Kinetic Resonance Raman Studies Reveal Different Conformational States of Bacteriorhodopsin†

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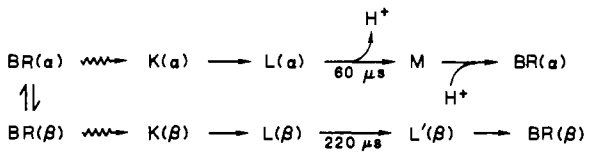
Received March 17, 1988; Revised Manuscript Received June 21, 1988

ABSTRACT: Kinetic resonance Raman (RR) experiments were designed to study the photoinduced cyclic reaction of the retinylidene Schiff base chromophore of bacteriorhodopsin (BR) which controls BR's function as a light-driven proton pump. Aqueous suspensions of BR-containing purple membranes of *Halobacteria* were used as samples. Time resolution in the RR experiment was achieved by flowing the sample through two parallel CW laser beams for pumping and probing. The time behavior of the two intermediates L-550 and M-412 of the photocycle was studied between 10 μs and 5 ms on the basis of their characteristic RR bands. It was found that L-550 decays with two different time constants (60 μs and ~220 μs at 23 °C). In the fast reaction channel a proton is released from the chromophore while in the slow reaction channel a new intermediate is formed whose chemical structure is only slightly different from that of its precursor L-550. The inhomogeneous kinetic behavior of L-550 is ascribed to two different conformational states ("α" and "β") of the apoprotein which control the different reaction pathways. It is argued that α and β already exist in the unphotolyzed species, giving rise to two different states, BR(α) and BR(β), of the chromoprotein which undergo light-induced reactions of the form



where L'(β) is the new intermediate which could be characterized by its RR spectrum. At 23 °C and pH 7.4 the population ratio of BR(α) and BR(β) is 1.4:1. It is suggested that BR(α) and BR(β) exist in a thermodynamic equilibrium whose time constant is much larger than the period of the cyclic reaction. The well-known biphasic decay of the M intermediate will also be discussed in terms of conformational substates of the apoprotein.

The retinal-binding protein bacteriorhodopsin (BR) which is found in the cell membrane of *Halobacteria* acts as a light-driven proton pump. The proton gradient that is established in this way is used by the cell as energy source to drive metabolic processes under anaerobic conditions. BR's unique function had first been elucidated by Oesterhelt and Stoeckenius (1973). Since then a considerable body of information was collected [for a review, see Stoeckenius and Bogomolni (1982)].

Light absorption initiates a cyclic reaction of the BR chromophore which runs through various intermediate states and finally is reconstituted within a few milliseconds (Figure 1). There is evidence that in the primary photochemical step of this reaction the retinal moiety isomerizes around the 13–14 double bond (Smith et al., 1985).

The linear reaction scheme in Figure 1 had first been suggested by Lozier et al. (1975) on the basis of optical transient- and low-temperature spectroscopy. In this scheme the primary photointermediate K-590 runs through a linear sequence of thermally controlled reaction steps via intermediates L, M (N), O, before the parent chromophore BR-570 is reconstituted. However, this simple model does not fit all experimental data satisfactorily. Various modifications, therefore, were suggested in the literature (Ottolenghi, 1980; Stoeckenius & Bogomolni, 1982).

The light-induced reaction of BR controls its function as a proton pump. A fundamental problem will be in which way the reaction center at the chromophoric site is coupled to the protein environment to induce proton pumping. The resolution of this problem requires an intimate knowledge of time-dependent structural changes at the chromophoric site. In the present work we used resonance Raman spectroscopy (RR) to investigate the structure and kinetic behavior of BR chromophores [for a review, see Smith et al. (1985) and Stockburger et al. (1986)].

We focused our interest on the transition between the L and M intermediate in the cyclic reaction. During this step a proton is released from the SB group. This had stimulated the idea that the SB proton might initiate the pumping mechanism. Experimentalists therefore were looking for a time correlation between the formation of M-412 and the appearance of protons in the aqueous phase or at the surface of the membrane (Lozier et al., 1976; Ort & Parson, 1978; Li et al., 1984; Ehrenberg et al., 1984). Complementary to such experiments, models for the pumping mechanism were suggested in which the chromophore is considered as a light-driven molecular switch (Stoeckenius et al., 1978; Schulten & Tavan, 1978; Tavan & Schulten, 1986).

In a previous work we had developed time-resolved RR techniques that enabled us to study the L-to-M transition

† This work was supported in part by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 93.

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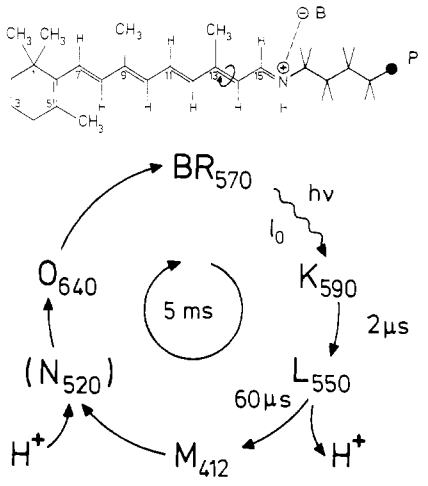

FIGURE 1: Photochemical cycle of bacteriorhodopsin as suggested by Lozier et al. (1975). The parent, BR-570, and the intermediates are designated by capitals BR, K, L, M, N, and O. The lower labels give the wavelengths of maximum absorption. The thermal decay constants refer to room temperature and neutral pH. The upper part shows the retinylidene Schiff base chromophore in all-trans form as found in the parent BR-570.

between a few microseconds and a few milliseconds (Alshuth & Stockburger, 1986). It turned out that this reaction step cannot be described correctly by a linear decay of L to M as proposed in the reaction scheme of Figure 1. In the present study it was found that L decays with two time constants. The question was if this is related to an inhomogeneous structure of the L chromophore or of the protein envelope.

The RR spectra of BR-570 exhibit a rich manifold of characteristic vibrational bands (Stockburger et al., 1986). The strongest one, the so-called ethylenic band, mainly consists of symmetric stretching motions along the central C=C double bonds of the retinal chain (Smith et al., 1985). Its frequency, intensity, and shape depend very sensitively on the geometry (cis–trans) of a chromophore and its electrostatic interaction with its environment, i.e., on its chemical structure as a whole. An inhomogeneous chemical distribution, therefore, would lead to an inhomogeneous structure in the ethylenic band region. The observation of a single Lorentzian in the case of the parent species BR-570 (Alshuth & Stockburger, 1986) allows the conclusion that, within narrow limits, the chemical structure of the chromophore is the same for all BR molecules. Within these limits only small deviations from a well-defined unique geometry of the chromophore may occur (Massig et al., 1985). From the RR spectra of intermediates of the photocycle (Figure 1) it is suggested that also in such states a unique structure of the chromophore in the whole ensemble is established.

The biological process in BR molecules is initiated in the chromophore and later carried into the protein envelope until a proton is released from the membrane. In the literature so far it was mainly accepted that the protein envelope like the chromophore has a homogeneous chemical structure in the whole ensemble of BR molecules. So it became usual to describe the state of the entire protein by the state of the chromophore alone. This point of view is clearly manifested by the assignment of the various states of the photocycle in Figure 1 which considers only the chromophore. In that notation the various states are distinguished by the absorption maxima of the chromophore. The capital letters, BR, K, L, M, and O, which are in general use are related to properties of the chromophore alone, like its temporal appearance during the cycle or its chemical structure. Such a description which is based on the assumption of a homogeneous structure of the protein envelope is misleading.

It may happen that structural variations occur at some sites in the protein envelope which are not "seen" in the RR spectra but which may change the reaction of the entire system dramatically. Thus the transition from L-550 to M-412 in which a proton-transfer reaction is involved may strongly be influenced by changes of the envelope structure.

In the analysis of our new data it was concluded that the ensemble of BR molecules has not a homogeneous structure. The initial state BR-570 mainly exists in two conformational substates of the envelope ("α" and "β"). These two species undergo different photoinduced cyclic reactions. While in the α-cycle the SB group is deprotonated during the L-to-M transition; this is not the case in the β-cycle. It will be discussed if the existence of these two species has some relevance for the biological function.

