EVIDENCE FOR THE PROTONATION OF TWO INTERNAL CARBOXYLIC GROUPS DURING THE PHOTOCYCLE OF BACTERIORHODOPSIN

Investigation by kinetic infrared spectroscopy

F. SIEBERT, W. MÄNTELE and W. KREUTZ
Institut für Biophysik und Strahlenbiologie, Universität Freiburg, Albertstrasse 23, 7800 Freiburg, FRG

Received 15 February 1982

1. Introduction

The amino acid sequence of bacteriorhodopsin (BR) [1,2], its three-dimensional structure at low resolution [3] and projected structure at 3.7 Å resolution [4] have been determined. Attempts have been made to fit the amino acid sequence into the three-dimensional structure [5,6] and the retinal binding site has been established [7–9]. To understand the proton pumping mechanism, molecular events in the chromophore and protein should be known as well as the structural information. Resonance Raman spectroscopy provides information on the chromophore (review [10]), whereas kinetic infrared spectroscopy [11,12] detects molecular changes in the chromophore as well as the protein. It has been applied successfully in investigations of rhodopsin [13] and bacteriorhodopsin. For the latter, the BR570–M412 difference spectrum has been measured over 1700–1500 cm^{-1} and 1300–1100 cm^{-1} [12]; a preliminary study on the BR570–L550 difference spectrum in the same spectral range has been published [14]. Having extended the spectral range beyond 1700 cm^{-1}, we now report evidence for the presence of two carboxylic acid residues being protonated and re-deprotonated during the photocycle. While the protonation of one group occurs simultaneously with the formation of M412, the time course for the second group is slower and does not reflect kinetics observed for chromophoric changes. In addition, the two groups can be identified by their different absorption maxima. The time constant for re-deprotonation is similar for both groups coinciding with that for the reformation of BR570 from M412.

These results will be discussed with respect to a possible involvement of carboxylic groups in the proton pumping mechanism [15,17] and with respect to their role regarding the spectroscopic properties of BR [15,16,18–21].

2. Materials and methods

The apparatus used for the kinetic infrared investigations has been described [11,12]. Purple membrane from *Halobacterium halobium* (mutant R_1M_1) was isolated as in [22]. A detailed description for the preparation of purple membrane samples for infrared spectroscopy is given in [23]. For measurements at high and low pH, dried film samples were overlaid with solutions of NaOH and HCl, respectively. These samples were then sealed with a second window at a spacing of 20 μm. The low pH was estimated from the relative yield of O640 [24], as measured at 1505 cm^{-1} and at 1525 cm^{-1}, representing the positions of the C=C-vibration of the chromophore in O640 and BR570, respectively [12]. The high pH was assessed from the slowing down of the decay of M412 [25]. In the other cases, films hydrated or deuterated via the vapor phase at 100% relative humidity, were used. Purple membranes were treated with pronase as in [27]. The digestion was controlled by SDS gel electrophoresis experiments [27].

3. Results and discussion

Four flash-induced signals at different wavenumbers for a hydrated and a deuterated BR film sample are shown in fig.1.

