Infrared Study of the L, M, and N Intermediates of Bacteriorhodopsin Using the Photoreaction of M†

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ABSTRACT: Infrared spectroscopy is used to characterize the transitions in the photocycle of bR involving the M intermediate. It has been shown previously that in this part of the photocycle a large protein conformational change takes place that is important for proton pumping. In this work we separate the spectra of the L, M, and N intermediates in order to better describe the timing of the molecular changes. We use the photoreaction of the M intermediate to separate its spectrum from those of L and N. At temperatures between 220 and 270 K a mixture of M and L or N is produced by illumination with green light. Subsequent blue illumination selectively drives M back into the ground state and the difference between the spectra before and after blue excitation yields the spectrum of M. Below about 250 K and L/M mixture is separated; at higher temperatures an M/N mixture is seen. We find that the spectrum of M is identical in the two temperature regions. The large protein conformational change is seen to occur during the M to N transition. Our results confirm that Asp-96 is transiently deprotonated in the L state. The only aspartic protonation changes between M and bR are the protonation of Asp-85 and Asp-212 that occur simultaneously during the L to M transition. Blue-light excitation of M results in deprotonation of both. The results suggest a quadrupolelike interaction of the Schiff base, Asp-85, Asp-212, and an additional positive charge in bR.

Bacteriorhodopsin (bR) is a retinal–protein complex from the purple membrane patches in the cell membrane of *Halobacterium halobium.* Upon light absorption the light-adapted form bR_{LA} pumps protons from the cell to the extracellular medium and the electrochemical energy of the resulting proton concentration difference is utilized by the cell.

Light-induced proton pumping by bacteriorhodopsin is coupled to a series of successive reactions, the photocycle. Light absorption initiates a sequence of transitions in bR through metastable states (intermediates): a light-driven first step is followed by a series of thermally activated reactions, and the final state is the initial bR form. The following reaction scheme is regarded as an appropriate approximate description of this photocycle (Lozier et al., 1975):

$$bR \rightarrow K \rightarrow L \rightarrow M \rightarrow N \rightarrow O \rightarrow bR$$

The letters denote the intermediates; they were originally distinguished by their characteristic visible absorption spectra. During this photocycle the proton is transported through the membrane in several discrete steps that are synchronized to those in the photocycle (Drachev et al., 1978; Keszthelyi & Ormos, 1980). The understanding of the mechanism of proton transport depends largely on a correct description of the reaction steps and the molecular transitions during these steps in the photocycle.

Vibrational spectroscopy has proved extremely useful in determining the structural changes of both the chromophore and the protein that occur during the photocycle and in assigning the groups that take part in the proton transport (Smith et al., 1985; Rothschild & Marrero, 1982; Engelhart et al., 1985; Dollinger et al., 1986; Braiman et al., 1988; Gerwert et al., 1989, 1990). The retinal is connected to the protein through a Schiff base. The Schiff base is protonated in the bR ground state. During the photocycle it deprotonates in the L → M step and subsequently reprotonates during the M → N transition. It is generally believed that this transient deprotonation is crucial for pumping: the proton is released to one side to Asp-85 (Braiman et al., 1988) toward the outside of the cell and reprotonation occurs from the opposite side to complete the pumping cycle. The retinal does not change its structure between the L and N states; therefore, a crucial protein conformational change was postulated by Fodor et al. (1988) that they called the T–C switch. This conformational change occurs between the deprotonation and reprotonation of the Schiff base and changes it connectivity, thereby making sure that deprotonation and reprotonation happen to different groups. By FTIR spectroscopy between 240 and 260 K we characterized a protein conformational change that follows M formation and is needed for pumping (Ormos, 1991). We showed that if it is prevented from taking place by freezing, reprotonation of the Schiff base occurs from the group to which the proton had been previously released, Asp-85, and pumping does not take place. Time-resolved FTIR spectroscopy suggests that at room temperature the timing of this protein transition is coincident with the M → N step (Braiman et al., 1991). Recent kinetic analysis of the photocycle based on visible absorption kinetic experiments reported on two M intermediates in sequence with a very large free energy difference between them (Váró & Lányi, 1991a). The transition between the two M intermediates was interpreted as the crucial conformational change that changes the connectivity of the Schiff base. In addition to previous works reporting more than one M (Renard & Delmelle, 1985; Kouyama et al., 1988; Dancshazy et al., 1988), where the distinction is based on purely kinetic analysis, with solubi-