Evidently there is no limitation to only two conformational substates of the apoprotein. We believe that the biphasic decay of the M intermediate which is well established manifests the existence of additional substates that control the decay channel of M.

Materials and Methods

Raman Spectra. The RR spectra were recorded by a conventional spectrometer (Spex 14018 double monochromator, EMI 9659 QB photomultiplier, Ortec amplifier and counter). CW krypton and argon lasers (Coherent CR 2000 and CR 15) were used for pump and Raman-probe radiation. The spectrometer was controlled by a microcomputer (Apple IIe) in combination with a Spex compudrive (CD 2). The Raman signals were first collected in the memory of a counter device (Ortec 874) as photon counts per second for each spectral position. Overview spectra in the region between 700 and 1700 cm^{-1} were recorded with a step width of 1 cm^{-1}. For recording band shapes in a narrow spectral region a step width of 0.2 cm^{-1} was used. The dwell time at each step was 1 s. In all cases the spectral bandwidth was $\sim$4 cm^{-1}.

In order to use the unphotolyzed species as a spectral standard, it was necessary to record pump–probe and probe-only spectra quasi-synchronously. This was achieved by chopping the pump beam with a repetition rate which was large compared with the stepping rate of the spectrometer. By means of an optoelectronic control device the Raman signals from the pump–probe and probe-only mode were fed into different memories of the counter. After the experiment the data were transferred to computer systems for subtraction and band-fitting procedures.

Sample Preparation. Purple membranes (PM) were isolated from *Halobacterium halobium* cells as was described by Oesterhelt and Stoeckenius (1974). In most experiments a highly diluted aqueous suspension of PM in 50 mM phosphate buffer was used. Its optical density was $\sim$1.0 at 570 nm. Different pH values were established by adding NaOH or HCl. All experiments were carried out at room temperature ($\approx$23 °C). A few test measurements were performed with PM suspensions at high salt concentration (4 M NaCl).

Time-Resolved RR Experiments. Pump–probe experiments with two CW lasers in combination with a rotating cell were described in detail by Alshuth and Stockburger (1986). In the present study the same technique was used (see Figure 2).

When the cell, which in our experiments was completely filled with aqueous purple membrane suspension, is uniformly rotated, the liquid approaches the angular velocity of the cell. The pump beam with diameter $\bar{d}_P$ ($1/e^2$ points of the Gaussian profile) thus illuminates a cylindrically shaped liquid layer that

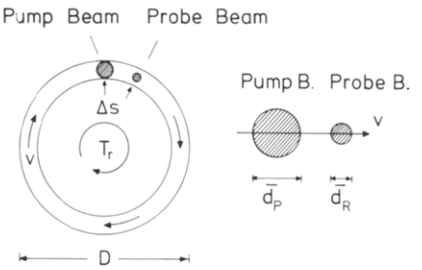

FIGURE 2: Layout of a pump–probe RR experiment using a spinning cell. Typical parameters were $v = 6$ ms^{-1}, $T_r = 20$ ms, $\bar{d}_P = 160$ μm, and $\bar{d}_R = 60$ μm.

moves with constant velocity v. The distribution of photoproducts across this layer follows the Gaussian intensity distribution of the laser beam. The Raman probe beam with diameter $\bar{d}_R$ ($\bar{d}_R \approx 0.3\bar{d}_P$) was set at a variable distance Δs from the pump beam which defines a delay time δ ($\delta = \Delta s/v$). For each distance Δs the Raman probe beam was moved across the liquid layer until a minimum in the RR signal of the unphotolyzed BR was reached which corresponds to maximum product formation. In this way pump and probe beam were aligned to each other in a reproducible way.

One advantage of the repetitive flow technique is that it allows one to sample the weak RR signals of intermediates in a quasi-continuous way. This requires that the parent species of BR is completely reconstituted when the circulating sample enters the pump beam again. For the rotating cell this means that the rotational period T must be large compared with the period τ_p of the photocycle.

When the pH is raised, τ_p increases significantly and it is difficult to fulfill the fresh-sample condition with a rotating cell so that the long-lived intermediates are accumulated in the illuminated liquid layer (Alshuth & Stockburger, 1986). In the present work we used this accumulation effect in experiments at elevated pH.

Typically, the diameters of the pump and probe beam were 160 and 60 μm, respectively. A sufficient degree of photolysis was obtained with pump powers between 300 and 600 mW at 647 nm (Alsuth & Stockburger, 1986). This line was used in order to avoid secondary photolysis of the L intermediate which only absorbs weakly at 647 nm. Various lines of an argon laser served as probe beams.

RR Spectra of L by Difference Formation and Band Fitting. The fraction of BR that can be converted into L in a single transit through the pump beam at 647 nm is limited to values between 30 and 40%. This is mainly due to photoreversal from the primary intermediate K (Alshuth & Stockburger, 1986). One therefore probes a mixture in which the unphotolyzed species is the dominating component. This has to be subtracted in order to obtain the pure L spectra.

The difference procedure is described in Figure 3 for the characteristic bands in the C=C stretching region. Probe-only and pump–probe spectra were recorded quasi-simultaneously as described. In a first approximation the probe-only spectrum was subtracted from the pump–probe spectrum to such an extent that a smooth shape of the difference spectrum (Figure 3, bottom spectrum) in the region of the strong RR band at 1527 cm^{-1} was obtained. This spectrum is characterized by two peaks at 1539 and 1551 cm^{-1}. These data together with the parameters of the BR band at 1527 cm^{-1} allowed us to fit the pump–probe spectrum in the middle of Figure 3 and to obtain the amplitude of the BR band that is involved. If this was subtracted, a spectrum of L was obtained that was identical within a few percent with the one deduced intuitively in the first approximation. Satisfactory signal-to-noise ratios

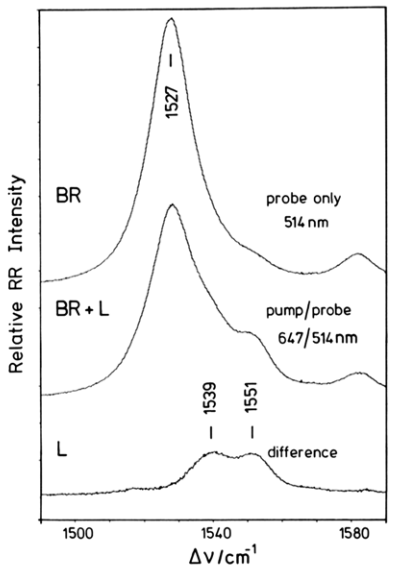

FIGURE 3: RR bands of BR-570 and L-550 in the C=C stretching region. The probe-only and pump–probe spectra were recorded quasi-simultaneously as described under Materials and Methods. The RR spectrum of the intermediate L was obtained by subtracting an appropriate fraction of BR from the pump–probe spectrum as described in the text. Other parameters were $\delta = 20$ μs, $\bar{d}_P = 160$ μm, $\bar{d}_R = 60$ μm, $v = 6.28$ ms^{-1}, $T_r = 20$ ms, $P_P = 350$ mW, and $P_R = 5$ mW.

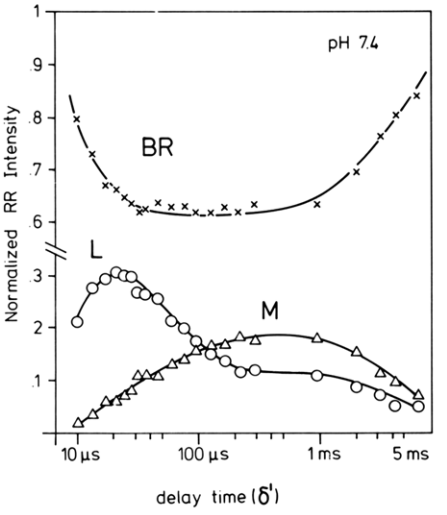

FIGURE 4: Normalized RR intensities of BR-570, L-550, and M-412 according to eq 2a as a function of the delay time. The intensities were deduced from the original data of Alshuth and Stockburger (1986). The intensities for the time domain $\delta > 1$ ms which had been measured under different pump conditions were normalized to the values in the time domain $\delta < 1$ ms. The time scale is given by $\delta' = \delta + 10$ μs.

were obtained by repetitive scanning. The spectral error between the different runs was less than 0.2 cm^{-1}.

