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Primary events in bacteriorhodopsin probed by subpicosecond spectroscopy

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The photochemical cycle of light-adapted bacteriorhodopsin at room temperature was initiated by 0.6-ps single pulses at 615 nm. In the spectral region from 410 to 750 nm the absorption difference spectra were measured. Three photoinduced states have been observed during the first 10 ps after excitation. The difference spectrum between the latest one and bacteriorhodopsin is similar to the difference spectrum between the earlier observed K intermediate (with nanosecond lifetime) and bacteriorhodopsin. The K intermediate is preceded by the primary photoproduct with 3-ps lifetime (the J intermediate). Both J and K have red-shifted spectra compared with the initial spectrum of bacteriorhodopsin. The earliest state has a lifetime of 0.7 ps, and a blue-shifted spectrum. We have designated this state as I_{460} in accordance with its maximum in difference spectrum, and considered that I_{460} is the electronically excited state of bacteriorhodopsin. The photoinduced dichroism measurements made are in good agreement with this consideration. The complete sequence of the primary photochemical events in bacteriorhodopsin can be written as:

$$\text{bacteriorhodopsin} \xrightarrow{h\nu} \text{bacteriorhodopsin}^*(I_{460}) \xrightarrow{0.7\ \text{ps}} J \xrightarrow{3\ \text{ps}} K$$

Introduction

Bacteriorhodopsin is a photoactive pigment of the purple membranes of *Halobacterium halobium*. It contains a retinal chromophore attached to a protein via a protonated Schiff base [1].

The research into the primary photoreaction of bacteriorhodopsin has begun by Kaufmann et al. [2] who measured with picosecond resolution the formation of the K intermediate, called so after the work by Losier et al. [3] who described the photochemical cycle of bacteriorhodopsin. K was the earliest intermediate observed by nanosecond flash-photolysis at a temperature of 1°C [3] and at room temperature [4]. The difference spectrum between the K state and the initial form of light-adapted [1] bacteriorhodopsin is characterized by a positive band with its maximum at 635 nm ($\Delta A > 0$, where ΔA is the absorbance change), a negative band ($\Delta A < 0$) with its minimum at about 550 nm and intersects the base line at 595 nm [3,4]. In both works the maximum-to-minimum ratio of difference spectrum was about 1.15.

A difference spectrum similar to the above was observed in experiments performed by picosecond flash-photolysis at room temperature. The difference spectrum measured by Kaufmann et al. [2] at 13 ps after 6-ps excitation did not change for at least 300 ps, and was similar to the spectrum between K and bacteriorhodopsin, but the maximum-to-minimum ratio of difference spectrum was about 1.6. Later, Applebury et al. [5] in a similar

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experiment studied in more detail the kinetics of formation of the intermediate observed by Kaufman et al. [2]. This intermediate was detected at 50 ps after excitation with the ratio that approximated the one observed by Kaufmann et al., and was equal to 1.45. It was preceded by an earlier state arising during the excitation, of which the difference spectrum was shifted to red (relative to the spectrum measured after 50 ps) and the lifetime was equal to 11 ps. The later state was identified by Applebury et al. [5] as the K state observed before, and the earlier one can be termed (according to the designations introduced by Dinur et al. [6]) as the J state.

In 1983 Shichida et al. [7] measured the difference spectra during excitation of bacteriorhodopsin by a 25-ps pulse and in a time range from 50 ps to 5 μs after excitation. There was a difference spectrum similar to the spectrum of the K state observed by Applebury et al. [5]. It was observed at 50, 100 and 500 ps after excitation. The positive band maximum was at 625 nm, the spectrum intersected the base line at 590 nm, and the maximum-to-minimum ratio of the difference spectrum was equal to 1.33. At 150 ns after excitation the difference spectrum changed somewhat. The point of intersection and the band maximum were red-shifted (up to 595 nm and 635 nm, respectively), and the amplitude ratio was about 1. On the whole, the difference spectrum became similar to the spectra obtained by Losier et al. [3] and Goldschmidt et al. [4]. Shichida et al. [7] concluded that the K state observed in a picosecond time interval differed from the state arising in a nanosecond time interval. The latter was designated as kL, and its lifetime was equal to 2.2 μs, which approximated the result obtained by Losier et al. [3] for the decay of K.

