Peptide Mimics of the M13 Coat Protein Transmembrane Segment

RETENTION OF HELIX-HELIX INTERACTION MOTIFS*

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Sequence-specific noncovalent helix-helix interactions between transmembrane (TM) segments in proteins are investigated by incorporating selected TM sequences into synthetic peptides using the construct CKKK-TM-KKK. The peptides are of suitable hydropobicity for spontaneous membrane insertion, whereas formation of an N-terminal S-S bond can bring pairs of TM helices into proximity and promote their parallel orientation. Using the propensity of the protein to undergo thermally induced α-helix → β-sheet transitions as a parameter for helix stability, we compared the wild type and mutant (V29A and V31A) bacteriophage M13 coat proteins with their corresponding TM peptide constructs (M13 residues 24–42). Our results demonstrated that the relevant helix-helix tertiary contacts found in the intact proteins persist in the peptide mimics. Molecular dynamics simulations support the tight "two in two out" dimerization motif for V31A consistent with mutagenesis data. The overall results reinforce the notion of TM segments as autonomous folding domains and suggest that the generic peptide construct provides a viable reductionist system for membrane protein structural and computational analysis.

Observations that excised or co-expressed fragments of the transmembrane (TM) segments of multi-spanning membrane proteins can reconstitute to a functional form (1–3) have led to the hypothesis that such segments constitute independent folding domains that can be elaborated from the properties of individual TM helices (4, 5). The task of their structural analysis can thus be essentially reduced to the examination of tertiary contacts between membrane-embedded helices. Therefore, it appears valid to choose a hairpin (helix-loop-helix) structure as the minimal tertiary contact unit of analysis for determination of the extent and nature of helix-helix interactions that may arise in polytopic membrane proteins. For such analyses, studies with de novo designed peptides have demonstrated the feasibility of generating milligram amounts of synthetic TM segments using peptide constructs carrying terminal positive charges (6, 7). As well, synthetic peptides allow for wide mutation possibilities without complications from requirements for cell viability.

To explore the properties of isolated TM segments in a systematic manner, we have chosen M13 major coat protein for a model study. This 50-residue protein (8) is found in the capsid of filamentous coliphage M13 (9). During phage assembly, the α-helical M13 coat proteins participate in an oligomerization process within Escherichia coli plasma membrane, which ultimately leads to the encapsulation of viral DNA (10, 11). In vivo, the coat protein is expected to be oligomeric during the life cycle of M13, with the minimal structure of such oligomers being a dimer (12). However, in vitro the oligomerization state of the isolated WT M13 coat protein (and its relative, IKε coat protein) on SDS-polyacrylamide gel electrophoresis and other gels have been variously reported as monomers (13–15) or dimers (10, 12, 16, 17), with the observed state a sensitive function of micelle concentrations, ionic strength, and pH (10, 18). That the wild type oligomeric state is condition-dependent suggests the likelihood of equilibration between various states but generally in the direction away from tight dimer formation to allow further propagation of oligomers.

Previous mutagenesis studies on the M13 coat protein demonstrated that various Val → Ala mutations in the TM region have contrasting position-dependent effects on its helical stabilities. In particular, mutant V31A acquired an unusually large amount of thermal stability from this mutation, whereas the V29A mutant behaved very similarly to the WT (19). Based on the mutagenesis results, the oligomerization-promoting helical interface of M13 coat protein was postulated to have a "two in two out" motif 24YAWAMVYVVGATIGIKLF42 (residues in the helix-helix interface are underlined) (19) conceptually analogous to that of the glycophorin A dimer (20, 21).

As indicated above, M13 coat protein presents the advantage as a model system that helix-helix interactions can be assessed by the propensity of the micelle-embedded M13 protein to undergo a thermally induced α-helix → β-sheet transition (19). In such experiments, heating disrupts both helix-membrane and helix-helix interactions, eventually allowing the TM segments to escape the micelle into the aqueous environment, where they aggregate rapidly to form β-sheets because of their extreme hydrophobic nature. Although the specific type and aggregation state of this β-form of M13 coat protein are not precisely known, Hemminga and colleagues (10) have reported that the β-aggregate consists of 440 strands upon heating WT M13 coat protein to 55 °C in 10 mM cholate. Thus, the clear β-sheet diagnostic CD spectra provide a useful assay for aggregation of the nascent water-based form of the protein as driven by the hydrophobic effect. Because helix-helix interactions become important only when relatively strong noncovalent dimers/oligomers pre-exist in the micelle, thermal stability can be used as
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a parameter to assess the sequence dependence of the intrinsic strength of inter-helical interactions.

