HYDRATION OF RETINAL AND THE NATURE OF METARHODOPSIN II

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1. Introduction

Accumulated evidence suggests that in the major vertebrate visual pigment, rhodopsin, the 11-*cis* retinal chromophore is covalently linked to a lysine residue of the protein via a protonated Schiff base (imine) linkage. After exposure to light, rhodopsin passes through a series of spectroscopically distinct intermediate steps culminating in the release of the free retinal aldehyde, as the all-*trans* isomer, from the apoprotein opsin [1]. The precise stage at which hydrolysis of the Schiff base occurs is not known but, using a variety of circumstantial evidence, we earlier proposed that this most likely takes place between the intermediates known as metarhodopsins I and II, implying that metarhodopsin II consists of the aldehyde form of retinal non-covalently bound to the active site of opsin [2]. However, a subsequent resonance Raman study of metarhodopsin II failed to show the anticipated aldehydic C=O stretching band at this stage [3], leading the authors to support the interpretation of metarhodopsin II as an unprotonated retinal–opsin Schiff base complex [4], despite the absence of a C=N band in the spectrum.

Interpretations of Raman spectra of visual pigments are commonly made by comparison with the spectra of retinals and model compounds dissolved in organic solvents, in which they are readily soluble. It occurred to us that this might, on occasion, be misleading. For instance, although rhodopsin is a membrane-bound protein, it is known that at the metarhodopsin stage of photolysis the chromophore is readily accessible to small water-soluble reagents like hydroxylamine [5,6] and borohydride [7,8] and, by extension, to water from the surrounding medium. Bearing in mind the known effects of hydrogen bonding on IR spectra [9] and the tendency for some aldehydes to form covalent hydrates [10], we have re-examined the vibrational spectra of retinal in the presence of water, and show that solvent markedly affects both Raman and IR bands in the important C=O and C=N stretch region.

2. Materials and methods

All-*trans* retinal from Fluorochem. and Sigma was used without further purification, since HPLC and proton NMR revealed no impurities other than traces of geometrical isomers. Acetonitrile (Hopkin and Williams) was stored over 4 A molecular sieves, D_2O (99.8%) was from Fluorochem., CD_3CN from CEA (France) and H_2O was glass-distilled. Emulphogene (Mulgofen) BC-720 was a gift of GAF (GB).

N-Retinylidene-*n*-butylamine was prepared by treatment of retinal with excess *n*-butylamine (redistilled) in acetonitrile over 4A sieves, and was protonated when required by addition of small volumes of conc. HCl. Retinal and its protonated Schiff base were found to be moderately soluble in acetonitrile/water mixtures, up to at least 30 mM for 1:1 (v/v) mixtures. The unprotonated Schiff base was less soluble. All solutions were freshly prepared for each experiment, working in dim light under an argon atmosphere.

Raman spectra were obtained on a Spex Ramalog IV instrument with spinning cell and back-scattering optics, excited at 488 nm (argon laser). No degradation of samples, other than some photoisomerization, was detected by UV/vis spectroscopy during the experiments. Samples were ~3 mM.

Infrared absorbance spectra in the 1500–1800 cm^{-1} range were recorded on a Perkin-Elmer 580 double-beam IR spectrophotometer with calcium

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fluoride cells, 0.05 mm pathlength. Retinal and Schiff base samples were ~30 mM in acetonitrile/D_2O mixtures.

100 MHz proton NMR spectra in CD_3CN/D_2O mixtures were measured on a Varian XL-100 instrument, operated in the pulsed Fourier transform mode.

3. Results and discussion

Both IR and Raman studies show that the position and intensity of the C=O stretch vibration of retinal are influenced by the presence of water (fig.1,2). The

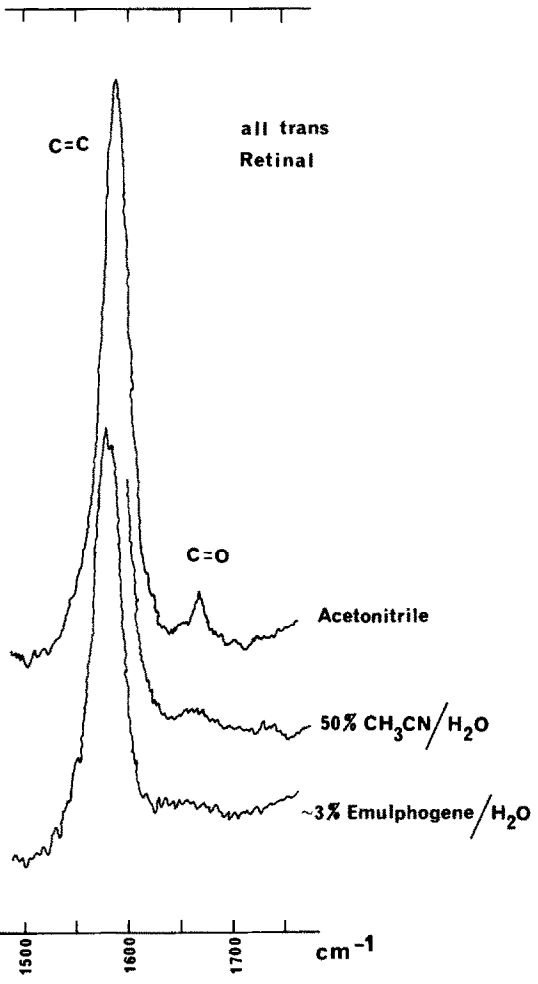

Fig.1. Representative Raman spectra of all-*trans* retinal in acetonitrile and aqueous mixtures, in the 1500–1750 cm^{-1} range. Note the disappearance of the small 1660 cm^{-1} band assigned to the aldehyde C=O stretch, in the presence of water.

