The pore-forming domain of Bacillus thuringiensis Cry1Ac insecticidal protein comprises of a seven α-helix bundle (α1-α7). According to the “umbrella model,” α4 and α5 helices form a hairpin structure thought to be inserted into the membrane upon binding. Here, we have synthesized and characterized the hairpin domain, α4-loop-α5, its α4 and α5 helices, as well as mutant α4 peptides based on mutations that increased or decreased toxin toxicity. Membrane permeation studies revealed that the α4-loop-α5 hairpin is extremely active compared with the isolated helices or their mixtures, indicating the complementary role of the two helices and the need for the loop for efficient insertion into membranes. Together with spectrofluorometric studies, we provide direct evidence for the role of α4-loop-α5 as the membrane-inserted pore-forming hairpin in which α4 and α5 line the lumen of the channel and α4 also participates in the oligomerization of the toxin. Strikingly, the addition of the active α4 mutant peptide completely inhibits α4-loop-α5 pore formation, thus providing, to our knowledge, the first example that a mutated helix within a pore can function as an “immunity protein” by directly interacting with the segments that form the pore. This presents a potential means of interfering with the assembly and function of other membrane proteins as well.

The major defensive and offensive strategies chosen by bacteria are based on the interaction of toxins with cellular membranes (1–3). This interaction frequently results in membrane permeation or translocation into the cytoplasm (1, 4–6). Consequently, toxins need to be soluble to reach their target cells. However, once the target is reached, the toxins are transformed enzymatically modified into their active form (6), after which the toxins bind to target cells through a two-stage process to form channels/ pores (11, 12). The first step involves reversible binding to a receptor followed by an irreversible step in which membrane insertion occurs (3, 6, 13, 14). The irreversible step is also implicated with toxicity; however, the mode of action of the toxin is not yet clearly understood. Biophysical and structural studies of the δ-endotoxins as a paradigm for membrane toxins may help elucidate the specific interactions, mechanism of insertion, and organization within the membrane of toxins and channel proteins.

A major part of the current knowledge concerning the mechanism of δ-endotoxin toxicity is through experience with three major proteins of the Cry family: Cry3A, Cry1Aa, and Cry1Ac. The Cry proteins are an extensive toxin family found within the δ-endotoxins as a paradigm for membrane proteins. Two highly hydrophobic helices of the Cry3A domain I, namely α4 and α5, were each shown to form homo- and hetero-oligomers in the membrane milieu (17–19). Helix α5 of Cry3A (17) and Cry1Ac (20) were shown also to have membrane permeation capabilities. These data suggest that α4 and α5 might be the region that is inserted into the membrane and actually participates in pore formation. This model is supported by mutagenesis analysis done on full-length proteins, where loss of toxicity is a common characteristic of most α4 and α5 mutations (16, 21–23). Previous studies indicate that although mutations in α4 usually cause loss of toxicity, they do not change the aggregation capabilities of the toxin (23). On the other hand, a strong correlation was found between toxicity and α5 oligomerization. Specifically, for mutations that did not affect toxicity, the protein retained its oligomerization ability, excluding one known exception H168R (23). Recent results based on site-directed mutagenesis place α4 in the aqueous interface of the pore, whereas the more hydrophobic α5 is located at the brane insertion to occur.

Bacillus thuringiensis, a ubiquitous Gram-positive bacterium, produces parasporal crystals (10). Within these crystals lie the δ-endotoxins, which are specific insect toxins. The δ-endotoxins are digested by the insects in the midgut and are enzymatically modified into their active form (6), after which the toxins bind to target cells through a two-stage process to form channels/ pores (11, 12). The first step involves reversible binding to a receptor followed by an irreversible step in which membrane insertion occurs (3, 6, 13, 14). The irreversible step is also implicated with toxicity; however, the mode of action of the toxin is not yet clearly understood. Biophysical and structural studies of the δ-endotoxins as a paradigm for membrane proteins may help elucidate the specific interactions, mechanism of insertion, and organization within the membrane of toxins and channel proteins.