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lized bR Váró and Lányi (1991b) also observed a slight spectral shift of M during its lifetime.

It is important to determine the timing of the crucial conformational change and characterize it in detail. In the present work we attempt to proceed toward this goal by separating the infrared spectra of the intermediates that precede and follow the M form in the photocycle and by trying to make a distinction between the possible two M forms using low-temperature FTIR difference spectroscopy. In this method the sample is illuminated at low temperature and depending on the temperature illumination produces a certain intermediate, the IR spectrum of which can be determined (Rothschild & Marrero, 1982). The problem is that the resultant state is typically a mixture of several intermediates. The IR spectrum reflects the mixture of the individual states and the separation of the component spectra is not obvious. This is partly the reason why various laboratories employ different experimental conditions to determine the spectrum of M (Dollinger et al., 1986; Braiman et al., 1988; Gerwert et al., 1989)).

To eliminate the uncertainty we apply a direct method for the separation of the spectrum of M from the spectra of other intermediates. The procedure is based on the photosensitivity of M: upon photoexcitation of M the molecule returns to the ground state very fast (Litvin & Balashov, 1977) [several microseconds at room temperature (Kalisky et al., 1978; Ormos et al., 1978)]. We also know that proton pumping does not occur in this short-circuited photocycle (Karvaly & Dancshazy, 1977; Ormos et al., 1978). Therefore, if the low-temperature photoproduct of bR that contains the M form is excited by blue light, the M form is driven away and the difference is just the spectrum of M. This procedure allows an unbiased determination of the M spectrum.

MATERIALS AND METHODS

Purple membranes were isolated from *Halobacterium halobium* strain ET1001 according to Oesterhelt and Stoeckenius (1974).

For the infrared spectroscopy hydrated dry films of pm-s were used. An unbuffered water suspension of pm-s (pH ~6) was dried on a Cleartran window in vacuum. The sample was then fully hydrated in a hygroscopic sample chamber for 24 h at room temperature. Samples with optical density of about 1 at 570 nm were used.

Infrared spectra in the wavenumber range 800–2000 cm^{-1} were collected using a Mattson Sirius 100 FTIR spectrophotometer at 2-cm^{-1} resolution. Before each experiment the sample was light-adapted at 295 K for 10 min with focused light from a 250-W tungsten lamp passing through a 15-cm path length water heat filter and a 530-nm glass high-pass filter. After light adaptation the sample was cooled to the temperature of the experiment. A closed-circuit helium refrigerator (CTI Model 21) with a Lake Shore Cryogenics 93C temperature controller was used to set the temperature to an accuracy of better than 0.2 K. Experiments were performed at temperatures between 210 and 271 K.

Photoreactions of bR were initiated by illuminating the sample with the optical arrangement used for light adaptation but the filters were varied: the bR and M forms were excited through 540-nm and 420-nm interference filters, respectively. The duration of the illumination was 10 s; preliminary tests indicated that a 10-s illumination of the bR form with 540-nm light results in saturating photocycling. In every case single-beam spectra of 100 scans were collected; the duration of 100 scans was about 1 min.

We used difference spectroscopy to detect the molecular changes during the transitions. In this method single-beam spectra are collected before and after the perturbation and the logarithmic difference of the two reflects the changes. In the case of bacteriorhodopsin, first a spectrum of the bR ground state (the background spectrum) is collected. Then, the sample is illuminated and a single-beam spectrum is collected again (the sample spectrum). The difference between these spectra reflects the change caused by the illumination, i.e., the difference between the intermediate produced and bR.

