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FTIR investigation of the blue to acid-purple transition of Bacteriorhodopsin by use of induced halide binding

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1. Introduction

Bacteriorhodopsin (bR) and Halorhodopsin (hR) are light energy converting ion pumps in the cytoplasmic membrane of *Halobacterium salinarium* [1, 2]. Both belong to the family of retinal proteins with the typical seven-helix-motif. The *all-trans* retinal chromophore is covalently linked to a lysine residue in helix G via a protonated Schiff base. The sequence homology of the two proteins is about 30 % but the specificity and the vectoriality of the pumps are different: Whereas bacteriorhodopsin drives an outwards directed proton transport, halorhodopsin mediates an inwards directed transport of chloride ions.

The absorption maximum of retinal proteins in the visible spectral range is mainly determined by electrostatic interactions between the chromophore and charged amino acids [3]. In particular, weakening the interaction or increasing the distance between the protonated Schiff base and its counterion, leads to a red shift of λ_{max} [4]. In bR this counterion is dicussed to be a complex consisting of the negatively charged aspartates D85 and D212 and of the positively charged arginine R82 [5]. In hR D85 is replaced by a neutral threonine, T111. Nevertheless both proteins exhibit similar absorption maxima in their initial states: λ_{max}= 570 nm for bR and λ_{max}= 578 nm for hR, reflecting almost the same degree of stabilization of the protonated Schiff base. Therefore it has been suggested that in hR a bound chloride ion contributes a negative charge similar to D85 [6], while the other residues of the counterion complex in bR are conserved in hR as D238 and R108.

A well known effect in bR chemistry is the "purple to blue transition" which is reflected by a shift of λ_{max} from 570nm to 605nm upon acidification below pH 3 [7]. This redshift mirrors protonation of D85 [8] and thus destabilization of the positively charged Schiff base. The blue bR-species bR_{605} has been considered to be similar to the redshifted O intermediate of the bR photocycle in which also D85 is protonated [9]. Binding of certain anions to bR_{605} recovers a purple form with a λ_{max}=572nm [10]. This blue shift to the so called "acid-purple" form can be induced by halide ions or perchlorate, but not by sulfate ions. According to a hypothetical model the anion binds close to the protonated Schiff base thereby reversing the effect of D85 neutralisation [10]. This constellation of the counterion complex in the acid-purple form might be an analogue to the anion binding site in hR, which is responsible for the uptake of transported anions. Indeed, light dependent chloride pumping has been reported for acid-purple bR [11]. Supporting the analogy between halorhodopsin and

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the acid-purple form of bacteriorhodopsin, an anion free blue form has been found for hR from *Natronobacterium pharaonis*, which undergoes a blue shift of the λ_{max} upon anion binding similar to bR_{605} [12].

Here we report on the investigation of the blue to acid-purple transition in bR. A continous gas flow cuvette is used to induce chloride binding to the protein. To investigate the possible role of single residues involved in chloride binding, the reaction is carried out with bR-wildtype and the mutant R82K where the arginine 82 is replaced by lysine.

2. Experimental

Measurement of static FTIR-difference spectra is described in [13]. Purple membrane suspensions were adjusted to pH 1.0 with H_2SO_4. After centrifugation at 100000 × g for 4 h, the pellet was applied on two CaF_2 windows. In order to induce rapid chloride binding a continous flow IR gas cuvette (Fig. 1) was used. After equilibrating the light adapted sample for 3-4 h at RT about 10 ml dried HCl gas were carefully injected in small steps into the cuvette until the reaction was observed. HCl addition was controlled by the rovibrational bands of gaseous hydrochloric acid between 2600 cm^{-1} and 3000 cm^{-1} (not shown). Positive bands in the difference spectra represent product bands after gas injection.

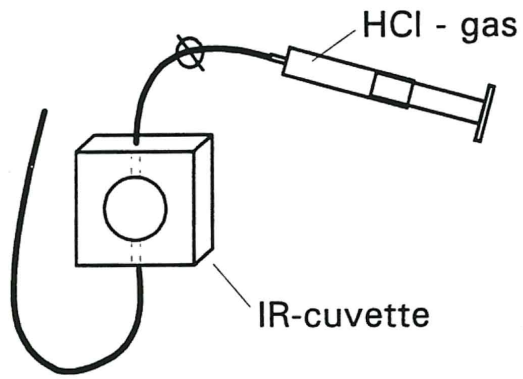

Fig. 1: Scheme of the continous flow IR gas cuvette. The HCl gas was taken from the gaseous phase of a standard hydrochloric acid bottle.

3. Results

The spectra in fig.2 demonstrate that the technique of HCl injection for induction of chloride binding is applicable to proteins in order to achieve high quality FTIR difference spectra. Baseline drifts, unspecific changes of continuum or water absorption bands and denaturation of the protein could be avoided.

