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In Vitro Studies of Membrane Protein Folding

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Abbreviations: **AFM**, atomic force microscopy; **bO**, bacterio-opsin; **bR**, bacteriorhodopsin; **CCT**, CTP:phosphocholine cytidylyl transferase; **CHAPS**, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; **CHAPSO**, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxyl-1-propanesulfonate; **CMC**, critical micelle concentration; **DGDG**, digalactosyl diacylglycerol; **DHPC**, L-α-1,2-dihexanoylphosphatidylcholine; **DM**, *n*-decyl β–D-maltoside; **DMPC**, L-α-1,2-dimyristoylphosphatidylcholine (C14 saturated chains); **DMoPC**, L-α-1,2-dimyristoyleoylphosphatidylcholine (C14:1 chains with one *cis* unsaturated bond at position 9); **DOPC**, L-α-1,2-dioleoylphosphatidylcholine (C18:1 chains with one *cis* unsaturated bond at position 9); **DOPE**, L-α-1,2-dioleoylphophatidylethanolamine (C18:1 chains with one *cis* unsaturated bond at position 9); **DOPG**, L-α-1,2-dioleoylphophatidylglycerol; **DPC**, dodecylphosphocholine; **DPoPC**, L-α-1,2-dipalmitoleoylphosphatidylcholine (C16:1 chains with one *cis* unsaturated bond at position 9); **DPoPE**, L-α-1,2-dipalmitoleoylphosphatidylethanolamine (C16:1 chains with one *cis* unsaturated bond at position 9); **DPPG**, L-α-1,2-dipalmitoylphosphatidylcholine (C16 saturated chains); **DSC**, differential scanning calorimetry; **GuHCl**, guanidine hydrochloride; **LHCII**, major photosynthetic light harvesting complex of higher plants; **MGDG**, monogalactosyl diacylglycerol; **OG**, octyl-β–D-glucopyranoside; **OmpA**, *Escherichia coli* outer membrane protein A; **OmpF**, *Escherichia coli* outer membrane protein F; **PC**, phosphatidylcholine; **PE**, phosphatidylethanolamine; **PEG**, polyethylene glycol; **PG**, phosphatidylglycerol; **PGP-Me**, 2,3-diphytanyl-sn-glycerol-1-phospho-3'-sn-glycerol-1'-methylphosphate; **PM**, purple membrane; **POPC**, 1-palmitoyl-2-oleoyl-phosphatidylcholine; **TM**, transmembrane

ABSTRACT: The study of membrane protein folding is a new and challenging research field. Consequently, there are few direct studies on the *in vitro* folding of membrane proteins. This review covers work aimed at understanding folding mechanisms and the intermolecular forces that drive the folding of integral membrane proteins. We discuss the kinetic and thermodynamic studies that have been undertaken. Our review also draws on closely related research, mainly from purification studies of functional membrane proteins, and gives an overview of some of the successful methods. A brief survey is also given of the large body of mutagenesis and fragment work on membrane proteins, as this too has relevance to the folding problem. It is noticeable that the choice of solubilizing detergents and lipids can determine the success of the method, and indeed it appears that particular lipid properties can be used to control the rate and efficiency of folding. This has important ramifications for much *in vitro* folding work in that it aids our understanding of how to obtain and handle folded, functional protein. With this in mind, we also cover some relevant properties of model, lipid-bilayer systems.

I. INTRODUCTION

Integral membrane proteins constitute around 30% of all cell proteins, are of fundamental biological importance, and form major targets for drug development. The study of these membrane proteins represents one of the major challenges in modern day molecular biology research. The number of gene sequences for these proteins is steadily increasing, which contrasts sharply with our lack of knowledge of the final three-dimensional structures of these proteins, as well as of their biosynthesis and folding within the membrane.

Protein folding has been at the forefront of molecular cell biology research for a number of years. However, the work in this field has been almost exclusively aimed at water-soluble proteins. Integral membrane proteins have been excluded largely because they are considerably more difficult to work with. This is due to their hydrophobicity and the problems involved in mimicking their native, anisotropic environment. Furthermore, with regard to folding work, even in the fruitful land of water-soluble proteins, the research has centered on small proteins of about 100 to 150 amino acids, while many membrane receptors and channels are two, three, or four times larger. No such integral mem-

brane receptor protein or channel protein has yet been refolded *in vitro* from a denatured state. Nevertheless, work on membrane proteins is indeed possible, and there has been a considerable amount of excellent work in this field, which has resulted in several impressive breakthroughs. The pioneering studies of Khorana and colleagues in the 1980s showed that an integral membrane protein (bacteriorhodopsin) could be refolded from a fully denatured state to a functional state *in vitro* (Landen and Khorana, 1982). This remains the only case where an α helical membrane protein has been fully unfolded and refolded *in vitro.*

This review covers *in vitro* membrane protein folding studies and reveals the rich variety of innovative approaches that have been taken. Membrane protein folding is a field in its infancy, and one that is expanding. This involves the development of novel techniques and the formation and testing of new hypothesis. This differs to certain aspects of water-soluble protein folding, where the success of many established methods means that much of the work has become a data collection exercise on the folding and misfolding of individual proteins. Hopefully, some of the large body of highly trained and talented protein folders can now be persuaded to face the challenges involved in elucidating the folding and function of integral membrane proteins. Nobody, however, should be under any illusion about the reality of membrane protein work, as can be gauged from an entertaining and informative review (von Heijne, 1999).

Studies on membrane protein folding *in vitro* are of interest at several levels. Intellectually it is an intriguing problem. Incorrect folding, maturation, and processing of integral membrane proteins are also associated with several disease states. However, perhaps the greatest challenges lie in bridging the gap between *in vitro* studies on individual proteins in simple systems to those on larger, and more intact, *in vitro* systems and ultimately *in vivo*. Ideally, we wish to know the biogenesis, structure, and function of any protein *in vivo*. The complexity of natural systems means that currently, however, membrane proteins have to be isolated from the membrane in order to obtain molecular level information on their structure and dynamics.

Another huge area of interest lies in drug development and biotechnology. Two of the largest problems with membrane proteins are, first, the difficulty in obtaining large amounts of wild-type and mutant proteins from overexpression systems and second, in maintaining protein solubility and function. Expression is usually in heterologous host organisms where the protein aggregates, or only a small amount of the protein inserts into the host membrane. Improvements in methods to solubilize and refold membrane protein aggregates or to improve the targeting to host membranes are required. The folded state also has to be maintained in some sort of detergent/lipid membrane mimic. An understanding of the factors responsible for correct folding therefore is vital for any biophysical or pharmaceutical study on a membrane protein.

Biosynthetic pathways in the cell make use of protein integration machinery to incorporate membrane proteins into the membrane. These are complex systems, and a number of fundamental questions await answers, including how transmembrane (TM) domains are recognized and folded in the bilayer in their correct orientation. There have been a number of reviews covering recent advances in our understanding of this protein incorporation in the endoplasmic reticulum, mitochondria, thylakoid, and bacterial membranes (High and Laird, 1997; Johnson and van Waes, 1999; Neupert, 1997; Rapoport, Rolls and Jungnickel, 1996; Robinson and Mant, 1997; von Heijne, 1997). Insertion of proteins into the endoplasmic reticulum occurs co-translationally

at sites termed translocons, which are also responsible for secretory protein translocation. These translocons consist of several membrane proteins that form a pore into which nascent polypeptide chains enter cotranslationally from the ribosome. It is not clear how the TM domains of multispanning membrane proteins exit this translocon pore into the bilayer. Some evidence points toward all TM domains exiting together, maybe in a largely folded state. There does, however, seem to be the distinct possibility that TM α helices fold individually within the translocon but exit as single helices or in pairs. This means that the final TM helix packing and protein folding occurs in the membrane bilayer. An idea that is relevant here is the two-stage model that simplifies the folding of α helical membrane proteins into two stages (Popot and Engelman, 1990; Popot and Engelman, 2000). Independently stable TM α helices form in the first stage as they are stable entities within a hydrophobic bilayer. These helices then pack together in the second stage and form the final protein fold. This model is based on thermodynamic arguments and therefore does not necessarily represent a mechanism that is followed *in vivo* or *in vitro*. The two-stage model does, however, imply that TM helices, which emerge individually from the translocon, would be individually stable in the membrane and could then pack to form the final protein. Furthermore, *in vitro* studies have also shown that not all TM helices of a protein have to be stable on their own (Hunt et al., 1997), but that bundles of 2, 3, or more helices are sometimes needed to help the remaining helices both form and pack.

The ability to manipulate α helical membrane proteins in the manner suggested by the two-stage model has important implications for *in vitro* work that involves solubilizing membrane protein aggregates during overexpression, or devising lipid/detergent systems that maintain protein solubility and function. It allows the problem to be broken down, and instead of aiming for a folded state in a single step it should be possible to use one solvent mixture, first to achieve an intermediate stage with some independently stable TM helices. The solvent may then be altered to allow these helices to pack and facilitate the final folding of the protein. The two-stage model applies only to helical proteins, and although TM α helical proteins may dominate membrane protein structures, structure based on β barrels also occur, particularly in outer membrane proteins (for a recent overview see [Schulz, 2000]). Some of these β proteins, like porins, seem to follow a fairly simple folding process partly because these proteins have a low hydrophobicity when compared with many α helical proteins. It has been suggested that partial folding of porins occurs in the aqueous phase, with the membrane-destined regions forming loops that then fold to give a barrel on insertion into the membrane.

The ever-increasing interest in membrane proteins and the constantly changing technology means that by no means all the relevant work on membrane protein folding can be covered here. This review covers work aimed at understanding the folding mechanisms of membrane proteins as well as achieving optimum folding and function *in vitro*. Membrane protein folding is still at a developmental stage, and methods need to be found both to unfold and refold proteins as well as to obtain many important folding parameters. The current status of methods used to refold proteins and to study the stability of the folded state and the folding kinetics are covered. At present, much of the work pertinent to membrane protein folding does not in fact come directly from folding studies, but also indirectly from nearly any membrane protein work. Isolation, solubilization, or molecular biology of these proteins generally involves working out how to fold, or maintain a fold, during

purification. Some of these approaches are highlighted. It is perhaps not surprising from the two-stage model that α helical membrane proteins can frequently be assembled from helix fragments, which allows information to be gained on the nature of helix interactions. This is discussed briefly together with an overview of site-directed mutagenesis studies that can also shed light on folding interactions.

Finally, the solvent is important for membrane proteins. The membrane environment actually offers the chance to control folding through manipulation of lipid forces, as well as using this to understand some of the driving forces for folding. Thus we can begin to decipher some of the weird and wonderful detergent/lipid mixtures used in protein isolation. Indeed manipulation of lipid properties by biological membranes seems to be important for function. Dynamic assemblies of certain lipids and cholesterol, known as lipid rafts, provide a relatively ordered lipid environment compared with that surrounding the raft where a different lipid mixture is present (Simons and Toomre, 2000). Current hypotheses include a central role for rafts in signaling by accumulating particular proteins for signal transduction. Thus, we devote a section to lipid properties and their interaction with membrane proteins, discussing what is known at a molecular level from model membrane studies, and how they can affect protein conformation and function.

II. HOW DO YOU UNFOLD AND REFOLD AN INTEGRAL MEMBRANE PROTEIN *IN VITRO*?

How to refold a membrane protein from a denatured state *in vitro* remains an unanswered question for the vast majority of integral membrane proteins. To date, only three proteins have been successfully refolded *in vitro* from a fully denatured state. However, much information on the folding problem can be gained from the vast number of studies on membrane protein purification and reconstitution, and some pertinent examples of this are discussed in this section. We begin this section with a brief outline of some of the detergent/lipid systems that have proven successful for folding membrane proteins *in vitro*. This is followed by details of the conditions for folding the fully, or partially, unfolded membrane proteins: bacteriorhodopsin (bR), the major light harvesting complex of higher plants (LHCII), *Escherichia coli* diacylglycerol kinase (DGK) and the *Escherichia coli* outer membrane proteins OmpA and OmpF. We then give a brief overview of some alternative approaches that have been used to fold or purify functional membrane proteins. These include results of overexpression studies and various methods that have been used to alter the protein stability or solubility, either through changes to the protein itself or to the lipid/detergent environment. The methods discussed in this section are summarized in tabular form at the end, together with some specific protein examples.

A. Denaturant-Induced Unfolding, Detergent/Lipid-Induced Refolding

Folding from a completely denatured state has currently only been achieved for three membrane proteins; the archaebacterial proton pump bR (Huang et al., 1981; London and Khorana, 1982) and the *E. coli* outer membrane proteins OmpA and OmpF (porin) (Eisele and Rosenbusch, 1990; Schweitzer, Hindennach, and Henning, 1978; Surrey and Jähnig, 1992). These successes demonstrate that the information

necessary to define the final fold is present in the amino acid sequence, or at least this is the case for these three proteins.

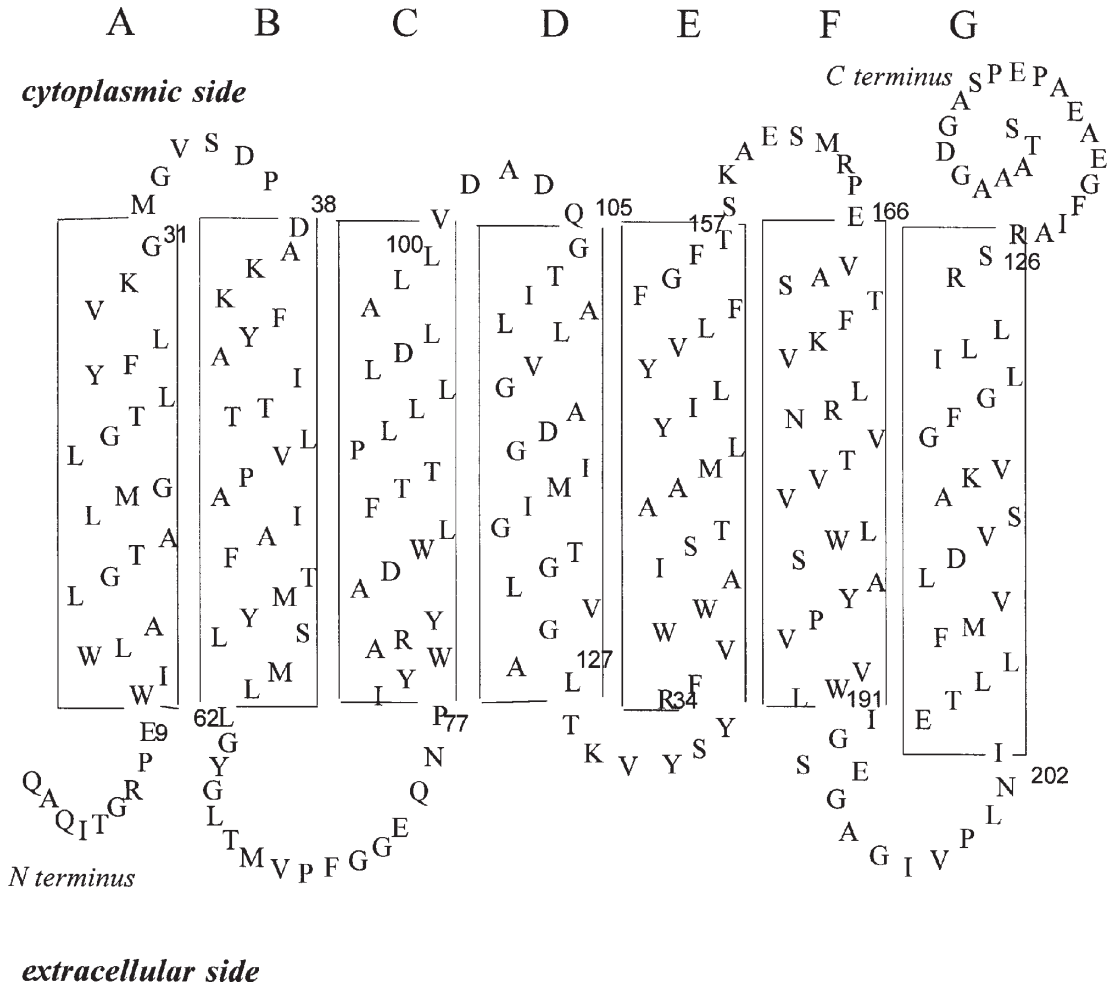
These proteins that can be folded *in vitro* have also been amenable to crystallographic study. BR naturally forms a crystalline array in the membrane, which made it ideal for 2D crystallization (Grigorieff et al., 1996; Henderson et al., 1990). More recently bR has been the focus of a new 3D crystallization technique using lipid cubic phases (Landau and Rosenbusch, 1996), and this has resulted in structures with resolutions of 1.9 Å (Belrhali et al., 1999) and 1.55 Å (Luecke et al., 1999). High-resolution structures of 2.5 Å and 2.4 Å have also been reported for OmpF (Cowan et al., 1992) and the membrane domain of OmpA (Pautsch and Schulz, 1998), respectively.

BR is an α helical protein, while OmpA and OmpF fold to β barrel structures in the membrane (see Plate 1*). This structural difference is reflected in the different methods that have been used to denature the proteins. The hydrophobic, α helical protein, bR (see Plate 1a and Figure 1a), cannot be folded in a single step from a fully denatured state into a lipid-bilayer environment. A partially denatured state in SDS has proven useful in studies of bR folding into both detergents and lipid systems (Huang et al., 1981; London and Khorana, 1982; Popot, Gerchman and Engelman, 1987; Curran, Templer, and Booth, 1999; Booth, 1997; Booth, 2000). Other α helical proteins have also been found to fold from an SDS- or LDS-denatured state with additional appropriate detergents, for example, the major light-harvesting complex of higher plants, LHCII (Paulsen, Rümler and Rüdiger, 1990; Plumley and Schmidt, 1987), minor light-harvesting complexes CP26 and CP29 (Ros, Bassi, and Paulsen, 1998) (Giuffra et al., 1996) and *E. coli* diacylglycerol kinase (DGK) (Lau and Bowie, 1997) (see Figure 1b and c). In contrast to the situation with α helical proteins, β barrel proteins such as OmpA and OmpF currently provide the only opportunities to study the folding of a membrane protein from a completely denatured state directly into a lipid-bilayer environment. About half of the residues in the membrane of OmpA and OmpF are hydrophilic and as a result these proteins can be completely unfolded in aqueous urea or GuHCl (Eisele and Rosenbusch, 1990; Schweitzer et al., 1978; Surrey and Jähnig, 1992; Surrey, Schmid, and Jähnig, 1996). These fully denatured β barrel proteins can then be refolded and inserted directly into lipid-bilayer vesicles merely by diluting the denaturant with the lipid vesicles (Jähnig and Surrey, 1997). A good summary of other β barrel proteins that are amenable to folding in this manner has been provided in Table 1 of Buchanan 1999a.

1. Bacteriorhodopsin, bR

BR is 248 amino acids long and consists of a tightly packed bundle of 7 TM α helices with a covalently bound retinal cofactor within the helix bundle (Plate 1a). It is the only protein constituent of the purple membrane (PM) of *Halobacterium salinarium,* where it exists as an hexagonal array of trimers. Here we focus on the ability to unfold and refold monomeric bR *in vitro* in detergents and lipids. There has also been excellent work, which falls outside the immediate scope of this review on the reversible binding of retinal and accompanying protein conformational changes in the native membrane environment (see, for example, Oesterhelt, Meentzen, and Schumann, 1973; Gärtner et al., 1983; Schreckenbach, Walckhoff, and Oesterhelt, 1977, and a review by Haupts, Tittor, and Oesterhelt, 1999) as well as the

* Plate 1 appears following page 528.



A.

FIGURE 1. Two-dimensional representations of the secondary structures of (a) bacteriorhodopsin, (b) the light harvesting complex, LHCII, and (c) Diacylglycerol kinase, DGK. Boxed regions approximate the regions of transmembrane and surface α helices.

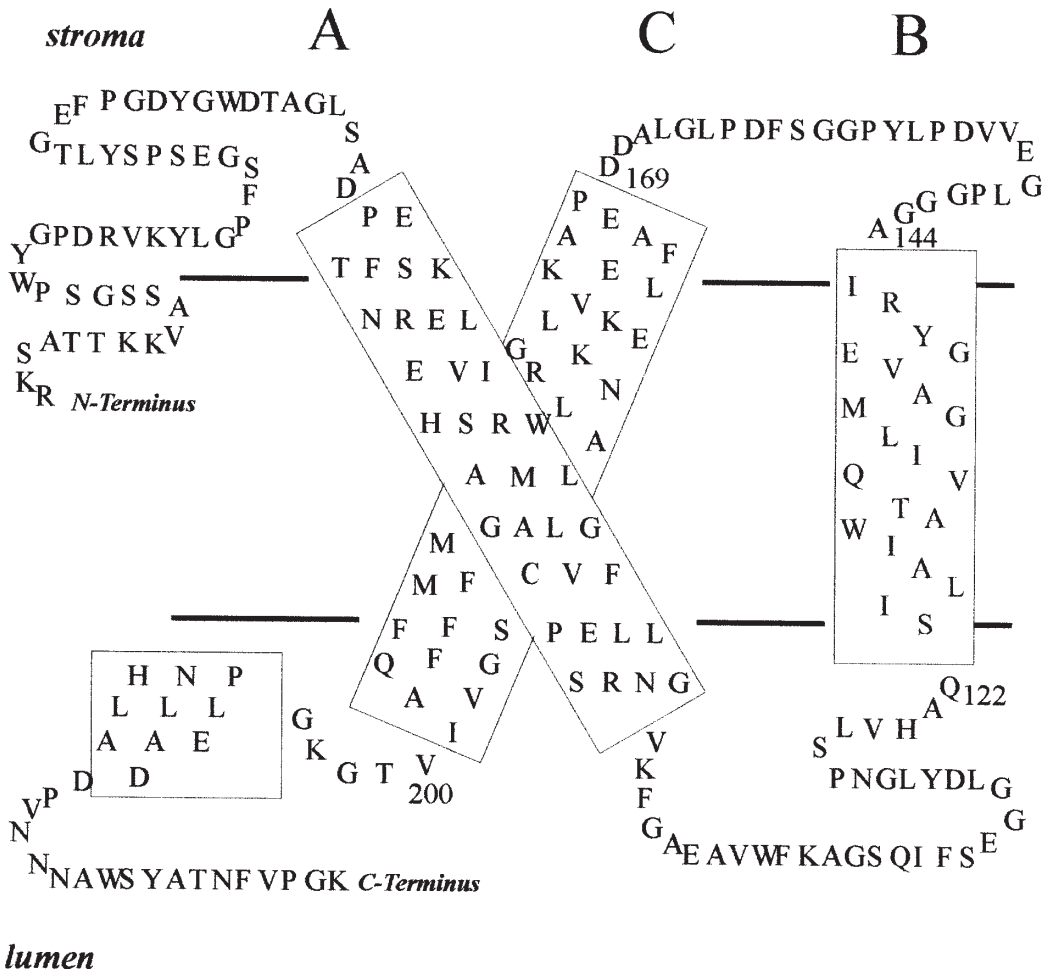


FIGURE 1B

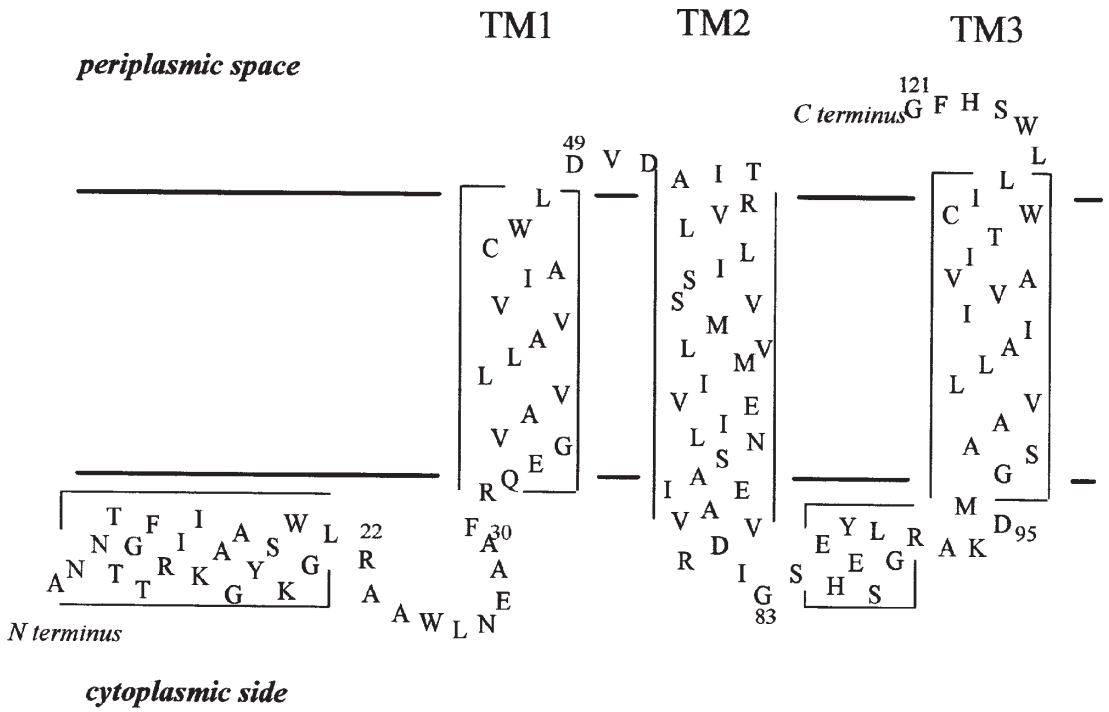


FIGURE 1C

TABLE 1
Some Successful Methods for Folding Overexpressed or Denatured Integral Membrane Proteins *in vitro*

Protein	**Size, structure & function**	**Solubilisation of overexpressed protein or denaturation of protein**	**Formation of native-like protein**	**Folding yields and assays**
Halobacterial bacteriorhodopsin bR	• 248 amino acids and a covalently bound retinal chromophore • 7 TM α helices • light-driven protein pump	• Chloroform/methanol/tricthylamine extraction of *E.coli* membrane fragments (Braiman *et al.*, 1987) • Urea solubilisation of maltose-binding fusion protein from inclusion bodies in E.coli (Chen & Gouaux, 1996) • Apoprotein can be fully denatured in trifluoroacetic acid (Huang *et al.*, 1981) • Apoprotein partially denatured in SDS, leaving ~55% of native helix content (Huang *et al.*, 1981; Riley *et al.*, 1997)	• Initially transferred from organic acid to SDS and then folded by mixing with DMPC/CHAPS or DMPC/DHPC micelles, lipid vesicles e.g. DMPC, DOPC, containing retinal (Booth *et al.*, 1997; Curran *et al.*, 1999; London & Khorana, 1982) • Denatured apoprotein can be co-dissolved with lipids in organic solvent, transferred to aqueous buffer and then retinal added (Braiman *et al.*, 1987; Huang *et al.*, 1981; London & Khorana, 1982). • Various method of regenerating PM from apoprotein in native membrane environment (Fischer & Oesterhelt, 1980; Gärtner *et al.*, 1983; Haupts *et al.*, 1999; Oesterhelt, Meentzen & Schumann, 1973; Schreckenbach, Walckhoff & Oesterhelt, 1977; Schreckenbach, Walckhoff & Oesterhelt, 1978) and following protein biogenesis and lattice formation *in vivo* (Dale & Krebs, 1999; Isenbarger & Krebs, 1999; Krebs *et al.*, 1997).	• Folding yields 80-100% (Booth, 2000; London & Khorana, 1982) • *In situ* functional assay of photocycle kinetics or proton pumping, also formation of purple chromophore (Booth, 2000; London & Khorana, 1982) • Native-like hexagonal bR lattice can be made from folded state (Popot *et al.*, 1987)

TABLE 1 (continued)

Major light harvesting complex of higher plants, LHCII	• 232 amino acids. 12 chlorophyll and 3 xanthophyll pigments non covalently bound • 3 TM α helices and 1 short amphipathic helix at membrane surface • photosynthetic light harvesting protein	• SDS solubilisation of E.coli inclusion bodies or urea solubilisation of His-tag protein from *E.coli* inclusion bodies (Paulsen *et al.*, 1990; Rogl. H *et al.*, 1998) • Apoprotein partially denatured in SDS, leaving about 30% of native helix content (Paulsen *et al.*, 1993)	• Reconstituted with thylakoid membrane extracts by freeze thaw (Plumley & Schmidt, 1987) • Reconstituted in OG micelles containing pigments, or mixed OG/lipid micelles (e.g. PG, DGDG) & precipitation of LDS (Booth & Paulsen, 1996; Paulsen & Hobe, 1992; Reinsberg *et al.*, 2000) • Ni affinity chromatography of urea solubilised His-tag apoprotein, wash with LDS and elution by OG and pigments (Rogl. H *et al.*, 1998)	• Folding yields of ~100% can be achieved e.g. on reconstituting LHCII with thylakoid membrane extracts (Paulsen *et al.*, 1990). • Energy transfer can be assessed *in situ* and non-denaturing SDS-PAGE gels of pigment-bound complex assessed, although difficult to quantify (Booth & Paulsen, 1996; Paulsen *et al.*, 1990; Reinsberg *et al.*, 2000) • Folded state can be crystallised and electron microscopy reveals structure similar to native (Hobe *et al.*, 1994)
E.coli diacylglycerol kinase, DGK	• 121 amino acids • postulated structure-3 TM α helices and 2 cytoplasmic α helices • Probably functions as trimer • Phosphorylates diacylglycerol to give phosphatidic acid	• His-tag protein overexpressed in *E.coli* solubilised in Empigen or DM (Gorzelle *et al.*, 1999; Lau & Bowie, 1997) • Apoprotein slightly denatured by SDS, leaving ~85% of native helix content (Lau & Bowie, 1997)	• Ni affinity chromatography of urea solubilised His-tag protein and elution with DM (Wen *et al.*, 1996). However low activity found for single Cys mutants in DM. Significant increase in activity obtained when eluted by DPC and reconstituted into DPC/POPC vesicles (Gorzelle *et al.*, 1999)	• Functional assay of enzyme activity • Native-like specific activity obtained (Gorzelle *et al.*, 1999; Lau & Bowie, 1997)
E. coli transporter, EmrE	• 110 amino acids • postulated structure-4 TM α helical structure • Probably functions as an oligomer • H^+-coupled multi-drug transporter	• Chloroform/methanol extraction of E.coli membranes (Yerushalmi *et al.*, 1996)	• Protein co-dissolved with lipids in organic solvent and transferred to aqueous buffer (Yerushalmi *et al.*, 1996)	• Functional assay-H^+ driven transport of ethidium or methylviologen (Yerushalmi *et al.*, 1996)