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

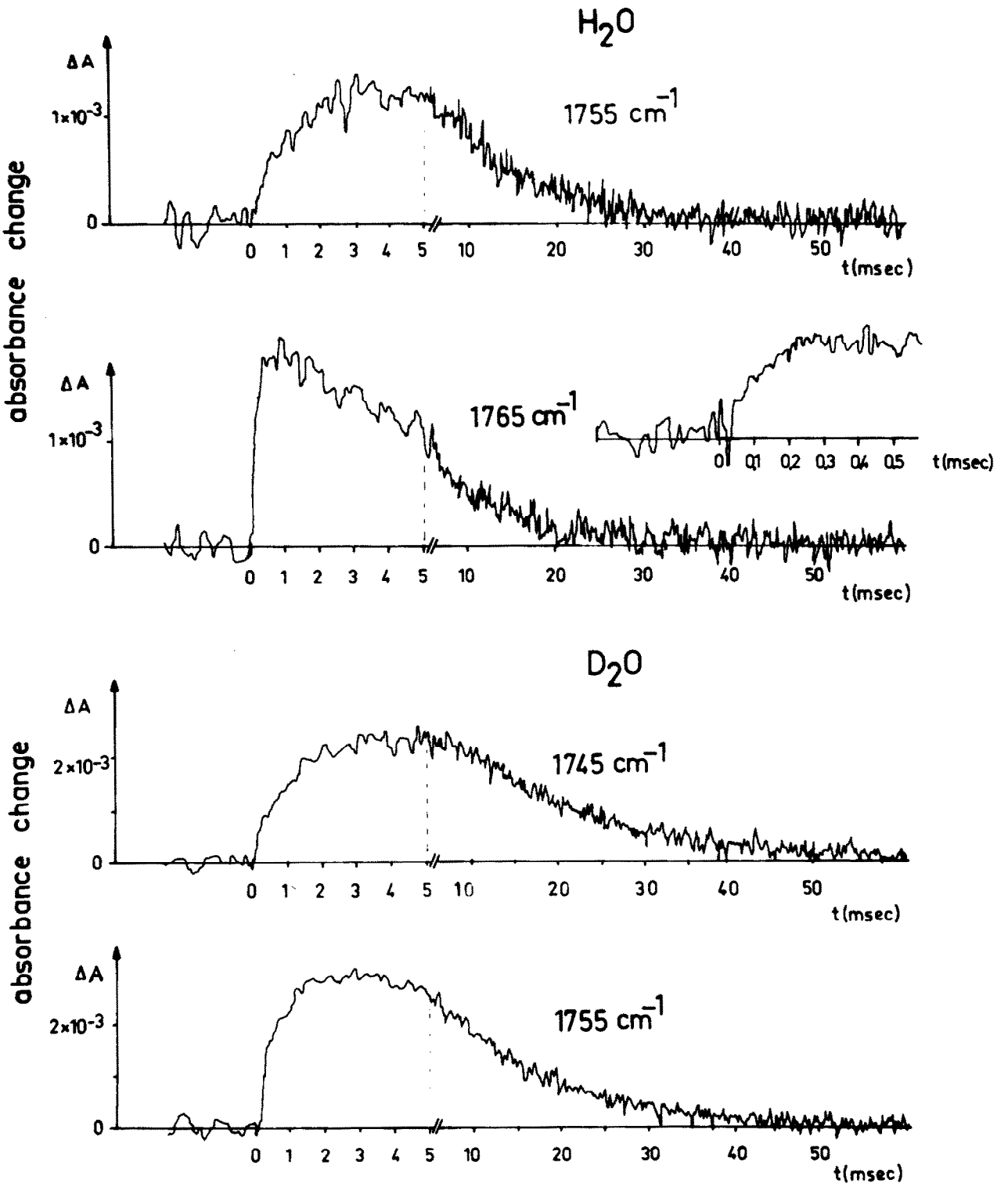

Fig.1. Kinetic signals of a purple membrane film in the hydrated and deuterated state: $T = 20°C$, the sample is excited at $t = 0$ with a Xenon flash at wavelengths > 530 nm; 100 signals were averaged to improve the signal-to-noise ratio. Note change in time base at 5 ms. Insert at 1765 cm^{-1} shows time-resolved absorbance rise.

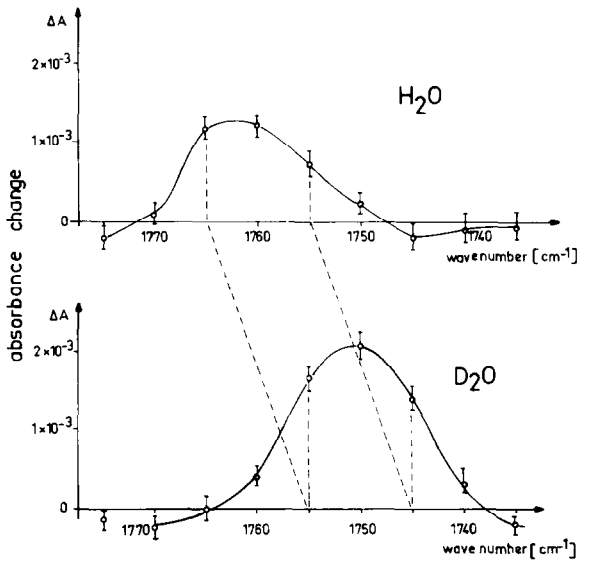

Fig.2. Difference spectra derived from the maximum absorbance change of kinetic signals over 1775–1735 cm^{-1}; (– – –) signals in fig.1.

