Biochimica et Biophysica Acta, 553 (1979) 438—449

BBA 78380

HYDROPHOBIC MEMBRANE PROTEIN FROM CHROMATOPHORES OF *RHODOSPIRILLUM RUBRUM*

STRUCTURAL AND SPECTROSCOPIC STUDIES OF MONOLAYERS AND MULTILAYERS

F. KOPP, P.A. CUENDET, K. MÜHLETHALER and H. ZUBER

Institut für Zellbiologie und Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule, CH-8093 Zürich-Hönggerberg (Switzerland)

(Received October 12th, 1978)

Key words: Chromatophore; Membrane protein; Molecular organization (Monolayer)

Summary

A hydrophobic, lipid- and pigment-free polypeptide from the chromatophore membrane of *Rhodospirillum rubrum* was spread from chloroform/methanol, pyridine and formic acid solutions at an air-water interface. Surface pressure versus area isotherms of the monolayers formed at the interface were partially dependent upon the spreading solvent used. From the surface area at 20 dynes/cm compression, an average molecular area of 12.9 nm^2/molecule was calculated for a polypeptide monolayer spread from chloroform/methanol. Multilayers built up on germanium plates at different surface pressures were subjected to attenuated total reflection infrared spectroscopy. In all cases the amide I and II absorption bands were typical of α-helical and random conformations. Electron microscopy of transferred monolayers replicated by rotary platinum shadowing revealed domains of regular texture in specimens prepared at 20 dynes/cm. Such domains were virtually absent in specimens prepared at 10 and 30 dynes/cm. Light optical diffractometry of the ordered arrays yielded a smallest repetitive area of 13.5 nm^2 which agrees well with the molecular area obtained from the monolayer surface. Although no drastic changes in secondary structure were detected in the course of this study, some conformational changes are indicated by solvent-dependent differences in the surface pressure versus area isotherms.

Introduction

Integral membrane proteins are markedly insoluble in aqueous solutions. As has been pointed out by Hwang et al. [1] one can consider that membrane pro-

teins operate at an interface between high dielectric (water) and low dielectric (membrane) phases. Structure-function investigations of isolated membrane proteins should therefore take place in similar environmental conditions. One approach in this direction is to study a specific membrane protein in interface films. Such films can be transferred onto solid supports for infrared spectroscopy and high resolution electron microscopy. These two methods allow the concomitant study of molecular structure and ultrastructural appearance of the specific protein (with or without other membrane components) present in the transferred film.

In a first attempt we have studied interface films of the major lipid- and pigment-free polypeptide component of the chromatophore membrane of the photosynthetic bacterium *Rhodospirillum rubrum*. This initial study is intended to provide information basic to the investigation of protein-lipid and protein-bacteriochlorophyll interactions in membrane model systems suitable for both infrared spectroscopy and high resolution electron microscopy. Photoreceptor complexes [2,3] prepared from chromatophores of *R. rubrum* contain, in addition to the major chromatophore polypeptide, the reaction center polypeptides, the bacteriochlorophyll and the carotenoids. They are physiologically active in converting absorbed radiation into chemical potential. The major polypeptide has been prepared from such complexes [3]. The same component can be detergent solubilized from reaction center-depleted chromatophores as a red-shifted bacteriochlorophyll-associated protein [4]. This complex is presumed to represent a subunit of the light-harvesting antenna system of *R. rubrum*. Accordingly, the polypeptide part of this complex has been designated light-harvesting polypeptide [5].

Light-harvesting polypeptide is insoluble in water but soluble in a mixture of chloroform and methanol. It can also be extracted into the organic solvent directly from the reaction center-depleted chromatophores [4]. The polypeptide is separated from pigment and lipid by gel filtration on Sephadex LH-60 in chloroform/methanol. Its mol. wt. is approx. 14 000 [4] and it contributes as much as about 50% of the chromatophore protein [3]. Light-harvesting polypeptide has been found to be partially exposed at the cytoplasmic surface of the chromatophore membrane [5,6]. The likely function of this polypeptide in the chromatophore membrane is in the organization of the light-harvesting bacteriochlorophyll into functioning red-shift units through which the excitation energy transfer to the reaction center takes place [3,7]. Antenna bacteriochlorophyll in the in vivo system is red shifted relative to the monomeric bacteriochlorophyll in organic solvents. An outstanding property of light-harvesting polypeptide is its highly lipophilic nature which is demonstrated by its abnormal binding capacity for sodium dodecyl sulfate [3,8] and its ability to dissolve in different non-aqueous solvents [3]. From such solvents it can be spread at an air-water interface to form a monomolecular layer.

