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Guided Reconstitution of Membrane Protein Fragments

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Abstract

Structural analysis by NMR of G protein-coupled receptors (GPCRs) has proven to be extremely challenging. To reduce the number of peaks in the NMR spectra by segmentally-labeling a GPCR, we have developed a Guided Reconstitution method that includes the use of charged residues and Cys activation to drive heterodimeric disulfide bond formation. Three different cysteine-activating reagents: 5-5'-dithiobis(2-nitrobenzoic acid) [DTNB], 2,2'-dithiobis(5-nitropyridine) [DTNP] and 4,4'-dipyridyl disulfide [4-PDS] were analyzed to determine their efficiency in heterodimer formation at different pHs. Short peptides representing the N-terminal (NT) and C-terminal (CT) regions of the first extracellular loop (EL1) of Ste2p, the *Saccharomyces cerevisiae* alpha-factor mating receptor, were activated using these reagents and the efficiencies of activation and rates of heterodimerization were analyzed. Activation of NT peptides with DNTP and 4-PDS resulted in about 60% yield, but heterodimerization was rapid and nearly quantitative. Double transmembrane domain protein fragments were biosynthesized and used in Guided Reconstitution reactions. A 102-residue fragment, 2TM-tail (Ste2p(G31-I120C)), was heterodimerized with CT-EL1-tail^{DTNP} at pH 4.6 with a yield of ~75%. A 132-residue fragment, 2TMlong-tail [Ste2p(M1-I120C)], was expressed in both unlabeled and ¹⁵N-labeled forms and used, with a peptide comprising the third transmembrane domain, to generate a 180-residue segmentally-labeled 3TM protein that was found to be segmentally labelled using [¹⁵N,¹H]-HSQC analysis. Our data indicate that the Guided Reconstitution method would be applicable to the segmental labeling of a membrane protein with 3 transmembrane domains and may prove useful in the preparation of an intact reconstituted GPCR for use in biophysical analysis and structure determination.

Keywords

GPCR; heterodisulfide formation; membrane protein fragments; Guided Reconstitution; segmental-labeling

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INTRODUCTION

Physiological signals are transduced from the outside of a cell to the cytoplasm through transmembrane receptors. Mutations in these receptor proteins, such as G protein-coupled receptors (GPCRs), can lead to many diseases¹ and many receptors drug targets^{2,3}. Structural insights into these proteins would enable the development of superior and more effective and specific therapeutics. Recent success in crystallization has yielded significant advances in our understanding of GPCR structure and function, with significant implications for drug discovery, as reviewed in ^{4,5,6}. However, understanding the dynamics of GPCR conformational changes upon activation requires the use of additional methodologies like nuclear magnetic resonance (NMR). Unfortunately, analysis of membrane protein structure by NMR has been difficult to perform due to these proteins' amphiphilic nature, which requires study in membrane-like environments, amino acid redundancy in TM domains resulting in spectral overlap, and challenges in the preparation of the isotopically labeled proteins required for high-resolution NMR measurements. In order to overcome some of these difficulties, we and others are attempting to study fragments of receptor proteins⁷⁻¹³.

The use of fragments of receptors for biophysical analysis is subject to uncertainty regarding whether the behavior of fragments of membrane proteins is similar to that of the full-length protein. In order to validate the approach of studying fragments as surrogates for the structure of such domains in the "native state," several groups have tried to reconstitute complete GPCRs from their fragments both *in vivo* and *in vitro*. A number of GPCRs can reassemble from fragments as judged by reconstitution of native signaling activity or spectroscopic properties¹⁴⁻¹⁷. This provided support for the study of isolated fragments. Nevertheless, a segmentally-labeled reconstituted receptor would be useful for the high-resolution NMR structural analysis of large domains of a membrane protein and would provide a test of whether the approach was valid by enabling biochemical assays. In our lab, initial attempts to reconstitute a GPCR from *Saccharomyces cerevisiae* (Ste2p) by mixing two fragments in a micellar environment resulted in mostly monomeric fragments¹⁸. Mixing of fragments during reconstitution can result in fragments adopting an incorrect orientation with respect to each other and in the formation of the undesired homodimeric products. The presence of such side products would be highly detrimental to structural analysis by techniques such as NMR.

Methods for linking membrane protein fragments at a specific point, such as native and expressed chemical ligation¹⁹⁻²¹ could ensure reconstitution in the correct orientation and decrease homodimer formation. Moreover, formation of a covalent linkage should drive the reaction to favor dimerization and allow reconstitution into the presumed thermodynamically stable conformation of the linked fragments. These approaches have been very useful for synthesizing soluble proteins from their parts (see reviews above) and have previously been used to isotopically label segments of these macromolecules²². However, less success has been reported for membrane protein fragments; we have found that with large fragments of membrane proteins the generation of the thioesters and the successful ligation of the resulting fragments are difficult to perform on a scale necessary to obtain the amount of product required to conduct NMR studies²³⁻²⁸.

