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Fourier transform infrared spectroscopic evidence for the existence of two conformations of the bacteriorhodopsin primary photoproduct at low temperature

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Fourier transform infrared difference spectroscopy of bacteriorhodopsin at low temperature reveals at least two stable forms of bacteriorhodopsin$_{570}$ and the K photoproduct. In the case of bacteriorhodopsin$_{570}$, warming from 81 to 135 K causes a reduction in absorption of several chromophore vibrations, but not the C=N stretching mode. These changes are consistent with a reorientation of the chromophore which leaves the angle of the C=N bond unchanged relative to the membrane plane. In the case of the K intermediate, two different forms can be isolated at 135 K on the basis of wavelength-dependent photoalteration. One form is identical to the low temperature K630 species, whereas a second blue-shifted form is present only above 135 K. This new form exhibits a 985 cm^{-1} peak in the hydrogen-out-of-plane bending region, which is similar to a reported room-temperature resonance Raman spectrum of K. Temperature-dependent changes in the conformation of the protein involving possible alterations in peptide hydrogen bonding are also detected.

Introduction

The bacteriorhodopsin photocycle involves both energy transduction and proton transport, two processes which are of great importance in biology [1,2]. At room temperature, photon absorption by the light-adapted state of bacteriorhodopsin, bacteriorhodopsin$_{570}$, results in the formation in approx. 10 ps of a red-shifted product designated K610 [3]. Additional intermediates in the bacteriorhodopsin photocycle are shown in Fig. 1. The early intermediate K ** is of particular interest, because subsequent steps in the photocycle occur in the dark, whereas the formation of K involves the absorption of light, and thus must be directly involved in the storage of the photon energy.

Numerous biophysical studies including visible and ultraviolet absorption [4–6], resonance Raman light scattering [7,8], and Fourier transform infrared absorption [9–11] have been carried out at

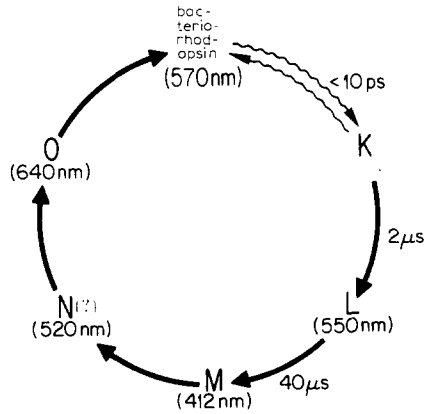

Fig. 1. Bacteriorhodopsin photocycle with wavelength of visible absorption maxima shown in parentheses. Approximate half-times for decay kinetics are also indicated.

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** Hereafter, we refer to all red-shifted primary photoproducts of bacteriorhodopsin$_{570}$ as K.

low temperature on K, particularly at 77 K where it is stable. One important question regarding these studies is whether the conformation of bacteriorhodopsin$_{570}$ and K at low temperature differs from the corresponding states at physiological temperatures. The fact that the visible absorption maxima of the bacteriorhodopsin$_{570}$ and K species are temperature sensitive indicates that at least the chromophore, and possibly the protein conformation, is affected. An additional complicating factor is that recent kinetic visible absorption and resonance Raman spectral studies both at low temperature and room temperature [12–15] reveal several forms of the K intermediate.

In order to investigate further the effect of temperature on the conformation of bacteriorhodopsin$_{570}$ and the K intermediate we have utilized Fourier transform infrared spectroscopy, which is an effective method for probing the protein and chromophore configuration [9–11,16,17]. Due to the high signal/noise ratio obtainable with Fourier transform infrared spectroscopy, the alterations which individual chromophore groups such as the retinal Schiff base undergo [17] during the photocycle can be detected. Since infrared absorption is sensitive to all the vibrations in purple membrane, individual protein groups such as the carboxyl group of aspartate or glutamate can also be probed [10,11,16].

In this study, we report evidence for differences between the conformation of bacteriorhodopsin$_{570}$ and the K intermediate at 81 and 135 K. At 135 K, two different forms of the K species can be distinguished on the basis of wavelength-dependent photoalteration. One form resembles the K630 intermediate measured at 81 K, whereas the second form has a blue shifted absorption maximum and exhibits alterations in several chromophore vibrations.