E. coli outer membrane protein OmpA	• 325 amino acids • membrane domain of 170 amino acids in 8 stranded β barrel • Exact function unknown, possibly gives cell structural stability, may form a channel	• Urea-solubilisation of *E.coli* inclusion bodies (Surrey & Jähnig, 1992) • Protein completely denatured in urea (Surrey & Jähnig, 1992)	• Folding in lipid vesicles (e.g. DMPC) by mixing the urea-denatured state with the vesicles (Surrey & Jähnig, 1992)	• No obvious functional assay • Yields of about 100% estimated from SDS-PAGE and protection from trypsin digestion (Jähnig & Surrey, 1997)
E. coli outer membrane protein OmpF (porin)	• 340 amino acids • 16 stranded β barrel • Functions as a trimer • Forms pore in outer membrane	• Urea-solubilisation of *E.coli* inclusion bodies (Surrey *et al.*, 1996) • Protein completely denatured in urea or GCL (Eisele & Rosenbusch, 1990; Surrey *et al.*, 1996)	• Poor folding in lipid vesicles on mixing the urea-denatured state with the vesicles. Folding yields increased if detergent used (e.g. DM/DMPC mixed micelles) (Eisele & Rosenbusch, 1990; Surrey *et al.*, 1996)	• Yields of about 80% obtained in lipid/detergent mixtures, e.g. DM/DMPC or octyl-pentaoxythelene /soybean lipids (Eisele & Rosenbusch, 1990; Surrey *et al.*, 1996) • Functional channels obtained after reconstitution into planar bilayers (Eisele & Rosenbusch, 1990)
Neisseria meningitidis class 3 porin PorB	• 331 amino acids • probably 16 stranded β barrel which functions as a trimer	• Urea-solubilisation of *E.coli* inclusion bodies (Qi *et al.* 1994) • Partially denatured in SDS, with ~15% α helix content (Minetti *et al.* 1997; 1998)	• Folding from urea-denatured state in zwittergent 3-14 and detergent concentration reduced to 0.05% by gel filtration chromatography (Buchanan, 1999a; Qi *et al.* 1994) • Reversible folding from SDS into zwittergent 3-14 (Minetti *et al.* 1997; 1998)	• Functional assay • Full reversible folding/unfolding in SDS/zwittergent 3-14 (Minetti *et al.* 1997; 1998)
E. coli outer membrane phosphlipase A OMPLA[1]	• 269 amino acids • 12 stranded β barrel • Activated by dimerisation	• Urea-solubilsation of *E. coli* inclusion bodies (Buchanan, 1999a; Dekker *et al.* 1995)	• Folding on dilution into Triton X-100.	• Functional assay-activity is regulated by reversible dimerization (Snijder *et al.*, 2001) • Crystals obtained for monomer and dimer (Snijder *et al.* 1999)
E. coli ferric enterobactin receptor FepA[1]	• 724 residues • 22 stranded β barrel • iron transporter	• Urea-solubilisation of *E.coli* inclusion bodies (Buchanan, 1999a;Buchanan, 1999b)	• Urea exchanged for SDS/zwittergent 3-14 mixture and detergent concentration reduced to 0.05% by gel filtration chromatography	• Functional assay

[1] Further details and examples of refolding procedures for β barrel membrane proteins are well summarized by Buchanan, 1999.

insertion and assembly of bR in PM *in vivo* (see, for example, Dale, Angevine, and Krebs, 2000; Dale and Krebs, 1999; Isenbarger and Krebs, 1999; Krebs, Li, and Halambeck, 1997).

Bacteriorhodopsin has a high average hydrophobicity and is completely denatured to a random coil-like state in organic acids, such as trifluoroacetic acid (Huang et al., 1981). Denaturants that destroy the structure of most water-soluble proteins have nowhere near the same effect on bR. For example, even completely delipidated apoprotein (bacterio-opsin, bO) is resistant to denaturation by 8 *M* GuHCl (Huang et al., 1981). Trifluoroacetic acid or 88% acetic acid are used to denature the delipidated protein to a random coil-like state. Such drastic denaturants have also been found to be effective at unfolding TM helices of other membrane proteins. In order to fold the protein, the acid-denatured bO state is first transferred into SDS, which is achieved by neutralizing the acid and dialyzing against SDS (Huang et al., 1981). The protein can then be transferred into a variety of renaturing detergent micelles, mixed detergent/lipid micelles or lipid vesicles, by diluting or precipitating the SDS (Booth et al., 1995; Huang et al., 1981; Liao, London and Khorana, 1983; London and Khorana, 1982; Popot et al., 1987). bR spontaneously folds under these conditions in the presence of retinal. Folding yields of about 95% can be readily obtained at pH6, for example, in mixed L-α-1,2-dimyristoylphosphatidylcholine (DMPC)/3-[(3-cholamidopropyl)dimethylammonio-1-propanesulfonate (CHAPS) micelles (Booth et al., 1995), DMPC/L-α-1,2-dihexanoylphosphatidylcholine (DHPC) micelles (Riley et al., 1997) native lipid vesicles (Popot et al., 1987), DPoPC vesicles (Curran et al., 1999), and DMPC vesicles (Curran et al., 1999; Popot et al., 1987). Reversible refolding of bR in mixed DMPC/CHAPSO/SDS micelles has also been demonstrated (Chen and Gouaux, 1999).

bR folding yields are generally determined from the extent of recovery of a native-like, purple chromophore absorption band at about 560 nm (Booth and Farooq, 1997; London and Khorana, 1982; Lu and Booth, 2000). The folded bR is monomeric and shows native-like activity. The formation and decay of the M intermediate, which is a key step in the proton-pumping photocycle, exhibits similar characteristics to wild-type protein. Proton pumping has also been demonstrated for folded bR in DMPC/CHAPS micelles (Alexeiv et al., 1994). The photocycle kinetics of folded monomeric bR, as well as that isolated from PM, are dependent on the detergent/lipid environment. Thus, while > 95% of the folded bR has native-like activity, the actual "folded state" of the protein has slight conformational differences in different folding environments. This probably reflects a change in the distribution of thermally accessible folded conformations as the detergent/lipid environment is altered.

SDS induces helix formation in proteins and forms mixed micelles with other detergents, or partitions into lipid vesicles. For example, the SDS-denatured apoprotein state of bR has an α helical content equivalent to about 3 or 4 of the 7 native TM helices. This is no doubt why SDS has proven successful in the folding of bR: it promotes α helix formation and then aids the direct transfer and insertion of these helices to a membrane-like environment. SDS is, however, not always needed in refolding α helical membrane proteins. It has been possible to omit SDS from the bR unfolding/refolding reaction by co-dissolving delipidated apoprotein directly with lipids in organic solvent. The solvent can then be evaporated and the bO/lipid mixture resolubilized in aqueous buffer. BR is regenerated by the addition of retinal and yields of about 85% can be achieved in DMPC or DOPC vesicles (Meijberg, W., Curran, A.R., Templer, R.H., and Booth, P.J.,

unpublished data). This type of approach has also been reported for the EmrE transporter of *E. coli* (Yerushalmi, Lebendiker, and Schuldiner, 1996). This is thought to be a 4 TM helix bundle protein that functions as an oligomer in the membrane. EmrE is soluble in chloroform/methanol mixtures and can be reconstituted from this organic solvent by first mixing with lipids, then evaporating the chloroform/methanol solvent, and finally rehydrating the protein/lipid mixture.

2. Light Harvesting Complex, LHCII

LHCII is 232 residues long and binds about 12 chlorophylls and 3 xanthophylls. It has 3 TM α helices and a short amphipathic helix on the membrane surface (Kühlbrandt, Wang, and Fujioshi, 1994) (see Figure 1b). About a third of this native helicity is present in its SDS-denatured state (Paulsen, Finkenzeller, and Kühlein, 1993).

The method used to fold LHCII *in vitro* is essentially the same as that described above for bR, although different detergents and pH are used. LHCII can be reconstituted in detergent solution by folding its previously denatured apoprotein in the presence of Chls and xanthophylls. The apoprotein is denatured in SDS or LDS and then transferred into renaturing octylglucoside (OG) micelles that contain LHCII's pigments, by diluting or precipitating the dodecylsulfate (Booth and Paulsen, 1996; Paulsen et al., 1993). The denatured protein does not fold unless the pigments are present. LHCII folding and assembly is accompanied by the establishment of efficient energy transfer between its bound Chls, which can be monitored by changes in Chl fluorescence. Formation of the folded, functional state is shown by native-like energy transfer from Chl *b* to Chl *a*, which can be measured by a loss of Chl *b* fluorescence, together with an increase in Chl *a* fluorescence after excitation of Chl*b*. The *in vitro* folding of LHCII is very sensitive to the experimental conditions and is critically dependent on the pigment-protein ratio and the reactant concentrations. Folding is performed in the presence of DTT, and the optimum folding conditions are pH 9 with 12.5% sucrose present. Lipids are not required for LHCII formation *in vitro*, as the complex can be assembled in OG alone. However, the inclusion of lipids in the OG micelles increases the thermal stability of the folded state (Reinsberg et al., 2000). This is not specific to a particular lipid and similar effects are seen on folding the protein in mixed OG/lipid micelles containing either the native thylakoid lipids MGDG or DGDG or synthetic lipids DPPG or DOPG. This is despite the fact that DGDG and a native PG lipid seem to have specific interactions with LHCII *in vivo*.

3. Diacylglycerol Kinase, DGK

DGK is shorter than LHCII, with only 121 residues and is thought to have 3 TM α helices and 2 cytoplasmic α helices at the membrane surface (see Figure 1c). Reversible unfolding has been demonstrated for DGK in mixed micelles of *n*-decyl β–D-maltoside (DM) and SDS (Lau and Bowie, 1997). Unfolding DGK with SDS results in only about a 15% decrease in the helix content and probably no or very little loss of the TM helices (Lau and Bowie, 1997).

4. Outer Membrane Proteins, OmpA and OmpF

OmpF is 340 amino acids long and forms a trimer in the membrane (see Plate 1b*).

* Plate 1 appears following page 528.

Each monomer consists of a β barrel of 16 antiparallel strands and forms a water-filled pore through the membrane. OmpA consists of 325 amino acids, is monomeric, and has a periplasmic domain as well as a membrane domain. The membrane domain consists of about 170 amino acids and is a β barrel with 8 antiparallel strands (Pautsch and Schulz, 1998). OmpA and OmpF can both be denatured to a random coil-like state in urea or to an α helical state in SDS (Eisele and Rosenbusch, 1990; Jähnig and Surrey, 1997; Surrey and Jähnig, 1992). Spontaneous insertion and folding of OmpA occurs on diluting the urea with detergent micelles (above the critical micelle concentration, CMC) or lipid vesicles (in the fluid phase) (Jähnig and Surrey, 1997; Kleinschmidt, Wiener, and Tamm, 1999b). These outer membrane proteins can also be refolded from as SDS-denatured state, although the SDS induces some helical content in the unfolded state. Reversible refolding of another outer membrane protein, PorB class 3 protein of *Neisseria meningitidis* has also been demonstrated from a SDS- or heat-denatured state to a functional folded state in the detergent zwittergent 3-14 (Minetti et al., 1997).

The exact function of OmpA is unknown, and thus there is no obvious functional assay for the folded state. Trypsin digestion is used to determine the amount of protein that has inserted into the bilayer and is protected from the aqueous phase. Urea-denatured OmpA is completely denatured by trypsin, while the native, membrane-incorporated form is digested down to a 24 kDa, membrane-protected fragment. The urea-denatured and native forms also run differently on gels, at about 35 and 30 kDa, respectively. These gel electrophoresis and trypsin results are used as a measure of the yield of folded protein, although they are not definitive as they only give an idea of the relative mobility on SDS PAGE gels and how much protein is protected from trypsin. For example, it may be that some folding of the protein occurs outside the membrane and this results in trypsin protection, as has been observed for water-soluble β sheet proteins. These assays also do not measure the folding of the C terminal domain of this protein (residues 172 to 325). One hundred percent "folding" yields (based on the trypsin and gel assays) can be obtained at pH10 in small unilamellar vesicles (~30 to 50 nm diameter) of DMPC, POPC, DOPC and mixed DMPC/DMPG and POPC/POPG vesicles (Jähnig and Surrey, 1997; Kleinschmidt and Tamm, 1996; Rodionova et al., 1995). Essentially no protein insertion is found for larger vesicles with diameters of about 200 nm.

OmpF can be folded in a similar manner to OmpA, although the optimum pH is 6 and low yields of about 15% are found for spontaneous folding from urea directly into lipid vesicles. The yield can be increased to about 75 or 80% when mixed detergent/lipid mixtures such as DM/DMPC are used (Eisele and Rosenbusch, 1990; Surrey et al., 1996). These high yields occur at a 1:1 molar ratio of DM to DMPC, which corresponds to the transition from a vesicle to micelle structure. Porin has also been denatured in 6 *M* GuHCl and renatured with yields of about 80% in octyl-pentaoxythelene and soybean PC lipid mixtures followed by removal of the detergent by dialysis (Eisele and Rosenbusch, 1990). Purified, renatured OmpF trimers form functional channels after reconstitution into planar bilayers.

B. Other Approaches to Unfolding/Refolding Membrane Proteins

1. Hints from Overexpression and Purification Studies

Alternative approaches to the folding of partially unfolded or misfolded states of

membrane proteins have resulted from the purification of overexpressed protein. This may well be where most research on integral membrane proteins has been focussed, but as it is not the main subject of this particular review only a few examples are highlighted here.

Not many membrane proteins have been overexpressed at high yields (i.e., in milligram amounts). It is not surprising that the proteins that can be overexpressed in a heterologous host are frequently found to insert rather poorly into the host membrane and generally form inclusion bodies or other insoluble aggregates (Grisshamer and Tate, 1995). These aggregates have to be solubilized and the protein folded into a functional state, if the overexpressed protein is to be useful. This task has turned out to be decidedly tricky, especially for α helical proteins.

a. Urea, SDS, and Inclusion Bodies

The conditions for folding overexpressed outer membrane proteins from such protein aggregates have been reviewed recently (Buchanan, 1999a). The simple, up and down β barrels such as OmpA (with antiparallel strands where each successive strand is adjacent) seem to be relatively easy to renature from inclusion bodies (Schulz, 2000). As discussed above for OmpA and OmpF, the protein is usually disaggregated and unfolded by urea denaturation (or sometimes GuHCl). A variety of methods (tabulated in Buchanan, 1999) have been used to fold urea-denatured or GuHCl-denatured outer membrane proteins in detergent (or lipid vesicles for OmpA and OmpF).

The fact that bR, LHCII, and CP26 can be solubilized in, and folded from, SDS has allowed efficient purification of functional protein from *E. coli* expression systems (Braiman et al., 1987; Chen and Gouaux, 1996; Khorana, 1988; Paulsen et al., 1990; Rogl. H. et al., 1998; Ros et al., 1998). BO can be extracted from *E. coli* membranes with chloroform/methanol/triethylamine, and the *E. coli* lipids are then removed by phase extraction in chloroform methanol at pH7 (Braiman et al., 1987). The resulting bO can then be purified by ion exchange on DEAE-trisacryl, again in chloroform/methanol, and the pure protein transferred to SDS. Regeneration efficiencies of over 80% are obtained when this SDS-solubilized protein is folded in DMPC/CHAPS micelles containing retinal.

The apoprotein of LHCII, LHCP, seems to form inclusion bodies in *E. coli*, which are solubilized by heating in LDS or SDS and then reconstituted in thylakoid membrane extracts by freeze thaw cycles (Paulsen et al., 1990; Plumley and Schmidt, 1987). It is possible to fold the SDS-solubilized LHCP by adding pigments in OG micelles and removing the dodecylsulfate by precipitation (Paulsen et al., 1990).

b. His-tags and Ni(II) Affinity Columns

Elution of proteins from Ni(II) columns with particular detergent/lipid micelles seems to provide an effective way of obtaining pure, folded protein from solubilized inclusion bodies. An approach reported for LHCP, involves solubilization of inclusion bodies of His-tagged LHCP in 8 *M* urea and the subseqent folding of the protein on the Ni(II) affinity column. After the LHCP has been loaded onto the Ni(II) column, the column is washed with LDS followed by OG. Finally, the column is washed with OG containing all of LHCII's pigments, and the folded LHCII is then eluted (Rogl., H et al., 1998).

DGK from *E. coli* can also be over-expressed with a His-tag in *E. coli* and the cell extracts solubilized in Empigen or OG and subsequently loaded onto a Ni(II) column. Functional DGK can then be recovered in DM micelles by equilibration and elution of the protein off the column with DM. Several single Cys mutants of DGK were, however, found to have low activities when purified by this method. Further activity losses occurred on reconstitution into 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) lipid vesicles. Many of the Cys mutants with low activity (55 out of 65 Cys mutants investigated) were found to fold to a state with a significantly increased activity if they were eluted off the Ni(II) column by dodecylphosphocholine (DPC) and then reconstituted into mixed DPC/POPC vesicles (Gorzelle et al., 1999).

c. Fusion Proteins and Inclusion Bodies

Overexpression of α helical membrane proteins is frequently hampered by the protein attempting to insert into the host membrane. Water-soluble fusion proteins have been used to increase overexpression yields through the formation of inclusion bodies.

Partial folding of a G-protein coupled receptor has been reported, following overexpression in *E. coli*. An oderant receptor was overexpressed to unusually high yields (about 20 mg of pure receptor per liter of culture) as a glutathione-*S*-transferase (GST) fusion protein together with a His-tag (Kiefer et al., 1996). The yield of expressed protein was apparently achieved by incorporating three positive charges into the short loop between the first two postulated TM α helices, which normally has no positive charges. Short loops with positive charges have been found to be very hard to translocate across bacterial membranes (Gafvelin and von Heijne, 1994). The introduction of these three positive charges into the first loop of the oderant receptor therefore would block translocation of this loop and encourage high-level expression of the protein as inclusion bodies. The GST fusion was also found to be necessary for expression. The fusion protein was solubilized in sarcosine and digitonin and purified on a Ni(II) affinity column. The GST fusion was then cleaved, the receptor reconstituted into phosphatidylcholine (PC)/phosphatidylglycerol (PG) lipid vesicles, and the digitonin removed by an Extractigel column. This seemed to result in formation of at least part of the receptor's tertiary structure as some binding of a native-ligand analogue was demonstrated. However, this ligand binding could not be quantified, and it is not possible to assess how much of the purified receptor attained tertiary structure, or how much native tertiary structure resulted.

bO has also been expressed as a maltose-binding fusion protein that forms inclusion bodies that can be solubilized in urea (Chen and Gouaux, 1996). Following cleavage of the maltose-binding protein in the presence of urea and SDS, the protein is purified by size exclusion chromatography and subsequently folded in DMPC/CHAPS micelles containing retinal.

2. Modifying the Membrane Protein

Membrane proteins frequently exhibit low stabilities and activities in detergent micelles (Bowie, 2001). This is a major stumbling block to most biochemical and biophysical studies and is highlighted in folding studies where appropriate solubilization conditions must be found to both unfold and refold the protein, prevent ag_

gregation of the often highly hydrophobic proteins, and at the same time result in stable, active states.

Attempts have been made to modify membrane proteins to increase their stability outside the native membrane. A simple screening method has been developed to detect stable mutants of DGK, based on their thermal stability (Zhou and Bowie, 2000). Twelve single point mutations at 8 different sites in the protein were found to improve the protein stability. A combination of 4 of these mutations gave a protein that was about 18 times more stable in OG micelles than the most stable single point mutant, and significantly more stable than wild type (a half life of 35 min at 80°C as opposed to 6 min for wild type at 55°C) (see also Sections IV.A and VI.A)

Another approach to make integral membrane proteins more amenable to study has been to reduce their hydrophobicity and therefore alter their solubility in aqueous solution. As a first step to making bR more soluble in aqueous solution, 5 polar or charged residues have been introduced into helix D. These new residues, together with some native residues, give an uninterrupted stripe of polar groups on the lipid-exposed face of this helix (Chen and Gouaux, 1997). The resulting protein could fold to a functional state in lipid/detergent micelles but denatured more rapidly than wild-type protein at raised temperatures. This presumably reflects the poorer solubilization and stabilisation of the protein by the detergent micelle when one helix is more hydrophilic. Another attempt to increase the solubility of membrane proteins in water has been to attach polyethylene glycol (PEG) to bR (Sirokman and Fasman, 1993) and porin (Wei and Fasman, 1995). Both PEG-protein conjugates are water soluble and can be folded to a native-like state despite the PEG. For example, bR-PEG was found to be partially denatured in water, with CD spectroscopy indicating an α helical content that was about 75% of that of folded bR. The bR-PEG conjugate could be folded in DMPC/CHAPS micelles, but only if it was incubated in SDS first. Thus, effectively this is similar to the folding discussed above for bR, where SDS-denatured bR is folded in DMPC/CHAPS micelles.

3. Modifying the Refolding Detergent/Lipid System

The protein stability in detergent micelles can be improved by adding lipids, or by moving away from micelles altogether and working in lipid vesicles. This is conventionally done in reconstitution work where detergents are used frequently to isolate and purify membrane proteins. The protein is then transferred to detergent/lipid vesicles and the detergent removed by dialysis. With regard to folding studies, the incorporation of several different lipids into OG micelles have been found to increase the thermal stability of folded LHCII (Reinsberg et al., 2000). SDS-denatured bR can be folded directly into lipid vesicles, thus allowing the folding to be studied in these vesicles, rather than mixed detergent/lipid micelles (Curran et al., 1999).

The properties of the lipids can also be used to increase bR stability. For example, the thermal stability of bR increases as the amount of L-α-1,2-dioleoylphosphatidylcholine (DOPC) in the vesicles is increased (W. Meijberg, A.R. Curran, R.H. Templer, and P.J. Booth, unpublished data). BR can be regenerated in mixed DMPC/DOPC vesicles in the absence of any SDS. In 100% DMPC vesicles the midpoint of the unfolding transition is about 80°C, and virtually no folded protein can be detected above 85°C. However, increasing the mole fraction of DOPC to about 0.05 increases the unfolding transition to 88°C,

with a substantial amount of folded protein being present at 85°C. The increase in DOPC content could increase the stored curvature elastic energy of the bilayer and the lateral pressure that the lipid chains impose on the helix bundle of the folded protein. This may in turn increase the stability of the helix bundle (see also Sections IV.A and VI.E).

The lipid properties also affect the efficiency of bR folding. Folding yields of about 95% are obtained when SDS-denatured bO is folded in lipid vesicles consisting solely of DOPC, L-α-1,2-dipalmitoleoylphosphatidylcholine (DPoPC) or DMPC (in the fluid, L_α phase). However, the yield drops as phosphatidylethanolamine (PE) is introduced into the lipid vesicles (Curran et al., 1999). For example, the folding yield is 56% in DOPC/L-α-1,2-dioleoylphophatidylethanolamine (DOPE) vesicles with a 0.16 DOPE mole fraction. There are several possible explanations for this that are currently under investigation. In these PE/PC mixtures an increase in the DOPE content of the PC/PE vesicles increases the lateral pressure imposed on the protein by the lipid chains (see Section VI.B). Such an increase in pressure could hinder the insertion of the protein into the bilayer. Alternatively, the pressure increase could hinder folding within the bilayer and thereby affect the folding yield. Increasing the PE content does seem to slow a rate-limiting folding step of bO (A.R. Curran, S.J. Allen, W. Meijberg, R.H. Templer, and P.J. Booth, unpublished data, and see Section VI.E).

The optimization of lipid properties may also explain the behavior of DGK in DM or DPC/POPC mixtures discussed earlier (see section II.B. on overexpression and His tags). Reconstituting DPC-solubilized DGK mutants in POPC vesicles leads to higher protein activities than either in DM micelles or when reconstituting from DM-solubilized protein (Gorzelle et al., 1999). Presumably an appropriate lipid lateral pressure is required to retain an optimal activity of the mutant proteins and prevent any misfolding, and this is easier to achieve in a lipid-bilayer vesicle rather than a detergent micelle. Furthermore, the protein fold has to be retained while incorporating the protein into the lipid vesicle. A lyso lipid like DPC may be better at aiding this incorporation than a detergent like DM. Including lyso lipids in a POPC bilayer vesicle will lower the lateral pressure in the chain region of POPC and thus facilitate insertion of the protein.

The matching of the hydrophobic thickness of the protein and the lipid bilayer may be another important factor in membrane protein folding. Whereas this question has yet to be addressed directly with regard to folding, the effect of hydrophobic mismatching between membrane proteins and the host membrane system has been studied for a number of systems. The effect of a hydrophobic mismatch between bR in PM (i.e., membrane patches with native lipids present) on reconstitution into different lipid bilayers has been investigated (Piknova, Marsh, and Thompson, 1997). The PM sample was incorporated into PC lipid systems of different chain lengths (C12-C18 chain lengths) and the phase behavior of lipid system investigated. When the hydrophobic thickness of the lipid was less than that of the protein (e.g., C12 and C14 PC), the lipids were able to compensate by elongating their alkyl chains and becoming more rigid. This led to an increase in the gel to fluid phase transition temperature. Conversely, if the hydrophobic thickness of the host lipid (e.g., C18 PC) was greater than that of the protein, the reverse effect was seen. The alkyl chains attempt to reduce their length by increasing the fluidity of the chain region, thus resulting in a lower than expected gel to fluid phase transition temperature. The phase transition temperature of the C16 PC remained constant, indicating that the hydrophobic thickness of the PM and the lipids were

closely matched. SDS-denatured bO has also been refolded into PC lipid vesicles of different chain length (Curran et al., 1999). No hydrophobic mismatch effect was found for folding into DMPC (C14 chains), DPoPC (C16:1 chains), or DOPC (C18:1 chains), with over 90% folding yields in each case. (The hydrophobic thickness of these lipid systems probably differs by about 4 Å.) The effect of hydrophobic mismatch on gramicidin, a small peptide that forms a well-defined channel, has been examined recently (Harroun et al., 1999). Gramicidin was also able to stretch or thin its host membrane by as much as 2.5 Å to reduce any mismatch. Several other studies have addressed the many possible effects of hydrophobic mismatches for membrane-inserted peptides, as reviewed by Killian (1998).

4. Mechanical Unfolding

Atomic force microscopy (AFM) provides an alternative to the chemical denaturant approaches described above, and as a single molecule force microscopy can be used to measure the force required for the unfolding of individual proteins. The potential of this method has been realized recently with bR. PM can be imaged by AFM to reveal the array of bR trimers and the cytoplasmic surface can be differentiated from the extracellular surface (Möller et al., 1999). Pushing an AFM tip onto the cytoplasmic surface of a protein with a contact force of about 1 nN for 1 s results in adsorption of a single protein to the tip in 15% of all cases (Oesterhelt et al., 2000). The single protein hole left in the bR lattice can then be imaged. This approach offers the chance to investigate the mechanical unfolding of individual bR molecules (discussed further in Section IV.A).

C. Summary of Methods Used to Unfold/Fold Membrane Proteins: Urea or Guanidine Hydrochloride vs. SDS

Some of the methods that have been discussed here for the folding of integral membrane proteins are summarized in Table 1. For the proteins presented in this table, successes have come from SDS denaturation or solubilization of overexpressed protein for α helical proteins or urea solubilization for the β barrel proteins. SDS only partially denatures bR, LHCII, and DGK, while many β barrel proteins are more or less fully denatured in urea.

The very nature of hydrophobic, α helical membrane proteins such as bR means that it is unfeasible to obtain a structureless state in a predominantly aqueous environment. Moreover, any denaturants that denature the hydrophobic protein will also destabilize the renaturing membrane, and equally an unfolded helix inside a membrane bilayer is energetically unfavorable (Lemmon et al., 1997; Popot and Engelman, 1990). Single-span (or slightly longer) helical peptides/proteins may at present provide a more informative approach to investigate the spontaneous folding and insertion of TM α helices. These short peptides can have sufficient hydrophilic residues to render them denaturant/water soluble.

