Metal cation assisted protonation of retinylidene Schiff base in aqueous acetonitrile and in reverse micelles

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Metal cations (Ca^{2+}, Mg^{2+}, Zn^{2+}) cause water to protonate all-*trans*-*N*-retinylidenebutylamine when solubilised in (i) acetonitrile containing aqueous $MgBr_2$ and (ii) CaA_2, MgA_2 and ZnA_2 [where A = bis(2-ethylhexyl) sulfosuccinate anion] solubilised water pools in heptane. The results are discussed in terms of the role of metal cations and water in wavelength regulation in the opsin family of proteins.

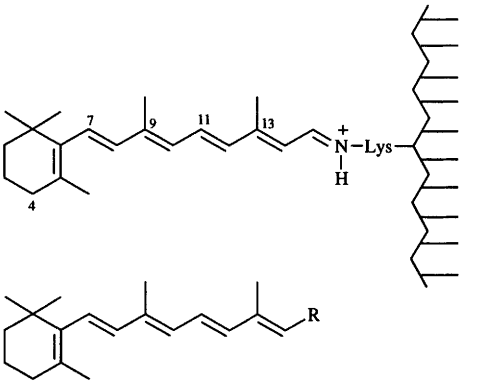
Introduction

The opsin family of proteins[1] are a unique class of photoreceptor pigments in which retinal (vitamin A aldehyde) is covalently attached as a chromophore to the ε-amino group of a lysine residue *via* a Schiff base linkage (Fig. 1). Visual rhodopsin[2] and halobacterial bacteriorhodopsin[3] are the most studied photopigments of the opsin family. These proteins are membrane-bound and show unique structural and functional properties.[4] One of the properties which has attracted much attention in recent years is the varying extent of absorption spectral red shifts found in different pigments.[5,6] Closely associated to this is protonation and stability of the retinal Schiff base chromophore.[7–10] A complete understanding of the protonation and stability of the Schiff base chromophore, the control of acidity constants of the protonated Schiff base during light induced changes and an overall molecular mechanism for wavelength regulation in these proteins have been subjects of a great deal of interest in recent years.

It has recently been found that the retinal pocket in bacteriorhodopsin has specific metal cation (Ca^{2+} or Mg^{2+}) binding sites.[11,12] The near atomic resolution of the tertiary structure of bacteriorhodopsin has shown the presence of an inhomogeneous electric field within the retinal pocket.[13] It is also believed that water molecules and hydrogen bonding play important roles in controlling spectral, structural and light induced functions of these proteins.[14–23]

The present investigations have been undertaken to evaluate the role of metal cations and water on the protonation and spectral shift of retinal Schiff base. For this purpose retinal Schiff base has been incorporated into AOT [sodium bis(2-ethylhexyl) sulfosuccinate] based reverse micelles. As is well known, reverse micelles containing a surfactant solubilised water pool mimic the hydrophilic pockets of enzymes, whilst the alkyl chains of surfactants provide a hydrophobic domain.[24] Recently it has been demonstrated that reverse micelles can, to some extent, mimic the microenvironment in which the retinal Schiff base resides in these proteins.[25,26] It has also been shown that water bonding with anions and cations in organic solvents increases its acidity through a cooperative effect.[27–29] It has been further demonstrated that organic bases like diazabicycloundecene in lithium and magnesium salt solution in acetonitrile may be protonated by water.[30]

We report here the protonation and absorption spectral features of all-*trans*-*N*-retinylidenebutylamine **1** in aqueous acetonitrile containing Mg^{2+} cations and in reverse micelles prepared from bis(2-ethylhexyl) sulfosuccinate containing divalent cations (Ca^{2+}, Mg^{2+}, Zn^{2+}) as counterions and sodium bis(2-ethylhexyl) sulfosuccinate (AOT) in heptane.



1: R = CH=N–Bu

2: R = CH=$\overset{+}{\underset{H}{N}}$–Bu

Fig. 1 The chromophore of retinal proteins. Bacteriorhodopsin: all-*trans*/13-*cis*. Rhodopsin: 11-*cis* and the structure of the retinylidene Schiff base **1** and its protonated salt **2**.