Results and Discussion

A New L Intermediate. In a previous work we had studied the kinetic behavior of the L–M transition on the basis of the characteristic C=C stretching bands (Alshuth & Stockburger, 1986). The results for neutral pH are summarized in the diagram of Figure 4. It can be seen that L does not decay completely into M as would be expected from the linear reaction scheme in Figure 1 but levels off to a residual value in an intermediate time domain between 300 μs and 1 ms. This residual amplitude decays with a time constant of ~5 ms. Within the accuracy of the measurements this is the same value as was found for the decay of M and the reconstitution of BR.

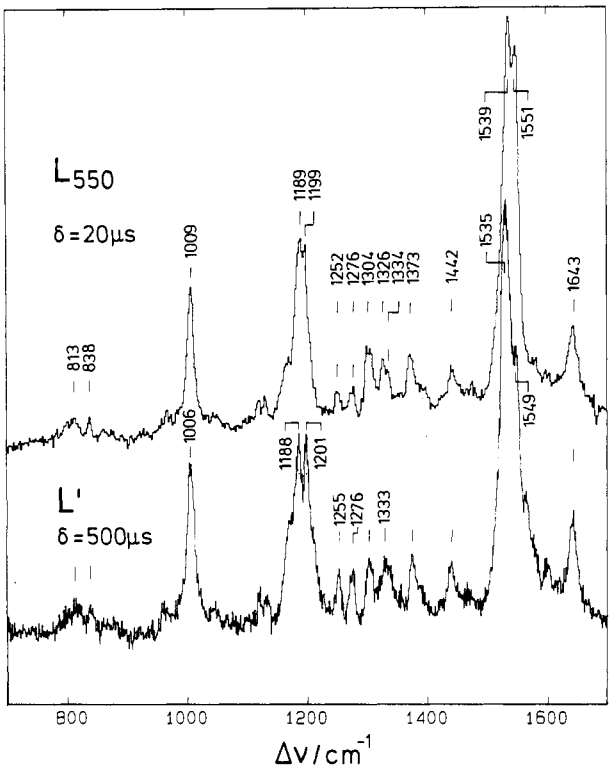

FIGURE 5: RR spectra of L intermediates at delay times of 20 and 500 μs. The other experimental conditions were the same as given in the legend of Figure 3. The spectra were obtained by the difference procedure as described in Figure 3 and in the text.

From the data in Figure 4 it had been suggested by Alshuth and Stockburger (1986) that an intermediate equilibrium betwen L and M is established with a time constant of ~62 μs. On the basis of a "relaxation" experiment during our present study this interpretation had to be rejected. It turned out that in the intermediate time domain where the equilibrium reaction had been assumed no interaction between L and M takes place (Diller, 1988).

From the time behavior in Figure 4 it is suggested that L exists at least in two forms, one that decays fast between 20 and 300 μs and another that decays slowly within a few milliseconds. The question therefore arises if these two kinetically different L chromophores can be distinguished by their chemical structure.

In order to clarify this point, we recorded RR spectra in pump–probe experiments for delay times of 20 and 500 μs, respectively. The two spectra depicted in Figure 5 are very similar. Indeed, each band in one spectrum has its related band in the other. But it is also evident that the two spectra are not identical. The most distinctive differences occur in the C=C stretching region where the low-frequency peak of the "L doublet" is shifted from 1539 to 1535 cm^{-1} and the relative intensity of the two peaks is changed. It is well established that the frequency of the "in-phase" C=C stretching mode of the retinal chain is extremely sensitive to π-electron delocalization (Stockburger et al., 1986). This effect mainly depends on electrostatic interaction between the positively charged SB group and negative charges in its environment. The observed spectral changes therefore indicate that this interaction must be slightly different in the two L components.

Some differences between the spectra in Figure 5 also occur in the fingerprint region between 1180 and 1280 cm^{-1}. These, however, cannot be correlated with isomerization about a double bond which would lead to more pronounced spectral changes (Smith et al., 1985; Stockburger et al., 1986). We therefore conclude that the "crude" geometry of the retinal chain in the two "L" components is the same and the spectral changes in the fingerprint region reflect only minor conformational distortions of the retinal chain.

The same isotope effects were observed in the two L spectra when the purple membranes were suspended in D_2O and consequently the proton at the nitrogen of the SB group was replaced by a deuteron (Diller, 1988). This proves that the SB group in the two L species has a similar structure and in particular that the SB group is protonated in both species.

In the following we assign the long-lived L chromophore by L′ which takes into account the great structural similarity with the early L component for which the conventional notation L-550 or sometimes simply L will be used.

Here we note that the spectrum of the long-lived L component obtained at pH 9.6 by Alshuth and Stockburger (1986) is closely related to the spectrum of L′ in Figure 5 at neutral pH. This relationship will play a role later in this paper.

The Transition from L to L′. In order to study the time dependence of the transition from L to L′, a series of RR spectra were recorded in pump–probe arrangement for delay times between 20 and 600 μs in the region of the characteristic C=C stretching bands. The pure spectra of the L intermediates were obtained by the difference procedure. Spectra for 10 different delay times are presented in Figure 6.

Let us first consider the spectral changes as a function of time. During the period between 20 and 160 μs the two L bands remain nearly unchanged. For delay times longer than 160 μs changes in the L spectra can be clearly identified. Thus the peak at 1539 cm^{-1} shifts down, and its intensity with respect to the peak at 1551 cm^{-1} increases. Above 400 μs the spectrum approaches its final shape which can be assigned to the new species L′. From the spectral changes in Figure 6 it is estimated that the time constant for the transition from L to L′ lies between 200 and 300 μs.

The decay of the originally populated L chromophores therefore has to be described by at least two time constants. The fast decay refers to the transition from L to M as proposed in the conventional photocycle (Figure 1). Its time constant can be very accurately measured by monitoring the population of M. In this paper we adopt a value of 60 μs for τ_1, which had been obtained by Hanamoto et al. (1984) under similar conditions as we used. The slow decay from L to L′ is reflected by a time constant τ_2 that lies between 200 and 300 μs (Figure 6). Since M and L′ appear with different time constants, they cannot be populated from a common L precursor. So we are led to the conclusion that two different L species are involved which react independently of each other. If the two Ls are formally distinguished by labels "α" and "β", their decay can be described by

$$L(\alpha) \xrightarrow{\tau_1} M(\alpha) + H^+ \qquad \tau_1 = 60\ \mu s$$

$$L(\beta) \xrightarrow{\tau_2} L'(\beta) \qquad 200 < \tau_2 < 300\ \mu s \qquad (1)$$

It is important to note that, according to eq 1 and Figure 6, the RR spectra of the two chromophores L(α) and L(β) cannot be much different. This can be concluded as follows. At δ = 20 μs a mixture of L(α)/L(β) is probed which, as will be shown below, is present in a ratio of 1.4:1. At δ = 120 μs L(α) has nearly vanished and the contribution of L′(β) is still small so that L(β) dominates at this delay time. Since, however, the spectral features between 20 and 120 μs in Figure 6 are nearly unchanged, this argues that the spectra of L(α) and L(β) and consequently also the structure of their chromophores cannot be much different.

