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TIME-RESOLVED STEP-SCAN FTIR INVESTIGATIONS ON THE $M_1 \rightarrow M_2$ TRANSITION IN THE LIGHT-DRIVEN PROTON PUMP BACTERIORHODOPSIN

OLAF WEIDLICH, CHRISTOPH RÖDIG
and FRIEDRICH SIEBERT*

Institut für Biophysik und Strahlenbiologie, Albert-Ludwigs-Universität Freiburg, Albertstr. 23, D-79104 Freiburg

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Using time-resolved step-scan FTIR spectroscopy, it has become possible to identify two different M states in the 5 to 300 µs time range, which clearly differ from the M_N state. The identification has become possible by measuring a pure BR → KL difference spectrum at 100 ns, which can be used to correct for the contributions of this intermediate in the later difference spectra. The subtraction of the later corrected difference spectra thus represent L → M_1, M_2 difference spectra with varying relative amount of the two M states. The comparison of these spectra clearly reveals differences in the amide-II spectral range, and possibly also in the amide-I range. From these observations it can be concluded that the two M states do not differ in the chromophore structure but in the protein structure.

Keywords: Bacteriorhodopsin; photocycle; M intermediates; step-scan FTIR spectroscopy

INTRODUCTION

In the light-driven proton pump bacteriorhodopsin (BR), the absorption of a photon by the chromophore *all-trans* retinal, which is bound to the protein *via* a protonated Schiff base to lysine 213, causes its isomerization to a distorted 13-*cis* geometry within 500 fs.

* Corresponding author. e-mail: frisi@ruf.uni-freiburg.de

Subsequently, the chromophore relaxes and the protein undergoes structural changes. The cyclic photoreaction is characterized by the intermediates J, K, KL, L, M, N and O (in the order of their appearance). In the M photoproduct, the Schiff base is deprotonated by proton transfer to aspartic acid 85 (Asp85). In the N intermediate, the Schiff base is reprotonated from Asp96. In the O species, the chromophore is thermally back-isomerized to the *all-trans* geometry and its decay to the initial state closes the photocycle. Protonation of Asp85 initiates the ejection of a proton to the extracellular side, whereas reprotonation of the Schiff base from Asp96 triggers proton uptake from the cytosolic side (Refs. [1, 2] for review). It is generally accepted, that the proton of the Schiff base is directly involved in proton pumping, and therefore, the formation of the M intermediate by deprotonation of the Schiff base is one of the key steps in the photocycle. It has been shown that by chemical inhibition of the deprotonation proton pumping is abolished [3, 4]. The reaction scheme of the photocycle has been intensively studied in recent years, and models involving linear and branched pathways have been developed. However, it appears that a linear model is sufficient if backreactions between succeeding intermediates are included [2], although more complicated models involving branched pathways cannot be excluded on the basis of experimental data. Most studies in this respect comprise time-resolved UV-vis investigations, which provide precise informations on kinetic constants. Such studies have revealed an important conclusion: there must be one reaction step without a backreaction or with strongly biased forward reaction that links two states with deprotonated Schiff base, *i.e.*, two M states [5].

The existence of two M states has been also deduced from mechanistic considerations. If a vectorial proton transport has to be realized, the Schiff base has to be first connected to the extracellular side for deprotonation and subsequently to the cytosolic side for reprotonation. Therefore, two M states have been inferred which differ in the accessibility of the Schiff base [1, 2, 6]. Up to now it has not been possible to determine the molecular differences between the two M states. This is mainly due to the low M_1 amplitude resulting from an L/M_1 equilibrium favouring the L state. In addition, the coexistence of several intermediates caused by the equilibrium conditions makes the analysis of spectra difficult.

More detailed information on the molecular events during the photocycle can be obtained from vibrational spectroscopy, and FTIR difference spectroscopy is able to detect molecular changes of the chromophore and the protein [7, 8]. We have recently introduced the method of time-resolved step-scan FTIR spectroscopy, enabling the measurement of precise time-resolved difference spectra with a time-resolution determined by the detector rise time and by the bandwidth of the acquisition electronics (500 ns) [9, 10]. By the use of a photovoltaic detector and a 200 MHz AD converter, we have now increased the time resolution to approx. 30 ns. This allows the measurement of a pure BR → KL difference spectrum. Using a large memory into which the signals are stored, transients covering a time range up to 500 μs can be captured. In order to increase the time resolution at later times after the flash, the linear time base is converted to a quasi-logarithmic one [11].

