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A New Method for D_2O/H_2O Exchange in Infrared Spectroscopy of Proteins

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Abstract. In this paper, we describe a new method to obtain D_2O/D_2O exchange in photosynthetic reaction centres from *Rhodobacter sphaeroides*. The method is characterized by: (i) a very high efficiency of the isotopic replacement; (ii) an extremely low amount of D_2O needed; (iii) the short time required for dehydration and D_2O rehydration; (iv) the possibility of controlling concomitantly the hydration state of the sample. The proposed method can be applied to other proteins.

Keywords: D_2O/D_2O exchange, photosynthetic reaction center, FTIR spectroscopy, isopiestic method, protein hydration

1. Introduction

Reaction-induced Fourier transform infrared difference spectroscopy (FTIR-DS) in the mid-IR region (4000–800 cm^{-1}) is an important tool for biochemical and biophysical studies of protein processes [1]. The potentiality of FTIR-DS is fully exploited when the structure of the protein is known and at least some of the bands in the difference spectrum have been assigned to a vibration of a given chemical moiety of a specific amino acid or cofactor inside the protein. A series of strategies exists for correct assignment [1]. Among them, the comparison of FTIR difference spectra recorded in H_2O, D_2O is often the first step. In fact, H_2O replacement by D_2O entails exchange of accessible N–, O–, S– bound protons leading to band shifts in the spectrum. The main drawback arises from the incomplete exchange (even for water-accessible residues), leading to "mixed" D_2O/H_2O spectra.

The photosynthetic reaction center (RC) from *Rhodobacter (Rb.) sphaeroides* represents a model system in bioenergetics, especially suited to investigate electron transfer (ET), proton transfer, and quinone redox chemistry in proteins [2]. It is also an excellent system to study matrix effects on the

dynamics of membrane proteins and ET processes [3]. The structure of the *Rb. sphaeroides* RC is known at atomic resolution and its photochemistry has been characterized in detail [4, 5].

FTIR-DS studies on *Rb. sphaeroides* RC have been carried out during more than 20 years (see [6] for a recent review), leading to the marker bands for cofactors, amino acid side chains and internal water molecules [6, 7, and references therein]. The RC has also been studied by time-resolved FTIR-DS ([8–12] and references therein) and used as a "case study" to develop and test new data analysis techniques ([12, 13] and references therein). Despite this large amount of data, several issues are still debated (see e.g., [10, 11, 14]).

In a recent work [15] it has been shown that the hydration state of the RC controls the protein dynamics associated to ET reactions. The hydration state has been controlled accurately by using an isopiestic method, which consists in equilibrating a dehydrated RC film in the presence of saturated salt solutions providing definite values of the relative humidity, *r* [15]. In the present work we show that the isopiestic method mentioned above can be used to obtain a very high efficiency of H_2O/D_2O exchange for *Rb. sphaeroides* RCs. The method can be extended to other proteins.

2. Materials and Methods

RCs were extracted from *Rb. sphaeroides* strain 2.4.1 using lauryldimethylamine N-oxide (LDAO) as detergent and purified following the procedure described in [16]. FTIR spectra were recorded on a Bruker IFS 88 spectrometer. A Globar source and a DTGS detector were used. The intactness of the RC was checked in the sample compartment by recording a FT-UV-Vis spectrum in the 15000–10000 cm^{-1} range using as a detector a silicon photodiode. Temperature was set to 281 K by a N_2 cryostat (Oxford Instrument).

RC films were prepared on CaF_2 window using 40–60 μL drops of a 60 μM RC solution (10 mM TRIS HCl, pH 8.0, 0.025% LDAO, and 10 mM o-phenanthroline). A small compartment (volume $\sim$ 1 mL) was obtained by inserting the CaF_2 window carrying the film and a second one, separated by an O-ring, in a clipping sample holder. The relative humidity r within the compartment containing the RC film was controlled by a few μL drops of saturated NaCl or LiCl solutions to achieve at 281 K values of r equal to 76% and 11%, respectively [15].

