Resonance Raman spectroscopy of sensory rhodopsin II from *Natronobacterium pharaonis*

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Abstract Sensory rhodopsin II (pSRII), the photophobic receptor from *Natronobacterium pharaonis*, has been studied by time-resolved resonance Raman (RR) spectroscopy using the rotating cell technique. Upon excitation with low laser power, the RR spectra largely reflect the parent state $pSRII_{500}$ whereas an increase of the laser power leads to a substantial accumulation of long-lived intermediates contributing to the RR spectra. All RR spectra could consistently be analysed in terms of four component spectra which were assigned to the parent state $pSRII_{500}$ and the long-lived intermediates M_{400}, N_{485} and O_{535} based on the correlation between the C=C stretching frequency and the absorption maximum. The parent state and the intermediates N_{485} and O_{535} exhibit a protonated Schiff base. The C=N stretching frequencies and the H/D isotopic shifts indicate strong hydrogen bonding interactions of the Schiff base in $pSRII_{500}$ and O_{535} whereas these interactions are most likely very weak in N_{485}.

Key words: Archaeal phototaxis; Deuterium shift; Photocycle; Schiff base

1. Introduction

Sensory rhodopsin II from *Natronobacterium pharaonis* (pSRII) functions as a photophobic receptor which enables the bacteria to avoid damaging photo-oxidative conditions. This seven helical membrane protein belongs to the family of bacterial rhodopsins which has been first identified in *Halobacterium salinarum*. Representatives of this protein family include the light activated ion pumps bacteriorhodopsin (BR) and halorhodopsin (HR) as well as the two phototaxis receptors sensory rhodopsin I (SRI) and sensory rhodopsin II (SRII). SRII and its natronobacterial counterpart pSRII reveal extensive similarities in structure and function [1–3].

Like other bacterial rhodopsins, pSRII possesses an all-*trans* retinal bound to a lysine (Lys-206 on helix G) via a protonated Schiff base [2]. Upon light excitation, an all-*trans*→13-*cis* isomerisation of the retinal chromophore occurs. Thereby, intramolecular reactions like e.g. proton transfer from the Schiff base to an Asp residue [4] are triggered which eventually lead to the activation of the transducer pHtrII and subsequent components of the physiological reaction cascade. Finally, the protein relaxes back to the initial state to allow for a new photo-induced reaction cycle. The reaction sequence of the photocycle is quite similar to that as described for e.g. BR [5], consisting of archetypical K-, L-, M-, N-, and O-like intermediates [6] (see Fig. 1 for a simplified version of the pSRII photocycle) [7]. The early species (K and L) are formed in the μs-range. The M-like state (M_{400}) exhibits a deprotonated Schiff-base as indicated by its blue shifted absorption maximum around 400 nm. This intermediate decays to the parent state via the N- and O-intermediates in about 500 ms in pSRII which is substantially slower compared to the 10-ms M decay in BR. This difference in the turnover rate is crucial for the particular function of the pigments. Whereas ion-pumps need a high turnover for an efficient energy conversion, an effective signal transduction requires longer time scales.

The absorption maxima of the parent states of the photophobic receptors SRII and pSRII are considerably blue shifted as compared to those of other bacterial rhodopsins. Furthermore, the absorption spectra display a considerable fine structure. Due to its importance for the physiological function, colour regulation in retinylidene pigments has been extensively analysed for rhodopsin and BR (e.g. [8]) and recently also for SRII, particularly by reconstituting the apoproteins with retinal analogues [9,10]. It was found that the strength of hydrogen bond of the Schiff base with the protein environment is crucial for controlling the opsin shift. In the present study on pSRII, this issue is now addressed by employing resonance Raman (RR) spectroscopy which may provide detailed information about hydrogen bonding interactions of the Schiff base.