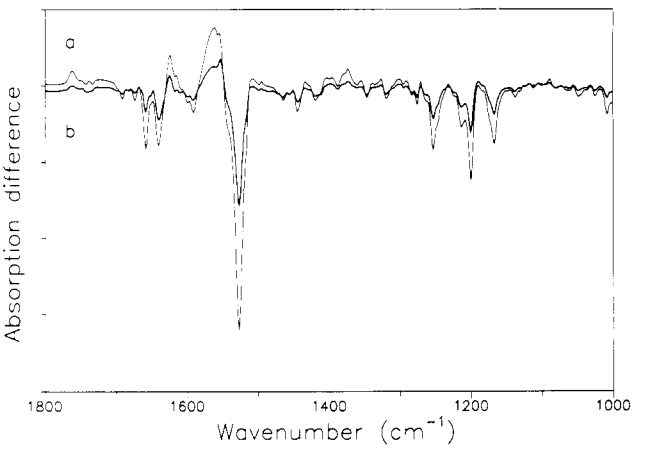

FIGURE 1: Infrared difference spectra of bacteriorhodopsin at 230 K following different illumination protocols. (a) Difference spectrum following illumination by green light. (b) Difference spectrum following green and subsequent blue illumination. The duration of the illumination in each case was 15 s. After green illumination, the spectrum collection of spectrum a was started after a 15-s waiting period to ensure identical timing in spectra a and b.

To determine the effect of blue-light excitation of M, difference spectra following two illumination protocols were compared. First, after dark background spectrum collection the sample is illuminated with green light for 10 s; this is immediately followed by illumination with blue light also for 10 s. In this procedure the green illumination results in a photoproduct that we expect to be a mixture of M and some other form (depending on the temperature). The subsequent blue illumination excites M and drives it (at least part of it) back to the bR state. The sample spectrum is then immediately collected and the difference absorption spectrum is calculated. We compare this spectrum to a control spectrum: after collecting the dark background spectrum, the sample is illuminated only with green light to see the total original amount of photoproduct of the green illumination. To ensure identical timing in the two cases, the sample spectra in the latter experiments are collected after a wait identical in length with the time of the blue illumination. Between each cycle of illumination and spectrum collection the sample was heated to 271 K to facilitate completion of the photocycle. The sample was equilibrated for 5 min and cooled back down to the temperature of the experiment. This procedure resulted in well-reproducible spectra. Due to the insufficient signal-to-noise ratio of the individual difference spectra, cycles of such experiments were repeated and corresponding spectra were averaged; typically 50 spectra pairs were used. The experiment was automated: the illumination shutter, the filter changing, and the temperature controller were PC-controlled, allowing accurate timing and reproducible experiments.

RESULTS

Typical difference spectra following only green and green + blue excitation are shown in Figure 1. Indeed, illumination by blue light causes a marked decrease in amplitude as well as a line shape change. Since the difference between the two difference spectra is the effect of blue illumination that drove