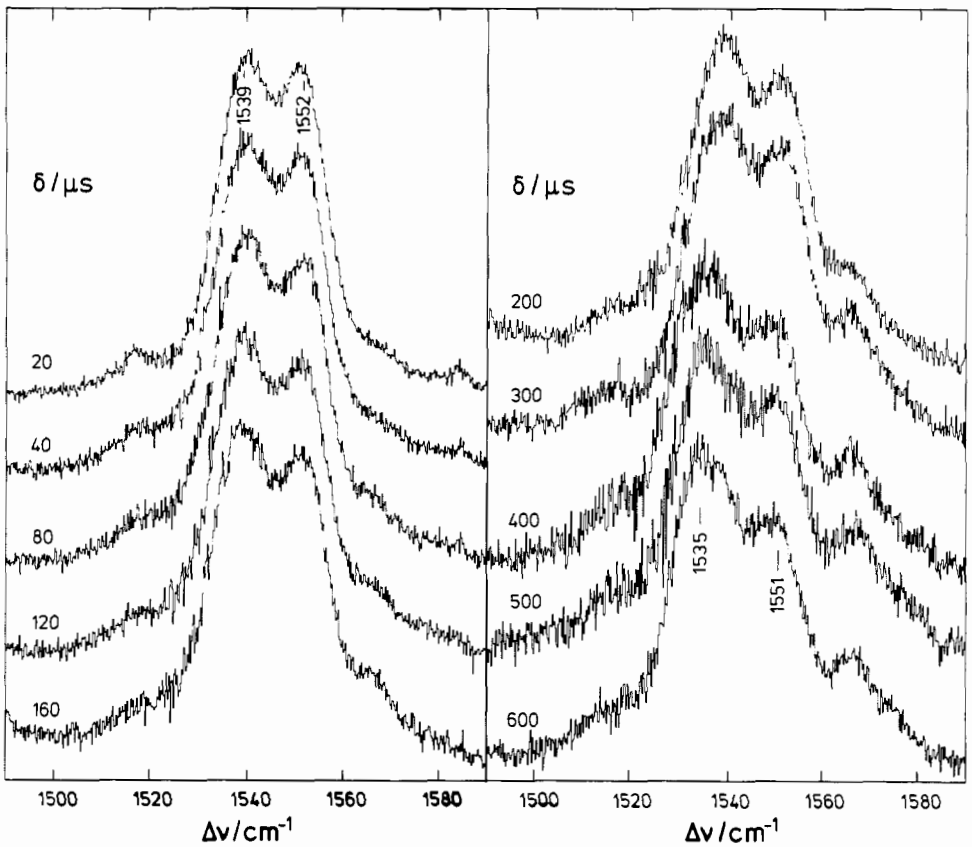

FIGURE 6: RR bands of L intermediates in the C=C stretching region for various delay times between 20 and 600 μs. Other experimental parameters and difference procedure as given in Figure 3.

On the other hand, as suggested by eq 1, L(α) and L(β) undergo such completely different reactions that there must be some specific sites with different chemical structure in the protein envelope which are responsible for the different reactivity. Such sites obviously do not significantly influence the chemical structure of the L chromophore. Only when L(β) relaxes to L′(β) is this structure slightly modified. In this sense the labels "α" and "β" are related to site-specific structural differences in the protein envelope of the L species.

Relative Concentration of L(α) and L(β). In eq 1 the decay of L(α) and L(β) into M and L′(β) is considered separately from the preceding and consecutive reaction steps in the photocycle. This is justified since the formation of the two L species is fast (~2 μs; see Figure 1) and the decay of M and L′(β) is slow (~5 ms, Figure 4) compared with the time constants τ_1 and τ_2 of eq 1. On this basis the relative population of L(α) and L(β) shall now be determined.

In Figure 7 the data for neutral pH are displayed as "normalized" RR intensities:

$$I_i(\delta) = \bar{I}_i(\nu_{C=C},\delta>0)/\bar{I}_{BR}(\nu_{C=C},\delta<0) \qquad (2a)$$

which means that the RR intensity of a species "i", as set proportional to the intensity of the strongest C=C stretching band and measured at any positive delay time ($\delta > 0$), is normalized to the intensity of the parent BR measured in the same way before the pump event ($\delta < 0$).

In an analogous way normalized concentrations are defined that are related to the normalized RR intensity by

$$\begin{aligned} [BR] &= I_{BR} \\ [L(\alpha)] &= f_L I_{L(\alpha)} \\ [L(\beta)] &= f_L I_{L(\beta)} \\ [L'(\beta)] &= f_{L'} I_{L'(\beta)} \\ [M] &= f_M I_M \end{aligned} \qquad (2b)$$

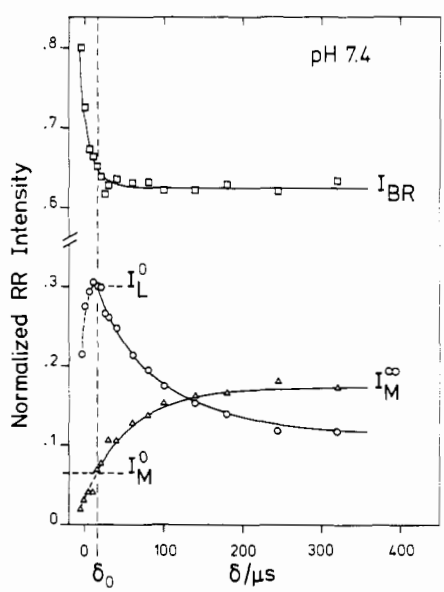

FIGURE 7: Normalized RR intensities according to eq 2b in the microsecond time domain. The data are the same as in Figure 4 (pH 7.4).

It is obvious from this definition that in the case of the parent BR the two normalized quantities are equal. In the case of L and M the proportionality factors f_i take into account the different RR scattering cross sections σ_i of the various intermediates with respect to the parent. These factors are given by

$$f_L = \sigma_{BR}/\sigma_L \qquad f_{L'} = \sigma_{BR}/\sigma_{L'} \qquad f_M = \sigma_{BR}/\sigma_M \qquad (3)$$

and depend on the RR probe wavelength. Since, as we had argued above, the chromophores of L(α) and L(β) have a

similar if not identical chemical structure, the same factor f_L was used in eq 2b.

We now consider the temporal evolution of the three L intermediates starting from a delay time δ_0 at which pump and probe beam no longer overlap. It can be concluded from the diagram in Figure 7 that δ_0 is about 16 μs.

According to eq 1 the sum of the normalized RR intensities of the three L intermediates is given by

$$\begin{aligned} I_L &= I_{L(\alpha)} + I_{L(\beta)} + I_{L'(\beta)} \\ &= I^0_{L(\alpha)}e^{-\delta'/\tau_1} + I^0_{L(\beta)}e^{-\delta'/\tau_2} + I^\infty_{L'(\beta)}(1 - e^{-\delta'/\tau_2}) \end{aligned} \quad (4)$$

where $\delta' = \delta - \delta_0$ and the superscripts "0" and "∞" refer to $\delta' = 0$ and $\delta' \rightarrow \infty$.

Since according to eq 1 only L(α) is converted into M, it follows from the definitions in Figure 7 and eq 2b that

$$I^0_{L(\alpha)} = (f_M/f_L)(I^\infty_M - I^0_M) \quad (5)$$

This relation takes into account that at $\delta_0 \sim 16$ μs a part of L(α) is already converted to M ($\tau_1 = 60$ μs). On the other hand, it can be assumed in good approximation that the amount of L'(β) which at δ_0 has already been formed from L(β) can be neglected since τ_2 (200–300 μs) is much larger than δ_0. One therefore obtains

$$I^0_{L(\beta)} = I^0_L - I^0_{L(\alpha)} \quad (6)$$

and with eq 1 and eq 2b

$$I^\infty_{L'(\beta)} = (f_L/f_{L'})I^0_{L(\beta)} \quad (7)$$

From the diagram in Figure 7 we deduce

$$I^\infty_M = 0.174 \qquad I^0_M = 0.065 \qquad I^0_L = 0.3 \quad (8)$$

When these values in combination with eq 5, 6, and 7 are inserted into eq 4, a good fit to the experimental data in Figure 7 is obtained with the following values

$$\begin{aligned} &\tau_1 = 60\ \mu s \qquad \tau_2 = 220\ \mu s \qquad f_M/f_L = 1.28 \\ &\qquad\qquad f_{L'}/f_L = 1.52 \end{aligned} \quad (9)$$

giving

$$I^0_{L(\alpha)} = 0.14 \qquad I^0_{L(\beta)} = 0.16 \qquad I^\infty_{L'(\beta)} = 0.105 \quad (10)$$

If the decay of L(α) during δ_0 is still considered as a correction, one obtains for the "initial" (index "i") concentrations

$$[L(\alpha)]^i = f_L I^0_{L(\alpha)} + f_M I^0_M = f_M I^\infty_M \quad (11)$$

$$[L(\beta)]^i = f_L I^0_{L(\beta)} = f_{L'} I^\infty_{L'(\beta)} \quad (12)$$

From eq 11 and 12 and with the values given in eq 8–10

$$\left[\frac{L(\alpha)}{L(\beta)}\right]^i = 1.4 \quad (13)$$

This important quantity can be obtained more directly from the data in Figure 8, left panel, which represent the decay of M and L'(β) in the millisecond time domain. Since within the limits of error these two species decay with the same time constant ($\sim$5 ms), one obtains from an averaged value of $I_M/I_{L'(\beta)}$ and with eq 9

$$\left[\frac{L(\alpha)}{L(\beta)}\right]^i = \frac{f_M}{f_{L'}}\left[\frac{I_M}{I_{L'(\beta)}}\right]^{\delta>1ms} = 1.36 \quad (14)$$

in good agreement with eq 13.