Experimental

Materials and methods

AOT and all-*trans*-retinal were obtained from Fluka and Sigma Chemical Co., respectively. Butylamine, magnesium bromide and tetrabutylammonium bromide were from Aldrich Chemical Co. Calcium chloride, magnesium chloride, zinc chloride were from s.d.fine chemicals, Bombay. Acetonitrile (s.d.fine chemicals, Bombay) was dried according to the literature procedure.[31] All-*trans*-retinylidenebutylamine **1** was prepared according to the literature procedure.[32] Final solutions of **1** were made in heptane and acetonitrile separately. All experiments involving **1** were carried out under a nitrogen atmosphere and in dim red light. An Impact 400 FT–IR spectrophotometer (Nicolet, USA) was used to record FT–IR spectra. A CaF_2 cell was used for recording the spectra of aqueous samples. UV–VIS spectra were recorded on Hitachi-U-2000 UV–VIS spectrophotometer. Inductive coupled plasma atomic emission spectroscopic analyses were carried out on ICP-AES, LABTAM-8440, Plasma Lab. Karl-Fischer titrations were carried out in a Systronics (Bombay) Auto KF Titrator model 349.

Preparation of bis(2-ethylhexyl) sulfosuccinates containing divalent cations (Ca^{2+}, Mg^{2+}, Zn^{2+})

Solutions of the corresponding metal chloride ($CaCl_2$, $MgCl_2$ or $ZnCl_2$) and AOT in deionised water were mixed in a beaker with an excess (1.5 equiv.) of the metal chloride. The mixture was stirred for 0.5 h and was allowed to settle. The white

precipitate obtained was washed repeatedly with deionised water to remove excess metal chloride and filtered through Whatman-42 filter paper. The precipitate was dried over P_2O_5 under high vacuum (10^{-5} Torr) for 48 h and then subjected to atomic emission spectroscopic analysis and Karl-Fischer titrations for determining metal and water content, respectively. The inductive coupled plasma atomic emission spectroscopic analyses of the prepared surfactants showed the presence of the respective metal cations (Ca^{2+}, Mg^{2+}, Zn^{2+}) with very low sodium content. AOT is denoted as NaA to distinguish it from calcium, magnesium and zinc salts of bis(2-ethylhexyl) sulfosuccinic acid which are denoted as CaA_2, MgA_2 and ZnA_2, respectively. The values of the water to cation molar ratio obtained from the Karl-Fischer titration were 1.86, 1.52, 2.0 and 1.54 for NaA, CaA_2, ZnA_2 and MgA_2, respectively.

Intercalation of retinal Schiff base 1 in heptane solubilised water pools of AOT and bis(2-ethylhexyl) sulfosuccinate salts of Ca^{2+}, Mg^{2+} and Zn^{2+}

To a surfactant solution in heptane (1×10^{-2} mol dm^{-3}) was introduced a heptane solution of **1** (1.2×10^{-5} mol dm^{-3}). An appropriate amount of deionised and double distilled water required for various ω values ($\omega = [H_2O]/[surfactant]$) was added to the above solution. The contents were vigorously stirred (Teflon) until the solution became optically clear and subsequently used for further studies.

Results and discussion

The FT–IR spectrum of **1** in acetonitrile shows the presence of a band at 1622 cm^{-1} due to C=N stretching vibrations. This band shifts to 1652 cm^{-1} when the IR spectrum of **1** in acetonitrile is recorded in the presence of trifluoroacetic acid or hydrogen bromide, and to 1655 cm^{-1} in the presence of hydrogen chloride. The band at 1652/1655 cm^{-1} is assigned to the C=N stretching of the protonated Schiff base because in the presence of dry acid, the 1622 cm^{-1} band disappeared and 1652/1655 cm^{-1} is the only new band to appear in the 1500–1700 cm^{-1} region. A similar extent of frequency shift is observed for Schiff base **1** in $MgBr_2$–H_2O–CH_3CN solutions (Fig. 2). For Schiff base **1** the observed values of the intensity ratio of C=C and C=N bands are 1.15, 1.60 and 0.6 in CH_3CN–HCl, CH_3CN–Mg^{2+}–H_2O and CH_3CN respectively. An increase in the intensity ratio of C=C and C=N of **1** in acid solutions further supports protonation and confirms the observations made earlier.[15] However, cations of lithium bromide or non-metallic tetrabutylammonium bromide do not cause protonation of **1** even when present in large excess (30 mol excess, Table 1).