Structural and spectroscopic characteristics of bacteriorhodopsin in interface films have recently been reported by Hwang et al. [1]. These authors spread a mixture of lipids and purple membrane fragments from *Halobacterium halobium* at an air-water interface. In contrast, we have been able to form mono- and multilayers of lipid-free membrane protein. Moreover we have employed different solvents used for spreading synthetic water-insoluble homo-

polypeptides (e.g. glutamic ester polymers), for which it had earlier been shown that their conformation in interface films is related to the type of the spreading solvent [9].

Materials and Methods

Materials. Light-harvesting polypeptide was isolated from reaction center-depleted chromatophores of the carotenoidless mutant G-9$^+$ of *R. rubrum* in chloroform/methanol as described earlier [4]. The freeze-dried polypeptide preparation used in this study was free of salt, lipid and pigment [4]. The dry weight was determined after 14 h at 100° C over phosphorus pentoxide; it was lower by 8% than the weight after freeze-drying alone. All reagents were analytical grade. Doubly quartz-distilled water was used throughout.

Monolayers and film balance. Monolayers were formed by spreading protein solutions, usually at a concentration of about 0.5 mg/ml, in chloroform/methanol (1 : 1, v/v), pyridine, or formic acid (98—100%) at an air-water interface. To form a polypeptide monolayer, droplets of 1—2 μl were applied successively with a micropipette onto the water surface. This was done alternately at three different places allowing for better evaporation of the solvent. 50—60 μg of protein were applied/film (0.7 mg/m^2) to obtain an area sufficiently large to allow transfer onto solid supports under constant surface pressure. In order to achieve comparable results all films were compressed at a constant barrier speed of 10^{-3} m^2/min on a tray of 0.075 m^2. Before compression was started, the solvent was allowed to evaporate for at least 20 min and usually a pressure of 20 dynes/cm was reached 60 min after spreading was completed.

Sample preparation for infrared spectroscopy. Polypeptide multilayers were prepared at three different film pressures by repetitive mechanical apposition of a germanium reflection plate (20 × 50 × 1 mm) onto the compressed film, with the planes of the plate closely parallel to the interface. The film pressure was kept constant to within 0.15 dyne/cm. Three to six monolayers were transferred onto both sides of the germanium plate according to the molecular density of the film (film pressure). Multilayers on germanium were analyzed by attenuated total reflection infrared spectroscopy [10] in order to obtain sufficient infrared absorption. A Perkin Elmer 580 spectrophotometer equipped with an internal reflection device was used. The angle of incidence was 30°, resulting in about 55 internal reflections. Spectra were scanned without compensation for the absorption of germanium. The atmosphere in the spectrophotometer was kept dry by continuous recycling through a molecular sieve.

Electron microscopy and optical diffraction. According to the method of Blodgett [11], single monomolecular layers of light-harvesting polypeptide were deposited at three different film pressures onto small pieces of freshly cleaved mica. To prevent rearrangement of the molecules the samples were then immediately frozen in liquid nitrogen. For electron microscopic investigation the monolayers were replicated in a Balzers freeze-etch device BA 500 M. The method used was based on earlier work of Kopp et al. [12,13]. While still

under liquid nitrogen the specimens were first placed in a small brass container which was then fitted onto a cooled stage in the freeze-etch unit. This stage permitted rotation of the specimen. After a vacuum better than 10^{-7} Torr was achieved, the brass container was opened and the exposed ice-free specimen replicated by conical shadowing. The monolayers were shadowed with platinum-carbon at a shallow angle of 9°. The replica thickness was measured with a quartz crystal film-thickness monitor positioned in the shadowing direction and calculated for a direction vertical to the specimen plane. The average thickness was 0.28 nm. Additional 15 nm of carbon evaporated vertical to the specimen plane reinforced the replicas. The latter were floated off on water and viewed in a Siemens 102 electron microscope equipped with an anticontamination device. Pictures were taken at 100 000 times magnification. Calibration was performed with a cross-grating of 2160 lines/mm. The negatives were directly used for light optical diffractometry [14,15]. For image reconstruction the background noise spectrum was filtered out. The filtering mask placed in the diffraction plane was made by punching a piece of paper according to the positions of the diffraction spots. A millimeter scale of the type used for light microscopy served for internal calibration of the diffractometer.

