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RESONANCE RAMAN SPECTROSCOPY OF CHEMICALLY MODIFIED AND ISOTOPICALLY LABELLED PURPLE MEMBRANES

II. KINETIC STUDIES

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Summary

Kinetic resonance Raman spectra of native and isotopically labelled purple membranes are compared. Using these data and the assignments of the previous paper in this sequence, we have confirmed that the Schiff base is deprotonated at times that are short in comparison to M_{412} evolution. In addition, by monitoring the kinetic resonance Raman spectra in ^2H_2O with 488.0 nm excitation we have been able to characterize in more detail the vibrational features associated with this unprotonated intermediate that precedes M_{412}. Furthermore, the kinetic spectra of fully deuterated purple membranes in H_2O have allowed us to assign the 1465 cm^{-1} band in these spectra to the C=C stretching frequency of BR_{570} and the 1512 cm^{-1} band to the C=C stretching frequency of M_{412}. These spectra have also provided an indication of a Raman spectral feature associated with O_{640} and, finally, our kinetic spectra have provided evidence that there is a significant alteration in the rate constants for the evolution of the various intermediates when the non-exchangeable protons on the membrane are replaced by deuterons.

Introduction

This investigation uses the assignments of the C=N and C=$\overset{\oplus}{N}$-H vibrational modes in bacteriorhodopsin that have been made in the previous paper in this

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Abbreviation: BR, bacteriorhodopsin.

sequence [1] to monitor the rate of Schiff-base deprotonation in native and isotopically labelled membranes. In addition, correlations are made between the observed rate of deprotonation and changes in other regions of the resonance Raman spectrum of purple membrane. These correlations were aided by recording kinetic resonance Raman spectra as a function of different laser excitation wavelengths to enhance the spectral features of specific intermediates in the proton pumping cycle. The analysis of these data lend further support to the existence of an additional unprotonated intermediate X and pinpoints specific alterations in the structurally sensitive fingerprint region of the resonance Raman spectra.

Experimental

The method of Kanner and Racker [2] was used to grow *Halobacterium halobium* (S-9) cells and to isolate the fully protonated purple membrane. These purple membrane suspensions were washed 3—4 times with a 1% solution of deoxycholic acid to free the membrane fragments of carotenoid contamination. The purity of these samples was evident by the absence of Raman bands of 1001 cm^{-1}, 1157 cm^{-1} and 1515 cm^{-1} with all excitation frequencies except 488.0 nm. These bands are characteristic of β-carotene [3]. The fully deutereated purple membranes were grown at Argonne National Laboratories by established procedures [4]. The purity of these samples was evident by the absence of the strong band at 1303 cm^{-1} which is characteristic of fully deuterated carotenoids [5]. The purple membrane suspensions had concentrations $\sim 2.5 \cdot 10^{-5}$ M, based on an extinction coefficient $\epsilon_{570} = 6.3 \cdot 10^4$ $mol^{-1} \cdot cm^2$. For ^2H_2O suspensions 99.8 atom% ^2H_2O (Aldrich) was used.

The kinetic resonance Raman spectra were obtained in the following way. The purple membrane suspensions were flowed by a variable speed pump (Micropump, model No. 14-21-303), through a closed system of tubing which included a capillary tube of known internal diameter. A laser beam was then focused on the capillary to a known diameter, so that from the bulk rate of flow the average time a molecule spent in the illuminated area could be calculated. The laser lines used were 457.9 nm and 488.0 nm of an Ar^+ laser and 568.2 nm of a Kr^+ laser. The laser beam initiated the photochemical cycle of bacteriorhodopsin and also excited the Raman spectrum. The Raman scattering was collected through a Spex 1401 monochromator and detected by a cooled RCA C31034 photomultiplier tube followed by home built photon-counting electronics, and the data were processed on a Modcomp II computer. The spectra were obtained with steps and resolution of 1 cm^{-1} or 2 cm^{-1} and a counting time of 10—20 s per channel.