RESULTS

The following strategy has been developed to circumvent the difficulties mentioned above in determining the two M states. (1). The pure KL difference spectrum can now be used to correct for the admixture of this state to the later intermediates. (2). Time-resolved difference spectra are obtained in the time range from 2 to 300 μs, which are corrected for the respective contributions of the KL state. Later times have not been analyzed here, since then the N state would contribute to the M_2/N equilibrium. (3). Differences are formed between the corrected spectra, *i.e.*, between the later spectra and the spectrum obtained at 2 μs after the flash. The differences represent then the L → M transition at various times after the flash, *i.e.*, with varying relative amount of M_1 and M_2. Although at early times only little M (mainly M_1) is formed, an L → M_1 difference spectrum is obtained of sufficient accuracy, circumventing the problem of low M_1 amplitude.

Figure 1, upper trace shows the KL difference spectrum obtained at 100 ns after the flash. It exhibits the characteristics of the early KL state [12, 13]: the difference band due to Asp115, the strong ethylenic mode of the chromophore at 1512 cm^{-1}, the finger print band at 1189 cm^{-1} and the 15-HOOP mode at 982 cm^{-1} [10]. The lower trace

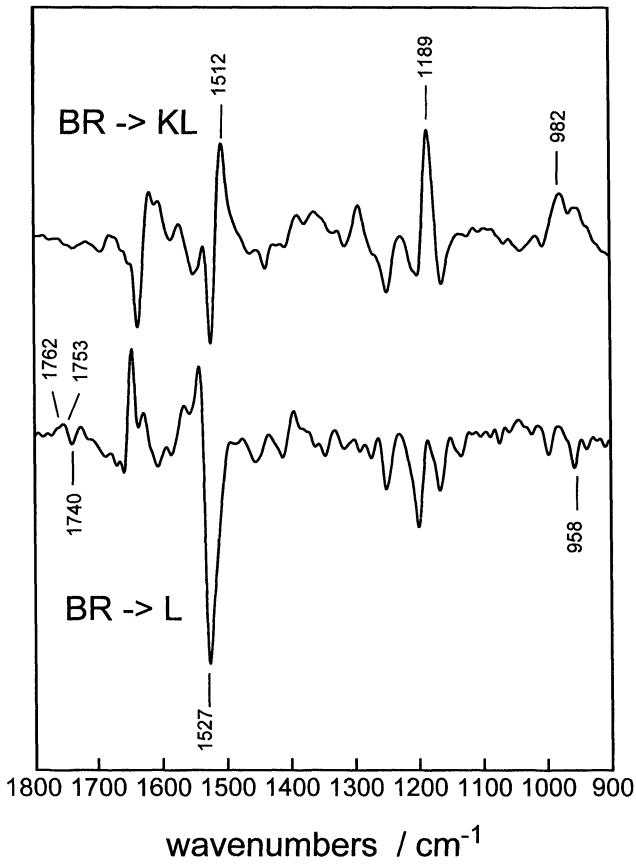

FIGURE 1 Time resolved infrared difference spectra of the BR → KL and BR → L transitions. Upper trace: spectrum obtained at 100 ns after the flash, representing the BR → KL transition. Lower trace: difference spectrum obtained at 2 μs after the flash, corrected for the contribution of the KL intermediate. It represents an almost pure BR → L spectrum.

in Figure 1 represents the spectrum measured 2 μs after the flash, corrected for the KL contribution. As a criterion for the subtraction, the HOOP mode at 982 of the KL state has been used. Thus, the trace represents an almost pure BR → L difference spectrum, especially characterized by the features between 1750 and 1730 cm^{-1}, due to protonated Asp96 and Asp115, and by the band around 1400 cm^{-1},

due to the NH bending mode of the protonated Schiff base coupled to the C-15H bending mode of the chromophore [10] In a similar way, spectra at 5, 20, 75 and 270 μs have been corrected.

As an example for the formation of the differences, the two traces in Figure 2 represent spectra obtained at 5 and 270 μs. They are mormalized to each other with respect to the extent of the transition. Clear differences can be seen at 1559 cm^{-1} (positive band) and