Materials and Methods

Purple membrane was isolated from the *Halobacteria halobium* strain S9 as reported previously [9]. Fourier transform infrared difference measurements were made on films of purple membrane deposited on AgCl windows by the isopotential spin-dry method [18]. A film is hydrated by direct exposure to a drop of H_2O for 3–5 min followed by shaking to remove excess water. The sample is then sealed in a chamber formed by adding a second AgCl window, light-adapted for 15 min with yellow light, and cooled rapidly to 81 K using a Helitran cryostat (Air Products, Allentown, PA).

Measurements were made with an MX-1 spectrometer interfaced to a 1200S data analysis system (Nicolet Inst., Madison, WI). Many aspects of the experiment including temperature control, illumination and interference filter selection, data acquisition and storage were automatically controlled using our program 'Multicon'. A Fourier transform infrared-difference spectrum is normally computed by subtracting the spectrum of a film recorded for 15 min in the dark from a second spectrum recorded in the dark after irradiating the film with green light for 15 min (Ditric Optics, Hudson MA, 500 nm wide-band interference filter). The sample is then photoreversed by red light (Ditric, 650 nm wide-band interference filter) for 15 min. Under these conditions of illumination it was found that the sample was driven maximally to K (which normally consists of 30% K and 70% bacteriorhodopsin$_{570}$ at 77 K [19]). In order to increase the signal-to-noise ratio of the final difference spectra, a sequence of off (bacteriorhodopsin$_{570}$) → green → off (K) → red is repeated several times. A Fourier transform infrared difference spectrum is computed by first subtracting the two 'off' spectra of each cycle, and then averaging each difference with the corresponding difference spectra from each of the other cycles. Using this procedure peaks as small as $2 \cdot 10^{-4}$ absorbance can be routinely detected above the noise level.

Results

Figs. 2A and B and 3A and B compare the difference spectra at 81 and 135 K in the regions 1300–1800 cm^{-1} and 800–1300 cm^{-1}, respectively. These data reveal changes in the conformation of both bacteriorhodopsin$_{570}$ and K at the two temperatures. For example, if we compare the negative peaks in Fig. 1A and B which reflect the conformation of bacteriorhodopsin$_{570}$, we find close agreement in frequency and intensity of the 1640 cm^{-1} protonated C=N stretching vibration of the Schiff base attachment between the retinal