The difference spectrum for a hydrated and a deuterated sample, representing the maximum absorbance changes, are shown in fig.2. The absorbance changes were normalized to the absorbance changes at 1525 cm^{-1}, representing the amount of BR photolysed [12]. The band is asymmetric, exhibiting a shoulder at the low wavenumber side. Upon deuteration the whole structure is shifted by 10 cm^{-1} to lower wavenumbers and the feature becomes more symmetric. Thereby the intensity of the band is increased. The changes caused by deuteration are a strong evidence that the band is due to the C=O vibration of carboxylic acids [26]; the shoulder indicates that 2 different groups are involved. The signals in fig.1 therefore represent the protonation respectively the deuteration and subsequent dissociation of carboxylic acids. A closer investigation of the signals shows that the rise time at the shoulder is considerably longer as compared to the maximum of the band, especially in H_2O. The kinetic data obtained by a computer evaluation of the signals are given in table 1. The signals are fitted by a model, in which they represent the rise and decay of an intermediate in a consecutive reaction. The table also contains the kinetic data for the rise and decay of M412. The rise of the signal at 1765 cm^{-1} is shown in an expanded time scale in the insert of fig.1. From these data, especially from the same influence of deuteration on the rise of

Table 1
Kinetic data of the signals shown in fig.1 and of the M412-intermediate measured under the same conditions

20°C		$\tau_{1/2}$ (rise)	$\tau_{1/2}$ (decay)
H_2O	1755 cm^{-1}	1.2 ms	7 ms
	1765 cm^{-1}	60 μs	5.5 ms
	M412	60 μs	6 ms
D_2O	1745 cm^{-1}	1.4 ms	11 ms
	1755 cm^{-1}	450 μs	10 μs
	M412	400 μs	12 ms

M412 and the rise of the signal at 1765 cm^{-1}, it can be deduced that the carboxylic group with the band at higher wavenumbers is protonated and re-deprotonated simultaneously with the formation and decay of M412. The different rise times corroborate the spectral finding, that two different kinds of groups are protonated. The decay times, however, coincide within experimental error. The low intensity of the band at lower wavenumbers can be partly explained by the slow rise time, which leads to a reduced measured maximum amplitude of the signal. (The computer evaluation gives a difference of 30% between the apparent and the calculated amplitudes.) This effect also explains the more symmetric feature of the spectrum measured in D_2O.

For further characterization the absorbance changes were measured at different pH and temperature. Over the pH-range investigated (3.5–9.5), no influence on the amplitude of the signals was observed. At pH < 3.5, the formation of the blue BR inhibited the signals but also inhibited the formation of M412. Between 5–25°C no amplitude changes were observed, if corrected for kinetic effects. To exclude the possibility of the involvement of the 4 carboxylic residues at the C-terminus [1,2], purple membranes were treated with pronase, which cleaves off the C-terminus [27]. The amplitudes were not affected; the decay kinetic was slowed down by a factor of ~2, as also observed for the decay of M412.

The above data provide evidence that the spectral changes described correspond to the protonation and deprotonation of carboxylic groups. To conclusively prove this finding it would be desirable to demonstrate changes corresponding to the disappearance of the ionic form. Ionized carboxylic acids show 2 strong bands over 1300–1420 cm^{-1} and 1500–1610 cm^{-1} [28]. In addition to the variability of the band posi-

tion, the presence of other bands makes the assignment difficult. In the spectral regions under consideration we observed changes with corresponding kinetics [12], which may be interpreted as the disappearance of ionized carboxylic groups. Without additional evidence, however, the assignment remains uncertain.

