STRUCTURE AND STABILITY OF MEMBRANE PROTEINS

By MICHAEL H. B. STOWELL and DOUGLAS C. REES

Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125

I. Introduction 279
II. Membrane Protein Structure 280
A. Bacteriorhodopsin 281
B. Photosynthetic Reaction Center 283
C. Matrix Porin 285
D. Light-Harvesting Complex II of Photosystem II 287
E. Other Membrane Proteins 288
F. General Structural Features of Membrane Proteins 289
III. Biological Membranes and the Fluid Mosaic Model 290
IV. Theoretical and Experimental Aspects of Protein Stability 292
A. General Two-State Thermodynamic Model of Protein Stability 292
B. Water-Soluble Proteins and the Hydrophobic Effect 294
C. Experimental Studies of Membrane Protein Stability 297
D. General Features of Membrane Protein Stability 305
V. Energetic Basis of Membrane Protein Stability 305
A. Packing Interactions 306
B. Solvophobic Effects 306
C. Increased Secondary Structure Stability 307
D. Entropic Effects 307
E. Concluding Remarks 307
References 308

I. Introduction

It is a fundamental tenet of present-day structural biology that the unique tertiary structures of proteins reflect the strongly stabilizing influence of hydrophobic interactions (Kauzmann, 1959; Tanford, 1980; Baldwin, 1986; Dill, 1990). According to this view, protein folding is driven by the positioning of apolar residues in the protein interior, where they are shielded from exposure to the aqueous solution by surrounding polar residues. Relative to the unfolded state, the native state of a protein will be stabilized by the sequestering of nonpolar residues from the aqueous environment; estimates of the contribution of these hydrophobic interactions to protein stability obtained from calorimetry (Baldwin, 1986) and transfer experiments of amino acid analogs (Tanford, 1980) are in the range of a few hundred kilocalories/mole. Because water-soluble proteins are stable by only ~15 kcal/mol relative to the unfolded state (Privalov, 1979; Privalov and Gill, 1988), hydrophobic effects are

expected to provide an indispensable contribution to protein stability. Accordingly, if hydrophobic effects could be somehow "turned off," one would predict that stable tertiary protein structures could not possibly be formed. As a corollary, it would also seem that proteins could only adopt stable tertiary structures in an aqueous environment, because by definition, hydrophobic effects cannot exist in the absence of water. Despite such predictions, however, many proteins do adopt stable tertiary structures in predominantly nonaqueous environments. Examples of such proteins include membrane proteins, which fold with the bulk of their tertiary structure located within the nonpolar environment of the lipid bilayer, as well as nonmembrane proteins, such as the plant seed proteins crambin and zein, which are quite soluble and stable in nonaqueous solvents (Llinás *et al.*, 1980). Because these proteins exist in primarily apolar environments, the contribution of the hydrophobic effect to their stability is expected to be greatly diminished (Engelman, 1982). Thus, the critical question arises: If the hydrophobic theory of protein stability is correct, how is it possible for stable proteins to exist in nonaqueous environments where hydrophobic effects should be quantitatively less significant? This question will be addressed in light of the available structural and stability data on integral membrane proteins, emphasizing a comparison of these properties to those of better characterized water-soluble proteins.

II. Membrane Protein Structure

The past few decades have witnessed four important developments in the structural analysis of membrane proteins: (1) The 7-Å resolution structure of bacteriorhodopsin (BR) determined by Henderson and Unwin (1975), followed by the subsequent high-resolution structural analysis at 3.5 Å resolution (Henderson *et al.*, 1990). (2) The 2.3-Å resolution structure of the photosynthetic reaction center (RC) from *Rhodopseudomonas viridis* (Deisenhofer *et al.*, 1984, 1985; Deisenhofer and Michel, 1989), and the homologous reaction center from *Rhodobacter sphaeroides* (Allen *et al.*, 1986, 1987; Chang *et al.*, 1986, 1991; Ermler *et al.*, 1992). (3) The 1.8-Å resolution structure of the outer membrane protein, porin (POR) from *Rhodobacter capsulatus* (Weiss *et al.*, 1990), and the homologous *Escherichia coli* porin structures (Cowan *et al.*, 1992). (4) Finally, and most recently, the structure of the light-harvesting complex from photosystem II (LHCII), determined at 3.4 Å resolution by Kühlbrandt and coworkers (Kühlbrandt *et al.*, 1994). Each of these landmarks in membrane protein structure analysis will be briefly discussed, with an emphasis on their implications for membrane protein stability.

A. *Bacteriorhodopsin*

BR is present in the purple membrane of the salt-loving *Halobacterium halobium,* where it functions as a light-driven proton pump. The initial structural investigation of BR at 7 Å resolution revealed seven rodlike features that were identified as transmembrane-spanning α helices (Henderson and Unwin, 1975). This groundbreaking work established the significance of the α helix as an important structural element of integral membrane proteins, and catalyzed efforts both to understand the important elements of membrane protein folding and to predict the topologies of membrane proteins based on sequence analysis (Engelman *et al.,* 1980; Kyte and Doolittle, 1982; Eisenberg *et al.,* 1984; Engelman *et al.,* 1986). Those efforts directed toward the sequence-based identification of potential membrane-spanning α-helical segments have been relatively fruitful and are routinely used today in the analysis of membrane protein sequences.

BR forms a trimer in the membrane, with each monomer composed of seven transmembrane-spanning α helices that contain 20–25 amino acids/helix. The helices are designated A through G, in order of appearance in the amino acid sequence. The topology of the monomer can be described as an up–down multihelical bundle, with segments adjacent in the sequence located nearby in the structure, thus minimizing the length of the loop regions. The contact surfaces in the BR trimer are formed between each monomer and the adjacent monomers in a head-to-tail fashion (Fig. 1). The intermonomer contacts form a loose network of hydrogen bonds and hydrophobic interactions generated primarily by contacts between helix B from one monomer and helices E′ and D′ of the neighboring monomer. There are no intermolecular contacts about the threefold trimer axis, which results in a sizable cavity in the central core of the trimer. The external surface of the trimer is very hydrophobic, with the majority of exposed residues consisting of nonpolar, aliphatic residues. Transfer free energy calculations (Yeates *et al.,* 1987; Rees *et al.,* 1994) estimate the thickness of the bilayer-spanning region of BR to be 35 Å. Tryptophan residues, the most abundant aromatic amino acid in BR, are clustered primarily within the interior of a single monomer, whereas tyrosine residues participate in a number of hydrogen-bonding interactions at the monomer–monomer interface. There is a distinctive ring of aromatic residues (Fig. 2) located at the presumed water–bilayer interface, as is observed in both the RC and POR structures (see below). The BR core may be divided into two regions, the interior of the proton channel (which includes the retinal chromophore Schiff base) and a hydrophobic core (which also serves as the

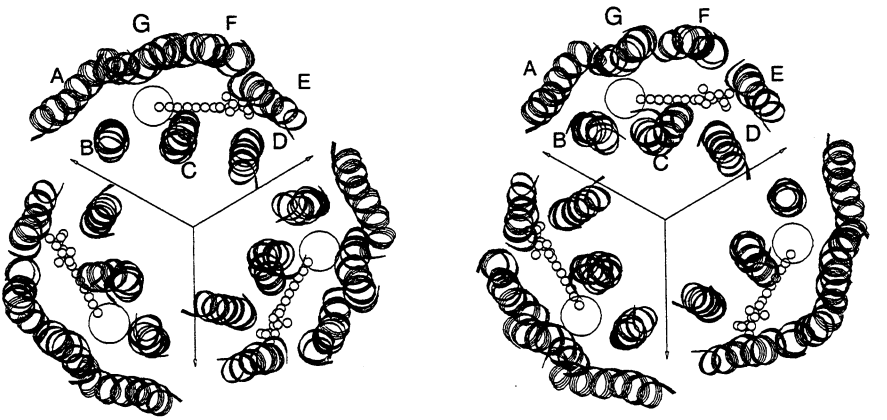

FIG. 1. Stereoview of bacteriorhodopsin. Helices A through G are labeled and the retinal chromophore is illustrated in ball and stick form. The approximate location of the proton channel is indicated by the circle that encloses the retinal chromophore Schiff base. Coordinate set 1BRD of the Brookhaven Protein Data Bank (Bernstein *et al.*, 1977) was used for this figure.

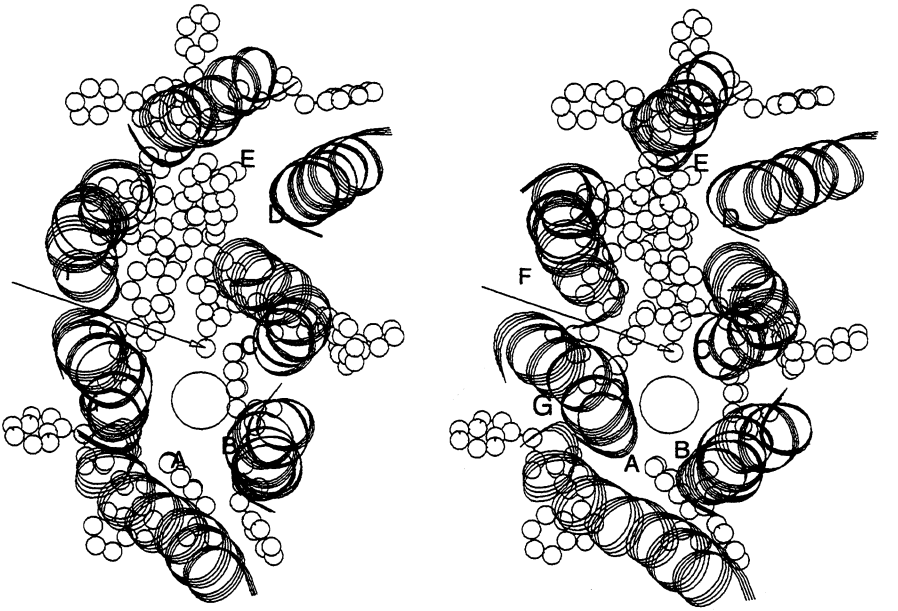

FIG. 2. Stereoview of a single bacteriorhodopsin monomer showing the location of the hydrophobic aromatic residues, depicted in ball and stick format. A ring of aromatic residues surrounds the bacteriorhodopsin monomer. A large number of tryptophan residues comprise the hydrophobic core located adjacent to the proton channel, approximated by the circle. The retinal chromophore Schiff base is located at the arrow.

pocket for the retinal chromophore) (Fig. 2). The hydrophilic region undoubtedly reflects the proton pumping function of BR and represents the only hydrophilic part of the protein interior. The hydrophobic core comprises the major part of the internal volume, however, so that overall the BR interior is similar in nature to the hydrophobic core of water-soluble proteins (Rees *et al.*, 1989a).