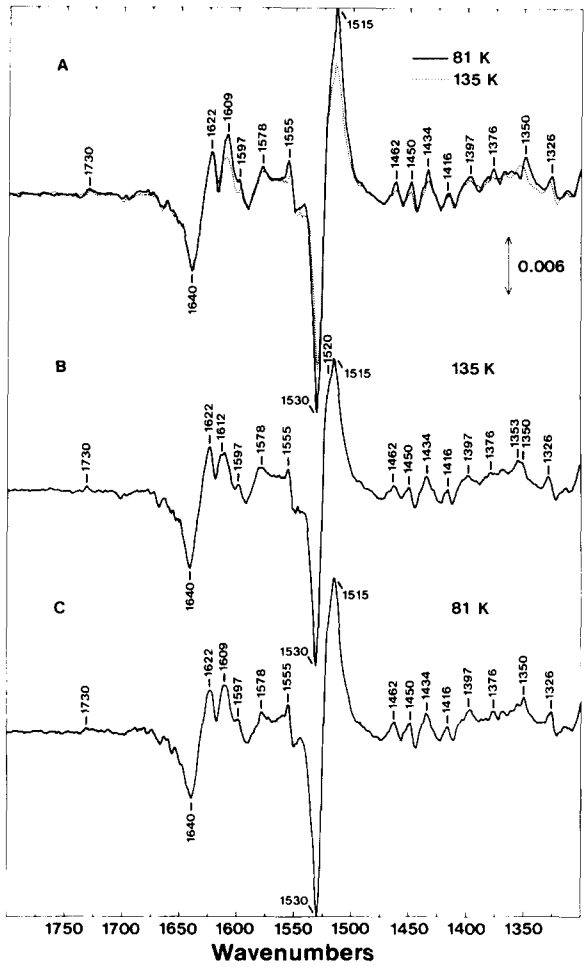

Fig. 2. Fourier transform infrared difference spectra in the range 1300–1800 cm^{-1} of purple membrane film at 2 cm^{-1} resolution. (A) 81 K, solid-line; 135 K, dotted line: four cycles of the sequence (light off (bacteriorhodopsin$_{570}$) → 500 nm → light off (K) → 650 nm) were repeated, where a 15 min spectrum was recorded for each part of the sequence (16 spectra in total for four cycles). A difference spectrum was determined by subtracting the first light-off (bacteriorhodopsin$_{570}$) from the second (K) for each cycle yielding four difference spectra which were averaged together. the 135 K difference spectra were recorded immediately after the 81 K spectra on the same sample by warming to 135 K in the dark in the pure bacteriorhodopsin$_{570}$ state. (B) The 135 K difference spectrum shown as dotted line in A is repeated for clarity. (C) The 81 K difference spectrum was recorded immediately after the 135 K difference spectrum on the same sample by cooling back to 81 K in the dark in the bacteriorhodopsin$_{570}$ state. The arrow indicates absorbance scale. Each one minute of 'scanning' consisted of the acquisition of 32 interferograms. Fourier transforms were computed with triangular apodization.

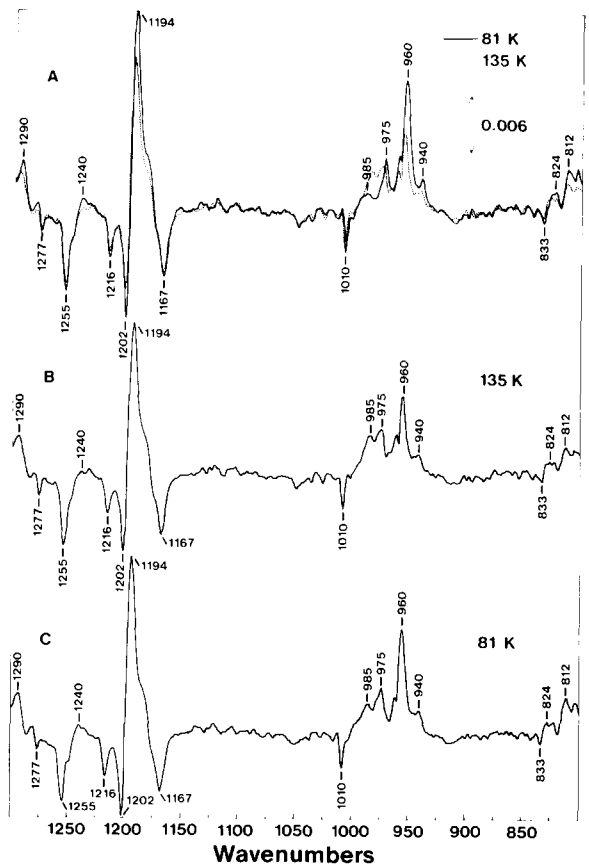

Fig. 3. Fourier transform infrared difference spectra in the region 800–1300 cm^{-1} recorded at 2 cm^{-1} resolution. All conditions in A, B and C are the same as in Fig. 2.

chromophore and the protein. In contrast, the negative peak at 1530 cm^{-1} due to the C=C stretching 'ethylenic' mode is diminished in intensity at 135 K relative to 81 K. A similar reduction in intensity is found for several other prominent negative peaks including those at 1255, 1216, 1202, 1010 and 833 cm^{-1}. All of these peaks with the exception of 833 cm^{-1} have been assigned to chromophore vibrations [7,20]. The 833 cm^{-1} peak has been shown recently on the basis of isotope substitution to be due to a tyrosinate Fermi resonance (Rothschild, K.J. et al., unpublished results). Significantly, none of the intensity changes noted above are accompanied by a significant frequency shift of more than 2 cm^{-1}.

In contrast to the situation for the negative bacteriorhodopsin$_{570}$ peaks, we find changes in

both frequency and intensity for many of the positive peaks in Figs. 2 and 3 which reflect the conformation of the K intermediate. For example, the integrated intensity of the 960 cm^{-1} peak assigned to the hydrogen out-of-plane bending mode in the retinal chain [7] is reduced by over 50% and a new peak appears near 985 cm^{-1}. The 1609 cm^{-1} peak, which has been assigned recently to the protonated C=N stretching vibration in K at 81 K [17], is reduced in intensity, leaving a residual peak at higher frequency. In the case of the 1515 cm^{-1} peak assigned to the C=C ethylenic stretching mode, a reduction in intensity occurs along with the appearance of a more pronounced high-frequency shoulder near 1520 cm^{-1}. Additional changes in intensity and/or frequency are found near 1350, 1290, 1240 and 1194 cm^{-1}. All of these peaks are present in the resonance Raman difference spectrum of the bacteriorhodopsin$_{570}$ to K transition at 77 K [20], and are therefore directly assignable to the chromophore.