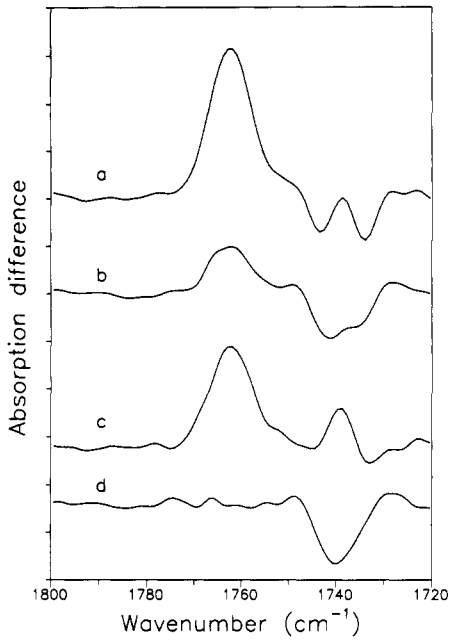

FIGURE 2: Illustration of the separation of the spectra due to the different intermediates in the photoproduct from the spectra in Figure 1. (a) Difference spectrum following green illumination (identical to spectrum a in Figure 1). (b) Difference spectrum following green and blue illumination (identical spectrum b in Figure 1). (c) Calculated difference spectrum for M – bR: the difference between spectra a and b. (d) Calculated difference spectrum due to the other component (L – bR): from spectrum a, a constant times spectrum c was subtracted. The constant was chosen such as to minimize the positive band at 1762 cm^{-1}.

part of M back to bR, simple subtraction of the spectra in Figure 1 therefore directly gives the difference spectrum M – bR (we will demonstrate later that this is correct). The separation of the other spectrum component is not so obvious, since the product following green + blue excitation still contains M (only a smaller amount—blue light excitation does not drive M back to bR with 100% efficiency). A comparison of the determined M – bR spectum with the one after only green excitation helps in the separation. A prominent, very well-defined difference is seen in the protonated carboxylic vibration region around 1760 cm^{-1}, as shown in Figure 2. It is clear that the 1762-cm^{-1} positive band is the main difference. The spectra suggest that this band is only present in the M – bR spectrum, in agreement with previous assignment of this band to Asp-85 protonated during the L → M transition (Braiman et al., 1988). We used this band for the separation. A constant times the M – bR difference spectrum is subtracted from the difference spectrum produced by green light: we chose the constant such as to eliminate the 1762-cm^{-1} band in the other spectrum component. This band was used to separate the difference spectra at all temperatures.

Depending upon the temperature, two types of mixture spectra were obtained. At low temperatures, below about 250 K, the spectra were the sum of the components shown in Figure 3, L and M. The component spectra obtained at the high temperatures, above 250 K, are shown in Figure 3, M and N. In both temperature domains the ratio of the components showed a temperature dependence; however, the spectra after separation did not differ significantly in the regions 220–250 K and 250–271 K.

DISCUSSION

Based on previous knowledge, the presented experiments are expected to cover the L–M–N part of the photocycle. At low temperatures an L/M mixture should be present, while