Results

Surface pressure versus area (π/A) isotherms of light-harvesting polypeptide monolayers

The isotherms obtained from interface films of light-harvesting polypeptide are partially dependent upon the kind of spreading solvent used. Fig. 1a, c and e gives the isotherms of films spread from chloroform/methanol, pyridine and formic acid. When normalized to the same molecular area at 10 dynes/cm, the compression curves prove to be quite similar up to this pressure. Solvent-dependent differences appear only at higher surface pressure. The compression diagrams of light-harvesting polypeptide films spread from both chloroform/methanol and pyridine are similar with a shoulder around 25 dynes/cm, whereas a continuous increase up to 40 dynes/cm was measured when formic acid was used as a spreading solvent. With all three spreading solvents part of the spread protein material was lost into the subphase. This was confirmed by sweeping the original film from the water surface: a new film formed by diffusion of polypeptide molecules from the subphase to the interface. The dispersion of molecules into the subphase was especially pronounced in the case of the pyridine spread films where it lead to an appreciably smaller film area, which is apparent from the shift of the pressure curve in Fig. 1b. More complete spreading of the polypeptide was obtained using chloroform/methanol or formic acid but also when using these spreading solvents, loss of molecules into the subphase amounted to about 9% of the total substance and could therefore not be neglected.

An average molecular area of 12.9 nm^2 was obtained at a film pressure of 20 dynes/cm from surfaces of polypeptide monolayers spread from chloroform/methanol. The calculation is based on the mol. wt. of 14 000 [4], the dry weight of the protein preparation, and the 'total' film area composed of the area of the original film and the area of the new film that formed from

