Time-Resolved Infrared Spectral Analysis of the KL-to-L Conversion in the Photocycle of Bacteriorhodopsin†

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ABSTRACT: Time-resolved infrared spectra of the hydrated film of light-adapted bacteriorhodopsin were recorded from earlier than 200 ns to 450 μs after light excitation in the 1800–900-cm^{-1} region on a newly designed dispersive-type infrared spectrometer [Iwata & Hamaguchi (1989) *Appl. Spectrosc. 44,* 1431–1437]. Both the KL-to-L and L-to-M conversions were detected in this time range. The spectral shape of KL is similar to K measured at 77 K except for the intense hydrogen out-of-plane vibrational band at 984 cm^{-1}. The kinetics of this band are different from those of the other KL-specific bands at 1510, 1296, and 956 cm^{-1}. Since the hydrogen out-of-plane vibrational bands are intensified by twists of the polyene chain, a change in the twist of the chromophore is suggested within the lifetime of KL. During the decaying process of L, the KL-specific vibrational bands are observed in parallel with L, indicating that KL and L are in equilibrium.

The light-adapted form of bacteriorhodopsin (BR)[1] has an *all-trans*-retinylidene Schiff base as the chromophore and transports protons across the membrane by utilizing light energy (Mathies et al., 1991). Upon light absorption, the chromophore is isomerized to the 13-cis form through a process from J to K in about 3 ps (Polland et al., 1986; Mathies et al., 1988; Doig et al., 1991). K then converts to KL, which has a slightly shifted absorption maximum in less than 10 ns (Shichida et al., 1983; Milder & Kliger, 1988). Time-resolved resonance Raman experiments (Doig et al., 1991) showed that KL has a more twisted chromophore than K in view of the intensity of hydrogen out-of-plane (HOOP)[1] vibrational bands.

The KL-to-L conversion occurs in an early microsecond range (Váró & Lanyi, 1991; Lozier et al., 1992). Low-temperature Fourier transform infrared (FTIR)[1] spectroscopic studies have shown the perturbation of Asp^{96} and Asp^{115} in L (Braiman et al., 1988). Recent studies on L revealed a stronger hydrogen-bonding interaction at the Schiff base, Asp^{96}, and internal water molecules (Maeda et al., 1991, 1992a,b). These changes close to the Schiff base may induce the twists of the chromophore proximal to the Schiff base (Fahmy et al., 1989), and may give rise to the first step in the proton-pumping process, the deprotonation of the Schiff base in the L-to-M conversion.

Time-resolved IR spectra of BR in the time range later than 10 μs, which covers the late life span of L, have been presented by use of stroboscopic FTIR spectroscopy (Braiman et al., 1991; Chen & Braiman, 1991; Gerwert et al., 1990), and also by step-scan FTIR spectroscopy (Uhmann et al., 1991). A recent step-scan FTIR spectroscopic experiment by Noelker et al. (1992) provided a spectrum at 500 ns. Diller et al. (1991, 1992) presented the infrared spectra of K from 100 ps to 14 ns by use of an infrared probe from a CO laser combined with the flash light from a dye laser for the frequency region 1760–1550 cm^{-1}. We report here the KL-to-L and L-to-M conversions by using a newly designed dispersive-type infrared spectrometer (Iwata & Hamaguchi, 1989; Kato et al., 1991) which allows the time resolution of 0.2 μs in the spectral region from 1800 to 900 cm^{-1}.

MATERIALS AND METHODS

Bacteriorhodopsin in purple membrane prepared by the standard method (Oesterhelt & Stoeckenius, 1974) was dried on a BaF_2 window (16 mm in diameter) in room air. The dried bacteriorhodopsin was covered with 1 μL of water and sealed with the second window and a silicone spacer. This set was mounted into a copper block and left overnight for the hydration of the purple membrane. Light adaptation was attained by exposure to a laser beam before the recordings.

Excitation was made by a second harmonic (532 nm) from a cw Q-switched Nd:YAG laser (Spectron Laser Systems, SL-902 TQ, 100-ns pulse width, 300 μJ/pulse, 50 Hz) which was focused on a circle of ~2 mm in diameter.