The Cry proteins are an extensive toxin family found within the δ-endotoxins as a paradigm for membrane proteins. Two highly hydrophobic helices of the Cry3A domain I, namely α4 and α5, were each shown to form homo- and hetero-oligomers in the membrane milieu (17–19). Helix α5 of Cry3A (17) and Cry1Ac (20) were shown also to have membrane permeation capabilities. These data suggest that α4 and α5 might be the region that is inserted into the membrane and actually participates in pore formation. This model is supported by mutagenesis analysis done on full-length proteins, where loss of toxicity is a common characteristic of most α4 and α5 mutations (16, 21–23). Previous studies indicate that although mutations in α4 usually cause loss of toxicity, they do not change the aggregation capabilities of the toxin (23). On the other hand, a strong correlation was found between toxicity and α5 oligomerization. Specifically, for mutations that did not affect toxicity, the protein retained its oligomerization ability, excluding one known exception H168R (23). Recent results based on site-directed mutagenesis place α4 in the aqueous interface of the pore, whereas the more hydrophobic α5 is located at the
back of the oligomer and interacts mainly with α4 and the lipid bilayer (16). Furthermore, it was shown that mutations I32S and Q33R, located on the polar surface of α4, substantially decrease the pore-forming activity, whereas F134L results in a 3-fold increase in activity (23). However, there is still no direct evidence that α4 inserts together with α5 as a hairpin into the membrane to form a pore.

To directly support this hypothesis, we have synthesized and characterized the corresponding peptides derived from Cry1Ac, namely, α5, α4, and its active and non-active mutants and the full-length membrane-inserted domain α4-loop-α5 (Table I). Note that the benefits of studying the membrane-inserted segment of a protein were clearly demonstrated by revealing the crystal structure of the KcsA potassium channel, which was done using only the membrane-inserted segments, helix M1-pore region-helix M2, of the channel (24). Importantly, the structure and the organization of this region were in agreement with studies carried out with the isolated helix M1, the pore region H5, and the helix M2 (25, 26).

Membrane permeation studies, binding experiments, and competition assays between the α4-loop-α5 and the α4 and α5 peptides provide direct evidence of the role of α4-loop-α5 as the membrane-inserted pore-forming hairpin in which α4 and α5 lines the lumen of the channel, and α5 participates also in the oligomerization of the toxin. Furthermore, the striking finding that an α4 mutant can inhibit α4-loop-α5 pore formation provides, to our knowledge, the first example that a mutated helix of a pore-forming toxin can function as an immunity protein by directly interacting with the segments that form the pore, similar to what has been proposed for the immunity proteins of colicin E1 (27). This presents a potential means of interfering with the assembly and function of other membrane proteins as well. These results are discussed also with regard to the importance of understanding protein-protein interaction within the membrane milieu (28, 29).

**EXPERIMENTAL PROCEDURES**

**Materials**—Butyloxy carbonyl amino acids were obtained from Bachem (Bubendorf, Switzerland), and t-butyloxy carbonyl-Leu-OCH$_3$ peptide glycylic α-amidating monooxygenase resin was purchased from Applied Biosystems (Foster City, CA). Other reagents used for peptide synthesis included trifluoroacetic acid (Sigma), N,N-diisopropylethylamine (Aldrich; distilled over n-hexane), benzotriazol-N-oxys(dimethylaminophosphonium hexafluorophosphate (Sigma), N-methylmorpholine (Aldrich), and dimethylformamide (peptide synthesis grade, Aldrich) in order to separate the free calcein. A final concentration of 2.5 μM SUV was used. Trapped inside vesicles, the calcein dye is self-quenched. Thus, membrane permeation can be detected by an increase in fluorescence as peptides are added. The percentage of fluorescence recovery, $F_r$, was defined as $F_r = (F_f - F_0)/F_{max}$, whereas $F_0$ = initial fluorescence, $F_{max}$ = the total fluorescence observed after the addition of paradaxin, and $F_f$ = the fluorescence observed after adding the peptide, at time t. Paradaxin was used at a concentration of 0.6 μM, which caused 100% calcein leakage from the vesicles.