Results and Discussion

The intermediate states between BR_{570} and M_{412}: Verification of species X

Kinetic absorption spectra have suggested that the only intermediate between K_{610} decay and M_{412} formation was L_{550} [6,7]. However, previous work from our laboratory using KiRRS of bacteriorhodopsin at pH = 7.0 indicated that the Schiff-base deprotonation proceeded at a rate which is faster

than M_{412} production and slower than the rise of the L intermediate [8]. This suggested the existence of another intermediate with an unprotonated Schiff base. However, this conclusion was based on the appearance of a band at 1620 cm^{-1} at times which were earlier than the detection of the C=C stretching frequency of M_{412} at 1566 cm^{-1}. Two questions remain about this conclusion. Firstly, is the band at 1620 cm^{-1} associated with the carbon-nitrogen stretch of an unprotonated Schiff base? Secondly, is the 1620 cm^{-1} band detected at early times associated with the same vibrational mode detected at later times and in steady state? The first of these questions has been answered in the previous paper of this sequence [1]. Indeed the steady state spectra of isotopically labelled membranes do indicate that the steady state 1620 cm^{-1} band is associated with the unprotonated Schiff base. The second question is addressed in this paper by comparing the kinetics of isotopically labelled membranes to KiRRS spectra of fully protonated purple membranes.

Let us establish at the beginning of this comparison the characteristics of the kinetic resonance Raman spectra of fully protonated membranes. In Fig. 1A and B kinetic spectra of such membranes obtained with 568.2 nm and 457.9 nm excitation are compared in the carbon-carbon and carbon-nitrogen stretching region. It is obvious from the 457.9 nm spectra in this figure that a band at 1620 cm^{-1} is detected when there is only weak scattering of the normally strong 1566 cm^{-1}, M_{412} C=C stretching frequency. This was the sole basis of our first suggestion of an additional unprotonated intermediate in the proton

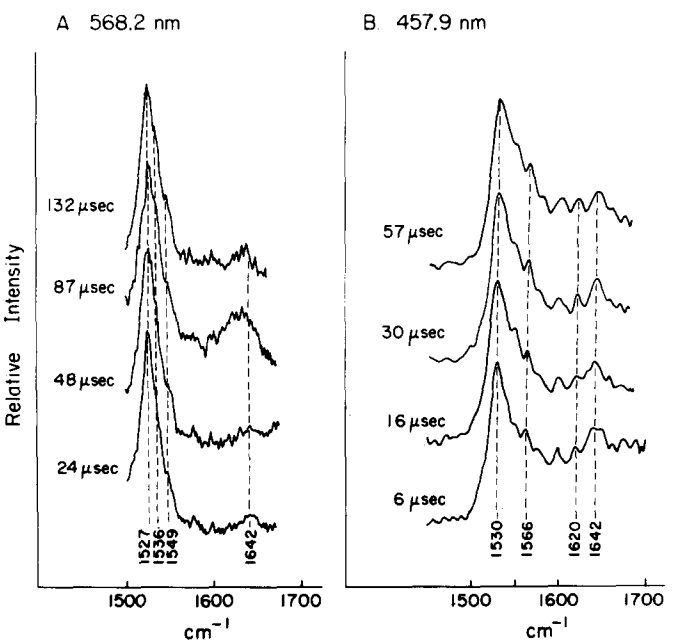

Fig. 1. Kinetic resonance Raman spectra of bacteriorhodopsin in H_2O suspension, obtained with 50 mW of 568.2 nm (A) and 457.9 nm (B) excitation, with various laser beam transit times.

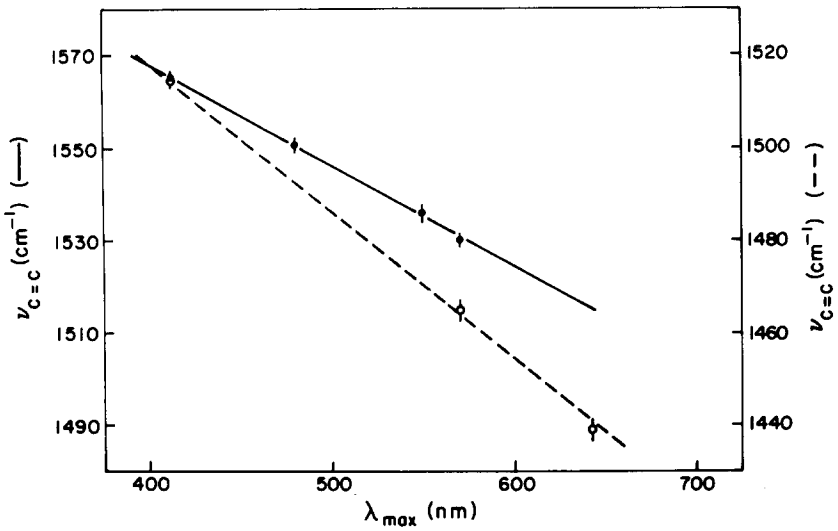

Fig. 2. Linear correlation plots of $\nu_{C=C}$ vs. λ_{max} for various species in the photochemical cycle of native (solid line) and fully deuterated (dashed line) bacteriorhodopsin.

