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Infrared studies of water induced conformational changes in bacteriorhodopsin

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Abstract. Fourier transform infrared difference spectroscopy has been used to study the effect of water on the conformation of bacteriorhodopsin. The infrared spectra as a function of water content show a conformational change at about 0.06 g H_2O/g bacteriorhodopsin. By an interference method the thickness of the sample was measured and shows similar behavior as a function of water content. This study gives insight into the process of water absorption by purple membrane. The observations are in good agreement with those found for other proteins.

Key words: Infrared, bacteriorhodopsin, hydration, conformational changes

Introduction

In order to understand the role of water in the structure and function of proteins many studies have been made on different systems using various methods (for review see Finney 1979; Kuntz and Kauzmann 1974). One of the methods is infrared (IR) spectroscopy which gives information about the conformation of the protein (Careri et al. 1979; Ruege and Hani 1975). Some of these studies were made at different water content and a conformational change at a water content of 0.05–0.09 g H_2O/g protein was found. This conformational change is thought to be related to the appearance of the protein function at high water content (Rupley et al. 1983). We began such a study on bacteriorhodopsin because our kinetic measurements (Váró and Keszthelyi 1985) showed changes in the Arrhenius parameters of the bacteriorhodopsin photocycle in the same range of water content. Bacteriorhodopsin is a membrane protein found in *Halobacterium halobium* which transforms absorbed light to a proton gradient across the membrane. This gradient is built up by translocating protons from inside the cell to the extracellular medium during a photocycle initiated by the absorption of light. This photocycle and the other related events in bacteriorhodopsin are sensitive to the water content (Korenstein and Hess 1979; Lazarev and Terpugov 1980). The kinetic parameters measured at high water content are very close to those measured in suspension. Their change as a function of water content is not continuous (Váró and Keszthelyi 1985).

This suggests that bacteriorhodopsin may undergo a conformational change at a well defined water content. This study shows the existance of the conformational change at 0.06 g H_2O/g bacteriorhodopsin. At the same time it gives a better understanding of water absorption by the purple membrane which contains bacteriorhodopsin.

Materials and methods

Halobacterium halobium strain S-9 was used to prepare purple membrane by the procedure described (Becher and Cassim 1975). Purple membrane was suspended in distilled water at a pH around 6.5. For IR measurements these purple membrane suspensions were dried on an IR transmitting Ge window. One type of sample was dried at room temperature, the other type at a temperature around 37 °C. The dried film was sealed in a sample holder in which the humidity of the atmosphere was varied using the vapours of different saturated salt solutions (Pethig 1979). The sample was incubated for a day at the given humidity before the measurement. From water uptake kinetic measurements it is known that the approximately 10 μm thick bacteriorhodopsin

Abbreviations: IR, infrared; FTIR, Fourier transform IR

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film stabilises its water content in a few hours. The spectra taken after 2 or 3 days incubation were the same. All the incubations and measurements were carried out at room temperature, on dark adapted bacteriorhodopsin to avoid any change in the 13-*cis/all-trans* retinal ratio during the water content change (Korenstein and Hess 1979; Váró and Keszthelyi 1983).

The data collection and analysis were performed using a Nicolet 7199 FTIR spectrometer with a detector sensitive above 800 cm^{-1}. The spectra were taken with a resolution of 2 cm^{-1}. In the process of data analysis the spectrum of the water vapours and baseline shifts of the instrument were eliminated by subtracting the spectrum of the sample holder containing only the vapours of the saturated salt solutions. From the spectra of bacteriorhodopsin obtained at different humidities the spectrum of dried bacteriorhodopsin (Fig. 1) was subtracted. These difference spectra show the changes of bacteriorhodopsin due to the different water content of the sample (Figs. 2–4). To minimise errors introduced by eventual baseline distortions in the amide regions the absorbance changes were read between the maximum and nearest minimum (see Figs. 6 and 7). This method proved to be very effective as demonstrated by the reproducibility of the experiments and is reflected in the errors. On a carefully prepared sample with parallel surfaces it was possible to determine the sample thickness using the interference pattern which appears in the region of the spectrum where there is no absorbance for bacteriorhodopsin and water (3,700 – 5,000 cm^{-1}). The interference appears only when all the active surface of the sample (about 1 cm^2) is highly homogeneous and it has perfectly parallel surfaces. For calculation we used the formula

