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Proline residues undergo structural changes during proton pumping in bacteriorhodopsin

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The role of proline residues in the proton pump mechanism of bacteriorhodopsin is investigated by Fourier-transform infrared (FTIR) difference spectroscopy using [^{15}N]-proline labelled bacteriorhodopsin. Due to the isotopic shifts of absorbance bands, proline vibrations can be assigned to bands at 1616 cm^{-1} and between 1460 cm^{-1} and 1400 cm^{-1}. At 1616 cm^{-1} a decrease of an absorbance band is observed in the intermediates K, L, M and in the dark adapted state (DA). This absorbance change can be correlated to a light induced H-bond alteration of a X-Pro amide carbonyl group. Isotopic shifts of difference bands between 1450 cm^{-1} and 1400 cm^{-1} are detected in K and M but are not observed in L and DA. From these frequency shifts of proline vibrations it is concluded that two proline residues undergo structural changes of their X-Pro C-N peptide bonds during proton pumping. The results support the suggestion that the more flexible X-Pro C-N bonds are used as hinges for a functional important structural motion of the protein backbone.

Proton transfer; Pump mechanism; Fourier-transform infrared spectroscopy; Proline isotopic labeling

1. INTRODUCTION

The retinal protein bacteriorhodopsin acts as a light driven proton pump (for reviews see [1–3]). After light excitation the light adapted protein (BR) undergoes a photocycle with intermediates designated K, L, M, N and O. The photocycle is accompanied by a vectorial proton transfer from the cytoplasmic to the extracellular side. It is well established that crucial steps in the proton pump mechanism are an isomerization around the $C_{13} = C_{14}$ double bond [4] and the deprotonation of the Schiff base in M. Recent results indicate at least a twist around the C_{14}-C_{15} single bond [5,6] which can explain the light-induced pK reduction of the protonated Schiff base [7], the linkage site between the chromophore and Lys^{216} of the opsin moiety. Thereby, the initial Schiff base counterion complex becomes instable. Now, the proton from the Schiff base can be transferred probably to Asp^{85} in the L to M transition [8,9]. In contrast to Asp^{85}, which is assumed to be a proton binding site in the proton release pathway, Asp^{96} is a catalytic binding site in the proton uptake pathway [8]. Exchange of these aspartic acids by asparagines reduces drastically the proton pump efficiency [10,11]. However, Asp^{85}, Asp^{96} and the protonated Schiff base cannot be the only member of the proton translocation chains. Other functional elements might be involved.

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Taking structural information into account it is remarkable that three proline residues Pro^{50}, Pro^{91} and Pro^{186} are well in the middle of the membrane spanning helices B, C and F [12]. Conserved proline residues within helical segments are a common motive in transport proteins and may be of functional relevance [13].

In order to understand the role of proline residues in the proton pump mechanism of bacteriorhodopsin, ^{15}N-Pro was biosynthetically incorporated into BR and the photocycle of the labelled compound was investigated by FTIR difference spectroscopy. BR-K, BR-L, BR-M and BR-DA (light-dark adaptation) difference spectra were recorded. Isotopic changes are detected at 1616 cm^{-1} in the intermediates K, L, M and DA in the difference spectra of ^{15}N-Pro labelled bacteriorhodopsin compared to the one of the unlabelled sample. Furthermore, changes due to labelling are measured between 1450 cm^{-1} and 1400 cm^{-1} in the intermediates K and M but not in L and DA. The correlation of these spectral changes to molecular events and their tentative assignments to specific prolines will be discussed.

2. MATERIALS AND METHODS

^{15}N-Pro was biosynthetically incorporated into bR using the method of Engelhard et al. [14]. *Halobacterium halobium* (strain R_1M_1) was grown in a synthetic medium containing ^{15}N-Pro. The yield of incorporation was determined radioactively by replacing ^{15}N-Pro by ^{14}C-Pro using the method as described in [15].