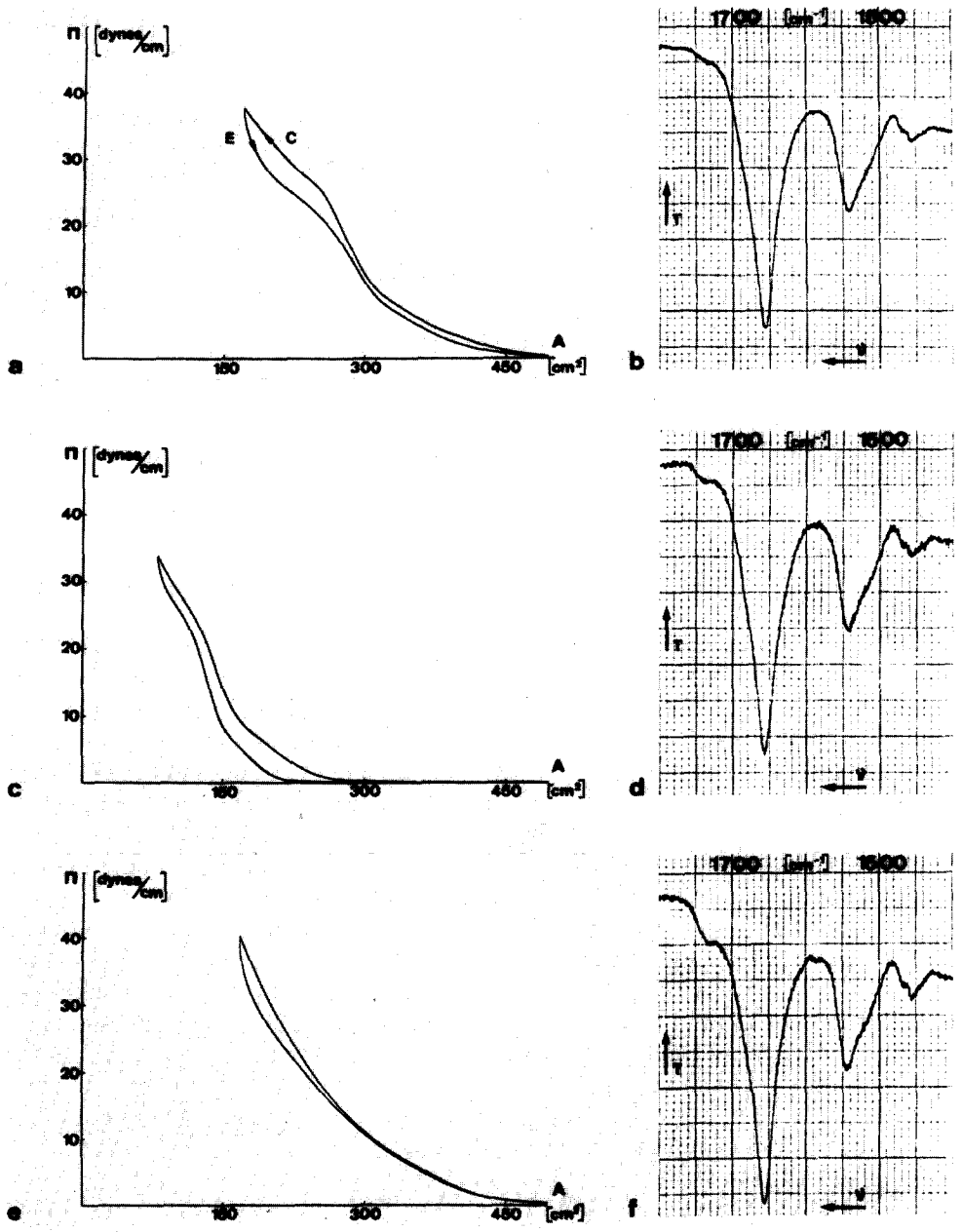

Fig. 1. Surface pressure/area (π/A) isotherms of monomolecular interface films of the hydrophobic bacteriochlorophyll-binding polypeptide (LHP) from *R. rubrum* G-9$^+$ chromatophores. Films were spread from (a) chloroform/methanol, (c) pyridine and (e) formic acid. The infrared spectra of multilayers prepared from the three types of interface films are shown by (b), (d) and (f), respectively. C, compression; E, expansion.

dispersed substance within 16 h after the original film had been removed from the interface.

Infrared spectra of light-harvesting polypeptide multilayers

Multilayers on germanium were prepared, at 10, 20 and 30 dynes/cm for each type of film, from light-harvesting polypeptide monolayers spread from chloroform/methanol, pyridine and formic acid. Spectra of the multilayers were scanned from 1800 cm^{-1} to 1400 cm^{-1}. For all layers, regardless of the degree of compression, the amide I band (C=O stretching vibrations) and amide II band (C-N stretching and N-H bending vibrations) show single maxima at approx. 1655 cm^{-1} and approx. 1545 cm^{-1} indicating α-helical and random coil conformations [16]. A single spectrum is shown for each type of film since no pressure dependence was detected (Fig. 1b, d and f). Identical absorption maxima were also found in spectra of samples of light-harvesting polypeptide dried from water dispersions and from organic solvents.

An accurate estimate of the α-helical content indicated cannot be obtained by curve analysis of the amide I band since the basis spectra for the helical and the random conformations are not separated well enough [17]. A qualitative estimation of helical conformation is possible by comparing the half-widths of the amide I absorption bands. For multilayers of light-harvesting polypeptide prepared from films spread from formic acid an increased order (helical content) is suggested, since their spectra show a smaller half-width of the amide I band as compared to those of the other films investigated (Table I). Bacteriorhodopsin, which makes up 75% of the purple membrane of *H. halobium*, gives a half-width of 50 cm^{-1} when measured in a dried suspension of the latter membrane fragments (Table I). Henderson and Unwin [18] estimated a helical content of 70—80% for this membrane protein. Thus the comparatively smaller half-widths obtained from light-harvesting polypeptide multilayers (Table I) suggest an even higher helicity for this polypeptide.

It is also possible to spread light-harvesting polypeptide directly from the dry bulk phase. Multilayers built up from such films are spectroscopically similar to those prepared from chloroform/methanol and pyridine spread monolayers (data not shown).

Electron microscopy and optical diffraction

Electron microscopy allows indirect observation of the organization of interface films. After conical coating with platinum, that part of the mica covered with

TABLE I

Membrane protein	Spreading solvent	Amide I absorption band, half-width (cm^{-1})
Light-harvesting polypeptide from *R. rubrum* G-9$^+$ chromatophores	chloroform/methanol	38
	pyridine	38
	non (bulk phase)	38
	formic acid	34
Purple membrane fragments (bacteriorhodopsin) from *H. halobium*	dried from aqueous solution	50

