and $^{15}NO_2^-$ (Figure 9C,D). The faster periodicity arises from ambient or weakly coupled protons while the slower periodicities arise from weakly coupled ^{14}N.[30] The differences observed between $^{14}NO_2^-$ and $^{15}NO_2^-$ of **1** are not found for the hemocyanin derivatives. Once again, there is no evidence that nitrite is equatorially coordinated to divalent copper through oxygen in the nitrite-treated protein.

This measurement, however, does not rule out the possibility that nitrite is axially coordinated to Cu(II). Axial coordination is expected to produce a smaller electron-nuclear coupling by roughly a factor of 30-50, by analogy with other Cu(II) complexes.[20,30,31] Therefore, nitrite that is axially coordinated to Cu(II) through oxygen would likely give rise to extremely shallow modulations because A_{iso} would be so much smaller than the electron-nuclear coupling and its spectral contribution would then not be distinguishable. Were nitrite to bind axially through nitrogen, its spectral contribution would be overwhelmed by those from coordinated imidazole nitrogens, and once again, not be recognized.

Conclusion. Nitrite binding in the model **1** is through oxygen, both in the crystalline state and in solution. The electron-nuclear coupling of ^{14}N and the unpaired electron of Cu(II) is comparable to the ^{14}N nuclear Zeeman interaction at X-band, and thus $^{14}NO_2^-$ modulations can be detected by ESEEM spectroscopy. The electron distribution on ^{14}N of Cu(II)-bound nitrite in frozen solution is very similar to that in solid, diamagnetic nitrite salts, as indicated from the quadrupole parameters (e^2qQ = 5.66 MHz, η = 0.31), suggesting similarities in metal-nitrite binding. This ESEEM investigation also suggests that in frozen solution the conformation of nitrite in **1** is the same as that in the crystal. The ESEEM study of nitrite-treated hemocyanin shows that nitrite does not coordinate to Cu(II) through oxygen as an equatorial ligand, as it does in the model.

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Supplementary Material Available: Complete structure information including anisotropic thermal parameters for non-hydrogen atoms, atomic coordinates, and intramolecular distances and angles (12 pages); observed and calculated structure factors (29 pages). Ordering information is given on any current masthead page.

Lipidic Cubic Phases as Transparent, Rigid Matrices for the Direct Spectroscopic Study of Immobilized Membrane Proteins

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Abstract: Lipidic cubic phases composed of 1-palmitoyl-*sn*-glycero-3-phosphocholine and water were used as structured, transparent, rigid matrices in an attempt to develop novel media for simultaneous structural and functional investigations of membrane proteins. Bacteriorhodopsin and melittin, immobilized in the cubic phase, resulted in very stable materials, whose circular dichroism (CD) spectra exhibit contents of α-helicity akin to those expected from the crystal structures. As judged from CD, the native conformations were temperature independent in the range of existence of the cubic phase (0-50 °C). Addition of NaCl to the bacteriorhodopsin-containing cubic phase stabilized the binding of retinal to the immobilized protein. Without added salt, a slow (i.e., days long) dissociation of retinal at ambient conditions was observed, which however takes place without impairing the native protein's conformation.

Introduction

Compared to soluble proteins, our understanding of the structure-function relationship of membrane proteins is still quite rudimentary, due to the fact that membrane proteins carry out their specific functions when immobilized in their native environment—the membranes—and often undergo denaturation when extracted out of the membrane and solubilized. Moreover, very little structural information is available on membrane proteins, mainly due to the great technical difficulty in obtaining well diffracting crystals. An alternative approach—the use of electron microscopy for obtaining high-resolution structures—has only recently been successfully applied to bacteriorhodopsin.[1] In order to gain a better understanding of the structure-function relationship of membrane proteins, it would be necessary to perform both structural and functional studies in the immobilized state of the protein under conditions which mimic the native ones. We wish to report here on a class of materials—lipidic cubic phases—which exhibit a combination of useful properties, which render them very suitable for such studies.