We also note that the above-mentioned temperature-dependent alterations of the difference spectra cannot be attributed to alterations in the level of light–dark adaptation. In particular, the difference spectrum of a fully dark-adapted sample at 81 K exhibits characteristic peaks at 1178 cm^{-1} (positive) and 1348 cm^{-1} (negative) which are not found in the 81 or 135 K difference spectra of light-adapted samples.

Isolation of two different forms of K at 135 K

The results presented above suggest that more than one form of K exists at 135 K. For example, although the 1515 cm^{-1} peak is identical in frequency at 81 and 135 K, at 135 K a more pronounced shoulder appears near 1520 cm^{-1}. On the basis of the established correlation between the frequency of the ethylenic C=C stretch and λ_{max} of various photocycle intermediates and retinal based pigments [21] one would predict that this second form of K is blue shifted approx. 20 nm from the 81 K form (K630). As shown below, both K630 and the second form of K, tentatively designated K610, can be isolated.

In order to photoselect for K610, the following procedure is used. (i) The sample is driven to 'K' with 15 min illumination using 500 nm light. (ii) The sample is photoreversed for 5 min with 700 nm light which is preferentially absorbed by K630 over K610. (iii) The remaining K is photoreversed for 5 min with 650 nm light.

Fig. 4C shows the difference spectrum between the first and second five min of 500 nm illumination. It can be seen from the 985 cm^{-1} peak that significant amounts of K610 are produced. The Fourier transform infrared difference spectrum obtained in the second photoreversal, step (iii), (cf. Fig. 4A) clearly shows that the species pushed back to bacteriorhodopsin$_{570}$ has an ethylenic C=C stretch frequency at 1520 cm^{-1} as well as a large 985 cm^{-1} peak with very little intensity at 960 cm^{-1} indicative of a predominant photoreversal of

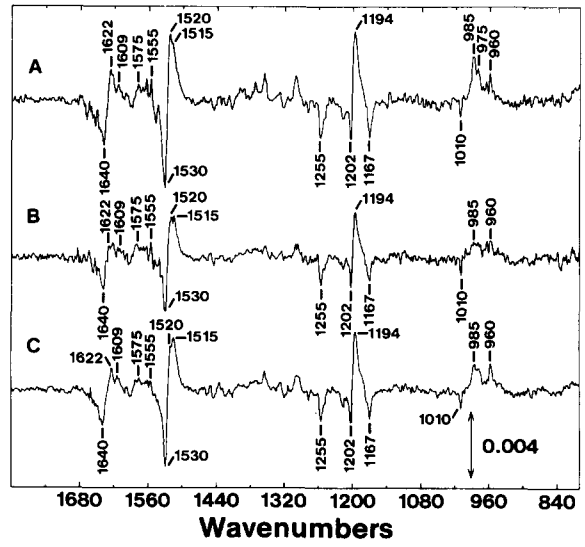

Fig. 4. Fourier transform infrared difference spectra at 135 K. 5-min scans were taken during each step of the illumination sequence 500 nm → 500 nm → 500 nm → 700 nm → 650 nm. (A) Spectrum obtained by computing the difference between the last two illumination steps. Note the order of subtraction for photoreversal (K → bacteriorhodopsin$_{570}$) [spectrum (i) – spectrum ($i+1$)]. (B) An identical procedure as in (A) was used except that the order of the 700 and 650 nm illumination in the sequence was reversed. (C) Spectrum obtained by computing the difference between the first two 500 nm illuminations after one complete cycle. All spectra are at 2 cm^{-1} resolution. The low signal-to-noise ratio exhibited in these spectra is due to two factors: (i) the use of shorter scanning time, which is necessary due to the kinetics of the respective K photoreversals at this temperatures, and (ii) the fact that less K is converted to bacteriorhodopsin in this time period. The arrow indicates absorbance scale.

K610. As a control, we also followed the same procedure outlined above but reversed the order of the 700 nm and 650 nm illumination. In this case the K610 species is expected to be driven more strongly to bacteriorhodopsin$_{570}$ in the first photoreversal. As shown in Fig. 4B, both the 1520 and 985 cm^{-1} peaks are now reduced relative to the 1515 and 960 cm^{-1} peaks indicating a greater amount of the K630 form relative to K610.