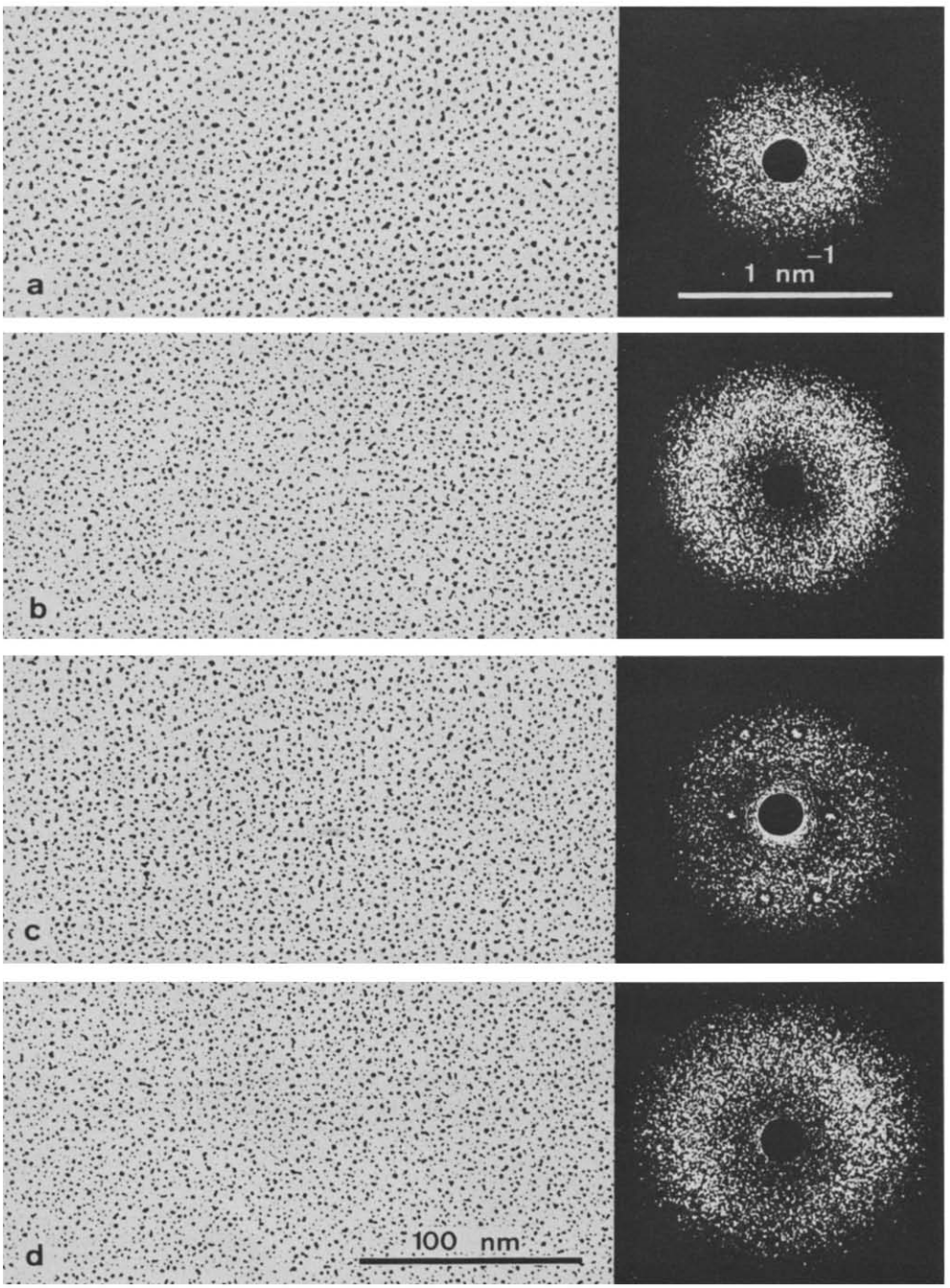

Fig. 2. Electron micrographs of light-harvesting polypeptide monolayers deposited onto mica. The specimens were replicated by rotary platinum shadowing. Control experiment with a clean mica surface (a). Light-harvesting polypeptide monolayer at a surface pressure of 10 dynes/cm (b), 20 dynes/cm (c) and 30 dynes/cm (d). Magnification 300 000×. The light optical diffractograms on the right were obtained from corresponding micrographs. The diffraction spots (marked by black circles) in (c) correspond to spacings of 2.5, 2.7 and 4.9 nm in the replica of the monolayer.

a light-harvesting polypeptide monolayer can easily be distinguished from the clean mica surface. Replicas of polypeptide monolayers on mica exhibit a finer granularity and a smaller mean distance between the platinum granules than a replica of the clean mica surface. This is shown in the electron micrographs and the corresponding diffractograms in Fig. 2. A predominantly random distribution of the platinum granules is found for films deposited at 10 dynes/cm and 30 dynes/cm (Figs. 2b and d). In the specimen prepared at 20 dynes/cm (Fig. 2c), however, domains of regular texture are visible. From these results a pressure-dependent change of the intermolecular order in the polypeptide monolayer is indicated.

