A TIME-RESOLVED SPECTRAL STUDY OF THE K AND KL INTERMEDIATES OF BACTERIORHODOPSIN

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ABSTRACT Nanosecond time-resolved absorption measurements on the photolysis products of bacteriorhodopsin (BR) in intact membranes are reported. At room temperature in fluid solution a single intermediate (KL) is seen 10 ns after excitation. Both spectral and kinetic results are consistent with the KL intermediate converting to the L intermediate by a single first order reaction. The observed temperature-dependent rate has the Arrhenius parameters: E_a = 10.5 kcal/mol, $A = 5 \times 10^{13}$ s^{-1}. The precursor to the KL intermediate is also observed. Its spectral character is consistent with the K intermediate which has been previously reported. The current data is consistent with a linear sequence in the BR photocycle for K, KL, and L in room temperature fluid solution. Differences in the spectral characteristics of the K intermediates described here and elsewhere are discussed in terms of differences in the microenvironment around the retinal moiety and the affect this may have on the conformation of the chromophore.

INTRODUCTION

The photocycle of bacteriorhodopsin (BR) has been studied under a number of conditions by many laboratories. One of the interesting aspects of BR is the similarity, at least at a superficial level, of its photocycle with reactions of photolysis intermediates of rhodopsin (Ottolenghi, 1982). Both proteins have protonated retinal Schiff base chromophores and both produce a sequence of intermediates with different absorption spectra subsequent to photolysis. One interesting question for both photolysis sequences is to what extent they are either sequential or branching in nature.

Recent work on the BR photocycle has centered either on the initial events, which occur on the femto to picosecond time scale (Gillbro and Sundstrom, 1983; Hsieh et al., 1983; Ippen et al., 1978; Nuss et al., 1985), or on later events, starting with the M intermediate (Beach and Fager, 1985a; Kalisky et al., 1981; Ort and Parsons, 1978). Investigations of the K to L transition indicate that the number of K intermediates observed and the kinetics they exhibit is dependent on solvent conditions and temperature (Kalisky and Ottolenghi, 1982; Lozier et al., 1975; Nagle et al., 1982; Shichida et al., 1983; Smith et al., 1983; Stern and Mathies, 1985). In room temperature fluid solution Shichida et al. (1983) showed that the absorption spectrum of the photointermediate changed significantly between 900 ps and 150 ns. They attributed this to the existence of two intermediates over this time. They proposed a K intermediate which is sequentially followed by a KL intermediate. Time-resolved resonance Raman work by Stern and Mathies (1985) showed that the ethelynic stretch of the chromophore shifts from 1531 to 1519 cm^{-1} between 200 ps and 20 ns. They attributed this shift to BR going from the K to the KL intermediate. It should be noted that in these studies the K-like intermediate which is observed on the nanosecond timescale has been named KL. This contrasts with much previous literature, where this intermediate has been named K. We will follow the more recent convention and call the K-like intermediate which occurs on the nanosecond to microsecond timescale KL and call the intermediate previously observed in subnanosecond times K.

Recent work in our lab has shown that in rhodopsin the spectra and kinetics of the batho to lumi transition in room temperature solution can best be described by the presence of two batho intermediates, each with a distinct spectrum and lifetime (Einterz et al., 1987a, b). The current work was motivated by the question of whether, in a similar fashion, photolysis of BR produces two or more K-like intermediates. We found, as in previous work, that there is only one spectrally distinct intermediate which uniformly decays on the 10 ns to 3 μs time scale. We did, however, see a shift in the spectrum within the first few nanoseconds after photolysis, consistent with the K to KL transition previously reported.

MATERIALS AND METHODS

Halobacterium halobium strain Et-1001 was grown and the purple membrane purified after the procedure of Becher and Cassim (1975). Samples were stored in 0.1% sodium azide at 4°C and were diluted with distilled water to give an optical density of ~0.9 in a 1 cm cell at the visible absorption maximum after light adaptation. Samples for photolysis were

placed in a standard 1 cm temperature-controlled glass cuvette and were lightly stirred while measurements were carried out.