Based upon this overall knowledge of WT and mutant M13 coat proteins, we have de novo designed and synthesized a series of peptides which correspond to the presumed transmembrane segment (residues 24–42) of the M13 coat protein. If TM helices act as autonomous folding domains, with self-contained recognition faces that guide helix-helix packing, we are in a position to test the hypothesis that peptides comprised of isolated TM sequences should exhibit properties similar to those characteristic of the corresponding segments within the intact proteins.

MATERIALS AND METHODS

Synthesis and Purification of Model Peptides—Peptide correspond- ing to bacteriophage M13 major coat protein transmembrane segment positions 24–42, designated WT, V29A, and V31A (see Fig. 1), were synthesized by the continuous flow Fmoc (N-(9-fluorenyl)methoxycarbonyl) solid phase method (22). Peptides were synthesized with PALPEG-PS resin (PE Biosystems, Foster City, CA), so that cleaved pep- tides were automatically amidated at C termini. The crude peptides were desalted on a Sephadex G-10 column eluted using 25 mM NH4HCO3. Peptide purification was carried out on a reverse-phase Jupiter C4 semi-preparative column (10 × 250 mm, 300 A, from Phenomenex) using a CH3CN/H2O system, with trifluoroacetic acid as counter ion. Purified peptides were characterized by mass spectrometry and amino acid analysis.

Preparation and Purification of M13 Coat Proteins—WT M13mp18 ssDNA was purchased from Amersham Pharmacia Biotech. The V29A and V31A mutants were prepared as described previously using the site-directed mutagenesis kit provided by Amersham Pharmacia Biotech (19, 23). The identity of each phage was checked by DNA sequencing prior to and after large scale preparation. Confirmed M13 phages were amplified at 1-liter scale using E. coli strain JM101 as the host cell. Yield of phage from 1 liter of culture was typically 2.7 mg of purified coat protein was obtained from 1-liter phage preparations.

TCEP Reduction—TCEP (purchased from Roche Molecular Biochemicals) was used to reduce disulfide bonds formed between terminal Cys residues (24). Reduction was carried out in Tris buffer (10 mM Tris–HCl and 10 mM NaCl, pH 7) with 32 mM of peptide and 10 or 40 mM of TCEP at ambient temperature for 1 h. Gel electrophoresis was performed at neutral pH using a precast 4–12% NOVEX NuPage gel (Helixx Technologies, Inc.).

Spectroscopic Measurements—CD spectroscopy was performed on a Jasco-720 spectropolarimeter using a 1-mm quartz cell embedded in a water jacket. Spectra were taken with resolution = 0.2 nm and speed = 50 nm/min. Response time and accumulation were set to 4 and 2 s, respectively, for peptides, and to 2 and 4 s for proteins. Concentrations of the peptide stocks were determined by amino acid analysis in triplicate. Protein concentration was calculated according to A280 = 1.659 for 1 mg/ml WTProt (25). Extinction coefficients of the mutant proteins were determined with the assistance of ProtParam. Peptide concentrations were dissolved to 40 μM in micellar solutions prepared with 10 mM lysyl-phosphatidylcholine (LPC; Avanti Polar Lipids, Inc.) and phos- phate buffer (10 mM phosphate, 10 mM NaCl, pH 7). Coat proteins were dissolved at a concentration of 1 mg/ml in deoxycholate (DOC) micelles (30 mM DOC, 25 mM Na2SO4, pH 9). Fluorescence measurements were performed on a Hitachi F-4500 fluorescence spectrophotometer with the excitation wavelength set at 295 nm to minimize interference from Try. Emission spectra were recorded from 300–400 nm with a bandpass of 5 nm and a speed of 60 nm/min. Peptide concentration was typically 2.7 μM, in aqueous or micellar solutions.