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Each FTIR-measurement was repeated three times with three different samples. Only those spectral changes were considered to be caused by ^{15}N-Pro labelling which were observed for each individual spectrum. Difference spectra are taken as described in [8]. The subtraction procedure is described in [5] and [8].

3. RESULTS

In order to assign the bands which are caused by vibrations of the proline residues BR-M, BR-K, BR-L and BR-DA difference spectra of unlabelled and ^{15}N-Pro labelled bR were measured.

3.1. *BR-M difference spectra*

In fig.1(a,b) the BR-M difference spectra of the unmodified and ^{15}N-Pro labelled bacteriorhodopsin samples are shown. In general, both difference spectra are very similar indicating the reproducibility of the absorbance changes. However, deviations are observed around 1650 cm^{-1}, 1640 cm^{-1}, at 1616 cm^{-1} and in the spectral region from 1460 cm^{-1} to 1380 cm^{-1}.

Between 1660 cm^{-1} and 1530 cm^{-1} the amide I vibration at 1650 cm^{-1}, the OH bending vibration of H_2O at 1640 cm^{-1} and the amide II vibration at 1550 cm^{-1} cause strong background absorbances. This high optical density could provoke phase errors in the Fourier Transformation leading to intensity shifts of difference bands not caused by isotopic labelling in this spectral region. For example, the change in the difference band shifting from 1640 cm^{-1} to 1651 cm^{-1} could be due to such phase errors and therefore cannot be unequivocally assigned to the ^{15}N-Pro labelling.

An expansion of the region between 1670 cm^{-1} and 1590 cm^{-1} is shown in fig.2A. The intensity loss of the band at 1616 cm^{-1} in the difference spectrum of the labelled sample is also seen in the BR-M difference spectra taken in D_2O (fig.2A c,d) which was taken as control. In D_2O the overall structure of the difference spectra is not conserved, mainly due to the shift of the C=N$^+$H stretching vibration from 1641 cm^{-1} to 1622 cm^{-1}. The intensity loss at 1616 cm^{-1} is also found at lower temperatures (220 K; data not shown) indicating that this spectral feature is not due to e.g. sample preparations but finds its origin in ^{15}N label of Pro residue. From these results it can be concluded that part of the band at 1616 cm^{-1} is made up by a proline vibration. Since the higher atomic mass of ^{15}N compared to the naturally abundant ^{14}N shift vibrations to lower frequencies one should observe in the difference spectra of the labelled compound an additional downshifted band. However, this spectral region is dominated by strong absorbance bands of other molecular groups, which seem to obscure the possibly small shifted band.

The spectral region from 1500 cm^{-1} to 1300 cm^{-1} of the same samples are shown in fig.2B(a,b and c,d). Effects of labelling which are marked by broken lines are seen for the different samples at 1455 cm^{-1}, 1428 cm^{-1}, 1413 cm^{-1}, 1406 cm^{-1} and 1380 cm^{-1}. These small deviations can be better visualized by subtracting the difference spectra of the labelled and unlabelled compounds from each other. This is depicted in fig.3. Fig.3A presents the control in which two BR-M difference spectra of two unlabelled (a) and of two labelled (b) bacteriorhodopsin samples are subtracted. In these control subtractions an almost flat baseline is observed between identical sample preparations. Contrary, subtractions of BR-M difference spectra of labelled and unlabelled bacteriorhodopsin taken in H_2O (c), in D_2O (d) and at low temperature (e) show in the difference spectra of the ^{15}N-Pro labelled bacteriorhodopsin a small increase of intensity around 1455 cm^{-1}, increase around 1417 cm^{-1}, and a small in-