During excitation Shichida et al. [7] observed a difference spectrum red-shifted by 10 nm relative to the K formation spectrum. It was interpreted as the spectrum corresponding to the formation of the J intermediate.

The J intermediate and the formation of the K intermediate were observed at low temperatures [5,8] where the K state is metastable. The results, on the whole, are similar to the results of measurements at room temperature, but the lifetime of J increased up to 36 ps at 4 K [5].

First of all, the present paper is concerned with the processes preceding the formation of the J state as well as with more precise determination of some parameters of the $J \rightarrow K$ process at room temperature. In this case the time resolution must be better than 1 ps. To date the photoinduced absorption changes in bacteriorhodopsin which occur at 615 nm, with a time constant of 1 ± 0.5 ps after the action of a 0.5-ps pulse, have been observed by Ippen et al. [12]. It can be assumed that 1 ps is the formation time of the J state, and that another state, I, preceeds J [6]. But measurements must be carried out over the entire spectral range where absorption changes may take place, and the sample must be excited by a single ultrashort pulse rather than by a pulse train.

Materials and Methods

The source of ultrashort pulses in the experiment was a rhodamine-6G ring dye laser, operating in the colliding pulses regime [13]. It was pumped by 6–8 W argon laser radiation. The dye laser radiation was a continuous pulse train with a pulse duration of less than 0.1 ps, and with intervals of 12 ns. The wavelength was 615 nm, and the spectral width was equal to 8 nm. The pulse train passes through four cells with rhodamine B, where the laser pulses were amplified at the moment that the dye was excited by second-harmonic pulses of the Nd:YAG laser. Since the pumping pulse duration was 10 ns and the repetition rate was 1 pps, only one ultrashort pulse was amplified every second. The amplified pulse energy was 0.5 mJ, and its duration was equal to 0.6 ± 0.1 ps. The fact that the amplified pulses were longer than the ones from the master oscillator is explained by group velocity dispersion in the optical elements of the amplifiers.

After being amplified the pulse was divided into two channels (exciting and probe), and in the probe channel the radition at 615 nm was converted to picosecond continuum (in water) as it is usually accepted in ultrafast spectroscopy experiments [9]. The diameter of the exciting beam on the sample cell was 2 ± 0.5 mm, and the maximum excitation energy was $(5 \pm 1) \cdot 10^{-5}$ J. Due to the optical delay line the probe pulse could pass through the excited part of the sample at variable

time intervals after (or before) excitation. The delay line position at which the exciting and the probe pulses coincide in the sample corresponds to the zero point on the time scale. It should be noted that the zero point differs for different wavelengths, as the blue part of the continuum is delayed in relation to the red one [9]. We determined the zero point in measuring the kinetics at different wavelengths. In measuring the difference spectra the dependence of the zero point on wavelength was assumed to be linear. Over the spectral region, where the measurements were performed (410–750 nm), the delay correction was equal to $1.3 \cdot 10^{-2}$ ps/nm.

After the sample cell the probe light passed through the monochromator and was recorded by a photomultiplier.

Measurements were not taken in the spectral region from 595 to 640 nm because of scattering of exciting radiation with $\lambda = 615$ nm. To obtain 100% linear polarization, polarizers were placed in front of the cell, in the probe and the exciting channels. The polarization of both the beams was parallel. If required, the exciting radiation polarization was turned by 90° with a quartz plate (4.6 mm thick).