2. Materials and methods

pSRII was purified as described in [5]. The solubilised protein (0.075% dodecyl maltoside) was measured in a solution containing 10 mM Tris–HCl (pH 8) and 150 mM KCl. H_2O/D_2O exchange was performed by concentrating a 1-ml sample solution three-fold and subsequently diluting it with D_2O (99.9%) buffer (10 mM Tris, 100 mM NaCl) to 3 ml. This procedure was repeated three times. In the last step the sample was diluted to a final volume of 1 ml. The optical density of the solutions used for the RR experiments was 1.5 at 500 nm. RR spectra were measured with 514- and 488-nm excitation (Ar^+ laser) using a spectrograph equipped with a CCD detection system. The spectral slit width was 2 cm^{-1} and the wavenumber increment per data point was 0.15 cm^{-1}. Details of the experimental set-up

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Abbreviations: RR, resonance Raman; pSRII, sensory rhodopsin II (from *Natronobacterium pharaonis*)

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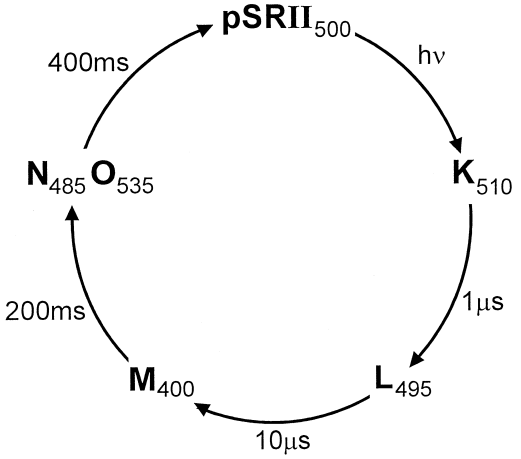

Fig. 1. Simplified version of the photocycle of pSRII. The 'N_{485} O_{535}' state indicates that the decay of M_{400} constitutes various fast equilibria between M_{400}, N_{485} and O_{535}. For a more detailed description of the photocycle see [5].

are described elsewhere [11]. RR measurements were carried out by using the rotating cell technique in order to reduce the accumulation of long-lived intermediates in the probe beam [12].

3. Results and discussion

In order to probe the RR spectrum of the parent state of pSRII, the photochemical rate constant 1_0, controlled by the laser power P_0, and the dwell time Δt of the sample in the laser beam, determined by the rotational frequency of the cell, have to be chosen such that the photoconversion parameter $1_0 \cdot \Delta t \ll 1$ ($P_0 = 10$ mW; $\Delta t = 10$ μs) [12]. The RR spectra obtained in that way from the samples in H_2O and D_2O are shown in Fig. 2. The fingerprint region between 1100 and 1300 cm^{-1} that is characteristic for the configuration and conformation of the retinal polyene chain is very similar to that of the parent states of BR and HR which includes an all-*trans* retinal with a protonated Schiff base [12–15]. In contrast, there are substantial differences compared to the dark-adapted form of BR (13-*cis* retinal) and the parent state of rhodopsin (11-*cis*) [15]. Thus, the chromophore structure pSRII is assigned to a protonated all-*trans* retinal Schiff base which confirms the results of recent experiments, in which is was shown that 13-*cis* retinal does not bind to the opsin [16]. The frequency of the C=C stretching at 1547 cm^{-1} agrees well with the value predicted from the $\nu_{C=C}/\lambda_{max}$ correlation well established for retinal proteins and model compounds [17,18].

The N–H in-plane bending contributes to a weak band at 1347 cm^{-1} which in D_2O shifts down to 981 cm^{-1}. This latter band which originates from a mode dominated by the N–D in-plane coordinate [19] is at a slightly lower frequency compared to the corresponding bands of the parent states of BR and HR [12,14]. Furthermore, the C=N stretching vibration of the Schiff base is attributed to a broad band at ca. 1650 cm^{-1} which is upshifted as compared to related retinal chromophores [12–14]. Specifically, this mode has been shown to respond sensitively to hydrogen-bonding and electrostatic interactions of the Schiff base with its immediate environment which in turn affect the absorption maximum of the chromophore [14,17,20].