Laser flash photolysis experiments were performed with an apparatus previously described (Lewis et al., 1987; Milder and Kliger, 1985). Excitation was with either a Quanta Ray DCR-1 or DCR-2 Nd:YAG laser (Spectra Physics, Mountain View, CA) at 532 nm (7 or 4 ns pulse width, respectively). Detection was carried out in either a kinetic or spectral mode. In the kinetic mode either a xenon flash lamp (5 μs pulse width) or a He/Ne laser was used as the probe source. The probe light was collected through a monochromator and detected by an EMI D279 photomultiplier (Thorn EMI, Middlesex, UK) whose output was recorded by a 7912 digitizer (500 MHz) (Tektronix, Inc., Beaverton, OR) for signal averaging and analysis. In the spectral mode the output of a xenon lamp was used as the probe beam. The probe light was collected through a Jarrell-Ash Monospec 27 spectrograph (Anes, Inc., Concord, MA) with a 150 groove/mm grating and dispersed across a gated intensified diode array (PAR 1420) optical multichannel analyzer (OMA). The intensifier was typically gated on for 5 ns using a PAR 1302 pulser (Princeton Applied Research, Princeton, NJ).

RESULTS

Fig. 1 shows transient difference spectra for BR after photolysis in water at 20°C. Spectra measured during 5 ns time windows are shown at specified times between 50 ns and 3 μs after photolysis. Similar transient difference spectra were obtained at 13, 27, and 34°C. As the temperature increased, the rate of change in the transient spectrum increased. At all temperatures an isosbestic point is seen in the BR difference spectra near 555 nm. That there is an isosbestic point is consistent with a single-transient species on this time scale being transformed to another single species which is stable on this time scale.

Fig. 2 shows the kinetics of the decay of the red shifted transient absorption at 632.8 nm. Again, results are shown for temperatures between 13 and 34°C. At all temperatures the decay fits a single exponential, with lifetimes of 0.54 μs at 34°C, 0.79 μs at 27°C, 1.23 μs at 20°C, and 1.87 μs at 13°C. These results give a linear Arrhenius plot (r = 0.9998) with E_a = 10.5 kcal/mol and $A = 5 \times 10^{13}$ s^{-1}. Again, these results are consistent with BR having only one process in the 50 ns to 3 μs time regime.

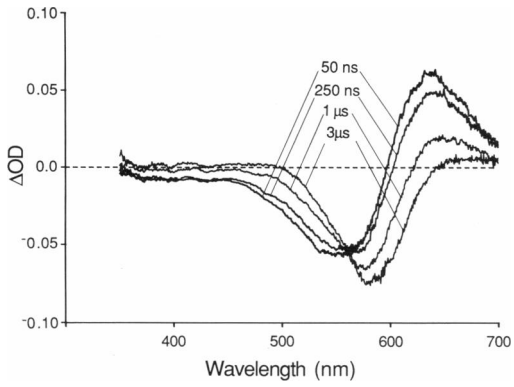

FIGURE 1. Time-resolved absorption difference spectra for the photolysis of intact membrane BR taken at 20°C. Spectra were measured with a 5 ns OMA gate and were taken at the times specified after photolysis by a 4 ns, 532-nm laser pulse. Each spectrum represents 128 averages.

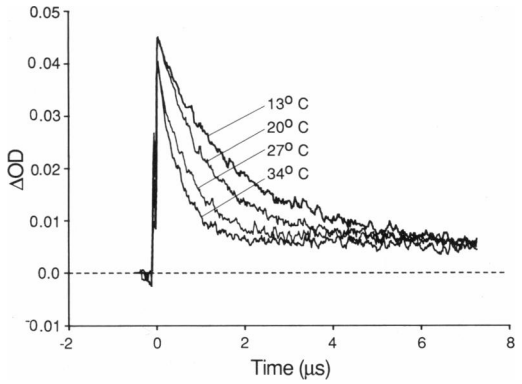

FIGURE 2. Kinetics of the transient absorption seen at 632.8 nm after photolysis of intact membrane BR at various temperatures. Each trace represents 256 averages.

In studies of rhodopsin, which exhibits two forms of the batho intermediate, the relative amounts of the two batho intermediates depends on the power of the exciting laser (Einterz et al., 1987b). Thus we measured the transient difference spectrum of BR as a function of laser power. With a pump beam diameter of 0.5 cm we excited the sample with laser powers from 0.24 to 17.5 mJ/pulse. At low powers (<1 mJ/pulse) the transient absorption signal size increased linearly with pulse energy. Above this pulse energy the signal size did not increase as quickly as the laser power and it leveled off at powers above 12.5 mJ/pulse. Fig. 3 displays part of the transient difference spectra taken 50 ns after the laser pulse for four representative laser energies. In all cases increasing the laser energy only increased the signal size, it did not shift the spectrum of the intermediate.