Lipidic cubic phases, first described by Luzzati et al.,[2] are highly viscous, isotropic, and thermodynamically stable rigid materials composed of (phospho)lipids and water. Moreover, these materials are transparent, which makes them perfect matrices for spectroscopic investigations. Depending on the particular conditions and choice of lipids, different cubic structures can be formed. These are divided into two groups—bicontinuous and closed aggregates.[3] The bicontinuous group is based on a three-dimensional, curved bilayer structure of lipids surrounded by water, in which both components diffuse freely. The bilayer can be described by infinite periodic minimal surfaces, which have at each point a mean curvature of zero. The closed-aggregate group is built up of an ordered array of micelles, made up of diffusionally restricted lipids, surrounded by water. These remarkable properties should enable the immobilization and spectroscopic investigation of membrane species. Moreover, based on the fact that these materials are composed of crystallographically well-defined polar, nonpolar, and interfacial regions, we have anticipated that they

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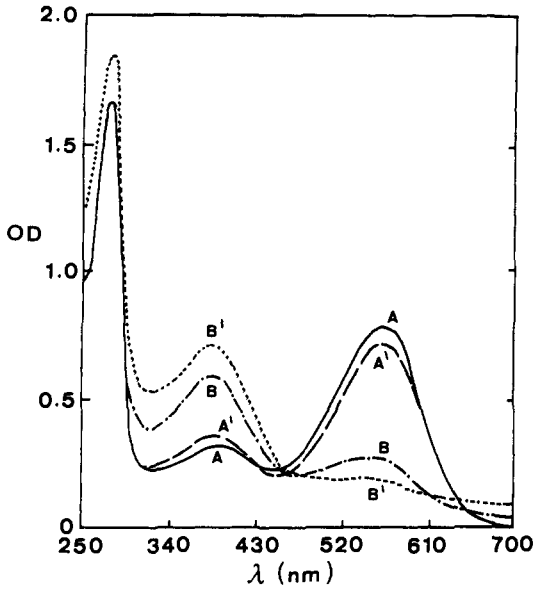

Figure 1. UV–vis spectra of $1.7 \cdot 10^{-5}$ M bacteriorhodopsin in a 44% (w/w) PLPC cubic phase (10 mM p-buffer, pH 8.0): A = with 1 M NaCl, 3-day old sample, A′ = with 1 M NaCl, 6-day old sample, B = without NaCl, 3-day old sample, and B′ = without NaCl, 6-day old sample.

should function as a general type of structured, "solid solvents", which in principle should be able to solubilize compounds with nearly any chemical composition.

Ericsson et al.[4] have reported the incorporation of lysozyme and other globular proteins into a monoolein-based cubic phase, and cubic phases containing casein and gliadin were also described.[5,6] However, no detailed structural or functional studies have been described. We have recently reported on the immobilization, spectroscopic, rheological, and enzymatic studies of α-chymotrypsin, a water soluble enzyme, in a lipidic cubic phase composed of 1-palmitoyl-*sn*-glycero-3-phosphocholine (PLPC) and water.[7] It was shown that the enzyme retains its native conformation and exhibits enzymatic activity, and that the viscoelastic properties of the cubic phase remain unchanged upon incorporation of the protein. In this paper we will present evidence demonstrating that this type of membranous material is indeed capable of incorporating membrane proteins such as bacteriorhodopsin and melittin. Bacteriorhodopsin (bR), a light driven proton pump, is a 248 amino acid integral membrane protein found in the purple membrane of the halophilic bacterium *Halobacterium halobium*,[8] consisting of seven α-helices that span the membrane. Melittin, the main component of the honey bee venom, is a water soluble, 26 residue polypeptide which is a strong membrane lytic agent known to bind spontaneously to membranes.[9] Moreover, we will show that the cubic phase remains stable and transparent so that the protein's properties can be conveniently studied and controlled in the immobilized form.