The monitoring light source for the measurement of the IR spectrum was a globar. The light after passing the sample was dispersed with a Hitachi I-3000 grating infrared spectrometer and was focused on a mercury–cadmium–tellurium (MCT) detector. The time resolution of the system is 0.2 μs. Only the intensity changes by the photoreaction of the sample were extracted by an AC-coupled amplifier, and were recorded on a digital oscilloscope (Tektronix, DSA 602, 11A4). For the details of the instrument, see Iwata and Hamaguchi (1989) and Kato et al. (1991).

Excitation and the recording were repeated 1024 times in a rate of 50 Hz, and the data were averaged. The scanning

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[1] Abbreviations: BR, light-adapted form of bacteriorhodopsin; HOOP, hydrogen out-of-plane; FTIR, Fourier transform infrared.

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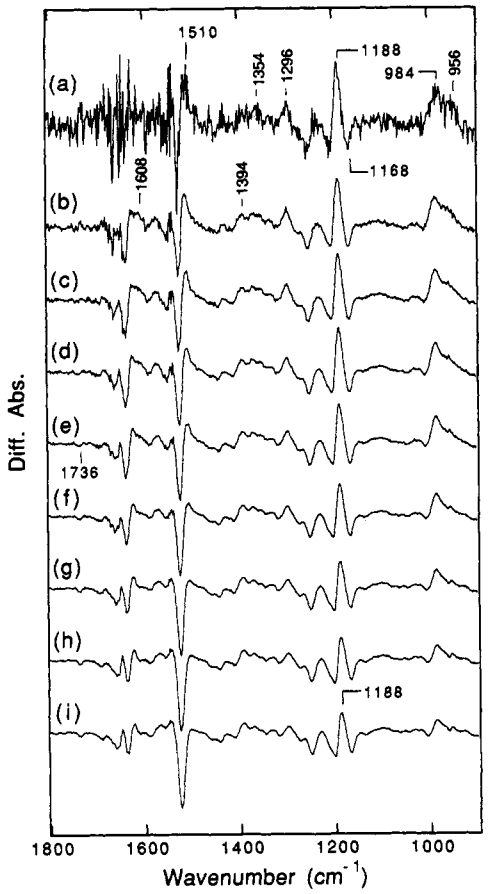

FIGURE 1: Spectra in the time region of (a) 0–200, (b) 200–400, (c) 400–600, (d) 600–800, (e) 800–1000, (f) 1000–2000, (g) 2000–3000, (h) 3000–5000, and (i) 5000–9000 ns. The full scale of the vertical axis is 0.057.

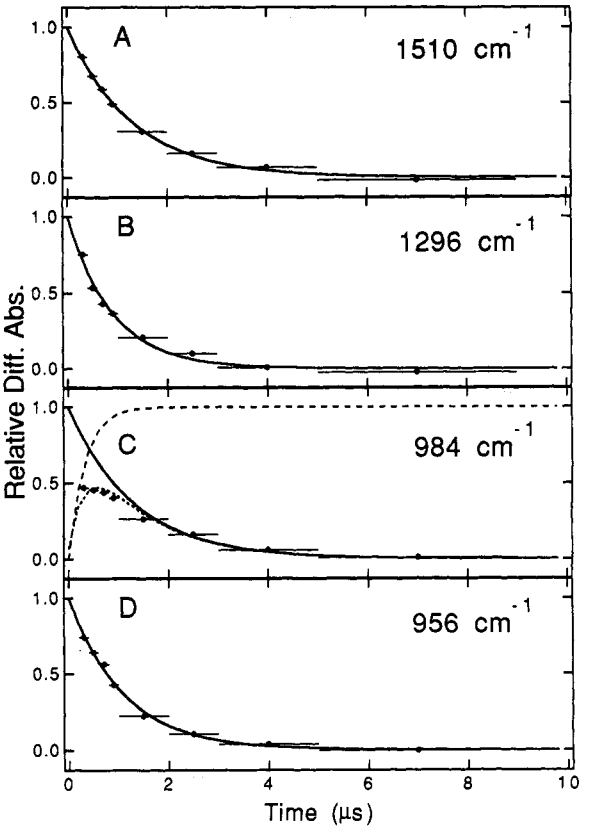

FIGURE 2: Changes in the spectra in Figure 1 at (A) 1510, (B) 1296, (C) 984, and (D) 956 cm^{-1} are plotted against time. Panels A, B, and D are fitted by single-exponential curves (solid line) which are extrapolated to the relative intensity at zero time to 1.0, respectively. The time constants were about 1 μs. The dotted line in panel C is the simulated sum of two exponential curves. One is the same exponential curve as in panel A (solid line), and the other is a calculated increasing exponential curve (dashed line) with the time constant of about 400 ns.