**Rhodamine Fluorescence Quenching Measurements**—Rhodamine fluorescence is highly sensitive to self-quenching but poorly affected by the dielectric constant of its environment. Therefore, the tendency of the peptides to oligomerize in aqueous solution was tested using Rhodamine-labeled peptides. Changes in the fluorescence were measured after adding protease K (10 mg/ml final concentration) to Rh-labeled peptides (0.1 μM) previously dissolved in PBS. More protease K was added until there was no further change in the fluorescence emission. Excitation was set at 530 nm, and emission was set at 580 nm. All the fluorescence measurements in the present study were done on an entrance of a Bowman Series 2 SLM spectrophotometer (SLM-Aminco Spectronic Instruments).

**Binding Experiments**—The degree of peptide association with PC or PS SUV was measured by adding increasing amounts of vesicles to 0.1 μM NBD-labeled peptides dissolved in PBS. The fluorescence intensity was measured as a function of the lipid/peptide molar ratio, with excitation set at 467 nm, and emission set at 530 nm. The fluorescence values were corrected by subtracting the corresponding blank (PBS with the same amount of vesicles). The binding isotherms were analyzed as partition equilibria (31–38) using the formula $X_{f}^*$ = $K_t^* C_r$, where $X_{f}^*$ is defined as the molar ratio of bound peptide per 60% of the total lipid, assuming that the peptides were initially partitioned only over the outer leaflet of the SUV, as has been previously suggested (38); $K_t^*$ corresponds to the partition coefficient; and $C_r$ represents the equilibrium concentration of the free peptide in the solution. The value $X_{f}^*$ was calculated by extrapolating $F_r$ (the fluorescence signal obtained when all the peptide is bound to lipid) from a double-reciprocal plot of $F$ (total peptide fluorescence) versus $C_r$ (total concentration of lipids) (35). Knowing the fluorescence intensities of unbound peptide, $F_u$, as well as the bound peptide, $F_b$, the fraction of membrane-bound peptide, $f_b$, could be calculated using the formula $f_b = (F_b - F_u)/(F - F_u)$.

Having calculated the value of $f_b$, it is then possible to calculate $C_r$ as well as the extent of peptide binding, $X_{f}^*$. The curves that result from plotting $X_{f}^*$ versus free peptide, $C_r$, are referred to as the conventional binding isotherms.

**Fluorescence Resonance Energy Transfer (FRET) Measurements**—FRET experiments were performed by using NBD-labeled peptides serving as energy donors and Rh-labeled peptides serving as energy acceptors.

---

**Table I**

* Sequences, designations and molecular weights of the synthetic peptides derived from Cry1Ac protein

| Peptide name | Sequence | $M_r$ |
|-------------|----------|------|
| WT α4       | LREEMR1QFNDMNSSLALTA1PLFA | 2780 |
| 7S α4       | LREEMR1QFNDMNSSLALTA1PLFA | 2754 |
| QR α4       | LREEMR1QFNDMNSSLALTA1PLFA | 2808 |
| F9L α4      | LREEMR1QFNDMNSSLALTA1PLFA | 2746 |
| α4-loop-α5  | LREEMR1QFNDMNSSLALTA1PLFAVQCYQVPLSVVYQAAANLHLSLVDVSFG | 6079 |
| WT α5       | PLLSVVYQAANLHLSLVDVSFG | 2596 |

---

1 The abbreviations used are: PC, phosphatidylcholine; PS, and phosphatidylserine; NBD-CI, N-[7-nitrobenz-2-oxa-1,3-diazole-4-yl] chloride; Rho, carboxytetramethylrhodamine; HPLC, high performance liquid chromatography; SUV, small unilamellar vesicles; PBS, phosphate-buffered saline; FRET, fluorescence resonance energy transfer; WT, wild type.
at the wavelengths of the maximal donor emission. The percentage of SUV (350 mgles) added to calcein containing SUV PC and PS/PC. Increase in the fluorescence of rhodamine is quenched when several Rho-labeled molecules are in proximity, and the increase in fluorescence after proteinase K was added indicates that 4 peptides to cause calcein release is a result of their inability to bind phospholipid membranes, we performed additional binding experiments.