pumping cycle [8]. This suggestion was further confirmed [9] by the detection of multiple C=C stretching frequencies occurring with different evolution times and different resonance enhancement characteristics. This observation can also be seen by comparing Fig. 1A and B. As can be seen in Fig. 1A, the 568.2 nm spectra have bands in the C=C stretching region at 1530 cm^{-1}, 1535 cm^{-1} and 1549 cm^{-1}. On the other hand, the 457.9 nm spectra (Fig. 1B) have bands at 1530 cm^{-1}, 1550 cm^{-1} and 1566 cm^{-1}. It has been suggested [10] that these vibrational frequencies are correlated with the visible absorption maximum of the chromophore. In fact, as is seen in Fig. 2, a correlation can be established between visible absorption and the above C=C stretches if the 1530 cm^{-1} and 1566 cm^{-1} bands are associated with the BR_{570} and M_{412} as has been suggested [11]. This correlation associates the 1536 cm^{-1} band with an absorption maximum at 550 nm in good agreement with the intermediate L_{550}, and the same correlation suggests that the 1550 cm^{-1} band arises from a species with an absorption at ~470 nm which we call $X_{\sim 470}$. It should be noted in this regard that long transit times are required to detect L_{550}, since the C=C stretch of this intermediate overlaps strongly with the intense 1530 cm^{-1} C=C stretch of BR_{570}. Thus, BR_{570} has to be significantly depleted before the 1536 cm^{-1} band of L_{550} can be observed. This is in agreement with the calculations of Marcus and Lewis [9]. On the other hand, the 1550 cm^{-1} band in Fig. 1 occurs at a position where initially no strong Raman features are observed and, therefore, its time evolution is more accurately represented in these kinetic spectra. In fact, Fig. 1B clearly shows that the 1550 cm^{-1} mode occurs at times that are faster than M_{412} and slower than L_{550} production. Thus it is logical to assume that the 1550 cm^{-1} band is associated with the unprotonated intermediate suggested in the experiments of Marcus and Lewis [8].

With this summary before us for comparison, let us now turn to the kinetic Raman spectra of fully deuterated purple membrane in H_2O suspensions at

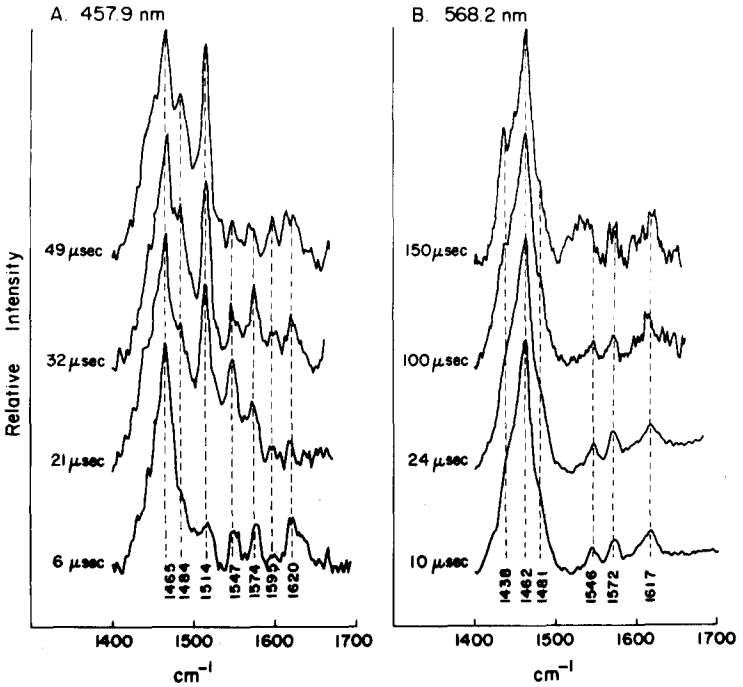

Fig. 3. Kinetic resonance Raman spectra of fully deuterated bacteriorhodopsin in H_2O suspension, obtained with 50 mW of 457.9 nm (A) and 568.2 nm (B) excitation, with various laser beam transit times.

