saccharomyces cerevisiae* to a hydrophilic, 40-residue fragment of the carboxyl terminus of this G protein-coupled receptor. Peptides corresponding to (a) the 40-residue portion of the carboxyl tail (T-40), (b) the tail plus a part of TMD7 (M7-12-T40), and (c) to the tail plus the full TMD7 (M7-24-T40) were chemically synthesized and purified. The molecular mass and primary sequence of these peptides were confirmed by mass spectrometry and tandem mass spectrometry procedures. Circular dichroism (CD) revealed that T-40 was disordered in phosphate buffer and in the presence of 1,2-dimyristoyl-sn-glycero-3-phosphocholine/1,2-dimyr

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1 The abbreviations used are: GPCR, G protein-coupled receptor; CD, circular dichroism; DMDP, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-sn-glycer-3-phospho-racemic-[1-glycero]-sodium salt; HBTU, O-benzotriazolyl-N,N,N',N'-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; MPG, 1-myristoyl-2-hydroxy-sn-glycero-3-phospho-racemic-[1-glycero]-sodium salt; PPG, 1-palmitoyl-2-hydroxy-sn-glycer-3-phospho-racemic-[1-glycer]-sodium salt; Ste2p, α-factor receptor encoded by the STE2 gene of *S. cerevisiae*; TFE, 2,2,2-trifluoroethanol; TMD, transmembrane domain; MS, mass spectrometry; Fmoc, N-(9-fluorenyl)methoxycarbonyl.
thetic TMDs of Ste2p revealed that the solubility of the third and fourth TMDs was very low, and they could not be effectively purified on HPLC without solubilization by the addition of several lysine residues at both termini of the peptides (13). Furthermore, the sixth TMD of Ste2p had a high aggregation tendency in SDS-PAGE and exhibited \( \beta \)-structures in phospholipid bilayers as judged using CD or IR spectroscopy (13, 14).

To improve water solubility, a number of laboratories have placed solubilizing sequences at the termini of peptides predicted to represent the transmembrane domain of the membrane protein. An often used strategy is to place oligomers of lysine at these positions, and significant improvements in solubility and sample preparation have been achieved (13, 15–17). Nevertheless, in the native state Lys residues are rarely found at both ends of a transmembrane domain, and the inside-positive rule suggests a high preference for Lys and/or Arg residues on the cytoplasmic side of transmembrane domains (18). Furthermore, the use of Lys residues eliminates native residues that are adjacent to the TMDs, and studies on such model peptides provide no insights into the conformational preferences of the native loop or tail residues in the context of one contiguous molecule.

Recently we showed that the residues at the termini of the second transmembrane domain of Ste2p (a GPCR involved in mating) can affect the integration of this domain into the membrane and can influence the tilt angle of the peptide (19). Specifically, we found that a 30-residue peptide corresponding to transmembrane domain two (TMD-2; 25 residues) plus 5 residues extending into the cytosolic compartment integrated into bilayers was highly helical and tilted at an angle of 34° to the bilayer normal. In contrast, a 30-residue peptide corresponding to the same transmembrane two plus 5 residues extending into the extracellular space did not integrate into bilayers and formed \( \beta \) structures. A 35-residue peptide containing the same transmembrane core and both of the above cytosolic and extracellular residues was helical with a tilt angle of 36°. It is clear that residues at the termini of transmembrane peptides influence the structure assumed by the transmembrane region, and this influence may have biological relevance.

To extend our previous investigations on single TMDs we decided to explore peptides corresponding to these regions of the receptor attached to residues that contain appreciable hydrophilic tendencies. In the case of GPCRs it struck us that nature has provided two hydrophilic domains surrounding the seven-transmembrane core of these polytopic membrane proteins. We reasoned that these termini might aid in the overall folding of the receptor and, most importantly, might act as hydrophilic end groups that have evolved to allow the intact receptor to achieve its biologically active conformation. We anticipated that the combination of these hydrophilic tail residues and a hydrophobic TMD would result in a multidomain peptide with sufficient solubility to conduct conformational analyses in aqueous-organic and membrane mimetic solvents.

This communication reports the synthesis and characterization of a 64-residue peptide containing 40 residues of the cytosolic carboxyl terminus and 24 residues comprising the seventh transmembrane domain of Ste2p. Our results clearly demonstrate that this multidomain peptide exhibits distinct conformational preferences for the TMD and tail residues. The use of such hydrophilic appendages may enable the study of multiple transmembrane domains of GPCRs under membrane mimetic conditions. Such high resolution analyses should provide important information on the three-dimensional structure of a large region of Ste2p.