The sample was produced from *Halobacterium halobium* (353п strain) using the technique described by Oesterhelt and Stoeckenius [10] and modified by Chekulayeva [11]. The cell in which measurements were performed was 2 mm thick, the optical density of the sample at the maximum of light-adapted bacteriorhodopsin absorption band (570 nm) being equal to 1. The purple membranes were kept in water (pH 5.7). Before being measured the sample was adapted for 5 min to the light from a slide projector passed through a thermal filter. Additional illumination was done during the experiment every 15 min. After 8 h of continuous work there were no changes anymore in the specturm of the sample or in its optical density which would point to its destruction.

Results

Fig. 1 shows difference spectra with wavelength correction of the zero point. The spectrum measured in the wavelength region 410–595 nm at 0.5 ps after excitation has a positive band with its

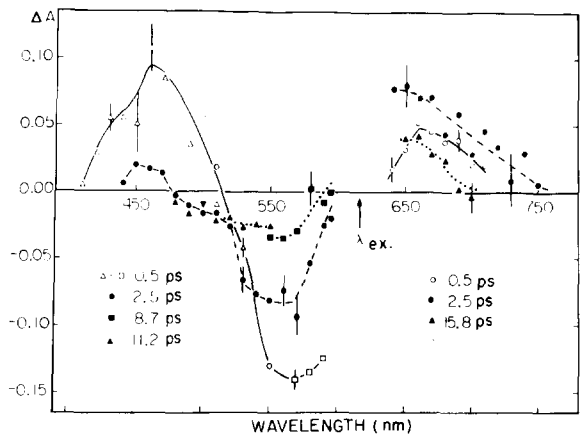

Fig. 1. Bacteriorhodopsin difference absorption spectra at room temperature measured at the indicated moments of time after excitation with 0.6-ps pulses at 615 nm (exciting fluence, $P_{ex} = 2.5 \cdot 10^{15}$ photon/cm^2). each point is an average of 20–40 measurements. Standard error bars are shown. △○□, three independent series of measurements at 0.5 ps after excitation. ΔA, absorbance change.

maximum at 460 nm and a negative band with its minimum at 570 nm (solid line). The difference spectrum intersects the base line at 515 nm. The negative band has apparently its origin in the bleaching of the initial absorption band of bacteriorhodopsin, and the absorption increase in the blue spectral region is related to the formation of the state not observed in the photochemical

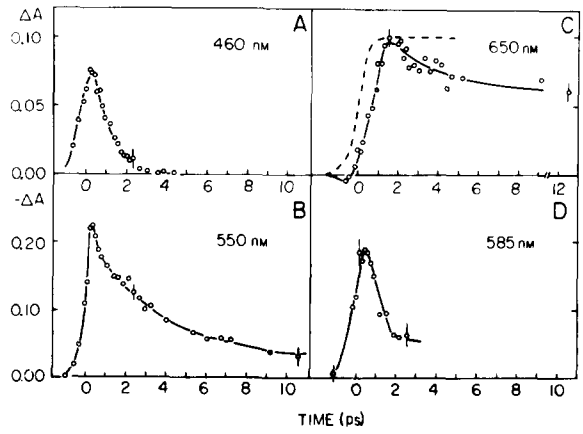

Fig. 2. Ultrafast absorption kinetics in bacteriorhodopsin measured at 460 nm, $P_{ex} = 2 \cdot 10^{15}$ photons/cm^2 (A); 550 nm, $P_{ex} = 4 \cdot 10^{15}$ photons/cm^2 (B); 650 nm, $P_{ex} = 2.5 \cdot 10^{15}$ photons/cm^2, the dotted line represents instantaneous response to 0.6-ps pulse (C); 585 nm, $P_{ex} = 3.5 \cdot 10^{15}$ photons/cm^2 (D).

cycle of bacteriorhodopsin before. The time dependence of absorption at 460 nm is shown in Fig. 2a. The same kinetics in the semilogarithmic scale is presented in Fig. 3a. It can be seen that the increase of optical density arising during excitation then exponentially decays with $\tau_1 = 0.7 \pm 0.3$ ps to the zero level of ΔA.

