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POLARIZED ATTENUATED TOTAL REFLECTANCE SPECTRA OF ORIENTED PURPLE MEMBRANES

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Summary: The technique of polarized Fourier transform infrared attenuated total reflectance spectroscopy has been applied to the study of oriented purple membranes of Halobacterium cutirubrum. This method offers a fast and simple approach for probing conformations of proteins in-situ and capable of obtaining polarized infrared spectra at an angle of incidence that is much greater than the Brewster angle.

Polarized infrared spectroscopy is an effective tool, complementary to X-ray diffraction, for probing the structure of oriented macromolecular assemblies (1-5). The purple membrane is composed of a single integral membrane protein, bacteriorhodopsin, and one type of lipid, diphytanyl glycerol derivatives of phospho- and glycolipids, at a very high protein to lipid ratio, i.e. 4:1 by weight (6,7). It is known that the protein bacteriorhodopsin has seven α-helical segments, oriented nearly perpendicular to the membrane plane. Estimates of the α-helical content depend on the method used (8,9) and vary from 45 to 80%. Pure purple membrane sheets can be prepared easily and form highly oriented films on a glass slide (8,9). The simple chemical composition, together with the ease of preparation of oriented samples, renders the purple membrane an ideal system for the study of oriented membrane assemblies.

Linear dichroic infrared spectra of purple membranes were previously measured using a single-axis goniometer with which the sample plate was tilted to an angle α_0 relative to the XY plane (see Figure 1a). Infrared radiation with the electrical field parallel to the Z-axis (E vertical, E_v)or parallel to the Y-axis (E horizontal, E_h) was passed through the sample and the spectrum recorded (10). This type of arrangement, however, is limited by the fact that the angle $(90-\alpha_0)$ can never exceed the Brewster angle (11) and thus the maximal dichroic effect cannot be achieved (i.e. a spectrum measured at $\alpha_0=90°$).

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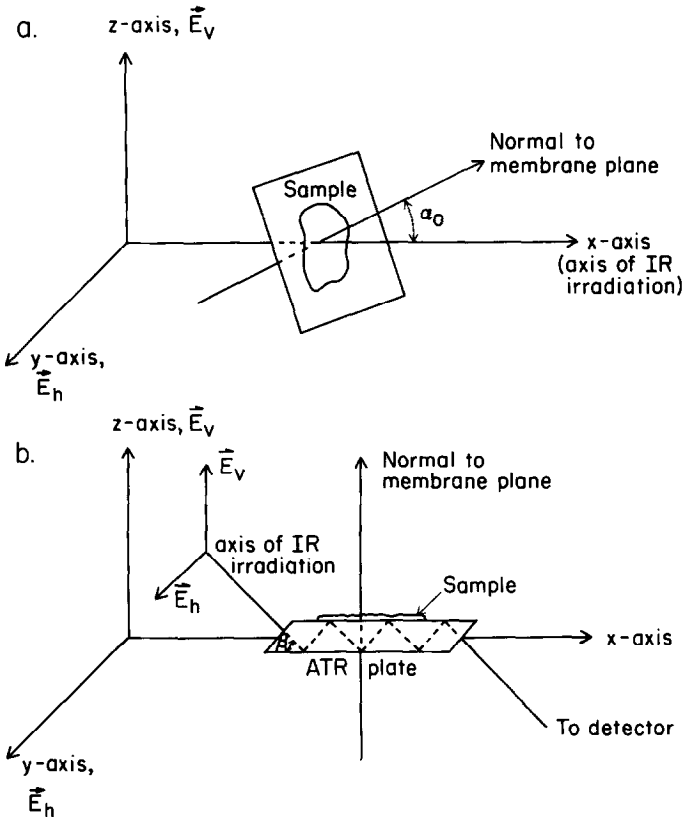

Figure 1. Experimental set up for polarized infrared transmission (a) and polarized ATR infrared experiments (b).