To overcome some of the above difficulties we are exploring a method that would be applicable to the Guided Reconstitution of GPCRs and other classes of membrane proteins from fragments. Guided Reconstitution, as presented here, drives assembly of intact proteins through the introduction of cysteine residues placed near the C-terminus and N-terminus of two protein fragments (Scheme 1). Although there are some GPCRs that contain native disulfide bonds important for maintaining the structure and function of the receptor, not all GPCRs contain important disulfides²⁹. In cases where the formation of endogenous disulfide bonds is not critical, Guided Reconstitution could be used to help to simplify the determination of the NMR structure by generating segmentally-labeled proteins. Cysteine is well-tolerated as a substitution for all amino acid residues in many membrane proteins and the cysteine SH groups are highly reactive. However, disulfide bond formation may result in generation of homodimers in addition to the desired heterodimers. Preactivation of the Cys residue of one of the fragments would, in principle, reduce the formation of homodimers, by favoring disulfide formation with the free -SH containing fragment^{30,31}. This preactivation approach was previously used to facilitate heterodimerization in integrin $\alpha\text{IIb}\beta 3$ ³². In designing our strategy we noted that the successful synthesis and biosynthesis of membrane protein fragments often requires placement of charged residues, most often a string of lysine residues, at the termini of the transmembrane domains^{33,34}. We incorporated these by positioning negative charges near the C-terminal Cys and positive charges near the N-terminal Cys to decrease homodimer formation and increase heterodimer formation through repulsive and attractive electrostatic forces, respectively³⁵.

In this paper, we report a comparison of heterodimerization of Ste2p-derived peptides by activation with three Cys activators; Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid); DTNB), 2,2'-dithiobis(5-nitropyridine) (DTNP), and 4,4'-dipyridyl disulfide (4-PDS). Although some of these were used previously in peptide dimerization, the yields and conditions for both activation and cystine formation were not systematically evaluated^{30-32,36}. We chemically synthesized short model peptides representing the N-terminal (NT) and C-terminal (CT) portions of the first extracellular loop (EL1) of Ste2p. Each of these contained a Cys substitution (I120C and S121C, respectively). The peptides (see Table 1 for nomenclature and structures) were used to compare the activating agents, examine the relative kinetics of the disulfide exchange reaction at different pH values, and evaluate the contribution of charged tags to heterodimerization. We also generated a peptide that contained the CT sequence extending through TM3 into the second intracellular loop (IL2) to allow us to generate a three-transmembrane "protein" fragment of Ste2p.

In parallel with the chemical synthesis we biosynthetically synthesized two versions of a double transmembrane (TM) domain Ste2p fragment. Both of these contained an N-terminal His₆ tag, the first two TM domains, intracellular loop 1, and two-thirds of the first extracellular loop followed by the Cys residue and the charged tail residues in order to promote electrostatic interactions. The difference between the two proteins is the length of the N-terminal extracellular sequence. Originally we investigated a protein fragment containing an N-terminal truncation with expression beginning at G31. This was designed to simplify the NMR spectrum and to mimic the 2TM protein that we have already used in structural studies in micelles¹¹ and organic:aqueous media¹³. Later, to better replicate the true protein structure, we generated a 2TM Ste2p construct with its N-terminal at the

beginning of the full-length receptor. For both 2TM polypeptides we have been able to isolate multiple milligram quantities per liter of culture. Using conditions optimized for the model peptides as a guide, we formed heterodimers of both 2TM protein fragments with either CT-EL1-tail or TM3. Guided Reconstitution of the activated 2TM fragment of Ste2p with the third transmembrane domain (TM3) peptide yielded a 180-residue protein in segmentally-labeled and unlabeled forms demonstrating that this approach is suitable for reconstitution of membrane proteins.

MATERIALS AND METHODS

Reporter Gene Assay for Ste2p Signalling

Induction of expression of the *FUS1-lacZ* reporter was used to monitor signaling responses of intact receptors and the Ste2p(I120C,S121C) mutant was performed as previously described³⁷. A similar assay was used for the split receptor constructs³⁸. Briefly, the desired constructs were expressed in *Sacharomyces cerevisiae* and grown in the synthetic media lacking either leucine, tryptophan, or uracil to the desired density. The cells were incubated with the desired concentrations of α -factor, then permeabilized and β -galactosidase activity assayed based on fluorescence of a fluorescein-di- β -D-galactopyranoside (FDG) substrate. The amount of β -galactosidase produced was quantitated and recorded. See Supplemental Material (Supplementary Table S1) for a table of constructs and growth conditions.

Growth Arrest Assay

The growth arrest assay was used to measure the biological response of various receptor constructs and was performed as described with slight modifications³⁹. Briefly, cells were grown overnight in SDTrp. The cells were washed, and resuspended to a final density of 1×10^6 cells/ml. Lawns of cells were mixed with soft agar, then poured on SD-Trp plates. Paper discs were spotted with 1.0 μ g, 0.5 μ g, 0.25 μ g, or 0.125 μ g α -factor. The plates were incubated for 48 hours at 30°C, and then the halo diameter measured.

Peptide Synthesis and Purification

The synthesis and purification of all synthetic peptides used herein are given in Supplementary Materials and the RP-HPLC and ESI-MS analysis is shown (Supplementary Figures S1-S3).

Activation of NT-EL1-tail and NT-EL1 Peptides

Optimization of the peptide activation is described in the Supplementary Material (Supplementary Figure S4). DTNB Reagent: The NT-EL1-tail peptide was solubilized in DMSO: ACN(0.1% TFA):water(0.1% TFA) (1:2.5:5) and incubated with 10-fold molar excess of DTNB reagent at room temperature for 1-2 hours. DTNP Reagent: The NT-EL1-tail and NT-EL1 peptides were each solubilized in DMSO: ACN(0.1% TFA):water(0.1% TFA) (1:7:2) and mixed with 2.5 molar excess DTNP solubilized in DMSO:ACN(0.1%TFA) (1:5) and incubated at room temperature overnight. 4-PDS Reagent: The NT-EL1-tail lyophilized peptide was mixed with 2.5 molar excess 4-PDS and both were solubilized together in DMSO:ACN(0.1% TFA):water(0.1% TFA) (1:4.7:0.2) and the reaction was incubated overnight at room temperature. The NT-EL1

peptide was activated in a similar manner with DMSO:ACN(0.1%TFA):water(0.1%TFA) (1:4.8:1.2). The reactions were followed by analytical RP-HPLC and ESI-MS. The activated peptides were all purified on a Waters DeltaPak C-18 column (19x300mm, 5 mL/min) with varying gradients. The NT-EL1-tail activated with DTNB and DTNP was purified using a 25-70% ACN(0.1%TFA) gradient over 70 minutes, the NT-EL1-tail activated with 4-PDS was purified using an 18-70% gradient, and the NT-EL1 peptide activated with both DTNP and 4-PDS was purified using a 10-70% gradient over 70 minutes.