Peptides Binding to Membranes—Characterization of vesicle binding by WT, I7S, Q8R, and F9L. 4 was facilitated through the attachment of an NBD moiety to the peptides. NBD is very sensitive to the dielectric constant of the environment. Therefore, membrane binding can be detected as a substantial increase in NBD fluorescence. The attachment of the NBD did not alter the membrane-permeating activity of the peptides, which has also been shown in other studies (19). A constant concentration of peptide (0.1 μM) was titrated with the desired vesicles (e.g. PC, PS/PC). Increases in the fluorescence intensities of NBD-labeled peptides as a function of the lipid/peptide molar ratio yielded the conventional binding curves (see Fig. 2, panels A, B, C, and D for 4, Q8R, I7S, and F9L, respectively, for PC/PS vesicles). Similar curves were obtained with PC vesicles and, therefore, are not shown. The light scattering of the vesicles was subtracted by repeating the experiments with unlabeled peptides. Since all peptides apart from 5 are monomeric in aqueous solution, as revealed by rhodamine dequenching experiments (see next paragraph), we were able to analyze their binding isotherms as partition equilibria. The surface partition coefficients were estimated from the initial slopes of the curves shown in the insets of all panels in Fig. 2 (for PC/PS) (38). Table II shows the calculated partition coefficients (and the free energy of binding, −ΔG) for both PC and PC/PS phospholipid membranes. F9L shows positive cooperativity as the binding isotherm curves upward. The wild type and the rest of the 4 mutants give linear binding isotherm curves. WT-5 binding to vesicles was also facilitated through NBD-labeled peptides (Fig. 3). The binding isotherm was not extrapolated for 5, since it is an oligomer in solution (see next paragraph and Fig. 4). However, the saturation level of the 5 binding isotherm is similar to that of the 4 peptides, and therefore it is reasonable to assume a partition coefficient of the same scale (10⁻⁴ M⁻¹). Qualitatively, since the fluorescence of NBD-labeled 5 did not dequench upon binding, we qualitatively plotted the binding isotherm, which displayed strong positive cooperativity (Fig. 3, inset), similar to that previously shown for Cry3A 5 (15).

Peptide Oligomerization in Solution—To test whether helices 5 and 4 and its mutants are monomers or oligomers in aqueous solution, we used Rho-labeled peptides. The fluorescence of rhodamine is quenched when several Rho-labeled molecules are in proximity, and the increase in fluorescence after enzymatic cleavage is a result of dissociation of these aggregates. A final concentration of 0.1–1 μM Rho-labeled peptide was added to PBS, and fluorescence levels were measured at 580 nm. After equilibrium was reached, proteinase K was added, and the change in the fluorescence was recorded. Peptide 5 exhibited a strong decrease in fluorescence in PBS at all peptide concentrations, after which the addition of proteinase K caused an increase of almost 3-fold in fluorescence level. The difference in fluorescence before and after proteinase K was added indicates that 5 oligomerizes in aqueous solution. Rho-labeled 4 and its mutants did not show a significant change in fluorescence levels before or after adding proteinase K. Thus, 4 and its mutants seem to be monomers in solution. Fig. 4 shows, for example, the results obtained at a peptide concentration of 1 μM.

Inhibition of the 4-Loop-5 Hairpin Activity—We further tested whether 4 and its mutants or 5 can interfere with the functional assembly of the 4-Loop-5. In these experiments,
the a4-loop-a5 was used at a concentration in which it has ~50% calcein release activity (0.015 mM), and a5 has ~10% activity. The peptides a4 and all mutants were used at the maximal concentration tested in the calcein release assay. Fig. 5 shows the results for PC vesicles. Similar results were obtained with PC/PS vesicles and, therefore, are not shown. The results indicate that F9L a4 has a very strong antagonism on the activity of a4-loop-a5 (Fig. 5). Whereas peptides a5, a4, and mutants I7S and Q8R displayed only weak inhibition abilities, the F9L a4 inhibition of the hairpin was marked; specifically, the activity of a4-loop-a5 was completely abolished when mixed with F9L a4 (Fig. 5). This surprising result occurred even when F9L a4 was incubated with the vesicles before adding the hairpin.

