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Millisecond Fourier-transform infrared difference spectra of bacteriorhodopsin's M_{412} photoproduct

(rapid-sweep interferogram/tyrosinate/purple membrane/kinetics/time resolution)

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ABSTRACT **We have obtained room-temperature transient infrared difference spectra of the M_{412} photoproduct of bacteriorhodopsin (bR) by using a "rapid-sweep" Fourier-transform infrared (FT-IR) technique that permits the collection of an entire spectrum (extending from 1000 to 2000 cm^{-1} with 8-cm^{-1} resolution) in 5 ms. These spectra exhibit $<10^{-4}$ absorbance unit of noise, even utilizing wet samples containing ≈10 pmol of bR in the measuring beam. The bR → M transient difference spectrum is similar to FT-IR difference spectra previously obtained under conditions where M decay was blocked (low temperature or low humidity). In particular, the transient spectrum exhibits a set of vibrational difference bands that were previously attributed to protonation changes of several tyrosine residues on the basis of isotopic derivative spectra of M at low temperature. Our rapid-sweep FT-IR spectra demonstrate that these tyrosine/tyrosinate bands are also present under more physiological conditions. Despite the overall similarity to the low-temperature and low-humidity spectra, the room-temperature bR → M transient difference spectrum shows significant additional features in the amide I and amide II regions. These features' presence suggests that a small alteration of the protein backbone accompanies M formation under physiological conditions and that this conformational change is inhibited in the absence of liquid water.**

Infrared difference spectroscopy is a useful technique for measuring protein structural changes. Every residue has infrared-active group vibrations that are potentially sensitive to changes in covalent bonding (e.g., conformation, protonation state) and in noncovalent interactions with the surrounding environment (e.g., hydrogen bonding, steric hindrance). Although the presence of many IR-active groups in a large protein leads to a very complex IR spectrum, careful null measurements make it possible to observe only the small subset of vibrations that change during a biochemical transformation.

The photoreactive proteins bacteriorhodopsin (bR) and rhodopsin are ideally suited for observing such difference spectra. By photolyzing these proteins inside a spectrometer, it has been possible to make very precise measurements of the resulting IR absorbance changes (1–12). These IR difference spectra have provided a wealth of information. For example, it has been shown that during the photoreaction of bR (λ_{max} = 568 nm) to M (λ_{max} = 412 nm), an aspartate residue becomes protonated (1, 7); additional protonation changes of carboxylic acid residues occur at other steps in the bR photocycle (7, 9, 10). More recently, IR difference spectra (along with UV difference spectra) have detected changes in protonation of several tyrosines in the photointermediates between bR and M (8–10). Such spectra provide important experimental tests of proposed proton-translocation mechanisms for bR.

Early IR difference spectra of bR and rhodopsin photoproducts were obtained by using flash photolysis techniques and single-wavelength transient measurements with submillisecond time resolution (5, 6, 11). However, covering a significant IR spectral region with successive single-wavelength measurements requires extensive periods of signal-averaging. With Fourier-transform infrared (FT-IR) spectroscopy, it is possible to collect data simultaneously over a large spectral region. The time resolution of conventional FT-IR spectrometers is fairly slow; hence, previous FT-IR difference measurements on bR and rhodopsin photoproducts have relied on nonphysiological conditions [e.g., drying (1) and/or cooling (2–4, 8–10)] in order to trap photointermediate species.

To study protein reactions under physiological conditions, it is clearly important to develop time-resolved IR techniques that can take advantage of the intrinsically higher sensitivity of Fourier-transform spectrometers. A number of approaches to this problem have been described (12, 13). We recently presented a method that is based on sweeping the interferometer moving mirror rapidly enough to obtain a 512-point spectrum, extending from 0 to 2000 cm^{-1} with 8-cm^{-1} resolution, in just 5 ms (14). By triggering an external photolysis laser pulse at the start of data collection, it is possible to obtain IR spectra of short-lived photoproducts. We designate this approach "rapid-sweep" FT-IR spectroscopy.‡

We have applied the rapid-sweep method to the study of bR's M photoproduct, which has an ≈10-ms decay time at room temperature (15, 16). Our results support many conclusions about bR → M structural changes that were originally drawn from FT-IR experiments at low temperature—e.g., protonation changes of tyrosine and aspartic acid residues. However, at room temperature additional spectral features are observed that suggest that the protein backbone of bR requires a liquid water environment in order to attain the M state found under physiological conditions. A preliminary account of this work has been presented (17).