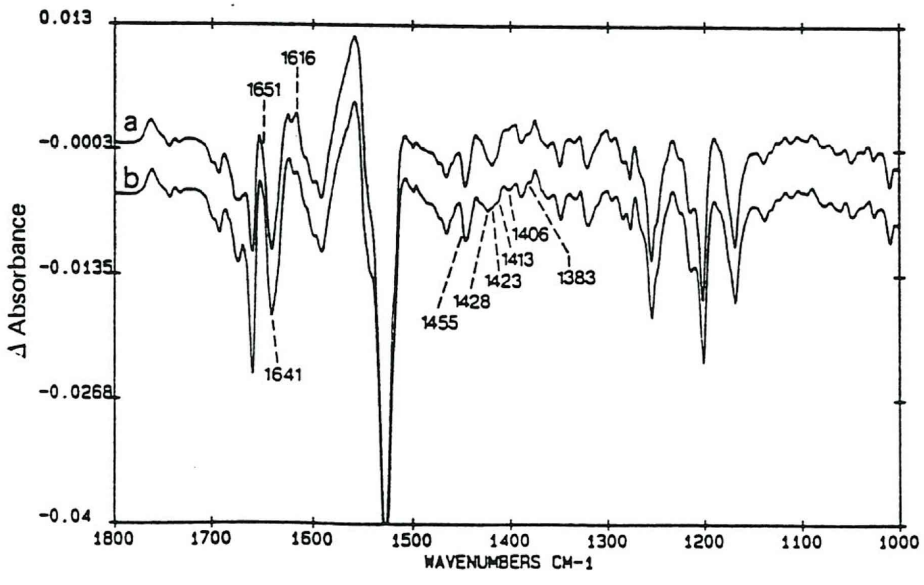

Fig.1. BR-M difference spectra taken at 270 K in H_2O of unlabelled (a) and ^{15}N-Pro labelled (b) bacteriorhodopsin.

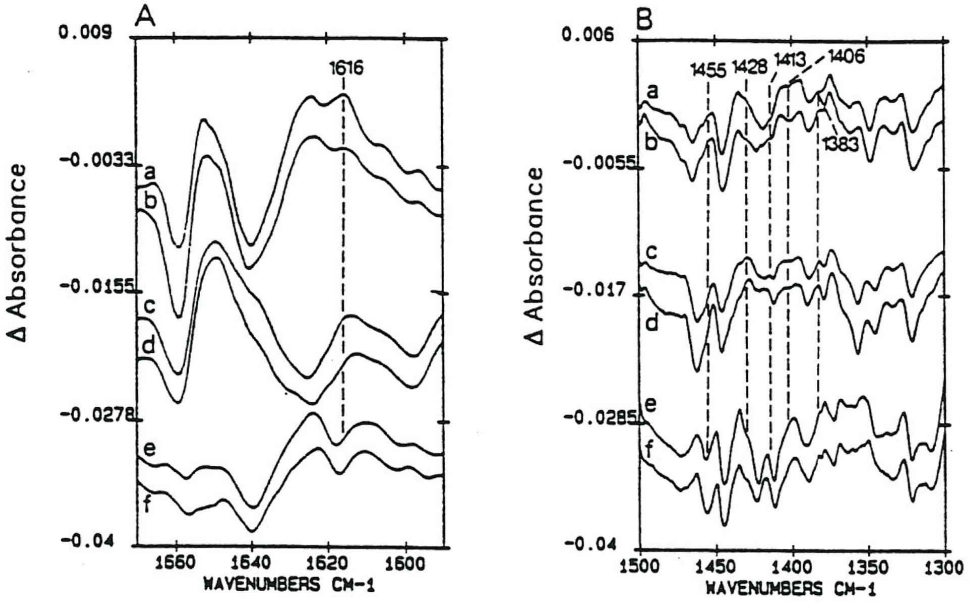

Fig.2. BR-M difference spectra (a,b,c,d) and BR-K difference spectra (e,f) from 1670 cm^{-1} to 1590 cm^{-1} in A and 1500 cm^{-1} to 1300 cm^{-1} in B. In (a,b), the same BR-M spectra of unlabelled (a) and labelled (b) compound are shown as in fig.1. In (c,d), BR-M-difference spectra of unlabelled (c) and labelled (d) compounds taken at 270 K in D_2O are shown. In (e,f), BR-K difference spectra taken at 77 K are shown. In (e) of unlabelled and in (f) of ^{15}N-Pro labelled bacteriorhodopsin are shown taken in H_2O.