Peptide-Peptide Interaction within the Membrane Milieu—
The ability of the peptides to oligomerize in their membrane-bound state was investigated using FRET. The experiments were performed both with PC and PS/PC vesicles at a molar ratio where 100% binding was expected (Fig. 2) using NBD-labeled peptides as energy donors and Rho-labeled peptides as energy acceptors. Fig. 6 depicts the curves of the experimentally derived percentage of energy transfer versus the bound acceptor/lipid molar ratio. When Rho-labeled Cry1Ac a5 was sequentially added to its NBD derivative, a decrease in the NBD fluorescence and an increase in the rhodamine fluorescence were revealed, suggesting that Cry1Ac a5 self-associates in its membrane-bound state (Fig. 6). Similarly, it was found that the Cry3A counterpart of a5 could also form oligomers when bound to the membrane (15, 17, 19).

The interaction of helices a4 with a5 in the membrane was studied as well. Peptide a4 and each one of its three mutants exhibited resonance energy transfer with a5, indicating that a4 and its mutants hetero-oligomerize with a5 (Fig. 6). In Fig. 6, the curve corresponding to a random distribution of monomers
mRNAs were added to PBS at a final concentration of 1 μM. Fluorescence was measured before and after digestion with protease K. Gray bars represent peptides before cleavage with protease K. Shaded bars represent peptides after cleavage with protease K.

In the current study we show that the Cry1Ac segment α4-loop-α5 is much more active in membrane permeation than either of the hydrophobic helices by themselves (Fig. 4). For example, helix α4 shows no pore-forming activity at all, whereas α5 has low activity (Fig. 4). This provides strong evidence that both helices and the loop connecting them are needed to form the channel and subsequently cause toxicity. According to the crystal structure of the homologue proteins Cry1Ac and Cry3A, the α4-loop-α5 segment is depicted as a dashed line Rho-Cry1Aα and Cry3A, the necessary to form the channel and subsequently cause toxicity. Furthermore, α4 and with WT α5 at a molar ratio of 0.15 peptide/lipid ratio.

(Fig. 4). This may indicate that the α4-loop-α5 is inserted into the membrane and that α4 faces the lum of the channel, whereas α5 is at an outer circle, facing the hydrophobic environment of the membrane milieu. There is concrete evidence to support this model. For example, Aronson and co-workers (23) show that the α4-loop-α5 Cry1Ac. The broken line represents a random distribution of monomers (42) using a Ro of 51Å (17).

Our results show that α5 can create pores (Fig. 4) and homologues (Fig. 6) and that it exhibits a cooperative binding isotherm curve (Fig. 3). In addition, α5 alone can release calcein molecules trapped inside vesicles (Fig. 4), causing perforation of the membrane. However, pore formation activity is extremely high when it is connected to α4. Furthermore, loss of toxicity in Cry1Ac with mutations in α5 was directly correlated with the loss of oligomerization, suggesting that α5 is crucial for membrane oligomerization (44, 23, 45). In short, α5 can play an important role both in membrane insertion and in the oligomerization of several proteins to form a channel. Helix α4 can also bind vesicles, although no cooperativity was observed (Fig. 2A). This can be explained by the more polar nature of α4 compared with α5, which makes it harder for α4 to insert into the membrane. Nevertheless, helix α4 has the ability to oligomerize specifically within the membrane environment, both with α5 and with itself (Fig. 6). In contrast, α4 and its mutants were incapable of membrane permeation, except for the slight ability of F9L α4 (Fig. 4). This may indicate that α4 plays a role in oligomerization within the phospholipid bilayers but is incapable of sufficient membrane insertion without the help of α5.

F9L α4 is a homologue peptide to the F134L mutation on the full-length protein, which was previously shown to have a

FIG. 4. Oligomerization of the peptides in solution. Rho-labeled peptides were added to PBS at a final concentration of 1 μM. Fluorescence was measured before and after digestion with protease K. Gray bars represent peptides before cleavage with protease K. Shaded bars represent peptides after cleavage with protease K.

FIG. 5. Inhibition of calcein release induced by α4-loop-α5 by the addition of shorter peptides. Fluorescence emissions were recorded at 515 nm with 4-nm slits. Shown are peptide α4-loop-α5 (0.015 μM) alone as a control, and combined with the short peptides WT, I7S, Q8R, and F9L α4 and with WT α5 at a molar ratio of 0.15 peptide/lipid molar ratio.