B. Photosynthetic Reaction Center

Photosynthetic reaction centers are integral membrane proteins responsible for the primary step in photosynthesis, the conversion of photochemical energy into electrochemical energy. The RC from *Rps. viridis* was the first membrane protein structure to be determined at atomic resolution and unambiguously demonstrated than an α helix could span the membrane bilayer. The structure also provided detailed insights into the folding pattern and molecular interactions of the RC, and provided a structural model for the spatial organization of the cofactors participating in the light-driven electron transfer reactions. The RCs from both *Rps. viridis* and *Rb. sphaeroides* are composed of three separate membrane-spanning subunits designated L, M, and H. A central feature

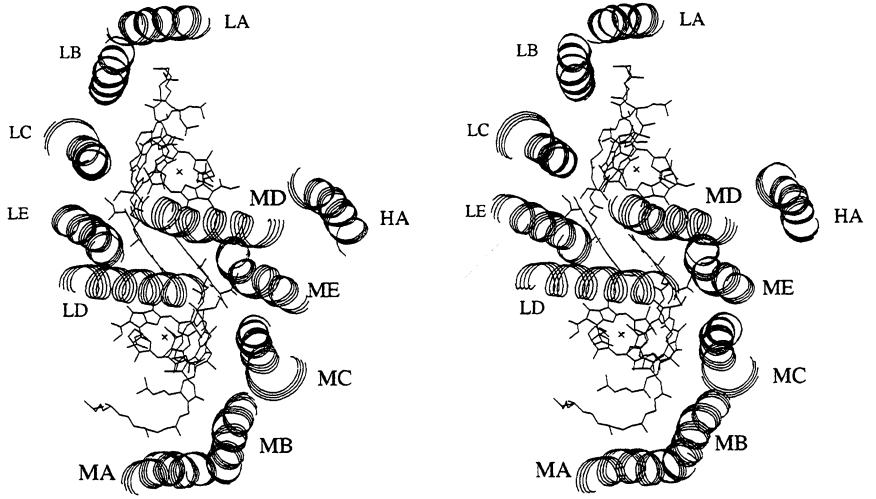

FIG. 3. Stereoview of the *Rb. sphaeroides* RC viewed down the pseudo-twofold axis. The helices are labeled according to the text and the chromophores are depicted in stick format. The central core of the protein is composed of helices LE, LD, ME, and MD, which surround the bacteriochlorophyll special pair. Coordinate set 4RCR of the Brookhaven Protein Data Bank (Bernstein *et al.*, 1977) was used for this figure.

of the RC structure is the presence of 11 hydrophobic α helices, each containing approximately 20–30 residues. The L and M subunits are homologous, and contain five transmembrane helices each. These helices are designated A through E in order of appearance in the protein sequence, prefixed with either L or M to designate the particular subunit. The H subunit contains a single transmembrane helix (HA) and adopts the majority of its fold outside the membrane bilayer (Figs. 3 and 4). Helices in the L or M subunits are arranged in a single layer. As with BR, helices adjacent in sequence tend to be adjacent in the structure, with the consequence that the majority of helix–helix interactions are antiparallel. The photosynthetic cofactors are predominantly positioned between the helices. The core of the RC is similar in hydrophobicity to the interior of water-soluble proteins, whereas the external surface is more hydrophobic (Rees *et al.*, 1989a). This characteristic difference

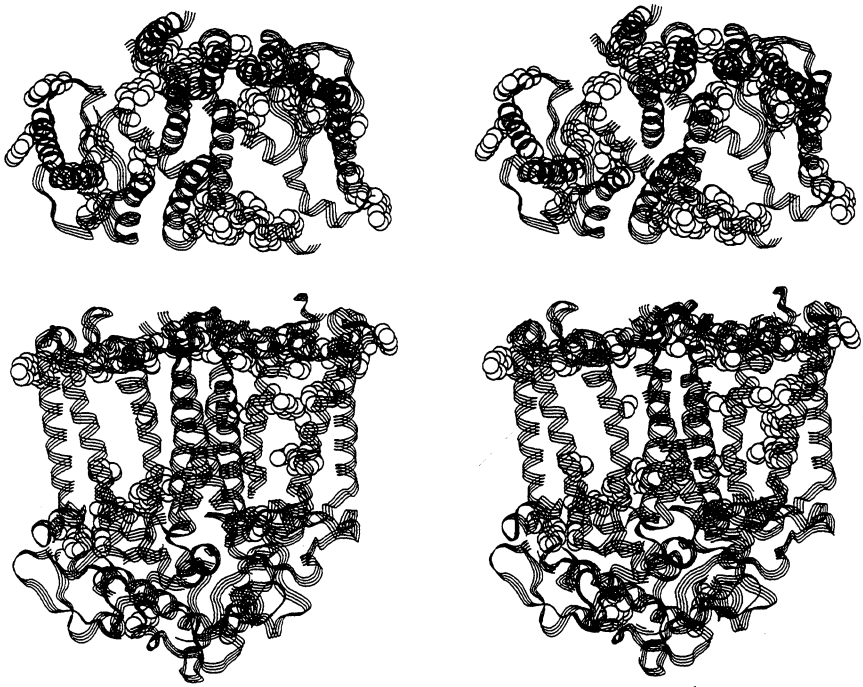

FIG. 4. Stereoview of the *Rb. sphaeroides* RC showing the ring of tryptophan residues located at the water–lipid interface. The top figure is viewed from the special pair toward the nonheme iron; the bottom figure is viewed in the plane of the membrane bilayer. The extramembranous portion of subunit H is evident in the bottom stereopair.

in hydrophobicity between the inner and outer surfaces of the helices generates an amphipathic pattern that can be detected by analysis of the protein sequence. Around the RC, there is a prominent ring of tryptophan residues localized at the presumed water bilayer interface (Fig. 4) (Deisenhofer and Michel, 1989; Schiffer *et al.*, 1992; Cowan, 1993), which may serve as a polarizable "buffer" between the high dielectric media of water and the low dielectric media of the membrane bilayer, or to help maintain the position of the protein in the bilayer.

C. Matrix Porin

Although the first membrane protein structures to be determined, BR and RC, are composed primarily of α helices, the structure of the outer membrane protein porin demonstrated that this folding topology is not a universal property of membrane proteins. The most striking feature of the porin structure is the formation of a 16-stranded, antiparallel β-barrel structure that traverses the membrane (Weiss *et al.*, 1991; Cowan, 1993). The inside of the β barrel forms an aqueous pore through which metabolites can pass. Because sequential residues along a β strand are oriented toward alternate sides of the β sheet, the barrel contains residues that, to first order, are hydrophobic on the external membrane-facing side and hydrophilic on the pore side (Fig. 5). It would seem that porin truly satisfies the requirements of an "inside-out" protein (Engelman

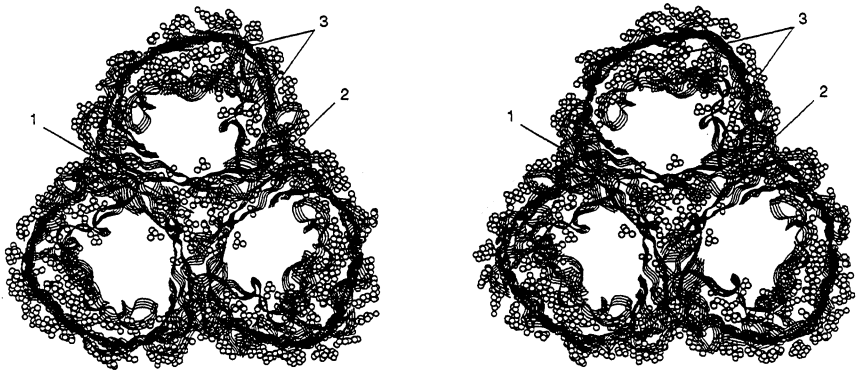

FIG. 5. Stereoview of the *Rb. capsulatus* porin viewed down the threefold trimer axis. Hydrophobic residues are shown in ball and stick format. The three hydrophobic core regions are labeled: 1, the hydrophobic core of the trimer axis; 2, the hydrophobic monomer–monomer interface; and 3 the hydrophobic core formed by the extended loop L3. Coordinate set 1POR of the Brookhaven Protein Data Bank (Bernstein *et al.*, 1977) was used for this figure.

and Zaccai, 1980), with a hydrophobic exterior and with the identification of the internal aqueous pore as forming the polar interior. Important aspects of the porin structure are the trimeric nature and an extended two-stranded sheet in the interior of the β barrel (designated L3, Fig. 5), which together give porin a tripartite hydrophobic organization consisting of an external membrane-facing side, a central mostly hydrophobic core, and a hydrophilic inner surface that forms the aqueous channel. The trimer contacts of porin comprise approximately 35–40% of the exposed surface of the molecule, whereas the two-stranded internal β sheet provides about 25% of the exposed internal surface. The trimer contact regions are tightly packed, with regions of stacked aromatic residues as well as interdigitated aliphatic residues. The extended two-stranded loop, designated L3, along with the internal surface of the monomer barrel, also generates a tightly packed hydrophobic core (Fig.

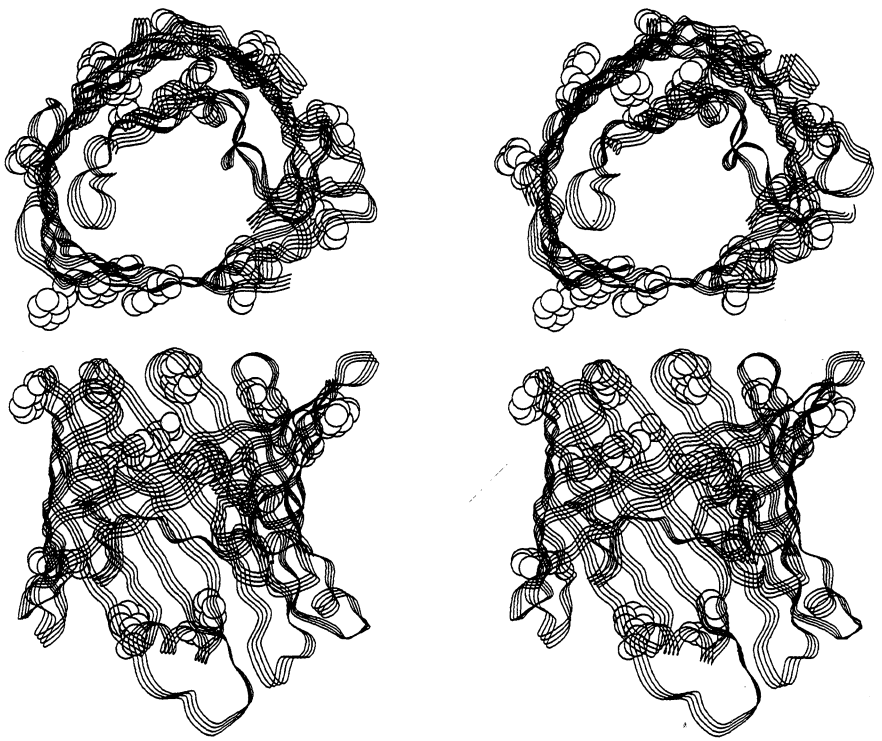

FIG. 6. Stereoview of the *Rb. capsulatus* porin illustrating the ring of phenylalanine residues located at the water–lipid interface. The top figure is viewed normal to the membrane bilayer; the bottom figure is viewed in the plane of the membrane bilayer.