Fig. 3A displays the RR spectrum ($1_0 \cdot \Delta t \ll 1$) of pSRII (H_2O) in the region between 1480 and 1680 cm^{-1} which, in addition to the C=N stretching, includes the prominent C=C stretching of the retinal chain centered at 1547 cm^{-1}. A careful inspection of this peak reveals a non-Lorentzian shape evidently due to additional bands at the high- and low-frequency wings of the peaks which are attributed to C=C stretchings of different states of the pSRII chromophore. Essentially the same spectrum is obtained upon 488-nm excitation (spectrum not shown here), i.e. in resonance with the high-energy shoulder of the absorption band, ruling out that the additional bands in the RR spectrum reflect a structural heterogeneity of the chromophore in the parent state. Instead, it is very likely that these bands originate from intermediates of the photocycle.

In fact, due to experimental constraints the accumulation of long-lived intermediates in the RR experiment was inevitable. Since the rotational frequency of the cell could not be reduced below 50 s^{-1} in order to keep photoconversion during a single probe event small, the rotational period (20 ms) was substantially shorter than the decay times of the long-lived intermediates M_{400}, N_{485} and O_{535} that are in the range between 200 and 400 ms (Fig. 1; [5]). Thus, even for a very low photoconversion parameter the 'fresh-sample' condition cannot be fulfilled [12,13] and these intermediates species are accumulated in the probe volume of the sample to a non-negligible extent. This interpretation is confirmed by comparison of the RR spectra obtained with a different photoconversion parameter. Raising the laser power to 50 mW reveals a substantial increase of the peak broadening (Fig. 3B) which results from

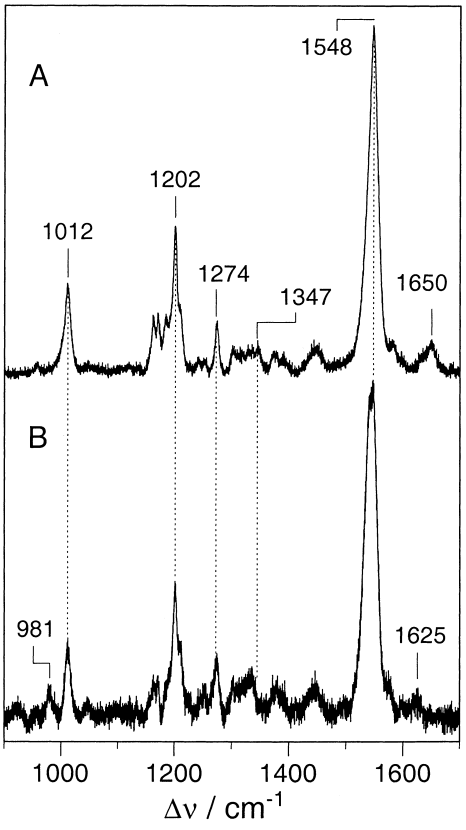

Fig. 2. RR spectra of pSRII in (A) H_2O and (B) D_2O obtained with low laser power ($P_0 = 10$ mW; Δt $1_0 \ll 1$). The excitation line was 514.5 nm. Further experimental details are given in the text.