Activation of CT-EL1-tail with DTNP and 4-PDS

The lyophilized CT-EL1-tail peptide was separately mixed with 2.5-fold excess DTNP and 4-PDS and each reaction was solubilized in DMSO:ACN(0.1%TFA):water(0.1%TFA) with (1:1:3) and (1:2:4.5) ratios, respectively. The reactions were followed by RP-HPLC and ESI-MS and conversion to activated peptide occurred within 1 hour. The samples were lyophilized when the reaction was complete and then resuspended in 10% ACN(0.1% TFA) prior to purification by preparative RP-HPLC on a Waters DeltaPak C-18 column using a 25-75% ACN(0.1%TFA) gradient over 80 minutes.

Expression and Purification of Transmembrane Fragments

MH₆-Ste2p(G31-I120C)-GMDDD (2TM-tail) was expressed in Rosetta *E. coli* cells grown at 37°C in Luria-Bertani (LB) broth with 200 µg/ml ampicillin to an OD₆₀₀ of 0.75, pelleted gently, resuspended in M9 minimal media (1 g/L NH₄Cl, 20 mM KH₂PO₄, 48mM Na₂HPO₄, 8.6mM NaCl, 0.4% glucose, 2mM MgSO₄, 0.1mM CaCl₂, and 200 µg/ml ampicillin) and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 22°C overnight. Inclusion bodies were isolated and solubilized as described previously⁴⁰ just prior to injection on the RP-HPLC column. The product was then isolated on a Zorbax-300SB C3 PrepHT column (21x150mm, 5 mL/min) at 60°C with a 40-90% Solvent B gradient over 50 minutes where Solvent A was 80% water, 20% isopropanol, 0.1% TFA and Solvent B was 80% acetonitrile, 20% isopropanol, 0.1% TFA. The peptide purity was analyzed by analytical RP-HPLC on a Zorbax-300SB C3 analytical column at 60°C with a 40-90% Solvent B gradient over 30 minutes. ESI-MS was used to verify the MW.

To generate a longer fragment that includes the N-terminal cytoplasmic domain of Ste2p, MH₆-Ste2p(M1-I120C)-GMDDD [2TMlong-tail] was expressed in T7Express *E. coli* cells (NEB) grown at 37°C in LB broth with 200 µg/ml ampicillin to OD₆₀₀ 0.5, pelleted gently, resuspended in M9 minimal media supplemented with either NH₄Cl or ¹⁵NH₄Cl and 200 µg/ml ampicillin followed by induction with 1 mM IPTG at 30°C overnight. Inclusion bodies were isolated and purified as described.

Heterodimerization Reactions of Small Model Peptides

The dimerization assays were performed in 6 M GnHCl/0.1 M NH₄OAc buffer (6M GnHCl buffer), pH 8.6, 6.2 or 4.6 at RT with 1.5 molar excess CT peptide. The reactions were followed by RP-HPLC on an Eclipse XDB-C8 column with a 10-70% ACN(0.1%TFA) gradient over 25 minutes at RT. The reactants, products, and any side products observed are indicated on the chromatograms and were verified using ESI-MS. The relative conversions were determined by comparing the area of the NT-EL1:CT-EL1 peak at each time point to

the theoretical yield of heterodimer based on the NT-EL1 HPLC peak area at 0 hour. Conversion error estimation for reaction with tail peptides were based on the average of at least 3 repeated experiments whereas the errors in the no tail experiments were based on averaging the heterodimer peak area after this had reached its maximum value for complete conversion.

Removal of the Charged Residues by CNBr Cleavage

The purified NT-EL1-tail:CT-EL1-tail heterodimer was lyophilized and solubilized in 70% TFA then mixed with excess CNBr. The reaction was analyzed by RP-HPLC and the MW of the new product without charged tails was verified by ESI-MS.

Guided Reconstitution of the 2TM-tail Fragment and the CT-EL1-tail-activated Peptide

The 2TM-tail protein fragment was solubilized in TFE:6 M GnHCl buffer, pH 8.6, 6.2 or 4.6 (1:1) then mixed with activated CT-EL1-tail (1:1.5). The reaction was followed over time by RP-HPLC on a Zorbax 300SB C3 column at 60°C with a 40-95% Solvent B gradient over 25 minutes. The MWs were verified by ESI-MS.

Guided Reconstitution of Activated 2TMlong-tail and TM3

The 2TMlong-tail or ¹⁵N-2TMlong-tail protein fragment was solubilized in TFE:water(0.1% TFA) (2:1) and mixed with 5-fold excess DTNP solubilized to 1mg/ml in DMSO by sonication. The final reaction mixture containing 2TMlong-tail and DTNP in TFE:water(0.1% TFA):DMSO in a (1:0.5:0.4) ratio was incubated overnight at room temperature and followed by analytical HPLC on a Zorbax 300SB-C3 column at 60°C with a 40-90% Solvent B gradient over 25 minutes and ESI-MS. The activated peptide was purified on a Zorbax-300SB C3 PrepHT column at 60°C with a 37-90% Solvent B gradient over 50 minutes. Following activation, the 2TMlong-tail-DTNP or ¹⁵N-2TMlong-tail-DTNP and ~2-fold molar excess TM3 were each solubilized in TFE, followed by sonication and the addition of an equal volume of water. After mixing, the reaction was followed by analytical HPLC and ESI-MS. Based on UV absorbance and weight, the final concentrations were ~0.4M 2TM and ~0.9M TM3, respectively. Purification of the final product was performed as described for the activated 2TM peptide fragment.