5). Thus, porin would appear to have a discontinuous hydrophobic core. The relative instability of porin monomers compared to porin trimers (Rosenbusch, 1974) would be explained if the trimer interactions do indeed form a hydrophobic core of this protein and are therefore partly responsible for the stability of porin. As with the RC, a very pronounced ring of aromatic residues (tyrosine in this case) is located at the presumed water–lipid interface (Fig. 6).

D. Light-Harvesting Complex II of Photosystem II

The LHCII is an important element of photosystem II (PSII), which is responsible for the funneling of photons to the reaction center of PSII. LHCII is composed of an approximately 25-kDa polypeptide together with a minimum of 12 chlorophyll and 2 carotenoid (lutein) molecules. LHCII contains three transmembrane helices and adopts a trimeric structure in the chloroplast membrane. The structure of LHCII has been determined at 3.4 Å resolution by electron crystallography (Kühlbrandt *et al.*, 1994). This structure has several important features with interesting ramifications for membrane protein structure and stability. The LHCII polypeptide contains three transmembrane helices, A, B, and C. There is also a small fourth helix, designated D, which is positioned perpendicular to the membrane normal. Helix A and helix B form a left-handed supercoil in the central core of the complex (Fig. 7). This supercoil is stabilized by a set of ion pairs between Arg^{70}-Glu^{165} and Glu^{65}- Arg^{185}. Additionally, two lutein molecules bind to the grooves of the supercoil, so that the A and B helices and the two luteins form a pseudo "four-helix" bundle at the central core of the complex. The important structural role of the luteins is evidenced by the observation that their presence is essential to the stability of the LHCII and is required for the protein to fold into a stable tertiary structure (Paulsen *et al.*, 1993). Helix C is somewhat removed from the internal core formed by the A and B helices, and exhibits the most sequence variability of the three transmembrane helices. Given the large number of bound chlorophylls, residues in the A and B helices do not actually contact the membrane. Consequently, helix C is the only polypeptide segment of LHCII that has substantial interactions with the membrane bilayer. Overall, less than 36% of the LHCII polypeptide is within the membrane bilayer region, resulting in the presence of equal masses of polypeptide and chromophore within the membrane bilayer. Furthermore, a large portion of the interaction of the complex with the membrane bilayer is mediated by the chlorophylls, and not protein, because only helix C maintains contact with the bilayer. This is a very different situation from that observed with either

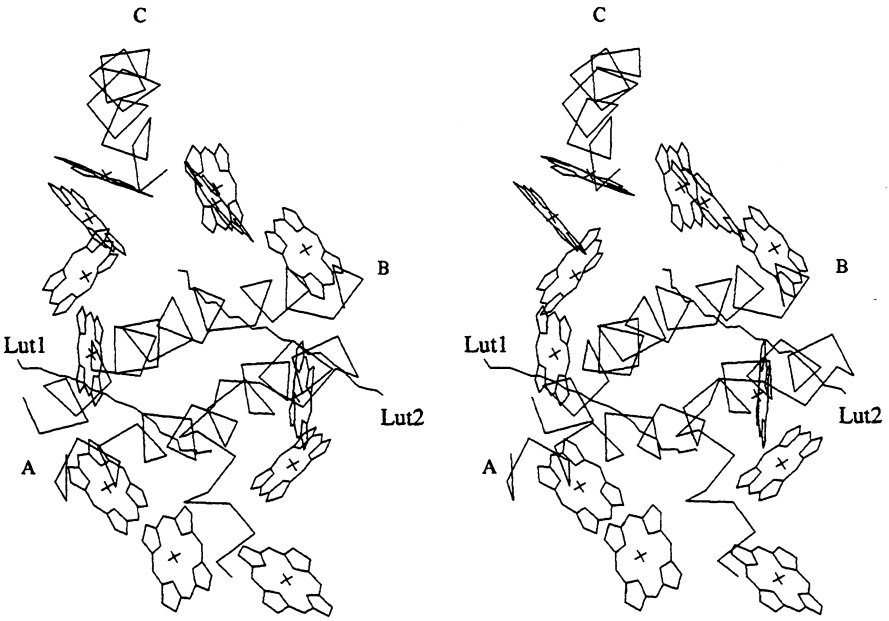

FIG. 7. Stereoview of LHCII viewed normal to the membrane bilayer. The helices are labeled according to the text; the luteins are labeled Lut1 and Lut2. Coordinates supplied courtesy of W. Kühlbrandt.

BR or RCs, wherein the various chromophores are surrounded by polypeptide and compose substantially less than 50% of the molecular mass contained within the membrane bilayer.

E. Other Membrane Proteins

At present, the structural data available for membrane proteins are limited, relative to water-soluble proteins. This lack of structural data reflects the current difficulty in obtaining well-diffracting crystals of membrane proteins. Much effort has gone into ameliorating this problem, and several new approaches to membrane protein crystallization are being investigated, including the use of "peptidetergents" (Schafmeister *et al.*, 1993) and novel fusion proteins (Prive *et al.*, 1994). The techniques of electron diffraction also continue to improve, and their ability to describe membrane proteins structurally at medium to high-resolution ensures that these methods will remain an important tool for such investigations. Several structural studies will offer more insights into membrane

proteins, such as the X-ray structure of photosystem I, described at 6 Å resolution (Krauss *et al.*, 1993). Electron crystallographic work on the acetylcholine receptor (Unwin, 1993) intriguingly suggests that the membrane-spanning regions of this protein may have mixed α-helical and β-sheet secondary structure. Studies on peripheral membrane proteins, including prostaglandin synthase (Picot *et al.*, 1994) and lipase–procolipase (Vantilbeurgh *et al.*, 1993), have provided structural information about this important class of membrane proteins. Undoubtedly, other studies are also in progress that will give new and important insights into the relatively uncharacterized realm of membrane protein structure.

F. General Structural Features of Membrane Proteins

Based on analyses of the available three-dimensional structures, the following general features of membrane proteins have been described (Rees *et al.*, 1989b, 1994; Yeates, 1993). Given the limited structural data available for membrane proteins, however, the reader should be keenly aware that these observations and deduced generalizations are based on a very limited sampling. Consequently, as much as the authors may hope that these views are correct, they may well turn out to need "some" modification as more detailed structural information on membrane proteins becomes available.

1. Membrane proteins with known three-dimensional structures exhibit a relatively simple up–down topology transversing the membrane, such that residues nearby in the sequence tend to be nearby in the structure. This behavior may reflect constraints associated with insertion of the folding polypeptide chain into the lipid bilayer (Engelman and Steitz, 1981). As suggested by the preliminary structural data for the acetylcholine receptor (Unwin, 1993), however, more complex tertiary folds almost certainly exist, and the details of these structures will be eagerly awaited.

2. Surface residues in the bilayer-spanning region of integral membrane proteins tend to be more apolar than the buried, interior residues. Although this is opposite to the hydrophobic organization characteristic of water-soluble proteins, the interior of membrane proteins (with the exception of torodial shaped proteins such as porin, which have aqueous-filled internal channels) is not polar. Rather, the average hydrophobicities of buried residues in both water-soluble and membrane proteins are nearly identical.

3. The packing volume of buried residues in the *Rb. sphaeroides* RC is very similar to that observed in water-soluble proteins, implying that

the same types of efficient packing interactions are seen in both classes of proteins. Furthermore, there is no significant difference in the accessible surface area of the *Rb. sphaeroides* RC and oligomeric, water-soluble proteins of comparable size.

Although the folding environments of water-soluble and integral membrane proteins differ significantly, the structural consequences of these different folding environments is surprisingly minimal. With the exception of surface residue polarity, membrane proteins and water-soluble proteins share strong similarities in general features such as hydrophobicity of buried residues, surface area, and interior packing efficiency. These observations are reminiscent of the solvent dependence of crystal morphology; while maintaining the same types of internal packing interactions, the nature of groups on a crystal surface can be altered, by changing the crystal morphology, to minimize the surface energy for interaction with a particular solvent (Rees and Wolfe, 1993). In the case of water-soluble and membrane proteins, the same type of efficient interior packing is maintained, while the surface polarity of a protein varies with the solvent environment. A striking example of this is found in a protein family with both hydrophobic and hydrophilic members (Baud *et al.*, 1993). An important challenge is to rationalize these similarities in view of the apparently different roles hydrophobic interactions should have in stabilizing the tertiary structure folds of these two classes of proteins.

III. Biological Membranes and the Fluid Mosaic Model

Any discussion of membrane protein structure and stability is incomplete without a discussion of the properties of the lipid bilayer. The composition of the lipid bilayer can exhibit extreme variations from organism to organism and from organelle to organelle (Table I). Variations in membrane composition constitute an important facet of the ability of an organism to adapt to the environment. This adaptability is necessary not only for maintaining the passive function of the bilayer as a semipermeable barrier, but also to maintain the proper function and stability of the membrane proteins contained within the lipid bilayer. To understand the role of the lipid bilayer in membrane protein function, it is necessary to have some knowledge of the structural and thermodynamic properties of membrane bilayers and the effects that can be caused by variations in lipid composition. Perhaps surprisingly, subtle changes in the membrane bilayer composition can have a dramatic effect on the myriad of possible phases that the membrane bilayer can adopt, as well

TABLE I
Selected Membranes and Corresponding Lipid Compositions[a]

Membrane	PE	PC	PG	PI	PS	PA	S	DPG	GD	SM
Nuclear	26	53	—	7.1	—	—	—	—	—	13
Mitochondrial (inner)	37	41	3	5	—	—	—	14	—	1.4
Mitochondrial (outer)	24	42	10	21	—	—	—	3	—	3.4
Chloroplast (thylakoid)	—	3	9	1	—	—	7	—	77	—
Erythrocytes (hen)	30.7	30.3	—	3.7	—	—	—	—	—	—
Erythrocytes (rat liver)	24.7	46.1	—	6.7	4.2	2	—	—	—	16.8
Golgi apparatus (rat liver)	17	45.3	12.3	6.3	8.7	—	—	—	—	4.2
Escherichia coli (inner)	74	—	19	—	—	—	—	3	—	—

[a]PE, Phosphatidylethanolamine; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; S, sulfolipid; DPG, diphosphatidylglycerol (cardiolipin); GD, galactosyldiglyceride; SM, sphingomyelin. Taken in part from Datta (1987) and Gennis (1989).

as the temperature at which these phase transitions occur (Quinn, 1981; Seddon, 1990).Two lipid phases of particular interest to biological membrane systems are the L_α and the H_{II} phases. The L_α, or liquid crystalline, phase, the fluid phase commonly observed for the lipid bilayer in biological systems, can be visualized by freeze–fracture experiments on whole cells. In the L_α phase, the fatty acid chains tend to be mobile and disordered. The H_{II}, or inverted hexagonal, phase, a lipid phase with a high radius of curvature at the head group–water interface, has been implicated in a number of cellular functions, including protein translocation and membrane fusion. Considerable effort has gone into understanding the thermodynamic forces that govern these two phases and the factors influencing their interconversion. The dominant properties that determine the phase of a lipid bilayer are the relative head group size and the length of the fatty acid acyl chain (Gruner, 1985). The head group size determines the spontaneous radius of curvature for the lipid bilayer, and the length of the acyl chain controls the hydrocarbon packing chain strain. Together, these two properties govern the phase-forming tendency of phospholipids, and will also influence how the phospholipids may pack against embedded membrane proteins.