The time dependence measured at 550 nm (Figs. 2b and 3b) is more complicated. The semilogarithmic plot points to a two-component decay of bleaching arising during excitation. The slower component has $\tau_2 = 3.0 \pm 0.5$ ps. The fast component prevails at 585 nm where a two-component dependence can be observed, too (Fig. 2d). The time τ_1 is estimated to be 0.6 ps that is consistent with the time τ_1 measured at 460 nm.

In the spectrum measured at 2.5 ps after excitation, along with the partially relaxing bleaching of the initial absorption band of bacteriorhodopsin and almost completely relaxing absorption in the blue region, one can observe a considerable increase of absorption in the red region with a maximum at 650 nm which extends up to 750 nm (Fig. 1, dashed line). The state absorbing in the red region is formed with a delay about the instant of excitation that is explained by the kinetics measured at 650 nm (Fig. 2c). At the beginning there is slight bleaching followed by increasing absorption. For comparison, Fig. 2c shows an instantaneous response (dashed line) which would be observed in the case of formation of an absorbing and long-lived state during excitation. But in our case an absorbing state is formed about 0.7 ps after excitation. This estimate results from analyzing experimental dependences with a computer (see Discussion) and agrees with the lifetime of the state arising during excitation.

As it has been pointed out, at 550 nm one can observe a 3-picosecond component of bleaching relaxation which results in long-lived (in our time scale) changes of absorbance. At 650 nm the relaxation of the transient absorption to a stationary value is described by $\tau_2 = 3.4 \pm 1.0$ ps (Fig. 2c) that is consistent with the results of measurements at 550 nm.

The long-lived state is characterized by the difference spectra measured at 8.7 ps after excitation in the spectral region from 550 to 595 nm, at 11.2 ps in the region from 480 to 550 nm and at 15.8 ps in the region from 640 to 750 nm.

At 0.5 ps after excitation the ratio $\Delta A_{\parallel}^{550}/\Delta A_{\parallel}^{460}$ equals 1.37 ± 0.06. The symbol $\parallel$ denotes the

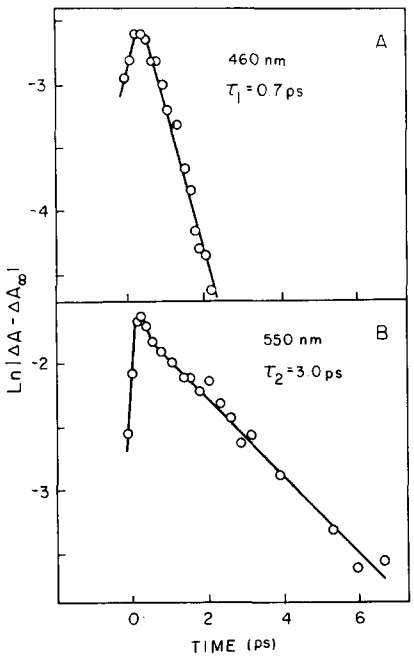

Fig. 3. Semilog plots of kinetics of Fig. 2. (A) Kinetics measured at 460 nm. Relaxation time $\tau_1 = 0.7 \pm 0.3$ ps. (B) Two-component kinetics at 550 nm. The slow component has a relaxation time $\tau_2 = 3.0 \pm 0.5$ ps. ΔA_∞, absorbance change measured at 10 ps after excitation and later.