NMR of Segmentally-labeled ¹⁵N-2TMlong-tail-TM3 Reconstituted Transmembrane Protein

The Guided Reconstitution product was solubilized in 150 µl TFE-d₂, followed by sonication, and 150 µl H₂O(0.1% TFA). The sample was transferred to a Shigemi NMR tube (Shigemi, Allison Park, PA) and heated to 45°C. Nitrogen-proton heteronuclear single quantum correlation ([¹⁵N,¹H]-HSQC) NMR spectra were recorded at 45°C on a three-channel Varian UNITY INOVA 600 MHz NMR spectrophotometer (Varian NMR Instrument, Palo Alto, CA) equipped with a z-axis pulse-field-gradient and a Varian 5-mm [¹⁵N,¹³C,¹H] triple resonance cryoprobe.

RESULTS

Choice of location of disulfide bond formation

In the early stages of this work we designed model peptides comprising the first extracellular loop (EL1) of Ste2p to test the strategy of Guided Reconstitution. Choice of the ligation point (formed by an introduction of a disulfide bond) was based on previous biochemical, mutagenesis and structural data^{41,42}. These data indicate that a helical segment (residues 106-114) in the N-terminal portion of EL1 may be important for biological activity and therefore would not be an optimum location for introduction of a disulfide bond. Furthermore, a previous cysteine-scan of the residues in EL1 indicated that mutating residues I120 and S121, which are C-terminal to the helix, did not affect function of the expressed protein⁴¹. Given these precedents in the literature, it was necessary to determine if Cys substitutions at these residues would 1) yield functional protein when both residues are mutated at the same time and 2) form a reconstituted receptor *in vivo* in yeast cells. To accomplish these goals a mutant Ste2p protein was generated containing the I120C,S121C mutations. In an assay based on transcriptional activation of the *FUS1-lacZ* reporter in response to the agonist, α -factor, the mutant Ste2p elicited a maximal response that was ~65% of the level observed for wild-type protein expressed under similar conditions (Figure 1 Top, Panel A). In addition, this cysteine-containing mutant exhibited only slightly decreased function in an assay of pheromone-dependent growth arrest as measured by halo size. This is an accepted measure of the biological activity of the receptor response to pheromone as only α -factor or closely related analogs will engender growth inhibition in a cell with a functioning receptor (Figure 1 Bottom). When two fragments comprising the first 2TM ending at I120C and the 5TM fragment starting with S121C were expressed simultaneously in yeast from two different plasmids they were able to reconstitute functional receptor at the yeast plasma membrane that exhibited ~45% of the maximal response of normal full-length receptors to α -factor, based on assays of induction of the *FUS1-lacZ* reporter (Figure 1 Top, Panel B). Coexpression of similar split receptors containing charged tails (see below) yielded only 50% of the maximal level of *FUS1-lacZ* induction exhibited by constructs without the charged tails (Figure 1 Top, Panel B). Since this indicates that the presence of the charged residues at the external surface of the receptors could hinder α -factor binding or otherwise affect receptor function, we added a Met residue to each fragment to allow cleavage of the charged residues using cyanogen bromide (see below).

Taking the results from the biological assays into account, two model peptides were designed to include the N-terminal EL1 residues with an I120C mutation (NT-EL1-tail; 27 residues) and the C-terminal EL1 residues with an S121C mutation (CT-EL1-tail; 17 residues) (Figure 2). To favor heterodimer formation, the N-terminal fragment contained negatively charged Asp residues and the C-terminal fragment contained positively charged Lys residues connected by methionine-containing linkers that would facilitate removal of the charged residues. Similar peptides were synthesized that did not contain the charged tails as controls [NT-EL1 (21 residues) and CT-EL1 (12 residues), respectively] (Figure 2). We also chemically synthesized a transmembrane (TM) containing peptide with the same N-terminal sequence as CT-EL1-tail and extending through the third TM and into the second intracellular loop [TM3 (48 residues)](Figure 2).

Heterodimer formation using the EL1 model peptides

Heterodimer formation was examined in 6M GnHCl buffer at three different pH conditions (8.6, 6.2 and 4.6). This strong denaturing solvent was chosen to simulate conditions we thought would be necessary for the dimerization of larger GPCR fragments which are insoluble in most organic aqueous solvent mixtures (*vide infra*). A benefit of using two of the activating reagents, DTNP and 4-PDS, is that due to the pKa of the SH in the pyridinyl moiety these reagents have been shown to facilitate formation of inter- and intra-molecular disulfide bonds under acidic conditions where the free unactivated Cys residues should not oxidize^{30,31}. The activated NT-EL1-tail peptides were reacted with a 1.5:1 molar excess of unactivated CT-EL1-tail (Figure 3A). At pH 8.6 the NT-EL1-tail^{4-PDS} rapidly reacted and by 3 hours had been completely consumed (Figure 3A). Under these conditions, even at short incubation times a significant fraction of the activated NT-EL1-tail peptide was converted to heterodimer as judged by HPLC. We also observed small amounts of both homodimers, NT-EL1-tail:NT-EL1-tail and CT-EL1-tail:CT-EL1-tail, as judged by ESI-MS of the HPLC peaks. Similar side products were found in all heterodimerizations with the relative amounts varying with the activator and the pH. The identities of each of these side products was determined by ESI-MS (see Supplementary Material Table S2).