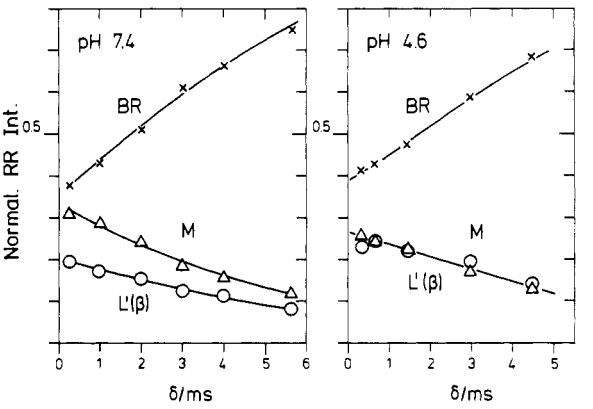

FIGURE 8: Normalized RR intensities according to eq 2b in the millisecond time domain at pH 7.4 and 4.6. The intensities are based on the original data of Alshuth and Stockburger (1986).

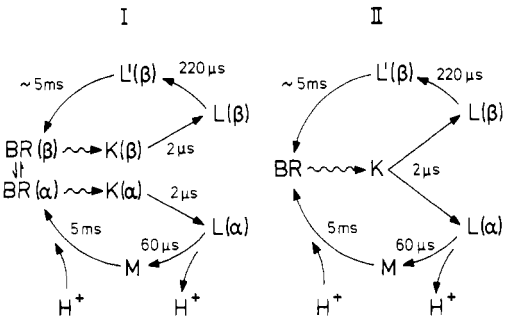

FIGURE 9: Two different reaction schemes as discussed in the text. The decay constants refer to room temperature and neutral pH.

The concentration of the initially formed L species is equal to the amount of BR which is converted in the pump beam. From Figure 7 one obtains

$$[L(\alpha)]^i + [L(\beta)]^i = f_M I^\infty_M + f_L I^0_{L(\beta)} = [\Delta BR] = 0.375 \quad (15)$$

and with eq 13

$$[L(\alpha)]^i = 0.219 \qquad [L(\beta)]^i = 0.156 \quad (16)$$

Equation 15 also allows us to deduce values for f_L, $f_{L'}$, and f_M. From eq 9 and 15 one obtains for excitation at 476 nm

$$f_L = 0.98 \qquad f_{L'} = 1.49 \qquad f_M = 1.26 \quad (17)$$

According to the definitions in eq 3 this implies that the RR cross section of L'(β) is 1.5 times smaller than that of L(β) for excitation at 476 nm. This suggests that the peak of the RR excitation profile of the C=C stretch of L'(β) is red-shifted with respect to that of L(β). This is in agreement with the expectation that the frequency downshift of the C=C stretch of L'(β) (see Figure 5) is accompanied by a red shift of its absorption maximum with respect to L(α) or L(β). The fact that different RR cross sections were found for L'(β) and L(β) or L(α) is independent evidence for the existence of the intermediate L'(β).

The Complete Cyclic Reaction Scheme. We concluded that two different intermediates L(α) and L(β) are formed. It was argued that the two species are related to different states "α" and "β" of the protein envelope. It was shown that L(α) and L(β) undergo independent reactions with different time constants although the structure of the chromophore in the two species is virtually the same. The question is now in which way the two L states are involved in the total cyclic reaction.

Two different reaction schemes are conceivable (Figure 9). In reaction scheme I it is assumed that the two apoprotein states "α" and "β" exist already in the unphotolyzed species,

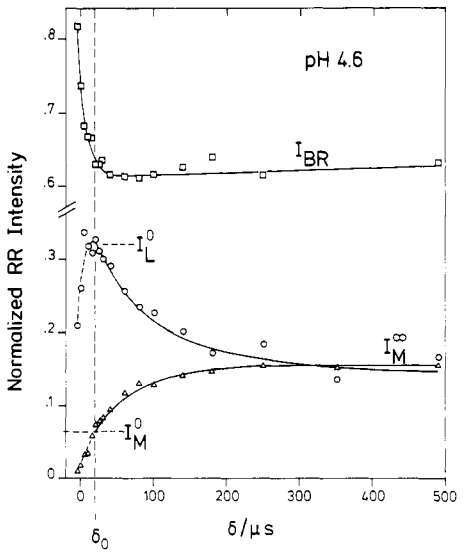

FIGURE 10: Normalized RR intensities according to eq 2b. Deduced from the original data of Alshuth and Stockburger (1986) for a PM suspension of pH 4.6.

giving rise to two different parent states BR(α) and BR(β) which are separated by a sufficiently high energy barrier which allows them to undergo largely independent reactions. In this scheme BR(α) and BR(β) are in a thermodynamic equilibrium

$$\mathrm{BR}(\alpha) \rightleftharpoons \mathrm{BR}(\beta) \tag{18}$$

which should depend on external parameters like temperature or pH.

In reaction scheme II of Figure 9 it is assumed that L(α) and L(β) descend from a common precursor K. This would imply that the two different apoprotein states "α" and "β" are formed during the photocycle and later decay during the reconstitution of the parent species.

Experiments at pH 4.6. Experiments at pH 4.6 were carried out in complete analogy to those at neutral pH. The results are displayed in Figure 10 for the microsecond and in Figure 8, right panel, for the millisecond time domain. For the analysis of the data the same procedure was used as for the data in Figure 7. From Figure 10 we deduce

$$I_M^\infty = 0.154 \qquad I_M^0 = 0.064 \qquad I_L^0 = 0.32 \tag{19}$$

A good fit according to eq 4 is obtained with the same parameters as used for pH 7.4 (eq 9). One finds

$$I_{L(\alpha)}^0 = 0.115 \qquad I_{L(\beta)}^0 = 0.205 \qquad I_{L'(\beta)}^\infty = 0.135 \tag{20}$$

and according to eq 11 and 12 with the values in eq 17, 19, and 20

$$[\mathrm{L}(\alpha)]^{\mathrm{i}} = 0.194 \qquad [\mathrm{L}(\beta)]^{\mathrm{i}} = 0.2 \tag{21}$$

giving

$$\left[\frac{\mathrm{L}(\alpha)}{\mathrm{L}(\beta)}\right]^{\mathrm{i}} = 0.97 \tag{22}$$

From the data in Figure 8, right panel, we derive in a similar way as described by eq 14

$$\left[\frac{\mathrm{L}(\alpha)}{\mathrm{L}(\beta)}\right]^{\mathrm{i}} = 0.85 \tag{23}$$

Reaction Scheme I Is Favored. We have tried to carry out the experiments at pH 7.4 and 4.6 under identical pump/probe conditions. It is found that the sum of the two initially formed L species is the same for the two pH values (compare eq 16 and 21) if one considers the limits of error within which identical conditions can be established in two different experiments. This suggests that for completely identical pump/probe conditions

$$[\mathrm{L}(\alpha) + \mathrm{L}(\beta)]_1^{\mathrm{i}} = [\mathrm{L}(\alpha) + \mathrm{L}(\beta)]_2^{\mathrm{i}} \tag{24}$$

is valid where the subscripts "1" and "2" refer to pH 7.4 and 4.6, respectively.