### EXPERIMENTAL PROCEDURES

#### Peptide Synthesis

Peptides T40 (NNASKNTTIDFTSTDFRYFPGTLSSFQTDSINDALKSS), M7-12-T40, and M7-24-T40 (for sequences and position in Ste2p see Table I and Fig. 1) were synthesized using the dicyclohexylcarbodiimide/4,4′-dimethylaminopyridine strategy in diethyl ether to obtain a resin with only 0.15 mmol/g of Fmoc amino acid substitution. The unreacted OH groups on the resin were capped using a 100-fold excess of acetic anhydride in the presence of 4-N,N′-dimethylaminopyridine. The resulting amino acids were coupled manually using HBTU/HOBt/diisopropylethylamine for activation of the Fmoc-Ser(butyl)-OH was loaded manually on a hydrophilic separations peptide synthesizer (Model 433A, Applied Biosystems). The coupling strategy utilized FastMoc chemistry with extended coupling times. Double coupling was carried out for each residue using HBTU/HOBt activation, and coupling was accomplished with acetic anhydride in the presence of diisopropylcarbodiimide. For comparison purposes the peptide M7-24 (VLTTVATLLAVLSLPLSSMWATAA) was synthesized on a preloaded Fmoc-Ala-Wang resin with 0.7 mmol/g substitution. Single coupling with HBTU/HOBt activation was used for the first 12 amino acid additions, and double coupling was carried on the completion of the synthesis. Coupling was accomplished as above. Resin weight gain was 60–70% relative to theoretical.

After the cleavage using 0.75 g of phenol, 0.5 ml of \( \text{H}_2\text{O} \), 0.5 ml of thiocyanate, 0.25 ml of 1.2-ethanediol, and 10 ml of trifluoroacetic acid, the crude peptides were isolated from the resin by filtration, and the filtrate was reduced to a low volume using a rotary evaporator, and the peptide was precipitated from diethyl ether and dried to give off-white solids. The yields of the crude peptides were 60–70% based on the resin weight gain, and overall, the yields of the syntheses were 40–50% based on the loading of the resin.

#### Peptide Purification

Crude peptides were purified on a semi-preparative Bondapak C18 (Waters) HPLC column (19 \( \times \) 300 mm) at room temperature with elution solvents of water (0.1% trifluoroacetic acid), acetonitrile (0.1% trifluoroacetic acid) at gradients from 10 to 80% acetonitrile over 90–120 min (T40, M7-12-T40, and M7-24-T40) or a gradient from 40 to 70% acetonitrile over 90 min (MT-24). In general 20 mg of crude peptide was dissolved in 2 ml of a solvent consisting of TFE/H\(_2\)O and injected directly onto the column. Fractions were collected, and homogeneity was judged by analytical reverse phase HPLC on a \( \mu \) Bondapak C18 column (3.9 \( \times \) 300 mm) with linear gradients of water (0.1% trifluoroacetic acid)-acetonitrile (0.1% trifluoroacetic acid).

#### Mass Spectrometry

The purity and amino acid sequence of peptides were evaluated using electrospray mass spectrometry (Thermo Finnigan LCQ DECA). All peptides were dissolved from a lyophilized powder directly into electrospray solvent (\( \text{H}_2\text{O}/\text{acetonitrile}/\text{acetic acid} 50:50:1 \)) and directly infused into an electrospray source (Thermo Finnigan) at 3 \( \mu \)l/min. The electrospray source was held at 4.5 kV, and the heated capillary was set at 125 °C. A total of 200 microscans were acquired and averaged for each peptide. Intact molecular masses for all peptides were obtained by deconvolution with the Bioworks software package (Thermo Finnigan).

To confirm the correct primary sequence, tryptic digestion and cyanogen bromide digestion were performed on the largest peptide (MT-24-T40) followed by MS/MS on the resultant peptide fragments. Peptides M7-12-T40 was first digested with trypsin (Promega), sequencing grade, 1:50 enzyme to peptide ratio following the manufacturer's protocol for 12 h at 37 °C, desalted with a C\(_18\) ZipTip (Millipore), and directly infused into the electrospray source at 3 \( \mu \)l/min. The resultant peptides were analyzed by normal MS followed by zoom scans (scan width 10 m/z, 200 microscans acquired and averaged). To determine the correct monoisotopic molecular masses for all peptides were performed on all peaks using the MS/MS to obtain sequence information (resolution 3 m/z, normalized collision energy 35%, 200 microscans acquired and averaged). Fragment ions from being a large portion of the amino terminus were not verified from the tryptic digestion experiment. To address this, a CNBr digest was performed on a separate aliquot of the M7-24-T40 peptide.
The M7-24-T40 lyophilized peptide was directly dissolved in 70% formic acid and digested with 1 M CNBr (Sigma) for 12 h in the dark at room temperature. The sample was repeatedly dried in a Spin Vac (Savant Instruments) and redissolved in HPLC grade H2O to remove excess CNBr. The sample was then resuspended in electrospray solvent, and the resultant amino-terminal peptide fragment (residues 1–19) was analyzed for intact monoisotopic mass. MS/MS was performed to verify sequence information.