We also performed dimerization experiments with NT-EL1 and CT-EL1 peptides that did not contain the charged tail regions. When these peptides were activated with DTNP and 4-PDS and then used in the heterodimerization reactions at pH 8.6, the reactions proceeded to completion within 1.5 hours. Conversion to the heterodimer product began immediately upon mixing especially in the presence of NT-EL1^{4-PDS} (42% formation at the earliest HPLC time point at pH 8.6 [Figure 4D, right panel]). The reactions were slightly slower at lower pHs for both activated peptides, but the maximum yield in all cases was reached by 3 hours. Although the heterodimer formation appeared to be more efficient for peptides without the charged tails, we continued our work with the tail-containing peptides because the presence of the charged residues is likely to be important for expression and solubility of larger reconstituted receptors (see Discussion).

In time course analyses conducted for up to 72 hours, the NT-EL1 and CT-EL1 unactivated peptides lacking charged tails formed very little heterodimer at any pH, and after 24 hours less than 2% heterodimerization had occurred (Figure 4A). The presence of the charged residues on NT-EL1-tail and CT-EL1-tail increased the heterodimer formation to ~20% at pH 8.6 and less than 10% at the lower pHs. The reaction of the DTNB activated NT-EL1-tail was slow at all pH values that we examined with a maximum conversion of ~40% at 72 hours at the lower pHs (Figure 4B, and data not shown). Moreover, as seen in the traces, yields of the heterodimer in this reaction were maximal at about 3 hours at pH 8.6 and then slowly decreased. Because of this the reactions using the NT-EL1-tail peptide that was activated with DTNB were not very reproducible at pH 8.6 and a variety of side products were observed which increased with reaction time. At pH 8.6 the heterodimer was not very stable as disulfide exchange reactions readily occurred and the release of the free activator upon heterodimerization led to a complex product mixture. Based on these results we did not analyze the DTNB activator for the peptides without the charged tails. Both the DTNP and 4-PDS activated NT-EL1-tail rapidly formed heterodimer at pH 8.6 peaking at 84% and

98% heterodimer, respectively, at about 3 hrs (Figure 4C,D). Subsequent to this time the yields either decreased (pH 8.6) or stayed about the same or slightly increased (DTNP pH 4.6 and pH 6.2).

CNBr removal of charged tags

The NT-CT heterodimer was purified, lyophilized, and solubilized in 70% TFA in order to remove the charged tails by CNBr cleavage (Figure 3B). The cleavage reaction went to completion after 1 hour and we were able to purify EL1 heterodimer that no longer contained the charged tail and verify this compound by ESI-MS (Experimental MW: 3889.60 Da, Expected MW: 3887.37 Da; Supplementary Table S2).

Heterodimer formation with a transmembrane containing fragment of Ste2p

The CT-EL1-tail peptide was activated with DTNP and 4-PDS as described above. The activation reaction for both small molecules was complete within 1 hour and resulted in 100% conversion to the activated CT-EL1-tail peptide. Activation of CT-EL1-tail was significantly faster and more complete than that of the corresponding NT-EL1-tail peptide. This could be due to a difference in the environment surrounding the SH nucleophilic center. The CT-EL1-tail peptide contains positively charged residues close to the introduced Cys residue in contrast to the environment of the cysteine in the NT-EL1-tail, which contains negative charges. The positively charged environment should favor formation of the thiolate anion, a strong nucleophile, whereas the negative carboxylates should suppress thiolate formation. Furthermore, the highly aqueous activation conditions that were required for the CT-EL1-tail peptide should favor thiolate formation and increase nucleophilicity.

A fragment containing the first two TM domains of Ste2p, G31-I120C, with an N-terminal His₆-tag, a Cys residue near the C-terminus, and a negatively charged tail containing Asp residues (2TM-tail) was expressed in *E. coli* and purified as described in the Materials and Methods section. The expression of this protein fragment was optimized in numerous *E. coli* strains and at different temperatures (Supplemental Figure S6). The C-terminal 27 residues of this fragment are similar to the NT-EL1-tail peptide that was activated in the above analysis (see Table 1). The final yields after expression and purification of this protein fragment were 2-4 mg per liter of culture in M9 minimal media (Figure 5A). The 2TM-tail protein fragment was solubilized in TFE:6M GnHCl buffer (1:1) and then the solution was mixed with a 1.5-fold molar excess of activated CT-EL1-tail peptide. The reaction was followed over 24 hours by RP-HPLC and ESI-MS at the three different pHs described above (Figure 5B-C and Supplementary Figure S8). The best yield (74%) of the heterodimer 2TM-tail:CT-EL1-tail 119-residue protein fragment was observed with the DTNP activated CT-EL1-tail peptide at pH 4.6 after 24 hours (Figure 5B and C). Calculation of yield was carried out by analyzing the area of each peak in absorbance (at 220 nm) of the traces in Figure 5C. The absorbance at this wavelength is correlated with the number of peptide bonds present in the polypeptide. Since the small activated CT-EL1-tail peptide is very hydrophilic compared to the membrane peptides, it elutes near the solvent peak and could not be quantitated. The 2TM-tail:2TM-tail homodimer contains 204-residues whereas the 2TM-tail:CT-EL1-tail heterodimer contains 119-residues. Accounting for the difference in the number of peptide bonds in each product the estimated yield of heterodimer was obtained. For 4-PDS

activation the best yield was 60% at pH 6.2. Based on these results, heterodimerization with larger protein fragments was performed using the DTNP activator. In these studies the MWs for all synthetic peptides and products of their heterodimerization were within 0.01% of the calculated values, which is the precision of the method.