The above results are consistent with a simple transformation between two intermediates. However, other workers have suggested the existence of a more complex process from the picosecond to nanosecond time domain. Results were thus obtained at the fastest time resolution of our

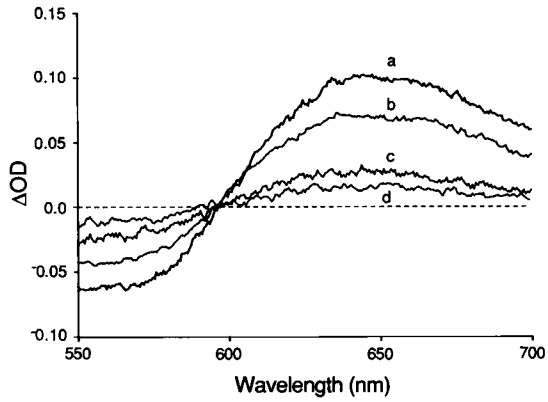

FIGURE 3. Transient absorption spectra seen at 50 ns after photolysis of intact membrane BR at 20°C with different exciting laser powers. The actinic beam was 0.5 cm in diameter and the traces correspond to excitation energies of: *a* 17.5 mJ; *b* 2.4 mJ; *c* 0.32 mJ; *d* 0.24 mJ. Each trace represents the average of 128 signals.

instrumentation. With the gate on the OMA set at 5 ns, spectra were measured during the rise time of the exciting laser pulse. Shown in Fig. 4 *a* are the results taken for a sample at 20°C at times starting 10 ns before the peak of the laser pulse (the leading edge of the laser pulse) to times 10 ns after the peak of the laser. Changes in these spectra are subtle. One can see, however, a shift in the isosbestic between spectra on this time scale. Changes are seen more clearly when the scale of the difference spectrum obtained at −5 ns is increased to more directly compare it with the difference spectrum taken at +10 ns. This is shown on Fig. 4 *b*. Shichida et al. (1983) directly obtained the relative signal size of the spectra at 900 ps and 150 ns after photolysis and showed that the signals for both near 700 nm are about the same. This criterion was used to scale the spectra shown in Fig. 4 *b*. Our spectra look similar to those previously reported. The difference spectrum taken at the shorter time shows a transient absorption which is more intense and blue shifted relative to the later time spectrum. The actual spectra of the intermediates are obtained by adding appropriate amounts of the difference spectra to the light adapted BR spectrum. Shichida et al. (1983) have done this and have shown that although the later difference spectrum appears red shifted relative to the earlier difference spectrum, the later intermediate (KL) is blue shifted and less intense than the one that precedes it (K). This is due to the relative intensities of the two difference spectra. From these results for BR at 20°C we are able to set an upper limit to the lifetime of the previously described K to KL transition as ~10 ns.

This conclusion is also consistent with measurements of the kinetics of transient absorption. At 625 nm we observed a large growing in of transient absorption concommitant with the laser pulse and no subsequent changes larger than the noise level (Fig. 5). From previous work (Shichida, 1983) and the data on Fig. 4, one would expect to see a significant increase in the transient absorption signal at this wavelength when the K to KL transition occurs. At 592.5 nm there is no change in the absorption on this time scale. At 560 nm, there is a large growing in of the transient bleaching concurrent with the laser pulse and no subsequent changes larger than the noise level. Thus, it also seems from the kinetic data that the K to KL transition takes place within 10 ns of photolysis.

Difference spectra were also taken of photolyzed BR at 6°C in the −10 to 10 ns time regime. Again, the −5 ns difference spectrum showed an absorption peak which was somewhat blue shifted from that obtained at 10 ns, but no new features were observed. Both the −5 ns spectrum and the +10 ns spectrum at 6°C were shifted by ~5 nm to the red relative to the spectra obtained at 20°C.

DISCUSSION

The above results are consistent with previous work (Shichida et al., 1983; Stern and Mathies, 1985) which suggest the existence of an intermediate, K, which must have a lifetime of between 1 and 10 ns. They are also consistent with the fact that this K intermediate decays to another intermediate, KL. There appears to be only a single KL intermediate in the photocycle of BR in intact membranes on the 10 ns to 3 μs time scale in room temperature fluid solution. The evidence to date is consistent with the intermediates K, KL, and L occurring in linear sequence in room temperature fluid solution. However, the details of the K to KL transition are not known.