The standard framework for describing the molecular details of biological membranes is the fluid mosaic model of Singer (Singer and Nicolson, 1972) that represents the membrane bilayer as a mosaic, composed of lipids interspersed with integral membrane proteins that span the lipid bilayer. Depending on the membrane system, lipids may constitute between 20 and 80% of the membrane mass. Both lipids and membrane proteins can diffuse laterally in the plane of the bilayer, in addition to

rotational motions around the membrane normal. The fluid mosaic model suggests an important role for the lipid in regulating membrane protein function, and also in stabilizing membrane protein structure. A role for the bilayer lipid composition in regulating the functioning of membrane proteins has been demonstrated in several studies. Experiments on rhodopsin have shown that the MI–MII transition in the rhodopsin photocycle is markedly effected by the presence of H_{II} forming phospholipids (Gibson and Brown, 1991; Mitchell *et al.*, 1992). Experiments with cytochrome c oxidase have demonstrated the important role of cardiolipin in the turnover cycle of this enzyme (Abramovitch *et al.*, 1990). Similar experiments with Ca^{2+}-ATPase have also demonstrated a functional role for H_{II} lipids (Navarro *et al.*, 1984). These experiments all demonstrate the significant relationship between the lipid bilayer composition and integral membrane protein function, and suggest that the regulation of membrane bilayer composition may be a method of regulating membrane protein function. These data also suggest that the lipid bilayer may play an important role in the stability or instability of membrane proteins (Section IV,C).

IV. Theoretical and Experimental Aspects of Protein Stability

A. *General Two-State Thermodynamic Model of Protein Stability*

Questions of protein stability are fundamentally thermodynamic in nature. Because the most extensive studies of protein stability have been conducted for water-soluble proteins, it is relevant to explore the thermodynamics of these systems in some detail to provide a framework for the corresponding discussion of membrane protein stability. For many water-soluble proteins, the thermodynamics of protein stability may be described by a two-state equilibrium model between the native (N) and denatured (D) forms of the protein: N $\leftrightharpoons$ D. For a two-state process with constant ΔC_p, the free energy of unfolding (ΔG_{ND}) for the N to D transition may be expressed as a function of temperature and pressure (Hawley, 1971):

$$\Delta G_{ND}(T,P) = \Delta H_m\left(\frac{T_m - T}{T_m}\right) - \Delta C_p\left\{T_m - T\left[1 - \ln\left(\frac{T}{T_m}\right)\right]\right\} + \frac{\Delta\beta}{2}(P - P_m)^2 + \Delta\alpha(P - P_m)(T - T_m) + \Delta V_m(P - P_m) \quad (1)$$

where T_m and P_m are the transition and pressure for unfolding at a point where $\Delta G_{ND} = 0$; ΔH_m, $\Delta\alpha = (\partial\Delta V/\partial T)_P$, $\Delta\beta = (\partial\Delta V/\partial P)_T$, and ΔV_m rep-

resent the enthalpy change, thermal expansivity factor, isothermal compressibility, and volume change for unfolding at (T_m, P_m), respectively. The entropy change at T_m is given by $\Delta S_m = \Delta H_m/T_m$. Provided ΔC_p, $\Delta\alpha$, and $\Delta\beta$ are independent of temperature and pressure, ΔG_{ND} is completely specified by the six parameters, T_m, ΔH_m, ΔC_p, $\Delta\alpha$, $\Delta\beta$, and ΔV_m. The dependence of ΔG_{ND} on T and P for a given set of solution conditions (pH, μ, etc.) defines a stability surface for a protein (Becktel and Schellman, 1987). Although pressure effects are often neglected, they are significant for the theoretical understanding of the thermodynamics of protein stability (Kauzmann, 1987). Additionally, pressure effects are relevant to more practical problems associated with marine barophilic organisms that grow under conditions of high hydrostatic pressure, and have possible applications to biotechnological processes.

In the physiological temperature range, to a good approximation, Eq. (1) may be expanded to second order in $(T - T_m)$ and $(P - P_m)$, yielding an expression for ΔG_{ND} with a quadratic dependence on T and P:

$$\Delta G_{ND}(T,P) = -\frac{\Delta C_p}{2T_m}(T - T_m)^2 - \frac{\Delta H_m}{T_m}(T - T_m) + \frac{\Delta\beta}{2}(P - P_m)^2 + \Delta\alpha\,(P - P_m)(T - T_m) + \Delta V_m(P - P_m) \quad (2)$$

This quadratic form implies, at least mathematically if not always physically, that for a given pressure (or temperature), there will be two T values (or P values) where the protein undergoes denaturation. From Eq. (2), the temperature and pressure of greatest protein stability, T^* and P^*, occur at

$$T^* - T_m = -\frac{\Delta H_m \Delta\beta + T_m \Delta\alpha \Delta V_m}{\Delta C_p \Delta\beta + T_m(\Delta\alpha)^2} \quad (3)$$

$$P^* - P_m = -\frac{\Delta C_p \Delta V_m - \Delta H_m \Delta\alpha}{\Delta C_p\Delta\beta + T_m(\Delta\alpha)^2} \quad (4)$$

with the free energy of maximal stability given by:

$$\Delta G_{ND}(T^*, P^*) = \frac{\frac{\Delta H^2_m}{2T_m\Delta C_p} - \frac{\Delta V_m}{\Delta\beta}\left(\frac{\Delta V_m}{2} - \frac{\Delta H_m\Delta\alpha}{\Delta C_p}\right)}{\left(1 + \frac{T_m\Delta\alpha^2}{\Delta C_p\Delta\beta}\right)} \quad (5)$$

or equivalently,

$$\Delta G_{ND}(T^*, P^*) = \frac{\frac{\Delta H^2_m}{2T_m\Delta C_p} + \Delta P_{max,T_m}\left(\frac{\Delta V_m}{2} + \Delta T_{max,P_m}\Delta\alpha\right)}{\left(1 + \frac{T_m\Delta\alpha^2}{\Delta C_p\Delta\beta}\right)} \quad (6)$$

where $T_m + \Delta T_{max,P_m} \equiv T_m - \Delta H_m/\Delta C_p$ is the temperature of maximal protein stability at $P = P_m$, and $P_m + \Delta P_{max,T_m} \equiv P_m - \Delta V_m/\Delta\beta$ is the pressure of maximal protein stability at $T = T_m$. In the absence of pressure effects, the maximal stability of a protein is given by

$$\Delta G_{ND}(T^*) \cong \frac{\Delta H^2_m}{2T_m\Delta C_p} \quad (7)$$

where $T^* = T_m - \Delta H_m/\Delta C_p$.

Formally, the maximum stability of a protein at T^* can be increased by some combination of increasing ΔH_m, decreasing T_m, and/or decreasing ΔC_p. If pressure effects are included, then maximal stability can be enhanced for increased values of ΔV, negative values of $\Delta\beta$ that approach 0, or smaller values of $\Delta\alpha$.

B. Water-Soluble Proteins and the Hydrophobic Effect

Although this review focuses on membrane proteins, given the paucity of experimental data on membrane protein stability and the relatively extensive experimental studies on the stability of water-soluble proteins, this latter group provides an appropriate starting point for developing a framework for understanding membrane proteins. From calorimetric studies of the stability of small, globular, water-soluble proteins, the following general observations have emerged [reviewed in Privalov (1979, 1990); Privalov and Gill (1988); and Murphy and Freire (1992)]:

1. The unfolding of proteins can often be characterized thermodynamically in terms of a transition between two states, N and D. Although intermediates between N and D can exist that may be significant for folding kinetics and pathways, their populations are often too low to contribute to the overall unfolding thermodynamics of these proteins. A notable exception to this generalization is the existence, for proteins such as lactalbumin, under some conditions of a relatively highly populated molten-globule intermediate between the N and D states [reviewed in Christensen and Pain, 1991].

2. The unfolding of water-soluble proteins is associated with a large, positive ΔC_p of unfolding. Thermodynamic studies of hydrophobic interactions demonstrate that the exposure of apolar groups is characterized by a large, positive ΔC_p. Accordingly, observation of a large, positive ΔC_p during protein denaturation is widely interpreted as reflecting the contribution of hydrophobic interactions to water-soluble protein stability. Consistent with this interpretation, the magnitude of ΔC_p has been shown to be proportional to the estimated exposure of nonpolar surface area during protein unfolding. More recent work has extended these initial observations to include a negative contribution to ΔC_p generated from the exposure of buried polar surface (peptide bonds, etc.) during protein unfolding (Murphy and Freire, 1992).

Due to the nonzero ΔC_p, the enthalpy of unfolding for a given protein measured at T_m, ΔH_m will change with variation in conditions (pH, denaturants, etc.) that alter the stability of the protein. In particular, because $\Delta C_p > 0$, ΔH_m will decrease with decreasing T_m. The slope of the dependence of ΔH_m on T_m provides a convenient mechanism for estimating ΔC_p for protein denaturation. Strikingly, when the temperature dependence of the specific enthalpy of unfolding (calories/gram) is examined for a range of water-soluble proteins, a nearly universal convergence toa value 13 cal/g at $T = 110°C$ is observed (Privalov, 1979). Although the interpretation of this convergence value is still debated, it appears to reflect a characteristic energy associated with nonsolvation interactions such as packing and hydrogen bonding (Privalov, 1979; Fu and Freire, 1992). Additionally, this convergence temperature enthalpy value should provide a useful benchmark for comparison of the experimental unfolding enthalpies of both water-soluble and membrane proteins.