The ability of water to protonate the Schiff base in acetonitrile in the presence of Mg^{2+} can be explained in terms of the cooperative effect wherein water forms solvent shared ion-pairs in acetonitrile (Scheme 1).

The protonation behaviour of **1** has also been examined in reverse micelles of bis(2-ethylhexyl) sulfosuccinate salts of Ca^{2+}, Mg^{2+} and Zn^{2+} in heptane. Schiff base **1** (1.24×10^{-5} mol dm^{-3}) when incorporated into reverse micelles of various salts, shows different absorption behaviour (Table 2, Fig. 3). The reverse micellar matrix of NaA does not cause protonation and hydrolysis of **1** under the present experimental conditions. NaA solubilised **1**, with water pool ω = 0–5, showed an absorption maximum at 356 nm (Table 2). Addition of trifluoroacetic acid to NaA intercalated **1** resulted in the appearence of a band at 431 nm due to protonated salt **2**. When Schiff base **1** was intercalated in the CaA_2 system, absorption bands at 357–360 to 419–426 nm, depending on the water pool size (ω = 0–5), were observed. Upon addition of trifluoroacetic acid to **1** in CaA_2, absorption bands at 357–360 and 419–424 nm shifted to 429 nm due to protonation of the Schiff base. The absorbance of the red shifted band (419–426 nm range) of the protonated Schiff base was at a maximum (82%) when the water content was ω = 3.75 and it decreased to *ca.* 46% when the

Table 1 FT–IR stretching vibrations (ν) of C=N of **1**[a] in different media at 25 °C

Medium	$\nu_{C=N}$/cm^{-1}
CH_3CN	1622
CH_3CN–Li^+–H_2O[b]	1622.8
CH_3CN–TBR–H_2O[c]	1622.2
CH_3CN–Mg^{2+}–H_2O[d]	1652
CH_3CN–HBr	1652
CH_3CN–HCl	1655
CH_3CN–TFA	1652

[a] [**1**], 1×10^{-2} mol dm^{-3}. [b] [LiBr], 30×10^{-2} mol dm^{-3}. [c] TBR = tetrabutylammonium bromide; [TBR], 30×10^{-2} mol dm^{-3}. [d] [$MgBr_2$], 1.2×10^{-2} mol dm^{-3}.

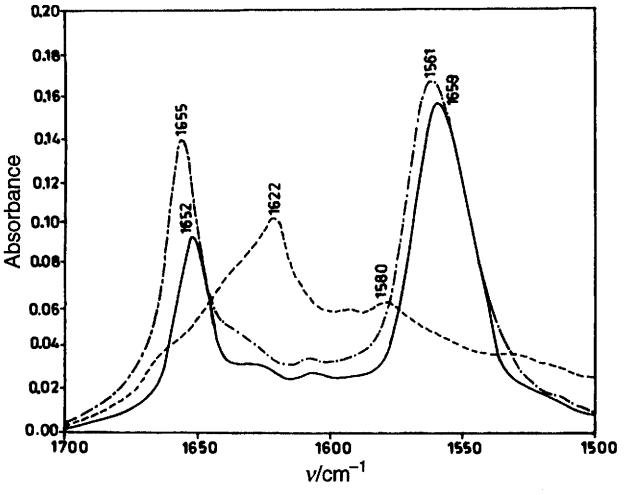
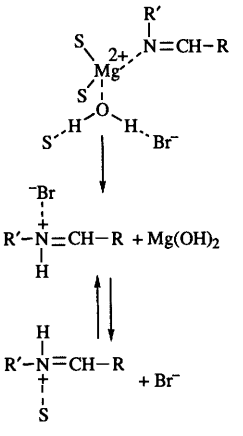


Fig. 2 FT–IR difference spectra of **1** in different media. (**1** + CH_3CN) – CH_3CN, ----; (**1** + CH_3CN + HCl) – (CH_3CN + HCl), —·—·; (**1** + $MgBr_2$ + H_2O + CH_3CN) – ($MgBr_2$ + H_2O + CH_3CN), ——. Concentrations: [**1**], 1.0×10^{-2} mol dm^{-3}, [$MgBr_2$], 1.2×10^{-2} mol dm^{-3}, [H_2O], 20×10^{-2} mol dm^{-3}.