Furthermore, in the case of a transmission spectrum measured at an angle α_0, where $90° > \alpha_0 >$ Brewster angle, the longitudinal optical modes become active (12). When compared with the regular transmission spectrum, the observed frequencies will be shifted to the high frequency side as α_0 increases. Hence, the data interpretation becomes more difficult.

Through the use of attenuated total reflectance (ATR) (13,14) at an angle of incidence which is much greater than the Brewster angle, the above mentioned disadvantages can be avoided (15). This is illustrated in Figure 1b. The infrared radiation enters the ATR plate at an angle of incidence β. With E_v parallel to the Z-axis (parallel to the sample axis) and E_h parallel to the Y axis (perpendicular to the sample axis) a spectrum with the maximal dichroic effect is measured. Also, since the angle of incidence can be adjusted to be larger than the corresponding Brewster angle, the effect of longitudinal optical modes can be eliminated (15).

EXPERIMENTAL

Spectra: Infrared spectra were collected on a Digilab 296 interferometer (Digilab Div. of Bio-Rad, Cambridge, MA) equipped with a wide band mercury cadmium telluride detector (with specific detectivity of 11.4×10^9 cmHz$^{\frac{1}{2}}$/W at 1 kHz) and a high intensity water cooled globar source. The ATR attachment was obtained from Wilks Sci. Corp. (South Norwalk, CT). The reflection plate was zinc selenide (Harrick Sci. Corp., Ossining, N.Y.) cut in a 45 degree rhombus (50×20×10 mm). The angle of incidence was 48 degrees, a geometry which results in approximately 18 active internal reflections. Also, because the angle of incidence used was much larger than the Brewster angle (approximately 24° in this specific case) the longitudinal modes are eliminated. Polarized light was generated by means of a grid polarizer (Perkin-Elmer Corp., Norwalk, CT) placed in front of the sample and behind the interferometer to discriminate against the possible phase retardation which could be imposed by the germanium oxide coated potassium bromide beamsplitter (16). 250 scans were accumulated at a resolution of 4 cm^{-1} (corresponding to a maximum optical retardation of 0.25 cm). ATR infrared spectra are plotted as -log(Rs/Ro), where Rs is the sum of the reflectivities of the sample from the internal reflection plate, and Ro is the measured background of the spectrometer.

Materials: Purple membrane preparations were obtained from Halobacterium cutirubrum NRC strain 34001 as previously described (7). The purple membrane was purified on a 1.3M/1.5M sucrose gradient, dialyzed against distilled water and pelleted at 50,000g for 60 minutes. Sodium dodecylsulfatepolyacrylamide gel electrophoresis showed the presence of a single protein band. This pelleted purple membrane was then spread 0.5 to 1.0 mm thick on a zinc selenide ATR plate and dried under vacuum. This procedure resulted in a preferential stacking of the membrane fragments in the plane of the ATR plate.

RESULTS AND DISCUSSION

Figure 2 shows ATR infrared spectra of purple membranes obtained with E_h and E_v infrared radiation. The assignment of the major peaks is straightforward (1,10) and is indicated in Figure 2. The Amide I peak at 1656 cm^{-1} is at a freqeuncy approximately 3 cm^{-1} higher than that of the regular α-helix band. This could either be due to defects in the α-helices of the protein (10), or due to the relatively high proportion of non-ordered protein (8,9). Because the Amide II band appears at 1540 cm^{-1}, a typical absorption for α-helices, the shift of the Amide I peak most likely results from a combination of inhomogeneously oriented sample, and the presence of random-coil structure within the protein. The frequencies of the Amide I and Amide II bands obtained from ATR spectra are the same as those obtained from normal transmission measurements.