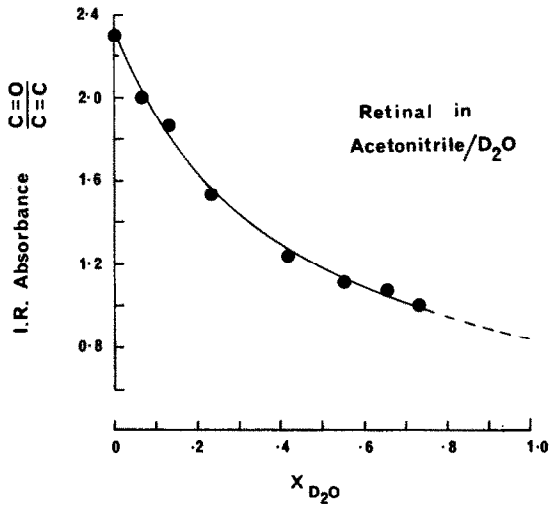

Fig.2. The variation of IR absorbance peak height of the 1660 cm^{-1} carbonyl band of all-*trans* retinal (as the ratio with the 1580 cm^{-1} C=C band) with mole fraction (X_{D_2O}) of deuterium oxide in acetonitrile/D_2O mixtures.

Raman spectrum of retinal in anhydrous acetonitrile was found to be identical to published spectra [11] showing, in particular, the strong C=C stretch at 1580 cm^{-1} and a well-resolved C=O stretch at 1659 cm^{-1} (fig.1). On addition of water, however, the C=O peak progressively shifts to lower frequencies and broadens until, at high [H_2O], it is scarcely perceptible. The frequency shift amounts to ~10 cm^{-1} in 50% (v/v) water, and is paralleled by a similar shift to lower frequencies in the C=C peak, which is otherwise relatively unaffected by water. No major changes are seen in other regions of the spectrum (down to 900 cm^{-1}). Similar disappearance of the carbonyl peak is seen in detergent solutions (fig.1), and in acetonitrile/D_2O and ethanol/H_2O mixtures.

The C=O band of retinal is much more prominent in IR absorbance than in Raman scattering, because of the different selection rules involved; more than double the height of the C=C absorbance in non-aqueous solution. Yet, here again, addition of water, as D_2O, produces a marked decrease in the 1660 cm^{-1} carbonyl absorbance (fig.2) without apparent effect on the C=C band at 1580 cm^{-1}. Again, as in the Raman spectra, this decrease in peak height is accompanied by broadening and a shift in position to about 1650 cm^{-1} for 1:1 acetonitrile/D_2O mixtures (i.e., $X_{D_2O} = 0.74$).

Water appears to have little effect on vibrational

spectra of the protonated Schiff base, *N*-retinylidene-*n*-butamylamine–HCl, the Raman spectra showing a well-resolved $C{=}N^+$ band at ~1657 cm^{-1} even at high $[H_2O]$. The C=N stretch of the unprotonated model Schiff base is poorly resolved at ~1622 cm^{-1} in our Raman spectra in pure acetonitrile, and is completely obliterated on addition of water because of strong fluorescence observed with these samples. This band is much clearer in IR absorbance spectra, which show a small decrease in intensity on addition of D_2O; ~30% reduction at maximum water content.

Hydrogen bonding seems the most likely of several possible causes of the specific effect of water on the retinal carbonyl vibration. The variations in IR absorbance with mole fraction of water (fig.2) are consistent with the formation of a 1:1 retinal–water complex, and the data fit quantitatively with an association constant:

$$K_a = \frac{[\text{Ret} \ldots D_2O]}{[\text{Ret}] \cdot X_{D_2O}} = 2.0\ (\pm 0.2)$$

Whilst this is in the same range as known K_a values for covalent hydrates (*gem*-diols with simple aldehydes [10], no evidence for such compounds was seen in proton NMR spectra and, indeed, they would be unusual in a molecule as devoid of electronegative groups as retinal. Aggregation of the retinal in aqueous solutions could affect the vibrational spectra, though it is unlikely that only the carbonyl band would be so affected. The similarity of the effects in both IR and Raman experiments, at a 10-fold difference in concentration, and the much smaller effect with the less-soluble unprotonated Schiff base, argue against this explanation. On the other hand, hydrogen bonding to the polar carbonyl group is to be expected. Early studies of the Raman spectrum of retinal [12] demonstrated the dependence of the C=O stretch on solvent, and a marked broadening of this band in ethanol was attributed to hydrogen bonding. Such phenomena are well known from IR studies of H-bonded systems [9], and have been exemplified in a study of the carbonyl band of simple amides in H_2O/D_2O mixtures [13].

Our observations cast doubt on the ability of resonance Raman spectroscopy to determine uniquely the chemical state of the retinal chromophore at intermediate stages in the photolysis of rhodopsin. The absence of an observable carbonyl vibration in metarhodopsin II [3] may simply reflect environmental effects and does not, by itself, rule out the existence of retinal as the aldehyde at this stage.

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