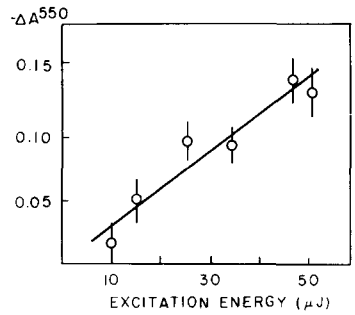

Fig. 4. Dependence of absorbance changes at 550 nm on excitation energy measured at 1.5 ps after excitation. The fluence $P_{ex} = 4 \cdot 10^{15}$ photons/cm^2 corresponds to the maximum excitation energy ($5 \cdot 10^{-5}$ J). At such fluence the deviation from the linear dependence shown in the figure was no more than 10% because of saturation.

parallel polarization of exciting and probe beams. When the exciting radiation polarization plane is turned by 90° with a quartz plate, the ratio $\Delta A^{550}/\Delta A^{460}$ becomes equal to 1.09 ± 0.09.

The dependence of optical density on exciting pulse energy was measured at 1.5 ps after excitation (Fig. 4). The experimental results over the whole energy range can be described by a linear dependence (the solid line in the figure). In the measurements performed at maximum exciting energy the fluence $P_{ex} = (4 \pm 2) \cdot 10^{15}$ photons/cm^2. With such fluence about 15% molecules in the irradiated space will be excited during each pulse.

Discussion

Sequence of primary events

The measurements performed show that throughout the first 10 ps after excitation three photoinduced states can be observed. The difference spectrum between the last and the initial bacteriorhodopsin state is similar to the difference spectrum between K and bacteriorhodopsin observed by Kaufmann et al. [2], Applebury et al. [5] and Shichida et al. [7] at room temperature in the picosecond time range. At 10 ps after excitation and later the ratio $\Delta A^{650}/\Delta A^{550} = 1.55$ that is close to the results of the previous measurements in the picosecond time range [2,5,7].

In accordance with the results obtained by Shichida et al. [7], in a time range from 1 to 100 ns one should expect the decay of the K state to the next one observed in the nanosecond measurements [3,4].

The difference spectrum recorded at 2.5 ps after excitation is close to the spectrum between J and bacteriorhodopsin measured by Applebury et al. [5]. Some features of the J state preceding the K intermediate was also observed by Shichida et al. [7]. On the other hand, the lifetime of the state recorded at 2.5 ps after excitation equals 3 ps, while that of the J intermediate measured by Applebury et al. [5] equals 11 ps. But the fact that the difference spectra are close in shape enables us to believe that we and Applebury et al. [5] observed one and the same state (J) preceding the K intermediate. It should be noted that in both works the spectra behave identically in the region of 500 nm where the difference spectrum between J and bacteriorhodopsin almost does not differ from the difference spectrum between K and bacteriorhodopsin.

To explain the different lifetimes of the J intermediate we carried out calculations which simulated the kinetics obtained in the experiments.

If we excite a state with its lifetime τ_{rel} that results in a long-lived photoproduct, the response of such a system to the instantaneous excitation (δ function) performed at the instant of time $t = t'$ will have the form:

$$H(t - t') = a_1 \, e^{-(t-t')/\tau_{rel}} + a_2$$

where the a_1 coefficient characterizes the absorbance change at the moment of excitation and a_2 characterizes it at the instant $t - t' \gg \tau_{rel}$. The equation is valid for any instant of time t' during the action of the real exciting pulse if the amount of molecules excited per pulse may be neglected in comparison with their total amount in the excited space.

The kinetics observed in the experiment, $\Delta A(\tau)$, is described in this case by the convolution:

$$\Delta A(\tau) = \int_0^\infty H(\eta) B(\tau - \eta) d\eta, \quad \eta = t - t' \qquad (1)$$

where τ is the delay between the probe and the exciting pulses and $B(\tau - \eta)$ is the correlation function of their intensities:

$$B(\tau - \eta) = \int_{-\infty}^{\infty} I_{pr}(t' - (\tau - \eta)) I_{ex}(t') dt'$$