Guided Reconstitution between 2TMlong-tail and TM3

In order to determine whether Guided Reconstitution was amenable to the creation of proteins containing larger numbers of transmembrane domains, we examined whether a 3TM polypeptide could be prepared from a two TM and a one TM containing fragment of Ste2p. A double transmembrane domain fragment containing the entire N-terminus with a Cys residue at position 120 [2TMlong-tail] was cloned. A schematic comparison of the 2TM protein fragments is presented in Figure 2D. Large-scale growths of *E. coli* cells expressing 2TMlong-tail or ¹⁵N-2TMlong-tail in minimal media yielded approximately 20 mg of purified protein fragment per liter of culture (Figure 6 and Supplemental Figure S7). This is a 10-fold increase over expression of the N-terminally truncated 2TM protein fragment. Activation of this protein fragment with DTNP in TFE:water(0.1%TFA):DMSO (Materials and Methods) resulted in 85-95% conversion (Figure 6 and Supplementary Table S3). The activated protein fragment was purified by preparative HPLC and used for Guided Reconstitution reactions (see below). The TM3 transmembrane peptide was generated by chemical synthesis (Supplemental Figure S3). This peptide was created with the charged tail at its N-terminus because the 5TM portion of the Ste2p receptor cannot be expressed without these charged residues (data not shown). Due to this observation we decided to optimize the Guided Reconstitution system for formation of a 3TM containing domain of Ste2p using constructs with the charged tails.

When Guided Reconstitution of the 3TM polypeptide was performed in GnHCl buffer, pH4.6, the samples precipitated over time (Figure 7A). In order to increase the yield of the Guided Reconstitution product, solubility tests were performed and it was observed that both TM fragments and the TM3 polypeptide were soluble in the membrane mimetic solvent TFE:water. Previous structural analysis from our lab with the 2TM protein fragment indicates that the helical hairpin formed in TFE:water is similar to, though less stable than, that formed in 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phospho-(1'-*rac*glycerol) micelles ^{11,13}. A small scale reaction was performed and monitored by analytical HPLC. Reconstitution of a three transmembrane domain protein fragment was observed and verified by ESIMS (Supplementary Table S3). In order to generate segmentally labeled protein for use in NMR analysis, a large-scale Guided Reconstitution reaction was performed. Five mg of ¹⁵N-2TMlong-tail was activated with DTNP, purified and then reacted with the unlabeled TM3 peptide in ~1-fold excess (Figure 7B-C). By UV absorption analysis, the weight of the final segmentally-labeled protein, 180-residues, was approximately 1 mg. This was about 15% of the theoretical yield. However, in manipulating membrane proteins it is not uncommon to encounter losses at various stages in the process. For example, the 5 mg of ¹⁵N-2TMlong-tail that was activated had 94% conversion and during the purification on the preparative column we expect ~40% loss due to the hydrophobic nature of the protein fragment. We have observed this loss in control purification experiments (data not shown). Therefore, ~2.8 mg of the 2TM protein fragment was used in the Guided Reconstitution

reaction with 3 mg of TM3. The conversion to heterodimer was 80% (3.8 mg) and the purification was then expected to have a 40% loss (3.1 mg). After all of the processing, the reaction resulted in ~55% of the expected yield (1.8 mg). The experimental ESI-MS determined mass for the peptides containing the 2TMlong-tail were from 0.05% to 0.2% higher than the calculated values. The latter number, found for the 29.3 KDa homodimer is clearly outside the precision of ESI-MS. The additional weight may be a consequence of bound small molecules such as water or due to small amounts of oxidation that occur during the harsh HPLC purification conditions (i.e. high temperature, 20% isopropanol). Despite these small differences the masses determined verify the identity of the products formed.

After lyophilization, the segmentally-labeled protein was solubilized in TFE-d₂:H₂O(0.1% TFA) and a [¹⁵N, ¹H]-HSQC spectrum was obtained to characterize the final product. The two-dimensional NMR of the heterodimeric 3TM product in which the N-terminal region and the first two TMs are labeled resulted in the expected number of peaks (130) (Figure 8). Further work will need to be done to assign all of the peaks in the segmentally-labeled spectra and to determine whether the chemical shifts for the first two TM domains are similar to those previously published ¹³.

CONCLUSIONS

In this paper we have shown that we can form a disulfide bond between an activated 2TM GPCR protein fragment and a peptide containing the third TM domain of Ste2p to generate a 180-residue protein fragment using a Guided Reconstitution reaction. The product resulting from heterodimerization of the fragments was the predominant (80%) product in the reaction as judged by HPLC. The Guided Reconstitution strategy included the use of oppositely charged residues on the 2TMlong-tail and TM3 containing protein fragments as well as the use of a low molecular weight activating reagent. Success in producing a new membrane “protein” with three transmembrane domains was aided by optimization of disulfide formation using model peptides.