crease around 1380 cm^{-1} (positive bands in the subtraction). An intensity decrease is found at 1435 cm^{-1} and at 1400 cm^{-1} (negative bands in the subtraction). These observations can be explained by downshifts of two difference bands with negative peaks at about 1454 cm^{-1} and 1417 cm^{-1} and positive peaks at about 1435 cm^{-1} and around 1400 cm^{-1} by about 15 cm^{-1} to 20 cm^{-1}. The shifts of two difference bands cause largest effects in the subtraction at positions where shifted bands of the higher frequency difference band and unshifted bands of the lower frequency difference band overlap. Therefore, the isotopic effects are most prominent at 1435 cm^{-1}, *1417 cm^{-1}* and 1400 cm^{-1}. The possibility that only one difference band with negative peak at around 1417 cm^{-1} and positive peak at about 1435 cm^{-1} is shifted down by about 15 cm^{-1} seems less

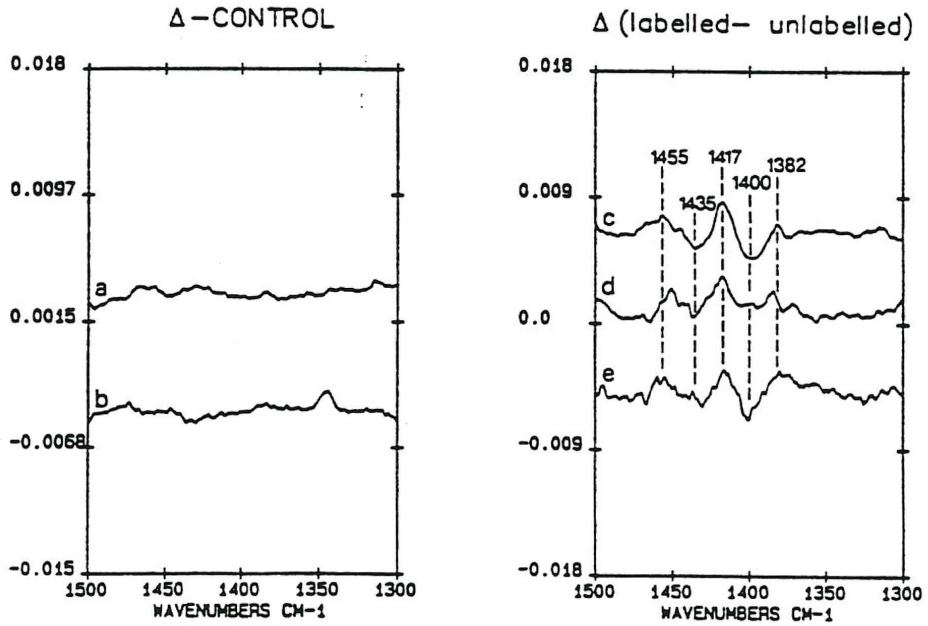

Fig.3. Subtraction of difference spectra to reveal the effect of isotopic labelling. By minimizing the parameter Δ^2, which Δ = difference spectrum of the wild type − ax (difference spectrum of the mutant) + b with a and b being the variables for minimization. In (A), control subtractions are performed of difference spectra of two unlabelled (a) and two labelled (b) bacteriorhodopsin samples. In (B), the BR-M difference spectra shown in fig.2a–d are subtracted (c,d). Negative absorbance bands in the difference spectra of unlabelled sample corresponds to positive bands in the subtraction and vice versa. In (e), low temperature BR-M difference spectra of labelled and unlabelled bacteriorhodopsin are subtracted.