The micrographs were subjected to light optical diffraction. The diffraction pattern obtained from the specimen prepared at 20 dynes/cm exhibits regular spots. From these first-order spots visible in the noise spectrum (Fig. 2c) angles and lattice spacings of the arrays visible in the corresponding micrograph can be measured. Taking into account the primary magnification of the micrograph, spacings of 2.5, 2.7 and 4.9 nm were obtained. The reconstructed image of the regular texture is given in Fig. 3. The smallest repetitive area in the reconstruction covers 13.5 nm^2.

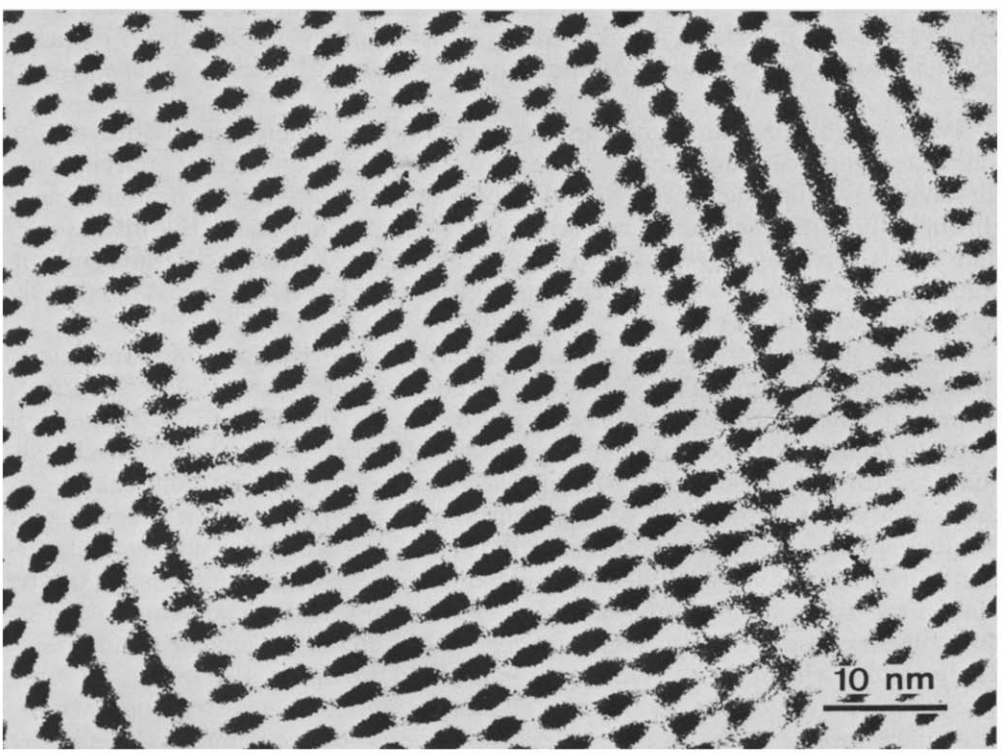

Fig. 3. Light optical reconstruction. The regular texture in a light-harvesting polypeptide monolayer deposited onto mica under 20 dynes/cm compression is shown. Magnification 1 500 000X.

Discussion

Neither drastic solvent effects nor surface pressure-dependent conformational changes were detected by infrared spectroscopy in light-harvesting polypeptide multilayers; the α-helical conformation of this membrane polypeptide is retained. Also no marked solvent-dependent differences are found in the shapes of the compression curves when light-harvesting polypeptide monolayers spread from chloroform/methanol or from pyridine (Fig. 1a and c) are compared. In contrast, two types of infrared spectra are obtained from interface films of the hydrophobic homopolypeptide polymethyl-glutamate spread either from chloroform or from pyridine-rich solutions [9,19]; they can be assigned to α-helical conformation in the first case and to β-pleated sheet structure in the latter. Furthermore polymethyl-glutamate films from chloroform exhibited a plateau of easy compressibility at a surface pressure of 20 dynes/cm, but no such plateau was observed when polymethyl-glutamate was spread from a pyridine-rich solution. Although the secondary structures of polymethyl-glutamate and light-harvesting polypeptide (both hydrophobic molecules) in monolayers are found to be independent of pressure, a surface pressure-dependent conformational transition (shift of the amide I band from 1655 to 1635 cm^{-1}) has been reported by Loeb [20] for interface films of the water-soluble β-lactoglobulin. The preserved α-helical conformation shown throughout the present study indicates that at least as far as the secondary structure of light-harvesting polypeptide is concerned a considerable structural stability is observed at the interface. An extensive α-helical content has also been reported for this molecule in solution (sodium dodecyl sulfate, different organic solvents) [7].