3. Energetically, the stabilizing and destabilizing interactions involved in protein folding are rather closely balanced, with a net difference of about 15 kcal/mol favoring protein folding under optimal conditions. Neglecting pressure effects for the moment, the free energy of maximal protein stability occurs at the temperature T^* and, from the derivation above, is approximated by Eq. (7). For a "typical" water-soluble protein such as lysozyme at pH 4, $\Delta H_m = 140$ kcal/mol, $T_m = 350$ K, and $\Delta C_p = 1.6$ kcal/mol/K, giving $T^* = 263$ K and $\Delta G_{ND}(T^*) = 17.5$ kcal/mol (Privalov and Khechinashvili, 1974). Similar stabilizing energies have been observed for a variety of water-soluble, globular proteins, which leads to the somewhat surprising conclusion that, to a reasonable approximation, the stabilities of different proteins are independent of molecular size.

4. At low pressures, the volume change associated with protein denaturation is often positive ($\Delta V > 0$), which means that the volume of the

D state is greater than the N state. Under these conditions, increasing pressure will stabilize the N form of the protein. The D state is more compressible than N ($\Delta\beta < 0$), however, so that as the pressure increases, the volume difference between N and D, ΔV, approaches zero and eventually becomes negative. Consequently, in this regime, increasing pressure destabilizes N, ultimately leading to pressure denaturation. As noted by Kauzmann (1987), this behavior is quite different than expected from the solubility of low molecular weight, apolar compounds in water. For these systems, ΔV for transfer of apolar compounds to water is negative at low pressures and positive at higher pressures, which is just the opposite of that observed for protein denaturation. A satisfactory interpretation of this behavior has yet to be provided.

The pressure dependence of the stability of water-soluble proteins has been investigated in relatively few cases (Brandts *et al.*, 1970; Hawley, 1971; Zipp and Kauzmann, 1973; Heremans, 1982; Weber and Drickamer, 1983; Samarasinghe *et al.*, 1992; Royer *et al.*, 1993), despite the significance of these effects for understanding the contribution of the hydrophobic effect to protein stability. Hawley (1971) experimentally determined the relevant parameters in Eq. (1) for chymotrypsin at pH 2.07: T_m = 315 K, P_m = 1 atm = (1/41) cal/cm^3, ΔC_p = 3.8 kcal/mol/K, ΔH_m = 100.2 kcal/mol, $\Delta\beta = -1.24 \times 10^3$ cm^6/kcal/mol, $\Delta\alpha$ = 1.32 cm^3/mol/K, and $\Delta V_m = +41$ cm^3/mol. Based on these values, the contribution of pressure-dependent effects to the maximal stability of this protein is small; at $T^* \cong 289$K and P^* = 1.25 atm, $\Delta G^*(T^*, P^*)$ = 4.21 kcal/mol. Neglecting pressure-dependent effects, $\Delta G^*(T^*, 1 \text{ atm})$ = 4.19 kcal/mol. Consequently, although pressure effects can significantly influence the stability of proteins, at least in this case, the pressure of maximal stability is shifted only slightly from 1 atm, so that the effect of pressure on the maximal stability of chymotrypsin at pH 2.07 is minimal.

5. Neglecting the pressure-dependent terms, and assuming that the measured ΔC_p for unfolding of water-soluble proteins reflects the exposure of apolar groups to water, the contribution of hydrophobic interactions, ΔG_{hyd}, to the free energy of protein unfolding is given by (Baldwin, 1986)

$$\Delta G_{hyd} = \Delta C_p(T - T_h) + \Delta C_p T \ln(T_s/T) \tag{8}$$

where T_h and T_s are the temperatures at which the enthalpy and entropy changes associated with the hydrophobic effect vanish, respectively. Based on the thermodynamic properties of liquid hydrocarbons dissolved in water, Baldwin proposed that T_h and T_s equal 22.2°C and 112.8°C, respectively. Because ΔC_p is positive for protein unfolding,

ΔG_{hyd} from Eq. (8) is consequently found to be large and positive (i.e., stabilizing the native structure). The residual contributions to the observed thermodynamic parameters, from sources other than the hydrophobic interaction, were found to be largely temperature independent for lysozyme. According to Baldwin's analysis, ΔH_{res} = 52 kcal/mol lysozyme = ~3.7 cal/g and ΔS_{res} = 543 cal/mol lysozyme/deg = ~0.039 cal/g/deg.

How much, and even whether, hydrophobic interactions stabilize protein structures depends entirely on the assumptions used to assigned values for ΔC_p, T_h, and T_s that are inserted into Eq. (8), however. Privalov and Gill (1988; Murphy *et al.*, 1990) argue that the most appropriate value for T_h and T_s is ~110°C. With this parameter value, hydrophobic interactions are predicted to be destabilizing for all $T < 110$°C. This is a very different picture than traditionally envisioned for the origins of water-soluble protein stability. In some sense, these different interpretations amount to different choices of standard states that reflect the types of molecular interactions included in the definition of hydrophobic effects. What is clear, however, is that the contribution of hydrophobic interactions to protein stability decreases with decreasing temperature and that this can ultimately lead to the phenomenon of cold denaturation (Privalov, 1990).

C. Experimental Studies of Membrane Protein Stability

In contrast to the situation for water-soluble proteins, relatively few experimental studies have been reported on the thermodynamics of membrane protein stability. Calorimetric studies of membrane protein stability, which can provide definitive, model-independent values for various thermodynamic parameters of protein stability, are complicated by the often irreversible nature of this transition. Still, the available experimental studies do provide important, initial glimpses into the thermodynamics of membrane protein stability. As the preceding discussion has indicated, the goal of any thermodynamic characterization is to determine the stability curve, defined as $\Delta G(T)$. $\Delta G(T)$ may be calculated once the values of ΔC_p, ΔH_m, and ΔT_m have been determined at the transition temperature for unfolding. From the stability curve, it is possible to determine quantities that are important for comparisons to water-soluble proteins, such as the free energy of maximal stability [from $\Delta G(T^*)$; Eq. (7)], the contributions of hydrophobic interactions to protein stability (from the value of ΔC_p), and an estimate of the specific enthalpy of unfolding at 110°C, which for many water-soluble proteins has a common

value of 13 cal/g. The complete evaluation of these parameters is not available for any membrane protein system, but the available studies do at least permit these general issues to be addressed.

1. Bacteriorhodopsin

The most extensive calorimetric studies of membrane protein stability have been conducted for BR, which exists as a trimer in the purple membrane of *H. halobium*. Studies from both Sturtevant's (Jackson and Sturtevant, 1978) and Brouillette's (Brouillette *et al.*, 1987, 1989) groups indicate that at pH 7 in phosphate, BR unfolds at $T_m \sim 100°C$, with $\Delta H_m \sim 100$ kcal/mol. This corresponds to a specific enthalpy of ~4 cal/g (using a subunit molecular weight of 26,000), which is (perhaps coincidentally) close to Baldwin's (1986) estimate for the residual enthalpy of unfolding due to nonhydrophobic interactions (3.7 cal/g). Monomeric BR prepared in detergent micelles unfolds with $T_m \sim 74°C$ and $\Delta H_m \sim 100$ kcal/mol. Consequently, monomeric BR is less stable than trimeric BR in a phospholipid bilayer, which may reflect the importance of monomer–monomer interactions and/or may be a result of a detergent effects (see below). A study (Brouillette *et al.*, 1989) of the variation in ΔH_m with T_m (achieved by variation of the pH) suggests that the heat capacity for unfolding of trimeric BR in the purple membrane is ~1.2 kcal/mol/K or ~0.046 cal/g/K, which is lower (on a per gram basis) than for many water-soluble, globular proteins. For example, ΔC_p for denaturation of ribonuclease and myoglobin are 0.09 and 0.16 cal/g/K, respectively (Privalov and Khechinashvili, 1974). From $\Delta H_m = 100$ kcal/mol, $T_m = 373$ K, and $\Delta C_p = 1.2$ kcal/mol/K, the maximal stability of trimeric BR is estimated from Eq. (7) as $\Delta G(T^*) \sim 11$ kcal/mol at $T^* \sim 290$ K, which is within the range often observed for water-soluble proteins.

An important question in these studies concerns the extent of BR unfolding following thermal denaturation. This can be conveniently monitored by CD studies that are sensitive to the secondary structure content of a protein. In the case of BR, CD studies in the far-UV region indicate a reduction of ~27% in α helicity as the protein thermally denatures at pH 8.5 in 190 m*M* phosphate buffer (Brouillette *et al.*, 1987). Consequently, there is significant residual secondary structure in thermally unfolded BR, so that it would be incorrect to consider the D state as approximating a random coil. It has been suggested that the low specific enthalpy of BR unfolding, relative to water-soluble protein, may reflect the retention of structure after denaturation "due to the constraints imposed by the lipid bilayer" (Jackson and Sturtevant, 1978).

The contributions of the interhelical loops to the stability of BR have been probed by Engelman and co-workers (Popot *et al.*, 1987; Kahn

and Engelman, 1992). Using an *in vitro* regeneration system, BR was reconstituted from three fragments—two synthetic peptides corresponding to helices A and B, and a chymotryptic fragment containing the five helices C to G. After addition of retinal, spectroscopic and X-ray diffraction studies indicated that the reconstituted BR had properties virtually identical to those of wild-type BR. Consequently, the connecting loops between these helices are not essential for BR to adopt a native structure. Two significant implications of this work are that (1) helix–helix interactions are important for the folding of at least this membrane protein, and (2) entropic effects related to the approximate two-dimensional nature of membrane systems are not critical for membrane protein stability. Because the number of degrees of freedom that are available to a true, random-coil denatured membrane protein are significantly reduced relative to a three dimensionally denatured form of a water-soluble protein, it is possible that membrane proteins could be stabilized, in effect, by destabilization of the denatured form. The observation that proteolytic fragments of BR can reassemble to form the native protein suggests, however, that these entropic considerations are not dominant, because otherwise the various fragments would diffuse independently through the bilayer and not associate to form the native structure. In a subsequent analysis (Kahn *et al.*, 1992), calorimetric studies of regenerated BR demonstrated that proteolysis of one loop does somewhat destabilize BR, resulting in a decrease of T_m by 6°C and ΔH_m by 44 kcal/mol. Interestingly, removal of the retinal chromophore causes a more pronounced destabilization of BR, with observed changes in T_m and ΔH_m of -16°C and -77 kcal/mol, respectively.

Recently, it was reported that dehydrated multilayers of membrane-bound BR are stable up to 140°C (Shen *et al.*, 1993), at which point the protein denatures irreversibly. Although no information was obtained about the thermodynamics of BR stability under these conditions (i.e., to establish that BR is actually *thermodynamically* more stable under these conditions, as opposed to *kinetically* more stable), this is still a remarkable observation. The authors suggest that the increased thermostability of BR in stacked, dehydrated multilayers may be due to the imposition of steric constraints that prevent large-scale changes to the protein structure, such as those occurring on denaturation. There is some evidence that steric constraints may not be essential for increased protein stability, however, because protein suspensions in anhydrous solvents have shown dramatic increases in the apparent stability, relative to aqueous solutions (Volkin *et al.*, 1991). It should be noted, however, that solvent-free (dry) protein may be even more resistant to thermal unfolding compared to organic solvents (Toscano *et al.*, 1990). The transfer of a water-soluble

protein from an aqueous to nonaqueous environment can be accomplished with apparently few structural changes (Fitzpatrick *et al.*, 1993). The potential of nonaqueous environments to increase protein stability, such as those seen for BR in dehydrated multilayers, appears significant, and it is essential that more detailed thermodynamic analyses of protein stability under these conditions be obtained to understand the origins of this enhanced stability.