$$d = \frac{N}{2n\,(f_1 - f_2)}$$

where d is the sample thickness, n the refractive index, f_1 and f_2 the wavenumbers of two maxima or minima, N the number of maxima or minima between f_1 and f_2.

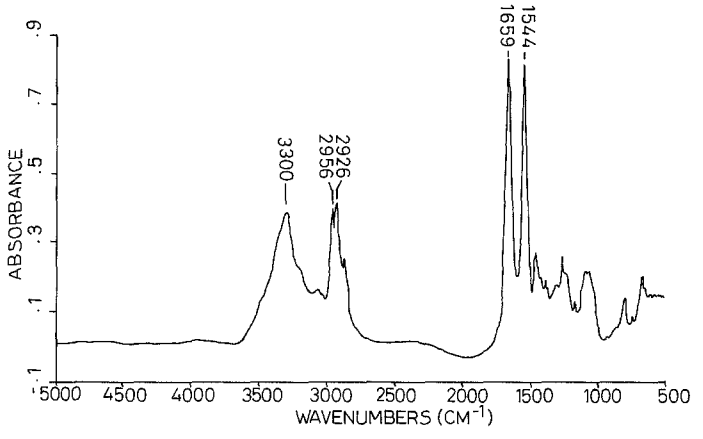

Fig. 1. The spectrum of dried bacteriorhodopsin

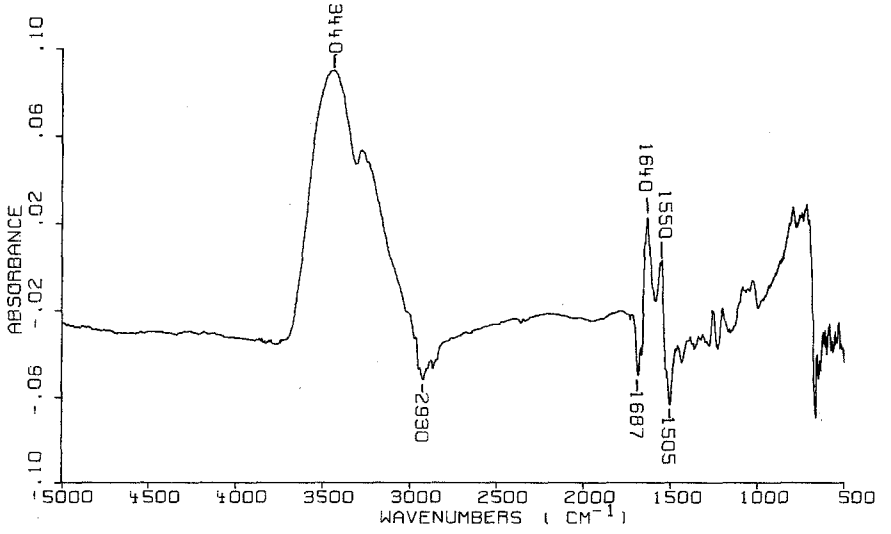

Fig. 2. Difference spectrum of bacteriorhodopsin containing 0.06 g H_2O/g bacteriorhodopsin and dried bacteriorhodopsin