was made in the range from 1800 to 900 cm^{-1} at a spectral resolution of 8 cm^{-1}. The whole scan process was repeated 4 times, with one scan taking about 3 h.

RESULTS

Spectra in the Nanosecond Range. Time-resolved IR spectra were recorded for –200–0-, 0–200-, 200–400-, 400–600-, 600–800-, 800–1000-, 1000–2000-, 2000–3000-, 3000–5000-, and 5000–9000-ns time domains. The spectrum of the photoproduct was obtained in the form of a difference spectrum with BR. The apparent intensities of the spectra in the early time domains were somewhat lower than in the late ones. This is due to the slower response of the detecting system. To compare the same molar amount of BR in the photochemical reaction, all the spectra were normalized by adjusting the amplitude of the negative band of BR at 1168 cm^{-1} to the same height. The difference spectrum in the –200–0-ns region was almost flat and was not normalized. The normalized spectra in the 0–200-, 200–400-, 400–600-, 600–800-, 800–1000-, 1000–2000-, 2000–3000-, 3000–5000-, and 5000–9000-ns regions are shown in Figure 1a–1i, respectively.

Since the K-to-KL conversion occurs in 1–10 ns at room temperature on the basis of time-resolved spectroscopy in the visible region (Shichida et al., 1983; Milder & Kliger, 1988), the contribution of K in the 200–9000-ns range is almost negligible. Thus, photointermediates in this time range are mainly assigned to KL and L. In the 0–200-ns and 200–400-ns domains (Figure 1a,b) where KL is dominant, the spectra resemble the K/BR spectrum recorded at 77 K (K^{LT}/BR); positive bands at 1510, 1608, and 1188 cm^{-1} correspond to those of K^{LT} at 1514 cm^{-1} for the C=C stretching mode (Siebert & Mäntele, 1983), 1609 cm^{-1} for the C=N stretching mode (Rothschild et al., 1984; Gerwert & Siebert, 1986; Maeda et al., 1991), and 1195 cm^{-1} for the C–C stretching mode (Gerwert & Siebert, 1986), respectively. The 1354- and 1296-cm^{-1} bands correspond to the relatively intense bands in the K^{LT}/BR spectrum at 1348 and 1294 cm^{-1}, respectively (Maeda et al., 1991). A broad feature with a peak at 984 cm^{-1} and a shoulder at 956 cm^{-1} must be due to HOOP modes. The same bands were observed recently by step-scan FTIR spectroscopy (Noelker et al., 1992). This feature is especially different from that of K^{LT} which shows a main band at 956 cm^{-1}. These KL bands gradually decrease, and all the features become similar to the L/BR spectrum recorded at 170 K (Maeda et al., 1991) in 5000–9000 ns (Figure 1a).

The band at 1394 cm^{-1} in the time-resolved spectrum, on the other hand, is not found in K^{LT} and does not decay in the KL-to-L conversion in the submicrosecond range. Thus, this band is common to KL and L but not to K^{LT}.

The time-dependent changes in the intensities of the 1510-, 1296-, 984-, and 956-cm^{-1} bands are plotted in Figure 2A–D, respectively. The first spectrum (Figure 1a) was omitted from the plots because of large uncertainties. The points for the 1510-, 1296-, and 956-cm^{-1} bands were fitted well by a single exponential with an apparent time constant of about 1 μs. In contrast, the 984-cm^{-1} band (Figure 2C) could not be simply fitted by the same single-exponential curve of about 1-μs time constant (solid line). The deviation could be explained by an overlap with a more rapidly increasing exponential curve

