Protonation changes during the photocycle of sensory rhodopsin II from *Natronobacterium pharaonis*

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Abstract The Fourier Transform Infrared (FTIR) spectra of photocycle intermediates of sensory rhodopsin II (pSRII) from *Natronobacterium pharaonis* were measured. The results of the FTIR experiments indicate considerable conformational movements of pSRII already at the stage of the early K-like intermediate which persist at least during the lifetime of the long lived intermediate. These changes in the amide bond region are more intense than those described for sensory rhodopsin I (SRI) and are quite similar to those observed for rhodopsin. Concomitantly with the deprotonation of the Schiff base a carboxyl group located in a hydrophobic environment is protonated. In analogy to bacteriorhodopsin, this carboxyl group might arise from Asp-75 which probably serves as counter ion to the Schiff base. The protonation reaction differs from the situation observed in SRI where the protonation is pH independent over the range of pH 5–8.

Key words: Fourier Transform Infrared (FTIR) spectroscopy; Phoborhodopsin; Sensory rhodopsin; Phototaxis; Signal transduction

1. Introduction

The phototaxis of the archaeon *Halobacterium salinarium* is mediated by two bacterial rhodopsins, sensory rhodopsin I (SRI) and sensory rhodopsin II (SRII), which are receptors for photophilic and photophobic responses. These two proteins belong to the family of retinal proteins with the ion pumps bacteriorhodopsin (BR) and halorhodopsin (HR) as additional members. Upon light excitation, all four pigments react in a cyclic manner, returning back to the original state. This photocycle consists of several intermediates which have been identified by their spectroscopic and kinetic properties. Functional key roles were attributed to the long lived intermediates which (with the exception of BR) have absorption maxima around 400 nm. In the case of the sensory rhodopsins these intermediates probably trigger the signal transduction chain which is quite similar to that of the chemotactic system, e.g. in *E. coli* (for recent reviews see [1,2]).

Whereas SRI has been studied in great detail, much less is known about the photophobic receptor SRII. It was first described by Takahashi et al. [3] and termed phoborhodopsin [4]. Due to its low concentration in the bacterial membrane and its sensitivity towards conditions of low ionic strength, SRII from *H. salinarium* has so far only been partially purified [5]. However, an SRII-like photophobic receptor has been described in *Natronobacterium pharaonis* [6,7] whose properties provided easier biochemical access [8,9]. The amino acid sequence which was deduced from the corresponding gene displayed considerable similarities to the other bacterial rhodopsins [10]. Interestingly, the amino acids in position 85, 96, 115, 118 (numbering according to the BR sequence) determine the function of the protein. For example, proton pumps possess Asp, Asp, Asp, Met in these sites, whereas for phoborhodopsins the pattern is Asp, Phe/Tyr, Asn/Gln, Val/Thr. In BR Asp-85 belongs to the counterion complex of the protonated Schiff base from which it receives the proton during the L⇒M transition. With the exception of HR, this Asp is conserved in all members of the retinal protein family and might be crucial for the respective function.

The photoreaction of SRII from *H. salinarium* and *N. pharaonis* (pSRII) has been studied by several groups [8,11–13]. Summarizing the data of these reports the intermediates of the pSRII photocycle bear considerable similarities to those of BR and seem to include K-, L-, M-, and O-like species. For example, the M state, which is formed in the sub-ms range, has an absorption maximum below 400 nm due to the deprotonated Schiff base. At low temperatures, a K-like species can be trapped whereas the L and O intermediates have only been kinetically identified.

In the present report the K- and M-like intermediates of pSRII from *N. pharaonis* are investigated by Fourier Transform Infrared (FTIR) spectroscopy. It is shown that the deprotonation of the Schiff base is accompanied by a protonation of a carboxyl group which is likely to be Asp-75. The protonation/deprotonation reaction is unaffected between pH 5 and pH 8.