FIG. 6. Peptide-peptide interaction within the membrane. Theoretically and experimentally derived percentage of energy transfer. Transfer efficiencies between donor and acceptor α5 Cry1Ac (empty circles) and Cry1Ac donor α5 and the following Cry1Ac acceptors: filled squares, WT α4; empty squares, I7S; semi-filled squares, Q8R; crossed squares, F9L. Cross circles, FRET between α4 as energy donor and α4 as energy acceptor; empty triangles, negative control donor N36 and acceptor α5 Cry1Ac. The broken line represents a random distribution of monomers (42) using a Ro of 51Å (17).
3-fold increase in toxicity (23). Importantly, the F9L a4 mutant is able to form oligomers within the membrane. It also has cooperative binding and slight membrane permeation capabilities. These facts may imply better insertion into the membrane milieu than the wild type (Fig. 2, A and D, insets). Substitution of an aromatic amino acid, which prefers the type two hairpins, thus interfering with channel formation. Wild

The pore-forming activity of F9L a

a protein undergoes a transformation. Domain I binds the mem-

E1 (27).

a interacting with the segments that form the pores. This mode providing the first example that a mutated helix of a pore-

a forming toxin can function as an immunity protein by directly

provides the first example that a mutated helix of a pore-

is able to form oligomers within the membrane. It also has

4 mutants showed an energy transfer with

4 and the loop play a major role in the insertion of the

membrane proteins as well.

REFERENCES

1. Cramer, W. A., Cohen, F. S., Merrill, A. R., and Song, H. Y. (1990) Mol.

Microbiol. 4, 519–526

2. Bayley, H. (1997) Curr. Biol. 7, 763–764

3. Lesieur, C., Voevo-Demjen, B., Abdali, L., Fivaz, M., and Gissou van der Goot, F. (1997) Mol. Membr. Biol. 14, 45–64

4. Bhakdi, S., Bayley, H., Valeva, A., Waley, I., Walker, B., Kehoe, M., and Palmer, M. (1996) Arch. Microbiol. 163, 73–79

5. Cabiaux, V., Wolf, C., and Ryssevacht, J. M. (1997) Int. J. Biol. Macromol. 21, 285–288

6. Rajamohan, F., Lee, K. M., and Dean, D. H. (1998) Prog. Nucleic Acids Res.

Membr. Biol. 60, 1–27

7. Lee, D., Carroll, J., and Ellar, D. J. (1991) Nature 352, 815–821

8. Grochulski, P., Masson, L., Borissova, S., Puszta-Carey, M., Schwartz, J. L., Brousseau, R., and Czyger, M. (1995) J. Mol. Biol. 254, 447–464

9. Li, J., Kon, P. A., and Ellar, D. J. (1996) J. Mol. Biol. 257, 129–152

10. Schesser, J. H., Kramer, K. J., and Bulla, L. A., Jr. (1977) Appl. Environ.

Microbiol. 33, 878–880

11. Slatin, S. L., Abrams, C. K., and English, L. (1990) Biochem. Biophys. Res.

Commun. 169, 765–772

12. Vachon, V., Paradie, M. J., Marsolais, S., Schwartz, J. L., and Laprade, R. (1995) J. Membr. Biol. 148, 57–63

13. Knight, P. J., Crickmore, N., and Ellar, D. J. (1994) Mol. Microbiol. 11, 429–436

14. Dean, D. H., Rajamohan, F., Lee, M. K., Wu, S. J., Chen, X. J., Alcantara, E., and Hussain, S. R. (1996) Gene 179, 111–117

15. Gazit, E., La Rocca, P., Sansom, M. S., and Shai, Y. (1998) Proc. Natl.

Acad. Sci. U. S. A. 95, 12289–12294

16. Masson, L., Tabashnik, B. E., Liu, Y. B., Brousseau, R., and Schwartz, J. L. (1999) J. Biol. Chem. 274, 31936–32000

17. Gazit, E., and Shai, Y. (1993) Biochemistry 32, 3429–3436

18. Gazit, E., Bach, D., Kerr, I. D., Sansom, M. S., Chejanovsky, N., and Shai, Y. (1994) Biochem. J. 304, 895–902

19. Gazit, E., and Shai, Y. (1995) J. Biol. Chem. 270, 2571–2578

20. Cummings, C. E., Armstrong, G., Hodgman, T. C., and Ellar, D. J. (1994) Mol.

Membr. Biol. 11, 87–92

21. Aronson, A. I., Wu, D., and Zhang, C. (1995) J. Bacteriol. 177, 4059–4065

22. Uawithya, P., Tuntitipawan, T., Katzenmeier, G., Panym, S., and Angsuthanaombo, C. (1998) Biochem. Mol. Biol. Int. 44, 825–832