Thermal Transition Studies—Conformational transitions were followed by CD spectroscopy using stepwise heating from 25 to 95 °C. During stepwise heating, samples were heated at increments of 5 °C and then held for 5 min at each temperature before heating to the next temperature.

Computational Modeling—Global conformation searches on likely M13 dimers were performed using the simulation program developed by Brünger and associates (20, 26, 27) for the study of glycoalix A and phospholamban. Simulation was performed with charges on Lys assumed to be 0 because the positive charge is likely to be screened because of interaction with the negative lipid head groups. The simulation starts with two idealized M13 TM (24–42) helices, placed at a crossing angle of either 25° (left-handed) or −25° (right-handed). Each of the two helices is then systematically turned with an increment of 45° and searched for the most stable conformation using standard simulated annealing protocol (5000 steps at 600,000, with time step = 1 fs, then 5000 steps at 300,000, with time step = 2 fs). The simulation is performed in vacuo, with dielectric constant (ε) = 1. For each initial conformation, the search was repeated four times with different initial velocities to increase sampling frequency, and thus 512 final structures were generated. To identify the convergence of structures having root mean square deviation ≤ 1 Å were averaged, energy minimized, and then subjected to the same simulated annealing protocol used in the systematic search.

RESULTS AND DISCUSSION

Peptide Design: Disulfide-linked Parallel Dimers—M13 model peptides were synthesized with the prototypical sequence: CKKK-TM-KKK, where TM represents the putative transmembrane segment of the corresponding M13 coat proteins in positions 24–42 (Fig. 1). In this design, the synthetic peptides lack both the amphipathic N-terminal domain and the C-terminal DNA binding segment of the full 50-residue coat protein, although the three C-terminal Lys residues in the peptides do in fact correspond to the Lys-rich character of this region in the wild type protein. Because the mean residue hydrophobicity of the core segment greatly exceeds the threshold requirement predicted by Liu and Deber (7), these peptides are expected to spontaneously insert into micellar membranes. We have found that terminal Lys residues are required to increase the solubility of the peptides, thereby facilitating their chemical synthesis and purification (7).

Full-length coat protein contains an amphipathic helix N-terminal to the TM segment (e.g. 14), which apparently acts as an anchor when the phage inserts into the plasma membrane and thereby restricts any noncovalent dimer/oligomer forma- tion to be parallel. To simulate this orientation preference, an N-terminal Cys was therefore included in the design of the model peptides to promote the formation of coherent parallel dimers via a disulfide bridge. With pairs of TM segment pep- tides held essentially parallel by these S-S links, noncovalent helix-helix interactions as a function of sequence within the peptide TM segments should, in principle, be maximized. All Cys-containing peptides formed dimers at neutral pH, which could be reduced to monomers with the addition of disulfide reducing agent TCEP (24). The complete resolution of peptide dimers by TCEP suggested that (i) the terminal Lys residues were indeed effective in preventing self-aggregation of the pep- tides and (ii) the dimer species observed were linked by S-S bridges. These results also indicated that lacking the disulfide linkage, the model peptides are monomeric in SDS, likely because of charge repulsion from the Lys residues.

Peptide Conformation as a Function of Environment—CD spectroscopy was used to elucidate the secondary structures of
the peptides. Because the peptides were stored in water, we sought to establish initially that they had not aggregated upon storage in stock solutions. Although all peptides assumed random conformations in pure water, indicating the apparent absence of aggregation, β-structures did form in phosphate buffer even at room temperature (not shown). β-Formation in the presence of phosphate is likely due to a "bridging" effect of the divalent anion in simultaneously "neutralizing" Lys amino groups, while also bringing pairs of them, and hence the peptide chains generally, into relative proximity.