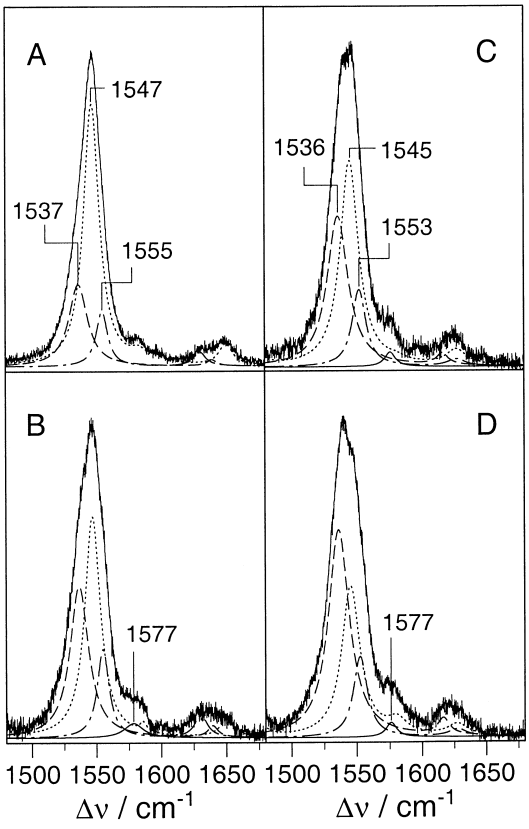

Fig. 3. RR spectra of pSRII in (A,B) H_2O and (C,D) D_2O in the C=C/C=N stretching region obtained with (A,C) low and (B,D) high laser power. The component spectra of $pSRII_{500}$, O_{535} and N_{485} are given by the dotted, dashed, and dashed–dotted lines, respectively. The excitation line was 514.5 nm. Further experimental details are given in the text.

the increase of the relative contributions from the intermediates. This effect is even more pronounced in the RR spectra measured from pSRII in D_2O (Fig. 3C,D). Also the C=N stretching region which is displayed in Fig. 4 in an expanded view reveals a similar behaviour in as much as the bandshapes vary significantly upon changing the excitation conditions.

It follows, that all measured RR spectra include contributions from the parent state and the three long-lived intermediates albeit in quite different portions depending on the experimental conditions. For a global analysis of these spectra, we have chosen a method in which the complete spectra of the individual components are fitted to the experimental spectra in the frequency range from 1480 to 1680 cm^{-1} [21]. Compared to conventional band fitting, this component analysis offers the advantage of substantially reducing the degrees of freedom in the fitting routine. Mutual subtraction of the RR spectra measured at high and low $1_0 \cdot \Delta t$ allows the determination of initial spectral parameters which were iteratively refined during the global fit to all experimental spectra. As an additional initial constraint for the analysis, it was assumed that the frequency of the C=C stretching is the same in the non-deuterated and deuterated species which appears to be justified in view of the negligible H/D shift of this mode also in related retinal chromophores [12–14]. For a refinement of the global fit, this constraint was eventually released and, in fact, no significant H/D shifts were obtained for this mode. The spectral parameters of the C=C and C=N stretching modes of all component spectra determined in this way are listed in Table 1. Based on the $\nu_{C=C}/\lambda_{max}$ correlation [18], the C=C stretching frequencies allow the assignment of the component spectra to the four species of the photocycle that are known to contribute to the spectra, i.e. the parent state and the three long-lived intermediates (vide supra). Thus, the component spectrum with the largest amplitude and a C=C stretching frequency of 1547 cm^{-1} is readily attributed to the parent state $pSRII_{500}$ whereas the spectra with $\nu_{C=C}$ at 1536.6 and 1554.9 cm^{-1} evidently correspond to the intermediates O_{535} and N_{485}, respectively. Note that the relative contributions of these intermediates differ in H_2O and D_2O indicating that the decay kinetics of O_{535} and N_{485} is slowed down upon H/D exchange which is in line with previous flash–photolysis studies [5]. Due to the more pronounced accumulation of intermediates in D_2O, at high laser power (Fig. 3D) a small contribution even of M_{400} is detected by its C=C stretching at ca. 1577 cm^{-1} despite the unfavourable resonance conditions for this species.

In contrast to the C=C stretching modes, the frequencies of the C=N stretching modes of the pSRII species do not reveal a reciprocal relationship with the absorption maxima (Table 1). Instead the downshift of the C=N stretching frequency in the order of $pSRII_{500}$, O_{535} and N_{485}, is paralleled by a substantial lowering of the H/D isotopic shift (ΔHD). Both parameters strongly depend on the interactions of the Schiff base with the protein environment [14,17,20]. Drastic