2. Erythrocyte Band 3

The principal erythrocyte anion transporter, or band 3, contains a 55-kDa transmembrane domain that can be separated from a 42-kDa cytoplasmic domain by proteolysis. The stability of the 55-kDa transmembrane domain has been studied following reconstitution into a series of different lipid environments by differential scanning calorimetry (Maneri and Low, 1988). In these different lipid environments, ΔH_m values for thermal denaturation were measured in a range from ~1 to 9 cal/g, with ΔC_p values of ~0 to 0.15 cal/g/K. If ΔH_m is plotted as a function of T_m, there is some tendency for the enthalpy of denaturation to increase with T_m (Fig. 8). The slope from a least-squares fit to this plot implies a

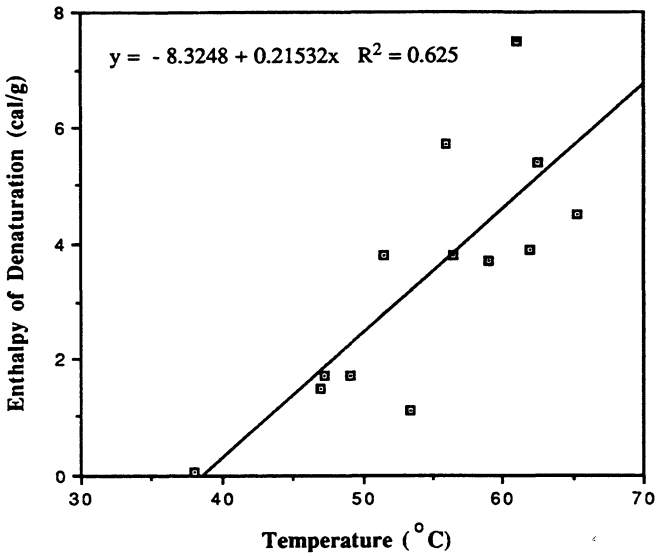

FIG. 8. Plot of the specific enthalpy of denaturation (ΔH_m) against the midpoint temperature for denaturation (T_m) for the membrane-bound domain of band 3 reconstituted in vesicles composed of different phospholipids. Data from Maneri and Low (1988).

ΔC_p of ~0.2 cal/g, and extrapolation of this line to 110°C yields a value for the specific enthalpy of unfolding of ~15 cal/g. These estimated values for both ΔC_p and the enthalpy of unfolding at 110°C are close to those expected for water-soluble proteins of comparable size (Privalov and Gill, 1988). Subsequent studies on band 3 in native membranes and in detergent micelles also found that the T_m and ΔH_m values obtained for thermal denaturation were "remarkably similar to those determined for concentrated solution of globular proteins," and further speculated that this reflects the presence of similar secondary structures (α helices) in both water-soluble and membrane proteins (Sami *et al.*, 1992).

3. Membrane-Associated Systems

Given the complexity of these systems, which contain a variety of different integral membrane proteins, it is difficult to extract quantitative thermodynamic parameters concerning the thermal denaturation of specific components. Nevertheless, some general observations have been reported that are relevant to the issue of membrane protein stability. Calorimetric studies of the erythrocyte ghost membrane system (Brandts *et al.*, 1986) revealed four structural transitions that occurred in the temperature range of 45–80°C. Of particular interest was the corresponding study of the temperature dependence of the circular dichroism spectrum for erythrocyte ghosts, which indicated that even at 90°C, ~50% of the α helicity measured at 30°C still remained. Consequently, both erythrocyte ghosts and BR retain considerable secondary structure in thermally denatured material. A scanning calorimetric study of photosystem II-containing membranes (Thompson *et al.*, 1986) observed five endothermal transitions in the 30–70°C temperature range. The enthalpy of denaturation for the entire complex, ΔH_m, was determined to be ~5–6 cal/g, which is within the range of values observed for denaturation of water-soluble proteins at these temperatures.

4. Glycophorin Dimer

Glycophorin A, a sialoglycoprotein found in erythrocyte membranes, forms homodimers mediated through interactions involving a single transmembrane-spanning α helix in each monomer. Although calorimetric or other types of thermodynamic characterizations have not been performed, the dimerization interactions are quite stable, persisting even in the presence of sodium dodecyl sulfate (Furthmayr and Marchesi, 1976). The glycophorin dimer can be disrupted, however, by addition of a synthetic peptide with the sequence of the transmembrane domain (Bormann *et al.*, 1989), which leads to complex formation between the

peptide and protein. Structural determinants of the helix–helix interactions responsible for dimerization have been explored by mutagenesis experiments (Lemmon *et al.*, 1992a,b), with analogous experiments recently reported for the transmembrane segment of phage M13 coat protein (Deber *et al.*, 1993). The helix–helix interface defined by these studies contains predominantly aliphatic side chains, with no highly polar groups. Consequently, noncovalent packing interactions between residues on the two helices appears to provide the principal driving force for dimerization. Furthermore, these packing effects appear to be sufficiently strong to generate a highly specific and stable dimer, even in the absence of other potentially stabilizing interactions such as connecting loops or polar (salt bridge) interactions. A calorimetric study of these membrane-localized helix–helix interactions would be quite informative for assessing the thermodynamic origins of the stability of this system.

5. Lipid and Detergent Influence on Membrane Protein Stability

The nature of the lipids and/or detergents that surround integral membrane proteins may have a profound influence on the structural stability of these proteins. The role of the membrane bilayer in membrane protein function was briefly discussed in Section III. A logical extension of these studies is the role of the membrane bilayer, or amphiphiles in general, in stabilizing or destabilizing membrane proteins. Only a few pioneering reports have investigated the role of the membrane bilayer lipids and detergents in membrane protein stability, and these are described below.

a. Lipid Studies. Early studies suggesting that the bilayer plays an important biological role in membrane protein stability were based on the observation that the thermostability of the Na^+, K^+-ATPase from brain membranes of various organisms, as measured by the rate of inactivation, was directly correlated with body temperature (Cossins *et al.*, 1987). These data implied that an increased body temperature corresponded to an increase in thermostability of the Na^+, K^+-ATPase. Although sequence identities of the Na^+,K^+-ATPase between the most diverse organisms in these experiments are greater than 85%, the remaining 15% differences could easily account for the increased thermostability at higher body temperature. More compelling support was provided by experiments performed with a single organism, the goldfish, acclimatized to different temperatures (Cossins *et al.*, 1987). In these studies, a significant increase was observed in the inactivation rate (74%) of 4°C acclimated goldfish Na^+,K^+-ATPase versus 25°C acclimated gold-

fish Na^+,K^+-ATPase. Although expression of two different Na^+,K^+-ATPases cannot be strictly ruled out, these data suggest that changes in the membrane bilayer composition, associated with growth at different temperatures, could be responsible for regulating the thermostability of the Na^+,K^+-ATPase.

Maneri and Low (1988) investigated the effect of variation in acyl chain length of phosphotidylcholine on the thermal stability of the erythrocyte anion transporter, band 3. This was the first calorimetric investigation of the thermodynamic role of the lipid bilayer in membrane protein stability. For a fixed head group type, the denaturation temperature of band 3 increased with increasing fatty acid chain length. Because the melting temperature of phospholipid bilayers increases with increasing fatty acid chain length, this indicates that the stability of the band 3 transporter increases with increasing stability of the bilayer. A more detailed description of this analysis is provided in Section IV,C,2.

b. Detergent Studies. A recent investigation of detergent effects on rhodopsin stability, although not rigorous, provides some interesting data on the thermostability of this protein and the effects of detergent (de Grip *et al.*, 1992). This study examined the role of 17 different detergents in stabilizing rhodopsin, as measured by the midpoint temperature associated with the loss of the native absorption spectrum for this protein. Although the data are limited, within a given class of detergents a lower critical micelle concentration (CMC) gives rise to a more stable protein. This result can most readily be explained by assuming that the free energy for micelle formation, ΔG°_{mic}, contributes to the stability of membrane proteins. ΔG°_{mic} for micelle formation can be expressed as (Gennis, 1989):

$$\Delta G^\circ_{mic} = RT \ln(X_{CMC})$$

where X_{CMC} is the mole fraction of the amphiphile at the critical micelle concentration. It must be stressed that for a rigorous calculation of ΔG°_{mic} the effects of counterions and activity coefficients should be included. Nonetheless, one can utilize this equation to calculate the differences in ΔG°_{mic}, $\Delta\Delta G^\circ_{mic}$, for several of the detergents utilized in the study, and to plot these data versus the corresponding ΔT for rhodopsin inactivation (Fig. 9). Although the general trend is clear, the observed correlation is far from ideal, however, which may reflect the simplified treatment of ΔG°_{mic} as well as the possibility of specific detergent protein interactions, which could be significant in cases such as the band 3 transporter in detergent micelles (Sami *et al.*, 1992).

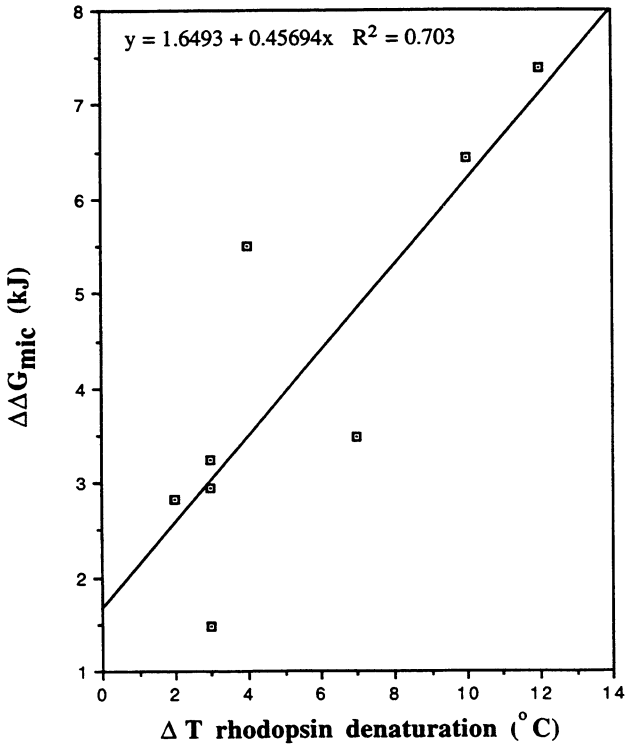

FIG. 9. Plot of $\Delta\Delta G_{mic}$ versus ΔT in the thermal denaturation of rhodopsin for a selected number of detergents. Data from de Grip *et al.* (1992).