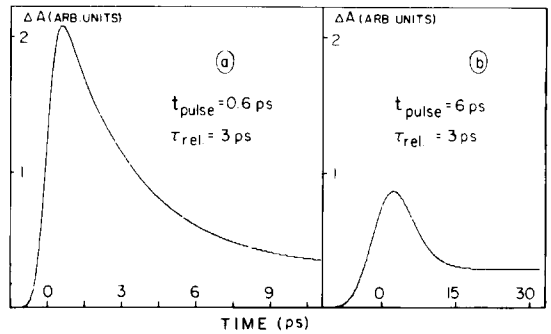

Fig. 5. Computer calculations of 3-ps relaxation kinetics. (a) The exciting and probe pulse duration is equal to 0.6 ps. (b) The pulse duration is equal to 6 ps. The kinetics alteration is related to the relaxation process during pulse action.

The value of τ_{rel} being the same, the form of the curve $\Delta A(\tau)$ calculated from Eqn. 1 will depend on pulse duration and shape. On the assumption that the durations of the probe and the exciting pulses are equal and the pulse shape is Gaussian Fig. 5 shows the results of calculations for two cases: the pulse duration on the level $1/e$, t_{pulse}, equals 0.6 ps (a) and 6 ps (b). The relaxation time in both cases is 3 ps.

When $t_{pulse} \ll \tau_{rel}$, one can observe simple exponential decay of the absorbance changes arising during excitation. When $t_{pulse} > \tau_{rel}$, the amplitude is smaller. The decay is nonexponential while the exciting pulse is acting and the zero point on the time scale lies near the maximum of the curve. The curves describe well the decay kinetics of J observed in the present work (curve a) and in the work by Applebury et al. [5] (curve b) in the spectral range from 550 to 570 nm (although, strictly speaking, the fraction of excited molecules in either experiment is not negligible). It must be said that both curves correspond to $\tau_{rel} = 3$ ps. The coefficients a_1 and a_2 in the calculations were the same for both curves.

For a better agreement with the kinetics, we measured at 550 nm the relaxation term with $\tau_{rel} = 0.7$ ps should be added to the function $H(t - t')$. This term indicates the process of relaxation of the state preceding J, which we designate as I. As a result, the absorbance changes amplitude increases a little and the decay becomes two-component. When $t_{pulse} = 6$ ps, the addition of the term with $\tau_{rel} = 0.7$ ps will not change the form of the curve b in Fig. 5. If we simulate the situation taking place at 650 nm, the coefficients of the terms with $\tau_{rel} = 0.7$ ps and $\tau_{rel} = 3$ ps must have different signs in the formula for $H(t - t')$. The dashed curve in Fig. 2d corresponds to the case $\tau_{rel} \to \infty$, and denotes the integral of the correlation function of exciting and probe pulses.

Gillbro and Sundstrom [14] studied the formation of the K intermediate when irradiating a flow of bacteriorhodopsin with a continuous train of pulses 4–8 ps long. At a sufficiently fast sample flow, when the accumulation of any photoproducts could be neglected, Gillbro and Sundstrom [14] observed fast (within 2 ps) relaxation of the initial bleaching in the region of 575 nm. According to our results, one should expect at this wavelength two-component relaxation, where the fast 0.7-ps component prevails. At shorter wavelengths Gillbro and Sundstrom seem to be able to resolve a 3-ps component.

The 1 ± 0.5 picosecond kinetics of absorption build-up at 615 nm observed by Ippen et al. [12] is similar to the kinetics we detected at 650 nm (Fig. 2c), and hence corresponds to the formation of the J intermediate as proposed in Refs. 6 and 28. Even though we did not take measurements at 615 nm, we may expect from interpolation of our spectra that the J and K states have similar extinctions at this wavelength, which explains the absence of 3-ps relaxation kinetics in the work by Ippen et al. [12].