Comparison of the three different activating reagents, DTNB, DTNP and 4-PDS, indicates that although the activation with DTNB is the most efficient (100% conversion), the dimerization reaction with this activated peptide was not very reproducible and the reagent is highly promiscuous, leading to both a complex dimerization mixture and a lack of stability of the heterodimer with time. Although the DTNP and 4-PDS activation reactions resulted in lower yields of activated NT-EL1-tail peptide (~60%), use of peptides activated with these reagents resulted predominantly in the desired heterodimers, providing suppression of homodimer formation at every pH tested. For the Ste2p EL1 loop peptide model system, both with and without the tails, coupling of fragments at pH 8.6 yielded heterodimer at a faster rate than at either pH 6.2 or 4.6. The peptides that contain the charged residues efficiently convert to heterodimeric product with both DTNP and 4-PDS. The highest yield (98%) was achieved with 4-PDS after 3 hours at pH 8.6 (Figure 4). Unexpectedly, the peptides without the charged tails converted to heterodimeric product even faster, so that the reaction was completed by 1.5 hours with both the DTNP and 4-PDS. While the DTNP activated reaction or peptides without charged tails was stable over a 24 hour reaction period, the heterodimeric product in the 4-PDS activated reaction eventually

converted to NT-EL1 monomer and NT-EL1:NT-EL1 homodimer, exhibiting up to 20% loss of the desired product overnight. When no activator was present, the presence of the charged residues increased the yield of heterodimer 10-30 fold when compared to the same peptides with no charged residues. The differences in the rate of heterodimer formation between similar peptides with and without the charged residues, may be due to differences in the electrostatic environment of the nucleophilic –SH centers in the different peptides, as also observed during the activation of these peptides. However, as described below, the charged residues can be important in the heterologous expression of the large receptor fragments.

The ultimate goal of our research is to generate a segmentally-labeled GPCR in which several of the TM domains are isotopically labeled for structure determination by NMR while the remaining unlabelled TMs remain invisible to NMR. Our first application of this approach to Ste2p will be the reconstitution of this receptor from a 2TM N-terminal fragment and a 5TM C-terminal fragment. To demonstrate the feasibility of this approach we expressed and purified two different double transmembrane containing Ste2p fragments (2TM-tail and 2TMlong-tail), that will be used in the reconstitution of the full receptor, and tested their ability to be used in the Guided Reconstitution system. Based on the optimum conditions found for the EL1 loop peptides, we activated CT-EL1-tail with both DTNP and 4-PDS and used these activated loop peptides to convert the 2TM-tail protein fragment to 2TM-tail-CTEL1-tail with a yield as high as 74% at pH 4.6. Notably the activation of the CT-EL1-tail peptide proceeded to a much greater extent than that of the NT-EL1-tail peptide under identical conditions. However, activation of the N-terminal domain is preferred in efforts to reconstitute longer fragments of Ste2p because the C-terminal 5TM fragment is difficult to purify. The best heterodimerization of the 2TMtail peptide with activated CT-EL1-tail was observed with DTNP at pH 4.6 whereas the shorter peptides exhibited little pH dependence with this activator. Previously model peptides were reported to be activated by DTNP in acetic acid:water (3:1) in 4-6 hours in excellent (90-95%) yields³⁰. We find that the actual yields of activated peptides are peptide-, activator- and pH-dependent. All of our reactions were run in the presence of ambient oxygen because the scales of the reactions and the method of sampling make it cumbersome to maintain an inert atmosphere (i.e. with argon). Thus, the effects of possible oxidation should be considered when strategizing these reactions.

The addition of the first 30-residues in the expression of the 2TM fragment increased the yield by ~10-fold. This protein was expressed in both minimal media and minimal media supplemented with ¹⁵NH₄Cl for downstream NMR analysis of a segmentally-labeled protein fragment. Activation of this peptide with DTNP resulted in >90% conversion. The activated 132-residue intermediate was purified and subjected to Guided Reconstitution with the chemically synthesized TM3 peptide, resulting in a segmentally-labeled 180-residue 3-transmembrane domain protein. [¹⁵N, ¹H]-HSQC analysis indicated that this product was segmentally-labeled and that the expected 130 crosspeaks were well-resolved. Further analysis, including chemical shift assignments and NOEs, is necessary to determine the structure of the final product.

To this point we have been working in denaturing conditions but many groups have succeeded in refolding membrane proteins into micelles and liposomes, including the recent refolding of a GPCR, CXCR1, into a functionally active form⁴³⁻⁴⁶. The Arseniev group has shown that transmembrane proteins expressed and/or purified in a denatured state can be refolded into functional proteins using lipid-protein nanodiscs⁴⁷. We plan to perform the Guided Reconstitution procedure with complementary fragments of Ste2p including the activated ¹⁵N-2Tmlong-tail and a 5TM construct that represents the remaining portion of the protein. The final segmentally-labeled receptor will be placed into non-denaturing conditions in which the protein will be folded, using α -factor binding assays will be used to determine functionality of the product. If necessary we will employ an affinity purification step using α -factor immobilized through the ϵ -amine of Lys⁷ to isolate a homogeneous population of reconstituted, functional Ste2p.

Guided Reconstitution would be an addition to the toolbox for membrane protein structure determination. There has been an increase in the number of membrane protein structures but this remains an understudied area. In the human proteome there are more than 6500 membrane proteins of which approximately 700 consist of 3TM or 4TM domains⁴⁸. The structures of most of these are not known. The generation of a segmentally-labeled 3TM polypeptide using the Guided Reconstitution method described herein should facilitate NMR studies on such proteins and may be applicable to larger proteins.