Sample Preparation

CD Samples in TFE/H2O—T40 (1.4 mg) and M7-24-T40 (0.83 mg) were dissolved in 0.29 ml of H2O and 0.2 ml of TFE/H2O (4:1) to obtain a stock solution of T40 and M7-24-T40, respectively. In all cases the peptides were first dissolved in 100% TFE and water was added to give the final ratio of solvents. All solvent ratios in this manuscript are volume to volume. The concentrations of stock solution were determined to be 0.82 and 0.42 mM, respectively, by measuring the UV absorbance at 280 nm using an extinction coefficient of 1340 M⁻¹ cm⁻¹ for the single tyrosine in T40 and 6890 M⁻¹ cm⁻¹ for a tyrosine and a tryptophan residue in M7-24-T40. Five aliquots of 20 μl of each stock solution were diluted to 0.4 ml with the appropriate amount of TFE and H2O to obtain 41 μM T40 and 21 μM M7-24-T40 solutions in 95, 75, 50, 25, and 5% TFE/H2O. Solutions of M7–24 (41 μM) used for CD analysis in TFE/H2O were prepared in an identical manner. Peptide concentrations determined using UV spectroscopy were about 37 μM, which was about 90% of the concentration calculated based on sample weight.

CD Samples in DMPC/DMPG (4:1) Vesicles—Peptides (0.58 mg of T40, 0.72 mg of T40-M7–12, and 0.86 mg of M7-24-T40 in 0.15 ml TFE/H2O) were added to 8 mg of DMPC/DMPG (4:1) in 1 ml of CHCl₃.

The resulting solutions were dried under N₂ flow. Residual traces of TFE/H₂O were diluted to 0.4 ml with the appropriate amount of TFE and H₂O, respectively, by UV spectroscopy. Aliquots (51.5 μl) of M7-24-T40 were added to three vials, and the solutions in these vials along with the remaining stock solution were lyophilized overnight. Then 100 μl of MPEG, PPG, and SPG (4 mg/ml) in 0.1 mM phosphate solution were added to the former three vials, respectively, to provide 50 μM peptide concentration, and 164 μl of the same PPG solution was added to the last vial to obtain a peptide concentration of 500 μM. Similarly, 50 μM solutions of T40 in MPEG, PPG, and SPG (4 mg/ml), a 500 μM solution of T40 in PPG (4 mg/ml), and 50 μM and 500 μM solutions of M7-24 in PPG were also prepared.

Fluorescence Samples—M7-24-T40 (1.4 ml of 30 μM), M7-12-T40 (1.1 ml of 41 μM), and T40 (0.92 ml of 50 μM) in DMPC/DMPG (4:1) vesicles (4 mg/ml) were diluted to 20 ml with 0.1 mM phosphate buffer, pH 6.3. DMPC/DMPG (4:1) vesicle solution to obtain 2.3 μM peptides and 0.3 mg/ml DMPC/DMPG (4:1) solutions for fluorescence emission measurements. Appropriate blank samples were prepared similarly starting with 4 mg/ml of DMPC/DMPG (4:1) vesicles. For fluorescence quenching measurements, 2.5 ml of the solutions used for the fluorescence measurements were further diluted to 10 ml to obtain 0.58 μM peptides in 0.075 mg/ml DMPC/DMPG (4:1) vesicles.

Circular Dichroism Measurements

The CD spectra of the peptides were recorded on an AVIV model 62-DS CD instrument (AVIV Associates, Lakewood, NJ). Two cuvettes with light path lengths of 0.2 and 1.0 mm were used for peptides in lipids (4 mg/ml) with concentrations of 30–50 and 500 μM, respectively. A cuvette of 1-mm light path length was used for peptides in TFE solution. All spectra were the average of five scans between 280 and 190 nm at an interval of 1 nm with a 1-s integration time at each wavelength. The bandwidth for each measurement was 1 nm. Before calculation of the final ellipticity, all spectra were corrected by subtracting the reference spectra of 4 mg/ml lipids of DMPC/DMPG (4:1), MPEG, PPG, and SPG without peptides or 4–95% TFE/H₂O, respectively. CD intensities are expressed as mean residue ellipticities (degrees cm²/dmol).

Assuming that the transmembrane and tail portions of M7-24-T40 behaved independently, we estimated the mean residue ellipticity of the 24 transmembrane residues of this peptide using the following equation.

\[ \theta_{\text{M7-24}} = \{\theta_{\text{M7-24}} \times 64 \} - \{\theta_{\text{T40}} / 40\} / 24 \] (Eq. 1)

Fluorescence Measurements

Fluorescence spectra were recorded using a photo-counting Fluoromax-3 (Jobin Yvon Horiba, Spex Fluorescence Division) equipped with a 1 × 1 × 3.4-cm quartz cuvette with a 2.6-ml sample volume. The emission spectra were scanned between 300 to 420 nm with an excitation wavelength of 280 nm at intervals of 1 nm with a 1-s integration time at each wavelength. Both the excitation and emission bandwidths were set to 3 nm. The fluorescence due to the transmembrane parts of M7–24 and M7–12 were obtained by subtracting the spectra of T40 in vesicles from those of M7-24-T40 and M7-12-T40 in vesicles.