2. Materials and methods

Sensory rhodopsin II from *N. pharaonis* was purified as previously described [14,15]. The pSRII preparation still contained about 30% cytochromes as indicated by the absorbance at 420 nm. Samples of pSRII suitable for IR spectroscopy were prepared by drying the protein solution on a CaF_2 window. The resulting film was rehydrated with 0.5 μl H_2O. Subsequently, the sample was sealed with a second window. H/^{2}H exchange was achieved by repeatedly drying and rehydrating the sample with ^2H_2O. Low temperature difference spectra were measured in a home-built cryostat at 80 K and 170 K with a Bruker IFS 88 FT-IR instrument. The photoreaction was initiated by illuminating the sample with blue light (420 nm $< \lambda <$ 500 nm). The difference spectra were obtained by accumulating 256 scans each before and after illumination and subtracting the resulting single beam spectra from each other. Using the photoreversibility of this reaction [11], the experiments were repeated eight times and the respective difference spectra were averaged. Difference spectra for steady-state illumination at 273 K ($\lambda > 500$ nm) were acquired by accumulation of

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Abbreviations: BR, bacteriorhodopsin; FTIR, Fourier transform infrared spectroscopy; HR, halorhodopsin; SR, sensory rhodopsin

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the scans during simultaneous illumination and subtracting the resulting single-beam spectrum from the spectrum of the same sample which was not illuminated. The spectra shown represent the average of four experiments. It should be mentioned that, due to protein impurities and the detergent present in the pSRII preparation the spectra are of poorer quality than those obtained from other retinal proteins, e.g. BR. For the measurements in ^2H_2O, the spectral range below 1500 cm^{-1} is obscured by additional absorbance of the detergent and the solvent.

3. Results and discussion

3.1. FTIR difference spectra of the K-like intermediate

If pSRII is irradiated with blue light (420 nm < λ < 450 nm) at temperatures below 170 K, a red-shifted intermediate is obtained which was described as a K-like species [11]. Fig. 1 (upper trace) shows the low temperature (170 K) difference spectrum obtained under blue light illumination. The most prominent bands are found between 1700 cm^{-1} and 1500 cm^{-1} in the amide bond region and at the position of the C=C stretching vibration. The C=C stretching mode of the ground state is indicated at 1558 cm^{-1} (negative) whereas the photoproduct absorbs at 1545 cm^{-1} (positive). The visible absorption maxima and the ethylenic stretching frequency for protonated retinylidene Schiff bases are linearly related to each other [16–18]. Taking the absorption maximum of pSRII at 498 nm [8,11] into account one would expect a difference band in the infrared spectrum at 1545 cm^{-1}. However, the position of 1558 cm^{-1} is too high to be in agreement with this value. A similar frequency shift is observed for the photoproduct at 1545 cm^{-1} which also does not correlate with the reported absorption maximum located between 525 and 535 nm [11]. These discrepancies may be caused by the very nature of difference spectra since in cases where the bands of the initial state and the photoproduct overlap, an apparent shift of the extrema may appear. Furthermore, the spectral range overlaps with that of amide-II vibrational modes which might give rise to composite bands. Therefore, an unequivocal assignment cannot be made without the use of isotopically labelled retinals which exhibit shifted ethylenic modes and allow the discrimination from amide-II bands. In preliminary Resonance Raman experiments a frequency of 1545 cm^{-1} was identified [19] indicating that the band observed in the FTIR measurements comprises several modes.

Hirayama et al. [11] reported that the K-state is composed of two red-shifted photoproducts with absorption maxima at 525 nm and 535 nm, respectively. Unfortunately, because of the problems described above the existence of the two components can neither be confirmed nor disproved by the present FTIR experiments. In summary, extrema at 1558 cm^{-1} and 1545 cm^{-1} are probably largely due to the ethylenic modes, but in addition, they may be influenced by amide-II spectral changes.

The fingerprint bands of the chromophore (spectral range between 1300 and 1100 cm^{-1}) bear similarities to the BR → K difference spectrum of BR [20]. Therefore it is plausible that an all-*trans* → 13-*cis* isomerization also takes place in phoborhodopsin. But it must be emphasised that these modes have considerably lower intensities than those observed for other retinal proteins or model compounds.

Strong bands of intensities comparable to or even greater than those of the ethylenic modes are observed in the amide-I spectral range (1680–1600 cm^{-1}). This is unlike the more familiar case of BR and rhodopsin, where amide-I spectral changes for the early photoproducts K and bathorhodopsin, respectively, have considerably lower intensities [20,21].