On the other hand, we found for the change of the relative population of the two L intermediates (mean values from eq 13, 14, 22, and 23)

$$\left[\frac{\mathrm{L}(\alpha)}{\mathrm{L}(\beta)}\right]_1^{\mathrm{i}} : \left[\frac{\mathrm{L}(\alpha)}{\mathrm{L}(\beta)}\right]_2^{\mathrm{i}} = 1.52 \tag{25}$$

If reaction scheme II of Figure 9 would be valid, it would follow from the two relations in eq 24 and 25 that the ratio of the two rate constants that are responsible for the formation of L(β) and L(α) from their precursor K increases by a factor of 1.52 when the pH is decreased from 7.4 to 4.6.

However, there are strong arguments against such a conclusion. In the pH range we consider here internal groups of the protein do not change their protonation state, which is reflected by our finding that the time constant τ_1 which describes the deprotonation of the SB group is the same for both pH values. We also did not find a change in τ_2 which describes the reaction step from L(β) to L'(β) which is localized at the chromophoric site. It is therefore very unlikely that the rate constant for the reaction step from K to L which is also localized at the chromophoric site should be pH dependent in this range. This implies that the observed pH-dependent changes as expressed by eq 25 have to be traced back to changes in the equilibrium population of the parent species BR(α) and BR(β) according to eq 18 and therefore reaction scheme I is valid.

This means that the equilibrium concentration of BR(β) with respect to BR(α) is increased by a factor of 1.52 when the pH is lowered from 7.4 to 4.6. An explanation for this phenomenon could be that for an increased density of protons at the membrane surface divalent cations are partially removed from their binding sites. It is well established that such cations play an important role for the structure and function of BR (Kimura et al., 1984). It is therefore conceivable that such changes might induce transitions from the α to the β form of the protein envelope.

Here we mention a more general argument against the validity of reaction scheme II. This scheme would require that the two states α and β of the apoprotein are formed during the photochemical cycle. This would imply that in the first two steps of the cycle which are fairly localized at the chromophoric site structural changes of the apoprotein were catalyzed at some distance. Such reactions are not very likely.

A Difference Experiment. Although the results do not provide any new information, this experiment is nonetheless of great value since it confirms all of our previous results in a completely independent way.

We start with the statement in eq 24 which we write in the form of a difference as

$$[\Delta \mathrm{L}(i)]_{12} = [\mathrm{L}(\alpha) + \mathrm{L}(\beta)]_1^{\mathrm{i}} - [\mathrm{L}(\alpha) + \mathrm{L}(\beta)]_2^{\mathrm{i}} = 0 \tag{26}$$

This says that the amount of initially formed L intermediates is the same for pH 7.4 ("1") and pH 4.6 ("2") under otherwise identical conditions. Equation 26 can be rearranged to

$$[\Delta \mathrm{L}(i)]_{12} = [\mathrm{L}(\alpha)_1 - \mathrm{L}(\alpha)_2]^{\mathrm{i}} - [\mathrm{L}(\beta)_2 - \mathrm{L}(\beta)_1]^{\mathrm{i}} = 0 \tag{27}$$

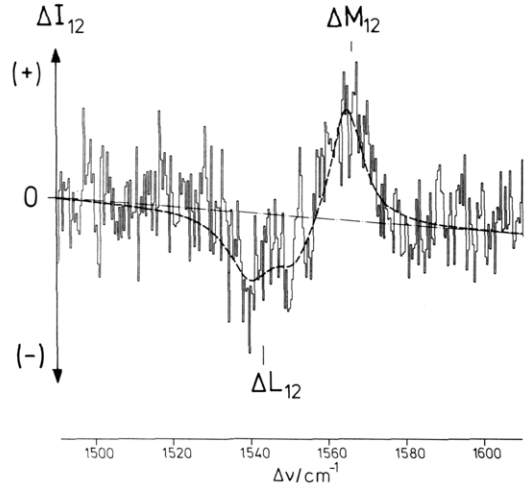

FIGURE 11: Difference of the RR signal from two PM suspensions at pH 7.4 ("1") and pH 4.6 ("2") on a relative scale. Details are described in the text. Experimental parameters: λ_P = 647 nm; λ_R = 476 nm; $\bar{d}_P$ = 160 μm; $\bar{d}_R$ = 60 μm; P_P = 350 mW; P_R = 5 mW; δ_1 = 20 μs.

where, as we know from our earlier analysis, the two brackets have positive values (see eq 16 and 21). If this difference is probed at some delay time δ, it will no longer vanish since the value of the first bracket decreases much faster (τ_1 = 60 μs) than that of the second one (τ_2 = 220 μs). We now chose a fixed delay time $\delta_1 \ll \tau_2$ so that in good approximation the decrease of the second bracket can be neglected. From eq 27 it follows that

$$[\Delta L(\delta_1)]_{12} = [L(\alpha)_1 - L(\alpha)_2]^i(e^{-\delta_1/\tau_1} - 1) < 0 \quad (28)$$

In a similar way one finds

$$[\Delta M(\delta_1)]_{12} = [\Delta L(\delta_1)]_{12} > 0 \quad (29)$$

For $[L(\alpha)]^i_1 = 0.219$ and $[L(\alpha)]^i_2 = 0.194$ as obtained under standard conditions (eq 16 and 21) and for δ_1 = 20 μs one obtains

$$[\Delta L(20\ \mu s)]_{12} = -0.007$$
$$[\Delta M(20\ \mu s)]_{12} = +0.007 \quad (30)$$

In order to test these predictions, one has to design an RR experiment in which spectra are recorded in state "1" (pH 7.4) and state "2" (pH 4.6) under identical pump/probe conditions and for otherwise identical samples. In the difference spectrum one then would expect negative peaks for the two strong L bands at 1539 and 1550 cm^{-1} and a positive peak for the M band at 1565 cm^{-1}.

In our experiments we used a spinning cell with a rotational frequency of 50 s^{-1}. The cell was divided into two compartments of equal size which were filled with purple membrane suspensions of the same concentration but at pH values of 7.4 and 4.6, respectively. A pump/probe experiment was carried out with δ_1 = 20 μs. The RR signals from each compartment were alternatively fed into separate parts of a memory. In this way spectra for the two pH values could be recorded quasi-synchronously, which allows one to obtain the difference spectra with high accuracy.

A difference spectrum obtained in this way is shown in Figure 11. It clearly shows what is predicted from eq 28–30, namely, a negative loop for the L bands and a positive for the M band. The size of the amplitudes obtained under standard conditions is consistent with the values in eq 30.

The fact that the results of our difference experiment are completely consistent with the predictions in eq 28–30 is an additional and independent evidence for the existence of two L intermediates of different reactivity.

The Photocycle in the Alkaline Region. We have carried out RR experiments with the rotating cell at pH values of the PM suspensions between 8.5 and 10.5 ("high-pH" or "alkaline" region). It is well established that the BR chromophore is chemically stable up to pH 12 (Druckman et al., 1982; Scherrer & Stoeckenies, 1984) and also preserves its biological function (Li et al., 1984). This behavior suggests that the principle reaction pathways of the chromophore are not changed at high pH.

The fact that the RR spectrum of L′(β) in Figure 5 is closely related to the spectrum of the long-lived L species at pH 9.6 (Alshuth & Stockburger, 1986) is in line with this conclusion. In a specially designed flow experiment we found that the relative population of L′(β) and M we had obtained at neutral pH is not markedly changed in the alkaline region (Diller, 1988).

These findings in particular indicate that at high pH the two species L(α) and L(β) are formed in the same way as at neutral pH and consequently the two related apoprotein states α and β also exist in the alkaline region. RR experiments at high pH therefore are useful in order to obtain additional information about these states.

On the other hand, drastic changes of rate constants occur at high pH. So it was found that the rate of formation of M from its precursor L is increased by a factor of ten when the pH is raised to values between 9 and 10 (Rosenbach et al., 1982; Hanamoto et al., 1984; Scherrer & Stoeckenius, 1985).