Fig.2 compares difference spectra of the blue to acid-purple transition for bR-wildtype and the mutant R82K at a resolution of 2 cm^{-1}. In general the two spectra are almost identical. A dominating difference band at 1515/1531 cm^{-1} occurs in the ethylenic stretching region of both spectra. A further difference band can be seen in both spectra at 1762/1731 cm^{-1} in the

carbonylic stretching region. In the fingerprint region between 1000 and 1400 cm^{-1} which mainly is indicative for the chromophore geometry both spectra are almost identical except a more intense band at 1336 cm^{-1} in the spectrum of the mutant. The band at 1633 cm^{-1} seems to be broader in the mutant compared to the wildtype spectrum and in addition a negative band at 1649 cm^{-1} disappears.

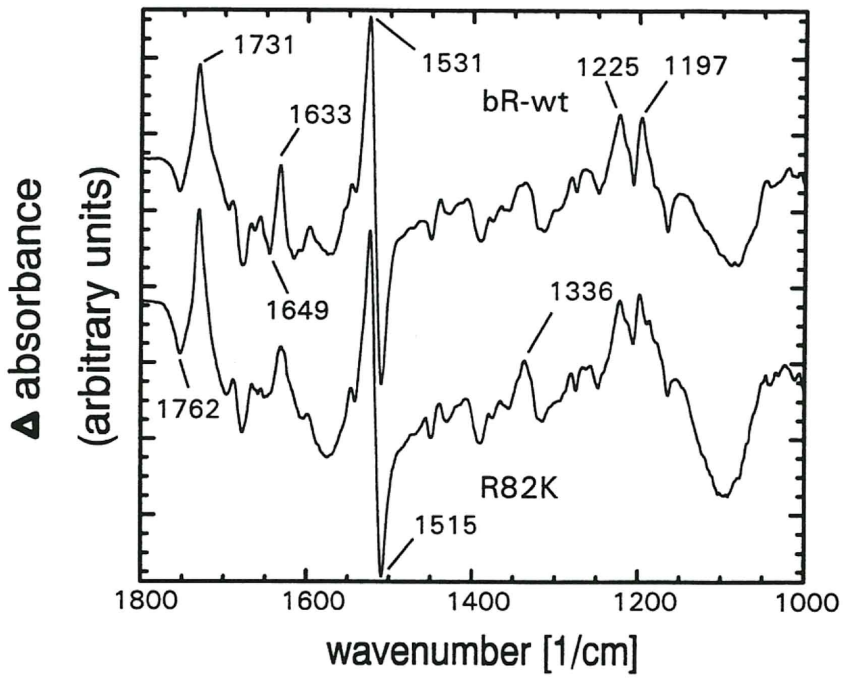

Fig. 2: Comparison of FTIR difference spectra representing the blue to acid-purple transition in bR-wt and R82K. Positive bands are due to the acid-purple form.

4. Discussion

The strong difference band of the ethylenic stretching vibration at 1515/1531 cm^{-1} corresponds to the shift of λ_{max} in the visible spectral range from 605 nm to 570 nm; the band is also in agreement with resonance Raman data on blue and acid-purple bR [14]. The difference band which shifts from 1762 cm^{-1} to 1731 cm^{-1} indicates involvement of D85 upon halide binding. The C=O stretching vibration at 1762 cm^{-1} is assigned to protonated D85, the only carboxylic acid with an unusual high frequency [15]. In a former FTIR study on the blue to acid-purple transition, where the reaction was induced by equilibration of the sample with different solutions, only the positive part, not the band at 1762 cm^{-1} could be seen [16]; the band is now detectable due to the better signal to noise ratio achieved by our method. All water exposed carboxyl groups which are protonated in bR_{605} absorb at lower frequencies at about 1720 cm^{-1} [13]. However, additional participation of other carboxylic acids cannot be excluded from our experiments.

Resonance Raman experiments on the blue to acid-purple transition of wildtype bR identified the 1633 cm^{-1} band as the C=N stretching vibration of the Schiff base [14]. The broadened 1633 cm^{-1} band in the R82K spectrum might reflect a more inhomogenous environment of the Schiff base compared to the wildtype. Participation of chloride binding by

R82 should cause a signal of the guanidino group in the wildtype spectrum between 1650 and 1690 cm-1 [17]. Even though a band at 1649 cm^{-1} disappears in the wildtype spectrum a clearcut assignment of this band to R82 remains an open question. Compared to the arginine C=N vibration in FTIR spectra of halorhodopsin the 1649 cm^{-1} band shows an unusual low frequency [17]. Furthermore this band might be masked behind the broadened band at 1633 cm^{-1} in the R82K spectrum in fig. 2. Thus some influence of the mutation on anion binding is observed. On the other hand, the general agreement between the FTIR difference spectra and the finding of identical blue to acid-purple shifts in the visible spectral range suggest that R82 does not play a specific role in anion binding, since it can be replaced by lysine.

Our results show that chloride binds in the acid-purple form of bR close to the Schiff base thereby affecting the protonated D85. Arginine 82 might contribute to the complex with its positive charge, but no indication for a specific interaction between the guanidino group and the anion was found. This is different to the anion binding site in halorhodopsin, where ion pair formation between arginine and halide ions has been shown [17]. Experiments on R108 mutants demonstrate a specific role of this arginine residue in halorhodopsin [Rüdiger *et al.*, in preparation].

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