Two possible explanations can be given for the smaller half-width of the amide I band of multilayers prepared from light-harvesting polypeptide dissolved in formic acid (Table I). (1) The α-helical content may have increased through the influence of he spreading solvent. (2) Changes in the intramolecular distortion may have led to helical structures of different thermodynamic stability (the helical content remaining constant) as has been discussed recently by Chirgadze et al. [21].

Possible conformational changes are also indicated by data on formic acid-spread interface films. The shape of the compression curve in Fig. 1e differs from the chloroform/methanol and pyridine-type diagram (Figs. 1a and c). Possible reasons for the changed compression characteristics (mean area/molecule) are the increased helicity (decrease of half-width, Table I) and changes in the tertiary structure [22] of light-harvesting polypeptide. An influence of residual spreading solvent in the monolayer and the subphase on the compression curve cannot be excluded. Repeated compression and expansion of the films after different intervals (to allow time for any additional evaporation of the solvent) resulted in a slight decrease of film area (smaller than 5% at 20 dynes/cm within 24 h). This difference could, however, also be due to some adsorption of molecules to the trough border and the edges of the barriers. The characteristic shape of the compression curves, the continuous slope in the case of the formic acid-spread film and the shoulder around 25 dynes/cm in the chloroform/methanol and pyridine-spread film, were not affected, however.

If compression and expansion curves (hysteresis loop) are considered, the three diagrams in Fig. 1 are seen to be different from each other. Although all films were compressed and expanded with the same low barrier speed, there was not enough time to reach thermodynamic equilibrium at each point of the pressure curves. Nevertheless, the π/A hysteresis loops show characteristic differences in their shape which are interpreted as reflecting mainly changes in tertiary structure. The thereby implied structural flexibility of light-harvesting polypeptide is consistent with the absence of cysteine residues in its amino acid composition [4]. It is known that proteins lacking cystine cross-bridges are able to adapt their conformation quickly and spontaneously to their environment [23].

An important question is how well a single transferred polypeptide monolayer represents the molecular arrangement at the interface. To date no conclusive answer can be given. However, from the electron micrographs it can be concluded that with the method used, areas of the monolayer could be transferred to mica with no obvious disturbance detectable down to the resolution limit of better than 3 nm. There remains the problem of stability of these transferred monolayers. For some lipid interface films [12] it has been shown that, after deposition on a solid, the original extended layer may break down and the molecules may rearrange into microcrystals depending on the type of molecules, number of layers, temperature, substrate, etc. Against the possibility of such a rearrangement in a polypeptide layer one can argue that the half-time of the rearrangement would be long for a 14 000 mol. wt. polypeptide. In the case of the comparatively smaller tripalmitin (mol. wt. 807) the half-time was found to be in the range of 10^5—10^6 s at room temperature.

Conical coating of deposited light-harvesting polypeptide monolayers was used instead of unidirectional shadowing. This in order to avoid directionality artefacts in the replicas [24]. The micrographs in Fig. 2 suggest a higher degree of order in the packing of the polypeptide molecules in specimens prepared at 20 dynes/cm. Ordered arrays are virtually absent in films transferred below and above 20 dynes/cm. The drastic decrease of ordered domains observed in the 30 dynes/cm film (Fig. 2d) might be related to a possible phase transition indicated by the shoulder at approx. 25 dynes/cm in the compression curve in Fig. 1a.

The smallest repetitive feature in the optically reconstructed image (Fig. 3) is assigned to one light-harvesting polypeptide molecule since its average area of 13.5 nm^2 agrees well with the average molecular area of 12.9 nm^2 obtained from film balance measurements. The likely conclusion from the observed correspondence are that (a) the transferred monolayers closely represent the molecular arrangement at the interface and (b) that light-harvesting polypeptide molecules deposited on mica do function as nuclei for growth of platinum granules. The molecular basis for this selective nucleation leading to the observed decoration effect is not understood at present. The molecular area obtained appears at first glance somewhat low for a 'spread' polypeptide of 14 000 mol. wt. Instead of about 0.18 nm^2/residue expected for a helical polypeptide lying flat at the interface, our data yield a value of 0.105 nm^2/residue. Compression from several lower initial surface concentrations (down to 0.07 mg/m^2) did not yield significantly different molecular areas. This ensures

that the relatively high initial surface concentration used in this study did not result in a low molecular area because of incomplete spreading. Concerning the small molecular area obtained, we assume that some folding up out of the interfacial plane occurs naturally. It is possible that a more polar part of the molecule penetrates into the subphase since it is known that part of the polypeptide is accessible at the polar surface of the membrane [5].