In LPC micelles buffered by phosphate to pH 7, the mutant peptides (V29Apep and V31Apep) formed well defined α-helical structures, whereas WT displayed an α-β mixture as suggested by the overall shape of the CD spectra (not shown). Despite β-sheet formation of peptides in phosphate buffer alone as mentioned above, addition of peptides in pure water to phosphate/LPC suspensions resulted in efficient uptake of peptides into micelles with concomitant helix formation. To demonstrate that α-helical structures induced in LPC were due to transmembrane insertion of the peptides, we recorded fluorescence emission spectra from Trp. We found that the maxima of Trp fluorescence emission spectra were blue-shifted to ~335 nm in LPC micelles from ~350 nm in water, confirming that Trp was transferred from water to the more hydrophobic lipidic environment (Fig. 2a). Interestingly, β-type aggregation of peptides in phosphate buffer produced a comparable blue shift, indicating the burial of the Trp residue within the peptide β-aggregate. However, the LPC-induced blue shift was accompanied by a concomitant increase in fluorescence intensity (Fig. 2b), typical of transmembrane insertion, whereas Trp fluorescence in phosphate was quenched as the peptides aggregated to form β-sheets.

**Thermally Induced α-Helix → β-Sheet Transition of M13 Peptides in LPC Micelles**—For the purpose of measuring intrinsic fluorescence to assess the propensity of various M13 peptides to undergo structural transitions in LPC micelles, we recorded fluorescence spectra of Trp26. We found that the maxima of Trp fluorescence emission spectra were blue-shifted to ~335 nm in LPC micelles from ~350 nm in water, confirming that Trp was transferred from water to the more hydrophobic lipidic environment (Fig. 2a). Interestingly, β-type aggregation of peptides in phosphate buffer produced a comparable blue shift, indicating the burial of the Trp residue within the peptide β-aggregate. However, the LPC-induced blue shift was accompanied by a concomitant increase in fluorescence intensity (Fig. 2b), typical of transmembrane insertion, whereas Trp fluorescence in phosphate was quenched as the peptides aggregated to form β-sheets.

**Peptide Mimics of Transmembrane Helices**—Thermal stability of M13 coat protein helical structure within membranes has been established as a parameter for the efficacy of noncovalent helix-helix interactions (19, 28). We therefore systematically examined the thermal transitions of the WT, V29A, and V31A M13 peptide mimics and compared their properties with those of the corresponding intact coat proteins. Of necessity, different lipids were employed for peptide versus protein experiments; coat protein purification is traditionally performed in negatively charged DOC micelles (17, 19), a requirement for disrupting protein-DNA interactions; however, DOC is soluble only under basic conditions (pH 9), at which charges on the terminal Lys residues in the peptide constructs become neutralized, and the peptides tend to precipitate. Hence, a zwit-terionic lipid at pH 7 (LPC) was employed for peptide experi-

**Fig. 2.** Peptide insertion into LPC micelles studied by Trp fluorescence measurements. Peptide concentration = 2.7 μM for all solvent conditions. Trp fluorescence spectra were recorded from 300 to 400 nm on a Hitachi F-4000 fluorescence spectrophotometer. Excitation was 295 nm. See text for further discussion.

**Fig. 3.** Thermally induced α-helix → β-sheet transition of M13 peptides (a) and M13 proteins (b), produced by stepwise heating, as monitored by circular dichroism spectroscopy. Peptides were dissolved at a concentration of 40 μM in LPC micelles; protein samples were prepared at a concentration of 1 mg/ml in DOC micelles (see text). Samples were heated to 95 °C at increments of 5 °C, with a 5-min incubation period at each temperature prior to recording spectra. In a given experiment, approximately 4 h elapses between recording of the first and last CD spectrum. Ellipticity values at 208 nm were normalized to 0 with respect to the [θ]208 value of each species at 25 °C. Each plot represents the average of three trials.
properties is solely due to the positional effect(s) of the substitution.