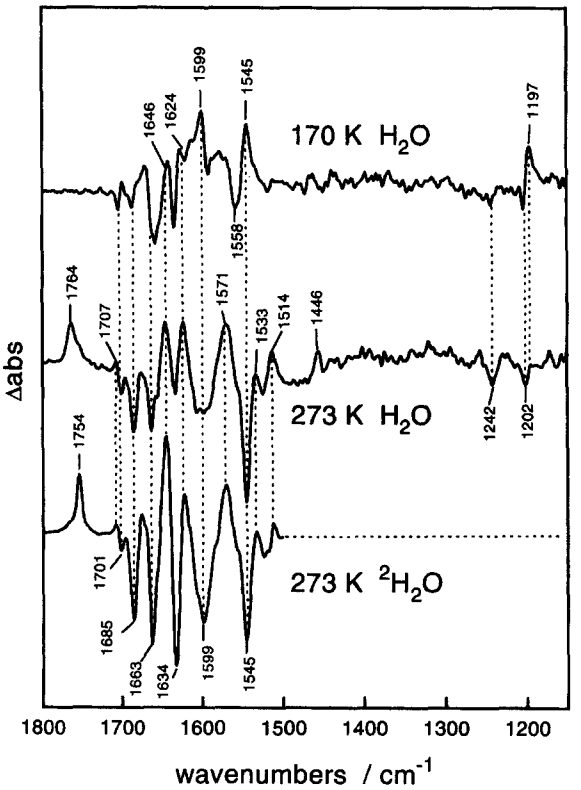

Fig. 1. FTIR difference spectra of pSRII. The upper trace depicts the difference spectrum between the early K-like intermediate (positive bands) and the ground state (negative bands). In the middle trace the difference spectrum between the sample irradiated during the measurements and the sample in the ground state is shown. Under these conditions mainly the M-like species (positive bands) is accumulated. The lower trace represents the difference spectrum taken in ^2H_2O. Otherwise the parameters are identical to those described for the spectrum in the middle trace.

At the intersection between the amide I and the C=O stretch region at around 1700 cm^{-1} a difference band is observed. Since it is not shifted towards lower frequencies by ^2H_2O it probably does not represent a C=O stretch of a protonated carboxyl group or a C–N stretch of the guanidinium group of an arginine. However, the residue giving rise to this band could be an amide of Asn or Gln. An example for similar properties of an amide side chain of asparagine was described for the Asp96Asn mutant of BR [22]. The corresponding vibrational mode was assigned to a difference band at 1704 cm^{-1}/1698 cm^{-1} which was not sensitive to ^2H_2O treatment.

It should be noted that the spectra taken at 80 K are essentially identical to those obtained at 170 K. Apparently, the K-like intermediate of pSRII has a greater thermal stability than the K-intermediate of the BR photocycle.

3.2. FTIR difference spectra of the M-like intermediate

The FTIR difference spectra of pSRII obtained under steady-state illumination (λ > 500 nm) at 273 K are depicted in Fig. 1 (middle and lower traces). The lower trace is obtained from a sample treated with ^2H_2O whereas the spectrum of pSRII in H_2O is shown in the middle trace. From the kinetics

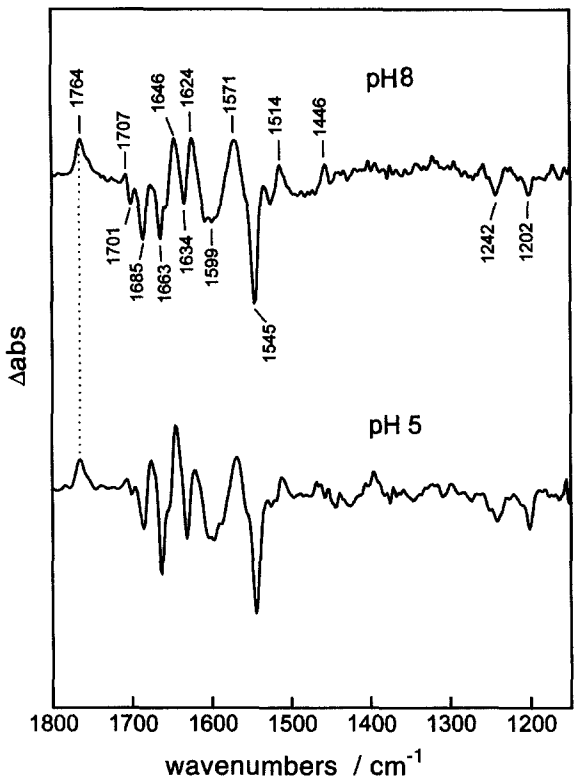

Fig. 2. FTIR difference spectra of pSRII taken at pH 5 and pH 8. The spectrum taken at pH 8 is identical to that of the middle trace of Fig. 1. The experimental conditions are as described in Fig. 1.