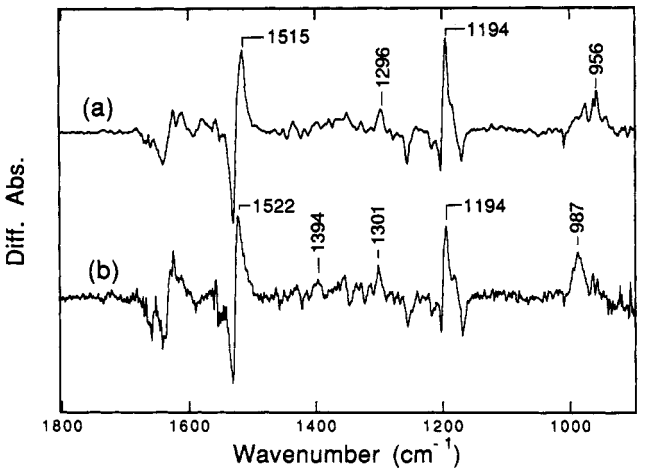

FIGURE 3: Difference spectra of the photoproducts of bacteriorhodopsin at 135 K against BR. BR was first irradiated with 500-nm light for 5 min to produce the mixture of photoproducts, K^{LT} and K-like photoproduct. Irradiation with 700-nm light for 5 min reverted K^{LT} to BR, and >630-nm light for 5 min reverted K-like photoproduct to BR completely. Spectrum a is the difference spectrum between before and after irradiation with 700-nm light. Spectrum b is the difference spectrum between before and after irradiation with >630-nm light. The full scale of the vertical axis is 0.06 for spectrum a and 0.013 for spectrum b.

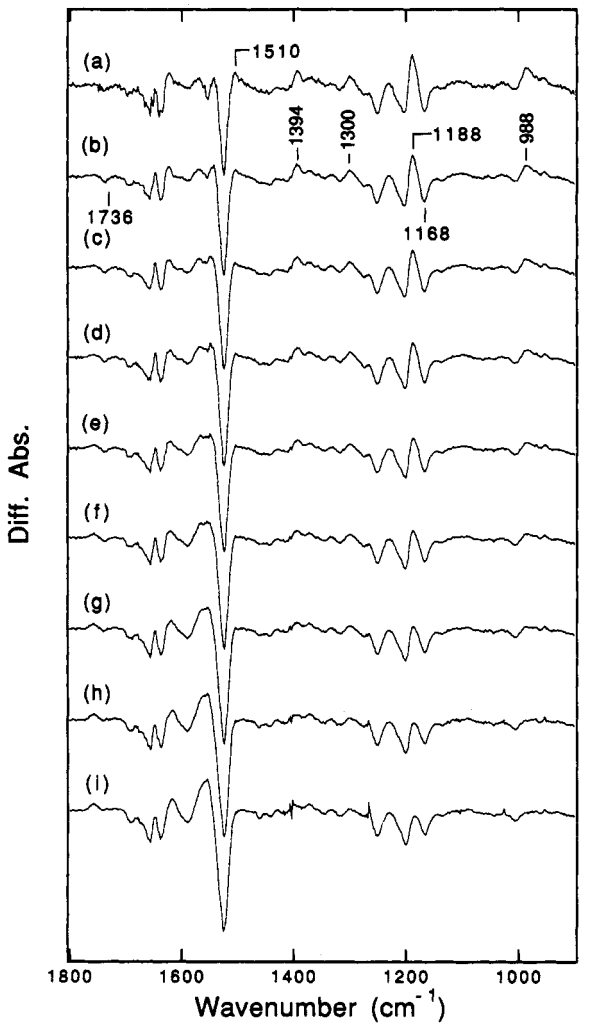

FIGURE 4: Spectra in the time region of (a) 0–5, (b) 5–10, (c) 10–15, (d) 15–20, (e) 20–30, (f) 30–40, (g) 40–80, (h) 80–160, and (i) 160–450 μs. The full scale of the vertical axis is 0.063.

(dashed line). The summed curve of these two (dotted line) roughly fits the experimental data points.