EXPERIMENTAL PROCEDURE

All spectra were obtained in the *f*/1 microbeam compartment of a Nicolet (Madison, WI) 60SX FT-IR spectrometer equipped with a liquid-N_2-cooled Hg/Cd/Te detector, using

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Abbreviations: bR, bacteriorhodopsin; FT-IR, Fourier-transform infrared; a.u., absorbance unit(s); sh, shoulder.

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‡We use the term "rapid-sweep" in distinction from "rapid-scan," which is often used to designate methods intended to increase the *repetition rate* of scanning rather than the speed of individual scans (e.g., rapid mirror turn-around or data collection during both directions of mirror motion).

our own modified version of the operating software provided with the system.

Rapid-Sweep Data Collection. By appropriate selection of interferometer mirror velocity and data sampling interval of the Nicolet 60SX spectrometer, it is possible to collect a 512-point interferogram, with a total retardation of 1.30 mm, in just over 5 ms (see Fig. 1). This interferogram, when apodized and Fourier-transformed, yields a spectrum extending from 0 to 1975 cm^{-1} with 8-cm^{-1} spectral resolution and 5-ms temporal resolution.

By illuminating a bR sample with a 15-ns laser photolysis pulse (540 nm, 3 mJ) just prior to the collection of the 5-ms interferogram, it was possible to obtain a time-resolved spectrum of the M_{412} photoproduct. The photolysis beam was directed into the microbeam compartment and was reflected along the IR optical path by a small 45° mirror placed in the center of the expanded and collimated IR beam. A weak lens ($f = 100$ mm) just outside the sample compartment shifted the focal point of the laser away from that of the IR beam, causing the defocused laser to illuminate the entire 1.5-mm-diameter region of sample probed by the IR beam.

Interferograms following a flash were collected alternately with no-flash interferograms, at a repetition rate of $\approx 4\ s^{-1}$. For wet samples at room temperature, this was slow enough to permit bR photoproducts to relax fully between successive mirror scans. However, for a very dry sample that exhibited slow M decay it was necessary to alternate flash and no-flash scans not one-by-one but rather in blocks of 2000.

After signal-averaging and Fourier transformation, the "flash-on" spectrum was ratioed to the "flash-off" spectrum, and the transmittance changes were converted to