Collisional Quenching Experiments—Fluorescence collisional quenching experiments with iodide were performed by adding an increasing amount of 4 mM KI solution in phosphate buffer (0.1 mM, pH 6.3) to a solution of the peptide under investigation to bring the final concentration of KI to 60.6 mM. Changes in fluorescence of the transmembrane region due to the addition of quencher were corrected by subtracting the fluorescence measured in parallel on T40 in a DMPC/DMPG (4:1) vesicle control. The quenching of the fluorescence intensities at maximum emission were calculated with the Stern-Volmer equation,

\[ F/F_0 = 1 + K_{sv}[I] \] (Eq. 2)

where \( F/F_0 \) is the ratio of fluorescence intensities in the presence of KCl and KI. The Stern-Volmer quenching constant \( K_{sv} \) was determined from the slope of \( F/F_0 \) as a function of the iodide concentration [I⁻].

RESULTS

Synthesis and Characterization of M7-24-T40

Peptides corresponding to the 40-residue portion of the carboxyl tail (T40), a 52-residue peptide (M7-12-T40) containing the tail portion plus half of the seventh transmembrane domain (12 residues), and a 64-residue peptide (M7-24-T40) corresponding to the 40-residue carboxyl portion of the tail plus the entire seventh transmembrane domain (24 residues) were synthesized using solid phase peptide chemistry. The sequences of these peptides are listed in Table I and represented in Fig. 1 with T40 denoting the 40-residue carboxyl terminus portion corresponding to the sequence from 300 to 339 of Ste2p. M7 represents the seventh transmembrane domain, and the

| Peptides | Sequences | Helicities % |
|----------|-----------|--------------|
| M7-12-T40 | SLPLSSMWATAANNASKTNITSSFTITSTDRFYPGLSSFQTDNSSDAKSS | 11 |
| M7-24-T40 | VLTITYVATLVLSTSLPSSWATAANNASKTNITSSFTITSTDRFYPGLSSFQTDNSSDAKSS | 36 |
| M7–12 | SLPLSSMWATAAN | 42 |
| M7–24 | VLTITYVATLVLSTSLPSSWATAAN | 94 |

The percent helicity of this region of Ste2p was calculated using CD data from Fig. 4B and the methods described in Wu et al. (25) and Chen et al. (26).
number after M7 represents the chain length of the transmembrane peptide fragment attached to T40. For comparison purposes, transmembrane fragment M7–24 was also synthesized.

Solid-phase peptide synthesis results in the accumulation of impurities along with the final product. In the case of peptides containing 30 or fewer residues, the resolving power of HPLC is usually sufficient to result in a distinct and highly pure final product. However, in the case of a 64-residue peptide containing a highly hydrophobic domain, such as M7–24–T40, it is critical to obtain independent proof of both homogeneity and sequence. To gain such information, we decided to employ a combination of reversed phase HPLC and mass spectrometric characterization.

HPLC profiles of crude T40, M7-12-T40, and M7-24-T40 were characterized by increasing heterogeneity (Fig. 2, panels A1, B1, and C1). Whereas the HPLC profile of T40 exhibited one major product (Fig. 2, panel A1), the profile of M7-24-T40 exhibited several significant peaks. Nevertheless, the peak at 17.72 min (Fig. 2, panel C1) was the first choice for the presumptive product because of its being the major peak in the crude peptide. It is worth noting that the crude products from previous syntheses of single transmembrane domains containing 30–40 residues were often significantly more complex than that observed for the 64-residue peptide M7-24–T40 (13). Thus, even as judged by the HPLC of the synthetic peptide, the advantages of the solubilizing tail peptide were evident. Purification of the three peptides using semipreparative HPLC resulted in isolation of products that exhibited a single peak on an analytical column with a homogeneity of >98% (Fig. 2, panels A2, B2, and C2). A highly homogeneous sample of M7–24 (≥95%) was obtained in a similar manner (data not shown).

Mass Spectrometry

The peptides were analyzed by electrospray mass spectrometry to confirm purity, intact molecular mass, and primary sequence. The peptides all had correct intact average molecular masses within ±2 Da (the T40 calculated average mass was 4335.6 Da, and experimental average mass was 4336.0 Da; the M7-12-T40 calculated average mass was 5551.9 Da, and the experimental average mass was 5551.0 Da; the M7-24-T40 calculated average mass was 6747.5 Da, and the experimental average mass was 6749.0 Da; the M7–24 calculated monoisotopic mass was 2428.4 Da, and the experimental mass was 2429.1 Da). The analysis of the MS peaks revealed that although there were impurities in the peptide, these were minor in amount, and no single impurity was greater than the background noise in the MS spectrum. Therefore, we are confident that the synthetic peptide is highly pure and that the biophysical studies on this peptide are relevant to this region of Ste2p.