The present study provides information on the molecular structure and organization of a specific polypeptide presumed to represent the structural unit of the antenna complex in *R. rubrum* chromatophores. This information will be the basis for future preparation and investigation of interface films containing the membrane components required for the reconstitution of a red-shifted antenna complex. In our opinion interface studies combined with infrared spectroscopy and high resolution electron microscopy will help to achieve a more complete understanding of molecular interactions (and their ultrastructural correlates) between the membrane components involved in the primary steps of bacterial photosynthesis.

Acknowledgements

We wish to express our gratitude to Dr. U.P. Fringeli for his comments related to infrared spectroscopy and Dr. E. Wielandt for helpful discussions. We are indepted to Dr. R. Bachofen and Dr. U. Müller for supplying the reaction center-depleted chromatophores from *R. rubrum* G-9$^+$ and the purple membrane fragments from *H. halobium*. Special thanks are due to Dr. D. Turner for critical reading of the manuscript and Mrs. Z. Zanivan for her expert technical assistance. This work was supported largely by the Swiss National Science Foundation grant 3.190-0.77.

References

1 Hwang, S.B., Korenbrot, J.I. and Stoeckenius, W. (1977) J. Membrane Biol. 36, 115—135
2 Hall, R.L., Kung, M.C., Fu, M., Hales, B.J. and Loach, P.A. (1973) Photochem. Photobiol. 18, 505—520
3 Tonn, S.J., Gogel, G.E. and Loach, P.A. (1977) Biochemistry 16, 877—885
4 Cuendet, P.A. and Zuber, H. (1977) FEBS Lett. 79, 96—100
5 Cuendet, P.A., Zürrer, H., Snozzi, M. and Zuber, H. (1978) FEBS Lett. 88, 309—312
6 Oelze, J. (1978) Biochim. Biophys. Acta 509, 450—461
7 Loach, P.A. (1976) Prog. Bioorg. Chem. 4, 89—191
8 Miyake, J., Ochiai-Yanagi, S., Kasumi, T. and Takagi, T. (1978) J. Biochem. 83, 1679—1686
9 Loeb, G.I. and Baier, R.E. (1968) J. Colloid Interface Sci. 27, 38—45
10 Harrick, N.H. (1967) Internal Reflection Spectroscopy, Interscience Publishers
11 Blodgett, K.B. (1935) J. Am. Chem. Soc. 57, 1007—1022
12 Kopp, F., Fringeli, U.P., Mühlethaler, K. and Günthard, Hs.H. (1975) Z. Naturforsch. 30, 711—717
13 Kopp, F., Fringeli, U.P., Mühlethaler, K. and Günthard, Hs.H. (1975) Biophys. Struct. Mech. 1, 75—96
14 Beeston, B.E.P., Horn, R.W. and Markham, R. (1973) Electron Diffraction and Optical Diffraction Techniques, North Holland/American Elsevier
15 Johansen, B.V. (1975) in Principles and Techniques of Electron Microscopy (Hayat, M.A., ed.), Vol. 5, pp. 114—173, Van Nostrand Reinold Company
16 Miyazawa, T. and Blout, E.R. (1961) J. Am. Chem. Soc. 83, 712—719
17 Eckert, K., Grosse, R., Malur, J. and Repke, K.R.H. (1977) Biopolymers 16, 2549—2563
18 Henderson, R. and Unwin, P.N.T. (1975) Nature 257, 28—32
19 Kopp, F., Cuendet, P.A., Mühlethaler, K. and Zuber, H. (1978) Experientia 34, 913 (Abstract)

20 Loeb, G.I. (1971) J. Polym. Sci., Part C 34, 63—71
21 Chirgadze, Y.N., Brazhnikov, E.V. and Nevskaya, N.A. (1976) J. Mol. Biol. 102, 781—792
22 Miller, I.R. and Bach, D. (1973) in Surface and Colloid Science (Matijevic, E., ed.), Vol. 6, p. 220, John Wiley and Sons, New York
23 Anfinsen, C.B. (1973) Science 181, 223—230
24 Slayter, H.S. (1976) Ultramicroscopy 1, 341—357