It is also possible to photoselect for the K630 species at 135 K. In this case, the sample was illuminated with 500 nm light for 3 min and then photoreversed with 700 nm light which selects for the K630 species. As shown in Fig. 5A and B, the resulting difference spectrum closely resembles that recorded at 81 K.

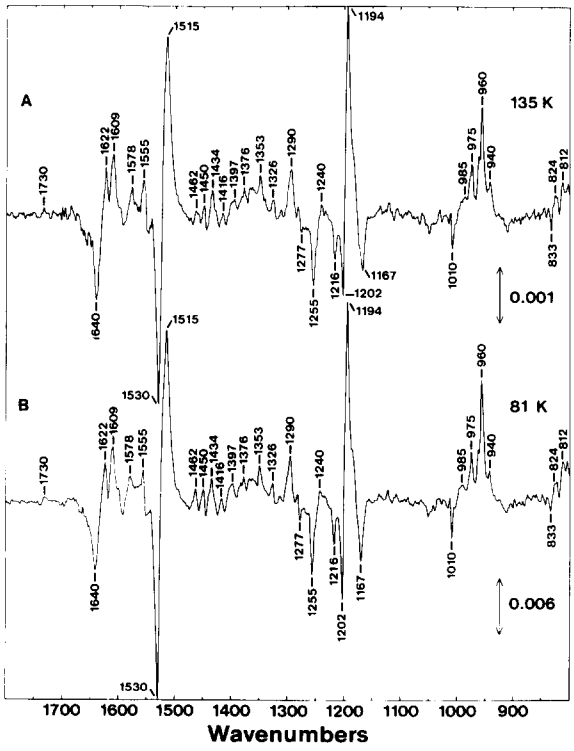

Fig. 5. (A) A Fourier transform infrared difference spectrum representing the average of 25 differences between a sample at 135 K illuminated cyclically for 3 min with 500 nm light and photoreversed with 3 min of 700 nm illumination. (B) Fourier transform infrared difference spectrum of sample recorded at 81 K using same data as in Figs. 2 and 3A. Note: absorbance scale for (A) is not the same as for (B).

Temperature-induced changes in the protein and chromophore

If the spectrum of light-adapted purple membrane cooled to 81 K is subtracted from a similar spectrum recorded at 135 K, the difference spectrum shown in Fig. 6A is obtained. If one compares this spectrum to the bacteriorhodopsin$_{570}$ to K difference spectrum at 81 K (Figs. 2A and 3A), there is agreement in the frequency of several negative peaks including those at 1530, 1256, 1202 and 1167 cm^{-1}, all of which have been previously assigned to vibrations in the bacteriorhodopsin$_{570}$ chromophore [7,20]. This comparison allows one to reach two conclusions: (i) the conformation of the bacteriorhodopsin$_{570}$ chromophore is altered between 81 K and 135 K; and (ii) the reduction in absorbance of many of the negative peaks in the bacteriorhodopsin$_{570}$ to K difference spectrum between 81 and 135 K can be attributed to an intrinsic reduction in absorbance of the bacteriorhodopsin$_{570}$ chromophore vibrations.

A similar procedure as described above can be used to study the changes occurring in the K intermediate upon warming. In this case, a spectrum is recorded of a sample driven partially to K at both 81 and 135 K and the difference computed (cf. Fig. 6B and C). Warming between the two temperatures was done both in the original bacteriorhodopsin$_{570}$ state (Fig. 6B) or in the K state (Fig. 6C). As seen, negative peaks appear in both cases at 1348, 1194 and 960 cm^{-1} which are characteristic of the K chromophore vibrations at 81 K [7,20]. In addition, positive peaks appear at 1354 and 985 cm^{-1}, which as seen in Figs. 2 and 3 are characteristic of the K intermediate at 135 K and most likely arise from chromophore vibrations. We therefore conclude from these comparisons that the intrinsic vibrational modes of the K chromophore are altered upon warming from 81 to 135 K. A related conclusion derived from Fig. 6C is that the K630 species appears to convert partially to K610 upon warming.