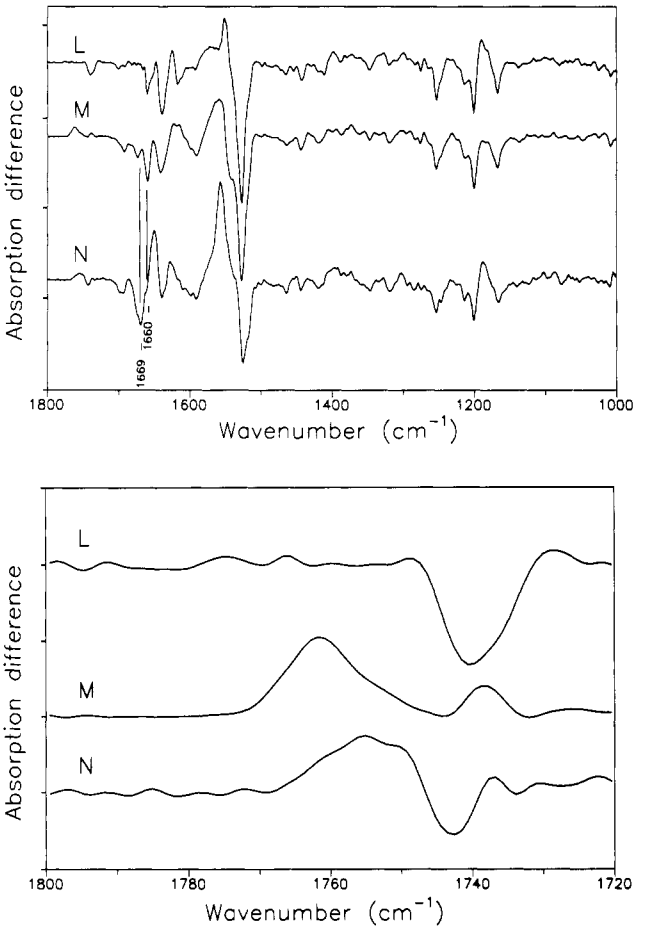

FIGURE 3: (Top panel) Difference spectra of the intermediates determined by the method illustrated in Figure 2. L, L – bR; M, M – bR; N – bR. The bars between spectra M and N mark the amide I bands, the exchange of which show the protein conformational change necessary for pumping. (Bottom panel) Same as in top panel, expanded to show the spectral region of the protonated carboxylic vibrations.

at the higher temperatures an M/N mixture is expected. Rough comparison of spectra L, M, and N in Figure 3 with those in the literature (Braiman & Rothschild, 1988; Pfefferle et al., 1991) confirms that indeed we see the L, M, and N forms. In the following sections we compare some characteristics of the resultant spectra with those obtained without this separation.

The L Intermediate. The obtained L – bR difference spectrum agrees very well with those published earlier. Comparison of subtle features of the protonated carboxylic vibrations is interesting, however. The characteristics of the spectrum are a large negative band with a peak at 1740 cm^{-1} and a shoulder at 1735 cm^{-1} assigned to Asp-96 (Braiman et al., 1988; Gerwert et al., 1989) and Asp-115 (Braiman et al., 1988), respectively. On the other hand, the positive shoulders at both sides of the double negative band observed in the low-temperature FTIR experiments at 1748 and 1729 cm^{-1} are practically missing. This fact is important since there is some controversy whether the negative band at 1740 cm^{-1} indicates a deprotonation of Asp-96 in the L state. According to Engelhard et al. (1985) and Gerwert et al. (1990), the positive shoulder at 1748 cm^{-1} together with the negative band at 1740 cm^{-1} rather indicate a spectrum shift and therefore a changed environment for the protonated Asp-96, as opposed to a deprotonation favored by Braiman et al. (1988 and 1991). The difference in the interpretation is not obvious to decide: the net negative band may be the result of an intensity change caused by a change, e.g., in hydrogen bonding. Although not

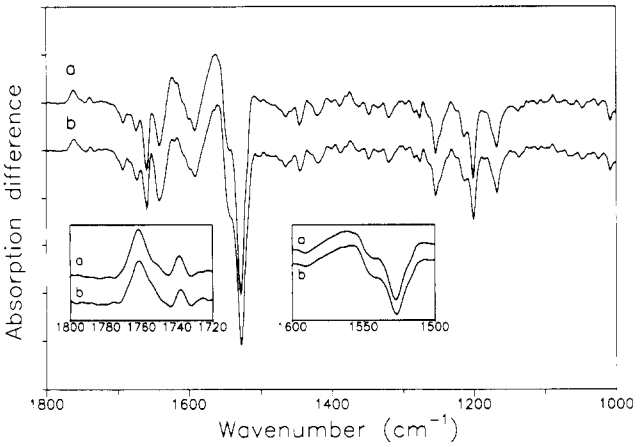

FIGURE 4: M – bR difference spectra obtained at different temperatures. (a) Average of M – bR spectra obtained below 250 K; at these temperatures M was separated from an L/M mixture. (b) Average of M – bR difference spectra obtained above 250 K; at these temperatures M was separated from an M/N mixture. The insets show the spectral regions of the protonated carbonyl and ethylenic vibrations enlarged for comparison.

compelling evidence, our results favor the latter interpretation. It seems that the negative band and the positive shoulder do not belong to the same group because while the negative band is identical to the one seen at low temperatures, the positive component is practically missing. It is not clear why in our spectra the shoulder disappears. In recent work by Chen and Braiman (1991) the spectra of L and M were separated from kinetic FTIR data at room temperature. There, too, the protonated Asp region of the spectrum of L looked similar to the one in this work. They proposed that the difference between the spectra at room temperature and low temperature (140 K) might be a temperature effect. If so, the change of bacteriorhodopsin with temperature that is represented by the change of the spectrum of L takes place below the lowest temperature we measured here: 220 K, much below physiological temperatures.