6. *Pressure Effects on Membrane Protein Stability*

No studies of the pressure dependence of membrane protein stability were uncovered in preparing this review. It is anticipated that pressure effects could be quite interesting and complex, however, due to pressure effects on both the protein and the bilayer (Heremans, 1982). Lee (1983) has proposed that the compressibility of a protein will increase as the surface tension of the surrounding solvent decreases, because it then becomes easier to expand the solvent cavity in which the protein is embedded to accommodate volume fluctations. Consequently, pressure effects on protein stability could be more important for membrane proteins than for water-soluble proteins, because the surface tension of water, ~100 cal/Å^2, is much greater than the surface tension of hydrocarbons,

~30 cal/Å^2. Clearly, the significance of these effects needs to be addressed experimentally.

D. *General Features of Membrane Protein Stability*

Although the experimental evidence is limited, it appears that the thermodynamic parameters describing the stability of membrane proteins, especially T_m and ΔH_m, and to a lesser extent ΔC_p and $\Delta G(T)$, are within the range seen for more extensively characterized water-soluble proteins. A possible exception to this behavior may be provided by BR, where ΔH_m and ΔC_p appear to be somewhat lower than expected for water-soluble proteins unfolding at a T_m ~100°C, which may reflect a decreased contribution of hydrophobic interactions to BR stability (Baldwin, 1986) and/or the presence of residual structure in unfolded BR (Jackson and Sturtevant, 1978) (see below). Overall, however, membrane proteins do appear to have stabilities comparable to those of water-soluble proteins.

There is evidence from several systems that thermally denatured membrane proteins still retain extensive residual secondary structure. It may be that the denatured form of membrane proteins shares some similarities to the "molten globule" state of water-soluble proteins (Christensen and Pain, 1991), which contains extensive secondary structure but poorly defined tertiary interactions.

The stability of membrane proteins is sensitive to the nature of the lipid and/or detergent in the surrounding environment. In general, membrane protein stability increases with decreasing critical micelle concentration in a homologous series of detergents or lipids, although specific protein–lipid interactions can occur that counteract this tendency. In the case of BR, a significant increase in thermostability has been achieved by the preparation of anhydrous multilayers.

V. Energetic Basis of Membrane Protein Stability

Although membrane proteins and water-soluble proteins exist in very different types of solvent environments, there are striking similarities in both the stabilities and structural organizations of these two classes of proteins. This poses a serious dilemma, because hydrophobic interactions are believed to provide the dominant contribution to the stability of water-soluble proteins, and yet the contribution of hydrophobic interactions to the stability of integral membrane proteins should be greatly diminished in the nonaqueous environment of the lipid bilayer. Alterna-

tive factors to hydrophobic interactions that could contribute to the stability of membrane proteins are discussed below.

A. Packing Interactions

Studies on BR (Kahn and Engelman, 1992) and glycophorin (Lemmon *et al.*, 1992a,b) have shown that packing (van der Waals) interactions between helices are sufficient to promote stable tertiary structure interactions, without the need for connecting loops and highly polar interactions (Lemmon and Engelman, 1992; Popot, 1993). These principles have been recently used, for example, to develop an amino acid sequence motif that promotes specific dimerization of transmembrane α helices (Lemmon *et al.*, 1994). The efficient interior packing seen for both membrane proteins and water-soluble proteins should promote protein stability by maximizing the contributions of favorable van der Waals interaction energies. The sufficiency of packing interactions to drive molecular assembly has also been demonstrated in model systems, with the synthesis of molecules that dimerize in nonaqueous solvents in the absence of hydrophobic, hydrogen-bonding, and ion-pair interactions (Bryant *et al.*, 1990; Cram *et al.*, 1992).

B. Solvophobic Effects

Because it is energetically unfavorable to create a surface in a liquid, minimization of the surface energy of a liquid-immersed structure will tend to favor more compact objects (Israelachvili, 1992). Similarities in the surface areas between the RC and water-soluble proteins of similar size suggest that the surface energies of these proteins could be similar, despite the difference in surface tensions between hydrocarbon liquids ($\sim$30 cal/Å^2) and water ($\sim$100 cal/Å^2). This suggests that the greater energy required to create a surface in water, relative to nonpolar solvents, can be offset by the greater favorable interaction between the polar surface residues and water, relative to the weaker interactions possible between nonpolar surface residues and the hydrocarbon chains in the bilayer. As a consequence of these compensating effects, the net result could be comparable surface energies for the interaction of the relevant solvents with either water-soluble or membrane proteins, so that the work associated with placing a protein in a solvent could be, to first order, independent of the solvent (Rees *et al.*, 1994). By analogy to the term "hydrophobic," this more general type of effect is termed "solvophobic." Like hydrophobic effects, solvophobic effects will tend to minimize the exposed surface area and stabilize compactly folded structures.

C. *Increased Secondary Structure Stability*

The presence of nonaqueous solvents often stabilizes the formation of hydrogen-bonded, regular secondary structures, because competing hydrogen bonds to solvent molecules are either no longer possible, or not as favorable. Consequently, α helices (and presumably β sheets) are stabilized in a membrane environment, relative to an aqueous solution (Popot and Engelman, 1990). It seems plausible that the tertiary structure of a protein will be stabilized under conditions whereby the secondary structure is more stable, and this effect has been used to derive scales of amino acid secondary structure propensities (Blaber *et al.*, 1993; Kim and Berg, 1993). Consequently, this effect could contribute to the stability of membrane proteins through the enhancement of secondary structure stability. Experimental tests of this hypothesis that have been conducted with water-soluble proteins are consistent with a small, but not negligible, contribution of secondary structure stabilization to protein stability (Blaber *et al.*, 1993; Kim and Berg, 1993; Lin *et al.*, 1993; Pinker *et al.*, 1993).

D. *Entropic Effects*

As described above, the denatured state of some membrane proteins still retains residual secondary structure. The presence of residual order suggests that the denatured state may have conformational restrictions that reduce the entropic favorability of the unfolding process. As a consequence, it is possible that membrane proteins may be stabilized by a destabilization of the denatured state. In this regard, the N to D transition for membrane proteins may be more directly comparable to the N to "molten globule" transition observed for some water-soluble proteins, because the molten globule form contains significant secondary structure, but little ordered tertiary structure.

E. *Concluding Remarks*

An important lesson from the study of membrane proteins is that water is not absolutely indispensable for the ability of proteins to adopt stable three-dimensional structures; proteins can also stably exist in nonaqueous solvents, whether in the membrane bilayer or in nonbiological systems. By definition, then, hydrophobic interactions are not indispensable for protein stability, because proteins with stable tertiary structures occur in nonaqueous solvents. In this regard, the role of hydrophobic interactions in protein stability may be analogous to the role of disulfide

bridges; whereas some proteins cannot exist without disulfide bridges, they are certainly not required by every protein. As has been appreciated for some time (Singer, 1962), the continued study of proteins in membranes and other nonaqueous environments is fundamental to defining the contributions of all solvents, including water, to the origins of protein structure and stability.

Acknowledgments

We thank our colleagues/collaborators S.I. Chan, D.S. Eisenberg, and G. Feher for sharing their insights into membrane proteins; W. Kühlbrandt for providing LHCII coordinates; and NIH GM45162 for support.

References

Abramovitch, D. A., Marsh, D., and Powell, G. L. (1990). *Biochim. Biophys. Acta* **1020,** 34–42.

Allen, J. P., Feher, G., Yeates, T. O., Rees, D. C., Deisenhofer, J., Michel, H., and Huber, R. (1986). *Proc. Natl. Acad. Sci. U.S.A.* **83,** 8589–8593.

Allen, J. P., Feher, G., Yeates, T. O., Komiya, H., and Rees, D. C. (1987). *Proc. Natl. Acad. Sci. U.S.A.* **84,** 5730–5734.

Baldwin, R. L. (1986). *Proc. Natl. Acad. Sci. U.S.A.* **83,** 8069–8072.

Baud, F., Pebay-Peyroula, E., Cohen-Addad, C., Odani, S., and Lehmann, M. S. (1993). *J. Mol. Biol.* **231,** 877–887.

Becktel, W. J., and Schellman, J. A. (1987). *Biopolymers* **26,** 1859–1877.

Bernstein, F. C., Koetzle, T. F., Williams, G. J. B., Meyer, E. F., Brice, M. D., Rodgers, J. R., Kennard, O., Shimanouchi, T., and Tasumi, M. (1977). *J. Mol. Biol.* **112,** 535–542.

Blaber, M., Zhang, X.-J., and Matthews, B. W. (1993). *Science* **260,** 1637–1640.

Bormann, B.-J., Knowles, W. J., and Marchesi, V. T. (1989). *J. Biol. Chem.* **264,** 4033–4037.

Brandts, J. F., Oliveira, R. J., and Westort, C. (1970). *Biochemistry* **9,** 1038–1047.

Brandts, J. F., Erickson, L., Lysko, K., Schwartz, A. T., and Taverna, R. D. (1986). *Biochemistry* **16,** 3450–3454.

Brouillette, C. G., Muccio, D. D., and Finney, T. K. (1987). *Biochemistry* **26,** 7431–7438.

Brouillette, C. G., McMichens, R. B., Stern, L. J., and Khorana, H. G. (1989). *Proteins Struct. Funct. Genet.* **5,** 38–46.

Bryant, J. A., Knobler, C. B., and Cram, D. J. (1990). *J. Am. Chem. Soc.* **112,** 1254–1255.

Chang, C.-H., Tiede, D., Tang, J., Smith, U., and Norris, J. (1986). *FEBS Lett.* **205,** 82–86.

Chang, C.-H., El-Kabbani, O., Tiede, D., Norris, J., and Schiffer, M. (1991). *Biochemistry* **30,** 5352–5360.

Christensen, H., and Pain, R. H. (1991). *Eur. J. Biophys.* **19,** 221–229.

Cossins, A. R., Behan, M., Jones, G., and Bowler, K. (1987). *Biochem. Soc. Trans.* **15,** 77–81.

Cowan, S. W. (1993). *Curr. Opin. Struct. Biol.* **3,** 501–507.

Cowan, S. W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, R. A., Jansonius, J. N., and Rosenbusch, J. P. (1992). *Nature (London)* **358,** 727–733.

Cram, D. J., Choi, H.-J., Bryant, J. A., and Knobler, C. B. (1992). *J. Am. Chem. Soc.* **114,** 7748–7765.

Datta, D. B. (1987). "A Comprehensive Introduction to Membrane Biochemistry." Floral Publishing, Madison, Wisconsin.