The intensity ratio of the Amide II/Amide I bands, determined from the E_h spectrum, was 1.17 and is the largest ratio so far observed in this type of study (10,19). As previously mentioned, our ATR spectra should be

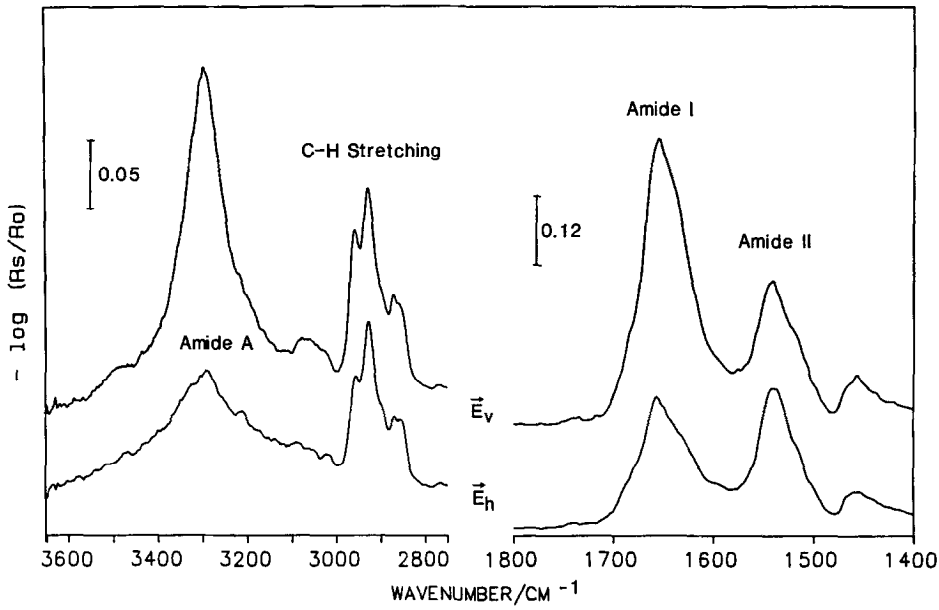

Figure 2. ATR infrared spectra measured at E_h (bottom trace) and E_v (top trace).

equivalent to those of transmission spectra measured at α_0=90° (cf. Figure 1a). The Amide II/Amide I intensity ratio obtained from the E_v spectrum was 0.47, which is in good agreement with that obtained from isotropic studies (17). The dichroic ratio of the three amide bands could then be obtained from the integrated intensities of the individual bands in the E_v and E_h spectra in Figure 2. The experimentally determined values for the dichroic ratios were 2.24 for the Amide I band, 0.98 for the Amide II band and 2.25 for the Amide A band. In an α-helical protein the main component of the transition dipole moment for the Amide I mode is mostly parallel to the helix axis, whereas the transition dipole moment for the Amide II mode is almost perpendicular to the helix axis (5). It is therefore expected that in the build-up films of oriented purple membranes the Amide I band is strong in the E_v spectrum whereas the Amide II band is strong in the E_h spectrum. Furthermore, the transition moment of the Amide I and Amide A bands have a similar tilt angle; hence, the dichroic ratios observed for these two vibrational modes should be close to each other. This is indeed the case in our experiment as shown by the polarized spectra in Figure 2. It can also be seen from Figure 2 that a certain percentage of the membrane lipids are oriented as well. The dichroic

ratio for the methylene C-H stretching mode was calculated at 1.21. Based on a simple model proposed by Zbinden (18), this value gives a tilt angle of approximately 42° for the main transition moment of the axis of the lipids.

In conclusion, the use of oriented multilammelar films in conjunction with ATR spectroscopy provides a fast and simple method to obtain polarized infrared spectra of oriented membrane proteins. This method might also prove useful in the study of protein conformational changes. In the case of the purple membrane, it has been shown that bacteriorhodopsin functions as a transmembrane light-induced proton pump (6,7), although the mechanism by which proton translation is achieved, is not known. The combination of flash kinetic studies (21) and determination of dichroic ratios may provide useful information on conformational changes in bacteriorhodopsin as a function of the photochemical cycle.

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