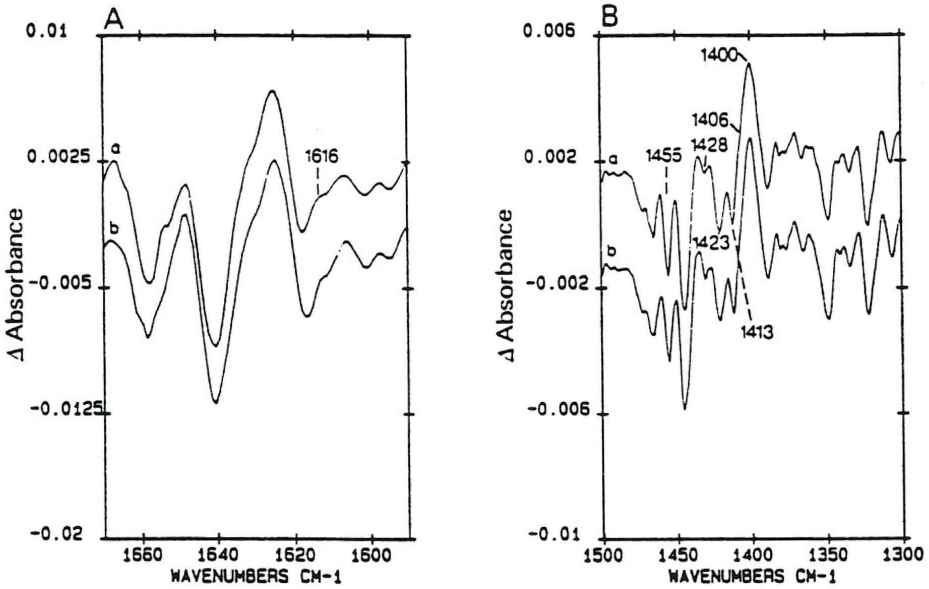

Fig.4. BR-L difference spectra taken at 170 K in H_2O for unlabelled (a) and ^{15}N-Pro labelled (b) bacteriorhodopsin. On an expanded scale from 1670 cm^{-1} to 1590 cm^{-1} in A and 1500 cm^{-1} to 1300 cm^{-1} in B.

likely because this does not explain the changes seen at 1455 cm^{-1} and 1390 cm^{-1}.

3.2. *BR-K difference spectra*

Fig.2e,f show the BR-K difference spectra from 1670 cm^{-1} to 1590 cm^{-1} in A and 1500 cm^{-1} to 1300 cm^{-1} in B for unmodified and ^{15}N-Pro labelled bacteriorhodopsin. As already discussed for the BR-M difference spectra the difference spectra of the labelled compound lacks a small absorbance band at 1616 cm^{-1}. Also, the spectral features at 1455 cm^{-1}, 1428 cm^{-1}, 1423 cm^{-1}, 1406 cm^{-1} and 1380 cm^{-1} are similar as in the BR-M difference spectra. They can again be explained by down shifts of two difference bands.

3.3. *BR-L difference spectra*

In fig.4 the BR-L difference spectra of unlabelled (a) and ^{15}N-Pro labelled bacteriorhodopsin (b) are represented. No evident deviations between both difference spectra are observed, except for a small change at 1616 cm^{-1} (fig.4A). This is contrary to the already discussed BR-K and BR-M difference spectra. If in L changes are present, they must be considerably smaller than in K and M.