of the photocycle [8,23] one can expect that the long-lived (M-like) intermediate absorbing around 390 nm is accumulated under steady-state illumination. The position of this absorption maximum strongly suggests that the Schiff base is unprotonated. This assumption is supported by the lack of positive fingerprint modes between 1250 cm^{-1} and 1190 cm^{-1}. It has been shown that the intensities of these chromophore modes are drastically reduced upon Schiff base deprotonation [24]. As in the 170 K spectrum, the negative fingerprint modes which are due to the parent state of pSRII also have low intensities. So far, there is no obvious explanation for this observation.

The ethylenic mode appears as a strong negative band at 1545 cm^{-1}. This position would be in agreement with the visible absorption maximum at 498 nm. Although Resonance Raman data [19] also support this designation one has to be careful in ultimately assigning this band. The same holds true for the broad positive band at 1571 cm^{-1} which is in agreement with the C=C stretch of the unprotonated Schiff base. However, its intensity appears far too great. It is known that the intensities of both the C=C stretch and the fingerprint modes are reduced upon deprotonation of the Schiff base. Therefore, it seems likely that amide II spectral changes contribute to a large part of this band, reflecting structural changes of the peptide chain.

As it was already observed in the 170 K spectrum the amide-I bands are remarkably profound. The intensity ratio of the amide I to the ethylenic mode is now similar to that observed in rhodopsin→metarhodopsin-II difference spectra. Thus, it can be concluded that the corresponding protein structural changes approach a similar size. In ^2H_2O these bands appear sharper but are essentially not shifted (Fig. 1, lower trace). This observation suggests that most of the sites which undergo changes are located in the interior of the protein and are therefore not accessible to H/^{2}H exchange. It has been shown that the M-like state in SRII from *H. salinarium* is responsible for triggering the signal transduction chain [25]. Therefore, it seems that the extensive conformational changes observed in pSRII are of physiological importance and capable of triggering further biochemical reactions. It should be noted, however, that the corresponding amide vibrations in SRI are of much lower intensity [26] which might be related to the different function of the two photoreceptors.

Adjacent to the amide I region a difference band (at 1701 cm^{-1}/1707 cm^{-1}) is seen which was already present in the 170 K spectra (at 1701 cm^{-1}/1699 cm^{-1}) and is probably caused by the same group. The different positions can be explained by altered environments in the K- and M-like intermediates which is recognized by this group.

The C=O stretching vibrations of protonated carboxyl groups are located between 1700 cm^{-1} and 1800 cm^{-1}. The positive band at 1764 cm^{-1} clearly indicates that in the M-like intermediate a carboxyl group is protonated. This is confirmed by its shift of about 10 cm^{-1} to 1754 cm^{-1} by ^2H_2O. Since no corresponding negative band is observed one can conclude that the carboxyl group becomes protonated with the formation of the long-lived intermediate. A mere environmental change of a protonated carboxyl group would cause a difference band. It is noteworthy that the frequency at 1764 cm^{-1} is similar to the one described for Asp-85 of BR which also becomes protonated during the formation of M. In analogy one can assume that Asp-75 of pSRII gives rise to this C=O stretching vibration. From its position one can also deduce that the environment of this protonated carboxyl group is quite hydrophobic not allowing for extensive hydrogen bonds.

The band presents a shoulder around 1754 cm^{-1}. This can be either caused by an additional group or by a special hydrogen-bonding environment. Since no shoulder is observed for measurements in ^2H_2O, we rather suggest the latter possibility. It should be mentioned that the C=O stretch of the carboxyl group, as compared to the ethylenic mode, has a considerably higher intensity than analogous groups in BR [27] and rhodopsin [28,29].

In order to test the possibility that the protonation state of this carboxyl group is influenced by pH, we repeated the measurements at pH 5. However, the difference spectrum is identical to that obtained at pH 8 (Fig. 2). Therefore, the corresponding group cannot be titrated in this pH range and it appears that, as in the case of rhodopsin [28,29], the counterion of the protonated Schiff base is the proton acceptor for Schiff base deprotonation. This observation is different from that reported for SRI. During its photocycle a protonated carboxyl group only changes its environment [26,30]. If these differences are connected to the distinctive functions of SRI and SRII remains to be elucidated.

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