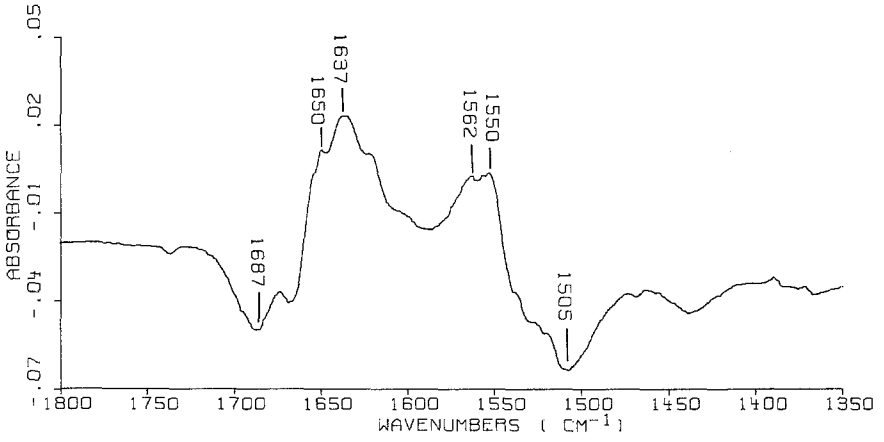

Fig. 3. Difference spectrum of bacteriorhodopsin containing 0.06 g H_2O/g bacteriorhodopsin and dried bacteriorhodopsin

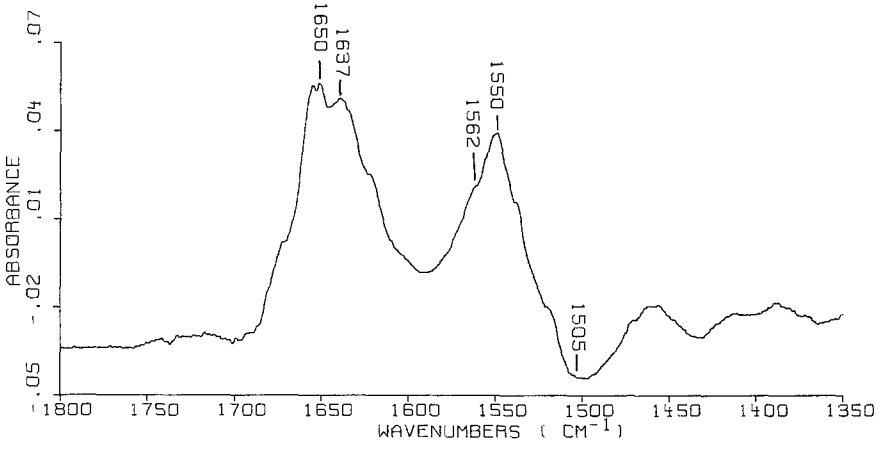

Fig. 4. Difference spectrum of bacteriorhodopsin containing 0.05 g H_2O/g bacteriorhodopsin and dried bacteriorhodopsin

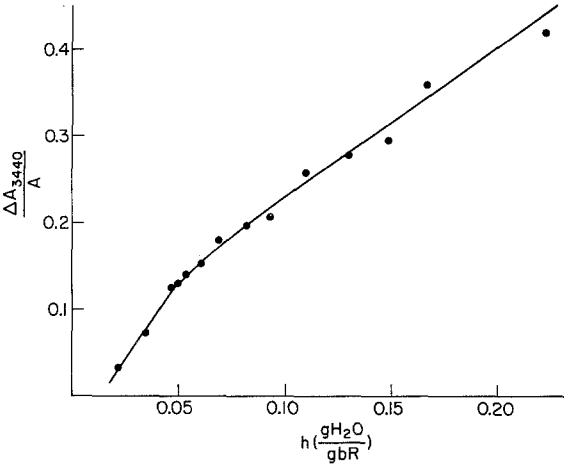

Fig. 5. The change of the absorbance of the 3,440 cm^{-1} band as a function of the water content normalized to the amide I absorbance of the dried film. The errors are less then ± 2%