The M Intermediate. On observing the spectra of M determined by our method, the first important thing to note is that we obtain identical spectra at all temperatures measured (220–271 K). It is reasonable to divide the spectra from our experiments into two principal groups depending on whether and L/M or M/N mixture was illuminated by the blue light. We averaged together the M – bR difference spectra from these two regions and they are shown in Figure 4: they are practically identical without any difference in features. Again, the protonated carboxylic region is interesting: there are two positive bands, one at 1762 cm^{-1} and one at 1738 cm^{-1}. Other studies (e.g., Gerwert et al., 1989; Chen & Braiman, 1991) indicate a shoulder around 1755 cm^{-1} and a negative band at 1742 cm^{-1}. They are missing here. The positive bands seen were assigned to the groups Asp-85 and Asp-212, respectively (Braiman et al., 1988). The protonation of these groups is the only aspartic difference between bR and M. In the ethylenic region the 1557-cm^{-1} amide II line, also attributed to M at high temperature (Chen & Braiman, 1991), is not pronounced.

The N Intermediate. The results are in very good agreement with the N – bR spectra measured recently by Pfefferle et al. (1991). All characteristic features are present: in the protonated carboxylic region, the 1753-cm^{-1} band that recently is believed to belong to Asp-85 in a changed environment as compared to M (Pfefferle et al., 1991) and the negative band at 1740 cm^{-1} assigned to the deprotonated Asp-96. The ethylenic mode determined by resonance Raman spectroscopy to be at 1530 cm^{-1} is seen as a shoulder at 1532 cm^{-1} in our difference spectra. In the C–C stretch frequency region the positive band at 1186 cm^{-1} identical to that in the spectrum of L is also seen, as well as the positive band at 1397 cm^{-1} that is probably the vibration of the unprotonated Asp-96 (Braiman et al., 1991).

The clear separation of these spectra permits us to clarify the scenario in the photocycle around the Schiff base deprotonation and reprotonation. In a previous paper we have shown that at 240 K after the formation of M the Schiff base reprotonates from Asp-85 to which it has been previously released and no pumping takes place. On the other hand, at 260 K formation of M was shown to be followed by a protein conformational change that is characterized by the exchange of two negative amide I bands at 1660 and 1670 cm^{-1} and consequently regular proton pumping takes place. The conformational transition was interpreted as the T–C protein transition that changes the connectivity of the Schiff base between deprotonation and reprotonation. The state following this transition was characterized as M-like in kinetic equilibrium with N. The conformational transition was assumed to be kinetically independent of known transitions of the photocycle. On the basis of the present results, the scenario has to be modified: The difference in the M-like spectra at 240 and 260 K in our previous paper (Ormos, 1991) is entirely due to the N form present at the higher temperature. The conformational change characterized by the exchange of the negative 1660- and 1670-cm^{-1} amide I bands and the change of the environment of the protonated Asp-85 represented by the shift of the carboxylic band from 1762 to 1754 cm^{-1} occur simultaneously with the M to N transition as seen in Figure 3. This also coincides with the appearance of the negative band at 1740 cm^{-1}: the deprotonation of Asp-96.

These results support the interpretation by Braiman et al. (1991) but apparently contradict the model of Váró and Lányi (1991) where the crucial conformational change is said to take place between two at least kinetically well separated M forms. At this time the disagreement is not understood. Our experiments resulted in a single M form at temperatures from 220 to 270 K, whether it was in equilibrium with L or N—it even did not show a temperature dependence (Figure 4). The question arises whether our method, the application of the blue-light sensitivity of M to define its spectrum, might introduce some confusion. However, since the photosensitivity of M has been observed at the lowest temperatures M is seen (Litvin & Balashov, 1977; this work) as well as at room temperatures as long as M is observed (Ormos et al., 1980), if there are two sequential Ms both are photosensitive and therefore our method should distinguish between them. It cannot be excluded, however, that a kinetic reason may account for the disagreement. A certain temperature dependence of the rates of formation and decay of intermediates may result in an apparent disappearance of a particular form (if its decay is much faster then the formation the concentration will never reach a noticeable level even though it is part of the reaction chain).