Here we are mainly interested in the decay of M which also changes in the alkaline region. There is evidence that the M decay follows biphasic kinetics (Hess & Kuschmitz, 1977; Ort & Parson, 1978; Ohno et al., 1981; Scherrer & Stoeckenius, 1985; Groma & Dancshazy, 1986) of the form

$$M^0_f e^{-t/\tau_f} + M^0_s e^{-t/\tau_s} \quad (31)$$

where "f" and "s" assign a fast- and slow-decaying component. The following data on the pH dependence are from the work of Scherrer and Stoeckenius (1985) and refer to a temperature of 20 °C:

	τ_f (ms)	τ_s (ms)	M^0_f/M^0_s
pH 7	3.8	6.5	26/74
pH 10.5	3.2	98	68/32

(32)

It is seen that the lifetime of the slow component increases dramatically at high pH while that of the fast component remains nearly constant. Also, a change of the relative amplitudes is observed with increasing pH.

In the context of this paper the question arises if the two M components could be related to two different substates of the apoprotein. In the following it will be proposed that two such states are populated.

RR Experiments in the Alkaline Region. RR spectra recorded at pH 10.5 are depicted in Figure 12. The upper spectrum was probed shortly after the sample had left the pump beam (δ_1 = 0.25 ms). The lower spectrum was probed after a full rotational period of the cell, i.e., just before the sample reentered the pump beam (δ_2 = 100 ms). The spectra, probed at 476 nm, mainly consist of contributions from BR, L′(β), and M. From a band-fitting analysis and with $f_{L'}$ and f_M from eq 17 the following relative concentrations were obtained:

	BR	L′(β)	M
δ_1 = 0.25 ms:	0.170	0.504	0.327
δ_2 = 100 ms:	0.348	0.527	0.125

(33)

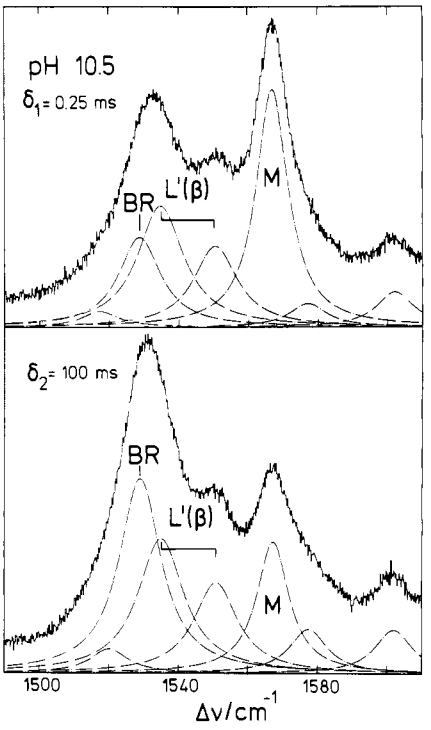

FIGURE 12: RR spectra from a pump–probe experiment with PM suspensions of pH 10.5 for two different delay times. Experimental parameters: λ_P = 647 nm; λ_R = 467 nm; $\bar{d}_P$ = 370 μm; $\bar{d}_R$ = 60 μm; P_P = 260 mW; P_R = 2.6 mW; T_r = 100 ms. Spinning cell as described in Figure 2. The spectra were deconvoluted by band fitting by using the spectral parameters of BR-570, M-412, and L'(β).

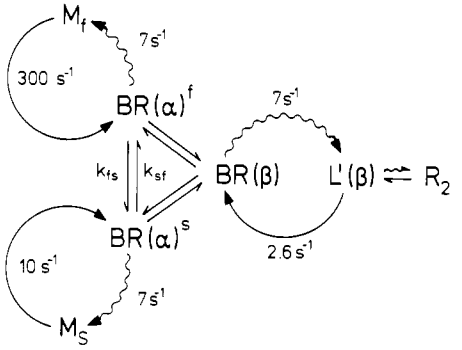

FIGURE 13: Reaction scheme suggested for the interpretation of the experiment at pH 10.5 in Figure 12. The rate constants refer to the experimental conditions in Figure 12.

For an interpretation of these results the reaction scheme in Figure 13 is proposed where it is assumed that M_f and M_s belong to two different parent species BR(α)^f and BR(α)^s which undergo cyclic reactions, i.e., the α form of the apoprotein is further subdivided. All substates of the apoprotein are assumed to be thermally coupled to each other.

It can be estimated that the PM patches stay for at least 30 s in the cylindrical liquid layer which is illuminated by the probe beam in the spinning cell (Diller, 1988). This period is a lower limit for the "observation time" of the RR experiment. The time needed to accumulate the spectra in Figure 12 was much longer (~12 min). Reactions between the various unphotolyzed BR components in Figure 13 may occur during this "observation time".

Some more insight into the reaction scheme of Figure 13 can be obtained if the experiment is "transformed" to a photostationary one. This can be done by distributing the illumination during the pump period over the full rotational period of the spinning cell. In this way a mean photochemical rate constant $\bar{l}_0$ is defined by

$$[BR]/[BR]_0 = e^{-\bar{l}_0 T_r} \quad (34)$$

where $[BR]_0$ and [BR] are the concentrations of the parent species before and after the pump event and T_r is the rotational period. A value of ~7 s^{-1} is obtained for $\bar{l}_0$ from the data of eq 33. The rate constants for the decay of M_s and M_f are obtained from eq 32. The decay time of L'(β) at high pH can be deduced from transient absorption experiments. From such data a value of ~380 ms was derived at pH 10.[1]

The experimental data in eq 33 shall now be discussed on the basis of the three-cycle scheme in Figure 13 where it is taken into account that L'(β) is photochemically coupled to a long-lived species "R_2" (Stockburger et al., 1979; Grieger, 1981). The presence of R_2 is documented in the spectra of Figure 12 by its C=C stretching band at ~1520 cm^{-1}. Although this band is weak, it may reflect a fairly high concentration of R_2 since, due to the red-shifted absorption of this species, its RR cross section at 476 nm is much smaller than that of the three other species. It can be assumed that R_2 does not change its concentration significantly during a rotational period of the cell. Then the data in eq 33 reflect the relative concentrations of BR, L'(β), and M at the two delay times in good approximation.

It is obvious that the decrease of M between 0.25 and 100 ms in eq 33 can be attributed solely to the slow component M_s (τ_s ~ 100 ms). This implies that M_f is depleted during the observation time of ~30 s. Following the reaction scheme in Figure 13, this would mean that BR(α)^f is converted to BR(α)^s or BR(β). From the equilibrium constant $k_{fs}/k_{sf} = M_s^0/M_f^0$ which at pH 10.5 is 32/68 (see eq 32) and from the rate constants in the reaction scheme of Figure 13 it can be concluded that only a minor part of BR(α)^f could be converted to BR(α)^s under photostationary conditions. The major part, therefore, must be converted to BR(β).

Although the analysis presented here is qualitative and therefore preliminary, it nevertheless leads to important conclusions. Thus BR(α)^s is fairly independent of BR(α)^f and BR(β). It should be emphasized that photostationary experiments as described here will allow us to study the interaction of the various states of the chromoprotein in detail.

Test Measurements at High Salt Concentration. All RR experiments described so far had been performed without additional salt. In order to approach the in vivo conditions of *Halobacteria*, a few test measurements were carried out with PM suspensions of 4 M NaCl concentration. Also under such conditions the long-lived intermediate L'(β) was observed with comparable intermediate concentration as in low-salt suspension. This demonstrates that the BR(β) cycle is significantly populated.

CONCLUSIONS

From kinetic and structural RR studies it was concluded that unphotolyzed BR exists at least in two different states BR(α) and BR(β). These are different with respect to the conformation of at least one specific site in the protein which in the α-state catalyzes proton transfer from the SB group while in the β-state it does not support or even impedes this reaction step.

Such sites obviously do not influence the geometry and electronic structure of the retinylidene chromophore significantly since so far we were unable to distinguish between

[1] Data from transient absorption experiments at pH 10 were provided to us by K. H. Müller and M. Engelhard.