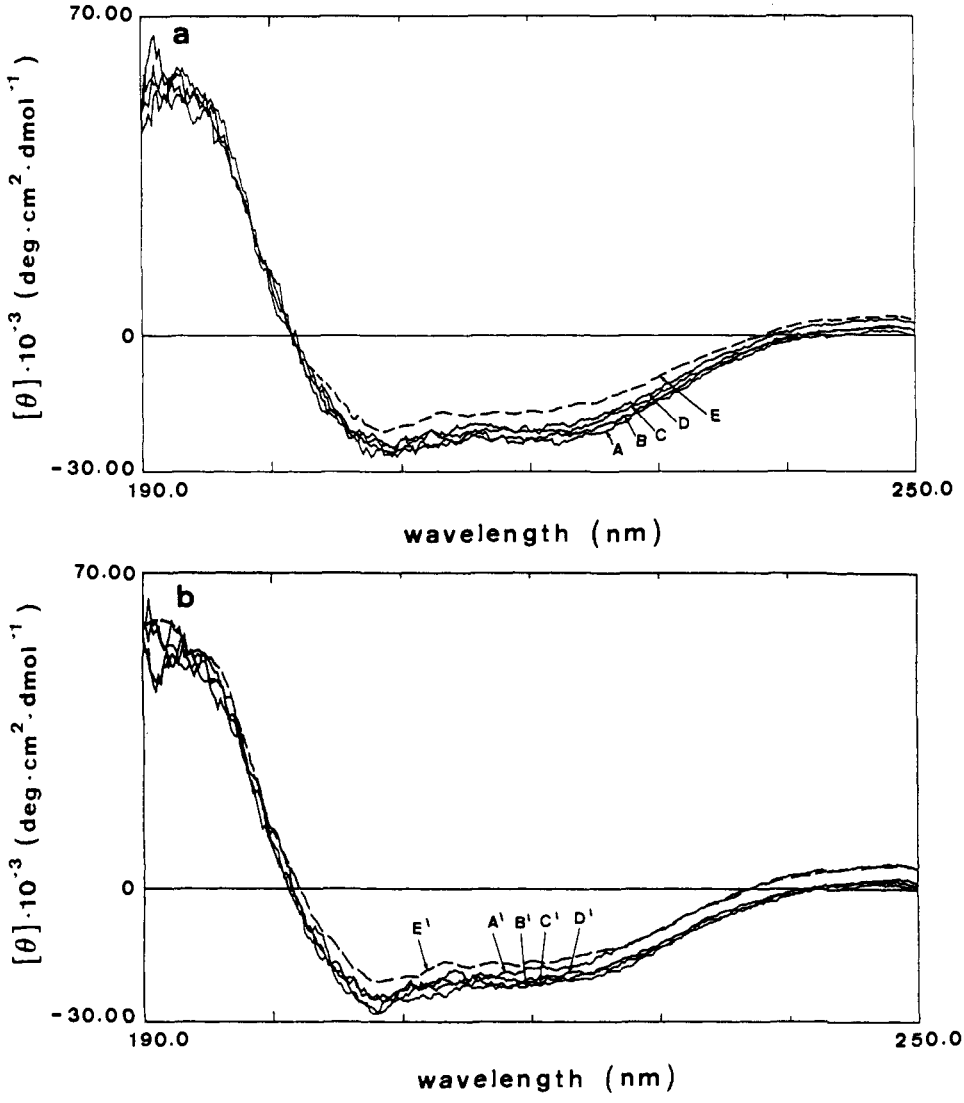

Figure 2. Temperature dependent circular dichroism spectra of $1.7 \cdot 10^{-5}$ M bacteriorhodopsin in a 44% (w/w) PLPC cubic phase (10 mM p-buffer, pH 8.0): (a) spectra taken during the heating cycle and (b) spectra taken during the cooling cycle, A = 5 °C, B = 15 °C, C = 25 °C, D = 35 °C, E = 45 °C. [θ] is the mean residue ellipticity in [deg·cm²·dmol⁻¹].