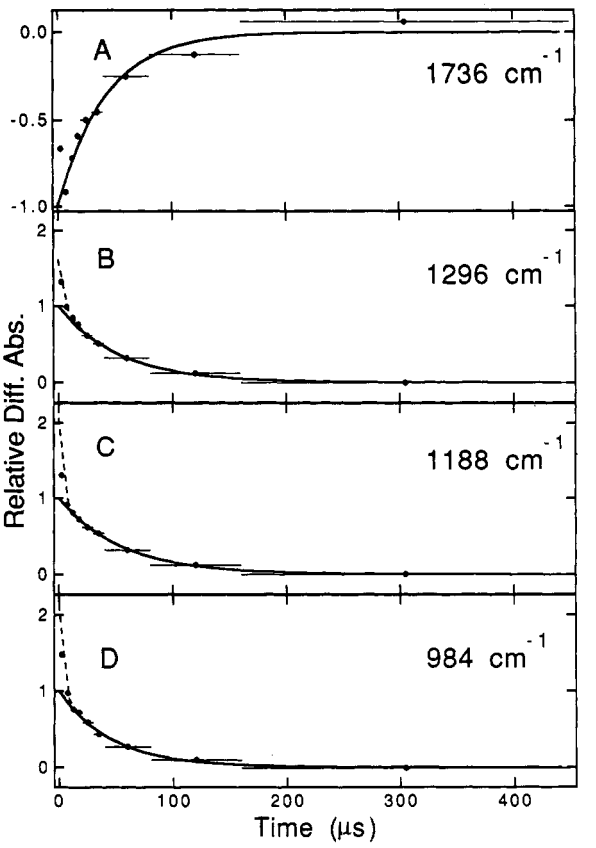

FIGURE 5: Changes in the spectra in Figure 4 at (A) 1736, (B) 1296, (C) 1188, and (D) 984 cm^{-1} are plotted against time. In panel A, the data points except for the first one are fitted by a single-exponential curve which is extrapolated to the relative intensity at zero time to −1.0. Each of panels B–D is fitted by a double-exponential curve (dotted line), and the slower one is depicted by a solid line which is extrapolated to the relative intensity at time zero to 1.0. The time constant of the slower phase is about 30 μs.

Rothschild et al. (1985) have described a K-like photoproduct which was produced as a mixture with K^{LT} at 135 K by irradiation of BR with 500-nm light. The latter reverted to BR by irradiation with 700-nm light whereas the K-like photoproduct did not. The K-like photoproduct was converted to BR by >630-nm light. Reproduced spectra for these processes are shown in Figure 3a,b, respectively, for the purpose of comparison with the time-resolved spectra. The spectrum in Figure 3a agrees with the K^{LT}/BR spectrum, and that in Figure 3b corresponds to the K-like product/BR. With respect to the presence of the 1394- and 987-cm^{-1} bands, the K-like photoproduct is similar to the KL detected in the time-resolved IR spectra (Figure 1). Since the K-like photoproduct was not produced at 77 K under the same light conditions for irradiation, it may be produced by thermal conversion of K^{LT} and the direct precursor of L. Thus, the K-like photoproduct at 135 K most probably corresponds to KL at room temperature.

Microsecond Time Domain. From a similar experiment, data in the microsecond time range were collected for the 0–5-, 5–10-, 10–15-, 15–20-, 20–30-, 30–40-, 40–80-, 80–160-, and 160–450-μs time domains. Each spectrum was normalized by the 1168-cm^{-1} band as described above. The spectra are shown in Figure 4a–i, respectively. The spectral changes in this time range are mainly attributed to the L-to-M conversion. However, the strong IR bands at 1300 and 988 cm^{-1}, which were not found in L or M at low temperature (Maeda et al., 1991), are now observed (Figure 4a–g). These bands are due to KL. They can be completely eliminated by

subtracting the KL/BR spectrum (Figure 1b) from the spectrum in Figure 4b. The spectrum after subtraction (not shown in figures) is essentially the same as the L/BR spectrum recorded at 170 K.