To confirm correct primary sequence order, tryptic digestion and cyanogen bromide digestion were performed on the largest peptide (M7-24–T40) followed by MS/MS on the resultant peptide fragments. From the tryptic digestion experiment all peaks above s/n matched predicted tryptic generated peptides from the M7-24–T40 sequence (the residue 1–29 calculated monoisotopic mass was 2942.6 Da, and the experimental monoisotopic mass was 2942.2 Da; the residue 30–43 calculated monoisotopic mass was 1558.7 Da, and the experimental monoisotopic mass was 1558.6 Da; the residues 44–62 calculated monoisotopic mass was 2103.9 Da, and the experimental monoisotopic mass was 2104.0 Da). A small peptide at the

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*F. Naider and F. X. Ding, unpublished results.*
carboxyl-terminal end consisting of the final two serines residues (residues 63 and 64) was not found presumably due to the fact that it was very small and did not bind efficiently to the C18 ZipTip.

To establish the amino acid sequence of the peptides, MS/MS was performed on the three tryptic-generated fragments from M7-24-T40. From these MS/MS experiments all product ions were manually verified to be consistent with the expected sequence and product ions from 42 of the 64 amino acids. Fragment ions from a large portion of the amino terminus were not verified from this experiment. To address this issue a CNBr digest was performed on a separate aliquot of the M7-24-T40 peptide to obtain a smaller amino-terminal piece. A fragment from the first 19 residues of the amino terminus was identified (the calculated monoisotopic mass was 1880.0 Da, and experimental monoisotopic mass was 1880.0 Da) and analyzed by MS/MS. All product ions from the amino-terminal peptide matched the predicted sequence, and the combination of these two data sets verified a total of 50 of the 64 amino acids, VLTTVATLLAVLSLPLSSMWATAANNASKTNTITSDFTTSTDVFYPGLSSFQTDSSINNDKSS. Taking all of this information into account and the fact that the shorter peptides, T40 and M7-12-T40, were obtained by withdrawing a portion of the resin in the course of the synthesis of M7-24-T40, we feel that the MS data confirms that T40 and M7-12-T40 also have the correct amino acids in the correct sequential order.

**CD Analysis of Peptides**

Studies in Trifluoroethanol/Water Mixtures—Previous studies on the transmembrane domains of Ste2p were carried out in membrane-mimetic environments including TFE/water, SDS micelles, and various vesicles (13, 19, 23). In these investigations a fragment corresponding to the seventh transmembrane domain of Ste2p was synthesized as a 30-residue peptide (GT-DVLTTVATLLAVLSLPLSSMWATAANNA) containing 24 residues presumed to represent the hydrophobic core and three residues from the third extracellular loop and the cytosolic tail. This peptide showed a marked decrease in helicity (from 75 to 45% helical) with decreasing TFE, 100 to 25%, and could not be studied in 100% water (13, 24).

To evaluate the effect of the 40-residue portion of the cytosolic tail on the solubility of the seventh transmembrane domain we carried out CD analyses on the 64-residue peptide and the 40-residue tail in TFE/water mixtures. T40 was found to be soluble in water and phosphate solution (0.1 mM, pH 6.3) at concentrations up to 0.8 mM. M7-12-T40 and M7-24-T40 were not soluble in the same solvents at room temperature and could be dissolved in 4% TFE, 96% water (Fig. 3, panel A). The CD pattern of T-40 in phosphate buffer manifested one broad negative trough centered below 200 nm. Similar CD patterns were observed in 4% TFE, 96% water (Fig. 3, panel A). Almost no change in the CD pattern of this peptide was found in 25% TFE, 75% water, and a small increase in ellipticity at
222 nm was observed in 50% aqueous TFE. However, in 95% TFE, 5% water the T-40 peptide had strong negative CD bands at 208 and 222 nm and a positive band near 195 nm (Fig. 3, panel A). In 4% TFE, 96% water the CD pattern of the 64-residue peptide was indicative of a partially structured peptide, and in 25% TFE, 75% water two clearly seen minima at about 205 and 220 nm, characteristic for a helical conformation, were evident in the CD spectrum (Fig. 3, panel B).

Estimation of the CD pattern for the transmembrane domain of the T40-M7–24 peptide by difference spectroscopy (see “Experimental Procedures”) revealed significant structure even in 4% TFE, 96% water, and in 25% TFE, 75% water the transmembrane region of the peptide was highly helical (Fig. 3, panel C). Most significantly, these solutions did not become turbid even after several weeks. The calculated helicities for M7-24-T40 and its component peptides are presented in Table II.

For comparison purposes we prepared the isolated transmembrane domain M7–24. We found that ~40 μM solutions of this peptide in TFE/water (96% TFE to 4% TFE) could be prepared. However, turbidity started appearing within a few hours in the 4% TFE solution, and after 3 days UV measurements showed that only 7% of the original peptide remained in solution. Measurements on freshly prepared solutions indicated that in 4% TFE/water this peptide had a CD pattern with a broad minimum centered at 214 nm and positive ellipticity below 203 nm (Fig. 3, panel D). Such a pattern is indicative of a partially aggregated peptide and the presence of β-like structures. At higher concentrations of TFE, CD patterns consistent with a partially helical peptide were observed.