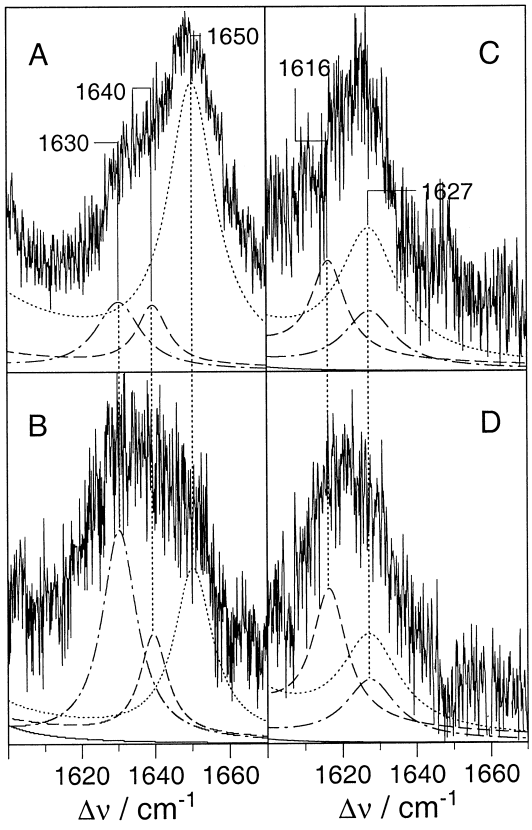

Fig. 4. Expanded view of the RR spectra of pSRII in (A,B) H_2O and (C,D) D_2O in the C=N stretching region obtained with (A,C) low and (B,D) high laser power. The component spectra of $pSRII_{500}$, O_{535} and N_{485} are given by the dotted, dashed, and dashed–dotted lines, respectively. The excitation line was 514.5 nm. Further experimental details are given in the text.

Table 1
Frequencies of the C=C and C=N stretching modes of the various states of pSRII[a]

State	$\nu_{C=C}$ (ΔHD)[b]	$\nu_{C=N}$ (ΔHD)[b]	Hydrogen bonding interactions
$pSRII_{500}$	1547.0 (−1.6)	1650.3 (−23.0)	strong
N_{485}	1554.9 (−2.3)	1630.3 (−2.8)	very weak
O_{535}	1536.6 (−0.3)	1639.6 (−23.2)	strong
M_{400}	1577	not detected	deprotonated

[a]Frequencies are given in cm^{-1}.
[b]Frequency shifts upon H/D exchange are given in parentheses.

variations of the electrostatic interactions which might account for the observed shifts of $\nu_{C=N}$ and ΔHD are not likely to occur in the protein as significant structural rearrangements in the chromophore binding pocket would be required. Thus, it is reasonable to assume that the changes of the C=N stretching frequency in H_2O and D_2O are due to alterations of the hydrogen bonding interactions with the counterion. For $pSRII_{500}$, the highest frequency (1650.3 cm^{-1}) and the largest H/D shift (−23.0 cm^{-1}) are observed which both are close to those observed for strongly hydrogen-bonded retinal Schiff bases [14,17]. A similar large H/D shift albeit at a lower C=N stretching frequency is found for O_{535} pointing to hydrogen bonding interactions that are comparable to those in $pSRII_{500}$. On the other hand, the much lower $\nu_{C=N}$ and ΔHD (−2.8 cm^{-1}) in N_{485} is very similar to that found for the parent state of HR where anion exchange experiments have indicated very weak hydrogen bonding interactions of the Schiff base [14]. Taking both spectral parameters as a measure for the hydrogen bond strength, it is, therefore, concluded that the interactions of the Schiff base proton with a hydrogen bond acceptor are weakened upon formation of N_{485}.

The strong hydrogen bond interactions of the Schiff base proton could be a source for the hypsochromic shift of the absorption maximum of pSRII ($\lambda_{max} = 500$ nm) as compared to that of the other bacterial rhodopsins ($\lambda_{max} > 560$ nm). However, that this factor cannot be the sole reason is evident from the spectrum of N_{485} which displays an even stronger blue shift, although the hydrogen bonding interactions are very weak. Further experiments are needed to explain the colour regulation in SRII and pSRII.

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