Two conclusions can be drawn from the position of the two bands at 1765 cm^{-1} and 1755 cm^{-1}:

1. The pK of the groups must be ~2.5 [29];
2. The carboxylic groups do not form hydrogen bonds which would shift the bands to lower wavenumbers. Indeed, the positions almost coincide with those of gaseous monomeric acids [28].

It cannot be established with certainty that only 2 different carboxylic groups/BR-molecule are represented by the difference spectrum (fig.2). Band intensities vary considerably for carboxylic acids, depending on compound and environment. It is noteworthy, however, that under the experimental conditions applied, identical band intensities were observed. This shows that a fixed stoichiometric ratio prevails between the amount of BR photolysed and the amount of carboxylic groups protonated. A comparison of the band intensity of the C=C vibration of protonated retinal-Schiff base model compounds [13] with that of the C=O vibration of carboxylic acids indicates that the assumption, that only two carboxylic groups are protonated, is reasonable. Therefore, this assumption will be maintained.

Despite of the low pK-value, the groups are protonated up to a pH $\geqslant$ 9.5. This indicates that both groups are protonated from intramolecular donors. Since they are not located at the C-terminus and do not form hydrogen bonds, the acid residues must be located in the inner part of BR. The low rate constant for deprotonation supports this view: If the deprotonation were a diffusion-controlled process in a homogeneous aqueous solution, a rate constant of $10^{10} \times 10^{-pK}$, i.e. $10^{7.5}\,s^{-1}$, would have been observed. Taking the fit of the amino acid sequence into the 7 helices according to [5] and lysine 216 as the retinal binding site [7–9], the most plausible candidates for the carboxylic acid residues are aspartate 85 and 96 on helix C and aspartate 212 as well as glutamate 204 on helix G.

In the further discussion the 2 groups will be treated separately. The most straightforward interpretation of the spectral changes at higher wavenumbers and with the M412 kinetics is that the corresponding

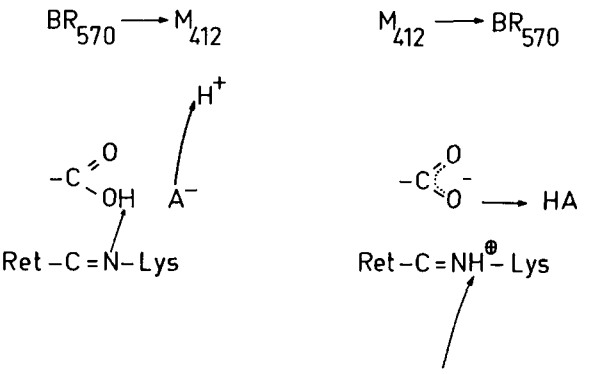

Fig.3. Schematic representation of the molecular steps involving carboxyl group protonation (BR570 → M412) and deprotonation (M412 → BR570). *Abbreviations:* Ret, retinal; Lys, retinal-binding lysine

carboxylic group represents the direct proton acceptor for the deprotonation of the Schiff base. Assuming that one of the protons pumped per photolysed BR is transferred via the Schiff base, the observation that proton ejection occurs on a time scale comparable to that of the rise of M412 requires the presence of an intermediate protonable group between the carboxylic group and the external medium. A model for the arrangement is shown in fig.3. The intermediate group A is protonated in BR570 and deprotonated upon protonation of the carboxylic group, i.e., deprotonation of the Schiff base. It is reprotonated by the deprotonation of the carboxylic group, which, in this model, would be located on the protein ejection side. Whereas the location on the proton uptake side, cannot be excluded altogether, the observation, that the group with pK ~ 2.5 is protonated at pH 9.5, makes it unlikely. The model implies that the pK of the Schiff base in M412 is very low, and also that deprotonation of the carboxylic group induces reprotonation of the Schiff base, which in BR570 has pK ~ 12 [38,39].