Studies in Lipid Vesicles—The peptides were reconstituted into DMPC/DMPG (4:1) bilayer using procedures previously described (14). As judged by its CD pattern, T40 is disordered in the presence of DMPC/DMPG vesicles in phosphate buffer, exhibiting one negative band centered at 198 nm (Fig. 4, panel A). Because the CD curves of this peptide in both phosphate buffer and in the presence of lipid vesicles are coincident, it is reasonable to conclude that T40 does not insert into the hydrophobic interior of bilayers. This is as expected for a region of the hydrophilic tail of an integral membrane receptor. In contrast to T40, M7-24-T40 showed a double minimum at 208 and 222 nm and positive absorbance at 190 nm that indicated some α-helical secondary structure. The percentage of α-helicity for M7-24 was estimated using the difference spectroscopy approach described under “Experimental Procedures” according to Equation 1. When the contribution of T40 was subtracted from M7-12-T40 and M7-24-T40, the difference CD spectra representing the transmembrane frac-

| Environments | Ratios | Helicitiesα | M7-24-T40 | T40 | M7-24 |
|--------------|--------|-------------|-----------|-----|-------|
| TFE/H2O      | 95/5   | 87          | 79        | 99  |       |
| TFE/H2O      | 75/25  | 68          | 50        | 98  |       |
| TFE/H2O      | 50/50  | 54          | 27        | 98  |       |
| TFE/H2O      | 25/75  | 37          | 15        | 73  |       |
| TFE/H2O      | 4/96   | NDβ        | ND        | 22  |       |
| DMPC/DMPG    | 4/1    | 36          | ND        | 94  |       |

α Helical contents of M7-24-T40 and T40 were calculated from CD spectra using the method of Yang and coworkers (25, 26). The helicity of M7–24 was estimated using the difference spectroscopy approach described under “Experimental Procedures” according to Equation 1.

β ND, not determined.
Fluorescence Spectroscopy on M7-12-T40 and M7-24-T40 in Vesicles

The interaction of the transmembrane regions of M7-12-T40 and M7-24-T40 with the lipid vesicles was assessed using fluorescence spectroscopy. Both of these peptides contain Trp residues in the transmembrane portion of the molecules. The fluorescence emission maxima of M7-12-T40 in phosphate buffer containing 4% TFE and in the presence of DMPC/DMPG bilayers were 355 and 352 nm, respectively (Fig. 6). This 3-nm blue shift suggests that the environment of the Trp residue of M7-12-T40 in the DMPC/DMPG suspension is slightly less polar than that in water. The Trp emission spectrum of M7-24-T40 in phosphate buffer containing 4% TFE and in DMPC/DMPG bilayers exhibited maxima of 352 and 339 nm, respectively. The 13-nm blue shift and 76% intensity increase of fluorescence intensity of the Trp residue of M7-24-T40 in the DMPC/DMPG bilayers as compared with aqueous TFE indicate that Trp residue of M7-24-T40 is in a highly hydrophobic environment in the presence of the vesicles.

Insertion of the peptides into the lipid was also evaluated by fluorescence quenching using KI. Linear plots of $F_0/F$ versus $[I]$ were obtained (data not shown), and the derived Stern-Volmer constants ($K_{sv}$) were $0.18 \pm 0.25 \text{ m}^{-1}$ and $3.12 \pm 0.16 \text{ m}^{-1}$ for M7-12-T40 in DMPC/DMPG bilayers and buffer, respectively. In the case of M7-12-T40, $K_{sv}$ values in the presence of DMPC/DMPG vesicles and buffer were $3.37 \pm 0.06 \text{ m}^{-1}$ and $4.18 \pm 0.14 \text{ m}^{-1}$, respectively. These results suggested that tryptophan residues of M7-24-T40 and M7-12-T40 in the presence of DMPC/DMPG vesicles were exposed differentially to the iodide ions.

DISCUSSION

Few attempts have been made to prepare and characterize peptides corresponding to more than one domain of a GPCR. Notable exceptions are the reconstitution studies on parts of bacteriorhodopsin, rhodopsin, and Ste2p (27–29). Although these studies expressed, and in some cases isolated, long pieces of these heptahelical proteins, no detailed biophysical analyses were conducted. A CD study has appeared on peptides containing two transmembrane domains from the cystic fibrosis transmembrane conductance regulator (30). However, to date no high resolution analysis has been reported nor was information on domain-domain interactions forthcoming. Our research using fragments of Ste2p have as a long range goal the determination of the three-dimensional structure of peptides corresponding to several domains of this GPCR. This paper represents the initial study on a 64-residue peptide from this integral membrane protein. The results of the CD and fluorescence experiments reported herein provide strong evidence that a multidomain peptide containing 40 residues from the cytosolic terminus and 24 residues from the seventh transmembrane domain of Ste2p manifests distinct structural preferences for these regions of the receptor. This is seen, in particular when the peptide was studied in the presence of micelles or lipid vesicles. In the latter case the blue shift of 13 nm in the fluorescent spectrum and the near doubling of the emission maximum indicated that the Trp residue is in a highly hydrophobic environment. However, under identical conditions the hydrophilic 40 residue cytosolic peptide remained disordered and outside the lipid microenvironment of the vesicle preparation. Although M7-12-T40 and M7-24-T40 had relatively low overall percentages of $\alpha$-helical residues in the presence of the lipid vesicles (Table I), this resulted from a major contribution of the disordered T40 portion of these peptides. The distinct domain behavior of the