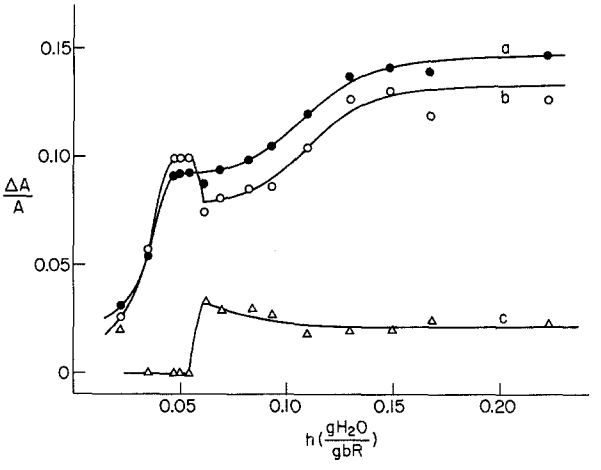

Fig. 6. The changes in the amide I region as a function of water content normalized to the amide I absorbance of the dried film: **a** difference between 1,637 cm^{-1} and 1,687 cm^{-1}, **b** difference between 1,650 cm^{-1} and 1,687 cm^{-1}, **c** difference between the baseline around 1,750 cm^{-1} and 1,687 cm^{-1}. The errors are less then ± 2%

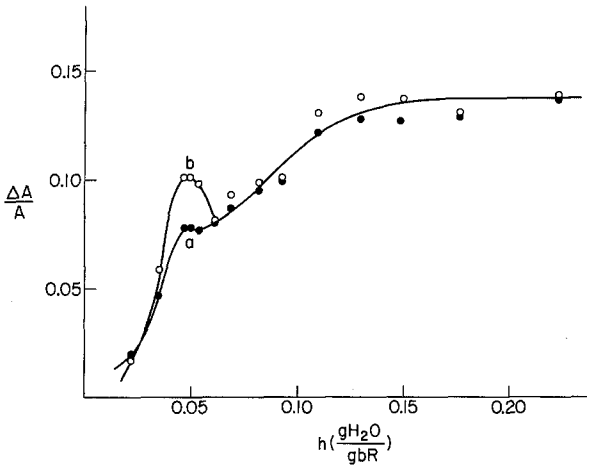

Fig. 7. The changes in amide II region as a function of water content normalized to the amide I absorbance of the dried film: **a** difference between 1,562 cm^{-1} and 1,505 cm^{-1}, **b** difference between 1,550 cm^{-1} and 1,505 cm^{-1}. The errors are less then ± 2%

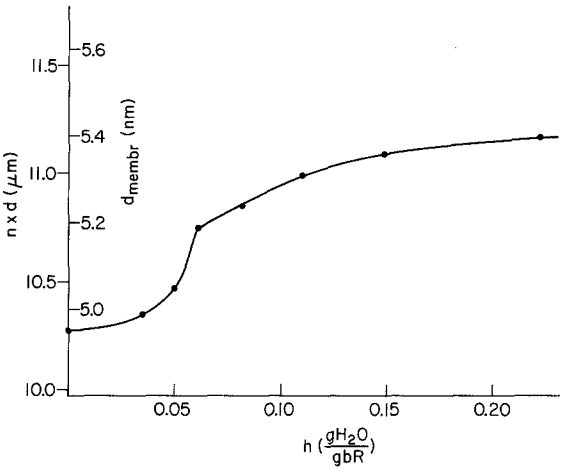

Fig. 8. The change of the sample thickness times refractive index ($n \times d$) and the purple membrane thickness (d_{membr}) as a function of water content. The errors are less then ± 1%

The relation between the humidity and water content of the sample was obtained from Váró and Keszthelyi (1983). All the data in the paper are ploted as a function of water content of the sample in units of g H_2O/g bacteriorhodopsin.