pH 7, which are shown in Fig. 3. Before discussing these spectra, let us recall from the previous paper in this sequence [1] that the steady state C=N stretch and C=N-H stretch in these membranes occurs at 1595 cm^{-1} and 1620 cm^{-1}, respectively. Once again, as in the fully protonated membrane, the 457.9 nm kinetic spectra of fully deuterated bacteriorhodopsin demonstrate that at times preceding significant accumulation of M_{412}, e.g., 6 μs, (as detected by only the extremely weak scattering of a band at 1514 cm^{-1}, the proposed C=C stretching frequency of M_{412}), the above mentioned C=N stretch band at 1595 cm^{-1} is already present. This adds strong support to the existence of an unprotonated species at early times, since other modes in this fully deueterated system are altered relative to fully protonated membranes by frequency shifts which are very different from those observed for the C=N stretch.

It should be pointed out that, unlike the assignments of the relatively localized end group vibrations in the deuterated membranes, identification of other vibrational modes in the spectrum with specific intermediates are more difficult. For example, even in the region of the intense stretching vibrations the only assignment that can be made with certainty is the 1465 cm^{-1} C=C stretch of fully deuterated BR_{570} in H_2O. In addition to this assignment it appears likely that the band at 1514 cm^{-1} can be assigned to M_{412} in the fully deuterated system. This is based on its intensity at long times and its disappearance with long wavelength excitation (compare Fig. 3A and B). However, its rate of appearance is considerably greater in this fully deuterated system in H_2O in which all exhangeable deuterons are replaced by protons leaving only the non-

exchangeable deuterons. This increase in reaction rate of this system is not understandable, since deuterons would normally be expected to slow down such molecular processes. Using the above assignments as a starting point, a new linear correlation of λ_{max} vs. $\nu_{C=C}$ can be established for fully deuterated purple membrane (see Fig. 2). This linear correlation has a slope which is different (increased) from the slope of the linear correlation established for fully protonated membranes. This is understandable since the C=C vibrational frequency is related to force constant by $m^{-1/2}$ where m is the reduced mass. Thus, we now attempt to assign the other bands in the C=C stretching region with the above correlation as a tentative guide.

With 568.2 nm excitation the only other well resolved band in the C=C stretching region appears at 1438 cm^{-1}, which is lower in frequency than the BR_{570} C=C stretch. Although deuterated carotenoids have a C=C stretching mode in this region [5], our data indicate that the 1438 cm^{-1} band is present even after exhaustive washings with deoxycholic acid which eliminates other bacterial carotenoid Raman bands [9]. In addition, the 1438 cm^{-1} band is present even at 568.2 nm excitation and does not exist at 457.9 nm excitation, while carotenoids exhibit the opposite resonance Raman enhancement. Thus, the intense 1438 cm^{-1} band is associated with a purple membrane intermediate and its lowered frequency, relative to the BR_{570} C=C stretch, indicates that this band is associated with an intermediate exhibiting increased electron delocalization in the retinal.

Such increased electron delocalization has always been associated in previous examples of polyene Raman spectra with a longer wavelength visible absorption. If indeed the linear correlation we have noted for deuterated systems is valid, then this C=C stretching frequency corresponds to a λ_{max} of 640 nm. Previous kinetic absorption spectroscopy of purple membranes have detected two intermediates with absorption maxima red shifted relative to BR_{570}. One of these intermediates is the primary photochemical product K which absorbs maximally at 610 nm [6] and is produced in ps [12] and decays in 2 μs [6] at room temperature. Our kinetic spectra clearly rule out this intermediate. The other long wavelength absorbing species is called O [6] and absorbs maximally at 640 nm and is the last intermediate produced in the proton pumping cycle. It appears likely that this is the intermediate which gives rise to the 1438 cm^{-1} band. Once again, as in the case of M_{412}, the rate of appearance of this band is significantly increased in the fully deuterated membranes. It is interesting that the 1438 cm^{-1} band is also present in stationary spectra of these deuterated membranes excited with 568.2 nm radiation (unpublished observations). This suggests that in the deuterated system, unlike the naturally occurring membranes, significant concentrations of O are accumulated in the photostationary mixture.