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3 M. Girvin, personal communication.
64-residue polypeptide allowed us to calculate difference spectra that reflected the structure of transmembrane regions of Ste2p. The finding that the 24-residue portion of the M7-24-T40 peptide was 94% helical and that it behaved similarly to the M7–24 peptide that we had independently prepared clearly showed that the structure of this transmembrane domain was not influenced notably by the hydrophilic peptide at the carboxyl terminus. The transmembrane portion of M7-24-T40 thus retained the structural preference predicted for this region of the GPCR.

A comparison of results from studies on M7-24-T40 and M7–24 in TFE/water mixtures indicated that a portion of the hydrophilic tail of Ste2p (residues 300–339) increased the water solubility of the hydrophobic core of transmembrane domain seven of this receptor. Indeed in 4% TFE, 96% water M7–24 was poorly soluble and showed a CD pattern indicative of a partially aggregated peptide (Fig. 3D). Moreover after 3 days 90% of the peptide had precipitated from solution. In contrast M7-24-T40 dissolved readily and stayed in solution for at least several weeks. The 64-residue peptide could also be dissolved in the presence of micelles at 0.5 mM concentrations and had the same conformation exhibited at low peptide concentration (Fig. 5D). This demonstrated that preparations of this peptide in the presence of detergent are suitable for high resolution analysis using NMR. Thus, the cytosolic terminus of Ste2p and possibly those of other GPCRs may behave as a hydrophilic template that facilitates the study of contiguous transmembrane domains in membrane-mimetic environments and the integration of such domains into membrane bilayers.

**Fig. 5.** CD spectra plotted as mean residue ellipticity versus wavelength for T40 (●) and M7-24-T40 (▼) in MPG (A), PPG (B), and SPG micelles (C). The concentration of peptides were 50 μM. D, calculated CD spectra for M7–24 at the peptide concentrations of 50 μM (●) and 500 μM (▼) in PPG micelles based on the difference between the CD of M7-24-T40 and T40. E, experimental CD spectra for M7–24 at the peptide concentrations of 50 μM (●) and 500 μM (▼) in PPG micelles. The lipid concentration was 4 mg/ml in all cases.

**Fig. 6.** Fluorescence emission spectra of the tryptophan residue of M7-12-T40 and M7-24-T40 in DMPC/DMPG (4:1) vesicles and in 4% TFE/H2O. The peptide concentrations were 2.5 μM. 1, M7-24-T40 in vesicles. 2, M7-12-T40 in vesicles. 3, M7-24-T40 in 4% TFE/H2O. 4, M7-12-T40 in 4% TFE/H2O.
Because the cytosolic terminus and the seventh transmembrane domain showed distinct and separable conformational tendencies, this approach can be used to study the biophysical tendencies and the inter-helix interactions in the transmembrane domains without concern that the template will disturb the native interactions. Moreover, these studies maybe carried out in the context of a peptide containing only amino acid residues corresponding to the receptor; no non-natural residues are introduced. Once an isotopically labeled version of M7-24-T40 becomes available we hope to determine the detailed structure of the transmembrane core and any conformational elements that are present in the tail. Although similar increases in solubility could be effected by adding lysine residues to the end of the transmembrane domain, no structural information on the cytosolic tail of Ste2p would be forthcoming from such model peptides.

In the case of the α-factor receptor it was critical to choose how much of the carboxyl-terminal tail to attach to the transmembrane domains. In deciding this issue we needed to balance the solubilizing potential of the tail with the contributions it would make to the spectroscopic method used in structure analysis. In this initial study on one transmembrane domain we reasoned that inclusion of the complete 105 residue cytosolic terminus would overwhelm the transmembrane region in both the CD and IR spectrum. Because previous molecular biological investigations showed that the entire cytosolic terminus was not necessary for Ste2p signaling (31), we chose to use the first 40 residues of the cytosolic tail as the hydrophilic template. In future studies with constructs expressing two or three transmembrane domains, larger regions of the cytosolic terminus may be required to obtain the desired solubility. If large regions of the tail are necessary, the difference spectroscopy procedures utilized in this report will be very important to determine the biophysical characteristics of the TMDs. Clearly, as the length of the tail is increased, additional details will be learned concerning the structure of the cytosolic domain of Ste2p. Finally, to carry out high resolution investigations by NMR it will be essential to incorporate isotopically labeled peptides into these peptides and perhaps to prepare hybrid peptides in which part of the molecule is labeled and the tail is unlabeled. Such hybrids will require a combination of biosynthetic and chemical procedures (20–22). Recently we completed the biosynthesis and expression of E–9–M7-24-T40 (nine residues of the third extracellular loop of Ste2p fused to M7-24-T40) as a fusion protein and are currently optimizing the release of this loop-TMD-tail peptide from the carrier protein. A high resolution structure of this peptide in detergent micelles should provide conformational aspects about three discrete regions of a GPCR.