The isolation of a 180-residue membrane peptide reconstitution product on a mg scale in relatively high yield validates the heterodisulfide approach for the preparation of a segmentally-labelled membrane protein and is encouraging for future efforts to reconstitute the GPCR Ste2p from two fragments containing two and five transmembrane segments, respectively. Furthermore, the possibility of reconstituting a receptor that can bind and respond agonist is supported by our demonstration of signaling responses by yeast cells expressing receptor fragments containing the relevant cysteine residues (I120C and S121C) and a discontinuity at the site of the split between the fragments used for Guided Reconstitution. Although we find that the charged tails appear to slow the rate of heterodimer formation, the presence of charges promotes solubility of fragments. The 5TM C-terminal fragment could not be expressed in bacterial culture without the positively charged lysine residues at the N-terminus (Fracchiolla and Naider, unpublished data). We have established a procedure for efficiently removing the added charged residues prior to structural analysis, after formation of the disulfide bond linking the polypeptide fragments into a reconstituted receptor yielding what we expect to be a biologically relevant surrogate for the intact GPCR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

NMR	nuclear magnetic resonance
GPCR	G protein-coupled receptors
DTNB	5-5'-dithiobis(2-nitrobenzoic acid)
DTNP	2,2'-dithiobis(5-nitropyridine)
4-PDS	4,4'-dipyridyl disulfide
NT	N-terminal
CT	C-terminal
EL1	extracellular loop
TM	transmembrane
Fmoc	FastMoc
TFA	trifluoroacetic acid
DMSO	dimethyl sulfoxide
ACN	acetonitrile
RP-HPLC	reverse-phase-high performance liquid chromatography
ESI-MS	electrospray ionization-mass spectroscopy
E. coli	Escherichia coli
MW	molecular weight
GnHCl	guanidinium hydrochloride
NH₄OAc	ammonium acetate
RT	room temperature

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Stage 1: Activation

Stage 2: Dimerization

Scheme 1.

Stage 1: Activation with small molecule Cys activators to increase the rate and selectivity of heterodimerization. Stage 2: Heterodimerization is performed with the activated 2TM protein fragment and the 3TM protein fragment to generate a larger, potentially segmentally-labeled protein fragment. Pink circles: His₆ affinity tags; Blue circles: 2TM protein fragment; Light Green circles: Cys residue; Dark Green circles: tail linker; Red circles: negative charges; Aqua circles: positive charges; Purple circles: TM3 protein fragment.

Figure 1.

Biological activity for Ste2p with the Cys mutations in the full-length and split receptors. Top) Signaling activity was observed by following the induction of *fus1-lacZ* - by 1 μ M α -factor as indicated by β -galactosidase (*lacZ*) activity. Panel A represents the full length receptor and Panel B represents the control and constructs from the split receptor assay. Bottom) Growth arrest as a function α -factor of concentration was observed for the full-length receptors.

Figure 2.

Schematics of the peptides used in the Guided Reconstitution Assays. For all of the peptides, the amino acid sequence is presented in the box. The underlined portion represents Ste2p receptor sequence. The italicized portions are the charged tails. The peptides without tails are generated without these sequences. A) NT-EL1 peptides with and without the charged tails. B) CT-EL1 peptides with and without the charged tails. C) TM3 peptide. D) Biosynthetically generated 2TM peptides.

Figure 3.

Heterodimerization of NT-EL1-tail⁴-PDS and CT-EL1-tail peptides at pH 8.6. A) Analytical HPLC chromatograms of the dimerization reaction at 0 and 3 hours after mixing. The yellow star indicates the Cys-activating moiety. B) Removal of charged residues from the purified NT-EL1-tail:CT-EL1-tail heterodimer by CNBr cleavage was performed in 70% TFA. The MWs for the products were verified by ESI-MS (see Supplementary Material Table S1).

Figure 4.

Time course of the formation of the NT-EL1-CT-EL1 heterodimer at different pH values. The reactions were performed between the NT-EL1-tail and CT-EL1-tail peptides (closed points) and between the NT-EL1 and CT-EL1 peptides (open points) with A) no activator, B) DTNB (tail peptides only), C) DTNP, and D) 4-PDS.

Figure 5.

Expression, Purification and Heterodimerization of MH₆-Ste2p(G31-I120C)-GMDDD (2TM-tail). A) Inclusion bodies were isolated and purified on a Zorbax-300SB C3 column at 60°C as described in the Methods. Inset: SDS-PAGE analysis of directly expressed 2TM-tail as described. B) Heterodimerization of 2TM-tail and DTNP-activated CT-EL1. The reaction was followed over time and the 0 h and C) 24 hour time points are shown.

Figure 6.

Expression, Purification and Activation of MH₆-Ste2p(M1-I120C)-GMDDD (2TMlong-tail). Top panel: analytical HPLC of the peptide prior to addition of the DTNP. Inset: SDS-PAGE analysis of directly expressed 2TMlong-tail as described. Bottom panel: analytical HPLC of the peptide after overnight in cubation with DTNP as described in Methods. The yellow star indicates the Cys-activating moiety.

Figure 7.

Guided Reconstitution of 2Tmlong-tail. The Guided Reconstitution reaction was performed with 2Tmlong-tail^{DTNP} and TM3 peptide in A) TFE:GnHCl buffer, pH 4.6 (1:1) and B) TFE:water (1:1). The yellow star indicates the Cys-activating moiety. The reaction precipitated when performed in the presence of GnHCl, whereas heterodimer formation was observed in TFE:water. C) Analytical HPLC of the purified segmentally-labeled ¹⁵N-2Tmlong-tail:TM3 180-residue protein fragment.

Figure 8. $[\text{}^{15}\text{N}, \text{}^1\text{H}]$ -HSQC analysis of segmentally-labeled ^{15}N -2Tmlong-tail:TM3. Left panel: ^{15}N -2Tmlong-tail:TM3 was solubilized in TFE- d_2 : H_2O (0.1% TFA) and analyzed by $[\text{}^{15}\text{N}, \text{}^1\text{H}]$ -HSQC at 45°C . Right panel: Schematic of segmentally labeled 3TM protein. The residues of the 2Tmlong-tail are observed in the HSQC.