All of the C=C stretches discussed thus far in both protonated and deuterated systems can be explained in terms of known intermediates and the linear correlation of C=C stretching frequency versus the intermediate absorption maximum. However, there is one vibrational mode in this region at 1484 cm^{-1} which cannot be rationalized using the above criteria. This band occurs in the kinetic spectra but is absent in variable temperature (95—210 K) steady state spectra we have obtained. In these variable temperature spectra bands at

1478 cm^{-1} and 1495 cm^{-1} were observed which could be correlated with the intermediates L and X, respectively [13]. The absence of these bands and the presence of the 1484 cm^{-1} band in the kinetic spectra are at present unexplainable.

Kinetic resonance Raman spectra of native membranes in ^2H_2O chromophore structural alterations preceding M formation

We now attempt to further characterize other regions of the kinetic resonance Raman spectrum of native purple membranes and correlate the kinetic changes in the spectral features with chromophore conformational alterations in the intermediates preceding M formation. In order to observe the vibrational spectra of these intermediates in more detail we slowed down the rate of formation of M by resuspending the fully protonated membranes in ^2H_2O [14] and spectra of such suspensions obtained with 457.9 nm and 488.0 nm excitation are shown in Fig. 4A and B. Notice that relative to the kinetic spectra of native membranes in H_2O obtained with 457.9 nm excitation (see Fig. 1B) the rate of appearance of the M_{412} C=C stretch at 1566 cm^{-1} is significantly decreased in ^2H_2O (see ^2H_2O 457.9 nm excitation, Fig. 4A). Thus, we now probe the vibrational spectrum of this ^2H_2O suspension with 488.0 nm excitation to attempt to excite the resonance Raman spectrum of $X_{\sim 470}$.

As can be seen in the 488.0 nm spectra (Fig. 4B), the 1566 cm^{-1} M_{412} C=C stretch is strongly suppressed and a band at 1550 cm^{-1} which correlates with an absorption at 470 nm is clearly observed. This C=C stretch in the 457.9 nm spectra in H_2O appeared to follow the rise of the 1620 cm^{-1} C=N stretch. Thus, in these 488.0 nm kinetic spectra we appear to have excluded M_{412}, and based on the observed C=C stretches our spectra seem to be composed mainly of BR_{570} and $X_{\sim 470}$. Although, contributions from L_{550} should also be reflected in these spectra.

Two changes in the 488.0 nm ^2H_2O kinetic spectra can be noted when these

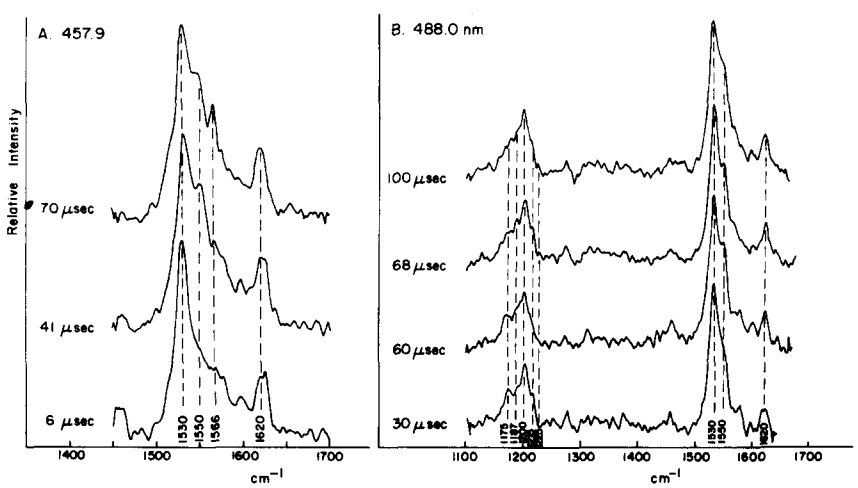

Fig. 4. Kinetic resonance Raman spectra of bacteriorhodopsin in ^2H_2O suspension, obtained with 50 mW of 457.9 nm (A) and 488.0 nm (B) excitation, with various laser beam transit times.

data are compared to the spectra of BR_{570}. Firstly, an increase in the 1187 cm^{-1} band intensity relative to the 1200 cm^{-1} and the 1175 cm^{-1} bands is observed, and secondly, a band evolves at 1225 cm^{-1} which is not present in spectra of BR_{570} but is clearly and repeatably observed in all spectra taken with long transit times.