It is interesting to note that the only aspartic protonation differences between bR and M are the protonation of Asp-85 and Asp-212. In addition, blue-light illumination also causes reprotonation of both. The fact that the protonation changes in the two occur simultaneously in both cases strongly supports the idea that, instead of simple counterion interactions between the protonated Schiff base and a single not protonated Asp residue, a more complicated arrangement is present. Lin (1988) pointed out that the electrostatic interaction between a protonated Schiff base and a single ionized Asp residue does not provide enough stabilization energy in a hydrophobic

environment. He suggested instead that a quadrupole is formed with additional positive and negative charged groups. Such an arrangement was concluded also from nuclear magnetic experiments (DeGroot et al., 1989, 1990). The members of this quadrupole would then be Asp-85, Asp-212, the protonated Schiff base, and an additional positive group. In the structure published by Henderson et al. (1990), the side chain of Arg-82 was tentatively arranged to point away from the Schiff base; however, it is more likely that the orientation is the opposite that would make this quadrupole interaction possible (K. Schulten, personal communication). In this case Arg-82 is a prime candidate for the second positive charge in the quadrupole as recently suggested by Dér et al. (1991) and Marti et al. (1991). The present results strongly support this picture. It is important to determine the protonation changes of Arg-82.

The method applied in this work gives an additional way to separate the spectra of intermediates. It has certain advantages over separation based on purely kinetic analysis: in the latter case the resultant spectra may depend on the kinetic scheme applied for the analysis. For example, in the recent excellent work of Chen and Braiman (1991) the resultant spectrum M – bR seems to contain N as a close inspection of the protonated aspartic bands suggests: the shoulder at 1754 cm^{-1} and the negative band at 1742 cm^{-1}. Our method is free of these kinetic problems; the advantage is exactly in separating the components in a kinetic equilibrium. The possible problems lie in the characterization of the M photoreaction. It is assumed that, on the time scale of our experiments, on excitation of M the molecule returns into the bR state. Although this assumption is not proven, there are several indications that it is reasonable: (1) Comparison of the resultant spectra with those in the literature does not indicate a previously not observed intermediate—the spectra are rather more simple. (2) At 255 K after blue-light excitation the N-like difference spectrum has an amplitude about 5% of that after only green-light illumination (data not shown); therefore, at least above this temperature, the amount of the possible intermediate between M and bR is negligible. (3) At room temperature the decay rate of the intermediate between M and bR concluded from photoelectric experiments is about 20 μs (Ormos et al., 1980). An extrapolation of the rate from data between 5 and 40 °C to the temperatures of this work shows that the rate at every temperature studied is large enough to ensure the decay of the not-defined intermediate.

An additional question about the method has to be discussed. It was assumed in the data manipulation and analysis that blue light only affects the M form and that following the excitation of M there is no reequilibration of the L/M or M/N mixtures (only in this case does our method yield the true spectrum of M). It is the results that support these assumptions: we obtained identical M – bR difference spectra at all temperatures, whether M was in equilibrium with L or N. This is only possible (in addition of course to the temperature independence of the spectrum of M) if our initial assumptions are correct. Otherwise, due either to the photoreaction of L or N or to reequilibration, an apparent temperature dependence of the spectrum of M would result.

It can be concluded about the method itself that, due to the different advantages and disadvantages compared to a simple kinetic analysis, it is useful in separating spectra of mixtures of intermediates; with better time resolution it could be applied at room temperature to decide the still not quite settled question about the timing of crucial conformational changes.

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