3. Results and Discussion

Spectra recorded at two hydration levels ($r = 11\%$ and 76%) are shown in Figure 1(a). The amide A (at $\sim$3295 cm^{-1}), the amide I (at $\sim$1655 cm^{-1}), and the amide II (at $\sim$1550 cm^{-1}) bands are easily identified, in agreement with spectra obtained in air-dried RCs reconstituted in phospholipid vesicles [17]. The peaks around 2900 cm^{-1} are attributed to the various CH_2 stretching modes [17]. The amide A band overlaps largely with the OH stretching band of water. As a consequence the large band at $\sim$ 3300 cm^{-1} is strongly reduced when the sample is dehydrated by equilibration at $r = 11\%$ as compared to $r = 76\%$. The dehydration of the sample can be better evaluated from the $(\nu_2 + \nu_3)$ combination band of water, centred at 5150 cm^{-1}, which is shown enlarged in Figure 1(b). The area below this band has been shown to be proportional to the water content, independently of the H bonding organization [15, 18]. The peak at 4850 cm^{-1}, on the lower wavenumber side of the water combination band, is

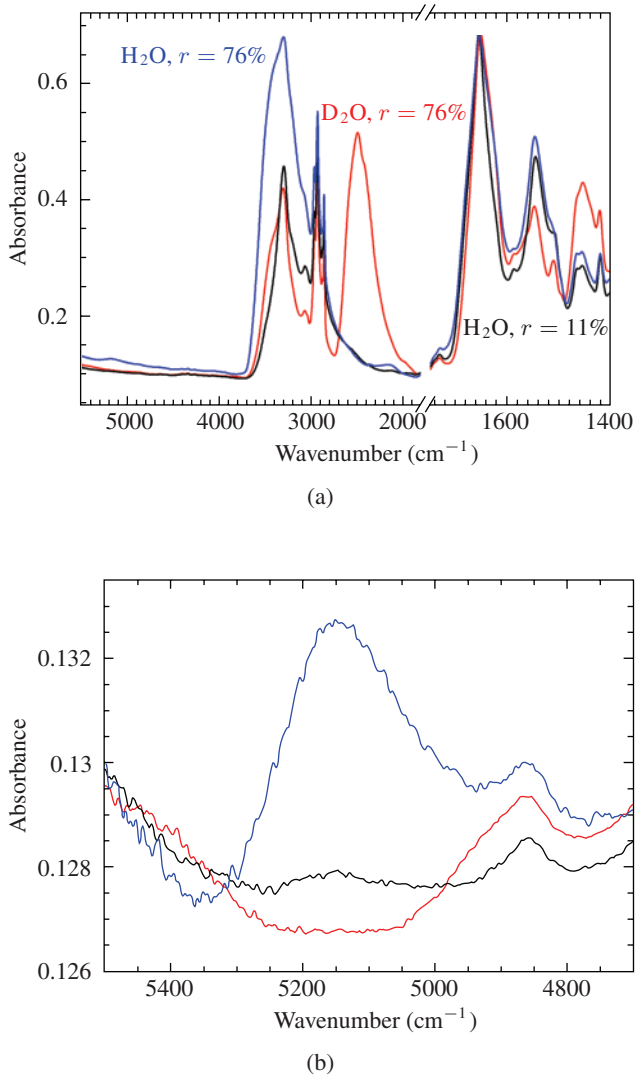

Figure 1: (a) FTIR spectra recorded in a RC film equilibrated at $r = 76\%$ (blue), dehydrated at $r = 11\%$ (black), and rehydrated at $r = 76\%$ in the presence of D_2O (red). (b) Enlargement of the 5500–4700 cm^{-1} spectral region, showing the $(\nu_2 + \nu_3)$ combination band of water at 5150 cm^{-1}.

attributed to a combination of the NH stretching frequency at 3280 cm^{-1} and the peptide frequency at 1550 cm^{-1} [15] and is clearly resolved at $r = 11\%$. When the water combination band is corrected for this contribution by subtracting a background [15], it can be estimated that at $r = 11\%$ less than 20% of the water content of the sample detected at $r = 76\%$ is retained. The association band of water visible at ~2100 cm^{-1} in the spectrum at $r = 76\%$ is also strongly reduced at $r = 11\%$ (Figure 1(a)).