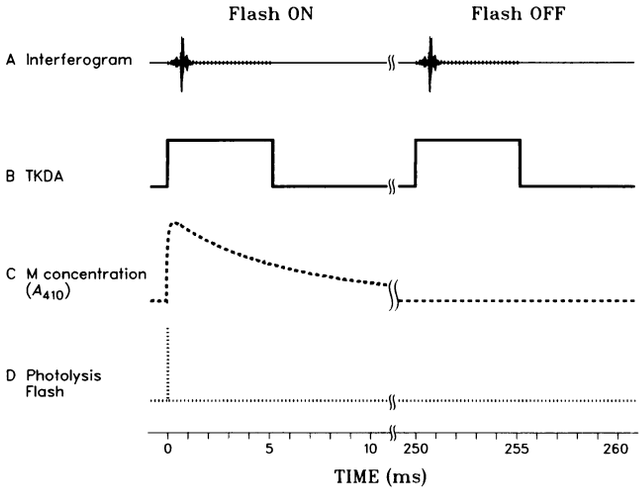

FIG. 1. Timing diagram for rapid-sweep technique. Shortly before the center burst of the interferogram (*A*), the TTL-level TKDA signal of the 60SX spectrometer (*B*) enables digitization of the data. The 100-kHz maximum digitization rate of the 60SX spectrometer limits the interferogram sampling to once every four He/Ne wavelengths when the maximum retardation velocity (25.1 cm/s) is selected. To avoid aliasing artifacts that would otherwise result from undersampling the interferogram, a long-pass interference filter with 5-μm cutoff (AGA Optics, Secaucus, NJ) is inserted in front of the detector. For alternate sweeps, the rising edge of TKDA triggers a laser flash (*D*) that photolyzes ≈30% of the bR in the sample to M. The rise time of M is ≈50 μs (15), and its decay back to bR is complete within several milliseconds, as shown by the transient absorbance change at 410 nm of a photolyzed bR sample (*C*). To eliminate spectral artifacts due to M precursors, the first 10 digitized points from each interferogram, corresponding to the first 100 μs after photolysis, were discarded prior to Fourier processing. The following 512 points, which include 38 points before and 474 points after the interferogram peak, were used to calculate the spectrum.

bR → M absorbance differences. Further details of the rapid-sweep technique will be published elsewhere.

Resolution Enhancement of Transient FT-IR Spectrum. The exponential decay of M back to bR during rapid-sweep interferogram collection causes line broadening in the difference spectrum. By using a procedure related to Fourier self-deconvolution (18), it is possible to deconvolute the broadened bandshape. Thus, by collecting 1024-point interferograms and correcting for the decrease in M concentration that occurred over the course of the 10-ms interferogram collection time, we could improve the resolution of our bR → M spectra to $\approx 4\ cm^{-1}$.

The decay-corrected absorbance difference spectrum $\Delta A(\bar{\nu})$ was calculated as

$$\Delta A(\bar{\nu}) = -\log\left[\frac{\mathcal{F}\{[I_f(t) - I_n(t)]/C_M(t)\} + \mathcal{F}\{I_n(t)\}}{\mathcal{F}\{I_n(t)\}}\right],$$

where I_f and I_n are the flash and no-flash interferograms, $C_M(t)$ is the relative M concentration at time t after a flash, and $\mathcal{F}$ represents the Fourier processing operation (including apodization and phase correction). We approximated $C_M(t)$ by a single-exponential decay function, $\exp(-t/\tau)$, using a value of 7 ms for τ. The sensitivity of the spectrum to this approximation was checked by recalculating the spectrum with other values of τ in the range of 5–10 ms and also by using a two-exponential decay with published values for the kinetic constants (16). These variations did not affect the positions of peaks and resulted in only minor changes in peak shapes.

Sample Preparation. Purple membranes, prepared from *Halobacterium halobium* strain S9 by standard methods (19), were washed several times with H_2O (or ^2H_2O, with several days between washes to permit isotopic exchange). A small amount of the wet pellet was squeezed between two 25-mm-diameter × 2-mm-thick CaF_2 windows (Harrick Scientific, Ossining, NY). A thin layer of silicone vacuum grease toward the circumference of the sandwich sealed the sample against water loss. We estimate that sample thicknesses produced by this method were 5–20 μm. The CaF_2 sandwich was held in a cell mount (Harrick), with one of the windows flush against a mask made by drilling a 1.5-mm-diameter hole in a 0.008-cm-thick stainless steel disk. The sample was aligned with the IR and photolysis beams directed coaxially through the hole in the mask.

Transient Visible Absorbance Measurements. Data were obtained from a home-built flash photolysis apparatus incorporating a Nicolet 1275 signal-averager, using bR samples prepared in an identical fashion, held in the same cell and photolyzed by the same laser employed for the transient FT-IR measurements.

RESULTS AND DISCUSSION

With our rapid-sweep FT-IR technique, it is possible to observe significant transient differences of ≈0.005 absorbance unit (a.u.) after <20 s of signal averaging (e.g., positive 1557- and negative 1527-cm^{-1} peaks in Fig. 2*A*). The noise level in Fig. 2*A* is ≤0.001 a.u., except from 1630 to 1680 cm^{-1} and below 1100 cm^{-1}, where the IR measuring beam was greatly attenuated by water and amide I and by CaF_2 window absorptions, respectively.