Just as with kinetics it is possible to calculate the contribution to the difference spectra of different states at different moments of probing with due regard to pulse duration. The difference spectrum, for example, measured at 0.5 ps after excitation is not purely a spectrum between I and the initial state, but the sum of the indicated spectrum (with a coefficient of 0.8) and the spectrum between J and bacteriorhodopsin (with a coefficient of 0.2). During the calculations one important assumption was made: the quantum yield of formation of the I state is equal to unity and the quantum yield of J comes to 0.3, that is, it is assumed to be equal to the quantum yield of *K* obtained by Goldschmidt et al. [4]. This assumption means that I is an excited electronic state rather than the ground state intermediate.

Assignment of the I state

The assumption made can be supported by the following arguments. First, at 0.5 ps after excitation one can observe considerable bleaching of the absorption band of bacteriorhodopsin, its maximum taking place at 570 nm. The measurements taken at 550 nm show that such big changes of absorbance may correspond only to the formation of a state with its quantum yield being close to unity. Indeed, with parallel polarization of the exciting and probe beams the absorbance change at 550 nm exceeds 0.2 at an excitation energy of $5 \cdot 10^{-5}$ J (Fig. 2b). It should also be taken into account that the pulse duration (0.6 ps) is comparable with the lifetime of the I state (0.7 ps). During excitation part of excited molecules is

relaxed, and the absorbance changes observed at the kinetics maximum are smaller in magnitude than in case of excitation by a shorter pulse with the same energy. With shorter pulses one can observe, according to our estimations, the value of ΔA^{550} exceeding 0.3. Such absorbance changes in the region far from saturation cannot correspond to the formation of a photoproduct with its quantum yield being 0.3, since the sample absorbance at 550 nm is about 0.9.

Secondly, the dependence of the $\Delta A^{550}/\Delta A^{460}$ ratio on the mutual polarization of the exciting and probe beams enables one to conclude that the transition dipole orientation of the blue-shifted band does not coincide with that of the initial absorption band. The observed blue-shifted band may be considered to be the α-band of the earliest ground state intermediate. But in this case, the results obtained correspond to such a structural change of retinal (occurring in less than 0.5 ps) that they lead to a considerable change of initial transition dipole orientation (at least 25°). It can be estimated using the photoselection theory [15] on the assumption that only monomolecular processes take place. Since the models, proposed to explain ultrafast structural changes of retinal in a restricted active site, do not allow such large changes of the dipole orientation [16,23], it is more likely that I is an excited state of bacteriorhodopsin. In this case, the polarization dependence of the $\Delta A^{550}/\Delta A^{460}$ ratio can be explained by the contribution to the excited state absorption at 460 nm of the transition (for example, the $^1B_u^+ \rightarrow {}^1B_u^-$ transition in terms of the C_{2h} group of symmetry) whose dipole is perpendicular to the dipole of the $^1A_g^- \rightarrow {}^1Bu^+$ transition from the ground state.

The fluorescence lifetime of bacteriorhodopsin, measured with a streak-camera as the sample is excited by single subpicosecond pulses, is less than 2 ps at room temperature [17]. The estimate for τ_{fl} obtained from the radiative lifetime $\tau_{rad} = 6$–7 ns [18,19] and the fluorescence quantum yield $\varphi_{fl} = 2 \cdot 10^{-4}$ [18,20,21] or $\varphi_{fl} = 2 \cdot 10^{-5}$ [19,22] will be 0.12–1.4 ps. Shapiro et al. [21] estimated τ_{fl} as 1.5 ± 0.4 ps by measuring the fluorescence lifetime at 77 K and determining the temperature dependence of φ_{fl}. Although the exact value of τ_{fl} at 300 K is still unknown, we believe that all the set of data available is not inconsistent with the statement about I as an excited electronic state. Assuming that the I state is fluorescent, the fluorescence lifetime must agree with the lifetime of I (0.7 ps).