Model for Noncovalent Dimeric Interactions between M13 TM Segments—In seeking a structural delineation that accounts for the experimentally observed differences in thermal stabilities, we examined the helix-helix interactions for hypothetical noncovalent parallel dimer formation between two M13 TM segments (24–42; dielectric constant = 1) using a global conformation search program (26, 31). The program executes an automated exhaustive two-body conformation search of all possible pairings between two helices and finds a set of probable secondary pairings between two helices and finds a set of probable structures; ultimately, the correct structure can only be selected with the guidance of experimental data. Because V31A is demonstrably the most thermally stable among the three M13 variants examined in this study, we expected its dimers to be the most compact. When the final structures were grouped by root mean square deviation, we found a cluster of minimized V31A dimer structures that conforms to the two in two-out dimerization motif proposed from mutagenesis data (19) (Fig. 4). The closest approach between the two helix backbones in this orientation is 6.5 Å, the shortest among all clusters found. The same dimerization motif was found for V31A dimers during simulations with dielectric constant = 2 and during simulations with shorter helices (24–39; eliminating Lys40).

The averaged structure of the V31A cluster is a symmetrical right-handed coiled-coil structure with a crossing angle of -19.5°. As highlighted in Fig. 4 (a and b), the dimerization motif YAWAMVYAIVGATIGIKL is consistent with seven residues, of which five are small residues (Ala or Gly). The residues comprising the motif create a smooth right-handed groove on each of the helices forming the dimeric interface, with the helices most tightly packed when the grooves are facing each other. The clear periodicity of the V31A dimerization motif is shown in the interaction energy profile presented in Fig. 4c.

When WT and V29A (residues 24–42) were simulated using the same parameterization, no structures conforming to the prescribed (V31A) dimerization motif were found. It appears that when Val31 is not mutated to Ala, the steric hindrance between the combined four Val (Val31) residues of the two helices brings the small residues in the upper part of the groove out of reach for interaction (Figs. 4b and 5). Here, the separation between helices is 8–9 Å, and typical dimer conformations of WT and V29A TM segments consist of dimer interfaces that resemble a knobs-into-holes type of packing similar to V31A, with the groove of one helix being utilized as “holes,” whereas some other (nonequivalent) face of the second helix being used as “knobs” (Fig. 5). Such an asymmetric arrangement affords the opportunity for helix-helix propagation and may thus present advantages to M13 phage during phage assembly stage, when coat proteins likely oligomerize to structures considerably higher than dimers within the plasma membrane of E. coli.

Despite the fact that V31A forms the most tightly packed dimer, the structure does not represent the global energy minimum in terms of residue interaction energy among the simulated structures (not shown). This circumstance, in turn, would suggest that the in vivo situation for bacteriophage M13 must be different from the glycoporphin A TM segment. For example, interfacial Leu Ala and Ile Ala mutations are found to destabilize the corresponding glycoporphin A dimers, as determined by analytical ultracentrifugation techniques (32). These overall observations highlight the dichotomous biological requirements of the two systems, viz., although maintenance of a “tight” dimer is apparently essential for glycoporphin A function, such that wild type residues will tend to be optimized for "tight" dimer is apparently essential for glycophorin A function, and may thus present advantages to M13 phage during phage assembly stage, when coat proteins likely oligomerize to structures considerably higher than dimers within the plasma membrane of E. coli.

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still be accommodated, albeit not optimally, by M13 bacteriophage mutants such as V31A.

Conclusion—Thermal transition analysis in this study demonstrates that the functional properties of the M13 TM segment are not altered when placed in a model system, indicating the persistence of the interfacial noncovalent dimerization motif. The implications and validity of these findings may extend to the systematic examination of dimeric interactions between any two TM segments in a polytopic membrane protein. In the latter instance, placement of the Cys residue in a given peptide can serve to promote parallel or antiparallel helix-helix structures as dictated by the disposition of adjacent helices in the system of interest. With the advantage of dual water membrane solubility and boundless mutation possibilities extending to non-natural amino acids, the construct CKKK-TM-KKK may provide a useful route to guide computational analysis and contribute insights on TM domains of proteins that can supplement information obtainable by traditional molecular biology approaches.

