

Membrane Active Peptides

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De novo design, synthesis and characterization of membrane-active peptides

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Abstract

Our current level of understanding of membrane-protein folding is primitive, but it is beginning to advance. Previously [Choma, Gratkowski, Lear and DeGrado (2000) *Nat. Struct. Biol.* **7**, 161–166], we described studies of the association in detergent micelles of short, simple-sequence hydrophobic peptides modified from the sequence of the water-soluble, homodimeric coiled-coil GCN4-P1 peptide using the principle that the interiors of membrane proteins are similar to those of water-soluble proteins. Here, we discuss more quantitative aspects of the association equilibrium and compare the free energies of association of a number of mutant peptides designed to explore specific features responsible for the association.

Introduction

A reasonable model for membrane-protein folding [1] posits that folding occurs in two kinetically separate steps. The first involves insertion of the helical transmembrane regions of the protein into the bilayer, while the second involves the formation of specific interactions between these helices to form a tightly packed native structure. The second step can occur in an intermolecular process, as in the folding/assembly of multimeric ion-channel proteins, or in an intramolecular process, as in the folding of monomolecular proteins. The features required for the insertion of peptides into

bilayers have been quantified through various model systems (reviewed in [2]). However, what drives the subsequent association of inserted helices is much less understood [3]. With water-soluble proteins, thermodynamic analysis of the reversible folding of proteins of known crystal structures has produced valuable insights into the relative importance of different inter-atomic forces governing structure formation (see, e.g. [4,5]). However, although an increasing number of high-resolution structures of membrane proteins are becoming available, folding studies on such systems are made difficult by the need to find reversible denaturing conditions effective on both the membrane-embedded and water-exposed portions of the proteins. Although some progress has been made in this area (e.g. [6,7]), much remains to be done.

Recently [8–10], studies of a small peptide from the transmembrane region of the homodimeric membrane protein glycoporphin [11] have shown exceptional promise in providing quantitative thermodynamic data that can be related to the experimentally determined structure. Statistical studies of polytopic transmembrane protein sequences [12] show a high occurrence of sequence patterns similar to the GVXXGV pattern shown (e.g. [13]) to be critical for glycoporphin dimerization. However, this is not the only motif governing transmembrane helix association (see, e.g. [14–17]). Consequently, we have started to study the folding of simple peptides as models for membrane proteins. These differ substantially from glycoporphin in that they do not rely on sequence features found in any particular naturally occurring membrane protein. Rather, we design them using a principle postulated from an earlier study [18], which suggested that the composition of the interiors of membrane proteins are similar to those of water-soluble proteins.

Key words: analytical ultracentrifugation, folding, helix association, membrane protein.

Abbreviations used: FRET; fluorescence resonance energy transfer; EAUC, equilibrium analytical ultracentrifugation; NBD, 7-nitrobenz-2-oxa-1,3-diazole; C-14 betaine, *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulphonate; MF, mole fraction.

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Previous results and methodology

In our first experimental test of this approach [19], we designed a membrane peptide based on the structure [20] of the water-soluble two-stranded coiled-coil from GCN4 (GCN4-P1). This dimeric protein is stabilized in aqueous solution by a series of hydrophobic interactions at the dimer interface. The only polar interaction between buried side chains involves a hydrogen bond between the carboxamide groups of two Asn side chains on neighbouring helices. Mutational studies indicate that although this interaction is actually destabilizing relative to a hydrophobic interaction, it specifies a dimeric state by producing even greater destabilization of higher aggregation states [5,21]. We converted GCN4-P1 and a number of variants to water-insoluble peptides by changing the exterior, polar side chains to a random selection of aliphatic, apolar side chains, while maintaining the buried side chains (here, B represents the unnatural amino acid β -alanine): GCN4-P1, MKQLEDKVEELLSKNYHLENEVARLKKLVGER; MS1, BQLIAAVLLLIHAVNLILLIHAVARLRYLVG; MS1-N14V, BQLIAAVLLLIHAVLILLIHAVARLRYLVG. The resulting peptides, MS1 and MS1-N14V, were indeed soluble to less than 1 μ M in aqueous buffer, but were found to dissolve readily in solutions of both anionic and neutral detergents at peptide/detergent ratios at least as high as 1:100 (typically we use detergent concentrations of at least 10 times the critical micelle concentration).