Model for primary events

The sequence of the primary processes in bacteriorhodopsin can be written as:

$$\text{bacteriorhodopsin} \xrightarrow{h\nu} \text{bacteriorhodopsin}^*(I_{460}) \xrightarrow{0.7\text{ ps}} J \xrightarrow{3\text{ ps}} K$$

where I_{460} is designated as corresponding to difference spectrum maximum at 460 nm. The radiationless electronic relaxation results in the J state formation, which in turn is followed by the J → K dark process in the ground state. This sequence is in a good agreement with that offered in Ref. 6. The work [6] took into consideration the data which were that time available [5,12].

We propose a model for primary events in bacteriorhodopsin (Fig. 6). The model is based on the assumption that two reaction coordinates are changed during the first 10 ps after excitation. Following earlier models [24,29] we suppose that the J intermediate contains a photoisomer (likely 13-*cis*) of the all-*trans* retinal, which is a chromophore of light-adapted bacteriorhodopsin. The isomerisation process results in a change of the Schiff base position relative to the protein matrix. The next step (J → K) is a coordinate change of a proton and most probably this proton occurs in the vicinity of the Schiff base [24,29]. The J and K intermediates are separated by a potential barrier.

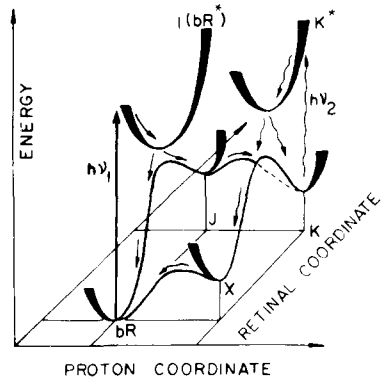

Fig. 6. Model for primary events in bacteriorhodopsin (see the explanations in the text).

This point is proved by the increase of the J state lifetime at low temperature [5,8].

In our model the bacteriorhodopsin excited state is common to both bacteriorhodopsin and J. Indeed, quantummechanical calculations [16,25] show that retinal photoisomerisation process must involve a common excited state for both isomers. Rosenfeld et al. [26] proposed a model of the common excited state for bacteriorhodopsin and K, which is based on the fact that the sum of the quantum yields of direct (bacteriorhodopsin → K) and back (K → bacteriorhodopsin) reactions are equal to unity. Our scheme (Fig. 6) differs considerably from that after Rosenfeld et al. In addition to the bacteriorhodopsin*-excited state we consider also the K*-excited state populated as a result of a photon absorption by the K intermediate. According to the results obtained by Kryuko et al. [27,28] on the kinetics of the direct and back reactions in deuterated bacteriorhodopsin at 13 K, bacteriorhodopsin and K cannot share any common excited state. In Refs. 27 and 28 the 80-ps kinetics of the direct reaction has been observed, the kinetics corresponding to the J → K process. If one assumes that the state reached by exciting the K intermediate is the same that reached by exciting the bacteriorhodopsin state, then after excitation of K the excited molecules would relax both to bacteriorhodopsin (with a quantum yield equal to that of the back reaction) and to the J state. So, the 80-ps kinetics for the J → K process would be observed in the back reaction as well. However, the results obtained in Refs. 27 and 28 suggest a more rapid (< 30 ps) kinetics of the back reaction.

We have introduced a hypothetical intermediate (at X in Fig. 6) which could not be observed in Refs. 27 and 28 because of the inadequate time resolution. With this intermediate the scheme of the direct and back reactions is sufficiently symmetrical. In order for the sum of the quantum yields of the direct and back reactions to reach unity, it is necesaary that the quantum yield of the bacteriorhodopsin* → J process should be equal to that of K* → K process. Similarly, the quantum yield of the bacteriorhodopsin* → bacteriorhodopsin process should be equal to that of K* → X process. If the sum is somewhat larger than 1 [30], our model is acceptable as well. Except the X intermediate other minima of the excited and ground states are based on a strong experimental evidence.

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