The interpretation of the slow component at lower wavenumbers is more difficult. The lower intensity of the corresponding band can partly be explained by kinetic arguments: the low rise time reduces the apparent amplitude. Also, intensity variations are frequently observed for carboxylic acids. One might assume that the slow component is correlated with the O640 intermediate, which also has a slow rise time. A closer examination of the corresponding time course [30], however, shows considerable differences. Further, while O640 increases at low pH and high temperature,

there are no amplitude changes for the carboxylic group. Even BR recombined with dimyristoyllecithin (kindly provided by N. Dencher, Biozentrum, Basel), still exhibits the protonation signals, although it does not produce an O640 signal below or above the lipid phase transition. The rate constant for the slow protonation is not observed in chromophoric transitions. This gives rise to the question regarding the trigger for the slow protonation. Since, at the time scale of interest, there is no delay corresponding to the M412 transition, M412 cannot be the trigger. Such a delay would have certainly been detected in measurements in D_2O, because the rise time of M412 approximately equals that of the slow component. Therefore, the protonation must be triggered at an earlier stage of the photocycle. A possible molecular event could be the postulated deprotonation of a tyrosine [31,32], which occurs between the formation of L550 and that of M412. This implies, however, that tyrosine cannot be the direct donor for the slow group. It is interesting to note that kinetics corresponding to the slow protonation have been observed at other wavenumbers [12]. Their interpretation requires further experiments. The slow carboxylic group may be involved in the mechanism for the pumping of an additional proton [33,34]. In addition to fast proton ejection [35,36], also a slower process has been observed [33].

In the model discussed above, the carboxylic group with the higher C=O-frequency could act as the counter-ion for the protonated Schiff base. Its protonation might induce the blue BR, for which a titration curve with $pK \sim 2.5$ has been measured [21]. The question on the mechanism of the pK-reduction of the Schiff base still remains open in this discussion. By demonstrating the participation of other specific amino acid chains, kinetic infrared spectroscopy may help to clarify this point. The use of isotopic labelling will serve as an important tool to interpret the spectra.

The protonation of carboxylic acids has also been demonstrated in [37] applying Fourier transform infrared difference spectroscopy. The decay of M412 was inhibited by using dry films of BR. In this way the BR570 – M412 difference spectrum was measured. Although there are many differences in this spectrum compared to our kinetic infrared BR570 – M412 difference spectrum [12], the agreement regarding the protonation of carboxylic groups is excellent. It is remarkable that protonation can also be observed in a dry film, where no protons can be exchanged with the external medium. This supports our assumption, that internal donors protonate the carboxylic groups.

Acknowledgements

We are grateful to Dr K. P. Hofmann for valuable discussions to Mrs S. El-Deeb for technical assistance, to Mrs W. Herbst for help in the preparation of the manuscript, and to Mr J. Spiegler and Mr Ch. Thies for their assistance in the preparation of pronase treated purple membranes.

References

[1] Ovchinnikov, Yu. A., Abdulaev, N. G., Feigina, M. Yu., Kiselev, A. V. and Lobanov, N. A. (1979) FEBS Lett. 100, 219–224.
[2] Khorana, H. G., Gerber, E. G., Herlihy, W. C., Gray, Ch. P., Anderegg, R. J., Nihei, K. and Biemann, K. (1979) Proc. Natl. Acad. Sci. USA 76, 5046–5050.
[3] Henderson, R. and Unwin, P. N. T. (1975) Nature 257, 28–32.
[4] Hayward, S. B. and Stroud, R. M. (1981) J. Mol. Biol. 151, 491–517.
[5] Engelman, D. M., Henderdon, R., McLachland, A. D. and Wallace, B. A. (1980) Proc. Natl. Acad. Sci. USA 77, 2023–2027.
[6] Engelman, D. M. and Zaccai, G. (1980) Proc. Natl. Acad. Sci. USA 77, 5894–5898.
[7] Bayley, H., Huang, K.-S., Radhakrishnan, R., Ross, A. H., Takagaki, Y. and Khorana, H. G. (1981) Proc. Natl. Acad. Sci. USA 78, 2225–2229.
[8] Lemke, H.-D. and Oesterhelt, D. (1981) FEBS Lett. 128, 255–260.
[9] Mullen, E., Johnson, A. H. and Akhtar, M. (1981) FEBS Lett. 130, 187–193.
[10] Mathies, R. (1979) Chem. Biochem. Appl. Lasers (Moore, C. B. ed) vol. 4, pp. 55–99, Academic Press, London, New York.
[11] Siebert, F., Mäntele, W. and Kreutz, W. (1980) Biophys. Struct. Mech. 6, 139–146.
[12] Siebert, F., Mäntele, W. and Kreutz, W. (1981) Can. J. Spectrosc. 26, 119–125.
[13] Siebert, F. and Mäntele, W. (1980) Biophys. Struct. Mech. 6, 147–164.
[14] Mäntele, W. and Siebert, F. (1980) Biophys. Struct. Mech. 6 suppl., 124.
[15] Honig, B., Ebrey, T. G., Callender, R. H., Dinur, U. and Ottolenghi, M. (1979) Proc. Natl. Acad. Sci. USA 76, 2503–2507.
[16] Warshel, A. (1979) Photochem. Photobiol. 30, 285–290.
[17] Herz, J. M. and Packer, L. (1981) FEBS Lett. 131, 158–164.
[18] Favrot, J., Leclercq, J.-M., Roberge, R., Sandorfy, C. and Vocelle, D. (1979) Photochem. Photobiol. 29, 99–108.