Folding studies of multispanning helical proteins from a completely denatured state therefore are an unrealistic goal. Fortunately, this is also an unnecessary goal, one reason being that most membrane proteins do not appear to fold from a completely denatured state and insert into the bilayer unassisted *in vivo*. Thus, the problems outlined above are avoided *in vivo* because as outlined in the introduction, the proteins seem to fold co-translationally inside a protein complex in the membrane,

termed the translocon (Johnson and van Waes, 1999). The details of this *in vivo* folding process are unknown, but the current evidence suggests that rather than folding spontaneously and inserting directly into the bilayer, TM α helices form inside the translocon and then move laterally into the bilayer. In some cases all the helices may emerge together, but it seems more likely that the helices leave the translocon either individually or in small groups with the final helix packing and tertiary folding then occurring in the bilayer itself. Thus, helix formation within the translocon and helix packing within the bilayer can to a certain extent be considered as separate events, as suggested by the thermodynamic-based, two-stage model for helical membrane protein folding (Popot and Engelman, 1990). This means that a denatured state, such as in SDS, with a substantial amount of native TM α helical structure is a good starting point for an *in vitro* folding study of a multispanning α helical membrane protein because it allows the second stage of helix packing in a mock membrane environment to be investigated.

The lipid/detergent environment used for folding is critical for high yields of functional, folded protein, for both α helical and β barrel proteins. It may be possible to gain some idea of which lipids will be useful. These can be synthetic lipids that are not native to the membrane of the protein in question. Section VI of this review is devoted to nonspecific lipid properties that are likely to be very relevant to solving the problem of obtaining high yields of functional membrane proteins *in vitro*. Assessing the final folded state of the protein can be decidedly tricky because structural data on membrane proteins are so hard to obtain. A functional test together with some structural information is probably the best solution to this at present.

III. *IN VITRO* EXPERIMENTAL STUDIES OF PROTEIN FOLDING KINETICS

Kinetic studies are the route to molecular and mechanistic information on folding, and the transition states and intermediates states that are involved. The folding kinetics of only two membrane proteins have been studied in detail: the α helical protein, bR and the β barrel protein OmpA. This is not surprising in view of the fact that there so few proteins have been shown to be amenable to *in vitro* folding. Transient folding intermediates have been identified for both bR and OmpA, but information on transition states has yet to emerge for membrane proteins. Some kinetic studies have also been performed on the folding of the major plant light harvesting complex, LHCII.

Here we review the experimental methods that can be used to determine the folding kinetics of membrane proteins. We also include a discussion of artifacts that can very easily arise during these studies, together with ways of dealing with such problems. We then discuss in detail the results obtained for bR and OmpA and finally give an overview of the information that has been gained for LHCII. Summaries are given as tables or diagrams in each section.

A. Experimental Methods

One problem in a kinetic study is how to trigger the folding reaction. This has been achieved for both bR and OmpA by mixing the denatured protein with the renaturing detergents or lipids and thereby diluting the denaturant. In the case of bR this has been adapted for stopped-flow mixing studies that have a millisecond dead time. Other problems in kinetic folding studies lie in follow-

ing and quantifying the formation of the final folded state. There is no method to measure a high-resolution structure of the protein under the experimental folding conditions, or is it always possible to measure the protein's function under these reaction conditions. Thus other, less specific, structural probes have to be used, ideally with some sort of functional assay. Formation of a native-like purple chromophore is used for bR, as this is indicative of a functional state. The ability of the folded state to pump protons in micelles or vesicles can also be measured. Far UV CD also indicates when native secondary structure is recovered during bR folding, but this occurs before the correctly folded, functional state forms so cannot be used to follow the final folding steps. The formation of native-like β structure (as determined by far UV CD) is used together with SDS gel electrophoresis and trypsin digestion to distinguish unfolded and membrane-inserted states of OmpA and OmpF. Unfortunately, these SDS gel and trypsin methods are not definitive measures (see Section II.A). The channel function of OmpF can be measured, but so far this has only been done after the folding reaction in DM/DMPC mixed micelles is complete and the protein has been transferred to planar membranes, which could conceivably alter the protein conformation.

There is currently no good method to follow protein structural changes during membrane protein folding. The methods that are generally used to follow the folding of water-soluble proteins are not immediately applicable to membrane proteins. For example, NMR coupled with H/D exchange is generally not possible because the protein/detergent/lipid species are too large for high-resolution NMR spectroscopy. Furthermore, H/D exchange rates within a membrane environment can be slow and occur at similar rates to those within a folded protein. Thus, there is not necessarily a large difference in exchange rates between folded and unfolded states in a membrane. Another method that has been very useful in folding studies of water-soluble proteins is ϕ value analysis (Fersht, 1998). This analysis relies on making single point mutations throughout the protein. A mutation tends to alter the free energy difference between the unfolded and folded states, and this is compared to any change that is found between the transition (or intermediate) state and the unfolded state. Several factors hinder ϕ value analysis of membrane proteins, which include the lack of atomic resolution structures, efficient overexpression systems, and reversible unfolding conditions. However, it may be worth embarking on this type of approach for bR, as there is now an atomic resolution structure for the protein. Furthermore, high yields of mutant protein can be obtained and the folding of SDS-denatured protein in DMPC/CHAPS is reversible. A quantitative analysis will still be limited by the difficulty in measuring forward and reverse rates for the folding/unfolding process as well as the lack of straightforward methods to assess the structures of the mutant bR proteins. The interpretation of equilibrium denaturation curves is also complicated by changes in the bR/SDS/DMPC/CHAPS interactions that occur during folding but are currently not fully understood.

Optical spectroscopy is the main method that has been used to follow the folding of bR and OmpA. Most emphasis has been placed on intrinsic protein fluorescence because of the convenience and high sensitivity of this technique. The quenching of Trps by brominated lipids, with the bromines located at different depths in the bilayer, can also be used to follow Trp movements within the bilayer. Transient absorption spectroscopy has also been used for bR to follow the changes in the retinal absorbance during binding and folding and recently atomic force microscopy (AFM) has been

used to remove and unfold single bR molecules from their native membrane.

B. Potential Artifacts

1. Light Scattering from Micelles and Vesicles

A potential problem with optical spectroscopy of membrane proteins is light scattering by the detergent micelles and lipid vesicles. Lipid vesicles are milky suspensions and frequently are of 200 nm, 100 nm, or 50 nm diameter and therefore scatter at UV and visible wavelengths. There is even significant scatter from micelles, which are usually of the order of only 5 to 10 nm diameter. The degree of light scattering also changes with time because the vesicles are not at equilibrium. Small changes in the light scatter are observed that correspond to changes in micelle or vesicle size of about ± 5 to 10% over a few seconds (although this depends on the lipids/detergents present). The use of stopped-flow mixing also seems to affect the vesicle size over short times, no doubt as a result of the rapid mixing under pressure. At very long times the size of the vesicles changes as the vesicles begin to fuse together.

It is not possible to remove the light scattering by micelles or vesicles, but it can be taken into account by the development of methods to monitor the changes in light scatter over time. Again this is not straightforward because the extent of light scatter from a protein-containing vesicle can behave differently to a non–protein-containing species (Curran et al., 1999). The changes in light scattering can be followed by time resolving the characteristic light-scattering absorption spectra (that can, for example, approximate to a Rayleigh function of λ^{-4}) (Booth and Farooq, 1997). Dynamic light scattering can be used to follow changes in vesicle size over longer times of several minutes to hours (Curran et al., 1999). Although this latter method is probably not sensitive enough to pick up small changes in scattering if there is more than one population of vesicles and the size distribution is large. Even when the light scattering or size of the micelles or vesicles can be measured over time, the underlying molecular changes in the species remain unknown, as does any resulting effect on the protein. Therefore, time constants determined from changes in protein fluorescence must be correlated with those determined from changes in light scattering of the micelles or vesicles. The protein fluorescence can change either as a result of protein folding events, or as a result of a change in the immediate detergent or lipid environment of the protein tryptophans or tyrosines. Further checks of the micelle or vesicle behavior should also be made, for example, by time-resolving changes in the fluorescence of dyes (in the presence and absence of protein) (Booth et al., 1995).

2. Circular Dichroism Spectroscopy

Circular dichroism spectroscopy can be applied to membrane proteins, although again this is potentially fraught with artifacts (Swords and Wallace, 1993). One problem is differential absorption flattening, which arises from an inhomogeneous distribution of the protein in the detergent micelles or lipid vesicles (Mao and Wallace, 1984). This can generally be overcome with high detergent or lipid to protein ratios that dilute the protein in the micelles or vesicles and reduce protein/protein aggregation. The lipid concentration must, however, be low enough to avoid saturating the UV detector,

as the lipids absorb light in the far UV where the measurements are made. Differential light scattering presents another difficulty for CD measurements. Any difference in the scatter of left and right circularly polarized light shows up as a signal in the CD spectra of both micelle and vesicle samples, particularly in the far UV. This is a significant problem for both micelles and vesicles, and cannot generally be taken into account by subtracting a baseline of the micelle or vesicle sample without the protein present, because the detector collects too little of the scattered light and the extent of light scattering can be different with and without the protein present. Differential scattering is actually a straightforward problem to solve. The detector should be moved close to the sample cuvette so that the detector angle increases to 90° (Mao and Wallace, 1984; Riley et al., 1997). This means that much of the scattered light is collected (because it is mainly scattered in the forward direction). Most CD spectrometers are not, however, adapted for membrane proteins, and unfortunately most CD studies overlook this problem. The scattering problem is assumed to be insignificant with small vesicles or micelles, and baselines are usually subtracted. This assumption is rarely justified. As outlined above, baseline subtraction is unlikely to solve the problem and the spectral distortions can be subtle from small vesicles and micelles. The lack of scattering artifacts therefore ought to be demonstrated.

Kinetic CD measurements suffer from an added problem because the micelles and vesicles can also change structure, shape, and size over time. Monitoring at a single wavelength such as 224 nm or 206 nm to follow changes in α or β content is insufficient, not only because of the overlap of CD signals from α and β structure, but because of all the potential pitfalls in membrane protein CD. It is desirable to increase the accuracy of the data by measuring over a larger spectral range, down to 190 nm (or below with synchrotron sources). This precludes the use of several detergents, or high lipid concentrations, which have a significant absorbance below 200 nm. Lipids generally absorb less in the far UV than detergents. Short-chain lipids such as DHPC, which also form micelle structures, can be used instead of detergents for measurements down to 190 nm.

Far UV CD can be a reliable method for following changes in membrane protein secondary structure when all the above precautions are taken into account (Riley et al., 1997). Measurements in lipid vesicles are also possible if the vesicles are small (<50 nm), and their concentration can be kept low enough to avoid saturating the detector, while maintaining an appropriate lipid/protein ratio (Mielke and Wallace, 1988).

3. Micelle or Vesicle Composition

The molecular nature and physical properties of the protein-containing micelles or vesicles that are used in any membrane protein work are generally unknown. This lack of knowledge prohibits a molecular level interpretation of much membrane protein work, including folding studies. Nevertheless, meaningful data can still be obtained. It is also worth aiming for a detailed molecular knowledge of folding process, and from this point of view lipid-bilayer systems provide the best refolding environment. Detergents, however, frequently provide a more practical solution at present, because they are easier to handle and their micelle structures scatter less light than lipid vesicles. The benefit of lipids is the wealth of information available on the structure, dynamics, and phase behavior of many

common biological lipids. Specific lipid properties therefore can be altered in a known manner to control and improve the efficiency of protein folding. This knowledge base is generally for purely lipid systems and although it can be applied to protein/lipid systems, it would be advantageous to have information on the structure and dynamics of the protein/lipid systems themselves.

Most of the kinetic studies on bR folding have been performed using an SDS-denatured state of the protein and folding in mixed micelles that contain DMPC and DHPC lipids (Booth, 1997). The extent of partitioning of the SDS into the DMPC/DHPC micelles and the structure of the resulting SDS/DMPC/DHPC/protein micelles is unknown. Similarly, this SDS partitioning is a unknown for the refolding experiments in lipid vesicles, which also involve an SDS-denatured state (Curran et al., 1999). Furthermore, very little is known about how the folding of a membrane protein in lipid vesicles affects the vesicle properties. For example, if two lipids are used, such as PE and PC, to alter the bilayer properties, it is unknown how the protein affects the distribution of the PE and PC in the vesicles. It is also clear from the bR vesicle work that the incorporation of the protein alters the light scattering by the vesicle. Counterintuitively, the scattering of the protein-containing vesicles appears less than that of the non-protein-containing vesicles. There is also an extremely subtle variation in the light scattering between different vesicle preparations that are made in an apparently identical manner. This is particularly evident in vesicles that are extruded to give a narrow size distribution. The broader distribution that is obtained from sonication presumably results in a broader scattering spectrum, and therefore it is harder to see the change in behavior of one particular vesicle size. Brominated lipids are sometimes incorporated into lipid vesicles and used as quenchers of protein fluorescence, but these will also alter the lipid vesicle dynamics, although to what extent is currently unknown. In general, more data are needed on the phase behavior of protein/lipid systems.

The above difficulties with lipid vesicles mean that it is necessary to be particularly vigilant with kinetic measurements. For example, the same vesicle preparation should be used for as many experiments as possible, the vesicles should be used a certain time after they have been extruded, or sonicated, the vesicle size distribution should be determined, and the experimental precision estimated using different vesicles and protein preparations. At present, temperature-dependent data ought to be interpreted cautiously. The experimental systems are complex and include several components such as protein, two lipid components/brominated lipids, detergent, buffers, etc. It is unknown how changes in temperature affect the dynamics of each individual component or the lipid vesicle as a whole. Thus, it is problematic to differentiate the activation energies of protein structural changes from those of the lipids, vesicles, or surroundings.

C. Summary of Methods Used to Study Folding Kinetics

The methods that have been used to study the folding kinetics of integral membrane proteins are summarized in Table 2.

D. Examples of Proteins Whose Kinetics Have Been Examined

1. Bacteriorhodopsin, bR

BR is a 7TM α helical protein that binds a retinal chromophore covalently. The

TABLE 2
Techniques Used to Monito the Folding Kinetics of Integral Membrane Proteins *In Vitro* as Well as Background Changes in the Lipid/Detergent Environment

Method	Information	Comments	Results & Examples
Light scattering	• Micelle and vesicle size distributions	• Dynamic light scattering can give sizes and distributions, but can be imprecise for large size distributions and if there is more than one size population • Time-resolved absorption spectra are very useful as they show small changes in light scattering over time, and are probably vital for stopped-flow studies. • Results depend on the lipids, buffer, pH etc and should be checked for each case.	• Changes in light scattering occur over timescales of milliseconds to hours for lipid vesicles and are more obvious for vesicle samples with narrow size distributions • Protein-containing vesicles scatter light differently to non-protein containing vesicles (Curran *et al.*, 1999) • Two time-constants of about 5ms and 200ms are observed on stopped-flow mixing of SDS micelles and mixed lipid/detergent micelles (Booth *et al.*, 1995)
Fluorescence dyes	• Change in fluorescence of Trp, Nile red or *N*-phenyl-1-napthylamine (NPN) as detergent or lipid environment becomes more hydrophobic	• Useful to monitor the mixing of micelles and vesicles.	• A time constant of about 5ms observed for stopped flow mixing of SDS micelles and mixed lipid/detergent micelles (Booth *et al.*, 1995)
Detergent and lipid composition	• Alter micelle or vesicle properties, e.g. hydrophobic thickness, bilayer lateral pressure profile and stored curvature elastic energy	• Insufficient known about molecular properties of detergent/lipid micelles • In principle the curvature elastic energy and lipid lateral pressures can be altered in lipid vesicles, although the effect of the protein on these properties requires investigation	• Measurements in mixed DMPC/DHPC micelles with different proportions of DMPC suggest that increasing the bending rigidity of these bilayer-type micelles slows down the rate-limiting apoprotein step of bR folding (Booth *et al.*, 1997), however further measurements are need in bilayer vesicles to avoid potential problems with altered micelle structures. • Increasing the PE content of PC/PE mixed bilayers reduces the folding yield of bR (Curran *et al.*, 1999) and also seems to slow the apoprotein folding. The exact structure and behaviour of the SDS/lipid/bR mixtures is unknown

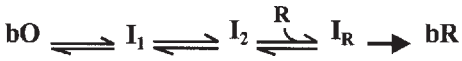
TABLE 2 (continued)

Stopped-flow mixing	• Initiate folding with a mixing dead time of about 4 ms	• The apparent rate(s) of micelle and vesicle must be measured by light scattering and fluorescence dyes	• SDS micelles and renaturing lipid/detergent micelles mix with a time constant of about 5 ms in both bR and LHCII folding experiments. Another time constant is also resolved in the case of bR.
Intrinsic protein fluorescence	• Change in fluorescence of Trp (or Tyr or Phe)	• Sensitive and less affected by light scattering than absorption measurements • Generally difficult to assign changes in fluorescence to a particular structural feature • Complicated by the fact that protein fluorescence changes as a result of either protein folding, or changes in the lipid/detergent environment. A further problem is the great difficulty in assigning kinetic phases if folding yield is less than 100%, as the proteins that do not fold can also contribute to the fluorescence changes.	• Multi-exponential time constants resolved in folding studies of bR, OmpA and LHCII, ranging from ms to hours. For bR some of the time constants have been shown to correspond to changes in lipid/detergent environment, e.g. 5ms and 200ms time constants associated with SDS and lipid/detergent micelle mixing and molecular rearrangement. The kinetic measurements of OmpA are complicated by folding yields of less than 100% and association of the protein with the lipid vesicles
Fluorescence quenching	• Brominated lipids	• Potential for information on relative distance of protein Trps and bromines on the lipid chains as protein inserts into lipids • Effects of bromines on insertion, folding yields and the lipid properties, especially at different temperatures, must be considered.	• Lipids with bromines at 4 different depths used to quench OmpA fluorescence, complicated results that are difficult to interpret (Kleinschmidt & Tamm, 1999). Data analysis improvements required and temperature dependence is unclear.
Far UV CD	• Changes in protein secondary structure	• The lack of differential scattering artifacts must be demonstrated. The best method is to increase detector acceptance angle to >90°. Subtraction of a lipid baseline is generally inadequate without a changed detector angle. • Spectra should be taken to 190nm, or below, and kinetics measured at more than one wavelength	• Kinetic measurements at 190nm and 224nm (with a detector angle >90°) show that about 30 amino acids fold from a random coil to α helical structure during the rate-limiting apoprotein folding step of bR (time constant of about 10-100s depending on lipid) (Riley *et al.*, 1997) • Measurements at only 206nm and without an altered detector angle show an increase in β structure during OmpA folding with time constants of 2 and 40min in DMPC/PG vesicles (Surrey & Jähnig, 1995)
Absorption spectroscopy	• Light scattering (see above) • Proteins that bind chromophores	• Light scattering by vesicles must be taken into account in data analysis. Subtraction of a vesicles background is insufficient and introduces artifacts	• Binding of bR retinal cofactor and formation of folded state (Curran *et al.*, 1999)

refolding kinetics of SDS-denatured apoprotein, bO in DMPC/CHAPS, DMPC/DHPC micelles, and DPoPC vesicles, in the presence of all-*trans* retinal, have been investigated by time-resolved fluorescence and absorption spectroscopy, together with some far UV protein CD (see two recent detailed reviews Booth, 1997; Booth, 2000). The folding reaction is initiated by mixing (either manually or by stopped-flow) the SDS-denatured protein with the renaturing micelles or vesicles containing retinal (Booth et al., 1995; Curran et al., 1999). The yield of folded protein is assessed *in situ* using absorption spectroscopy to monitor the formation of native-like, purple chromophore (Huang et al., 1981; London and Khorana, 1982). The folding reaction presumably occurs in mixed SDS/DMPC/DHPC micelles or SDS/DPoPC vesicles, although it remains to be determined how much of the SDS partitions into the micelles or vesicles.

One difficulty in kinetic studies is differentiating protein folding events from changes in the detergent/lipid environment. This is further complicated by the lack of definitive, specific probes for the changes in protein structure as well as the dynamics of the micelles and vesicles themselves. The apparent rate of stopped-flow mixing of the SDS micelles and the renaturing micelles or vesicles can be determined by two methods. First, using fluorescence dyes or tryptophan, the fluorescence of which increase in the more hydrophobic lipid moieties (Booth, Farooq, and Flitsch, 1996; Booth et al., 1995), and second, by time-resolving the light scattered by the micelles or vesicles (Booth and Farooq, 1997; Curran et al., 1999). These approaches have revealed only two kinetic phases with time constants of about 4 to 10 ms and hundreds of milliseconds on stopped-flow mixing of 0.2% SDS and 1% DMPC/ 1% CHAPS (w/v) micelles. The faster time constant seems to reflect mixing of the SDS and DMPC/CHAPS micelles, while the slower one involves some further molecular rearrangement of the SDS/DMPC/CHAPS micelles. Kinetic measurements in vesicles are even more complicated. Time constants similar to those for mixing of SDS and DMPC/CHAPS micelles are also observed on stopped-flow mixing of SDS micelles and lipid vesicles. Additional time constants (minutes to hours) can also be resolved that correspond to very subtle changes in light scattering and the degree of which depends on the vesicle composition. Not surprisingly, changes in bO fluorescence are also observed with these micelle/vesicle mixing time constants during the folding reaction, but it remains to be determined whether any protein folding actually occurs. An increase in protein fluorescence is observed during the fastest 4 to 10 ms phase. This could reflect an increase in the hydrophobic environment of the protein's aromatic residues as a result of either protein folding or the SDS-solubilized protein coming into contact with the more hydrophobic DMPC/CHAPS micelles or lipid vesicles. The hundreds of millisecond event is also observed as a change in protein fluorescence that has been tentatively assigned to the formation of an intermediate, I_1. The change in protein fluorescence with this time constant is lost when arginine at position 175 is mutated to glutamine. Arg175 has been suggested to be involved in correct tilting and orientation of some transmembrane (TM) helices in bR, through an interaction of its positive charge with the lipid headgroups (Henderson et al., 1990). Thus I_1 formation could be a folding event that involves helix orientation. Alternatively, it is conceivable that the micellar structural change that occurs during I_1 formation, alters the interaction between Arg175 and the detergent/lipid headgroups, and as a result no change in protein fluorescence is seen with this time constant in the Arg175Gln mutant.

The simplest, sequential reaction scheme that accounts for the kinetic data in DMPC/DHPC micelles is



BO is the SDS denatured state and I_1 and I_2 are intermediates that form prior to retinal binding. Parallel folding routes are omitted for simplicity. Although there seem to be at least two folding routes from I_2 to bR (Lu and Booth, 2000), and there are probably several parallel routes from bO to I_2. *All*-trans retinal (R) binds to I_2 noncovalently to give I_R. This is followed by formation of the covalent link with Lys216 (on a time scale of minutes) to give bR with covalently bound, all-trans retinal. Isomerization of retinal then occurs within the binding pocket to give bR that contains a mixture of all-trans and 13-cis retinal (Lu and Booth, 2000). I_R seems to consist of at least two observable states one where the retinal absorption band is similar to that of free retinal at about 380 nm (I_{R380}) and one where the retinal band is red-shifted to 440 nm (I_{R440}) (Lu and Booth, 2000). I_{R380} seems to form and decay in parallel with I_{R440} and with the same observed kinetics. This could result from a distribution of protein conformers in I_2 and I_R (and probably bO) that have slightly different protonation equilibria of their side chains and can interconvert by thermal energy. Alternatively, the I_R states could differ in polypeptide conformation or have altered protonation states of individual residues (or bound solvent ions) near the retinal with no difference in polypeptide conformation.

All the kinetic folding experiments thus far point to the apoprotein intermediate I_2 being key to the folding process and a prerequisite for retinal binding. Formation of I_2 is rate-limiting in apoprotein folding (Booth et al., 1996; Booth et al., 1995). Changes in intrinsic protein fluorescence also suggest the process is biexponential under certain lipid conditions, which probably reflects folding of slightly different conformations of the protein. The changes in protein secondary structure have been time-resolved during this stage of folding by far UV circular dichroism (CD) (Riley et al., 1997). The SDS-denatured bO state has an α helical content of about 4 TM helices, while the remaining equivalent of 3 TM α helices are disordered. The secondary structure of I_2 is native-like and corresponds to 7 TM α helices. The change in far UV CD during folding to I_2 has been monitored at both 190 nm and 224 nm. About half of the disordered SDS structure folds to α helices during the 20 s dead time of these particular far UV CD experiments, while the remaining 30 or so amino acids form α helices with a time constant equivalent to I_2 formation. The rate-limiting folding to I_2 has been suggested to reflect either the folding and insertion of parts of helices F and G, or the packing of the core regions of most of the TM helices, followed by formation of the helix ends (for reviews see Booth, 1997; Booth, 2000; Booth and Curran, 1999). In order to ascertain the effect of the helix-connecting loops of bR on protein folding, each loop has been mutated in turn to a repeating linker of GlyGlySer residues to give a structureless linker the same length as the wild-type loop (Allen et al., 2001; Kim et al., 2001). Mutation of the loop connecting helices C and D was found to cause a 10-fold reduction in the rate of I_2 formation in mixed DMPC/DHPC micelles. A 4-fold reduction in this rate was also noted for the FG-connecting loop. This suggests that the structure of the short CD loop, or an interaction between the CD and FG loops, is involved in the formation of I_2.

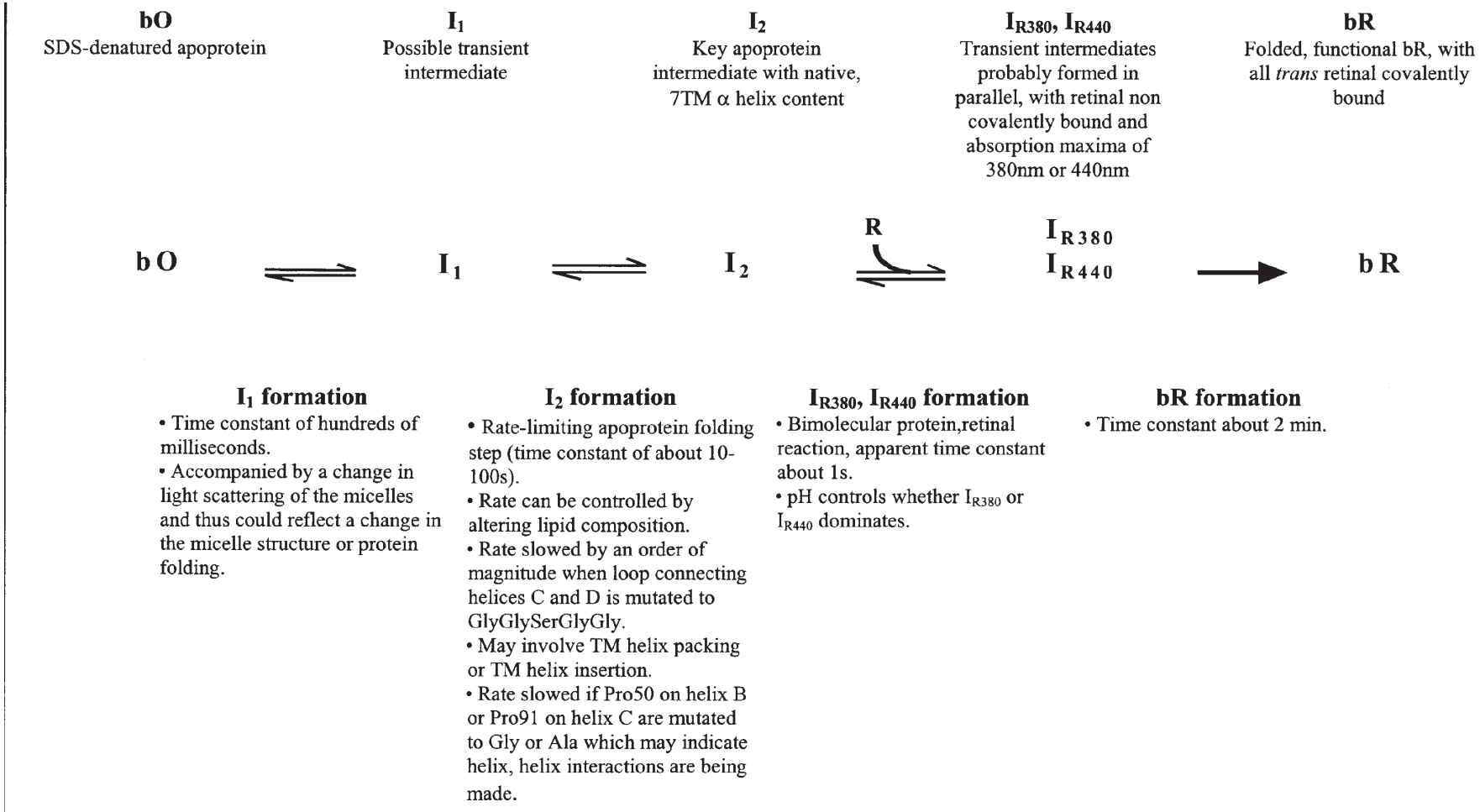
The rate of folding to I_2 can be controlled by manipulating particular characteristics of the refolding lipid environment, and its the time constant altered from 1 s to

minutes. Increasing the proportion of DMPC in DMPC/DHPC micelles slows the folding of this step, with the measured rate decreasing linearly with increasing mole fraction of DMPC (Booth et al., 1997). An increase in the longer, C14, chain PC lipid, DMPC, at the expense of the shorter C6 chain, DHPC, will increase the number of collisions in the lipid chain region of the bilayer-like micelle, which in turn will increase the lateral pressure imposed on the protein by the lipid chains. However, there are additional constraints imposed on the lateral pressure as a result of the DMPC/DHPC micelle structure, and the dynamics and shape of the micelles probably change as the relative amount of DMPC is increased. Lipid-bilayer vesicles overcome some of these problems. Mixed PC/PE lipid vesicles allow the lateral pressure to be altered by altering the PC/PE composition, and PC and PE lipids of the same chain length can be used to minimize differences in hydrophobic thickness and changes in the vesicle structure (see Section VI.B). Detailed kinetic studies of bR folding in DPoPC/L-α-1,2-dipalmitoleoylphosphatidylethanolamine (DPoPE) vesicles are currently underway. Preliminary data indicate that increasing the PE content, which increases the lipid chain lateral pressure slows down the bR folding (A.R. Curran, S.J. Allen, W. Meijberg, R.H. Templer, and P.J. Booth, unpublished data). As outlined above the rate-limiting folding step may reflect TM helix insertion or TM helix packing, both of which could be hindered by an increase in lipid chain lateral pressure (see Section VI.C) (Booth, 1997; Booth and Curran, 1999). Briefly, an increase in the lateral pressure near the bilayer center impedes the insertion of a helix that spans the bilayer, as well as effectively increases the microviscosity in the lipid chain region that could make it harder for TM helices to move together.