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REFERENCES

1. Kahn, T. W., and Engelmann, D. M. (1992) Biochemistry 31, 6144–6151
2. Loo, T. W., and Clarke, D. M. (1994) J. Biol. Chem. 269, 7750–7755
3. Groves, J. D., and Tanner, M. J. A. (1995) J. Biol. Chem. 270, 9007–9105
4. Popot, J. L., and Engelman, D. M. (1990) Biochemistry 29, 4031–4037
5. Deber, C. M., and Goto, N. K. (1996) Nat. Struct. Biol. 3, 815–818
6. Liu, L. P., Li, S. C., Goto, N. K., and Deber, C. M. (1996) Biopolymers 39, 465–470
7. Liu, L. P., and Deber, C. M. (1998) Biopolymers 47, 41–62
8. Nakashima, Y., Frangione, B., Wiseman, R. L., and Konigsberg, W. H. (1981) J. Biol. Chem. 256, 5792–5797
9. Russel, M. (1991) Mol. Microbiol. 5, 1607–1613
10. Spruijt, R. B., Wolfs, C. J., and Hemmingsa, M. A. (1989) Biochemistry 28, 9158–9165
11. Spruijt, R. B., and Hemmingsa, M. A. (1991) Biochemistry 30, 11147–11154
12. Haigh, N. G., and Webster, R. E. (1998) J. Mol. Biol. 279, 19–29
13. McDonnell, P. A., Shen, K., Kim, Y., and Opella, S. J. (1993) J. Mol. Biol. 233, 447–463
14. Williams, K. A., Farrow, N. A., Deber, C. M., and Kay, L. E. (1996) Biochemistry 35, 5145–5157
15. Shen, L. M., Lee, J. I., Cheng, S. Y., Jutte, H., Kuhn, A., and Dalbey, R. E. (1991) Biochemistry 30, 11775–11781
16. Makino, S., Woolford, J. L., Jr., Tanford, C., and Webster, R. E. (1975) J. Biol. Chem. 250, 4327–4332
17. Cavalieri, S. J., Goldthwait, D. A., and Neet, K. E. (1976) J. Mol. Biol. 102, 713–722
18. Stopar, D., Spruijt, R. B., Wolfs, C. J., and Hemmingsa, M. A. (1997) Biochemistry 36, 12268–12275
19. Deber, C. M., Khan, A. R., Li, Z., Joensson, C., Glibowicka, M., and Wang, J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11648–11652
20. Lemmon, M. A., Treutlein, H. R., Adams, P. D., Brünger, A. T., and Engelman, D. M. (1994) Nat. Struct. Biol. 1, 157–163
21. MacKenzie, K. R., Prestegard, J. H., and Engelman, D. M. (1997) Science 276, 131–133
22. Atherton, E., and Sheppard, R. C. (1990) (Rickwood, D., and Hames, B. D., eds) pp. 131–148, IRL Press, Oxford, UK
23. Li, Z., and Deber, C. M. (1991) Biochem. Biophys. Res. Commun. 180, 687–693
24. Burns, J. A., Butler, J. C., Moran, J., and Whitesides, G. M. (1991) J. Org. Chem. 56, 2448–2450
25. Nozaki, Y., Chamberlin, B. K., Webster, R. E., and Tanford, C. (1976) Nature 259, 335–337
26. Adams, P. D., Engelmann, D. M., and Brünger, A. T. (1996) Proteins 26, 257–261
27. Treutlein, H. R., Lemmon, M. A., Engelmann, D. M., and Brünger, A. T. (1992) Biochemistry 31, 12726–12732
28. Li, Z., Glibowicka, M., Joensson, C., and Deber, C. M. (1993) J. Biol. Chem. 268, 4584–4587
29. Chou, P. Y., and Fasman, G. D. (1978) Annu. Rev. Biochem. 47, 251–276
30. Deber, C. M., and Li, S. C. (1995) Biopolymers 37, 295–318
31. Adams, P. D., Arkin, I. T., Engelman, D. M., and Brünger, A. T. (1995) Nat. Struct. Biol. 2, 154–162
32. Fleming, K. G., Ackerman, A. L., and Engelmann, D. M. (1997) J. Mol. Biol. 272, 266–275
33. Papavonie, C. H., Christiansen, B. E., Folmer, R. H., Konings, R. N., and Hilbers, C. W. (1998) J. Mol. Biol. 282, 401–419
34. Koradi, R., Billeter, M., and Wuthrich, K. (1996) J. Mol. Graph. 14, 29–32