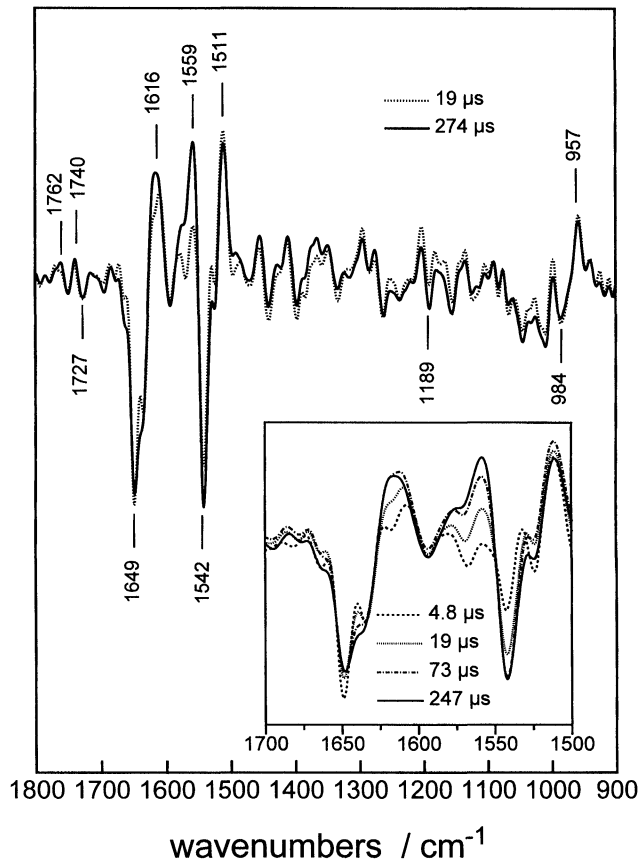

FIGURE 2 Upper two traces: $L \rightarrow M_1, M_2$ difference spectra obtained at 5 and 270 μs. The method for obtaining the spectra is described in the text. Insert: additional $L \rightarrow M_1, M_2$ difference spectra in the amide-I/amide-II spectral range, revealing clear differences between the two M states.

1542 cm^{-1} (negative band). These features reflect changes in the amide-II band, therefore reflecting structural changes of the peptide backbone. The temporal development can be seen in the insert. It shows that there may be additional changes in the amide-I spectral range at 1640 and 1616 cm^{-1}. One might argue that the difference band structure seen in the amide-II range reflects the C=C stretching mode of the L state (negative) and M state (positive), the differences thus only reflecting differences in the extent of the photoreaction. However, the otherwise excellent agreement in band position and intensity, including the band at 1762 cm^{-1} due to protonation of Asp85 demonstrates that the normalization has been performed correctly. Furthermore, the M_2 difference spectrum differs clearly from the M_N difference spectrum observed for the mutants lacking the proton donor Asp96 [14].

Thus, our results provide the first direct spectroscopic evidence for the existence of the two M states for wild type bacteriorhodopsin at physiological conditions which are responsible for the different accessibility of the Schiff base. The two M states differ in the amide-II, and perhaps also in the amide-I spectral ranges. This demonstrates that structural changes of the protein occur, but no changes of the chromophore.

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References

[1] Oesterhelt, D., Tittor, J. and Bamberg, E. (1992). *J. Bioenerg. Biomembr.*, **24**, 181.
[2] Lanyi, J. K. (1993). *Biochim. Biophys. Acta*, **1183**, 241.
[3] Longstaff, C. and Rando, R. R. (1987). *Biochemistry*, **26**, 6107.
[4] Kräutle, R. *et al.* (1990). *Biochemistry*, **29**, 3915.
[5] Váró, G. and Lanyi, J. K. (1991). *Biochemistry*, **30**, 5008.
[6] Ames, J. B. and Mathies, R. A. (1990). *Biochemistry*, **29**, 7181.
[7] Siebert, F. (1993). *Biomolecular Spectroscopy Part A*, Clark, R. J. H. and Hester, R. E., Eds. (John Wiley & Sons, Chichester), pp. 1–54.
[8] Siebert, F. (1995). *Israel J. Chem.*, **35**, 309.
[9] Uhmann, W., Becker, A., Taran, C. and Siebert, F. (1991). *Appl. Spectrosc.*, **45**, 390.
[10] Weidlich, O. and Siebert, F. (1993). *Appl. Spectrosc.*, **47**, 1394.

[11] Austin, R. H. *et al.* (1976). *Rev. Sci. Instrum.*, **47**, 445.
[12] Hage, W., Kim, M., Frei, H. and Mathies, R. A. (1996). *J. Phys. Chem.*, **100**, 16026.
[13] Sasaki, J., Yuzawa, T., Kandori, H., Maeda, A. and Hamaguchi, H. (1995). *Biophys. J.*, **68**, 2073.
[14] Sasaki, J., Shichida, Y., Lanyi, J. K. and Maeda, A. (1992). *J. Biol. Chem.*, **267**, 20782.