Recent work suggest that the 7TM apoprotein intermediate, I_2 also contains some helix, helix interactions, and possibly the inter-helix hydrogen bonds that occur via water between helices B and G, and C and D (Lu, Marti, and Booth, 2001.) Single point mutations of Pro 50 (near the center of helix B) or Pro 91 (near the center of helix C) to either Ala or Gly result in a 5- to 7-fold slowing of the rate of folding to I_2. Proline residues cause kinks in α helices, which are probably important in helix packing and cofactor binding sites. Furthermore, the backbone nitrogen of proline cannot hydrogen bond within the helix and therefore leaves a free backbone carbonyl group four residues higher up in the helix. These backbone carbonyl groups in bR, which are free of intrahelical hydrogen bonds as a result of prolines 50 and 91, are involved in inter-helix hydrogen bonds (via waters) between helices B and G, and helices C and D (Luecke et al., 1999). The presence of Pro50 results in hydrogen bonds between Thr46 of helix B and Lys216 of helix G (via water 502), and Pro91 results in hydrogen bonds between Leu87 of helix C and Asp115 of helix D (via water 511). The slowing of the rate-limiting step that occurs when either of these prolines is mutated could suggest that this folding step involves helix, helix interactions, and possibly these hydrogen bonds.

2. Summary of Current State of Understanding of bR Folding

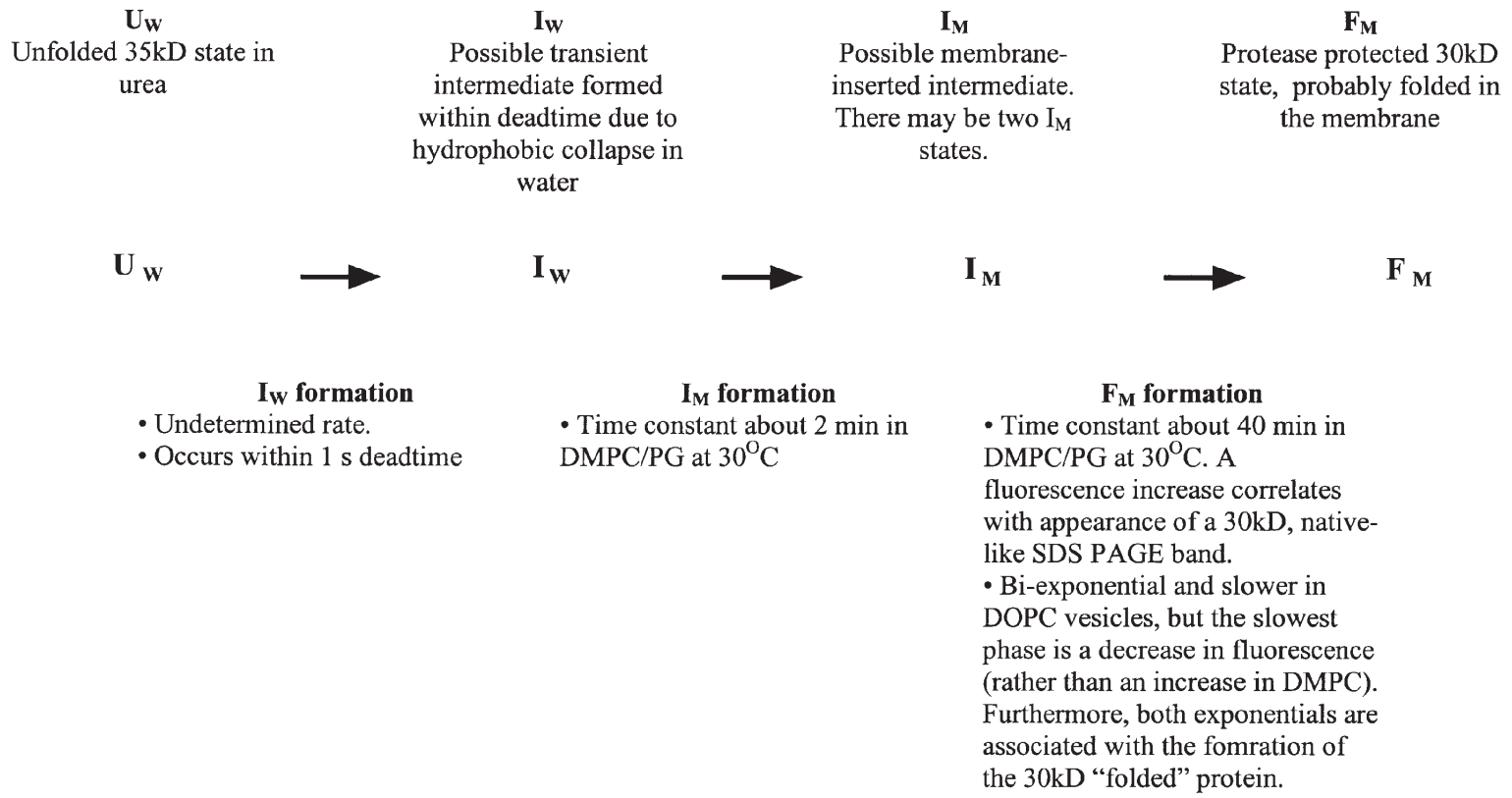
Figure 2a summarizes the current reaction scheme, experimental time constants and evidence for bR folding. Some of the major unresolved issues directly related to this scheme are also included.



Some key questions

- What protein structural changes are involved at each stage?
- What happens to the SDS?
- Does the bilayer bending rigidity and lipid chain lateral pressure control the rate of apoprotein folding and the overall folding yield?

FIGURE 2. Summary of the current understanding of *in vitro* folding of (a) bacteriorhodopsin and (b) OmpA.



Some key questions

- What happens to the protein that does not fold (about 25% in DMPC/PG vesicles)? Does it contribute to any of the above kinetic phases?
- Is a sequential route the most appropriate, particularly in DOPC vesicles where two widely spaced rates (time constants of about 40 mins and 500 mins) correlate with the appearance of the 30kD band ?
- Why are different fluorescence changes and rates observed in DMPC and DOPC vesicles and how does this really correlate with formation of a folded state?

FIGURE 2. (continued)

3. Light Harvesting Complex, LHCII

LHCII differs to the proteins discussed above in that it binds a large number of pigment molecules, which complicates studies of its folding kinetics. Furthermore, the binding of its 15 or so pigments appears to be largely cooperative. The method devised for studying the folding and assembly kinetics of LHCII was based on that used for stopped-flow studies of bR (Booth and Paulsen, 1996). Thus SDS-denatured apoprotein (LHCP) was mixed with OG micelles containing some PG lipids and the Chl*a*, Chl*b,* and xanthophyll pigments of LHCII. LHCII assembly can then be followed by a number of fluorescence signals, including intrinsic protein fluorescence and Chl*a* or Chl*b* fluorescence. The folded, functional state is characterized by efficient (100%) energy transfer from Chl*b* to Chl*a*, which is observed as an increase in Chl*a* fluorescence after excitation of Chl*b* (referred to as sensitised Chl*a* fluorescence), together with a decrease in Chl*b* fluorescence. The chlorophyll concentration has to be kept low enough to avoid inner filtering effects as high optical densities cause reabsorption of the emitted light occurs. This in turn means that the protein concentration and the stoichiometric excess of chlorophylls in the kinetic folding experiments is lower than that required for optimum protein folding and complex assembly. The maximum concentrations that could be used in the stopped-flow fluorimeter (with an excitation pathlength of 2 mm and emission measured at 90°C with a 2 mm pathlength) were found to be about 57 μ*M* total Chl and 3.25 μ*M* protein (a 1.5-fold stoichiometric excess of chlorophylls over protein). Under these conditions, only about 50% of the protein successfully assembles to functional LHCII. Several kinetic phases are resolved during the *in vitro* folding of SDS-denatured LHCII in OG micelles. A phase with a 10 ms time constant reflects the mixing of the denaturing SDS and renaturing OG micelles. A time constant of a few seconds is observed as a decrease in protein, Chl*a,* and Chl*b* fluorescence and is dependent on the presence of protein and all pigments (Booth and Paulsen, 1996). The origin of this phase is unknown. It could involve pigment binding to give an assembly intermediate, alternatively, it may be associated with the protein that does not fold to a functional state. Two further time constants of about 30 s and 174 s are resolved (the rates of which depend on the protein and pigment concentrations [see below]) both of which are associated with the formation of functional LHCII, as they are accompanied by a decrease in Chl*b* fluorescence and concomitant increase in sensitized Chl*a* fluorescence. The rates of both these latter time constants increase as the concentration of either protein, chlorophyll, or xanthophyll is increased, showing they both involve pigment binding (Reinsberg et al., 2000; Reinsberg et al., 2001). They could represent consecutive or parallel events in formation of LHCII. Alternatively, they could reflect a second- or higher-order pigment binding step that approximates to two exponentials. Increasing the relative proportion of Chl*a*, by increasing the Chl*a*/Chl*b* ratio above 1, also increases these two rates of LHCII formation, which could suggest that more Chl*a* molecules bind during the rate-limiting step than Chl*b* (Reinsberg et al., 2001). The resulting protein complexes with higher Chl*a* content are, however, less thermally stable than native protein.

LHCII has three xanthophyll binding sites, two of which seem to form a cross brace for the central two TM α helices, which also bind several chlorophylls (see Figure 2b). Thus binding at these two central xanthophyll sites could well be important for the association of the two central helices and formation of the chlorophyll binding pockets. Native LHCII seems to bind a mixture of xanthophylls, with lutein probably being the

one that forms the central cross brace. The stability of LHCII formed with only a single xanthophyll present decreases in the order lutein>zeanxanthin>violaxanthin>>neoxanthin (Hobe et al., 2000). The two rate constants associated with formation of LHCII also slow down in this order when only the individual xanthophylls are present (Reinsberg et al., 2001). This suggests that xanthophyll binding at the two central sites and formation of the cross brace is essential for LHCII folding and assembly.

Lipids are not required for LHCII formation *in vitro*, as the complex can be assembled in OG alone. LHCII binds digalactosyl diacylglycerol (DGDG) and phosphatidylglycerol (PG) lipids *in vivo,* although these do not seem to have any particular effect on the *in vitro* folding of the protein. Instead a nonspecific effect of lipids is observed in the folding rates (Reinsberg et al., 2000). The incorporation of DGDG, another thylakoid lipid monogalactosyl diacylglycerol (MGDG) or synthetic DPPG, DOPG lipids into the refolding OG micelles, all have the same effect on the measured rates. The rates of both kinetic phases associated with LHCII formation slow by the same amount as the concentration of any one of these lipid is increased. The exact origin of this lipid effect is unknown, but it could be partly due to a reduction in the effective concentration of protein and pigments. A similar effect of slowing both rates is also observed on decreasing reactant concentration, or increasing OG concentration, which also effectively decreases reactant concentration.

4. Outer Membrane Protein OmpA

The folding kinetics of the β barrel protein OmpA from a urea-denatured state to a state in lipid vesicles have been studied by time-resolving changes in protein fluorescence and far UV CD signals (Jähnig and Surrey, 1997; Surrey and Jähnig, 1995). The end state in the presence of lipid vesicles is taken to be a membrane-inserted, correctly folded state on the basis of trypsin digestion and SDS PAGE analyses, although this does not necessarily mean that the protein is fully inserted and folded (see Section II.A). These assays also do not correlate directly with the end state of the folding reaction because the experimental conditions are different.

The folding reaction was initiated by diluting OmpA (denatured in 8 *M* urea) into a solution of DMPC or DMPC/DMPG (95:5, mole ratio of PC to PG) vesicles at 30°C to give a final urea concentration of 16 mM. The mixing dead time was about 1 s. The DMPG was incorporated to prevent vesicle aggregation in the protease digestion and CD experiments, for which a relatively high protein concentration was used (2.4 μ*M* protein with 1.5 or 0.75 m*M* total lipid, which gives about 14 to 35 proteins per vesicle, of 30 to 50 nm diameter). Trp fluorescence kinetics were checked at this protein concentration of 2.4 μ*M* protein as well as at a lower concentration of 0.6 μ*M*. Far UV CD was monitored to follow the formation of β structure. Unfortunately, measurements were made at only 206 nm, and further wavelengths should have been monitored to increase the experimental accuracy, particularly in view of the fact no apparent precautions were taken for differential scattering artifacts. The negative ellipticity at 206 nm increased within the 1 s dead time. Folding experiments in the absence of lipid vesicles, where the urea-denatured state is diluted into aqueous buffer, also showed a similar CD change at 206 nm within the dead time. On this basis the dead-time change was assigned to partial folding or hydrophobic collapse in water prior to interaction of the protein with the membrane. Measurements on a faster time scale are

needed to confirm this assertion. Two further kinetic phases were observed when folding in the presence of lipid vesicles: a bi-exponential increase, with time constants of about 2 and 40 min, was seen in both protein fluorescence and far UV CD at 206 nm. The protein also became resistant to trypsin digestion during the 40-min phase. However, the time taken for the trypsin assay is too long to determine whether any trypsin protection occurs during the faster, 2-min phase. This 2-min phase was dependent on the lipid concentration. The kinetics were mainly measured at pH 7.3, for which the final folding and insertion yield according to the SDS PAGE and trypsin digestion assay was about 75%. Thus, some kinetics may reflect the remaining 25% of proteins that do not fold successfully.

The authors proposed a sequential model for OmpA folding, over a parallel or branched model:

$$U_W \rightarrow I_W \rightarrow I_M \rightarrow F_M$$

I_W is the intermediate state that is suggested to form within the 1 s mixing dead time as a result of urea dilution and hydrophobic collapse in water before the protein interacts with the membrane. A membrane-inserted intermediate is then proposed (I_M) to form with the 2-min time constant, and finally the folded state, inserted in the membrane (F_M) during the slow 40-min phase.

Kinetic measurements have also been carried out on the folding reaction of urea-denatured OmpA and DOPC vesicles (Kleinschmidt and Tamm, 1996). A bi-exponential increase in protein fluorescence was observed at 30°C, for which the two time constants of 11 min and 42 min were similar to those observed in DMPC/PG vesicles (2 min and 40 min assigned to I_M and F_M, respectively). However, an additional very slow decrease in fluorescence (linear on the measurement time scale of 500 s full scale) was observed in DOPC vesicles. A slightly different folding scheme was proposed where both the 11 min and 42 min phases were suggested to represent two sequential, membrane-bound intermediate states (I_{M1} and I_{M2}), with the very slow, linear phase reflecting the subsequent formation of inserted, folded OmpA (F_M). This seems oversimplistic. It is not clear why formation of the folded state is much slower in DOPC vesicles than DMPC vesicles or why it is accompanied by a decrease in fluorescence, whereas only an increase in fluorescence occurs in DMPC vesicles. Furthermore, the assignment of the 42-min phase to a membrane-bound intermediate in DOPC is inconsistent with the authors' interpretation of the SDS PAGE gels. Native OmpA and urea-denatured OmpA have different apparent molecular masses by SDS PAGE of 30 kDa and 35 kDa, respectively. The appearance of a 30-kDa band is taken as evidence that the very slow, linear phase leads to a correctly folded and inserted state. However, the data indicate that the 30-kDa band also occurs during the 42-min phase, which would imply that some "folded", 30-kDa state forms during this 42-min phase. Thus, a parallel folding scheme may be appropriate, with folded, inserted (30 kDa) OmpA forming in two phases: the 42-min phase and the very slow, linear phase. Some of the differences between the DMPC and DOPC results could be due to the different numbers of proteins per lipid vesicle (about 20 to 56, for DOPC vesicles, as opposed to 14 to 35 for DMPC/PG), the different properties of DOPC and DMPC, or vesicle aggregation.

The accessibility of Trp residues to membrane-bound fluorescence quenchers has also been assessed during folding of OmpA in DOPC vesicles in an attempt to obtain distance information during folding (Kleinschmidt and Tamm, 1999). The time course of Trp quenching by brominated

lipids, with bromines attached at four different depths to membrane lipid chains was measured over the temperature range 2 to 40°C. The native protein, together with single Trp mutants, were investigated. The data at all temperatures were interpreted according to the reaction scheme proposed above for OmpA folding in DOPC, although more supporting data for the applicability of the scheme at low temperatures seem to be needed. An apparent problem lies in the fact that the kinetics were only reported over a maximum of 80 min, which does not probe the formation of the 30-kDa "folded" state that was asserted to form in the previous work on a much longer time scale (linear over 500 s). Temperature effects on the lipids, bromine quenching, and bromine-lipid collision rates were not discussed. While there is little information on this point, 30 mol% of the lipids present in the vesicles are brominated lipids, which presumably has a large effect on the lipid phase behavior (and could in principle affect the yield of inserted protein). The effect of bromine quenching on the OmpA folding kinetics adds even more complications to the data interpretation, and many assumptions have been made. One of the main aims was to follow the change in position of the tryptophan(s) over time. Lipids were used with two bromines attached per lipid at positions 4 and 5, 6 and 7, 9 and 10, or 11 and 12 on the hydrocarbon chain. The two bromine atoms per lipid chain were treated as a single quencher and the extent of Trp fluorescence quenching was determined for each of the four bromine lipid samples. This gave changes in Trp quenching over time, with respect to bromines at four different positions in the bilayer. A distribution analysis was used to estimate the time dependence of the average position of the Trp(s) with respect to the bromine quenchers. This involved fitting the four points to a Gaussian distribution. These fits appear to have a very high margin of error, especially, for example, at 2°C, where the 4 points fall on a straight line, and fitting them to a Gaussian function is a questionable process. Nevertheless, the data show that overall changes in distance of the Trps with respect to the Br quenchers occur. Fitting the data to Gaussian functions, in the manner outlined above, showed that in the native protein at low temperatures of about 7 to 26°C the Trps seem to be about 10 Å from the bilayer center, at 27.5°C no change in position is seen and the Trps are constantly at the bilayer center. At 30°C the Trps are found to be near the center immediately after the 1 min mixing time, and then move away from the center to reach a distance of 10 to 11 Å after about 60 to 80 min. This type of approach has promise, but improvements in the data analysis are required.

a. Summary of Current State of Understanding of OmpA Folding

Figure 2b summarizes the current reaction scheme for OmpA folding, together with some of the major unresolved issues directly related to this scheme.

5. Outer Membrane Protein OmpF

Kinetic measurements on OmpF insertion are more problematic than those of OmpA. Kinetic experiments have been designed in the same way as those on OmpA (Surrey et al., 1996) (Jähnig and Surrey, 1997). Trypsin protection and conductance assays suggest that only 15% of the urea-denatured protein inserts as functional trimers into DMPC vesicles. Time-resolved protein fluorescence experiments resolved two

kinetic phases with time constants of 5 min and 50 min for this folding from urea into DMPC vesicles. The low yield of insertion hinders interpretation of these kinetic data, but largely by analogy with OmpA, the 5-min phase was suggested to represent formation of membrane-bound intermediate and the slow phase a folded, inserted monomer (that is not resistant to trypsin). Trimer formation subsequently occurs, but this was monitored by trypsin protection rather than fluorescence. The yield of OmpF trimers can be increased to 75% in DM/DMPC mixtures; however, the samples then become too turbid for fluorescence measurements, presumably because of aggregation. SDS gel electrophoresis of undigested and trypsin-digested protein from this DM/DMPC folding reaction showed that the fraction of dimers increases within about 30 min and then decrease; meanwhile, a delay is observed in trimer formation of a few minutes. This suggests the dimers are an intermediate in the assembly reaction.

IV. *IN VITRO* EXPERIMENTAL STUDIES OF THE THERMODYNAMICS AND STABILITY OF MEMBRANE PROTEINS

An understanding of the folding process of membrane proteins requires insight into the factors that determine the conformational stability of the native state of the protein. Much progress has been made in this area of protein stability for water-soluble proteins, but experimental studies of the thermodynamics of membrane protein stability have been few. This is due to their hydrophobicity, the different nature of their environment, and the fact that reversible folding/unfolding has only been achieved for membrane proteins in a few isolated cases (discussed briefly in Section II.A). Fortunately, a lot of information has been obtained from model studies using small peptides together with theoretical considerations. On the basis of these results some general ideas are starting to emerge (for recent reviews see White and Wimley, 1999 and Popot and Engelman, 2000).

This section aims to discuss some of the studies that have been performed on integral membrane proteins, focussing on the advantages and limitations of the methods used as well as the results obtained. We review biophysical methods (denaturant-induced unfolding, thermal denaturation with special emphasis on DSC and force spectroscopy), together with some examples of more indirect biochemical studies, such as mutational analysis of dimerisation interfaces, sometimes in combination with analysis of sequence databases. This is in not a comprehensive review of these experimental approaches and reflects our interests as much as anything else.

A. Experimental Methods: Membrane Protein Unfolding

One way to study the stability of proteins is to evaluate the forces that are required to unfold and/or denature these molecules. Two of the most common methods for water-soluble proteins are the use of chaotropic reagents (urea, guanidine hydrochloride [GuHCl]) or heat denaturation. Both these approaches have also been applied to membrane proteins, although the number of studies (and proteins studied) has been small.

1. Denaturant-Induced Unfolding

Denaturants have long been used in studies of protein folding *in vitro* and have

yielded a wealth of information on the folding mechanisms and stability of water-soluble proteins. Two of the most widely used compounds are GuHCl and urea, because they are relatively easy to handle and can result in complete unfolding of the protein under study, that is, to a largely random coil conformation. Of the two major classes of integral membrane proteins, α-helical and β-barrel, the latter is the most amenable to urea and GuHCl denaturation. The alternating hydrophobic-hydrophilic amino acid motif found in the TM strands of these β barrel proteins confers a relatively low overall hydrophobicity on their primary structure, and their solubility is maintained at high denaturant concentrations in water without any detergents. Detergents and/or lipids are, however, usually still needed to keep the β barrel protein in solution at low denaturant concentrations, which complicates the use of this method. To the best of our knowledge no attempt to measure the free energy of folding from an urea-denatured state has been published.

Several attempts have been made to unfold α helical membrane proteins by GuHCl or urea. It was found that the integral membrane domains of cytochrome b5 (Tajima et al., 1976), glycophorin (Schulte and Marchesi, 1979), Ca^{2+}-ATPase (Rizzolo and Tanford, 1978), band 3 from erythrocytes (Oikawa et al., 1985), and bovine heart cytochrome oxidase (Hill et al., 1988) are very resistant to denaturation by these compounds, which makes an analysis of their thermodynamic stability impossible. This resistance to GuHCl and urea probably arises in part from the use of the detergents that are needed to keep the very hydrophobic proteins in solution, irrespective of the denaturant concentration. The detergent micelles largely cover the hydrophobic parts of the protein, thereby shielding them not only from the aqueous solvent but also from the action of the denaturants. The hydrophilic domains of membrane proteins can usually be unfolded by GuHCl or urea, and exhibit a stability that is comparable to the stability of water-soluble proteins.

Alternatives to the traditional denaturants are denaturing detergents such as SDS or sarcosyl that are able to form mixed micelles with the detergent that is necessary to keep the α helical membrane in solution. This allows the SDS or sarcosyl to act directly on the protein under study, and unfolding occurs in a (denaturant) concentration-dependent manner. However, care has to be taken that the experiment probes the stability of the protein and not the concentration-dependent interaction of the denaturant (SDS) with the solubilizing-detergent micelle. In the former protein stability case, the results should be the same on a mole fraction basis, independent of the concentration of the solubilizing detergent, which indicates that mixed micelles are truly formed. The advantage of this SDS/mixed micelle method is that reversibility of the unfolding reaction can be readily achieved, allowing a quantitative interpretation of the results. On the other hand, denaturation by compounds such as SDS does not often lead to complete unfolding of the protein and can leave much of the α helical structure intact. In fact, SDS has been shown to induce α helical structure in small peptides and may even act by inducing non-native secondary structure in parts of the protein. This should always be kept in mind when comparing free energies of unfolding obtained from SDS-induced denaturation of membrane proteins and GuHCl or urea-induced unfolding of water-soluble proteins. Another obvious disadvantage is the fact that these SDS studies cannot be performed in the membrane, the native environment of the protein, as high SDS concentrations will lead to disruption of the bilayer.

a Bacteriorhodopsin, bR

BR is one of the most intensively studied membrane proteins, and conditions for reversible folding and unfolding have been known for a long time. Therefore, it is perhaps surprising that an attempt has been made only recently to estimate the thermodynamic parameters that describe the stability of bR from SDS-induced unfolding studies (Chen and Gouaux, 1999). BR is a 7 TM α helical protein that binds a retinal cofactor covalently within its helix bundle. Early work (Huang et al, 1981; London and Khorana, 1982) showed that 8 *M* GuHCl was insufficient to denature the protein, but that complete denaturation could be achieved in organic acids (trifluoroacetic or formic acid [see also Section II.A]). An intermediate in SDS (with ~55% of the native α helix content) was found to be key to refolding, and monomeric, functional protein could be produced in a large number of detergent or lipid systems (in the presence of retinal). The recent thermodynamic study of Chen and Gouaux followed directly from this earlier work. SDS titrations were carried out in DMPC/CHAPSO mixed micelles in the presence of retinal (1:1 DMPC,CHAPSO molar ratio) on SDS-unfolded apoprotein bO, and equilibrium was reached over the full titration range (0.2 to 0.7 mole fraction SDS) (Chen and Gouaux, 1999). In order to measure how much refolded, purple bR was present, UV absorption spectra were taken at each SDS concentration. These spectra did not exhibit an isosbestic point, indicating that one or more equilibrium intermediates exist. Unfortunately, no other spectroscopic probes were used that could give additional information on the number and nature of the intermediates, and therefore a quantitative interpretation of the data was not possible. In addition, as recognized by Chen and Gouaux, the physical state of the refolding micelle system is somewhat uncertain and might well change during the titration experiments, adding another layer of complexity to data interpretation. The unfolding transition of the apoprotein bO in the SDS/DMPC/CHAPSO micelles was, however, also monitored by fluorescence. The mid-point of the bO folding transition occurred at an SDS concentration of about 0.3 *M*, as opposed to that of about 0.45 *M* for the unfolding/folding transition of bR. Thus, retinal binding in bR leads to considerable stabilization of the folded structure as opposed to the folding of bO alone in DMPC/CHAPSO/SDS micelles. This is in good agreement with kinetic studies in a very similar detergent system, which estimate that the noncovalent binding of retinal to a partially folded apoprotein state contributes about +30 $kJ.mol^{-1}$ to the free energy of stabilization of bR retinal (Booth et al., 1996). Further stabilization comes from covalent binding of retinal.

SDS titrations have also been performed on bR fragments (Marti, 1998). The caveats mentioned above with regard to unknown intermediates and complexity of the refolding system also hold for these fragment studies. In order to evaluate the contribution of the helix-connecting loops to the structural integrity of the protein, SDS titrations of bR reconstituted from pairs of fragments were compared to titrations of intact bR, which was produced and reconstituted by the same method. In mixed micelles of DMPC/CHAPS (3:1 DMPC, CHAPS molar ratio) the unfolding midpoint of bR was about 1.1% SDS (w/v), corresponding to a molar ratio of 0.56 SDS in the SDS/DMPC/CHAPS mixture. A cut between helices B and C, or between E and F, resulted in a shift of the transition midpoint to about 0.7% (0.45 molar ratio) and a cut between helices C and D, or between helices D and E, to about 0.6% (0.41 molar ratio). The absorption spectra were not shown, but the varia-

tion in the steepness of the curves, as well as the width of the transitions, indicate that a quantitative interpretation of the results is not straightforward.

b. Diacylglycerol Kinase, DGK

DGK is a small (121 amino acid) trimeric integral membrane protein from *E. coli* involved in the formation of phosphatidic acid from diacylglycerol and ATP. No detailed structural information is available yet for this protein, but a topology model has been constructed based on fusion experiments and sequence analysis. This model consists of five α-helical segments, two of which (helices 1 and 4) are found on the cytoplasmic side of the membrane, whereas the other three have been identified as TM helices (Smith et al., 1994, and see Figure 2c). A method was developed to study the thermodynamic stability of this protein in detergent micelles, using SDS as the denaturant and UV absorption and CD as structural probes (Lau and Bowie, 1997). Two reversible unfolding phases were observed, a gradual change between 0.3 and 0.7 mole fraction SDS, and a cooperative change between 0.7 and 0.8 mole fraction SDS. Unfolding studies coupled with site-directed mutagenesis allowed the former of the two unfolding phases (0.3 to 0.7 mole fraction SDS) to be attributed to unfolding of the extra-membrane parts of the protein and the latter (0.7 to 0.8 mole fraction SDS) to the membrane-embedded regions. By analogy to the linear extrapolation model that is used for a quantitative interpretation of unfolding data of water-soluble proteins (see Pace, C.N., 1986), the data were analyzed assuming that the free energy of unfolding is linearly dependent on the SDS mole fraction. Free energy changes of +25 kJ.mol^{-1} and +67 kJ.mol^{-1} were determined for the unfolding of the cytoplasmic and membrane parts of the enzyme, respectively (although this probably does not include full unfolding of the TM helices, because DGK retains helix content in SDS [see Section II.A]). These values refer to the difference between the SDS-denatured state and a refolded, monomeric state that actually has a higher degree of helicity than the native (trimeric) enzyme. This highlights some of the potential difficulties in the use of SDS as a denaturant; nevertheless, it clearly allows significant progress to be made.