The ability of MS1 and MS1-N14V to associate in micelles was initially assessed by SDS/PAGE, as done concurrently and independently on similar peptides expressed as staphylococcal nuclease fusion proteins [22]. MS1 was found to migrate as a single band with a mobility similar to that expected for a trimer, and MS1-N14V migrated as a monomer (using fragments of membrane proteins as calibrants). This at once established the potential importance of polar interactions in driving membrane-protein association. Also, considering that the water-soluble GCN4-P1 peptide is a dimer and the corresponding N-to-V mutant is more strongly and less specifically associated in aqueous solution, there appear to be significant differences between forces driving helix association in water and membrane environments.

A more quantitative assessment of the association was done using fluorescence resonance energy transfer (FRET) and equilibrium analyti-

cal ultracentrifugation (EAUC) with various detergents. For FRET, we employed 7-nitrobenzo-2-oxa-1,3-diazole (NBD)-modified peptides (at the N-terminus) as fluorescence donors and tetramethylrhodamine-modified peptides as acceptors. The NBD-chromophore was also useful for high-sensitivity measurement of peptide concentration gradients in the centrifugation experiments. Regarding detergent choice, we found that the high-purity, homogeneous, oxidation-resistant detergent C-14 betaine (*N*-tetradecyl-*N,N*-dimethyl-3-ammonio-1-propanesulphonate; Sigma) was well suited for quantitative determination of equilibrium constants in both FRET and EAUC studies. Using both methods, we have determined that the MS1 peptide exhibits a rapidly reversible, co-operative monomer-trimer equilibrium such that about 50% of the peptide is trimeric at a peptide/detergent mole fraction (MF) of about 0.003.

Thermodynamic studies of association equilibria require that equilibrium constants be defined in terms of solution concentration units that faithfully reflect the entropic component of the chemical potentials. Because the water-insoluble transmembrane peptide is confined to the detergent phase, the thermodynamically significant concentration should be the peptide/detergent ratio. Indeed, in a recent FRET study of the glycoporphin peptide [8], dimerization equilibrium constants (determined by FRET, and expressed in aqueous concentration units) were found to change with detergent concentration in a manner approximately consistent with that expected for an equilibrium confined to the detergent phase.

In our own studies of the MS1 peptide [19], we found this also to be the case. Figure 1 shows this explicitly; calculating the equilibrium constant in units of peptide concentration gave a systematic increase with concentration (Figure 1A) but using peptide/detergent MF units provided constant values (Figure 1B). We also noted that, in the FRET experiment, equilibration of donor and acceptor fluorophores occurred within seconds (too fast to conveniently measure), unlike the much slower equilibrium noted for glycoporphin, especially in zwitterionic detergents [8].

Having a reversible, measurable equilibrium system invites studies of amino acid substitution effects on the equilibrium. Of primary interest is the effect on trimer stability of replacing the asparagine with other residues that might be accommodated in the core of a trimeric structure. We very recently investigated (by SDS/PAGE

and EUAC) the effect of replacing Asn-14 with the aliphatic residues Leu, Val and Ala, the small polar Ser and Thr, and the charged Lys, Glu, Asp and Gln. Our results [23] showed that only variants with carboxamide- or carboxylic acid-containing side chains at position 14 (Asp, Asn, Glu, Gln) showed significant association. Variants with Val and Leu in place of Asn-14 failed to strongly associate in the membrane, even though

molecular modelling suggests that these variants could form densely packed trimers. Variants with the smaller side-chain residues (Ala, Ser and Thr) also showed little or no association. Although small side chains, particularly Gly as in the glycoporphin dimer [9,10], have been shown to mediate transmembrane helix association, these small residues appear to require particular patterns of flanking residues to be effective [12].

Current studies

In the nomenclature of coiled-coils, position 14 in MS1 is an 'a' position, whereas 17 is a 'd'; both critical positions forming the interface between helices. In an analysis of three-stranded coiled-coils [24], it was noted that asparagines occur in d positions in the crystal structures of at least two known trimeric coiled-coil proteins [25,26], so it was of interest to determine to what extent this change might stabilize the trimer. Therefore, we investigated the effect of placing the asparagine at position 17 instead of 14 (shown in bold): MS1, BQLIAAVLLLI~~AV~~NLILLI~~AV~~ARLR~~Y~~LVG; MS1-N14V/L17N, BQLIAANLLLI~~AV~~VLINLI~~AV~~ARLR~~Y~~LVG.