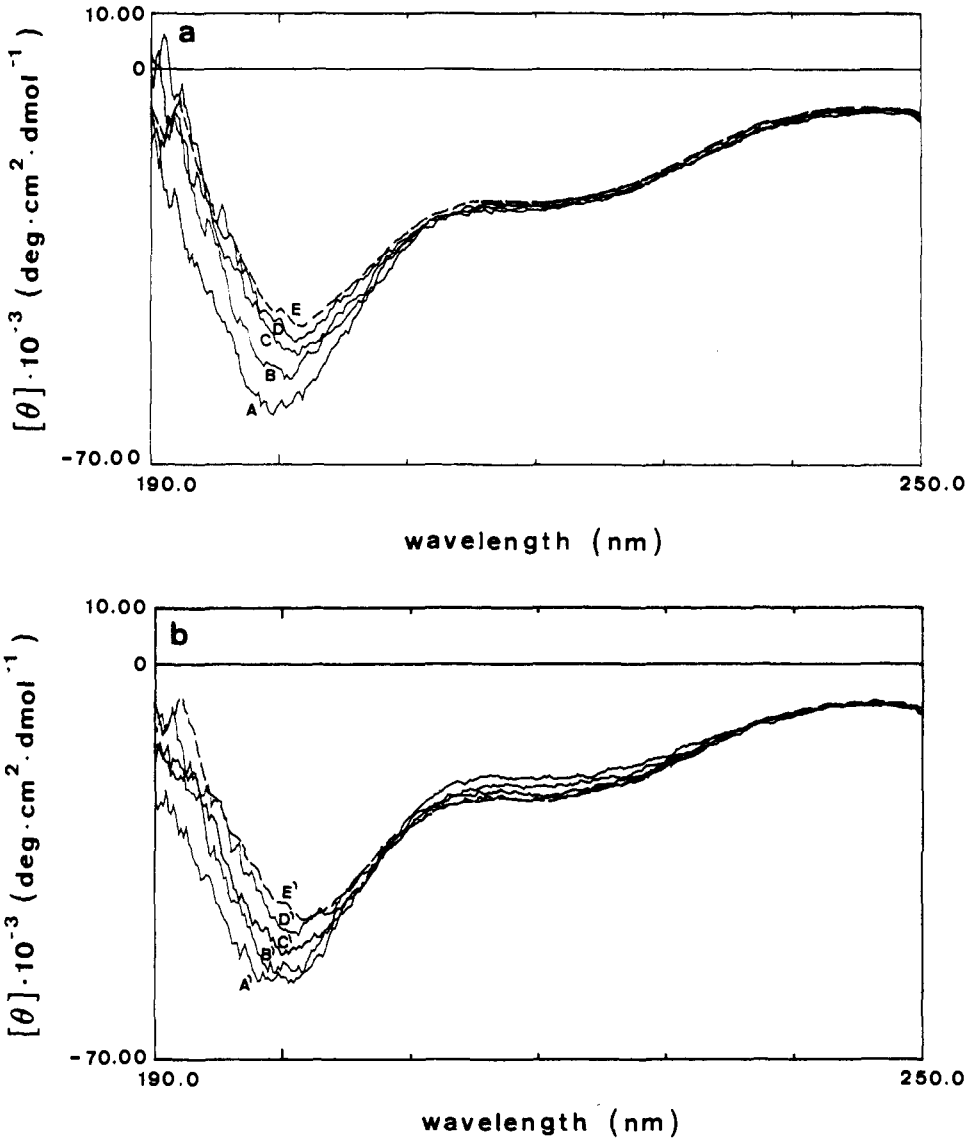

Figure 3. Temperature dependent circular dichroism spectra of $1.2 \cdot 10^{-4}$ M melittin in a 10 mM tris-HCl buffer, pH 7.4: (a) spectra taken during the heating cycle and (b) spectra taken during the cooling cycle, A = 5 °C, B = 15 °C, C = 25 °C, D = 35 °C, E = 45 °C. [θ] is the mean residue ellipticity in [deg·cm^2·dmol^{-1}].

Experimental Section

Reagents were as follows: L-α-PLPC (Avanti Polar); melittin (Sigma); NaCl (Merck). All were of highest purity grade and were used as received. Bacteriorhodopsin (purple membrane) was a gift from Prof. D. Oesterhelt, Max Planck Institute für Biochemie, Martinsried.

Cubic Phases. PLPC based samples were prepared by adding buffer solutions of melittin or suspensions of bacteriorhodopsin to PLPC in 1 cm, 1 mL UV-cuvettes (114-QS, Hellma) and centrifuging for 1–2 days (2900 g) at 25 °C. Complete formation of cubic phases was determined by appearance of highly stiff and transparent gels. The concentration in the solid system is given in moles per total volume.