Results

The difference spectra as a function of increasing water content show an increase almost everywhere. Only in three regions is there a decrease. The regions at around 1,687 cm^{-1} and 1,505 cm^{-1} were taken into account in the analysis of the amide I and amide II changes. The absorbance in the third region around 2,930 cm^{-1} is so small that it sometimes disappears in the noise. It cannot be properly analyzed. The three main positive bands at 3,440 cm^{-1}, 1,640 cm^{-1} and 1,500 cm^{-1} (Fig. 2) were studied carefully. The band at 3,440 cm^{-1} had the same shape under all conditions and was very similar to the spectrum of liquid water (Bayly et al. 1963). The absorbance change of this peak as a function of water content of the sample is given in Fig. 5. It shows the continuous increase of bulk water content of the sample.

The shape of the other two bands corresponding to the amide I and II changes of bacteriorhodopsin were sensitive to the preparation conditions (pH and ion concentration of the suspension, temperature of drying). The sample prepared at room temperature from suspension at pH 6.2 had difference spectra in the amide I and II regions of the form shown in Fig. 3 for all water contents of the sample. The maximum was at 1,637 cm^{-1} for amide I and 1,550 cm^{-1} for amide II. The absorbance changes as a function of water content for all the peaks in the amide I and II bands are shown in Figs. 6 and 7 (curve a) respectively. The negative peak at 1,687 cm^{-1} was almost the same size for all water contents. The thickness of this sample was measured as described above (Fig. 8).

The sample prepared from another suspension with pH 6.8 and dried at higher temperature had a slightly different behavior. At low water content of the sample (0–0.06 g H_2O/g bacteriorhodopsin) the two amide bands had the form shown in Fig. 4. They had maxima at 1,650 cm^{-1} and 1,550 cm^{-1} for amide I and II respectively. At higher water content (over 0.06 g H_2O/g bacteriorhodopsin) the shape of the peak changed back to that shown in Fig. 3 with maxima at 1,637 cm^{-1} and 1,550 cm^{-1} as in the previous case. The change of the lines at 1,650 cm^{-1} and 1,550 cm^{-1} as a function of water content is shown as curve b of Figs. 6 and 7, while the change of the lines at 1,637 cm^{-1} and 1,562 cm^{-1} follows curve a of the same figures. For both samples the peaks at 1,637 cm^{-1} and 1,562 cm^{-1} increased continuously with two saturation portions at around 0.05 g H_2O/g bacteriorhodopsin and over 0.15 g H_2O/g bacteriorhodopsin. The peaks at 1,650 cm^{-1} and 1,550 cm^{-1} in the second sample showed a decrease at 0.06 g H_2O/g bacteriorhodopsin. In this case at the same water content a negative band appeared at 1,687 cm^{-1} (Fig. 6 curve c). At high water content the sample behaves as the previous one.

From the interference pattern of the bacteriorhodopsin spectrum between 3,700–5,000 cm^{-1} the thickness and refractive index product ($d \times n$) of the

sample at different water content was directly determined (Fig. 8, external scale). From the absorbance of the sample the number of membrane layers was calculated. Using for the refractive index $n = 1.5$ which is reasonable for lipids and proteins the thickness of the membrane (inner scale of the Fig. 8) was estimated. It is in good agreement with the expected value.

While the peak at 3,440 cm^{-1} increases continuously with a small slope change (Fig. 5), the change of the amide I and II bands showed saturation at around 0.05 g H_2O/g bacteriorhodopsin (Figs. 6, 7) and the membrane thickness increased more abruptly in this region of sample water content (Fig. 8).

Discussion

From absolute IR spectra it is difficult to draw correct conclusions about the nature of the changes occurring in the spectra. Ruegg and Hani (1975) observed a change in the 3,300 cm^{-1} region as a function of hydration of casein, but they did not estimate the real location and shape of this change. By representing the area increase of the absorbance as a function of the relative humidity they got a curve similar to the water absorption isotherm of the protein (Pethig 1979) showing that the change is proportional to the absorb water. Careri et al. (1979) used IR difference spectra to study the hydration of lysozyme with D_2O and concluded that the band at 2,380 cm^{-1} can be caused by the accumulation of polymeric D_2O in clusters. In our case, the location of the peak (3,440 cm^{-1}), the shape of the band in the difference spectrum (Fig. 2), and its increase as a function of water content (Fig. 5) all support the conclusion that it is due to an accumulation of water with spectroscopic properties similar to that of liquid water. By using the absorption coefficient value of water (Bayly et al. 1963) it was possible to estimate that only a few percent of the absorbed water can cause this peak. This means that a small part of the absorbed water does not bind directly to bacteriorhodopsin or lipids but forms liquid water clusters. The amide A and B changes lying in the same region only cause small distortion of the water band.