Two recent papers on DGK illustrate a further useful application of the SDS approach (Lau et al., 1999; Zhou and Bowie, 2000). As outlined in Section II.B, in a search for a more thermostable protein a number of single point mutants of DGK have been identified from a random mutagenesis procedure with increased stability in detergent at 50°C. A combination of four of these mutants yielded an enzyme with a deactivation half life of 35 mins at 80°C, compared to 6 min at 55°C for the wild-type protein. SDS denaturation experiments showed that the increased thermostability (i.e., kinetic stability or thermal denaturation) resulted from an increased thermodynamic stability (i.e., with respect to denaturation by SDS), although actual values were not given. Interestingly, the sites of the stabilizing mutations were found at the end of the putative TM helices rather than the loops or hydrophobic parts of the protein. Thus, they appear to stabilize the interface between monomers rather than the interactions between individual helices within a monomer. However, a detailed analysis of these results awaits the elucidation of the three-dimensional structure of this protein.

c. Outer Membrane Proteins

Bacterial outer membrane protein form TM β barrels with TM strands consisting of

alternating hydrophobic and hydrophilic residues, making them easier to handle than α helical membrane proteins. Many of these β barrel proteins have been refolded from urea-denatured states, usually as part of purification procedures from inclusion bodies (see Section II.B). Successful refolding conditions often employ zwitterionic detergents such as zwittergent or lauryldimethylamine oxide. Surprisingly, none of these studies have quantified the results to obtain thermodynamic data, even though in some cases perfect reversibility of the unfolding transitions has been achieved (Minetti et al., 1997, 1998).

The secondary structure of a number of porins has been determined under native and denaturing conditions by CD spectroscopy. GuHCl and urea can completely denature these proteins, but considerable secondary structure is still present for the denatured state of these proteins in SDS. Under these conditions an average α-helix content of around 15% can be estimated from the ellipticity at 222 nm (see, e.g., Markovic-Housley and Garavito, 1986; Eisele and Rosenbusch, 1990; Dekker et al., 1995; Minetti et al. 1997, 1998), part of which is likely to be non-native. The latter can be concluded from the fact that in some cases the ellipticity at 222 nm increases in going from the native to the SDS-denatured state.

The conformational stability of the PorB class 3 protein from *Neisseria meningitidis* has been studied by SDS-PAGE, protease accessibility, absorption, and CD spectroscopy (Minetti et al., 1998). The data are of very high quality and clearly demonstrate full reversibility of the unfolding transition that is induced by SDS or heat in the presence of zwittergent 3-14. Isosbestic points were observed in the absorption and CD spectra, indicative of a two-state transition, while SDS-PAGE showed that this transition was accompanied by conversion of the native trimer structure to monomers. Using the linear extrapolation method of Lau and Bowie described above for DGK (Lau and Bowie, 1997), we calculate a free energy change of about +300 $kJ.mol^{-1}$ for the SDS denaturation reaction. This free energy change corresponds to the difference between the native trimeric state in the absence of SDS and the monomeric, SDS-denatured state. Nevertheless, it appears exceptionally large and is about an order of magnitude larger than typical values for unfolding water-soluble proteins. Possible explanations of this result are that the model employed is not valid because true mixed micelles are not formed or that the zwitterionic detergent binds tightly to the trimer, but not the monomer. In fact, it has been suggested that zwitterionic detergents are successful with this type of outer membrane protein because they can mimic the role of lipopolysaccharides, components of the outer membrane that bind tightly to porins, thus increasing the stability of the protein *in vivo*.

2. Heat-Induced Unfolding

A wealth of information on the thermodynamics of water-soluble proteins has resulted from unfolding studies that involve an increase in the temperature of the protein solution. In particular, differential scanning calorimetric (DSC) studies have been useful because they involve a unique combination of the experimentally controlled variable, temperature, and the measured variable, enthalpy. Temperature and enthalpy are conjugated extensive and intensive variables of the system, and thus it is possible to determine the partition function for the temperature range studied. The thermodynamics of the system can then be readily calculated (for reviews see Privalov and Pothekin, 1986; Sanchez Ruiz, 1995; Freire, 1995).

Parameters that can be directly obtained from a DSC experiment are the melting temperature T_m (usually defined as the temperature where the maximum of the heat absorption occurs) and the calorimetric enthalpy, ΔH^{cal}. The latter is determined simply from the area under the heat absorption peak in the thermogram and is expressed on a molar basis. It is often useful to also determine the van't Hoff enthalpy, ΔH^{vH}, because this quantity is sensitive to the shape of the transition curve. An effective equilibrium constant is defined, based on the progress of the reaction as obtained from the transition curve, that is, the amount of heat absorbed at a certain temperature vs. the total heat absorbed after the transition is completed. Using the Van't Hoff equation:

$$\Delta H^{vH} = RT^2 \, d \, \ln K^{eff}/d \, T,$$

an effective enthalpy can be found. In the case of an unimolecular two-state equilibrium transition (i.e., native $\leftrightarrow$ denatured) the van't Hoff and calorimetric enthalpies are equal for equilibrium transitions where intermediate states are significantly populated ΔH^{vH} is smaller, while for a reversible two-state unfolding of a multimeric protein ΔH^{vH} is larger; in the latter case the ratio of van't Hoff and calorimetric enthalpies equals the association state of the native protein. However, one should bear in mind that an analysis of this type is only valid if the sample is in thermodynamic equilibrium during the complete experiment. For this to be the case two criteria should be met: the transition should be reversible (determined in a rescan of the sample) and independent of the scanning rate used in the experiment, the latter to ensure that the reaction is not under kinetic control. Generally, for the thermal denaturation of membrane proteins one or both conditions are not fulfilled. This either precludes a quantitative analysis or in some cases it is possible to invoke a model incorporating a reversible transition that is followed by an irreversible transition at high temperatures, $T>>T_m$.

An advantage of heat-induced over denaturant-induced unfolding studies is that the former allows measurements to be done in native-like environment of membrane proteins (i.e., lipid bilayers). The role of lipid-protein interactions in membrane protein stability can also be analyzed by altering the composition and properties of the lipid bilayer. Some examples of studies on heat-induced unfolding of membrane proteins are discussed below.

a. Bacteriorhodopsin, bR

The thermal unfolding of *bR* has been studied by scanning calorimetry, often in combination with spectroscopic methods (absorption, fluorescence, and CD). The native environment of bR is as a hexagonal array of trimers in the PM of *H. salinarium*. The protein unfolds irreversibly in PM samples at neutral pH, at around 95°C with a change in enthalpy of approximately +400 $kJ.mol^{-1}$ (Jackson and Sturtevant, 1978; Brouillette et al., 1987; Shnyrov and Mateo, 1992). In addition, a reversible pretransition around 70°C has been observed by spectroscopy, calorimetry, and temperature-dependent X-ray scattering (Shen et al., 1993; Koltover et al., 1999). This pretransition represents a cooperative change in the crystalline order of the two-dimensional lattice, resulting from a conformational change in the bR monomer. It was concluded on the basis of the ratio of van 't Hoff and calorimetric enthalpies (close to 3) that the major species undergoing the unfolding transition is the bR trimer. This analysis, however, does not hold if the transition temperature is dependent on the scanning rate used in the experiment, which Galisteo and Sanchez-

Ruiz found to be the case for PM (Galisteo and Sanchez-Ruiz, 1993). Nonetheless, X-ray scattering studies indicate that order on the scale of bR trimers still exists after the pretransition in purple membranes, and that some order remains even after denaturation (Koltover et al., 1999; Muller et al., 2000)

The T_m for monomeric bR in mixed detergent/phospholipid micelles drops to approximately 75°C, but the unfolding enthalpy remains the same (Brouillette et al., 1989). This is somewhat surprising, as bR is monomeric in this environment and therefore the subunit and lattice interactions are not present. Interestingly, the pretransition is also observed in monomeric bR, albeit at lower temperature, which agrees with the interpretation given above that this pretransition is associated with a conformational change of the monomer. Using these micellar bR data, Haltia and Freire (1995) have estimated a -20 $kJ.mol^{-1}$ contribution of the lattice to the free energy of stabilization of bR in PM.

The heat capacity increment of unfolding for bR in PM was determined from the slope of a plot of the enthalpy of unfolding versus T_m in buffers of varying pH and amounted to +0.19 $J.Kg^{-1}$ (Brouillette et al., 1987). Both this value and the enthalpy of unfolding are low compared with values observed for water-soluble proteins. This can be explained by the fact that bR, as well as other α helical membrane proteins do not unfold completely after exposure to high temperatures. Circular dichroism (Brouillette et al., 1987; Kahn et al., 1992) and FTIR data (Cladera et al., 1992; Azuaga et al., 1996) indicate that a large portion of bR retains its helical structure after thermal denaturation. This was confirmed independently by X-ray scattering data (Muller et al., 2000), which showed only partial unfolding of the helices.

Several authors have studied the contribution of the helix-connecting loops and the retinal cofactor to the stability of bR (Kahn et al., 1992; Azuaga et al., 1996). Two complementary fragments of bR with a break in on of its six loops can functionally regenerate bR (see, e.g., Huang et al., 1981; Liao et al., 1983, 1984; Popot et al., 1987; Sigrist et al., 1988; Kahn and Engelman, 1992; Kahn et al., 1992; Marti, 1998). It is then possible to evaluate the contribution of the loops to the thermodynamics. Cleavage at individual loops and/or removal of retinal leads to a decrease in the melting temperatures and enthalpies of unfolding under all conditions studied. Effects appear to be additive and can be considerable: for example, in purple membranes the breaking of the loop between helix B and C leads to a downward shift in T_m of 6°C, and a decrease in the enthalpy of unfolding of about 180 $kJ.mol^{-1}$ at neutral pH and 360 $kJ.mol^{-1}$ at pH 9 (Kahn et al., 1992). The same cut for bR reconstituted in vesicles at pH 7 reduces T_m and ΔH by 12°C and 140 $kJ.mol^{-1}$, respectively, whereas an additional break between helices A and B results in a T_m that is 24°C lower and a ΔH smaller by 420 $kJ.mol^{-1}$ than the wild type under the same conditions. Removal of the retinal interaction by bleaching results in large decreases in both T_m (by 16°C) and ΔH (from +420 to +340 $kJ.mol^{-1}$). The authors concluded from these data that the contribution of the loops to the stability of the protein is both enthalpic and entropic in origin, that is, the extramembrane loops do more than merely connect the helices (see also Haltia and Freire, 1995).

Another interesting aspect in thermal denaturation studies of bR is the dependence of the protein stability on its environment. BR can withstand temperatures up to 140°C in stacked sheets at low relative humidity (Shen et al., 1993). This property was later linked to inter-membrane interactions which could prevent bR unfolding by steric hindrance (Koltover et al., 1999; Muller et al., 2000). Recently, we have taken

a different approach in our lab and analyzed the thermal stability of bR in lipid-bilayer vesicles of different composition and used changes in the purple absorption band to monitor bR. Monomeric bO, free from detergent and endogenous lipids, was refolded into membranes consisting of mixtures of DMPC and DOPC in the presence of retinal. Both the refolding efficiency and the thermal stability were strongly dependent on the membrane composition. The efficiency decreased with increasing DOPC content, while the thermal stability increased. A possible explanation for this behavior is the lateral pressure exerted by the lipids on the protein. Increasing unsaturation of the chains in the hydrocarbon region of the bilayer, by increasing the DOPC content, leads to an increase in the lateral pressure in this area. This can hinder folding, but at the same time increase the stability of the membrane-embedded protein (Meijberg, W., Curran, A.R., Templer, R.H., and Booth, P.J., unpublished results).

b. Erythrocyte Anion Transporter, Band 3

One of the best studied membrane proteins with respect to its thermostability in different lipid environments is the principle anion transporter from erythrocytes, band 3 (Maneri and Low, 1988). The protein consists of a 55-kDa TM domain and a 42-kDa cytoplasmic domain that can be separated by proteolysis. The stability of the TM domain was studied by differential scanning calorimetry in membranes of different lipid composition. The transition temperature was strongly dependent on the length of the acyl chains in mono-unsaturated phosphatidylcholines, varying from about 47°C for 14 carbon chains with one unsaturated bond (C14:1) to 66°C for 24 carbon chains with one unsaturated bond (C24:1). At the same time the enthalpy of denaturation associated with these transitions goes through a maximum for 20 carbon chains (C20:1), rising from +400 kJ.mol^{-1} (7 J.g^{-1}; C14:1) to +1800 kJ.mol^{-1} (33 J.g^{-1}; C20:1), then dropping to +1000 kJ.mol^{-1} (19 J.g^{-1}; C24:1). The increase in the thermal stability if the protein is attributed to better matching of the hydrophobic parts of the protein and the lipid bilayer brought about by the increase in bilayer thickness as the chain length is increased. This also agrees with the increase in T_m that has been observed after addition of cholesterol to the DOPC membranes (where cholesterol will also increase the bilayer thickness) and the decrease in T_m going from single to double and triple unsaturated chains of 18 carbon atoms length. However, a plot of the enthalpy of unfolding as a function of T_m in a monounsaturated series of phosphatidylcholines yields a maximum, strongly suggesting that the experiments do not solely probe the stability of the protein, but that other factors also play a role. Haltia and Freire (1995) have hypothesized that the unfolding transition and the melting of associated lipids are coupled. Thus, with increasing bilayer thickness and hydrophobic matching, the number of boundary lipids associated with the protein could diminish, explaining both the decrease and the relatively high value of the observed enthalpy. Another possible explanation can be found in the bulk properties of the lipid bilayer. The lateral pressure exerted by the lipids on the protein increases with chain length, the more so if an unsaturated bond is close to the hydrophobic-hydrophilic interface (see Section VI.B). In the monounsaturated PC series used in the study by Maneri and Low (1998), the distance of the double bond from the middle of the bilayer increases from about 2 Å for DMoPC (C14:1, *cis* 9), to about 7 Å for DOPC (C18:1, *cis* 9), while the distance to the hydrophobic/

hydrophilic interface remains the same (distances estimated from a 28 Å hydrocarbon core for DOPC; Ren et al., 1999). Going from C18:1 (cis9) to C24:1 (cis15) the distance of the double bond to the middle of the bilayer remains at about 7 Å, but the distance from the bilayer middle to hydrophobic/hydrophilic interface increases roughly 1.7 Å for every two carbon atoms in the chain. This means that the changes in the lateral pressure profiles in the hydrocarbon region of the bilayer are very different in both halves of the series, with probably smaller values in the middle of the bilayer in the case of C24:1 phosphatidylcholine, leading to a lower enthalpy of unfolding for the protein. It would be very interesting to see how the T_m and enthalpy of unfolding for band 3 vary in mixtures of lipids, where the hydrophobic thickness can be kept (more or less) constant, but the lateral pressure is altered, for example, mixtures of PC and PE lipids with the same hydrocarbon chains.

c. Outer Membrane Protein OmpF

Porins appear ideal candidates to gain insight into the factors determining the stability of membrane proteins. The number of studies on porin thermodynamics has, however, been small. To the best of our knowledge, there has been only one study using DSC to obtain thermodynamic parameters of an outer membrane protein, in this case OmpF (Phale et al., 1998). In 1% SDS the DSC traces showed a single asymmetric heat absorption peak at 72°C, corresponding to a calorimetric enthalpy for the trimeric protein of +1800 $kJ.mol^{-1}$ (16 $J.g^{-1}$). Both parameters were independent of the scanning rate between $10^{o}C.h^{-1}$ and $45°C.h^{-1}$, indicating that the kinetics of the reaction did not influence the shape of the transition curve. On the other hand, the reaction was shown to be irreversible because rescans of heated samples did not show any heat absorption peaks. This can be understood if the irreversible reaction takes place at temperatures considerably higher than T_m and without a clear thermal effect. If this assumption is made, an equilibrium analysis as performed by the authors is permitted (see Manly et al., 1985; Sturtevant, 1987). It was found that the experimental data were well reproduced by a model describing concomitant protein unfolding and breakdown of trimers into monomers. This is in agreement with an SDS-PAGE analysis of OmpF samples incubated under the same conditions and at increasing temperatures that also showed the appearance of monomers at the temperature where the major heat absorption was observed. Mutations in the trimer interface and variations in pH allowed the authors to determine the heat capacity increment of unfolding of +43 $kJ.mol^{-1}.K^{-1}$ (0.4 $J.K^{-1}.g^{-1}$) from a plot of ΔH^{cal} vs. T_m.

The specific enthalpy and heat capacity of OmpF unfolding are low, as is also the case for α–helical membrane proteins. In the case of OmpF, part of this is due to the specific conditions used in the experiment. CD spectroscopy of denatured OmpF in SDS has shown that about 15% α-helix is present after denaturation, so unfolding is less complete than in the case of water-soluble proteins. The free energy of unfolding for the wild-type protein is approximately +100 $kJ.mol^{-1}$, which is considerably larger than values found for water-soluble proteins. This finding is in agreement with the resistance of proteins of the porin class toward harsh conditions like low pH, high temperature, detergents, and chaotropic reagents, all of which tend to denature other types of proteins.

3. Mechanical Unfolding on Single Molecules: bR

A completely different approach to study the unfolding of a membrane protein uses a combination of AFM and single molecule force spectroscopy. In a recent study (Oesterhelt et al., 2000) PM patches were absorbed to mica and their surface was imaged by atomic force microscopy, AFM. The stylus was then placed over a particular protein and pushed down to allow adsorption of this molecule to the tip. The force extension spectrum was recorded after the subsequent retraction of the tip. The surface was then imaged again and a hole in the two-dimensional crystal was observed, confirming the extraction of a single bR molecule from the membrane. The power of this method lies in the fact that single molecules are studied. Fifteen percent of the total number of retractions on bR left a hole in the crystal structure and in 33% of these successful retractions a final rupture peak was observed corresponding to the full length of the bR molecule. These criteria ensure that the observed event corresponds to pulling the bR molecule out of the membrane by the C terminus. The data were interpreted as a pairwise extraction of the helices from the membrane in pairs, except for the removal of the last helix A. At a pulling rate of 40 $nm.s^{-1}$ the helices were removed at 100 to 200 pN, compared with a force of only about 25 pN for the extraction of lipids under comparable conditions. This study demonstrates that information on intramolecular interactions can be obtained by AFM. A comparison of results obtained for different proteins and/or conditions, together with higher-resolution data, holds much promise for our understanding of membrane protein stability under native-like conditions.

B. Experimental Methods: Mutational Analysis of Dimerization

The denaturation studies described above use physical or chemical perturbation of the environment to shift the stability of the protein until unfolding occurs. Mutational analyses are a complementary approach where changes in the protein itself are used to alter protein stability in a given environment. The power of this somewhat indirect approach was demonstrated in some seminal work on the dimerization of the human erythrocyte sialyglycoprotein, glycophorin A, by Lemmon, Russ, Engelman, and co-workers.

1. Glycophorin A

The dimerization of glycophorin A is mediated solely through the membrane part of the protein, which consists of a single TM α helix. The native dimer is stable in SDS, which allows disruptive mutations in the TM helix to be monitored on SDS-PAGE gels. This approach provided the base of a series of studies by Lemmon, Engelman, and co-workers (Lemmon et al., 1992a,b; 1994; Treutlein et al., 1992) on the sequence specificity of the dimerization reaction. A chimeric protein was designed and purified that consisted of a water-soluble protein (Staphylococcal nuclease) and the TM helix of glycophorin. Saturation mutagenesis was then carried out on all residues in the TM helix (i.e., each residue mutated in turn to every other 19 natural amino acids), and the resulting mutant chimeric proteins were assayed for dimer formation. The periodicity of 'allowed' and 'forbidden' sites of mutation (3.9 residues) led them to predict a right-handed supercoil structure of two TM α helices in the dimer. A dimerization

motif was also identified in the native glycophorin dimer that consisted of seven amino acids, LIxxxGVxxGVxxT (where x is any amino acid). Furthermore, the incorporation of this dimerization sequence into the TM helix of the epidermal growth factor receptor, the Neu oncogene product, or a poly-leucine helix resulted in dimer formation in each case. The subsequent structure determination of the native glycophorin helix-dimer in SDS by NMR (Mackenzie et al., 1997) confirmed that all the residues of the LIxxxGVxxGVxxT dimerization motif participated in van der Waals interactions between monomers and showed a stereospecific interaction between the Gly and Val residues of the different monomers. On the basis of the NMR structure, the relative contributions of a number of different factors to the dimerization equilibrium were determined (MacKenzie and Engelman, 1998). The amount of dimer detected was scored in one of four classes ranging from wild-type level to no dimer at all, and the scores were compared to the best-fit scores of a number of different models. Each of these models consisted of a linear combination of contributions arising from the gain in side-chain entropy associated with a mutation, the difference in the number of favourable van der Waals contacts, the number of steric clashes introduced, the change in hydrophobicity, and side-chain volume. The best fit was found for a model that included a large penalty for steric clashes, positive contributions for van der Waals contacts and side-chain conformational freedom, and no hydrophobicity or side chain volume contributions. The calculated scores from the best-fit model correlated well with experimentally determined differences in the free energy of dissociation of glycophorin dimers and two mutants obtained from analytical ultracentrifugation experiments (Fleming et al., 1997), as well as with monomer/dimer equilibria of multiple site mutants measured by SDS-PAGE (Mingarro et al., 1996, 1997). Given the simplicity of both the scoring method and the calculated energy function, this is a remarkable result, but it should be treated with some care. As pointed out by the authors, the data refer to the monomer/dimer equilibrium in a detergent environment, and therefore changes in hydrophobicity and side-chain volume may still have considerable contributions to the stability of the monomer in a hydrophobic environment (micelles or the lipid bilayer) compared with a monomer in aqueous detergent solution. Furthermore, small rearrangements of the helices in the dimer were not taken into account in the modeling. Nonetheless, it is very promising that the helix-helix interactions can be described in such a simple way.

The importance of the membrane environment on the thermodynamics of membrane proteins is once again underlined by results obtained on the glycophorin homodimer in a membrane environment. A genetic screen was developed for the dimerization of a TM helix based on the transcriptional activator ToxR from *Vibrio cholerae,* which is only functional after dimerization mediated through the TM domain (Langosch et al., 1996; Brosig and Langosch, 1998). The TM domain of ToxR was replaced with the glycophorin TM helix and the whole construct was fused to the C-terminus of the *E. coli* maltose-binding protein. This protein replaces the ToxR periplasmic domain and ensures firm anchoring and the proper directionality of insertion in the membrane. The amount of dimerization of the chimera can be analyzed on the basis of the activity of a reporter gene whose transcription is mediated by the ToxR-dimer. Analysis of the sequence specificity indicated that not all residues identified in the dimerization motif in SDS micelles were also critical for dimerization in the *E. coli* inner membrane. In fact, it

was shown that the central GxxxG motif by itself is enough for efficient dimerization. This result was also obtained in a slightly modified system where the concentration of the protein in the membrane can be controlled by tuning the induction of the construct. In this case the coupled reporter gene was for chloramphenicol acetyltransferase, an enzyme responsible for the breakdown of the antibiotic chloramphenicol (TOXCAT assay; Russ and Engelman, 1999, 2000). Libraries of Ala- and Leu-based sequences were then generated that have a strong propensity to dimerize in the membrane environment. The GxxxG motif was present in 96% of the leucine-based library and 79% of the alanine-based library. An analysis of the SwissProt database has further shown that a GxxxG motif may be used frequently as a packing motif in membrane proteins (Senes et al., 2000).

C. Experimental Methods: Membrane Protein Stability by Design

The results obtained from the dimerization studies of the glycophorin TM domain have stimulated a new approach to the study of TM helix interactions. The well-known helix interaction motif of the leucine zipper found in water-soluble proteins has been adapted to a TM environment by changing the noninteracting hydrophilic residues into hydrophobic residues. The interaction propensity of a particular amino acid motif is then investigated in a membrane environment using the methods described in the previous section.

The signature of the leucine zipper motif is the heptad repeat sequence $(abcdefg)_n$ of interacting residues, where *a* to *g* denote positions on a 3.5 residue per turn helical wheel projection. Positions *a* and *d* are taken up by hydrophobic residues and form the core of the interaction surface, whereas positions *e* and *g* are often charged and sometimes form salt bridges to the neighboring helix. Positions *b*, *c,* and *f* are on the opposite side from the interaction surface and usually are taken up by hydrophilic residues. By changing these into hydrophobic residues, the sequence can be rendered membrane soluble, while keeping the interaction surface intact. This was first studied by Gurezka et al. (1999), who showed that a leucine zipper motif of *gaxxdexgaxxdexga* mediates dimerization of an alanine host sequence in natural membranes. Zhou et al. (2000) studied poly-leucine bases sequences containing leucine, valine, or three valines and one asparagine at the *a* positions of the heptad repeat sequence. They were able to show that efficient dimerization of a chimera of staphylococcal nuclease and a TM helix in detergent solution only occurred in sequences containing the Asn in the middle of the membrane spanning region. This was corroborated in a TOXCAT assay of related sequences, which additionally showed that the effect of two Asn residues on dimerization in the lipid bilayer was twice as large as that of a single Asn. Competition experiments with free peptides lacking the SNase protein in the chimera system indicated the formation of trimers. This study strongly suggests that hydrogen bonding of the central polar Asn residue is essential for the helix-helix interaction, which contrasts with the glycophorin A case where van der Waals interactions are the dominant forces. In a similar study to Zhou et al., the leucine zipper motif was incorporated into a random hydrophobic sequence and shown to result in protein oligomerization, although the oligomer size was not determined (Choma et al., 2000).

D. Summary of Stabilty Studies

This section indicates that membrane proteins in their native membrane in general are more stable than their water-soluble counterparts. This is due to their hydrophobic regions that lie within the membrane bilayer, rather than their extrinsic hydrophilic domains, which themselves have stabilities similar to water-soluble proteins. A quantitative interpretation of the data is difficult, and probably the most important message here is that quantitative results are strongly dependent on the environment (detergent micelles or lipid bilayers, type of lipid in the bilayer, etc.) that is chosen for the experiments. The glycophorin and GxxxG motif study, together with the TOXCAT assay discussed in this section, are good examples of an innovative and integrative biophysical approach that have been successful in addressing a specific question in membrane protein assembly, TM helix association.

V. MEMBRANE PROTEIN ENGINEERING

Protein engineering has been used to great effect in the study of water-soluble protein folding and stability. Protein folders have targeted numerous proteins with every conceivable form of mutation. The result is an extensive list of proteins that have been subjected to site directed (Fersht, 1998) or random mutagenesis, (Giver et al., 1998; Hennecke, Sebbel, and Glockshuber, 1999; Jung, Honegger, and Pluckthun, 1999) circular permutations (Hennecke et al., 1999; Iwakura et al., 2000; Otzen and Fersht, 1998; Zhang and Schachman, 1996; Viguera, Blanco, and Serrano, 1995) and fragmentation analysis (Kippen and Fersht, 1995; Neura et al., 1996).

The effects of mutations on soluble proteins can be measured relatively easily. Changes in the energetics along a folding pathway can be detected and assigned to mutations (Fersht, 1998). This, however, is restrictively difficult for membrane proteins as, apart from a few exceptions, the only real ways of monitoring the effects of mutations in a membrane protein is by assessing the protein's ability to insert into a membrane or micelle, together with a measure of their function and/or susceptibility to thermal unfolding (Chen and Gouaux, 1999; Chen and Gouaux, 1997; Lau et al., 1999; Zhou and Bowie, 2000). These crude methods preclude many of the analytical techniques used in water-soluble protein folding. Nevertheless, all of the mutagenesis techniques mentioned above have been applied to membrane proteins. There have been a large number of such studies, largely with the aim of addressing membrane protein function. Because it is not possible to address all this work here, this section aims to give only a very brief survey of some studies that are relevant to membrane protein folding, assembly and insertion, while indulging in some speculation as to their wider meaning. We highlight first site-directed and random mutagenesis approaches, giving specific protein examples and then go on briefly to mention some circular permutation and protein fragment studies. This section is summarized in two tables, one focussing on the proteins studied and the other on the techniques that have been used to investigate the co-assembly of protein fragments.