[19] Warshel, A. (1978) Proc. Natl. Acad. Sci. USA 75, 2558–2562.
[20] Warshel, A. and Ottolenghi, M. (1979) Photochem. Photobiol. 30, 291–293.
[21] Fischer, U. and Oesterhelt, D. (1979) Biophys. J. 28, 211–230.
[22] Becher, B. M. and Cassim, J. Y. (1975) Prep. Biochim. 5, 161–178.
[23] Mäntele, W., Siebert, F. and Kreutz, W. (1982) Methods Enzymol. in press.
[24] Lozier, R. and Niederberger, W. (1977) Fed. Proc. FASEB 36, 1805–1809.
[25] Becher, B. and Ebrey, T. G. (1977) Biophys. J. 17, 185–191.
[26] Pinchas, S. and Laulicht, I. (1971) Infrared Spectra of Labelled Compounds, Academic Press, London, New York.
[27] Gerber, G. E., Gray, Ch. P., Wildenauer, D. and Khorana, H. G. (1977) Proc. Natl. Acad. Sci. USA 74, 5426–5430.
[28] Bellamy, L. J. (1957) The Infrared Spectra of Complex Molecules, 2nd edn, Methuen, London.
[29] Bellamy, L. J. (1968) Advances in Infrared Group Frequencies, vol. 2, Chapman and Hall, London.
[30] Mäntele, W., Siebert, F. and Kreutz, W. (1981) FEBS Lett. 128, 249–254.
[31] Bogomolni, R. A., Stubbs, L. and Lanyi, J. K. (1978) Biochemistry 17, 1037–1041.
[32] Hess, B. and Kuschmitz, D. (1979) FEBS Lett. 100, 334–340.
[33] Govindjee, R., Ebrey, T. G. and Crofts, R. (1980) Biophys. J. 30, 231–242.
[34] Renard, M. and Delmelle, M. (1980) Biophys. J. 32, 993–1006.
[35] Lozier, R. H., Niederberger, W., Bogomolni, R. A., Hwang, S.-B. and Stoeckenius, W. (1976) Biochim. Biophys. Acta 440, 545–556.
[36] Ort, D. R. and Parson, W. W. (1978) J. Biol. Chem. 253, 6158–6164.
[37] Rothschild, K. J., Zagaeski, M. and Cantore, W. A. (1981) Biochem. Biophys. Res. Commun. 103, 483–489.
[38] Ehrenberg, B., Lewis, A., Porta, T. K., Nagle, J. F. and Stoeckenius, W. (1980) Proc. Natl. Acad. Sci. USA 77, 6571–6573.
[39] Doukas, A. G., Pande, A., Suzuki, T., Callender, R. H., Honig, B. and Ottolenghi, M. (1981) Biophys. J. 33, 275–280.