The changes in the amide I and II regions are more complex. These bands cannot be attributed to the liquid water spectrum because water has only a single small band at 1,645 cm^{-1} (Bayly et al. 1963). If the water spectrum multiplied by a constant were subtracted from the bacteriorhodopsin difference spectrum so that the band at 3,440 cm^{-1} disappeared, the amide I band was slightly shifted down without a big change in its form. Over the difference between the maxima and minima used in calculations had little effect. The slope of the curves in Figs. 3 and 4 calculated from this data remained unchanged. For this reason in further calculations we used the original difference spectra which contained less noise. The shape of the amide I and II bands is very sensitive to bacteriorhodopsin conformation because it contains the vibrations of the atoms from the protein backbone. These conformations should depend on the conditions of preparation (pH, ion concentration of the purple membrane suspension, temperature of drying) and the conditions of measurement (temperature and water content) of the sample.

By changing the water content of the sample the peaks inside the amide bands do not shift, only their relative amplitudes change. The appearance of a hydrogen bond between the water and protein does not introduce new types of vibrations, it only changes their relative probability. The saturation effect of the absorption change at around 0.05 g H_2O/g bacteriorhodopsin can be explained by the fact that the highest affinity binding sites of purple membrane are on the protein. At low water content the H_2O molecule binds preferentially to these sites on the protein and they are saturated. After this, the water binds to the polar groups of the lipids and this has no strong effect on the bacteriorhodopsin spectrum. The above explanation is supported by the observation that the thickness of the membrane increases more rapidly in this region, until the membrane thickness change reaches the value of 0.25 nm at around 0.06 g H_2O/g bacteriorhodopsin, which is roughly the diameter of a water molecule. From other measurements (Váró and Keszthelyi 1983) it is known that at this water content the first continuous water layer on the membrane appears. At higher water content the amide I and II absorbance changes reappear, with a smaller slope. This shows that a change in the conformation of bacteriorhodopsin occurred and additional water molecules can bind. This conformational change is more prononced in the second sample as evidenced by the appearance of the negative peak and by a decrease in the intensity of some peaks. Similar changes were observed in lysozyme, α-lactalbumin (Careri et al. 1979; Poole and Finney 1984) and other proteins in the range 0.05 – 0.1 g H_2O/g protein. Some theories explain this behavior of IR spectra as local effects in the protein (proton redistribution, vibrational perturbations). However, such theories cannot explain the thickness change and the results of the kinetic measurements.

The Arrhenius parameters (activation enthalpy and frequency factor) of the photocycle of bacteriorhodopsin measured as a function of water content

(Váró and Keszthelyi 1985) show a sudden change around 0.06 g H_2O/g bacteriorhodopsin. This change appeared in optical and charge displacement measurements at every measurable step of the photocycle, showing that not only a small part of the bacteriorhodopsin but the whole protein underwent a change. These Arrhenius parameter changes together with the IR data can be explained only by considering conformational changes in the whole of bacteriorhodopsin.

The hydration study of purple membrane using IR spectroscopy has demonstrated the existence of a conformational change of bacteriorhodopsin at a well defined water content which was suspected earlier from the changes occurring in the potential barriers of the transitions during the photocycle. Conformational changes have also been observed in other proteins in the same range of water content, showing that this phenomenon is more general. The water not only binds to the protein but it is built into the structure of the protein and plays an essential role in its function.

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