Aqueous Stock Solutions. Bacteriorhodopsin suspensions were prepared in 18 mM p-buffer, pH 8.0. Melittin solutions were prepared in 20 mM Tris-HCl buffer, pH 7.4. The concentration of melittin solutions was determined spectrophotometrically,[10] using $\epsilon_{280} = 5570$ M^{-1}·cm^{-1}.

Spectroscopic Measurements. For UV measurements, the cubic phase samples were prepared directly in the spectroscopic cuvettes. For CD measurements, the samples were prepared in 1-mL vials and transferred to the measurement plates just prior to measurement. Bacteriorhodopsin containing cubic phases were kept at ambient conditions and without light protection at all times.

UV. All measurements of stock solutions and cubic phases were carried out in a Beckman DU-68 spectrophotometer.

CD. CD measurements were carried out in a Jasco J-600 instrument. The samples were measured as films pressed between quartz plates, thickness 0.01 and 0.05 cm (124-QS Hellma). The temperature of the samples was controlled to ±0.5 °C. The samples were allowed to equilibrate for 20 min at each temperature. [θ] represents the mean ellipticity per residue in deg·cm^2·dmol^{-1}.

Results and Discussion

PLPC/water form a cubic phase at a PLPC concentration range of 39–45% (w/w). Its proposed structure, based on X-ray studies[11] and NMR pulsed-field gradient measurements,[12] consists of a cubic array of closed, rod-like micelles with an axial ratio of approximately 2, surrounded by water. In this structure, each corner of the unit cell is occupied by a micelle, each surface has two micelles, and one micelle is in the center. The phospholipid

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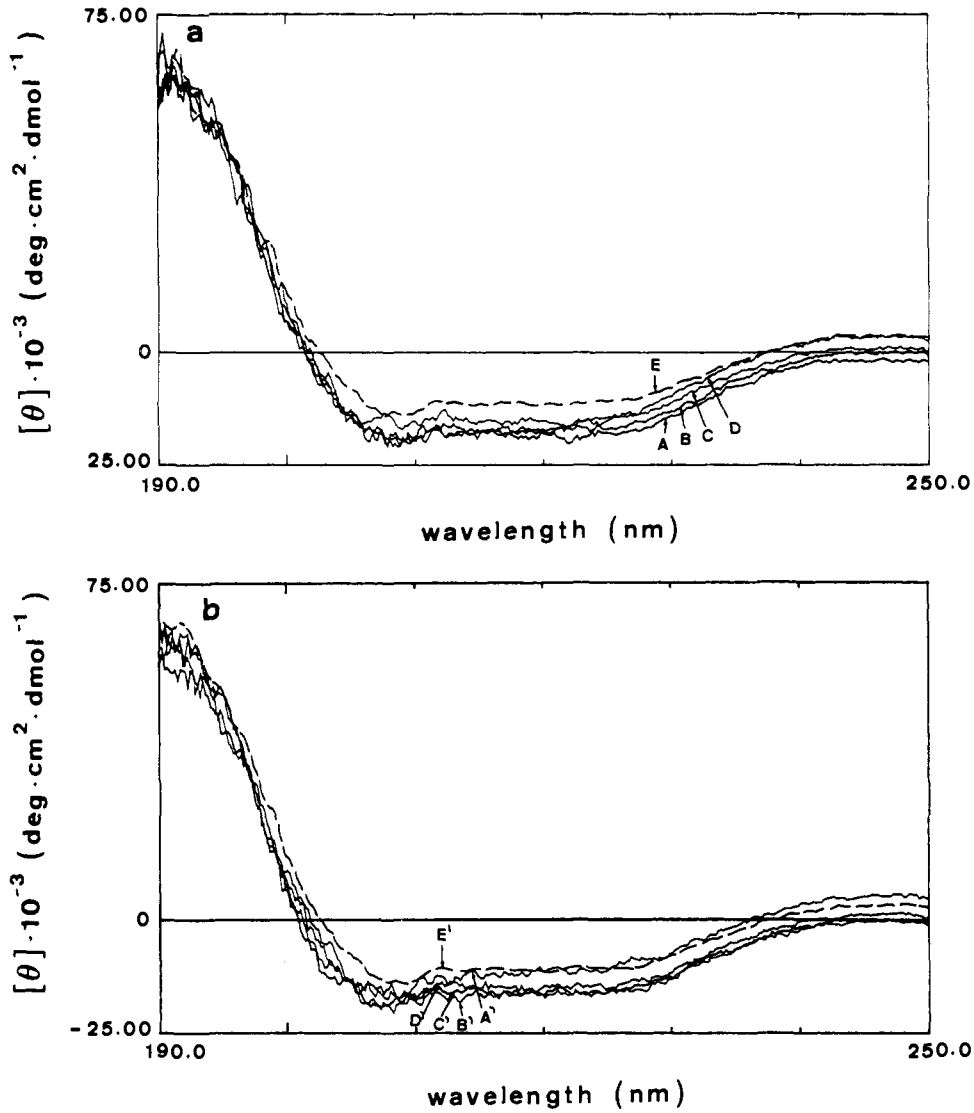