We found, by both EUAC and FRET (Figure 2), that although this variant did trimerize (in C-14 betaine micelles), the equilibrium was shifted towards monomer by about 1.7 kJ (0.4 kcal)/mol per monomer. As with the N14V variant, this emphasizes that studies of water-soluble peptides are unreliable for predicting the detailed association behaviour of membrane-soluble ones.

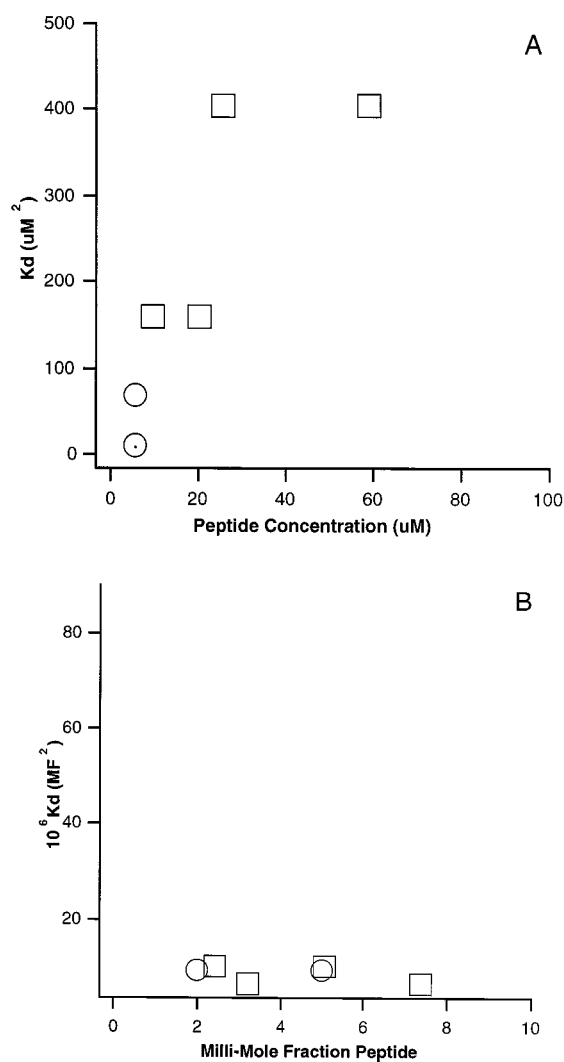
In a typical coiled-coil protein structure, helix-helix interfaces show certain residues from each helix packing into cavities formed by the others. Replacement of one of these residues by a smaller one should, therefore, either create a cavity or induce a structure change. In either case, one might expect the packed structure to be destabilized. To explore this, we synthesized the MS1 variant L10A: MS1, BQLIAAVLLLI~~AV~~NLILLI~~AV~~ARLR~~Y~~LVG; MS1-L10A, BQLIAANLLAI~~AV~~NLINLI~~AV~~ARLR~~Y~~LVG.

For the L10A peptide, we found, again by both EUAC and FRET in C-14 betaine micelles (Figure 3), that this variant formed significantly tighter trimers [$\Delta\Delta G = -2.1$ kJ (-0.5 kcal)/mol per monomer] than MS1. This result shows that the attempt to introduce a cavity, rather than causing a loss of trimer stability, actually produces a gain. To interpret this result, detailed structural information is needed, particularly since the effect

Figure 1

Calculated values of MS1 monomer-trimer dissociation constant (K_d) measured by FRET (○) and by EUAC (□) at C-14 betaine detergent concentrations of 1.1 and 2.75 mM in FRET, and 4 and 8 mM in EUAC experiments

(A) K_d was computed using concentrations expressed in units of mol/l of aqueous volume. (B) K_d values were calculated in units of mol of peptide/mol of detergent. The y axes of both panels have approximately the same proportional range.



is rather small. It would be interesting to know if the stabilization originates from differences between detergent-peptide and peptide-peptide packing or if the trimer structure changes to better accommodate the smaller residue.