The negative band at 1736 cm^{-1} is specific to the L/BR spectrum and shows the perturbation of Asp^{96} and Asp^{115} in L (Braiman et al., 1988). It grows in about 1 μs (Figure 1e), reaches a steady state in 10 μs (Figures 1i and 4b), and decays after 20 μs (Figure 4d). The 1394-cm^{-1} band which is common to KL and L as described above is attributed to the 1400-cm^{-1} band of L at low temperature. The strong band at 1188 cm^{-1} in this time range corresponds to the 1192-cm^{-1} band of L at low temperature (Gerwert & Siebert, 1986), although KL displays a stronger band at the same frequency. The bands of KL and L disappear in the last spectrum (Figure 4i), which is almost identical with the M/BR spectrum at 230 K.

Time-dependent decreases in intensity in the microsecond range for the bands at 1736, 1296, 1188, and 984 cm^{-1} are shown in Figure 5A–D, respectively. The 1300- and 988-cm^{-1} bands were plotted at 1296 and 984 cm^{-1}, respectively, where the KL shows their maxima. The increase in the negative intensity of the 1736-cm^{-1} band from the first to second point shows the formation of L. The decay is fitted roughly by a exponential curve with a half-lifetime of about 30 μs. The decay of each of the 1296-, 1188-, and 984-cm^{-1} bands is expressed by a double-exponential curve. The rapid phase (dashed line) probably reflects the conversion of KL to L. The time constants in the slow phase are also about 30 μs. The similarity of the decaying processes of KL and L indicates that the KL-to-L conversion is a reversible reaction.

DISCUSSION

The newly designed dispersive-type IR spectrometer (Iwata & Hamaguchi, 1989; Kato et al., 1991) measures time-resolved spectra with a time resolution better than 200 ns. The time-dependent IR spectra of the KL intermediate were obtained for the first time. The apparent time constants of the KL-to-L and L-to-M conversions, both of which were obtained with the hydrated film of bacteriorhodopsin, were nearly coincident with those obtained by visible absorption spectroscopic experiments for the purple membrane suspension (Shichida et al., 1983; Milder & Kliger, 1988; Xie et al., 1987; Váró & Lanyi, 1991; Lozier et al., 1992). The spectral feature of KL was similar to the K-like photoproduct produced by irradiation at 135 K. The spectra of L and M were coincident with those recorded at low temperatures (Maeda et al., 1991).

Shichida et al. (1983) distinguished KL from K by time-resolved visible spectroscopy and presented evidence for the K-to-KL conversion between 900 ps and 150 ns. Milder and Kliger (1988) have set the upper limit to the lifetime of K as 10 ns and shown no further spectral change up to 100 ns.

The KL-specific bands at 1510, 1296, and 956 cm^{-1} decayed exponentially with a time constant of about 1 μs. The 984-cm^{-1} HOOP band, however, shows a different kinetic feature from the other bands: Several points up to 2 μs in Figure 2C do not fit the exponential decay of other KL bands at 1510, 1296, and 956 cm^{-1}. Though the decay of the 984-cm^{-1} band appears to be delayed, it is more reasonable to explain it by a combination of exponential curves as shown in Figure 2C. In this case, although unresolved in the present experiment, the 984-cm^{-1} band may arise in the early nanosecond range. This change in HOOP bands can be correlated with the visible spectral changes of K-to-KL conversion. Similar bands at 986 cm^{-1} have been detected in the resonance Raman spectra in the nanosecond time range (Doig et al., 1991; Lohrmann et al., 1991). Kinetic experiments in an earlier time range will solve this problem.

The enhancement of HOOP intensity in the IR spectra is caused by the twists in the chromophore (Fahmy et al., 1989). The present results indicate changes in the twists of the chromophore in the submicrosecond time domain. In such a rapid process, the chromophore will not fit into a protein environment, which changes more slowly, and can accommodate to it only by local twist of the polyene chain. Assignments of these HOOP bands must be a problem for the future.

The KL-specific bands remain in the microsecond region and decay with a time constant similar to that of L. These results suggest that the KL-to-L conversion is reversible. This means that the free energy level of L is not so much different from that of KL (Váró & Lanyi, 1991) even though the former has more relaxed chromophore conformation. Therefore, L has some instability in the protein moiety, which may explain the observed perturbations of Asp^{96}, Asp^{115}, and water. These perturbations may induce subsequent dislocation of the proton on the Schiff base to Asp^{85} in the L-to-M conversion.

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