Figure 4. Temperature dependent circular dichroism spectra of $1.2 \cdot 10^{-4}$ M melittin in a 43% (w/w) PLPC cubic phase (10 mM tris-HCl buffer, pH 7.4): (a) spectra taken during the heating cycle and (b) spectra taken during the cooling cycle, A = 5 °C, B = 15 °C, C = 25 °C, D = 35 °C, E = 45 °C. [θ] is the mean residue ellipticity in [deg·cm^2·dmol^{-1}].

diffusion coefficient ($0.06 \cdot 10^{-12}$ m^2 s^{-1}) was found to be 2 orders of magnitude smaller than the corresponding one in the lamellar phase ($6 \cdot 10^{-12}$ m^2 s^{-1}),[12b] defining this phase as belonging to the class of closed aggregate cubic phases.

Bacteriorhodopsin and melittin, two membrane-affine species having otherwise quite different properties, were chosen in order to study their specific interaction with the cubic phase—the artificial membrane—and to explore the feasibility of using these materials as general matrices for membrane species.

Incorporation of bR into the PLPC cubic phase at concentrations around $2 \cdot 10^{-5}$ M, pH 8.0, resulted in homogeneous, transparent purple materials. The purple form of bR (λ_{max} = 561 nm) is the one in which the retinal is bound via a protonated Schiff base to the ϵ-amino group of lysine 216. Under these pH conditions, at room temperature and exposed to light, this form is unstable in the cubic phase and undergoes bleaching within 6 days (Figure 1). The retinal dissociates from the bR, forming bacterioopsin and free retinal (λ_{max} = 376 nm). Thus the color of the material changes from purple to yellow. Upon addition of 1 M NaCl to the preparation, leaving all other conditions unchanged, a marked stabilization of the native bR is observed: Under these conditions, almost no dissociation of bound retinal was detected within 6 days. In fact, at higher bR concentrations ($1.6 \cdot 10^{-4}$ M) cubic phases containing 1 M NaCl were found to be stable for at least 9 months at ambient conditions. Since bR is found in bacteria that exist in highly concentrated salt water, this stabilization can be rationalized in terms of creating conditions native to the protein. These observations are in accord with the reported restoration of the purple color of bR suspensions[13] as well as decrease in the rate of deprotonation of the protonated Schiff base[14] upon addition of salt.

Circular dichroism (CD) studies were performed on immobilized bR concomitant to the UV measurements. Based thereon, the conformation of bR in both the NaCl-containing and NaCl-free cubic phases was typically α-helical. The CD spectra of the immobilized proteins were unchanged as a function of time in both cases. This finding is especially interesting in the case of the NaCl-free cubic phase, as it suggests that the process of dissociation of retinal from the protein into the cubic phase medium, i.e., gradual formation of the apoprotein, takes place without affecting the protein's conformation.

The content of α-helicity of the immobilized protein, determined from measurements of three independent preparations at different temperatures using empirical formulas,[15] was 72.1 ± 10.7%, which is in excellent agreement with the value of around 75% determined from electron microscopy and electron diffraction on bR in the

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purple membrane.[1,16] Thus it is concluded that bR is immobilized in the PLPC cubic phase without impairment of its native conformation.