3.4. *Light-dark adapted difference spectra*

In fig.5 the BR-DA (light-dark adaptation) difference spectra of unlabelled and ^{15}N-Pro labelled bacteriorhodopsin are seen. Again an intensity decrease is noticeable at around 1616 cm^{-1}. But as in the BR-L

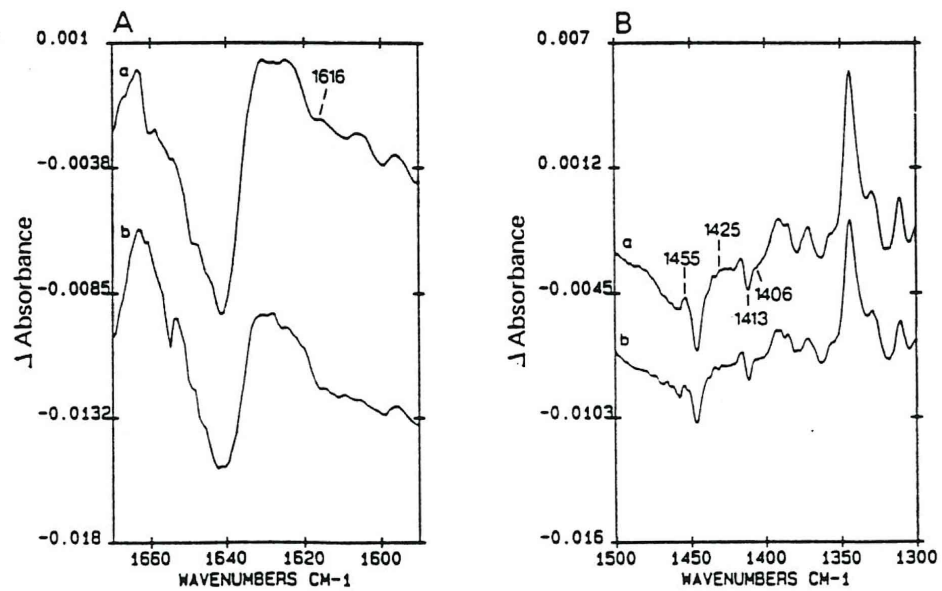

Fig.5. BR-DA (light-dark adaptation) of unlabelled (a) and ^{15}N-Pro labelled (b) bacteriorhodopsin taken in H_2O at 280 K. In (A), the spectral region from 1670 cm^{-1} to 1590 cm^{-1} and in (B), from 1500 cm^{-1} to 1300 cm^{-1} are shown.

difference spectra and in contrast to the BR-K and BR-M difference spectra, no further evident changes can be observed especially at 1455 cm^{-1}, 1425 cm^{-1}, 1413 cm^{-1} and 1406 cm^{-1}.

4. DISCUSSION

The comparison of native bR and ^{15}N-Pro labelled bR enabled us to identify different Pro absorbance bands: at 1616 cm^{-1}, at 1454 cm^{-1}, at 1435 cm^{-1}, at 1417 cm^{-1} and at 1400 cm^{-1}. The changes o bserved between 1460 cm^{-1} and 1380 cm^{-1} can be explained by downshifts due to isotopic labelling of two difference bands with negative peaks around 1454 cm^{-1} and 1417 cm^{-1} and positive peaks around 1435 cm^{-1} and 1400 cm^{-1}.

In the following, the assignment of these proline absorbance bands to particular proline vibrations will be discussed:

The proline vibration at around 1616 cm^{-1} is observed in the intermediates K, L, M and during dark adaptation. A normal mode analysis of a cyclopeptide Phe-Pro-Gly-Ala-Pro reveals a X-Pro amide I carbonyl vibration at 1616 cm^{-1} [16]. Based on the coincidence of the frequency position the proline absorbance band at 1616 cm^{-1} can be tentatively assigned to an X-Pro amide I vibration. In another infrared study of the polytripeptide $(Ala\text{-}Pro\text{-}Gly)_n$ an amide I vibration around 1620 cm^{-1} was observed to be characteristic for the H-bonding of this Ala-Pro amide carbonyl group [17]. The authors discuss the disappearance of this band as indication for a break in the H-bonding of the carbonyl group [17]. The band at 1616 cm^{-1} might be of similar origin. Interestingly, the effects are observed in intermediates, in which the chromophore adopts a 13-*cis* configuration. Therefore, this absorbance change reflects presumably an alteration of the H-bond pattern of an X-Pro-amide carbonyl group caused by the chromophore isomerization.