CONCLUSIONS

We have successfully synthesized four peptides with 24, 40, 52, and 64 residues corresponding to the sequence 276–399 of Ste2p. The 40-residue portion of the carboxyl tail solubilized the seventh transmembrane domain in aqueous solution containing 4% TFE. The 24 residues comprising the transmembrane portion of the 64-residue peptide inserted as an α-helix into DMPC/DMPG bilayers, whereas the carboxyl tail of 40 residues remained outside the bilayer in a disordered structure. This communication provides an example of the use of the cytosolic portion of a GPCR as a hydrophilic template useful for studying transmembrane regions of these receptors.

REFERENCES

1. Palczewski, K., Kumasaka, T., Horii, T., Behnke, C. A., Mosot ama, H., Fox, B. A., Le Trong, J., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Science 289, 739–745.
2. Venter, J. C. et al. (2001) Science 291, 1304–1315
3. Lander, E. S. et al. (2001) Nature 409, 860–921
4. Dowell, S. J. (2001) Drug Discov. Today 6, 884–886
5. Albert, A. D., and Yeagle, P. L. (2000) Methods Enzymol. 315, 107–115
6. Deber, C. M., Liu L. P., and Wang C. (1999) J. Pept. Res. 54, 200–205
7. Naider, F., Arshava, B., Ding, F. X., Arevalo, E., and Becker, J. M. (2002) Biopolymers 60, 334–350
8. Dohlman, H. G. (2002) Annu. Rev. Physiol. 64, 129–152
9. Burkholder, A. C., and Hartwell, L. H. (1985) Nucleic Acids Res. 13, 8463–8475
10. Konopka, J. B., Margarit, M., and Dube, P. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6764–6769
11. Hicke, L., and Riezman, H. (1996) Cell 84, 277–287
12. Chen, Q., and Konopka, J. B. (1996) Mol. Cell. Biol. 16, 247–257
13. Xie, H., Ding, F. X., Schreiber, D., Eng, G., Liu, S. F., Arshava, B., Arevalo, E., Becker, J. M., and Naider, F. (2000) Biochemistry 39, 15462–15474
14. Ding, F. X., Xie, H., Arshava, B., Becker, J. M., and Naider, F. (2001) Biochemistry 40, 8945–8954
15. Melynk, R. A., Partridge, A. W., and Deber, C. M. (2001) Biochemistry 40, 11106–11113
16. Zhou, F. X., Merianos, H. J., Brunger, A. T., and Engelma, D. M. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 2350–2355
17. de Planque, M. R., Goormaghtigh, E., Greathouse, D. V., Koeppe, R. E., Il, Krujitzer, J. A., Liskamp, R. M., de Kruif, B., and Killian, J. A. (2001) Biochemistry 40, 5090–5095
18. von Heijne, G. (1992) J. Mol. Biol. 225, 487–494
19. Ding, F. X., Schreiber, D., Ver Berkmoes, N. C., Becker, J. M., and Naider, F. (2002) J. Biol. Chem. 277, 14483–14492
20. Muir, T. W., Sundhi, D., and Cole, P. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 6705–6710
21. Tam, J. P., Xu, J., and Eom, K. D. (2001) Biopolymers 60, 194–205
22. Dawson, P. E., and Kent, S. B. (2000) Annu. Rev. Biochem. 69, 925–960
23. Arshava, B., Liu, S. F., Jiang, H., Bressmav, M., Becker, J. M., and Naider, F. (1998) Biopolymers 46, 343–357
24. Reddy, A. P., Talon, M. A., Becker, J. M., and Naider, F. (1994) Biopolymers 34, 679–689
25. Wu, C. S., Reda, K., and Yang, J. T. (1981) Biochemistry 20, 566–570
26. Chen, Y. H., Yang, J. T., and Chau, K. H. (1974) Biochemistry 13, 3350–3359
27. Marti, T. (1998) J. Biol. Chem. 273, 9312–9322
28. Ridge, K. D., Lee, S. S., and Abdulaev, N. G. (1996) J. Biol. Chem. 271, 7860–7867
29. Martin, N. P., Zemb, L., Sommers, C. M., and Dumont, M. E. (1998) Biochemistry 37, 682–695
30. Therien, A. G., Glibowicka, M., and Deber, C. M. (2002) Protein Expression Purif. 25, 81–86
31. Bencze, E. Z., Blumer, K. J., Courschesn, W. E., and Thorner, J. (1988) Cell 55, 223–234