A. Site-Directed and Random Mutagenesis

Numerous membrane proteins have been subjected to mutagenesis, with bR, rhodop-

sin, DGK, and lactose permease standing out as the most extensively studied. The inherent difficulties in determining membrane protein structures by high-resolution techniques, such as X-ray crystallography or NMR spectroscopy, have led researchers to combine lower-resolution methods such as electron paramagnetic resonance spectroscopy with site-specific labeling (via cysteine residues), especially in the study of protein conformational changes (Farahbakhsh, Altenbach, and Hubbell, 1992; Flitsch and Khorana, 1989; Frillingos et al., 1998; Jung et al., 1993; Rink et al., 2000; Wang et al., 1998). In addition, protein engineering has been used to try and modify membrane proteins and increase their stability, for use in conventional structural studies, unfortunately unsuccessfully (Prive and Kaback, 1996; Zhou and Bowie, 2000). However, Bowie and colleagues have succeeded in creating stabilized versions of DGK (Zhou and Bowie, 2000). A huge number of mutations of different membrane proteins have been produced as a consequence of these various protein engineering attempts to obtain structural information, and the results for α helical proteins can be summarized in the following, oversimplistic manner. The vast majority of mutations have little or no detectable effect on the structure of the protein and often only a minor effect on the function, unless the residue is involved in the active site or function of the protein, or in a specific structural interaction such as the GxxxG packing motif discussed in Section IV.B. Moreover, membrane proteins are often unaffected by extreme mutations that would not be tolerated by their water-soluble counterparts. We highlight in this section some of the studies on larger TM α helical proteins, where this rather remarkable tolerance to mutation has been noted. We do not, for example, include the saturation mutagenesis study of glycophorin, where certain TM residues were found to be key to the dimerisation of this protein (discussed at length in IV.B).

1. Bacteriorhodopsin, bR

Nearly all the residues in bR have been subjected to site-directed mutagenesis, largely with the aim of studying the structure and function of the protein (for a recent overview see Lanyi, 2000). In the course of these studies all the threonines (Marti et al., 1991), tryptophans (Mogi, Marti, and Khorana, 1989a), tyrosines (Mogi et al., 1987), aspartates (Mogi et al., 1988; Otto et al., 1990), prolines (Lu et al., 2001; Mogi et al., 1989b) and membrane-embedded serines (Marti et al., 1991) have been mutated to several other residues. Not surprisingly, mutations of residues involved in the retinal binding pocket result in proteins showing altered rates of chromophore formation, along with blue-shifts in the visible spectra. Overall, however, the studies highlight the tolerance of α helical residues in bR to mutagenesis. In addition, bR is unique as it is the only membrane protein for which mutagenesis has been used to gain information about folding kinetics (Allen et al., 2001; Lu et al., 2001).

In one mutagenesis study, Chen and Gouaux replaced five non-polar, lipid-exposed residues on helix D with polar and charged amino acids (Chen and Gouaux, 1997, also briefly mentioned in Section II.B). These mutations significantly altered the hydrophobicity of the helix to the extent that most structural prediction algorithms are no longer able to predict the presence of a TM helix. It would be reasonable to expect these nonconservative mutations to have serious detrimental effects on the stability and folding of the protein. However, the protein still functions perfectly adequately, and heat denaturation measurements demonstrate that

the mutants are only slightly more susceptible to thermal unfolding than wild-type protein (the enthalpy of activation for thermal unfolding of the mutant protein is +168 kJ. mol^{-1} when compared with +182 kJ.mol^{-1} for wild-type bR). Similarly, studies show that a double mutant (G113Q, G116Q) is only slightly more sensitive to acid- or SDS-denaturation than wild-type bR, but otherwise is unaffected by this double mutation (Chen and Gouaux, 1999).

A recent kinetic study has focused on the role of membrane-embedded proline residues (Pro50, Pro91, and Pro186) in folding of bR (Lu et al., 2001). The three membrane-embedded prolines were mutated to glycine or alanine. All six mutants functioned more or less normally (Mogi et al., 1989b) and folded from SDS into mixed lipid/detergent micelles in the absence of retinal to form an apoprotein intermediate with wild-type secondary structure (akin to I_2 in Figure 2a and see Section III.D). The rate of formation of I_2 was slowed by the mutations at positions 50 and 91. This result shows that *cis-trans* isomerization of the peptidyl-prolyl bond of neither Pro50 nor Pro91 is rate-limiting in this particular folding reaction. Furthermore, the results suggest that the helix kinks and helix, helix interactions caused by these proline residues are involved in I_2 formation. Interestingly, the only mutation to have no effect on the folding rates (P186G) resulted in a significant blue-shift in the chromophore absorption maxima. Thus, the nature of the folded state does not dictate the rate of folding in this case. Because all the proline mutations affect retinal binding and final formation of bR, the kinks in the TM α helices caused by the prolines are important for the formation of the retinal binding pocket.

Another recent kinetic study in which loop residues of bR were replaced with structureless linkers has highlighted the roles of loop residues in folding and stability of bR (Allen et al., 2001; Kim et al., 2001) (and see Sections II.A and IV.A). The loop residues have been found to contribute to the overall stability of the protein (Kahn, Sturtevant, and Engelman, 1992), and in addition particular loop structures play specific roles in folding. These include the BC and FG loops, whose replacements cause a blue-shift in chromophore formation, indicating importance for native-like retinal binding, and the CD and EF loops, which are thought to be required for the specificity of helix packing, possibly due to electrostatic interactions between these two loops (Marti, 1998). The differing roles of loop residues are also apparent from steady-state loop deletion and insertion studies (Gilles-Gonzalez, Engelman, and Khorana, 1991), which show that mutants containing deletions in the BC or EF loops or with the C-terminal sequence of the Sendai virus L-protein inserted into four of the six helix-connecting loops (Teufel et al., 1993) can be synthesized and folded *in vitro* to form native-like bacteriorhodopsin, albeit with lower stability in some cases, compared with wild type. However, the AB or EF loops cannot tolerate insertion of the Sendai virus epitope, indicating specific roles for these structures.

2. Rhodopsin

Retinitis pigmentosa (RP) is an autoimmune disease leading to the degeneration of the retina and blindness. It is associated with a number of point mutations in the transmembrane and intradiscal regions of rhodopsin that are thought to lead to incorrect folding and chromophore formation of rhodopsin. Therefore, mutagenesis has been used extensively (Garriga, Liu, and Khorana, 1996; Hwa et al., 1997; Hwa et al., 1999;

Liu, Garriga, and Khorana, 1996; Ridge et al., 1995b) to try to determine the importance of these residues in rhodopsin folding. One such study focused on the TM residues and used mutant proteins expressed in COS-1 cells (Hwa et al., 1997). From the results, the authors suggest that TM mutations cause packing defects, which are relayed to the intradiscal domain and induce formation of a non-native disulfide bridge. This is important as naturally occurring point mutations of the cysteine residues involved in this non-native bridge (C110 and C187) are also associated with RP and form mis-folded protein in COS-1 cells (Hwa et al., 1999).

3. Diacylglycerol Kinase, DGK

DGK has been the subject of several mutagenesis studies. Initially, a random library of over 600 mutations at all 121 residues was created (Wen, Chen, and Bowie, 1996), and the selection criterium was 5% to 100% of wild-type enzymatic activity. The strategy employed was such that in the absence of selection criteria, an average of 830 mutants would be expected. Therefore, about three-quarters (600/830) of the mutants possessed some DGK activity. Water-soluble proteins also demonstrate this level of tolerance (Bowie et al., 1990), but there is a clear discrepancy between the tolerance of highly solvent exposed positions and buried residues, which are extremely resistant to polar substitutions. In contrast, DGK is able to accommodate apolar to polar substitutions at all sites within the putative TM helices. This tolerance also applies to other TM α helical membrane proteins, providing the residues are not directly involved with the protein's function.

The tolerance of DGK to mutation is particularly well demonstrated by its "passive" TM α helix (Zhou, Wen, and Bowie, 1997). Bowie's lab mutated the whole of the putative first TM helix (TM 1, Figure 1c) to poly-alanine, with the resulting protein retaining 50% of wild-type activity. These studies suggest that specific interhelical contacts are not always important for determining the final fold of the protein.

The thermal stability of DGK has also been investigated via mutagenesis studies by Bowie and colleagues (Lau et al., 1999) (see also Sections II.B and IV.A). First, the two native cysteine residues were replaced with alanines, then two further mutations were made, which resulted in increasing the half-life of activity at 70°C from less than 1 min to 51 min. Furthermore, a library of mutants were screened for thermal stability (Zhou and Bowie, 2000) and another quadruple mutant was discovered, with a half-life of activity at 80°C of 35 min compared with the wild-type protein, which is active for less than 1 min. The trade off of this increase in stability is a mere 35% reduction in activity relative to wild type. These studies appear to demonstrate that DGK, and by association other membrane proteins have not been optimized for stability. However, when making assumptions about membrane proteins, one must bear in mind the importance of the environment, as the mixed lipid/detergent micelles often used are a poor imitation of lipid bilayers. A study of the effects of cysteine mutants on the activity of DGK demonstrates this point (Gorzelle et al., 1999). Sixty-five single cysteine mutations in the cysteine-less background resulted in a universal drop in activity, with 26 mutants showing activity of below 20% relative to wild type, when the proteins were refolded into DM micelles. However, if the proteins were refolded from DPC (a lyso lipid) micelles into POPC vesicles, 55 of the mutants showed increased activity, and 17 of the 26 severely impaired mutants had ≥60% of wild-type activity (see also Sections II.A and II.B).

4. Lactose Permease

There have been no studies to date on lactose permease that have been directed primarily toward the effect of mutations on the folding of lactose permease. However, the protein has been subjected to a vast number of mutations and therefore represents a mine of information concerning the effects of protein engineering on an integral membrane protein.

Lactose permease is a 12 TM, α helical transport protein and contains 417 residues, of which 390 have been mutated, mainly by replacement with cysteine residues. Protein activity is retained after mutagenesis of any of the 6 tryptophan, 12 proline, 14 tyrosine or 34 glycine residues. Only 4 of the sidechains (Glu269, Arg302, His322, Glu325) are absolutely critical for the function of the protein, suggesting that they are directly involved in the activity of the protein (Kaback and Wu, 1997). Other mutations that disrupt protein function tend to be extreme, for example, replacing Gly64, within helix II, with proline (Jung et al., 1995). However, this mutant, along with all the others reported, still folds and inserts into the membrane. Furthermore, large-scale mutations of lactose permease have demonstrated that the protein can fold with duplications of parts of helices, or even the complete N-terminal helix (Wrubel, Stochaj, and Ehring, 1994).

5. Photosynthetic Reaction Center: A Role for Proline Residues?

Second-site suppressor studies in a bacterial photosynthetic reaction center (Schiffer et al., 1995) have been particularly interesting because they have provided clues to the function of proline residues during folding. These experiments demonstrated the restoration of photo-competence of the photosynthetic reaction center when proline residues were introduced into mutants containing cavities caused by the removal of bulky aromatic residues from neighboring helices. Proline residues are relatively common in TM regions of membrane proteins, where they may promote folding by increasing protein stability. This is in contrast to the traditional role of proline residues as helix-breakers in water-soluble proteins.

6. E. coli Outer Membrane Protein OmpA

The following study on the N-terminal domain of OmpA was carried out predominantly *in vivo* and does not necessarily fall within the scope of this review. However, as it is the only example of the effects of point mutations on the folding and assembly of a TM β-structure, it deserves a mention.

A 2.5 Å crystal structure (Pautsch and Schulz, 1998), along with a recent NMR structure (Arora et al., 2001), shows that OmpA is an 8-stranded anti-parallel β-barrel, with large extracellular loops and small periplasmic turns between each strand. Koebnik replaced either lipid facing or inwardly facing sidechains in 3 of the 8 strands with randomly selected sidechains to study the sensitivity of this protein to mutagenesis (Koebnik, 1999). Three parameters were found to be important for correct protein insertion into the outer membrane of *E. coli*. First, the protein could not tolerate more than one polar, lipid facing sidechain per strand, and no charged sidechains in the center of a strand. Second, inwardly facing sidechains were intolerant to large increases in size and finally, unlike in TM helices, membrane-embedded proline residues were not acceptable in any position. Because

this study was carried out *in vivo,* it is quite possible that the assembly problems associated with mutants were not a direct result of the inability of the protein to fold, but instead a consequence of processing problems downstream of folding within the membrane. Despite this, it allows us to begin to compare the tolerances of helical and β-barrel TM proteins to mutations and seems to suggest that TM β-structures are more sensitive to mutations than their helical counterparts.

7. Circular Permutation Analysis

Circular permutation analysis is an interesting alternative mutagenesis strategy that consists of producing variants of the protein of interest with the wild-type N- and C-termini connected by a native peptide bond and new termini introduced elsewhere within the protein. To date, this strategy has only been applied to the study the folding of the $IICB^{Glc}$ subunit of the glucose transporter (Beutler, Ruggiero, and Erni, 2000b) and OmpA (Koebnik and Kramer, 1995). Both studies were carried out *in vivo*, but they are interesting because they are consistent with the *in vitro* studies described above, demonstrating the ability of membrane proteins to fold and function despite "rewiring". However, the fact that some circular permutants will not fold means that it would be useful to study this form of mutation *in vitro* to enable us to separate any processing problems from the innate ability of a protein to assemble in the membrane.

8. Summary of Some Key Findings of Mutagenesis Methods

Table 3 summarizes the key findings relating to folding for the proteins discussed above using mutagenesis approaches.

B. Analysis and Co-Assembly of Protein Fragments

Membrane protein fragments are particularly useful to investigate the stability of individual secondary structural elements when expressed in isolation, or the importance of interactions between two or more fragments when they are co-expressed. Fragment studies can be used to determine the roles of particular regions of the protein such as aqueous loops and co-factor binding sites in folding and stability of the folded protein. Following a general introduction, this section concentrates on the α-helical proteins bR and vertebrate rhodopsin, which have been studied fairly extensively by the use of fragments.

Fragment co-expression studies have been used widely *in vivo* and *in vitro* to determine the effects of splitting membrane proteins at various positions after folding and assembly. An extensive list of α-helical membrane proteins can assemble functional protein with splits or deletions in at least some of the loop regions (see Table 4). These include the m^2/m^3 muscarinic acetylcholine receptors, in which functional complexes can be formed from pairs of genes split in all three intracellular and extracellular loops, when co-expressed (Maggio, Vogel, and Wess, 1993; Schoneberg, Liu, and Wess, 1995), along with the sugar transporters GLUT-1 (Cope et al., 1994), lactose permease (Zen et al., 1994), and the $IICB^{Glc}$ subunit of the *E. coli* glucose transporter (Beutler et al., 2000a).

The yeast α-factor, the red cell anion exchanger (AE1 band 3) (Groves and Tanner, 1995; Groves, Wang, and Tanner, 1998), the voltage-gated Cl^- channel clc-1 (Schmidt-Rose and Jentsch, 1997) and P-glycoprotein (Loo and Clarke, 1994) are among helical channels and receptors that are capable of assembling with splits in one or more of the

TABLE 3
Examples of Some of the Key Findings Relevant to Membrane Protein Folding from Mutagenesis Methods

Protein	Type of mutation	Key findings	Reference
Bacterio-rhodopsin	Polar substitutions in helix D	High tolerance or bR to polar mutations in apolar regions	(Chen & Gouaux, 1997)
	Mutagenesis of membrane-embedded proline residues	Proline residues 50 and 91 are important in formation of 7 TM bundle and Pro50, Pro91 and Pro186 for retinal binding.	(Lu *et al.*, 2001)
	Loop deletions/insertions and replacement of loop residues with structure-less linkers	Differing roles of loop residues in folding and stability of bR. For example, the CD and EF loops are important in the formation of the 7TM bundle. A cut or replacement of the BC loop causes a blue-shift in the bound retinal chromophore band.	(Allen *et al.*, 2001; Gilles-Gonzalez *et al.*, 1991; Greenhalgh *et al.*, 1993; Kim *et al.*, 2001; Teufel *et al.*, 1993)
Rhodopsin	Deletion/reversal mutants in intradiscal loop residues	Structure of intradiscal loops important for correct folding. Disulfide bridge involved.	(Anukanth & Khorana, 1994; Hwa *et al.*, 1997; Hwa *et al.*, 1999; Ridge *et al.*, 1995b)
Diacylglycerol Kinase	Random mutagenesis of apolar residues	Highly resistant to mutations in apolar region	(Wen *et al.*, 1996)
	Mutation of TM Helix I to poly-alanine	Presence of a passive TM helix	(Zhou *et al.*, 1997)
Lactose permease	cysteine scanning mutagenesis	Only 4 residues absolutely essential for activity	(Kaback & Wu, 1997)
	Helix duplications	Tolerance of protein to helix duplications	(Wrubel *et al.*, 1994)
Photosynthetic reaction centre	Second-site suppressor analysis	Ability of proline residues to restore photo-competence in cavity-generating mutations	(Schiffer *et al.*, 1995)
Shaker K^+ *channel*	Charge reversal mutations	Specific interactions involved in assembly/oligomerisation	(Tiwara-Woodruff *et al.*, 1997)
OmpA	Random mutagenesis of lipid/inward facing residues	Sensitivity of barrel residues to mutations	(Koebnik, 1999)
	Circular permutation	Correct order of strands required	(Koebnik & Kramer, 1995)
$IICB^{Glc}$	Circular permutation	Orientation of TM helices determined by sequence, not by order of translation	(Beutler *et al.*, 2000b)
Glycophorin	Saturation mutagenesis and TOXCAT assay	Identification of LIxxxGVxxGVxxT dimerisation motif, with GxxxG being a frequent packing motif for TM helices.	See section 3.2 (Lemmon *et al.*, 1992a,b; Russ & Engelman, 1999)

TABLE 4
Some Approaches Used in the Co-Assembly of Membrane Protein Fragments

Method	Protein	Reference	Some techniques used
(Co-)expression in insect and mammalian cell lines	Rhodopsin	(Ridge *et al.*, 1995a; Ridge *et al.*, 1999)	• Western blotting/radiolabelling to demonstrate stability of individual fragments and complementarity • Functional assays • Localisation and topology studies
	m2/m3 muscarinic acetylcholine receptor	(Maggio *et al.*, 1993; Schoneberg *et al.*, 1995)	
	GLUT-1 glucose transporter	(Cope *et al.*, 1994)	
	P-glycoprotein	(Loo & Clarke, 1994)	
(Co-) expression in bacterial/native cell lines	Lactose permease	(Wrubel *et al.*, 1994; Zen *et al.*, 1994)	
	bR	(Kahn & Engelman, 1992; Liao *et al.*, 1983)	
	OmpA	(Koebnik, 1996; Koebnik, 1999)	
	α factor	(Martin *et al.*, 1999)	
	$IICB^{Glc}$	(Beutler *et al.*, 2000a)	
Cell-free translation	Red cell band 3 (AE1)	(Groves & Tanner, 1995; Groves *et al.*, 1998)	
Injection of cRNA into frog oocytes	Chloride channel CLC-1	(Schmidt-Rose & Jentsch, 1997)	• Radiolabelling to demonstrate expression • Electrophysiology to measure channel activity/gating
	Rell cell band 3 (AE1)	(Groves & Tanner, 1995; Groves *et al.*, 1998)	
	Shaker K^+ channel	(Tiwara-Woodruff *et al.*, 1997)	
Structural studies of TM peptides or fragments	CFTR	(Wigley *et al.*, 1998)	• FTIR spectroscopy • CD spectroscopy • Amide exchange • Mass spectrometry
	bR	(Hunt *et al.*, 1997; Luneberg *et al.*, 1998)	
	Shaker K^+ channel	(Peled-Zehavi *et al.*, 1996)	

loops. β-barrel proteins can also assemble from fragments, as has been demonstrated for the 8-stranded barrel OmpA (Koebnik, 1996) which functions when split at the 2nd or 3rd turn. However, for many of these proteins only particular fragments are stable in isolation and only certain combinations can co-assemble to form functional protein. In addition, in some cases such as in $IICB^{Glc}$ (Beutler et al., 2000a) assembly to form functional protein can only occur when the fragments are co-expressed on a single replicon. Therefore, there seem to be minimum requirements for fragments to co-assemble to form functional proteins before they are degraded. These requirements include interactions between residues within individual fragments as well as between fragments, the structure of certain loops, and/or spatial proximity of the fragments.

The sugar transporter lactose permease is a 12 TM α-helical protein and, as mentioned above, can be split in a number of loops, including that between TM segments 6 and 7 to produce fragments that assemble to form functional protein when co-expressed (Zen et al., 1994). However, if the two fragments are expressed separately, the 6 N-terminal helical fragment (N6) is observed inconsistently, while the 6 C-terminal helical fragment (C6) is not observed at all. This illustrates an example of interaction between the two halves of the protein to promote stability. This association is specific to lactose permease, because expression of N6 fragments from structurally related sucrose or tetracycline transporters are unable to stabilize the C6 fragment (Sahin-Toth, Kaback, and Friedlander, 1996).

Fragment studies have provided information about the stability of individual TM segments in membrane proteins, which can give clues to the possible structures *in vivo*. An example of this is cystic fibrosis transmembrane conductance regulator (CFTR), which is predicted to contain 12 TM segments, organized into two domains (TMD1 and TMD2), with six spanning regions each. Fragments studies suggest that the membrane spanning regions of TMDI adopt a stable α-helical structure (Wigley et al., 1998). This work has also led to hypotheses relating the secondary structures of these peptides to functional 'switching' roles. Furthermore, fragments have been used to study the importance of specific and general interactions between helices during folding in the Shaker K^+ channel (Peled-Zehavi et al., 1996) and bacteriorhodopsin (see below).

1. Bacteriorhodopsin, and Rhodopsin

Early fragment studies showed that native-like bR can be formed by mixing peptides containing helices A and B or separate A and B α-helical segments with the complementary five-helix fragment in the micelles (Liao et al., 1983) or lipid vesicles (Kahn and Engelman, 1992). These experiments suggest that helices A and B are independent folding domains, and the covalent loops connecting these helices to one another and to the rest of the protein are not essential for correct helix association. The idea that helices may act as autonomous folding domains prompted researchers to further examine the relative contributions of helix-connecting loops, helix-helix interactions, individual helices, and particular residues to folding and stability of bacteriorhodopsin.

Fragment studies have been used recently (Hunt et al., 1997) to study secondary structure and stability of each individual helix of bacteriorhodopsin by expressing seven polypeptides corresponding to each

transmembrane α helix. As predicted, helices A, B, D, and E formed α-helices, with differing stabilities, while that corresponding to the G helix formed a hyperstable β-sheet structure and the F helix peptide formed no discernible secondary structure. The C helix adopted a number of conformations in a pH-dependent manner. It has been postulated from these and other results that the F and G helices may have to interact with other regions of the protein before they can insert into the membrane. Experiments to study the secondary structure of fragments of bR with 2, 3, 4, or 5 TM segments produced very similar results (Luneberg et al., 1998). Enhanced structural stability of the N-terminal α-helices relative to the C-terminal helices was observed, indicating that the N terminal helices may provide a folding template in bacteriorhodopsin, inducing the correct secondary structure of the C-terminal fragments after association.

In the G protein coupled receptor rhodopsin, fragment and deletion studies (Ridge, Lee, and Abdulaev, 1996; Ridge, Lee, and Yao, 1995) have demonstrated that two or more fragments of the protein generated from cuts in the second or third cytoplasmic loop assemble and in some cases show native-like function when co-expressed in COS-1 cells. Further fragment studies (Ridge et al., 1999) containing deletions in the third cytoplasmic loop and C-terminus are consistent with this and have also shown that, as in bacteriorhodopsin, the loop residues are not always required for correct folding of rhodopsin. However, the intradiscal loop structures in rhodopsin (including the presence of an disulphide bridge) (Anukanth and Khorana, 1994) are thought to be important for correct folding and chromophore formation as deletion and reversal mutants form native-like chromophore very poorly if at all. In addition, at least some of the loops form ordered structures in solution when expressed in isolation (Yeagle et al., 1997).

C. Summary of Protein Engineering Methods

The folding and assembly of membrane proteins have been studied following the small- and large-scale mutations, taking the form of amino acid substitutions, insertions, deletions, or expression of truncated protein fragments. Membrane protein engineering has served to highlight the remarkable tolerance of α-helical membrane proteins to large-scale mutations, when compared with water-soluble proteins. Furthermore, in some cases there appears to be a breakdown in the tight sequence/structure/function relationship that is a central dogma of water-soluble protein folding. This is particularly well illustrated by the "passive" helix of DGK (Zhou et al., 1997). This phenomenon is most likely due to the restrictive nature of a membrane, which severely limits the structures a bilayer traversing polypeptide chain can adopt. Therefore, the number of sequences that will adopt similar structures is likely to be larger than for a soluble proteins. One could go on to conceive of situations where the only function of a TM region is to get the chain from one side of the membrane to the other, in this case the specific sequence would be irrelevant as long as it is capable of inserting into and spanning the membrane. There are, of course, situations where sequences on TM helices do matter, either because the residues are involved in the protein's function or because they aid necessary helix-helix interactions, such as the GxxxG sequences first highlighted in the study of glycophorin.

The fact that functional membrane proteins appear to be able to assemble from

fragments, assuming the fragments involved are independent folding unit, is in hindsight not surprising given that a collision event between two fragments is much more likely in the essentially 2-dimensional membrane when compared with the 3 dimensions that water-soluble proteins must work in. These fragment studies suggest that the interactions between the TM domains are enough to identify the protein fold, and that the loops connecting these domains therefore do not contribute to structural specificity. However, the situation is not as simple as this, and while TM helices may specify the overall fold, the loops can provide important fine details. The studies on the loop mutants of bR, where each loop was either cut or mutated to a Gly-Gly-Ser repeat sequence, demonstrate that these structures do contribute to stability, structural specificity, and the folding pathway.

VI. THE LIPID MEMBRANE

The examples we have given in earlier sections of this review (Sections II.A, III.D, and IV.A) indicate that the lipid membrane is not a passive bystander during protein refolding. Indeed, there is now substantial evidence to suggest that lipids play a significant role in modulating the final state behavior of many membrane proteins. It is important to recognise that the lipid membrane is more than just the impermeable fluid partitioner of the cellular space, but also an active component that may interact and help to control the behaviour of a range of membrane proteins. From a protein folding viewpoint, lipid properties can potentially be used to control protein folding and thus to design efficient folding systems for membrane proteins. We summarize at the end of this section how it has been possible to start designing PC/PE systems for bacteriorhodopsin. However, in order to make the best use of the lipid forces in protein folding, the nature of these forces must be well understood. Thus, in this section we give a detailed review of the relevant lipid properties, focusing on nonspecific interactions. This section begins with a description of the origins of internal mechanical stresses in membranes. We then explain how the complexities of the stresses in a lipid membrane can be translated into a set of simple material parameters. This is followed by a brief outline of how these parameters are measured in practice. This latter section (VI.D) is rather detailed and could be omitted on a first reading. It is however included in order to convey to membrane protein workers how we can begin to predict which lipid systems may be useful, and to quantify the relevant interactions. Finally, some examples of the ways in which these lipid mechanical stresses may affect protein folding are outlined. This last section is of course quite speculative given the gaps in our knowledge in membrane protein folding, but will, we hope, show how the correlations between lipid composition and protein folding and function might arise.

A. A Role for Nonspecific Interactions

There is general acceptance that certain lipids have very specific physical interactions that are used in cell regulation. For example, phosphoinositides are integral to cell signaling pathways (Janmey, Xian, and Flanagan, 1999; Shears, 1998), and there appears to be a need for sphingomyelin/cholesterol mixtures to form lipid rafts in signal transduction (Simons and Toomre, 2000). However, much of the recorded evidence of lipid/protein interactions suggests that nonspecific interactions may play an

equally significant role in cell regulation mechanisms (Bezrukov, 2000; Epand, 1996; Epand, 1998; Langner and Kubica, 1999; Seddon, 1990b). To clarify the difference between a specific and a nonspecific lipid/protein interaction, we follow Bezrukov's definition (Bezrukov, 2000) in which a nonspecific interaction is one that "does not involve any biochemical reaction or high selectivity with respect to fine chemical details". We have already discussed how nonspecific lipid/protein interactions can affect bR folding in PC/PE mixtures (see Sections II.A, II.B., III.D, and IV.A). There is similar evidence of nonspecific lipid/protein interactions from studies of proteins that are in their final folded state, including rhodopsin, Ca^{2+} ATPase, lactose permease, and alamethicin and gramacidin ion channels (Brown, 1994; Brown, Gibson, and Thurmond, 1996; Chen and Wilson, 1984; Keller et al., 1993a; Navarro, Toivio-Kinnucan, and Racker, 1984). As the physical source of these nonspecific interactions is universal, the findings from these studies are a valuable transferable source of insight into membrane protein refolding experiments.