Deber, C. M., Khan, A. R., Li, Z., Joensson, C., Glibowicka, M., and Wang, J. (1993). *Proc. Natl. Acad. Sci. U.S.A.* **90,** 11648–11652.

de Grip, W. J., van Oostrum, J., and de Caluwe, G. L. J. (1992). *J. Crystal Growth* **122,** 375–384.

Deisenhofer, J., and Michel, H. (1989). *EMBO J.* **8,** 2149–2170.

Deisenhofer, J., Epp, O., Miki, K., Huber, R., and Michel, H. (1984). *J. Mol. Biol.* **180,** 385–398.

Deisenhofer, J., Epp, O., Miki, K., Huber, R., and Michel, H. (1985). *Nature (London)* **318,** 618–624.

Dill, K. A. (1990). *Biochemistry* **29,** 7133–7155.

Eisenberg, D. S., Weiss, R. M., and Terwilliger, T. C. (1984). *Proc. Natl. Acad. Sci. U.S.A.* **81,** 140–144.

Engelman, D. M. (1982). *Biophys. J.* **37,** 187–188.

Engelman, D. M., and Steitz, T. A. (1981). *Cell* **23,** 411–422.

Engelman, D. M., and Zaccai, G. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 5894–5898.

Engelman, D. M., Henderson, R., McLachlan, A. D., and Wallace, B. A. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 2023–2027.

Engelman, D. M., Steitz, T. A., and Goldman, A. (1986). *Annu. Rev. Biophys. Biophys. Chem.* **15,** 321–353.

Ermler, U., Fritzsch, G., Buchanan, S., and Michel, H. (1992). *In* "Research in Photosynthesis" (N. Murata, ed.), pp. 341–347. Kluwer, Dordrecht, Netherlands.

Fitzpatrick, P. A., Steinmetz, A. C. U., Ringe, D., and Klibanov, A. (1993). *Proc. Natl. Acad. Sci. U.S.A.* **90,** 8653–8657.

Fu, L., and Freire, E. (1992). *Proc. Natl. Acad. Sci. U.S.A.* **89,** 9335–9338.

Furthmayr, H., and Marchesi, V. T. (1976). *Biochemistry* **15,** 1137–1144.

Gennis, R. B. (1989). "Biomembranes—Molecular Structure and Function." Springer-Verlag, Berlin and New York.

Gibson, N. J., and Brown, M. F. (1991). *Photochem. Photobiol.* **54,** 985–992.

Gruner, S. M. (1985). *Proc. Natl. Acad. Sci. U.S.A.* **82,** 3665–3669.

Hawley, S. A. (1971). *Biochemistry* **10,** 2436–2442.

Henderson, R., and Unwin, P. N. T. (1975). *Nature (London)* **257,** 28–32.

Henderson, R., Baldwin, J. M., Ceska, T. A., Zemlin, R., Beckmann, E., and Downing, K. H. (1990). *J. Mol. Biol.* **213,** 899–929.

Heremans, K. (1982). *Annu. Rev. Biophys. Bioeng.* **11,** 1–21.

Israelachvili, J. N. (1992). "Intermolecular and Surface Forces," 2nd Ed. Academic Press, London.

Jackson, M. B., and Sturtevant, J. M. (1978). *Biochemistry* **17,** 911–915.

Kahn, T. W., and Engelman, D. M. (1992). *Biochemistry* **31,** 6144–6151.

Kahn, T. W., Sturtevant, J. M., and Engelman, D. (1992). *Biochemistry* **31,** 8829–8839.

Kauzmann, W. (1959). *Adv. Protein Chem.* **14,** 1–63.

Kauzmann, W. (1987). *Nature (London)* **325,** 763–764.

Kim, C. A., and Berg, J. M. (1993). *Nature (London)* **362,** 267–270.

Krauss, N., Hinrichs, W., Witt, I., Fromme, P., Pritzkow, W., Dauter, Z., Betzel, C., Wilson, K. S., Witt, H. T., and Saenger, W. (1993). *Nature (London)* **361,** 326–331.

Kühlbrandt, W., Wang, D. N., and Fujiyoshi, Y. (1994). *Nature (London)* **367,** 614–621.

Kyte, J., and Doolittle, R. F. (1982). *J. Mol. Biol.* **157,** 105–132.

Lee, B. (1983). *Proc. Natl. Acad. Sci. U.S.A.* **80,** 622–626.

Lemmon, M. A., and Engelman, D. M. (1992). *Curr. Opin. Struct. Biol.* **2,** 511–518.

Lemmon, M. A., Flanagan, J. M., Hunt, J. F., Adair, B. D., Bormann, B.-J., Dempsey, C. E., and Engelman, D. M. (1992a). *J. Biol. Chem.* **267,** 7683–7689.

Lemmon, M. A., Flanagan, J. M., Treutlein, H. R., Zhang, J., and Engelman, D. M. (1992b). *Biochemistry* **31,** 12719–12725.

Lemmon, M. A., Treutlein, H. R., Adams, P. D., Brünger, A. T., and Engelman, D. M. (1994). *Nature Struct. Biol.* **1,** 157–163.
Lin, L., Pinker, R. J., and Kallenbach, N. R. (1993). *Biochemistry* **32,** 12638–12643.
Llinás, M., DeMarco, A., and Lecomte, J. T. J. (1980). *Biochemistry* **19,** 1140–1145.
Maneri, L. R., and Low, P. S. (1988). *J. Biol. Chem.* **263,** 16170–16178.
Mitchell, D. C., Straume, M., and Litman, B. J. (1992). *Biochemistry* **31,** 662–670.
Murphy, K. P., and Freire, E. (1992). *Adv. Protein Chem.* **43,** 313–361.
Murphy, K. P., Privalov, P. L., and Gill, S. J. (1990). *Science* **560,** 559–561.
Navarro, J., Toivio-Kinnucan, M., and Racker, E. (1984). *Biochemistry* **23,** 130–135.
Paulsen, H., Finkenzeller, B., and Kuhlein, N. (1993). *Eur. J. Biochem.* **215,** 809–816.
Picot, D., Loll, P. J., and Garavito, R. M. (1994). *Nature (London)* **367,** 243–249.
Pinker, R. J., Lin, L., Rose, G. D., and Kallenbach, N. R. (1993). *Protein Sci.* **2,** 1099–1105.
Popot, J.-L. (1993). *Curr. Opin. Struct. Biol.* **3,** 532–540.
Popot, J.-L., and Engelman, D. M. (1990). *Biochemistry* **29,** 4031–4037.
Popot, J.-L., Gerchman, S.-E., and Engelman, D. M. (1987). *J. Mol. Biol.* **198,** 655–676.
Privalov, P. L. (1979). *Adv. Protein Chem.* **33,** 167–241.
Privalov, P. L. (1990). *Crit. Rev. Biochem. Mol. Biol.* **25,** 281–305.
Privalov, P. L., and Gill, S. J. (1988). *Adv. Protein Chem.* **39,** 191–234.
Privalov, P. L., and Khechinashvili, N. N. (1974). *J. Mol. Biol.* **86,** 665–684.
Privé, G. G., Verner, G. E., Weitzman, C., Zen, K. H., Eisenberg, D., and Kaback, R. H. (1994). *Acta Cryst. D.* **50,** 375–379.
Quinn, P. J. (1981). *Prog. Biophys. Molec. Biol.* **38,** 1–104.
Rees, D. C., and Wolfe, G. M. (1993). *Protein Sci.* **2,** 1882–1889.
Rees, D. C., DeAntonio, L., and Eisenberg, D. (1989a). *Science* **245,** 510–513.
Rees, D. C., Komiya, H., Yeates, T. O., Allen, J. P., and Feher, G. (1989b). *Annu. Rev. Biochem.* **58,** 607–633.
Rees, D. C., Chirino, A. J., Kim, K.-H., and Komiya, H. (1994). *In* "Membrane Protein Structure: Experimental Approaches" (S. White, ed.), pp. 3–26. Oxford, New York.
Rosenbusch, J. P. (1974). *J. Biol. Chem.* **249,** 8019–8029.
Royer, C. A., Hinck, A. P., Loh, S. N., Prehoda, K. E., Peng, X., Jonas, J., and Markley, J. (1993). *Biochemistry* **32,** 5222–5232.
Samarasinghe, S. D., Campbell, D. M., Jonas, A., and Jonas, J. (1992). *Biochemistry* **31,** 7773–7778.
Sami, M., Malik, S., and Watts, A. (1992). *Biochim. Biophys. Acta* **1105,** 148–154.
Schafmeister, C. E., Miercke, L. J. W., and Stroud, R. M. (1993). *Science* **262,** 734–738.
Schiffer, M., Chang, C. H., and Stevens, F. J. (1992). *Protein Eng.* **5,** 213–214.
Seddon, J. M. (1990). *Biochim. Biophys. Acta.* **1031,** 1–69.
Shen, Y., Safinya, C. R., Liang, K. S., Ruppert, A. F., and Rothschild, K. J. (1993). *Nature (London)* **355,** 48–50.
Singer, S. J. (1962). *Adv. Protein Chem.* **17,** 1–68.
Singer, S. J., and Nicolson, G. L. (1972). *Science* **175,** 720–731.
Tanford, C. (1980). "The Hydrophobic Effect." Wiley, New York.
Thompson, L. K., Sturtevant, J. M., and Brudvig, G. W. (1986). *Biochemistry* **25,** 6161–6169.
Toscano, G., Pirozzi, D., and Greco, G. (1990). *Biotechnol. Lett.* **12,** 821–824.
Unwin, N. (1993). *J. Mol. Biol.* **229,** 1101–1124.
Vantilbeurgh, H., Egloff, M. P., Martinze, C., Rugani, N., Verger, R., and Cambillau, C. (1993). *Nature (London)* **362,** 814–820.
Volkin, D. B., Staubli, A., Langer, R., and Klibanov, A. M. (1991). *Biotech. Bioengin.* **37,** 843–853.
Weber, G., and Drickamer, H. G. (1983). *Q. Rev. Biophys.* **16,** 89–112.

Weiss, M. S., Wacker, T., Weckesser, J., Welte, W., and Schultz, G. E. (1990). *FEBS Lett.* **267,** 268–272.

Weiss, M. S., Kreusch, A., Schiltz, E., Nestel, U., Welte, W., Weckesser, J., and Schulz, G. E. (1991). *FEBS Lett.* **280,** 379–382.

Yeates, T. O., Komiya, H., Rees, D. C., Allen, J. P., and Feher, G. (1987). *Proc. Natl. Acad. Sci. U.S.A.* **84,** 6438–6442.

Yeates, T. O. (1993). *In* "Thermodynamics of Membrane Receptors and Channels" (M. B. Jackson, ed.), pp. 1–25. CRC Press, Boca Raton, Florida.

Zipp, A., and Kauzmann, W. (1973). *Biochemistry* **12,** 4217–4228.