When the dehydrated sample ($r = 11\%$) is rehydrated in the presence of NaCl in D_2O ($r = 76\%$) the spectrum exhibits strong alterations, diagnostic of an efficient deuteration. In Figure 1(a),

all the spectra have been normalized to the amplitude of the amide I band, which is less affected by D_2O replacement as compared to the amide II band. Although the region of the amide I includes some contribution from the water bending mode, normalization to the amplitude of the amide I band allows a better comparison between the spectra recorded in the presence of H_2O and D_2O at $r = 76\%$.

Figure 1(a) shows that upon rehydration with D_2O the band centred at 3300 cm^{-1} is almost halved in amplitude, as compared to the one recorded in H_2O at $r = 76\%$, consistently with a reduction of the water OH stretching contribution and the partial deuteration of the NH group of the amide A band. As expected, upon rehydration with D_2O, the OH stretching band of water is blue-shifted by about 800 cm^{-1} [19], resulting in a strong absorption band centred at 2500 cm^{-1}. In the hydrated samples at $r = 76\%$, both in H_2O and in D_2O, the amide A band exhibits a shoulder on the high wavenumber side. This shoulder, which essentially disappears in the dehydrated sample at $r = 11\%$, is attributed to the water OH stretching mode and to OH and NH groups of the protein. We propose that the disappearance of the shoulder in the dehydrated sample reflects not only water depletion but possibly also a shift to lower wavenumbers of the protein OH and NH groups, presumably due to a strengthening of the H-bonds. This interpretation would explain why the shoulder becomes again detectable upon rehydration with D_2O, although much reduced in amplitude and width. Since in D_2O the extent of deuteration is very high and H_2O is essentially absent (see below), the contribution of protein OH and NH groups is likely to be responsible for the band shoulder in the deuterated sample.

It is known that N-deuteration converts the amide II mode to largely a CN stretching vibration at 1490–1460 cm^{-1}, named amide II' band [1]. In line with this change, we observe a weakening of the band at 1550 cm^{-1} (amide II) and a large increase of the absorbance between 1420 and 1500 cm^{-1}, giving rise to a peak at 1460 cm^{-1}, which can be attributed to the appearance of the amide II' band. Interestingly a peak at 1550 cm^{-1} also appears, which corresponds to the wavenumber expected for the water association band upon D_2O replacement [19].

The extent of D_2O replacement can be evaluated from Figure 1(b). Following rehydration with D_2O, the spectrum between 5500 and 4700 cm^{-1} still exhibits the NH band at 4850 cm^{-1}, while the $(\nu_2 + \nu_3)$ combination band of water essentially disappears. We infer that the efficiency of D_2O replacement achieved upon rehydration in the presence of D_2O is larger than 95%. Since rehydration with D_2O occurs in samples which still retain some residual H_2O (see the spectrum at $r = 11\%$ in Figure 1(b)), it appears that equilibration with D_2O vapour not only leads to rehydration with deuterated water, but also results in the exchange of the residual H_2O with D_2O.

The isopiestic method for isotopic replacement discussed above offers significant assets: (i) the efficiency of the isotopic replacement is very high; (ii) the amount of D_2O needed is extremely low; (iii) dehydration and D_2O rehydration require a relatively short time (less than 6 hours as compared to, for example, more than 12 for dialysis); (iv) the hydration state of the sample can be concomitantly controlled through the $(\nu_2 + \nu_3)$ combination band of water [15].

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