The discussion presented in this section is confined to these nonspecific interactions. In particular we focus on to local interactions, that is, lipids that are a relatively near to the protein, (assuming protein refolding is unlikely to involve longer-range interactions between lipid and protein). The hydrophobic effect, charged headgroups, interfacial properties, and mechanical forces within the bilayer can all generate local interactions between protein and lipid that might affect refolding. It is well established that hydrophobicity is one of the primary physical effects driving the insertion of non-polar protein segments into the bilayer. The interfacial charge density is also critical to many protein-lipid binding events (Ahn, Guengerich, and Yun, 1998; Aivazian and Stern, 2000; Heimburg, Angerstein, and Marsh, 1999; Lundbaek, Maer, and Andersen, 1997; Martin, Epand, and Ruysschaert, 1998; Matsuzaki, 1998; Rostovsteva et al., 1998; Terzi, Hoelzemann, and Seelig, 1997). Furthermore, evidence is emerging that even where the interface is not charged the interfacial polarity influences the localization of certain proteins as well as particular amino acids (Giorgione, Kraayenhof, and Epand, 1998; Yau et al., 1998). The evidence that mechanical stresses stored in the lipid membrane affect protein behavior is now very substantial (Attard et al., 2000; Brown, 1994; de Cock et al., 1999; Dumas et al., 1997; Gilbert and Arena, 1998; Gruner, 1990; Gruner, 1994; Harroun et al., 1999; Keller et al., 1993a; Killian, 1998; Kroenke, 1999; Lewis and Cafiso, 1999; Lundbaek et al., 1998; Martin et al., 1998; Matsuzaki et al., 1998; Micol et al., 1999; Piknova, Perochon, and Tocanne, 1993; Ryba and Marsh, 1992; Veiga et al., 1999; Webb et al., 1998). Such evidence has prompted a range of theoretical papers that deal with ways of modeling the effect of lipid mechanical stresses on the function and localisation of membrane-embedded proteins (Arranda-Espinoza et al., 1996; Cantor, 1999a; Dan et al., 1994; Dan, Pincus, and Safran, 1993; Dan and Safran, 1998; Fattal and Ben-Shaul, 1993; Helfrich and Jakobsson, 1990; Huang, 1986; Kim, Neu, and Oster, 1998; Lundbaek and Andersen, 1999; Marcelja, 1976; May and Ben-Shaul, 1999; Mouritsen and Bloom, 1984; Nielsen, Goulian, and Andersen, 1998; Sperotto, 1997).

This section deals with the origins and effects of the stored mechanical stresses within the bilayer and will not directly address the hydrophobic effect, headgroup charge, or other interfacial properties. The effects of hydrophobicity, charge, and other interfacial properties all contribute to the stresses stored within the bilayer and there-

fore have both a direct and indirect effect on the refolding process. The present discussion on the effect of solely the mechanical stresses also allows hypotheses to be developed that can be experimentally tested.

B. The Lyotropic Phases of Natural Lipids

Our current understanding of the mechanical stresses that exist inside a bilayer lipid membrane have been derived from studies of lyotropic liquid crystalline phases and the physical forces that drive their stabilization.

There is a rich diversity in the liquid crystalline phases that may form when lipids and water are mixed (Luzzati, 1968; Seddon and Templer, 1995), but only one phase that can lead to the impermeable bilayer wall of the cell membrane. This is the fluid lamellar or L_α phase (Figure 3a). Nature has had to find a way of packing the lipids so that energetically costly hydrophobic contacts between hydrocarbon chains and water are minimized, while the polar headgroups are allowed to solvate. The general solution is to form an interface on which the hydrophilic headgroups pack rather closely, allowing them to sequester the hydrophobic chains behind them. In order to avoid aqueous contact with the chain termini, a second monolayer is packed back to back with the first. In the case of the L_α phase, the arrangement is for two back-to-back planar monolayers to form the basic element of the phase's structure. In the fluid phase lipids are free to diffuse laterally in the plane of the bilayer (diffusion rate $2 \cdot 10^{-8}$ cm^2 s^{-1} for natural lipids), but rarely jump out of it or flip from one side to the other (many hours to days for di-chain lipids). The lamellae stack periodically to balance the transverse repulsive and attractive forces that exist between bilayers. The L_α phase is liquid crystalline because of the combination of a two-dimensional fluid interface and the long-range periodic arrangement of the films. It is lyotropic because its structure depends on the addition of water to the amphiphilic lipid. From the biological perspective, the important point about this liquid crystalline phase is that with this structure we have the basic building block for the cell membrane wall, a continuous planar bilayer geometry, fluid in the plane of the membrane, and impermeable in the orthogonal direction (Rossof, 1996).

There is great diversity and variability in cell lipid composition; for example, there are over 250 lipid components in human erythrocytes (Myher, Kuksis, and Pind, 1989), including cholesterol, sphingomyelins, PCs, PEs, phosphatidylserines, and glycolipids with various chain lengths, degrees of chain unsaturation, and numbers of chains (see Table 5 and Figure 4). The amount of protein also varies between different membranes, from about 76% (by weight) in the inner mitochondrial membrane to about 50 to 60% in eukaryotic plasma membranes. Cholesterol is also a vital component of many biological membranes. Phospholipids are one of the main classes of lipids found in membrane and vary in both headgroup and length and unsaturation of their alkyl chains. Glycolipids are found in the plasma membrane and contain a sugar chain in place of the phospholipid headgroup. PC is the most abundant lipid in eukaryotic membranes and PE in prokaryotes. Archaebacteria, however, have membranes composed of ether-linked glycerides with branched alkyl chains.

Some naturally occurring lipids, such as the di-chain PCs, only ever form the lamellar phase, others, however, such as the di-chain PEs, will form nonlamellar phases under physiological conditions (Gruner et al., 1988; Seddon and Templer, 1995). Some of the more common non-lamellar phases

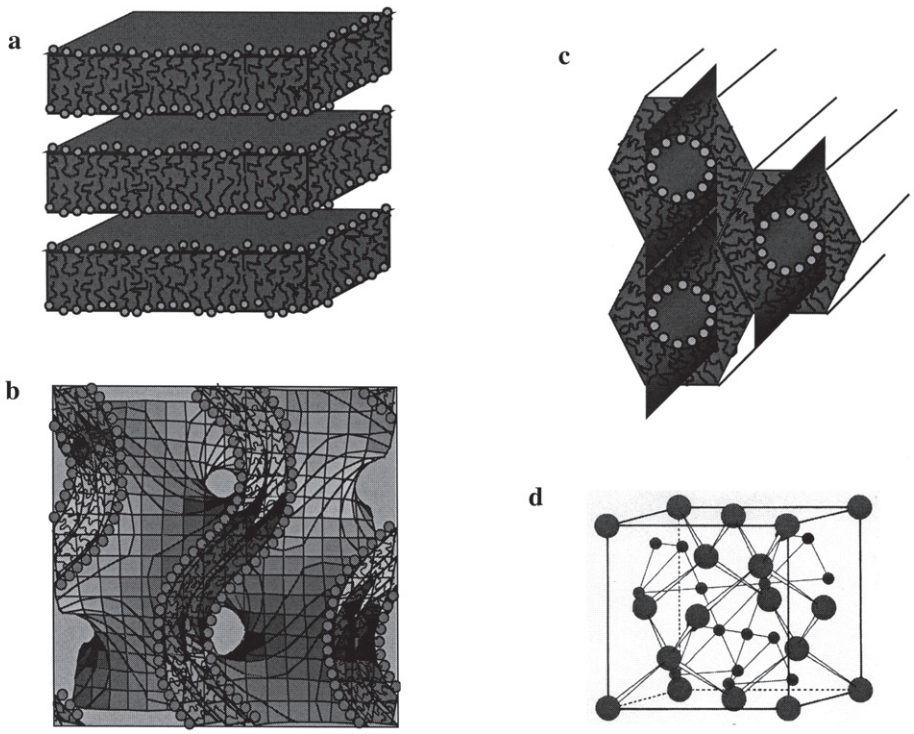
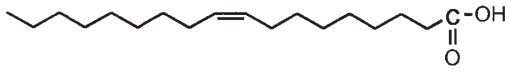


FIGURE 3. A selection of lyotropic liquid crystalline phases formed by biological lipids. The fluid lamellar phase, (a), consists of lipid bilayers separated by water layers. The bilayer thickness is of the order of 4 nm, but the water spacing can vary between approximately 1 and 100 nm depending on the nature of the forces between the bilayers. One of the inverse bicontinuous cubic phases called the gyroid is shown in (b). This structure divides space into two congruent sub-volumes of water. The mid-plane of the bilayer is a periodic minimal surface and the polar/apolar interface has average curvature towards the water. The inverse hexagonal phase, (c), is formed from a two-dimensional packing of monolayer cylinders. The curvature towards the water is now very clear. Only one packing of inverted micelles is known, (d), the Fd3m cubic phase. Here micelles of two sizes are to be found within the dodecahedra shown in the figure.

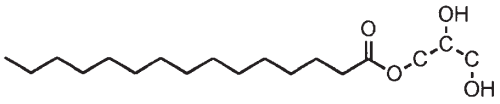
TABLE 5
Approximate Lipid Compositions of Some Biological Membranes

	Percentage of total lipid by weight (from Kates, *et al.* 1995; Alberts *et al.*, 1994; Devaux & Seigneuret, 1985; Kushwaha *et al.* 1975)				
Lipid	**Erythrocyte plasma membrane**	**Mitochondrial membranes (inner and outer)**	**Endoplasmic reticulum**	**Inner membrane of *E. coli.***	**Purple membrane of *H. salinarium***
Phospholipids PC	18	39	40		
PE	17	35	17	74	
PG			5	19	5
PS	7	2			
CL		20		3	
PGP-Me					52
Cholesterol	23	3	6		
Sphingolipids	18		5		
Glycolipds	3	trace	trace		30
Others	14	1	27	4	13

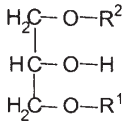
a)



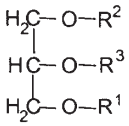
The fatty acid, *cis*-9-octadecanoic acid (commonly oleic acid)



The monoacylglycerol, 1-tetradecanoyl-*sn*-glycerol (commonly monopalmitin)

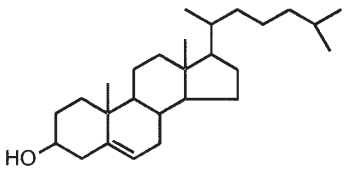


1,3-diacyl-*sn*-glycerol



triacylglycerol

R^1, R^2 and R^3 are acyl chains

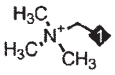


cholesterol

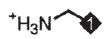
FIGURE 4. Chemical structures of some lipids

b)

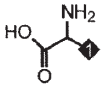
Some Glycerophospholipids



choline

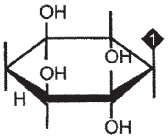


ethanolamine

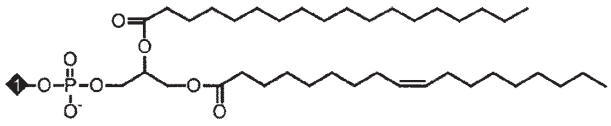


serine

glycerol



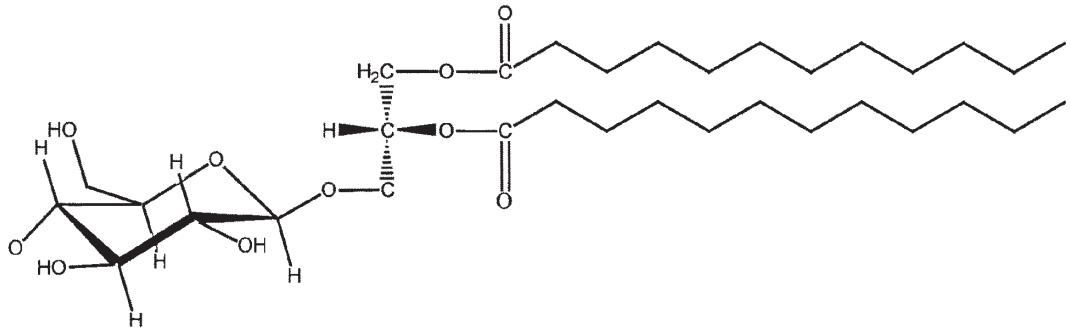
inositol



Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol and phosphatidylinositol lipids are formed by attachin the appropriate headgroup moiety to the phosphate group of the diacylglycerophosphate above. We show a stearic chain in 2 position and an oleic one in the 1 position.

c)

An example of a glycoglycerolipid



1,2-didodecyl-3-O-β-D-galactosyl-*sn*-glycerol (found in plants, animals and micro-organisms

FIGURE 4. (continued)

found with natural lipids are shown in Figure 3b. The di-chain PEs, for example, form both the inverse hexagonal or H_{II} phase and the inverse bicontinuous cubic phases, Q_{II}^{b}, in an excess of water; the phase formed depending on the details of chain structure and thermodynamic environment (Seddon, 1990b; Seddon et al., 1984). Monoglycerides tend to form Q_{II}^{b} phases (Briggs and Caffrey, 1994; Briggs, Chung, and Caffrey, 1996; Chung and Caffrey, 1995; Hyde and Andersson, 1984), while diglycerides in the presence of PCs tend to promote H_{II} phases and inverse micellar cubic or Q_{II}^{m} phases (Luzzati et al., 1992; Seddon, 1990a; Seddon, Bartle, and Mingins, 1990) and ceramides lower the transition temperature of the H_{II} phase transition when mixed with PEs (Kroenke, 1999; Pilar Veiga et al., 1999). In common with the diglycerides and ceramides, fatty acids, fatty alcohols, and fatty amines, form H_{II}, Q_{II}^{b} and Q_{II}^{m} phases when combined with PCs and PEs (Cevc et al., 1988; Huang, Seddon, and Templer, 1996; Seddon et al., 1997; Templer et al., 1998d). Cholesterol also promotes the formation of H_{II} phases when added to PCs (Chen and Rand, 1997). Many of the glycolipids also show a strong propensity for the formation of the H_{II}, Q_{II}^{b}, and Q_{II}^{m} phases (Duesing et al., 1997; Mannock et al., 1992; Mannock et al., 1994; Seddon et al., 1996; Turner et al., 1992). What characterizes the nonlamellar phases of the lipids we have mentioned in this paragraph is that their interfaces are no longer flat but bend toward the water. We term phases with such interfaces type II or inverse phases.

Single-chain PCs (lyso-PCs) display type I, or normal interfacial curvature, that is, they form interfaces that bend away from the water (Arvidson et al., 1985). The point to be noted here is that all the nonlamellar phases are characterized by having interfaces that are curved. Understanding the source of this interfacial curvature will lead us to an understanding of the origins of the mechanical stresses that are stored within a lipid bilayer.

C. The Origins of Internal Mechanical Stresses of Lipid Membranes

1. Lateral Pressure

The nonlamellar lyotropic liquid crystalline phases form because of the intrinsic propensity for interfacial curvature in all fluid monolayers (Helfrich, 1973; Kozlov and Markin, 1989). This propensity arises from the differential distribution of lateral pressures that occur within the monolayer (Helfrich, 1981) (Figure 5). Imagine that we were able to excise a patch of lipid monolayer from its native lamellar bilayer, hold it flat, and then probe the pressure as a function of depth through the monolayer. Because the hydrocarbon chains are molten in the liquid crystalline state and the lipids are crowded together, neighboring chain segments will suffer frequent collisions and one will observe a net positive outward lateral pressure in this region. Indeed, it has been possible to calculate the lateral pressure profile using statistical mechanical methods (Cantor, 1999b; Harris and Ben-Shaul, 1997; Szleifer, Ben-Shaul, and Gelbart, 1990a; Szleifer et al., 1990b; Szleifer et al., 1988), and profiles of the sort shown in Figure 5 are obtained in the case of fully saturated chains. Around the interface between the polar and apolar regions of the lipids we will find an inward interfacial tension (negative lateral pressure) as the hydrophobic effect drives the lipids together in order to reduce the exposure of hydrocarbon to water. Beyond this region is where the complex chemical interactions between

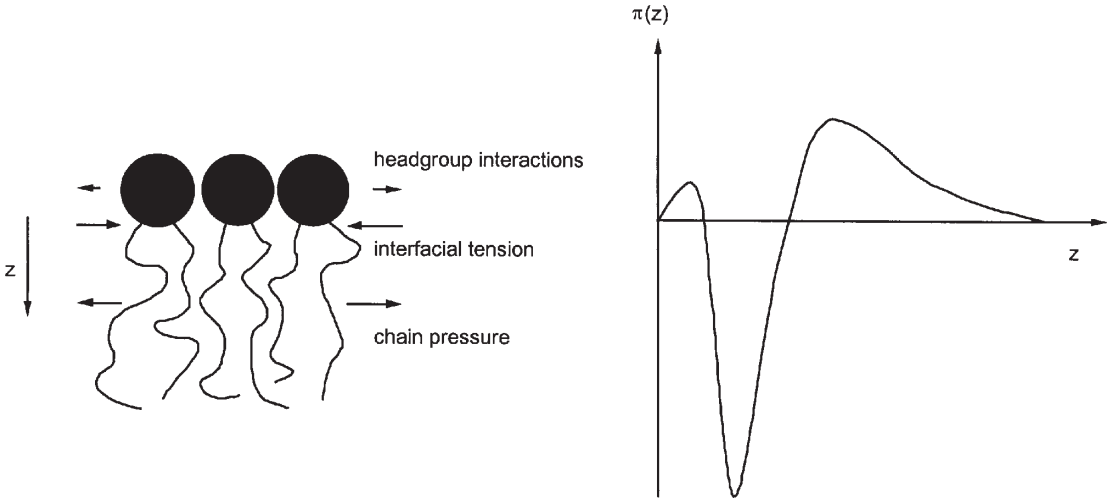


FIGURE 5. The lateral pressure. If one could sense the pressure within the section of flat monolayer shown, the qualitative result would be that shown in this figure.

polar headgroups take place. In Figure 5 we have shown the headgroup lateral pressure as being positive, and one could imagine this to be due to steric and hydration repulsions due to hydrogen bonding to water as well as electrostatic interactions in the case of charged headgroups. There is also evidence that some lipids will hydrogen bond from one headgroup directly to its neighbor, so in some cases the lateral pressure in the headgroup might be negative (Holmgren, Lindblom, and Johansson, 1988; Templer et al., 1998c). Very little is yet known about the precise nature of the chemical interactions in this region, but for the purposes of a general understanding of what drives the monolayer to bend this is in fact not necessary.

The total lateral pressure in the monolayer must be zero at equilibrium. In Figure 5 this simply means that the total pressure in the headgroup and chain region must be equal and opposite to that from the interface. If it is not then the monolayer will simply expand or contract in the lateral direction until the forces do sum to zero. Written formally this condition is expressed as

$$\int \pi dz = 0 \qquad (1)$$

where π is the lateral pressure and z is the distance perpendicular to the monolayer interface. This, of course, does not tell us anything about how much the monolayer would like to bend. For this the bending moment or torque, τ, of the monolayer has to be determined. The torque is defined as the product of the force acting on a body and the perpendicular distance to the axis of rotation. In our case this is the product πz that we must sum up over the thickness of the monolayer, or more formally

$$\tau = \int \pi \cdot z dz \qquad (2)$$

In general, this will be non-zero. This is because the plane about which the monolayer pivots (the two-dimensional equivalent of the axis of rotation) is located near to the molecular location with the least lateral compressibility, and this tends to be somewhere around the polar/apolar interface (Kozlov and Winterhalter, 1991a; Kozlov and Winterhalter, 1991b; Rand et al., 1990; Templer, 1995; Templer et al., 1998b). This molecular location is generally called the pivotal plane. Therefore, there is generally an imbalance between the torques from the chain region and the headgroups. This means

that if rather than holding the monolayer patch flat, as in our thought experiment, we were to remove any external forces on it, it would curl up, either toward or away from the water.

In type II lipids the torque due to the outward pressure in the chain region is far greater than any balancing pressure in the headgroup region. Indeed, where there is a desire for direct hydrogen bonding between headgroups this can add to the net desire for the interface to bend toward the water. Conversely, type I lipids have an excess of torque due to the high lateral pressure in the headgroup region and so they prefer curvature away from the water. Lipids with large polar headgroups and single chains normally fall into this category.

In nature the monolayer is normally not free to curl up in the way described above for our thought experiment. The hydrophobic effect means that monolayers must be held back-to-back, and in such a geometry the monolayers cannot simultaneously bend because they wish to do so by equal amounts, but in opposite directions (Figure 6). The system has only two choices: the monolayers may either remain flat or the system may change its structure into one of the lyotropic liquid crystalline phases with interfacial curvature.

2. Monolayer Torque Tension and Stored Curvature Elastic Energy

Biological membranes reflect the lamellar bilayer case, where both monolayers are held flat. In this case the monolayers are under torque tension, a phrase coined by

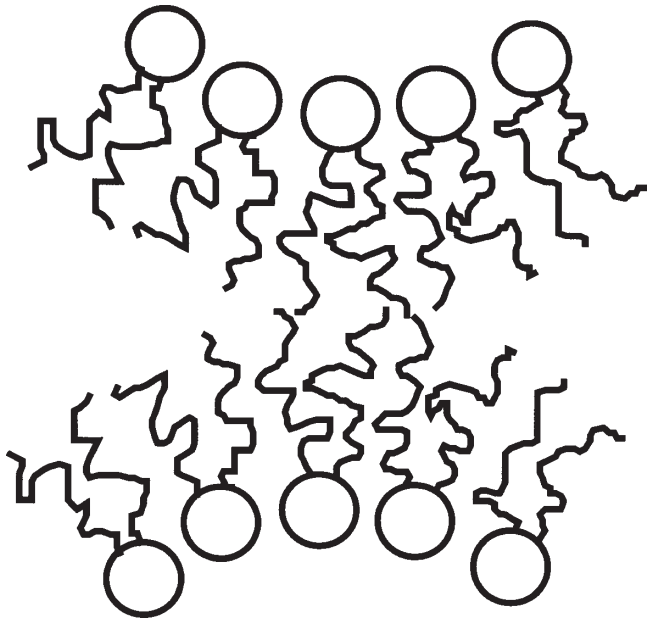


FIGURE 6. Curvature frustration. Although the differential distribution of lateral pressure does in general impart a desire for monolayer curvature this is frustrated in the biologically relevant Lα phase, because curvature of both monolayer leaflets cannot occur without the creation of vacuum. This is the general case even when a bilayer has an asymmetric distribution of lipids in each monolayer. Only when the 'inner' and 'outer' leaflets precisely complement each other in terms of their desire for curvature is the bilayer completely relaxed.

Helfrich (Helfrich, 1981) that aptly describes the fact that the monolayers are being held flat under tension due to the torque exerted by each. A good way to visualize the mechanical situation is to imagine slicing a tennis ball in half, placing each rubber hemisphere back to back, and then bending them simultaneously until they are lying back-to-back and flat. The torque tension can be released and the rubber hemispheres fly apart. This is the mechanical energy stored in the monolayers that are held flat, often called the stored curvature elastic energy.

3. Internal Stresses in PC/PE Mixtures Relevant to Membrane Protein Folding

The torque tension and the associated stored curvature elastic energy are present in all lipid lamellar bilayers. These properties may also be at the origin of some of the effects on membrane protein folding and function that have been discussed earlier in this review and is discussed in more detail in Section VI.E. Experiments on protein refolding at different lipid compositions inevitably involve the effect of different torque tensions on the protein. Different torque tensions reflect a different magnitude and distribution of lateral pressures in the bilayer as the lipid composition is altered (or indeed any other thermodynamic variable such as temperature, pressure, and pH is altered). Such effects of altered pressures are likely to be significant, because the magnitude of the chain pressures is really very high, at around 200 to 400 atm (Cantor, 1999b; Needham and Zhelev, 1996). Although the direct study of the lateral pressure is problematic and not without controversy, an informative picture has emerged out of our general understanding of lyotropic phase behavior that can be used to illustrate the sort of lipid compositional effects that might occur in refolding experiments.

PC/PE mixtures provide a good example of a biologically relevant system for which there is a substantial amount of data on the lipids themselves and that have been shown to affect the folding of bR. Currently, the most detailed knowledge of a mixed lipid system is for DOPC and DOPE mixtures. DOPE has an equilibrium inverse hexagonal phase under physiological conditions and DOPC an equilibrium fluid lamellar phase. DOPC/DOPE mixtures with small amounts of DOPE are in the L_α phase, but at some critical value (in this case at a mole fraction of DOPE of approximately 0.8 [Templer et al., 1992]) the system flips over into the H_{II} phase. It is the build up of monolayer torque tension, caused by the addition of DOPE, which eventually becomes too great to be sustained in the L_α phase and causes the system to undergo the phase transition.

The chains of DOPE and DOPC lipids are chemically identical, and thus when DOPE is added to DOPC it is the PE headgroups that cause the increase in torque tension. Measurements of lipid cross-sectional area reveal that the DOPE has a significantly smaller cross section than DOPC.

Molecular area measurements of lipids are surprisingly tricky (see, e.g., for a review and discussion Nagle and Tristram-Nagle, 2000), but current estimates make the cross-sectional area of DOPE approximately 65 $Å^2$ and of DOPC approximately 76 $Å^2$ at 37°C (Chen and Rand, 1997; Costigan, Booth, and Templer, 2000; Kozlov, Leikin, and Rand, 1994). It is thought that this is due to headgroup-headgroup hydrogen bonding being favorable for ethanolamine moieties, but headgroup-water hydrogen bonding being favored for choline moieties. Therefore, as DOPE is added to DOPC, the oleic hydro-

carbon chains become packed ever more closely together. This must result in a net increase in the lateral pressure of the chains. However, indirect measurements of changes in the lateral profile using a fluorescent probe (Templer et al., 1998a) indicate that the lateral pressure in the chain region also becomes redistributed, with excess pressure appearing toward the terminal end of the chains as DOPE is added. Recalling that the torque tension is the product of the lateral pressure and the distance from the pivotal plane (close to the glycerol backbone in this case), the redistribution of lateral pressure to this position maximizes its impact on the torque tension.

It is also possible to increase the membrane torque tension isothermally by manipulating not the average headgroup composition but the chains. For example, if one adds DOPC to DMPC the effect of mixing *cis*-unsaturated chains (oleic) with fully saturated chains (myristic) can be observed. The cross-sectional areas of these lipids at physiological temperatures are 76 and 67.5 $Å^2$ for DOPC and DMPC, respectively, with that of DOPC being greater, because the chain unsaturation increases the lateral pressure. Therefore, the addition of DOPC to DMPC increases the torque tension and the chain lateral pressure, but because the change is driven by the chain composition this is accompanied by an increase in lipid cross-sectional area, rather than the decrease in area seen with DOPE/DOPC mixtures. Increasing the torque tension and chain lateral pressure either by increasing the DMPC content of DMPC/DOPC mixtures, or the DOPE content of DOPE/DOPC mixtures, has the same apparent effect on bR folding; the folding yield is lowered and the overall folding rate slows down (see Sections II.A and III.D)

By way of an intermediate summary, we have seen how each monolayer making up a membrane bilayer is in general curvature frustrated, and this gives rise to internal membrane stresses that exist as a differential distribution of lateral pressures. Varying lipid composition, temperature, pH, or pressure may perturb the magnitude and distribution of lateral pressures, thereby increasing the internal stresses as the system approaches a phase boundary into a type II phase or decreasing them as it retreats from it.

D. Determining the Internal Mechanical Stresses of Lipid Membranes

1. Parameterizing the Effects of Internal Stresses in Lipid Bilayers: Bending Rigidity, Stretching Rigidity, and the Spontaneous Curvature

The mechanical basis for the interaction between proteins and the lipid membrane is via the deformations that each imparts to the other. To apprehend the full details of this is at present beyond the capabilities of either theory or atomistic computer simulation. Given the present state of knowledge, we are seeking qualitative to semiquantitative correlations between membrane properties and protein behavior (refolding, dynamics, and function). This in essence means that we need to simplify the physical pictures of both lipid membrane and protein.

The most widely used approach to obtain a tractable model of the lipid membrane derives from the work of Helfrich and Evans (Evans, 1974; Evans and Skalak, 1980; Helfrich, 1973). In their approach the thick monolayer is replaced by a thin sheet and then subsumes all of the molecular detail of the lipid monolayer into a set of elastic material parameters that imbue the sheet

with exactly the same elastic deformational response as the original monolayer. There are three important material parameters, k_c, the isothermal bending modulus for the monolayer, c_0, the spontaneous curvature for the monolayer and, k_a, the isothermal area dilation modulus for the monolayer.

The two curvature elastic parameters can be understood from our previous discussions. The spontaneous curvature of the monolayer is that curvature the monolayer would achieve if we removed the mechano-chemical constraints that normally hold it flat in the lamellar bilayer (Figure 7). The bending modulus tells us how rigid the monolayer is to being bent. The energy stored per unit area of monolayer while it is being held flat is, to first order, given by the expression $1/2 \cdot k_c c_0^2 \cdot$. This expression is essentially Hooke's Law for a thin film. It tells us that the more rigid the monolayer is or the more it wishes to bend, the greater the energy stored in the flat lamellar state. It is directly related to the torque tension (and hence the lateral pressure profile) by $t = 2k_c c_0$ (where we define spontaneous curvatures that are toward the water as being negative). The curvature elastic energy per unit area, g_c, required to bend the monolayer to some arbitrary curvature, given by c_1 and c_2 (Figure 8) is given by

$$g_c = \frac{1}{2} k_c (c_1 + c_2 - 2c_0)^2 \qquad (3)$$

at least as long as the monolayer topology is not altered (Helfrich, 1973; Helfrich, 1981; Helfrich, 1989). (Up to the present, only a few attempts to model protein-lipid interactions have considered the effect of changes in topology, see, e.g., Attard et al., 2000, and this additional complexity is not introduced here.)

The isothermal area dilation modulus simply tells us how stiff the membrane is to stretching in the lateral direction. If we stretch (or compress) the bilayer away from its equilibrium area per molecule, A_0, by an amount ΔA the energy required to do this is

$$g_a = 1/2 \cdot k_a (\Delta A / A_0)^2 \qquad (4)$$

Again this is in the harmonic approximation as was the case for the stored curvature elastic energy. It should be noted that because the lipid chain volume is incompressible, any dilation in molecular cross-sectional area results in compression of molecular length (Israelachilli, 1985; Tanford, 1980; Wennerstrom and Lindman, 1979). Therefore, we do not need to separately account for this, although it may at times be preferable to think of a bilayer deformation in terms of extensions in molecular length.