23. Manoj Kumar, A. S., and Aronson, A. I. (1999) J. Bacteriol. 181, 6103–6107

24. MacKinnon, R., Cohen, S. L., Kuo, A., and Chait, B. T. (1998) Science 280, 106–109

25. Ben-Efraim, I., and Shai, Y. (1996) Protein Sci. 5, 2287–2297

26. Ben-Efraim, I., and Shai, Y. (1997) Biophys. J. 72, 85–96

27. Zhang, Y. L., and Cramer, W. A. (1993) J. Biol. Chem. 268, 10176–10184

28. Choma, C., Gratkowski, H., Lear, J. D., and DeGrado, W. F. (2000) Nat. Struct.

Biol. 7, 161–166

29. Xiao Zhou, F., Cocco, M. J., Russ, W. P., Brunger, A. T., and Engelman, D. M. (2000) Nat. Struct. Biol. 7, 154–160

30. Merrifield, R. B., Vizioli, L. D., and Boman, H. G. (1982) Biochemistry 21, 5020–5031

31. Rapaport, D., and Shai, Y. (1991) J. Biol. Chem. 266, 23769–23775

32. Shai, Y., Bach, D., and Yanovsky, A. (1990) J. Biol. Chem. 265, 20202–20209

33. Allen, T. M., and Cleland, L. G. (1980) Biochemistry 19, 418–426

34. Pouy, Y., Rapaport, D., Mor, A., Nicolas, P., and Shai, Y. (1992) Biochemistry 31, 14216–14223

35. Schwarz, G., Gerke, H., Rizzo, V., and Stankowski, S. (1987) Biochem. J. 252, 685–692

36. Schwarz, G., Stankowski, S., and Rizzo, Y. (1986) Biochim. Biophys. Acta 861, 141–151

37. Rizzo, V., Stankowski, S., and Schwarz, G. (1987) Biochemistry 26, 2751–2759

38. Beschaaschivil, G., and Seelig, J. (1990) Biochemistry 29, 52–58

39. Gazit, E., and Shai, Y. (1995) Biochemistry 34, 12363–12371

40. Chen, X. J., Curtiss, A., Alcantara, E., and Dean, D. H. (1995) J. Biol. Chem.

270, 6412–6419

41. Schwarz, J. L., Juteau, M., Grochulski, P., Czyger, M., Prefontaine, G., Brousseau, R., and Masson, L. (1997) FEBS Lett. 410, 387–402

42. Fung, B. K., and Stryer, L. (1978) Biochemistry 17, 5241–5248

43. Kligler, Y., and Shai, Y. (2000) J. Mol. Biol. 295, 163–168

44. Wu, D., and Aronson, A. I. (1992) J. Biol. Chem. 267, 2131–2137

45. Aronson, A. I., Geng, C., and Wu, L. (1999) Appl. Environ. Microbiol. 65, 2503–2507

46. Sipos, L., and von Heijne, G. (1993) Eur. J. Biochem. 213, 1333–1340

47. Landolt-Marti, C., Wills, K. A., Deber, C. M., and Reithmeier, R. A. (1993) J. Mol. Biol. 229, 692–698

48. Manolios, N., Bonifacino, J. S., and Klausner, R. D. (1990) Science 249, 274–277

49. Engelmann, D. M. (1996) Science 274, 1850–1851

50. Cramer, W. A., Engelmann, D. M., Von Heijne, G., and Rees, D. C. (1992) FASEB J. 6, 3397–3402

51. MacKenzie, K. R., Prestegard, J. H., and Engelmann, D. M. (1997) Science 276, 131–133