Previous transient optical spectroscopy work (Kalisky

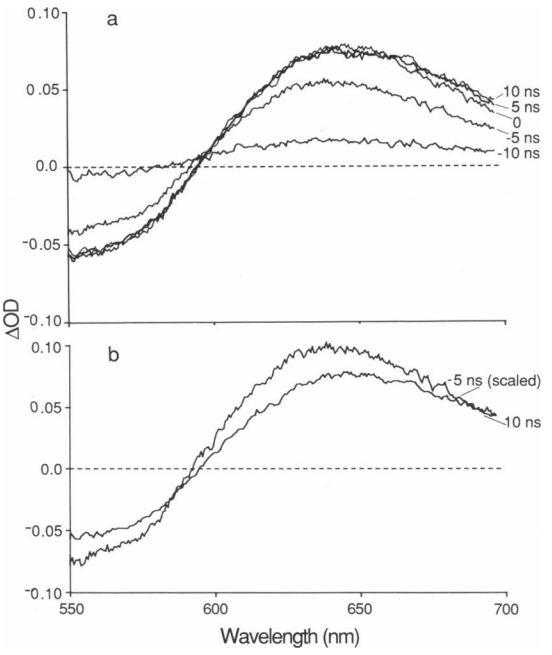

FIGURE 4. Transient absorption spectra taken during the 20 ns full width actinic laser pulse for intact membrane BR at 20°C. (*a*) Spectra were determined at times of −10, −5, 0, +5, and +10 ns relative to the peak of the laser pulse. (*b*) Spectrum taken at −5 ns scaled for comparison with the spectrum taken at 10 ns (see text). A 5 ns gate on the OMA was used and each spectrum represents 128 averages.

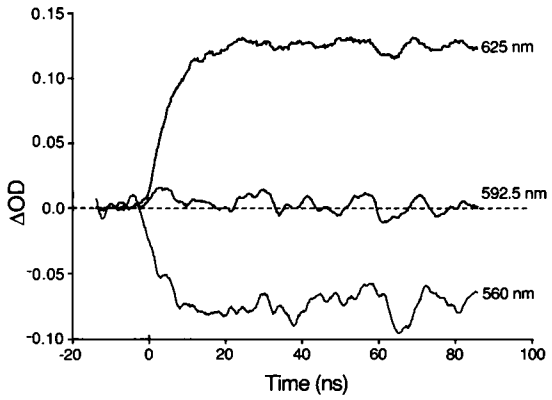

FIGURE 5. Kinetics of transient absorption and bleaching of intact membrane BR at 20°C at various wavelengths. The rise of the observed signals is consistent with the laser pulse width and rise time of the detection system. Each trace represents 128 averages.

and Ottolenghi, 1982) has shown that there are three K-like intermediates in fluid cryogenic solvents below −30°C. Under these conditions the K intermediates do not simply occur in a linear sequence, but rather occur in a branching sequence. Time-resolved resonance Raman spectra of BR show that neither the K nor the KL intermediate seen in room temperature fluid solution is the same as the K intermediate obtained as a steady-state photoproduct in low temperature glasses (Smith et al., 1983; Stern and Mathies, 1985). Thus, the reaction channels and the structure of the various K intermediates seem to be quite sensitive to temperature and solvent environment.

At least some of the differences between the various K intermediates may be due to differences in their single-bond conformations. Solvent conditions have been shown to significantly influence the conformation of BR (Draheim and Cassim, 1985b) and perhaps influence the microenvironment around the chromophore. This may then affect the routes of single-bond isomerization available to the retinal moiety after the initial all–trans to 13–cis double-bond isomerization. Resonance Raman spectra show that the C_{15}-HOOP frequency is different in the K, KL, and 77 K trapped K intermediates, indicating that they have different twisted structures (Smith et al., 1983; Stern and Mathies, 1985). Also, Fowner-transform infrared studies show that when a solution of BR is irradiated at 81 K to produce the K state and is then warmed to 135 K, the chromophore reorients and there are changes in its interaction with the protein (Rothschild et al., 1985). These changes are attributed to the BR going from the K to the KL state. Circular dichroism spectra of the various intermediates under various conditions may be useful in elucidating the structural differences of the K intermediates. Time-resolved circular dichroism techniques (Lewis et al., 1985) will be necessary to obtain these spectra, and appropriate experiments are currently planned.

We thank G. Rayfield for supplying us with the sample of purple membrane and for many helpful discussions. We also thank R. Bolgomolni and R. Mathies for helpful comments during the course of this work.

Finally, we thank the National Institutes of Health for support of this work through grants GM35158 and EY00983.

Received for publication 11 August 1987 and in final form 24 November 1987.

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