There are also major features in the difference spectra shown in Fig. 6 A–C which cannot be easily related to chromophore vibrations. In particular, the large negative and positive peaks at 1558 and 1543 cm^{-1}, respectively, are due to a downshift in frequency of the amide II mode

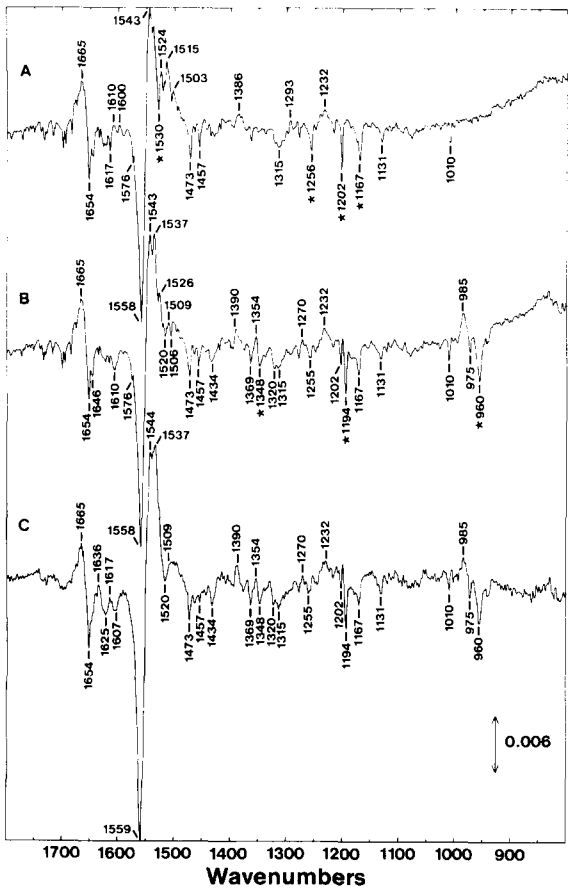

Fig. 6. Fourier transform infrared difference spectrum representing the average of four 15 min differences between a sample at 135 K and the same sample at 81 K. (A) Sample was warmed in the bacteriorhodopsin$_{570}$ state in the dark. Four cycles at both temperatures were completed and differences computed from the respective off (bacteriorhodopsin$_{570}$) spectra where the cycle consisted of: off (bacteriorhodopsin$_{570}$) → 500 nm → off(K) → 650 nm. * denotes some of the bacteriorhodopsin$_{570}$ vibrations which change during the bacteriorhodopsin$_{570}$ → K transition. (B) Same as (A) except difference was computed between the off(K) spectra. * denotes some of the K vibrations which change during the bacteriorhodopsin$_{570}$ → K transition. (C) Difference spectrum computed by subtracting the last off (K) spectrum at 81 K from the first spectrum at 135 K, after warming in the K state. Arrow indicates absorbance scale.

(peptide NH in-plane bend) as the sample is warmed. This can be seen directly by observing the frequency of the amide II or amide II′ (ND bending) as a function of temperature (Rothschild, K.J. and Roepe, P., unpublished data). Similarly, a set of positive and negative peaks at 1665 and 1654 cm^{-1} are due to an upshift in frequency of the amide I vibration near 1660 cm^{-1} [23]. The direction of the amide I and II frequency shifts would be consistent with an increase in the hydrogen bond length of the bacteriorhodopsin skeletal peptide hydrogen bonds. Since most of the protein is in the form of oriented alpha-helices [23], it would be reasonable to expect this to result in an overall lengthening of these helices. These results are not unexpected, since similar shifts have been observed in poly(L-alanine) [24].

We also note that the amide II bond shifts are different for warming in bacteriorhodopsin$_{570}$ (Fig. 6A) compared to warming in the K state (Fig. 6C). In the latter case the amide II appears to downshift to a lower frequency causing a splitting at 1544 and 1537 cm^{-1}. This suggests that the protein conformation is different in bacteriorhodopsin$_{570}$ and K.

Irreversible photoalteration of bacteriorhodopsin$_{570}$ at 135 K

It is important to establish whether the changes discussed in the previous sections are reversible. The photoreversability of K back to bacteriorhodopsin$_{570}$ at 81 K can be ascertained by subtracting a spectrum of bacteriorhodopsin$_{570}$, recorded in the dark, from a second spectrum, also recorded in the dark, after the sample has been sequentially illuminated with green light and red light for 15 min. As shown in Fig. 7A, this results in an essentially flat line. This result is in accord with visible-absorption low-temperature studies. In contrast, if this same procedure is followed at 135 K we find definite changes (Fig. 7B) which correspond to alterations in bacteriorhodopsin$_{570}$ before and after the first cycle of illumination. In particular, the positive peaks at 1530, 1255, 1202 and 1167 cm^{-1} all indicate an intrinsic reduction in the absorption of these bands after the first illumination cycle at 135 K. Note that if the sample is recooled back to 81 K we again find reversibility after a cycle of illumination (Fig. 7C).