The other two proline difference bands at 1454 cm^{-1}/1435 cm^{-1} and 1417 cm^{-1}/1400 cm^{-1} can be observed in K and M but are not detected in L and DA.

The assignment of the difference bands to CH bending vibrations which absorb in this frequency range [18], is less likely, because ^{15}N labelling is not expected to cause the observed isotopic shift on such vibrations. Another possibility of assignment stems from UV resonance Raman studies on polyproline and ribonuclease A [19]. In polyproline II a proline band at 1463 cm^{-1} and in ribonuclease A a proline band at 1466 cm^{-1} were observed which were attributed to *trans* configurations. The absorbance bands were assigned to C-N proline vibrations. These studies show that during *trans-cis* isomerization of prolines the C-N frequency is downshifted from 1463 cm^{-1} to 1435 cm^{-1} in polyproline and from 1466 cm^{-1} to 1458 cm^{-1} in ribonuclease A, indicating the sensitivity of this vibration to the configuration around the C-N bond. Because in bacteriorhodopsin proline vibrations are observed at similar frequencies, they can probably be correlated to C-N vibrations. Their downshift in the intermediates seems to reflect light induced conformational changes of two proline residues around this bond.

A global movement of the protein backbone during proton pumping was deduced from strong difference bands in the amide I/amide II region [20,21]. The higher flexibility around the C-N amide bond of prolines compared to other amino acids [13] might be the hinge for functional important conformational motions which facilitate the vectorial transport of a proton. However, if the observed frequency shifts are actually caused by a full isomerization or by a partial twist around the C-N peptide bond needs further investigations because frequency shifts could also be due to other perturbations, affecting the electron distribution of this bond. A full isomerization could establish a transient proton-binding site [22].

The observed absorbance bands seem to be caused by three different proline residues. In contrast to the proline absorbance band at 1616 cm^{-1}, which is observed in K, L, M and DA, the two proline difference bands between 1450 cm^{-1} and 1400 cm^{-1} are only observed in K and M but not in L and DA. Therefore, the different absorbance bands are due to three rather than two different proline residues.

The question remains to which of the 11 prolines contained in bacteriorhodopsin the observed absorbance bands can be assigned. Due to its position in the middle of the membrane, Pro^{50}, Pro^{91} and Pro^{186} might be of functional and/or structural relevance. Indeed, site directed mutagenesis of Pro^{186} to Ala and Gly show an impact on the chromophore and the proton pump ability [23]. However, mutation of Pro^{50} or Pro^{91} to Gly and Ala led to the reconstitution of the functional properties of the native system [23].

Interestingly, as the proline absorbance change at 1616 cm^{-1} also absorbance changes of Tyr^{185} are observed in the BR-K, BR-L, BR-M and BR-DA difference spectra [24]. Tyr^{185} is on the N-terminal site of Pro^{186}, thus, its α-carboxyl group forms the peptide bond with the nitrogen of Pro^{196}. Therefore, the molecular process leading to absorbance changes at Tyr^{185} presumably causes also absorbance changes at Pro^{186}. Consequently, the vibration at 1616 cm^{-1} is tentatively assigned to Pro^{186}.

In helix C Asp^{96} and Asp^{85} undergo protonation changes [8,9] and they seem to be part of the proton transfer chain [5,8–11,14]. Thereby, Pro^{91} on helix C and/or Pro^{50} on helix B may be affected and could cause the absorbance bands between 1450 cm^{-1} and 1400 cm^{-1}. However, further FTIR experiments with more specific isotopic labels or with site-directed

mutants are needed to confirm these tentative assignments.

Recently, similar experiments were performed by Rothschild et al. [25].

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