In fact, not only are changes in lipid cross-sectional area and length coupled, but so are k_c and k_a. To first order, both of these parameters derive from the way in which deformations of molecular cross-sectional area alter the lateral pressure profile. When a monolayer bends toward water, the molecular area dilates in the chain regions below the pivotal surface and shrinks above it and hence bending is a differential stretch. A simple mechanical model (Evans, 1974) predicts that $k_c \approx k_a d_l^2 / 12$, where d_l is the thickness of the monolayer. This means that changes in lipid composition that increase the bending rigidity also increase the rigidity to lateral stretching or compression.

The lipid-protein interactions within this model membrane that derive from deformations of the lipids imposed by the protein should depend on only three parameters, k_c, c_0, and k_a. The following section discusses how these material properties have been measured.

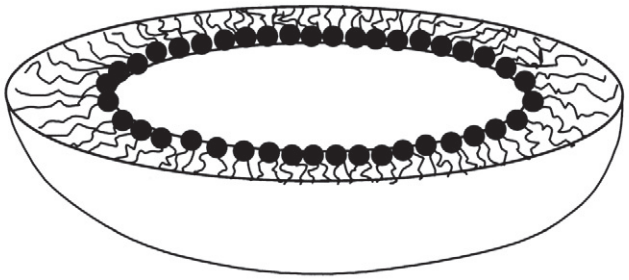


FIGURE 7. The spontaneous curvature can be visualized as the curvature a monolayer would achieve in the absence of any constraints as shown in this figure. In all systems that have been studied in depth it appears that the monolayer's inherent propensity is to curl up into a sphere. There are certain subtleties regarding the curvature elastic description that this observation implies, which are not dealt with here, for further information see Templer and co-workers (Templer, Khoo, and Seddon, 1998b).

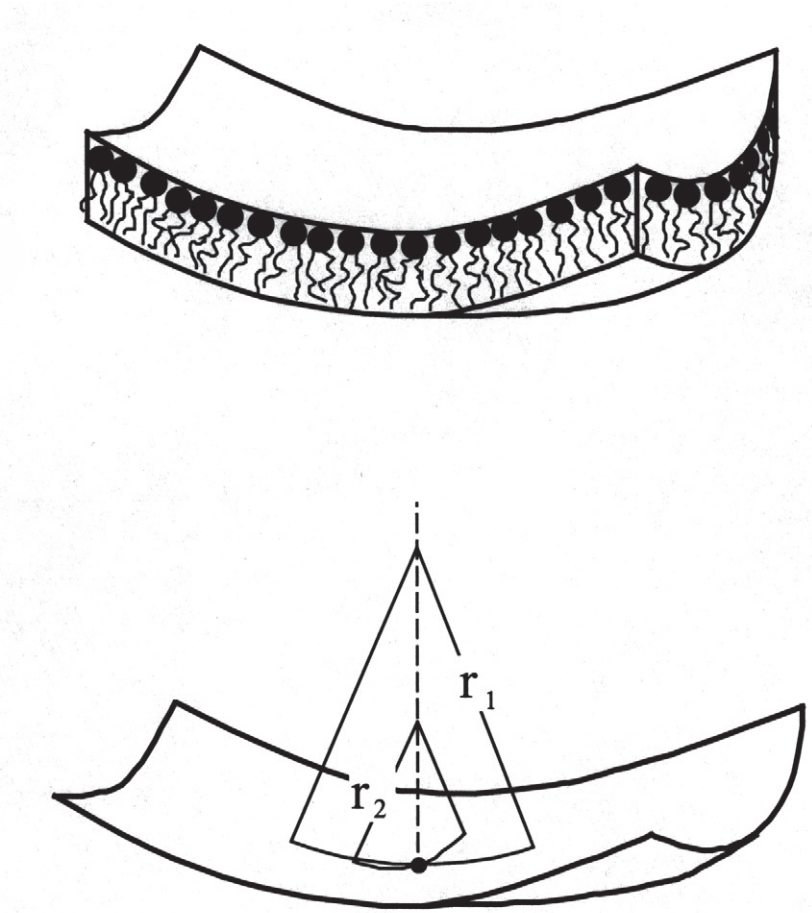


FIGURE 8. One replaces the thick monolayer with a thin mathematical sheet imbued with the curvature elastic properties of the monolayer when applying Equation 3. To do this the sheet is placed at the molecular location of the monolayer's pivotal surface (this means we need not consider stretching energy, but only curvature energy since this surface does not change its are during isothermal bending). Having done this the curvature at any point is determined by drawing a perpendicular line to the surface and then finding the smallest and largest diameter circles coincident with the perpendicular line and the surface. For the surfaces of interest these are always perpendicular to each other as shown in the figure. The inverse of the radii of these two circles are then called the principle curvatures.

2. Measurement of Monolayer Elastic Parameters

The elastic parameters have been measured at two different length scales, the macro-scale by looking at the deformation of large, single-walled vesicles and the micro-scale by measuring the deformation of interfaces in lyotropic liquid crystalline phases. The macroscopic measurements allow determination of k_c and k_a, while the microscopic methods additionally measure c_0.

To determine k_c in large unilamellar vesicles (20 to 30 μm diameter) one can take advantage of the fact that the membrane is in a state of constant thermally induced motion when under zero or very low tensions. The thermal agitation gives rise to bilayer undulations that wrinkle the surface of the vesicle. Direct microscopic observation and Fourier analysis of these undulations can be used to determine the bilayer bending modulus (Duwe, Kaes, and Sackmann, 1990; Duwe and Sackmann, 1990; Servuss, Harbich, and Helfrich, 1976), and more recently NMR relaxometry has been applied to measuring the spectrum of bilayer undulations. Alternatively, the vesicles can be manipulated by partial aspiration into a micropipette that enables measurement of both k_c and k_a (Needham and Zhelev, 1996). Applying a known suction pressure with a water manometer, which can be done with 10^{-6} atm precision, the membrane can be gently deformed and changes in its area and volume measured directly by observation on a microscope. With knowledge of the vesicle geometry and applied suction pressure, one can determine the applied tension and the bilayer strain that results. At low tensions (from 10^{-3} to 0.5 mN m^{-1}) the wrinkles in the bilayer are 'ironed out' and therefore one can determine k_c (Evans and Rawicz, 1990; Zhelev, Needham, and Hochmuth, 1995). Once all the bilayer undulations have been removed increasing the tension results in dilation in molecular area and k_a can be measured (Evans and Needham, 1987).

The energy cost of deformations of the monolayer interface can be determined by measuring the change in molecular structure, using small angle X-ray diffraction, after application of an osmotic stress. Knowing the applied osmotic pressure and measuring the change in the volume of water per lipid allows, it is possible to determine the change in the system's energy. k_a is determined from the change in molecular area as the L_α phase is dehydrated by the application of osmotic pressure. k_c and c_0 are determined from the variation in monolayer curvature after osmotic dehydration of the H_{II} phase. In those cases where the material of interest has a stable lamellar phase, one may attempt to determine the curvature elastic parameters by adding an appropriate amount of hydrophobic solvent, for example, alkane or alkene, to the L_α phase (Chen and Rand, 1997; Vacklin et al., 2000). The addition of the hydrophobic solvent relaxes molecular packing constraints that in its absence would normally destabilize the H_{II} phase with respect to L_α (Figure 9).

In general, the measurements made by these different techniques are in remarkably good agreement and we present a collection of them in Table 6.

E. Examples of Membrane Protein Folding and Function Depending on Lipid Mechanical Stresses

The fact that membrane proteins are so hard to refold *in vitro* means that there have been few direct studies of the effect of the lipid properties discussed above on protein

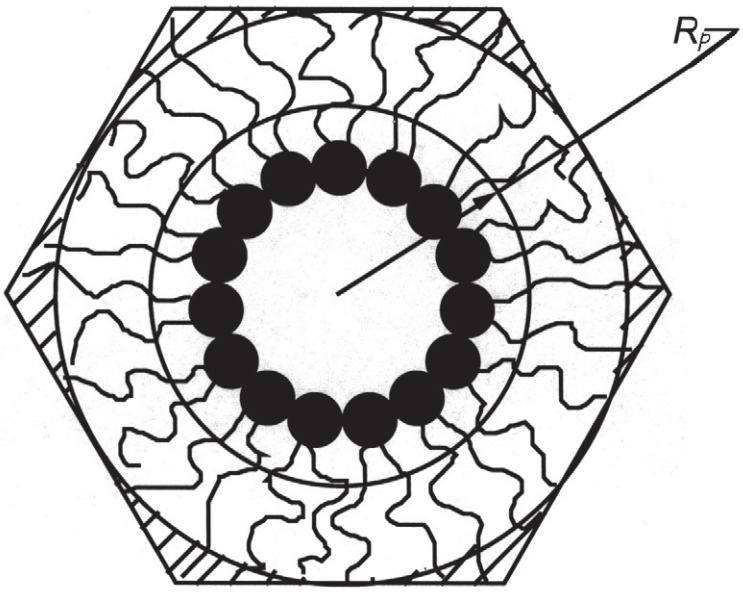


FIGURE 9. One can relieve the curvature elastic frustration stored in a lamellar phase by adding a hydrophobic component. The reason for this is clearly seen for the case of the inverse hexagonal phase. If each molecule wishes to have the same average length and same average interfacial curvature, then it is clear that this is incompatible with packing the monolayer cylinders on a two-dimensional hexagonal lattice. If we insist on the interface being cylindrical then chains must stretch into the hexagonal interstices and squash up against the hexagonal faces. These distortions constitute a considerable energetic cost, and it is only when the stored curvature elastic energy in a lamellar phase exceeds these costs that a phase transition to the H_{II} phase will occur. Adding sufficient hydrophobe to an otherwise stable lamellar phase will also give rise to an H_{II} phase transition, because the hexagonal interstices can now be filled (the hatched regions) thereby reducing the free energy of the H_{II} phase.

folding. Nevertheless, there is some evidence that points to these lipid effects playing a role. The only attempts to investigate the effect of lateral pressure and torque tension in PC/PE lipid mixtures on membrane protein folding events that we are aware of are those on the folding of bacteriorhodopsin and the insertion and channel formation of alamethicin. Two other examples of studies that are also pertinent to our discussions are those on glycophorin and lac permease. A search through the experimental literature also reveals a wide range of results that show further correlations between protein behavior and one or more of these lipid parameters discussed in this section. A couple of examples of this are included here.

1. PC/PE Mixtures Affect the Folding of Bacteriorhodopsin, bR

Studies on bacteriorhodopsin folding have been discussed at length in this review. The protein can be refolded from SDS micelles into PC/PE vesicles of either DMPC/DMPE, DPoPC/DPoPE, or DOPC/DOPE. In each case bR folds to over 90% yield in 100% PC vesicles, and the yield drops off as the PE content is increased (Curran et al., 1999). This suggests that the folding yield decreases as the chain lateral pressure and torque tension are increased, by incorporating more PE into the vesicle bilayer. Preliminary data also suggest that

TABLE 6
A Selection of Measured Lipid Elasticities

Lipid	k_a/mNm^{-1}	$k_c/10^{-19}J$	$-c_0/Å^{-1}$	T/°C	Reference(s)
DOPE		0.52	1/59	22	(Chen and Rand 1997)
DOPC	265	0.42 / 0.36	1/180	18 / 32	(Rawicz, Olbrich et al. 2000) (Chen and Rand 1997)
SOPC	230	0.45		18	(Rawicz, Olbrich et al. 2000)
DMPC	234	0.28		29	(Rawicz, Olbrich et al. 2000)
DEPC	263	0.60		21	(Rawicz, Olbrich et al. 2000)
MO		0.12	1/40	37	(Vacklin, Khoo et al. 2000)
didodecyl-β-D-glucopyranosyl-*rac*-glycerol			1/36	45	(Templer, Turner et al. 1995)
2LA/DLPC		0.6	1/30	35	(Templer, Seddon et al. 1998) (unpublished results A.M. Squires R.H. Templer and J.M. Seddon)
Chol/SOPC	640	1.23		15	(Evans and Rawicz 1990)
Chol/DOPE		0.60	1/46	22	(Chen and Rand 1997)
Chol/DOPC		0.44	1/54	32	(Chen and Rand 1997)

the rate of bR folding is slowed as the PE content and chain lateral pressure are increased. This agrees with data obtained from refolding SDS-bO into DMPC/DHPC micelles, where the rate of folding slowed as the DMPC content was increased (Booth et al., 1997). An increase in the longer chain lipid DMPC (C14 chains) over the amount of shorter chain DHPC (C6 chains) will increase the chain lateral pressure, although this effect is complicated by additional constraints on the pressure from the micelles. The stability of the folded state is also affected by the lipid chain lateral pressure. BR has been regenerated in DMPC/DOPC vesicles, in the absence of any SDS, and the thermal stability was found to increase as the DOPC content was increased (see Section II.A). This lipid system has been discussed previously, increasing the DOPC content, increase the torque tension and chain lateral pressure. Thus, it appears that increasing the chain lateral pressure and the monolayer torque tension slows down the folding process, lowers the folding yield of bR, but increases the thermal stability of the folded state. The exact nature of the lipid effects is currently under investigation; however, it can be interpreted as follows. The increased pressure within the bilayer could slow folding by hindering the insertion of a transmembrane helix. Insertion into the headgroup region may actually be easier (because an increase in chain lateral pressure is accompanied by a decrease in outward pressure in the headgroup region); however, the higher pressure near the middle of the bilayer will hinder insertion of a transmembrane segment. This higher pressure will also increase the effective microviscosity within the bilayer, which could and hinder the movement of transmembrane helices and therefore slow helix packing. The lower folding yield (at high PE) may result from either the increase in torque tension and chain lateral pressure preventing the insertion of SDS-denatured protein, or arise from the slower folding rates that allow other kinetic pathways to compete with folding. The increased thermal stability is probably due to the increased pressure (of high PE content) on the helix bundle of folded bR.

2. A Role for PE in Lactose Permease Folding

Lactose permease is a 12 transmembrane α helical sugar transporter found in the outer membrane of *E. coli*. Much work has been carried out on both the function and assembly of the protein *in vitro* and *in vivo* (Kaback, Voss, and Wu, 1997). The dependence of protein assembly on PE content of the *E. coli* membrane has also been investigated (Bogdanov and Dowhan, 1998; Bogdanov and Dowhan, 1999; Bogdanov et al., 1996). Initial studies utilized a conformationally sensitive monoclonal antibody (mAb4B1) directed against an epitope on a periplasmic loop between helices VII and VIII. *E. coli* mutant cells that lacked PE produced lactose permease that was not recognized by the antibody. However, recognition by the antibody, and thus correct folding of this loop between helices VII and VIII, was regained if the protein was then renatured in the presence of PE. Furthermore, the epitope was retained if PE was subsequently removed. A blotting procedure was developed for these experiments, involving transfer of the unfolded protein (i.e., lacking antibody recognition) from a SDS PAGE gel by Western blotting to a solid support coated with the lipids. Subsequent work investigated the effect of other lipids on the formation of epitope 4B1. This study showed that not all PE lipids were able to cause formation of the epitope. Saturated PE lipids could support formation of the epitope, while in the case of unsaturated PE lipids antibody binding could be regained by the addition of

saturated PG to the host membrane. PS was also found to cause epitope formation, but some unnatural diastereoisomers of PS lipids only caused epitope folding in the presence of saturated PG. PG alone was unable to support the formation of the epitope. The authors maintain that these results show a biological specificity for PE, or PS, in phospholipid-assisted folding of lactose permease (Bogdanov and Dowhan, 1999). The fact that the protein retains its epitope formation after PE is removed also suggests that PE is required during folding but not necessarily in the folded state. A major assumption in the work is that 4B1 epitope formation always reports for correct folding of the whole 12-helical protein and always correlates with full protein activity. It is an interesting thought that there may be a specific lipid requirement only during protein folding, and that this may involve a direct interaction of the lipid with the protein as it folds. As with everything in membrane protein research, a direct demonstration of this will be difficult. In view of the nonspecific lipid bilayer properties discussed in this section, it would also be extremely useful to see if nonspecific properties, such as the ability of PE to increase the desire for monolayer curvature, could equally be responsible for modulating the formation of epitope 4B1, or at least contribute to its formation.

A separate study has suggested that the helix tilt angle of lactose permease is also sensitive to the properties of the lipid membrane (le Coutre et al., 1997). However, this study has investigated the effect of lipid concentration on the tilt angle so the exact dependence on physical properties of the lipid bilayer is unclear.

3. PC/PE Mixtures Affect Alamethicin Channel Formation

Alamethicin, a 20 amino acid peptide, exhibits a voltage-dependent ion channel as a result of formation of a helix bundle on incorporation into lipid bilayers. The conductance state of the peptide in DOPC/DOPE planar bilayer has shown to be sensitive to the desire for monolayer curvature, exhibiting higher conductance states in bilayers with greater curvature (Keller et al., 1993b). Subsequent studies have shown that the insertion of the peptide into membranes is also sensitive to curvature (Lewis and Cafiso, 1999). Increasing the DOPE content of DOPC/DOPE mixtures lowered the binding constant of alamethicin (probably as monomers) to the bilayers and increased the binding free energy. This free energy increase was found to be linearly dependent on DOPE mole fraction, and on the spontaneous curvature of the DOPC/DOPE bilayer. The increase in DOPE does, however, increase the lifetime and conductance of alamethicin channels in the bilayer and stabilize alamethicin multimers (Keller et al., 1993b). Thus, increasing the propensity for monolayer curvature and hence the stored curvature elastic stress reduces the number of multimeric conductance channels within the bilayer but increases their stability. These results were interpreted as the monolayer curvature affecting the equilibrium between aqueous alamethicin, alamethicin monomers in the bilayer, and alamethicin multimers that act as channels in the bilayer. Thus, at low stored curvature elastic stress, the monomeric state in the bilayer is favored, while increasing the DOPE and hence the stored curvature elastic stress favors the channel state.

4. Insertion and Association of Gramicidin and Glycophorin A

Insertion of gramicidin into lipid membranes also results in channels that appear to be sensitive to the stored curvature elastic

stress of the host membrane (*Lundbaek* et al., 1997). The effect of different detergents environments on transmembrane α helix dimerization recently has been investigated using the glycophorin A transmembrane dimer as a model system (Fisher, Engelman, and Sturgis, 1999) (see also Section IV.B). Förster resonance energy transfer was used to measure the energetics of helix association in different environments. The observed equilibrium constant for dimer formation was determined to be about two orders of magnitude weaker in sodium dodecyl sulfate than in zwitterionic detergents. While it is difficult to ascertain how the properties of the different detergent systems modulate the process of helix association it does suggest that helix association is sensitive to the physical properties of the membrane.

5. Homeostatic Control of Bacterial Lipid Compositions and Nonbilayer Lipids

Many of the studies on the possible influences of mechanical stresses on bilayer proteins were prompted by the work of Lindblom and his co-workers on the homeostatic control of lipid composition in *A. laidlawii* and more recently *E. coli* (Lindblom et al., 1993; Lindblom and Rilfors, 1996; Lindblom et al., 1986) and Gruner's hypothesis of how the source of this control might be the lipid monolayer's spontaneous curvature (Gruner, 1985; Gruner, 1994). The story, however, begins with the first hints that the lipid membrane might have complex functional involvement in cell regulation from the measured diversity and variability of cell lipid composition. Look at the lipid composition of most cell membranes and you will see an enormous diversity of chemical species. For example, there are over 250 lipid components in human erythrocytes (Myher et al., 1989), including cholesterol, sphingomyelins, PCs, PEs, phosphatidylserines, and glycolipids with various chain lengths, degrees of chain unsaturation, and numbers of chains. Not only does membrane composition vary wildly from one cell species to the next, but also within one cell type the relative lipid composition is not maintained to a fixed recipe. Furthermore, there is enormous heterogeneity in lipid distribution within a single cell, with, for example, PCs being enriched on the outer wall of the plasma membrane and PE being enriched on the inside. Clearly, lipid distribution is of some importance to the cell and it is being finely controlled. This might at first not appear to be overly problematic, until one recognizes that many of the lipid components act to destabilize the impermeability of the membrane bilayer. Luzzati and his co-workers first demonstrated this over 30 years ago when they measured the lyotropic liquid crystalline behavior of fully hydrated natural lipids (Luzzati, 1968).

In any cell membrane it is usual to find a significant component of all the lipids will form such nonlamellar phases, indeed the total lipid extract from brain will form the inverse hexagonal phase under physiological conditions. Given the obvious dangers of using such lipids why are they so prevalent in nature?

It now appears that this is because nature controls membrane lipid composition interactively rather than via a set recipe. This is clearly a safer solution inasmuch as it allows the cell to respond to thermodynamic changes in its environment. The first evidence for this came from Lindblom and his colleagues who showed in a range of experiments on *A. laidlawii* and *E. coli* that the cell was actively controlling the lipid composition of the membrane. More importantly, they demonstrated that this control relied on the system controlling its lipid composition such that the total lipid extract was a constant temperature below the phase transition boundary between the L_α phase

and the inverted phases. Gruner hypothesized that the cell's control of lipid composition is based on its ability to sense the mechanical state of the membrane, in particular the stored curvature elastic stress. The idea here is that the cell maintains its lipid composition at some value of this stored stress by feedback. This is consistent with the presence of nonlamellar forming lipids in the membrane and with Lindblom and co-workers' measurements.

6. Stored Elastic Curvature Energy May Regulate CTP:Phosphocholine Cytidylyl Transferase (CCT)

A curvature elastic dependent mode of action has been identified for CTP:phosphocholine cytidylyl transferase (CCT) (Attard et al., 2000). CCT controls a rate-limiting step in the synthesis of PC lipids, and its catalytic activity is off when it is cytosolic and on when it is membrane bound. Because PC lipids invariably form the L_α phase they will generally reduce any stored curvature elastic stress in the cell membrane. Therefore, it is interesting to note that CCT activity rises with the stored elastic stress of the lipid membrane, providing nature with a means of interactively sensing and controlling the mechanical state of the membrane. This control appears to depend on the partitioning of CCT's amphipathic alpha-helical binding domain into the membrane monolayer leaflet. It has been hypothesized (Attard et al., 2000) that the cylindrical deformation imparted by the bound α helix on the monolayer releases stored curvature elastic energy, Figure 10, and this provides the necessary feedback mechanism.

F. Summary of the Role of Lipid Properties: Designing a Lipid-Based Refolding System

Nonspecific lipid properties clearly have a role in membrane function, in addition to any specific lipid, protein interactions. We have focussed on the stored mechanical stresses in the lipid membrane and shown how these can be measured and how we can begin to quantify their effect on membrane protein function. It appears that these stored mechanical stresses can also affect protein folding. In particular, the redistribution of lateral pressure across a PC bilayer, caused by the introduction of a "non-bilayer", PE lipid may affect the rate of TM helix insertion and packing, and in turn the efficiency of folding in a model membrane. Further data are required on protein structural changes to confirm this point. Taken together with the two-stage model discussed in the introduction, this is an important point for devising refolding system for membrane proteins.

The two-stage model implies the folding problem can be slit into two stages for practical purposes, while the known lipid properties allow manipulation of the lipid mechanical stresses to optimize the folding. In the case of bR, SDS has been used to give a partially denatured state with some helical content (the first stage of the folding method). This SDS-bO is then inserted directly into PC/PE lipid vesicles to give the folded state (the second stage of the folding method). Furthermore, altering the mechanical stresses of the PC/PE bilayer can be used to control the protein folding. Thus, it has been possible to design a simple, lipid refolding system, and control both the folding rate and efficiency.

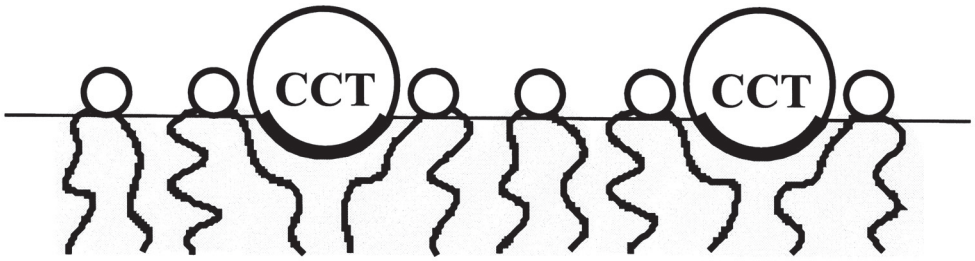


Figure 10. CCT binding helices are shown in cross-section embedded into a monolayer (the drawing is approximately to scale and the helix is of the order of 7.5 nm long and coming out of the page). The darker rim of the helix represent the extent of the hydrophobic amino acid residues and runs along the length of the helix. As shown the peptide enables the lipid chains in its vicinity to splay, thereby releasing stored curvature elastic energy.

VII. CONCLUSIONS: WHAT IS THE CURRENT STATE OF MEMBRANE PROTEIN FOLDING?

This review illustrates the scant number of direct studies on the folding mechanisms of integral membrane proteins and highlights the practical difficulties there are when working with these proteins. It is clear from the few studies that have been undertaken that even what looks to be a straightforward measurement turns out to be fraught with artifacts and problems. Frequently such problems are not at all obvious, but fortunately they can generally be overcome or at least corrected for during data analysis.

Most of the studies presented in this review may appear to have fairly substantial drawbacks. However, this depends on one's viewpoint. One simply cannot expect a problem as difficult as membrane protein folding to have a straightforward practical solution, and therefore working experimental systems are rarely going to be ideal. It is very important to recognize that we do have several working systems with which to study the partial refolding of several membrane proteins *in vitro*. These systems should be used to test ideas and to develop new techniques, while bearing in mind their drawbacks.

The most definitive and general conclusion that can be drawn from the biophysical studies presented in this review is that a GxxxG motif is often involved in TM helix association. A LIxxxGVxxGVxxT motif has been demonstrated in the specific case of glycophorin dimerization and been shown to induce dimerization of other TM stretches. GxxxG alone causes dimerization in synthetic Ala and Leu peptides in membranes, and this motif also occurs frequently in the SwissProt database where it seems to be involved in TM domain packing interactions.

Another lesson that has been learned so far is the importance of the detergent/lipid system used in the experiments. If the aim is a molecular level knowledge, then a lipid system whose properties are known should be chosen. An interesting aspect here to exploit is the fact that certain lipid properties can be used to control folding rates and yields. We have shown how this, together with the two-stage model of membrane protein folding, also allows us to design lipid refolding systems for α helical membrane proteins. Furthermore, it is possible to use the lipid properties to control the rate and efficiency of folding. Lipid lateral chain pressure may be important in controlling TM α helix insertion and helix packing rates, although we await definitive protein structural data on this point.

Currently, there are only two detailed studies on membrane protein folding *in vitro* (bR and OmpA). Thus, we have to be careful about drawing substantial general conclusions at this stage. It is also unrealistic to expect that detailed studies will suddenly emerge on other membrane proteins. These will occur, but will take time if we want informative, thorough, and artifact-free results. We therefore need to carry on testing specific hypotheses on existing refolding systems, while beginning work on new proteins. What is noticeably lacking from membrane protein folding studies are mammalian transport and receptor proteins, such as the 12 TM transporters, ion channels, ATPases, and G protein coupled receptors. While undoubtedly the results and methods presented in this review are relevant to these proteins to some extent, it is hard to say how much, in the absence of data on the proteins themselves.

From a practical point of view, there are many lessons that can be drawn from the existing studies highlighted in this review that can give hints as to what conditions may prove favorable to the successful refolding of membrane proteins. This is not only useful when working toward an understanding of the folding of the above classes of receptor and channel proteins, but indeed for much other work on membrane proteins *in vitro*. This is, of course, always assuming that significant amounts of the protein in question can be extracted either from native sources or following overexpression. There are two main approaches here, either trying to maintain a functional protein on extraction and purification, or allowing some partial denaturation and subsequent refolding. For hydrophobic α helical proteins, SDS seems to be useful for the latter, and His-tagged protein with elution off Ni(II) columns in appropriate detergents is useful in both cases. Another key seems to be using lipids in the refolding system. These could be native lipids (if there is a specific requirement) or nonnative lipids to alter the solvent properties. In particular it is worth looking at roles for PC/PE mixtures, or other bilayer systems that contain non-bilayer lipids, which allow the monolayer curvature and lipid chain lateral pressures to be controlled. Another approach that could prove useful is a combination of detergents or lyso (single chain) lipids prior to incorporation of the proteins into either mixed lipid/detergent micelles or vesicles.

Membrane proteins are arguably one of the most challenging and understudied areas of the proteome. This is a highly unsatisfactory situation, particularly in view of the tremendous biological importance of these proteins, their roles in diseases, multidrug transport, and as drug targets. This review shows that although difficult, studies on integral membrane proteins are possible, and there is much high-quality and informative work in this area. Nevertheless, it is a vast field and we desperately need more fundamental research on these proteins. With specific relevance to the folding problem, there is an obvious need for more direct studies on membrane protein folding. In addition, there is clearly a requirement for technique development, particularly to study protein structural changes and in the area of lipid-based refolding systems. The challenges thrown up by this area of science, together with the fascinating and often poorly understood function of the proteins themselves, means it is an unusually exciting research field that requires constant innovation to progress.

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