Although an unequivocal assignment can be made for the 1225 cm^{-1} band, as will be discussed below, the assignment of the relative change at 1187 cm^{-1} is partially in doubt since kinetic resonance Raman spectra of L_{550} evolution demonstrate that this relative intensity alteration is also a characteristic of L_{550} [9]. Therefore, it is not completely clear whether the observation of such an alteration in our 488.0 nm ^2H_2O spectra is the result of contributions from X or L or both. However, the close correlation of the rate of appearance of this band with the X_{1550} cm^{-1} C=C stretch (see Fig. 4B) clearly suggests that the 1187 cm^{-1} band is also a feature of X.

The 1225 cm^{-1} band appears not only in these ^2H_2O spectra but also in the spectra of M_{412} [9]. However, it can unequivocally be assigned to X_{470} as well. This is based on our exclusion of M_{412} in the ^2H_2O 488.0 nm spectra and the absence of this band in the 568.2 nm kinetic spectra of L_{550} measured by Marcus and Lewis [9]. Even though these workers used H_2O suspensions, we have recorded identical 568.2 nm kinetic resonance Raman spectra in ^2H_2O. The lack of a 1225 cm^{-1} band in L and the exclusion of M in the ^2H_2O spectra is another indication of the presence of an intermediate between L and M. In addition to this evidence, further support for an intermediate between L and M comes from comparing the significant decrease in the rate of appearance of M_{412} in ^2H_2O with the undetectable effect of ^2H_2O on the 568.2 nm kinetic resonance Raman spectra of L_{550} evolution. * The presence of the 1225 cm^{-1} band in this intermediate we have called X, gives additional support to our suggestion (based on the rate of appearance of the C=N stretch at 1620 cm^{-1}) that X is the first unprotonated intermediate, since a band at this frequency is a characteristic of unprotonated retinal Schiff bases [15] and, as a result, is also observed in M_{412}.

In summary, these data clearly support the existence of another unprotonated intermediate between L_{550} and M.

Acknowledgements

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References

1 Ehrenberg, B., Lemley, A.T., Lewis, A., Von Zastrow, M. and Crespi, H.L. (1980) Biochim. Biophys. Acta 592, 441—453
2 Kanner, B. and Racker, E. (1975) Biochem. Biophys. Res. Commun. 64, 1054—1061
3 Gill, D., Kilponen, R.G. and Rimai, L. (1970) Nature (London) 227, 743—744
4 King, G.I., Stoeckenius, W., Crespi, H.L. and Schoenbron, B.P. (1980) J. Mol. Biol., in the press

* We have obtained identical 568.2 nm kinetic spectra in ^2H_2O as were obtained by Marcus and Lewis in H_2O [9].

5 Rimai, L., Heyde, M.E. and Gill, D. (1973) J. Am. Chem. Soc. 95, 4493—4501
6 Lozier, R.H., Bogomolni, R.A. and Stoeckenius, W. (1975) Biophys. J. 15, 955—962
7 Kung, M.C., DeVault, D., Hess, B. and Oesterhelt, D. (1975) Biophys. J. 15, 907—911
8 Marcus, M.A. and Lewis, A. (1977) Science 195, 1328—1330
9 Marcus, M.A. and Lewis, A. (1978) Biochemistry 17, 4722—4735
10 Heyde, M.E., Gill, D., Kilponen, R.G. and Rimai, L. (1971) J. Am. Chem. Soc. 93, 6776—6780.
11 Lewis, A., Spoonhower, J., Bogomolni, R.A., Lozier, R.H. and Stoeckenius, W. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 4462—4466
12 Kaufmann, K.J., Rentzepis, P.M., Stoeckenius, W. and Lewis, A. (1976) Biochem. Biophys. Res. Commun. 68, 1109—1115
13 Marcus, M.A. (1978) Ph.D. Thesis, Cornell University
14 Korenstein, R., Sherman, W.V. and Caplan, S.R. (1976) Biophys. Struct. Mech. 2, 267—276
15 Cookingham, R.E., Lewis, A. and Lemley, A.T. (1978) Biochemistry 17, 4699—4711