BR(α) and BR(β) by their RR spectra. Even at the stage of L where the different reaction pathways originate such a distinction was not possible. These statements, however, are preliminary and do not exclude that minor differences in the RR spectra do occur which might be discovered by more extended studies.

Two independently reacting states of BR were recently also suggested by Balashov et al. (1986). They found in low-temperature experiments at 77 K a biphasic kinetics of the photoreversal of the primary intermediate K. Their interpretation was that two different states K_1 and K_2 are formed from two different states of BR. It is interesting that these authors proposed that two different cyclic reactions might occur at room temperature which were related to the two different BR states.

In the new reaction scheme I (Figure 9) the intermediates N-520 and O-640 were not considered. In their original work Lozier et al. (1975) had tentatively assumed that N-520 follows on M-412 in the linear reaction chain (Figure 1). The only basis for this assignment was that a residual "green" absorption was observed in the same time regime as the blue-absorbing M-412. On the basis of our experiments we conclude that the long-lived intermediate absorption in the green which is seen in flash photolysis experiments is due to the intermediate L'(β) which in the present work could be well characterized by its kinetic parameters and its RR spectra as an "L-like" species. It is just this species which played a key role in setting up the reaction scheme I (Figure 9). By no means can L'(β) be assigned as a sequential intermediate of M-412.

Until now the role of the intermediate O-640 in the cyclic reaction of BR could not yet be definitely clarified. It was argued that kinetic data are inconsistent with a sequential reaction mechanism (Sherman et al., 1979). On the basis of detailed kinetic and mechanistic studies Mäntele (1982) raised the question if O-640 and M-412 might originate from different parent species of BR. This stimulated the idea that O-640 might be a precursor of BR(β) in reaction scheme I. A few arguments based on deuterium effects and the pH dependence in favor of this assignment were given by Diller and Stockburger (1987). If an intermediate with the chemical structure of O-640 is a necessary precursor in the reconstitution process of the parent species, this may appear in the α- as well as in the β-cycle of reaction scheme I.

It is well-known that, under modified conditions, for instance, in a 25% NaCl solution adjusted to pH 10, BR can be completely converted to M-412 by illumination (Becher & Ebrey, 1977). Similar effects were observed in high concentrations of guanidine hydrochloride at elevated pH (Yoshida et al., 1979) or in concentrated salt solutions saturated with diethyl ether (Oesterhelt & Hess, 1973). In view of our interpretation this would mean that under such extreme conditions only the α-form of BR is finally populated by illumination.

From the results of the present paper the question is stimulated if the formation of M-412 in the photocycle is a prerequisite for proton pumping as has been assumed in the past by many authors. A crucial question in future work therefore will be if the BR(β) photocycle in reaction scheme I is also efficient in stimulating the proton pump mechanism. If this will be answered positively, the proton-transfer step in the BR(α) cycle would not be a "conditio sine qua non" for the biological activity.

It seemed that under the conditions of our experiments at neutral pH the two states BR(α) and BR(β) are the dominating ones. However, it must be considered that other states of the protein are populated. Under such auspices the biphasic decay of M-412 was interpreted by us.

While the chromophore of BR has a rigid and homogeneous structure, it follows from the present study that the protein envelope is much more flexible, giving rise to fairly discrete substates which control the proton-transfer step at the chromophoric site. In any case the BR molecules must be considered as entities whose reactivity is determined by interaction of all parts of the molecular system.

ACKNOWLEDGMENTS

We thank Professor A. Weller for encouragement and support. Purple membrane was a gift from the laboratory of Professor Dr. Oesterhelt, which is greatly acknowledged. We are grateful to K. H. Müller and Dr. M. Engelhard for leaving us data from flash-photolysis experiments prior to publication.

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Reaction of Pyruvate Kinase with the New Nucleotide Affinity Labels 8-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-Diphosphate and 5′-Triphosphate†

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Received March 22, 1988

ABSTRACT: Two new reactive nucleotides have been synthesized and characterized: 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-diphosphate and 5′-triphosphate (8-BDB-TADP and 8-BDB-TATP). ADP or ATP was converted to 8-thio-ADP (-ATP) via 8-bromo-ADP (-ATP), followed by condensation with 1,4-dibromobutanedione. Rabbit muscle pyruvate kinase is inactivated by both reagents in a biphasic manner with an initial rapid loss of 75% activity, followed by a slow total inactivation. The initial fast reaction with both compounds exhibits nonlinear dependence on reagent concentration, indicating formation of a reversible enzyme–reagent complex prior to covalent attachment. The presence of the γ-phosphoryl group improves the performance of the affinity label: K_I values for the fast phase are similar (about 100 μM), whereas k_{max} for 8-BDB-TATP is about three times greater than that of 8-BDB-TADP (0.286 min^{-1} vs 0.0835 min^{-1}). After an 80-min incubation with 175 μM of either reagent, about 2 mol/mol of subunit is incorporated with 76% inactivation caused by 8-BDB-TADP and 97% inactivation by 8-BDB-TATP. Loss of activity is prevented by substrates, with the best protection afforded by a combination of ATP, Mn^{2+}, K^+, and phosphoenolpyruvate. Reaction of pyruvate kinase with either compound in the presence of protecting ligands leads to incorporation of about 1 mol of reagent/mol of subunit with only about 15% loss of activity. These results suggest that 8-BDB-TADP and 8-BDB-TATP react with two groups on the enzyme, one of which is at or near the active site. The two new bromodioxobutyl derivatives may have general application as affinity labels of purine nucleotide sites in other proteins.

Pyruvate kinase is a key glycolytic enzyme which catalyzes the formation of ATP from ADP and phosphoenolpyruvate. Considerable information is now available on the structure of pyruvate kinase. NMR experiments have led to predictions about the spatial arrangement of substrates, essential metal ions, and the structural changes that occur upon substrate binding and catalysis (Mildvan & Cohn, 1966; Gupta et al., 1976; Mildvan et al., 1976; Rao et al., 1979; Dunaway-Mariano et al., 1979; Rosevear et al., 1987). Complete amino acid sequences are known for the muscle (Lonberg & Gilbert, 1983; Muirhead et al., 1986), liver (Lone et al., 1986; Inoue et al., 1986), and erythrocyte isozymes (Noguchi et al., 1987), as well as the yeast enzyme (Burke et al., 1983). The amino acid sequence of cat muscle pyruvate kinase has been fitted to the 2.6-Å resolution electron density map (Muirhead et al., 1986), and residues that participate in substrate binding and are near the active site have been identified. Affinity labeling offers a complementary technique for investigating the active site as it exists when the enzyme is in solution.

In this paper, we describe the synthesis and characterization of the new nucleotide analogues, 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-diphosphate (8-BDB-TADP)[1] and 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-triphosphate (8-BDB-TATP). The structures are shown in Figure 1. The placement of the bromoketo group adjacent to the 8-position of the adenine ring may allow the reagents to react in a different manner from 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-monophosphate (Colman et al., 1984) and 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-monophosphate (Kapetanovic et al., 1985), which have been shown to modify rabbit muscle pyruvate kinase. Furthermore, di- and triphosphate moieties of the new reagents are identical with those of the natural substrate and product, ADP and ATP. This paper presents evidence that 8-BDB-TADP and 8-BDB-TATP

† This work was supported by National Science Foundation Grant DMB-88-04706.

[1] Abbreviations: 8-BDB-TADP, 8-[(4-bromo-2,3-dioxobutyl)thio]-adenosine 5′-diphosphate; 8-BDB-TATP, 8-[(4-bromo-2,3-dioxobutyl)-thio]adenosine 5′-triphosphate; 8-TADP, 8-thioadenosine 5′-diphosphate; 8-TATP, 8-thioadenosine 5′-triphosphate; PEP, phosphoenolpyruvate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)-aminomethane; FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; FSBεA, 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine; 6-BDB-TAMP, 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-monophosphate; 2-BDB-TAMP, 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-monophosphate.

0006-2960/88/0427-7651$01.50/0