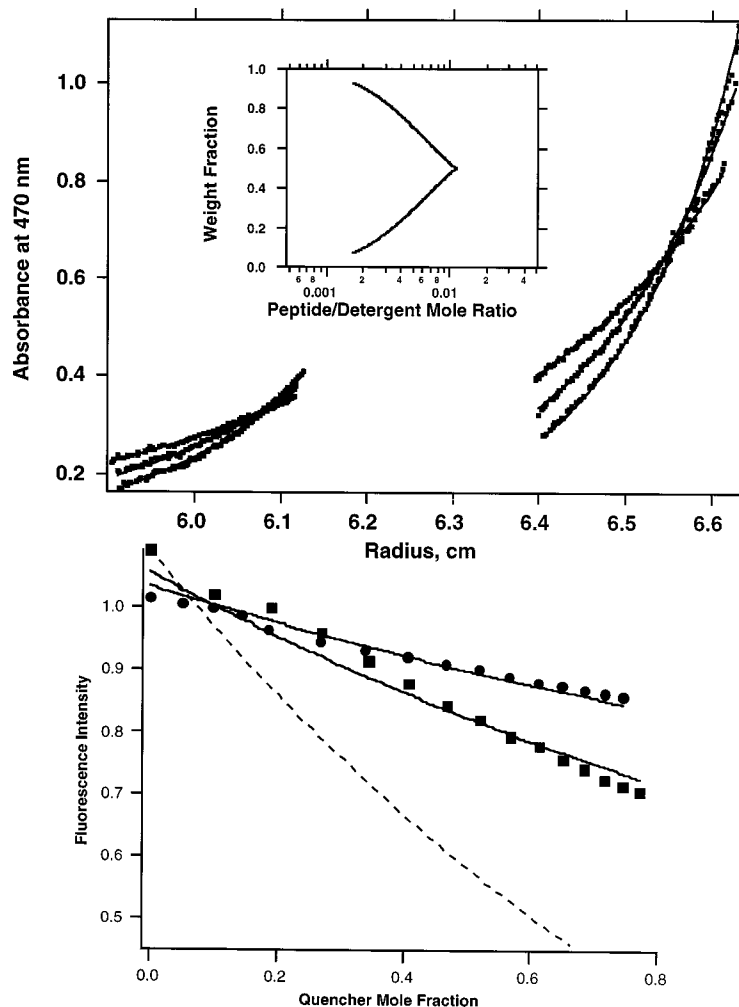
We are also investigating the effect of adding a second asparagine at either residue 7 (MS1-

V7N) or 21 (MS1-V21N): MS1, BQLIAAV-LLLIHAVNLILLIHAVARLRRLVVG; MS1-V7N, BQLIAANLLLIHAVNLILLIHAVARLRRLVVG; MS1-V21N, BQLIAAVLLLIHAVNLILLIAN-ARLRRLVVG. As can be seen by examining the sequence, residue 7 is well within the peptide's hydrophobic region, whereas residue 21 is within

Figure 2

EUAC (top panel) and FRET (bottom panel) results for MS1-NI4V/L17N

The top panel shows radial concentration data (points) and monomer-trimer equilibrium fits (global) for two different cell compartments containing 4 mM C-14 betaine and, respectively, 11.4 μM (left) and 23.7 μM (right) NBD-labelled MS1-NI4V/L17N in 100 mM sodium phosphate buffer, pH 7, with 12.8% $^2\text{H}_2\text{O}$ for density matching. Data were obtained using a Beckman XLI analytical ultracentrifuge at speeds of 40000, 45000 and 48000 rev./min in an An60 Ti rotor. Data fitting was done as described previously [19]. The inset shows calculated weight fractions of trimer (increasing) and monomer (decreasing) as a function of peptide/detergent MF over the range covered in the measurement and for the value of the monomer-trimer dissociation constant ($9.3 \times 10^{-5} \text{ MF}^2$) obtained by global curve fitting. The bottom panel shows fluorescence intensity as a function of the mole ratio of MS1-NI4V/L17N peptide labelled with tetramethylrhodamine to that labelled with NBD. C-14 betaine detergent concentration was constant at 1.65 mM. As described previously [19], the total peptide concentration was maintained constant at 5.5 μM (●) or 11 μM (■) using unlabelled peptide, and data (points) were globally fit (solid lines) to a monomer-trimer equilibrium model. The best-fit K_d was $6 \times 10^{-5} \text{ MF}^2$. The dashed line shows the result expected for 100% trimer.



one residue of the very positively charged C-terminal region, which is expected to reside within the headgroup region of micelles. In our work to date (J. Lear, H. Gratkowski, W. DeGrado, unpublished work), we have found by EUAC that the V7N variant shows a considerably stronger, co-operative monomer–trimer equilibrium ($K_d \approx 10^{-10}$ versus $\approx 10^{-5} \text{ MF}^2$ for MS1), whereas the V21N mutant associates no differently from MS1. Apparently, and as expected, the asparagine