We also note that changes in the K intermediate after photocycling at 135 K and then cooling to 81 K are deduced by comparing the bacteriorhodopsin$_{570}$ to K difference spectra in

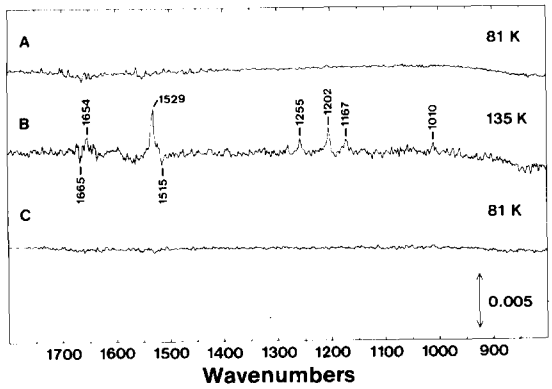

Fig. 7. Fourier transform infrared difference spectra computed by subtracting the two consecutive off (bacteriorhodopsin$_{570}$) scans at (A) 81 K prior to 135 K photocycling, (B) 135 K and (C) 81 K after 135 K photocycling. Warming and cooling was always performed in the off (bacteriorhodopsin$_{570}$) state. Complete photoreversability is seen in the case of the 81 K differences. The low signal-to-noise ratio in these spectra is due to subtraction of single 15 min scans. The arrow indicates absorbance scale.

Figs. 2A and C and 3A and C. There also exist differences in the positive K peaks. For example, a reduction of the peak at 1609 cm^{-1} relative to the 1622 cm^{-1} peak is found. Note, however, that not all of the changes observed at 135 K such as the appearance of the peak at 985 cm^{-1} are present. As a control we recorded a spectrum of a sample warmed, but not exposed to green light at 135 K, and then cooled back to 81 K. This resulted in no detectable change from the 81 K spectrum recorded prior to 135 K incubation.

Discussion

The data presented above indicates that at least two distinct forms of both bacteriorhodopsin$_{570}$ and K can exist at low temperature. The evidence includes the following.

(i) The bacteriorhodopsin$_{570}$-to-K difference spectra recorded at 81 and 135 K do not agree quantitatively. For example, most of the negative peaks associated with the bacteriorhodopsin$_{570}$ chromophore vibrations undergo a reduction in intensity at 135 K. Since the 1640 cm^{-1} band due to the C=N stretching mode remains unchanged, we can eliminate the possibility that less bacteriorhodopsin$_{570}$ is converted to K at the higher temperature. This possibility is also eliminated, since changes in both intensity and frequency of positive peaks associated with the formation of K are observed including a reduction of over 50% in the 960 cm^{-1} hydrogen-out-of-plane mode and the appearance of a new peak at 985 cm^{-1}. Interestingly, a similar change in the hydrogen-out-of-plane region is also found when one compares the resonance Raman spectrum of K at 77 K [7] with a room temperature spectrum recorded using a flowing sample and 656 nm illumination [12,13].

(ii) Warming a sample of bacteriorhodopsin$_{570}$ in the dark from 81 to 135 K produces changes in both chromophore and protein vibrations. In the case of the chromophore vibrations, an intrinsic loss of absorption is found in the same peaks which are reduced in the bacteriorhodopsin$_{570}$ to K difference spectrum. Temperature-induced protein conformational changes also occur as indicated by shifts in the amide I and II bands. These shifts, which are fully reversible, are consistent with the lengthening of the hydrogen bonds and subsequent alpha-helix elongation as the sample is warmed. We have also found that illumination of bacteriorhodopsin$_{570}$ at 135 K with green light and photoreversal with red light produces well-defined changes in the absorption of bacteriorhodopsin$_{570}$ chromophore vibrations which are not reversible and persist partially even upon recooling to 81 K.