The conformational stability of bR in the cubic phase is further demonstrated by investigations of the temperature dependence of the immobilized protein, which were carried out in the existence range of the PLPC cubic phase (0–50 °C). CD spectra taken at intervals of 10 °C, starting from 5 °C, up to 45 °C in the heating cycle (Figure 2a), and returning to 5 °C on cooling (Figure 2b), are shown. With the exception of the spectrum at 45 °C, the spectra at all other temperatures are virtually identical, indicating a temperature independent conformation of the immobilized protein. The thermal stability of bR's conformation in this temperature range has been reported for other media as well.[17] The observed shift in the spectrum at 45 °C is most probably due to changes in the cubic phase upon melting at 45 °C.

Whereas bR is a membrane spanning protein, melittin's basic C-terminal residues interact electrostatically with the polar head groups of phospholipids.[18] In water, melittin is unfolded at micromolar concentrations, low ionic strength, and neutral pH.[19] Under these conditions, electrostatic repulsion between positively charged residues dominate. Melittin undergoes a transition to a tetrameric α-helical conformation at higher melittin concentrations, acidic or basic pH and high ionic strength,[19] where anion binding screens the positive charges and reduces repulsion. Upon interacting with membranes, monomeric melittin adopts an α-helical conformation as well.[9,10,20]

Melittin ($1.2 \cdot 10^{-4}$ M) in aqueous solution, pH 7.4, exhibits a conformation corresponding to ca. 70% random coil, 30% α-helix at 5 °C (Figure 3a). Upon increasing the temperature to 45 °C, the absolute values of the ellipticity at ca. 200 nm, corresponding to the random coil conformation, decrease while shifting to ca. 202 nm. These changes are thermally reversible (Figure 3b). In contrast, melittin incorporated in PLPC cubic phases at the same concentration and under otherwise identical conditions exhibited an α-helical conformation, as judged by CD, which was temperature independent in the existence range of the cubic phase (Figure 4), with a possible exception at 45 °C, the reason being again the melting of the cubic phase (cf. the case of bR described above). Moreover, the content of induced α-helicity, calculated to be 98.3 ± 5.2% (from measurements of three samples at different concentrations and temperatures), is in good agreement with the value of about 90% deduced from the crystal structure.[21]

The nature of melittin's interaction with membranes has been a subject of widespread research. Weaver et al.[18] demonstrated recently that melittin acts biologically as a monomeric α-helical peptide. Generally, in the presence of micelles and liposomes, the induced α-helicity ranges between about 25 and 90%.[9,18,22] In our case, the induced α-helicity at all temperatures certainly indicates a most favorable interaction between immobilized melittin and the host lipidic/water matrix.

The two systems studied, bR and melittin, are significantly different, the former being a typical membrane spanning protein, the latter a peptide which binds to the membrane primarily through electrostatic interactions. Therefore, their interactions with the lipidic/water cubic phase must be governed by different forces. Nonetheless, they display some astonishing similarities. In both cases, spontaneous formation of the conformations which correspond to those expected from the crystal structures is observed. In both cases, these conformations are remarkably stable with respect to temperature. In both cases, incorporation of the guest proteins into the cubic phase results in very stable materials, which can be kept in sealed vials at ambient conditions for months. In addition, the fact that the conformation of bR remains unchanged upon dissociation of the retinal points to a most stable environment of the protein and concomitantly available medium for the release and solubilization of the latter. These observations, combined with our previously published results on α-chymotrypsin,[7] which is not a membrane protein, all suggest that cubic phases composed of phospholipids/water are indeed a class of structured, rigid materials capable of incorporating diverse chemical species in their native stable states—a general matrix. This remarkable property is a direct consequence of the existence in these materials of microscopically well-defined polar and apolar regions, separated by a membranous boundary.

We have demonstrated here a general principle of operation. Fine structural details of membrane–protein interactions in these materials are currently under investigation. We are also exploring the extent to which one can use these materials as matrices for the study of biochemical membrane processes.

Acknowledgment. We are indebted to Prof. D. Oesterhelt, Max Planck Institut für Biochemie, for the gift of bacteriorhodopsin.

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