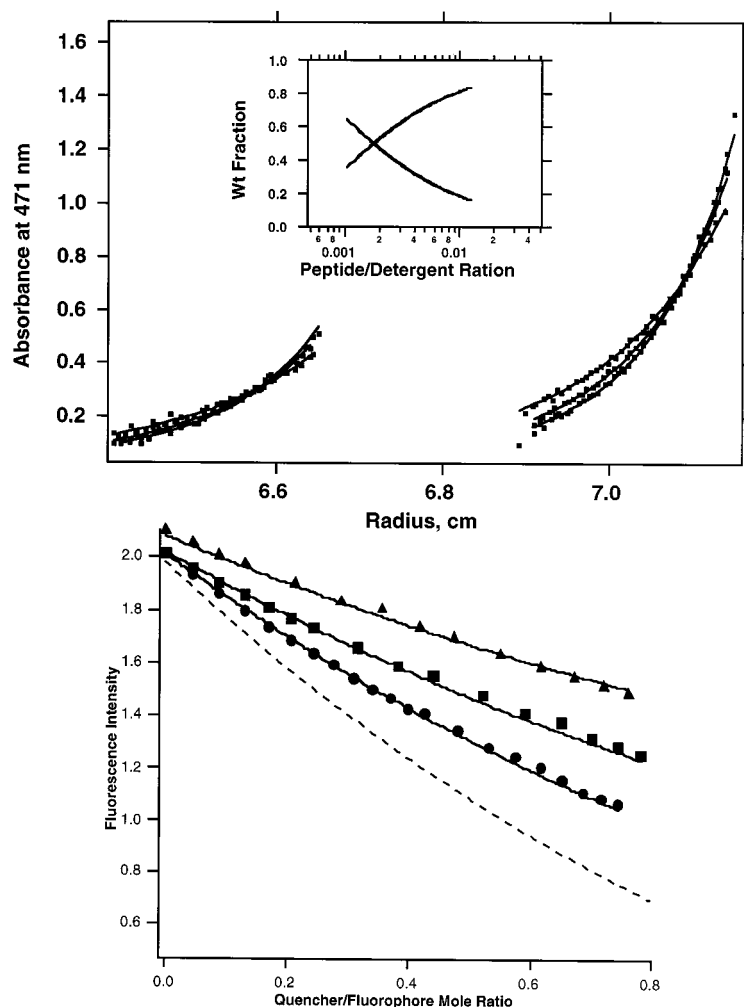
amides at position 21 are well enough solvated to virtually eliminate any driving force for their association.

It is interesting that the free energy of association of even the strongly associated MS1-V7N variant in the C-14 betaine detergent, expressed on a per-helix basis, is only a little more than half of what we have calculated from published data for the glycoporin dimer in SDS [8]. Glycoporin associates even more strongly in

Figure 3

EUAC (top panel) and FRET (bottom panel) results for MS1-L10A

As in Figure 2, the top panel shows data and fits for two different cell compartments containing 4 mM C-14 betaine and, respectively, 9.7 μM (left) and 20.5 μM (right) NBD-labelled MS1-L10A in 100 mM sodium phosphate density-matched buffer, pH 7. The inset shows, as in Figure 2, weight fractions of trimer and monomer calculated for a K_d of $2.3 \times 10^{-6} \text{ MF}^2$. The bottom panel shows fluorescence intensity as a function of the mole ratio of MS1-L10A peptide labelled with tetramethylrhodamine to that labelled with NBD. In this experiment, the total peptide concentration was maintained constant at 5.5 μM both within each determination (using unlabelled peptide) and also at the three different C-14 betaine detergent concentrations of 1.1 mM (●), 2.75 mM (■) and 5.5 mM (▲). The best global-fit K_d was $1.2 \times 10^{-6} \text{ MF}^2$. The dashed line shows the expected result for 100% trimer.



neutral detergents, such as those we employed in MS1 studies. Based on the experimentally determined structure [10] as well as on extensive molecular-dynamics simulations [27], glycoporphin association appears to be driven by close van-der-Waals contacts of residues around the Gly–Gly helical interface sites. Direct (non-water-mediated) hydrogen bonding does not appear to be involved. There appears, therefore, to be ample opportunity for designing model transmembrane helices not only with tighter association, but also with different and equally specific oligomerization states. We hope to obtain high-resolution structural information for some of these model systems to help interpret the free energy differences we observe and to help develop more reliable, sequence-based predictors of helical transmembrane protein folding. We also plan to apply what we have learned of the role of these polar interactions in determining transmembrane helix association to probe possible mechanisms of signal transduction by some classes of transmembrane receptor proteins.

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