Scheme 1 S = Acetonitrile, R = retinyl, R' = butyl

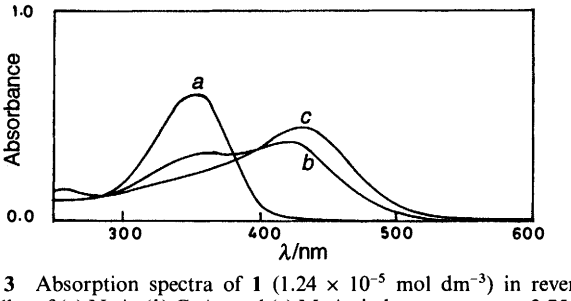


Fig. 3 Absorption spectra of **1** (1.24×10^{-5} mol dm^{-3}) in reverse micelles of (*a*) NaA, (*b*) CaA_2 and (*c*) MgA_2 in heptane at ω = 3.75

Table 2 Absorption maxima of retinal Schiff base **1** (1.24×10^{-5} mol dm^{-3}) in different media at 25 °C; values in the brackets indicate % protonation of **1**

Medium	λ_{max}/nm	Medium	λ_{max}/nm
Heptane	357	Heptane–TFA[a]	432
NaA–Heptane[b]		ZnA_2–Heptane[b]	
$\omega = 0$	356	$\omega = 0$	428
$\omega = 2.5$	356	$\omega = 2.5$	428
$\omega = 3.75$	356	$\omega = 3.75$	429 (97)
$\omega = 5.0$	356	$\omega = 5.0$	430
NaA–Heptane–TFA[b]		ZnA_2–Heptane–TFA[b]	
$\omega = 0$	431	$\omega = 0$	431
CaA_2–Heptane[b]		MgA_2–Heptane[b]	
$\omega = 0$	358 419 (45)	$\omega = 0$	424
$\omega = 2.5$	360 421 (75)	$\omega = 2.5$	361 427 (76)
$\omega = 3.75$	360 423 (82)	$\omega = 3.75$	429
$\omega = 5.0$	357 426 (46)	$\omega = 5.0$	360 428 (80)
CaA_2–Heptane–TFA[b]		MgA_2–Heptane–TFA[b]	
$\omega = 0$	429	$\omega = 0$	431

[a] TFA = trifluoroacetic acid. [b] [NaA], [CaA_2], [MgA_2] and [ZnA_2], 1×10^{-2} mol dm^{-3}.

water pool size was $\omega = 5$. Interestingly, the CaA_2 system is able to partially protonate the Schiff base **1** even when external water was not added. Similar absorption behaviour is observed when **1** is incorporated in the MgA_2 system. However, in MgA_2–heptane systems, while almost full protonation was observed at $\omega = 0$, the protonated Schiff base **1** is unstable, as it immediately starts hydrolysing. But at $\omega = 3.75$, it was found to be relatively stable. In the ZnA_2–heptane system, **1** shows an absorption band in the 428–430 nm range corresponding to the protonated Schiff base **2**. Almost full protonation of **1** occurs in ZnA_2–heptane reverse micelles containing water pools of ω = 0–5. The protonated Schiff base in the ZnA_2 system was found to be relatively more stable as compared with either CaA_2 or MgA_2 systems. Thus, while NaA does not cause protonation of Schiff base **1**, the ZnA_2 matrix is capable of protonating the Schiff base **1** and also providing a microenvironment in which Schiff base **1** remains reasonably stable. It is observed that the hydrolysis of the Schiff base occurs even at $\omega = 0$, when water has not been added to the surfactant solution. Hydrolysis of the Schiff base at $\omega = 0$ is due to resident water present in the surfactants. An estimation of resident water in the surfactants used in the present studies has been made by Karl-Fischer titration and water/cation molar ratios are found to be NaA, 1.86; CaA_2, 1.52; ZnA_2, 2.0; MgA_2, 1.54. Similar water–cation molar ratios have been found by NMR studies[33] (NaA, 1; CaA_2, ZnA_2 and MgA_2, 2). FT–IR studies have also shown that the vacuum-dried sample of AOT contains resident water molecules.[32] The presence of such residual water molecules in the surfactants causes hydrolysis of the Schiff base even if external water is not added to the surfactant solution in heptane.