(iii) The existence of two K intermediates at 135 K can also be deduced from the clear dependence of the photoreversal difference spectrum (K to bacteriorhodopsin$_{570}$) on the wavelength of irradiation. A long wavelength form is identical to K630 measured at 81 K. A second intermediate also present at 135 K exhibits a 5 cm^{-1} upshift in the ethylenic C=C stretch mode which would correspond to a downshift of 20 nm in the wavelength of the absorption maximum.

Several tentative conclusions can be reached about the nature of the conformational changes occurring in the two forms of bacteriorhodopsin$_{570}$ and K. In the case of bacteriorhodopsin$_{570}$ there are predominantly absorption changes without shifts in frequency or new peaks appearing. Since the sample consists of purple membrane sheets stacked in a multilamellar configuration [18], this effect could arise if there was a change in the

relative orientation of the chromophore relative to the membrane plane. At room temperature the chromophore tilt angle has been determined on the basis of linear dichroism to be close to 23 degrees with respect to the membrane plane [18]. Since there is a reduction in absorption of the in-plane C=C, C – C and CH bending vibrations, including the 1202 cm^{-1} peak associated with the $C_{14} - C_{15}$ stretching mode, this would be consistent with a tilting of the chromophore chain away from the membrane plane as the sample is warmed from 81 to 135 K. Such an effect might occur if warming produced a rotation about a single bond such as $C_{14} - C_{15}$ which left the orientation of the C=N bond unchanged. The apparent changes in α-helix conformation deduced in this study might be responsible for such an effect.

The change in the K intermediate which occurs between 81 K and 135 K is likely to involve more than a simple reorientation of the chromophore. For example, the upshift of the ethylenic C=C stretch from 1515 to 1520 cm^{-1} reflects a decrease in charge delocalization along the polyene chain and a concomitant blue shift of the K wavelength for maximum absorption. Similarly, the reduction in intensity and shift to higher frequency of the 1609 cm^{-1} peak in K610 is also consistent with a decrease in the charge delocalization of the chain. The reduction in intensity of the hydrogen-out-of-plane mode near 960 cm^{-1} and the appearance of a new peak near 985 cm^{-1} also may imply configurational change of the K chromophore. However, we cannot totally exclude at this point the possibility that some of these changes mainly reflect a change in the orientation of the chromophore relative to the membrane plane. For example, an increase at 985 cm^{-1} could arise from a hydrogen out-of-plane mode whose transition moment tilted further toward the membrane plane. Such an occurance for an out-of-plane chromophore vibration would be consistent with the movement of in-plane transition moments tilting further away from the membrane plane. We are currently exploring this possibility with polarized Fourier transform infrared difference spectroscopy.

We also note that our findings are in agreement with an earlier low-temperature visible-absorption study by Kalisky and Ottolengthi which found on the basis of multiple decay kinetics evidence for more than one form of K [14]. They concluded that at 180 K, the primary photoproduct, K610, rapidly equilibrates with a second blue shifted species. It is difficult, however, to make direct comparisons between their results and the present study due to the difference in temperature.

The existence of at least two forms of K at low temperature is significant for several reasons. First, many studies of the 'K' intermediate have been made at 77 K where it is stable and can be photoreversed back to bacteriorhodopsin$_{570}$. For example, laser photocalorimetric measurements have been performed at 77 K in order to determine the amount of energy storage in K [25]. In addition, a number of resonance Raman and Fourier transform infrared studies of K at 77 K have been made in order to determine the overall structure of the chromophore and protein [7–13,16,17,20]. The fact that there exists a different conformation of both bacteriorhodopsin$_{570}$ and K at 135 K relative to 81 K indicates that these studies should probably be repeated at a variety of temperatures.

Secondly, there exists the possibility of a correlation between the various room temperature and low temperature red-shifted intermediates. The earliest observed room temperature intermediate, J630, appears within 1 ps [26] and decays with a time constant of approx. 11 ps [3] to a second intermediate absorbing near 610 (K610). The J intermediate most likely corresponds to a ground state of the chromophore which is in a 13-*cis* configuration [27,28]. A third red-shifted intermediate which absorbs maximally near 596 nm has also been observed 150 ns after flash excitation which is distinct from the K610 intermediate measured between 50–900 ps [15]. Hence, the possibility exists that the two red-shifted intermediates detected in our experiment at 81 and 135 K correspond to these two different room temperature K intermediates, or that the 81 K form corresponds to the J intermediate. Future experiments will be aimed at testing these various possibilities and to investigate further the conformation of bacteriorhodopsin and its red-shifted intermediates.

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