This indicates that in the reverse micelle there is a delicate balance of charge between the Schiff base, water molecules and the metal cations which regulate the Schiff base protonation and stability. The varying percentage of protonation of **1** in various types of reverse micellar systems is due to the different degree of cooperativity effect and the influences exerted by metal cations. The degree of cooperative effect depends on the charge/radius values[34] of the metal cations which follows the order Na^+, 1.03 < Li^+, 1.47 < Ca^{2+}, 2.02 < Zn^{2+}, 2.70 < Mg^{2+}, 3.03. Magnesium with the highest value makes

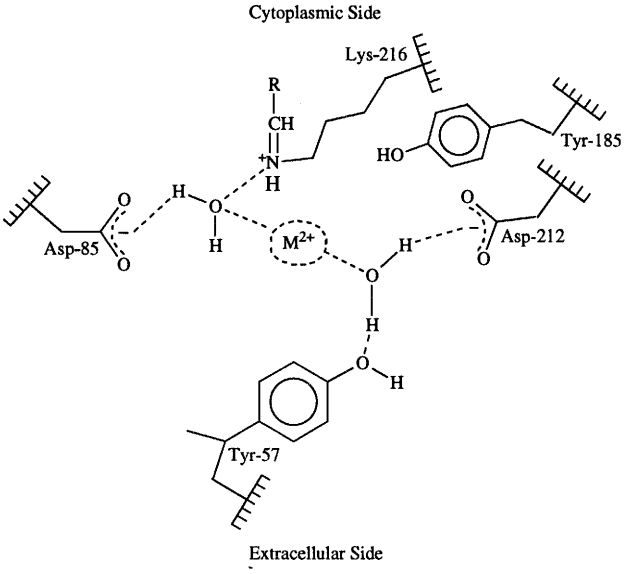


Fig. 4 Schematic model of the bacteriorhodopsin active site indicating the involvement of metal cations and water molecules, M = divalent cations, R = retinyl group

water acidic to a greater extent than the others which is reflected in the protonation and immediate hydrolysis of the Schiff base. However for sodium, with the lowest value, the cooperative effect is the lowest and hence no protonation occurs.

The observed protonation of the retinal Schiff base chromophore in the presence of metal cations bear considerable significance in view of the recent findings that the retinal pocket in bacteriorhodopsin has specific metal cation (Ca^{2+} or Mg^{2+}) binding sites.[11,12] As is well known,[13] in the retinal pocket of bacteriorhodopsin there are negatively charged Asp-85 and Asp-212, positively charged Arg-82 and the protonated Schiff base, strongly hydrogen bonded Tyr-185, unprotonated Asp-115 and available space for a few water molecules. The presence of all these charged species, including metal cations in not too large a volume, is expected to give large field effects. A schematic model of the bacteriorhodopsin active site indicating the involvement of water and the metal cation is depicted in Fig. 4. The protonation states of Asp-85, Asp-212 have already been derived from FT–IR, chemical modification, site-directed mutagenesis and many other studies.[35–37] The positioning of the water molecules is as suggested recently by FT–IR studies.[35] The metal ion is positioned so that it can coordinate with water molecules which are in turn hydrogen bonded with Tyr-57 and Asp-212 or with Asp-85 and the Schiff base. These interactions are sensitive to small light-induced protein conformational changes that occur during the photocycle. Thus, it can be concluded that metal cations working cooperatively with other ionic species in the retinal pocket render water molecules acidic, which protonates, stabilises and controls the acidity constant of the Schiff base chromophore, and contributes overall in the wavelength regulation of the opsin family of proteins.

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