After 2 hr of signal-averaging (Fig. 2*B*), the noise is reduced to $\leq 10^{-4}$ a.u. throughout the region from 1800 cm^{-1} to just below 1000 cm^{-1}. The features in this bR → M transient FT-IR difference spectrum are quite similar to those observed in static FT-IR difference spectra from dried (1) or frozen (3, 4, 7) bR samples as well as those observed in kinetic FT-IR spectra composed of single-wavelength transient measurements (5, 6). Many peaks in our transient bR → M

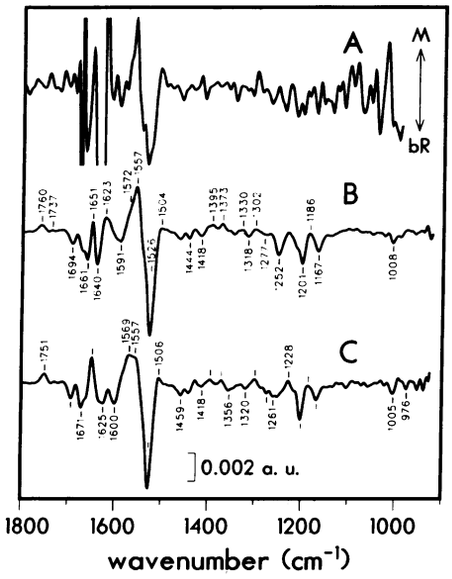

FIG. 2. (*A*) Five-millisecond transient FT-IR difference spectrum of the bR → M photoreaction obtained using 36 laser flashes. (*B*) The same spectrum as in *A* after averaging data from 14,000 flashes. (*C*) A spectrum similar to that in *B* but obtained from sample suspended in ^2H_2O. Unlabeled peaks in *C* correspond in frequency to labeled peaks in *B*. Water content of the samples corresponded to that in Fig. 5*B*.

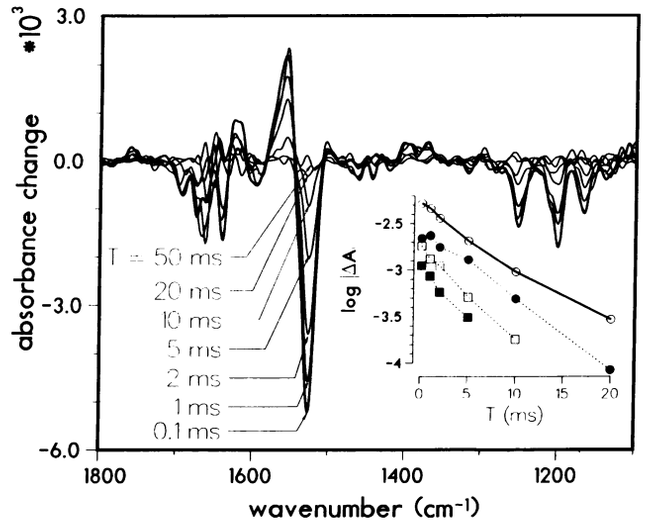

FIG. 3. Rapid-sweep FT-IR difference spectra of bR → M photoreaction obtained with various amounts of time delay (*T*) after the laser flash. The laser was triggered from the FWD (mirror forward) signal from the spectrometer, which occurred ≈70 ms before the TKDA signal. A variable delay generator and an oscilloscope then allowed control and measurement of the actual time interval *T* between the laser pulse and the start of data collection. All spectra were obtained within a 4-hr period on a single sample whose water content corresponded to that in Fig. 5*B*. Data for each value of *T* were collected in blocks of 1000 pairs of flash-on/flash-off scans. Each spectrum shown is the average of data from four such blocks, interspersed in a quasi-random fashion with blocks having different values of *T*. This strategy was taken to avoid drift artifacts due to photobleaching of sample or changes in alignment of photolysis beam. (*Inset*) Plot of log $|\Delta A|$ vs. *T* for several peaks in the difference spectrum: ○, 1526 cm^{-1}; ●, 1557 cm^{-1}; □, 1202 cm^{-1}; ■, 1168 cm^{-1}.

difference spectrum can be assigned to specific chromophore or protein vibrations, based on bR isotopic derivatives studied with FT-IR at low temperature (4, 7–9) or with time-resolved resonance Raman spectroscopy (20, 21). For example, negative (bR) peaks at 1640, 1526, 1252, 1201, 1167, and 1008 cm^{-1} and positive (M) peaks at 1623, 1572 (sh) (where sh indicates shoulder), and 1186 cm^{-1} are due to chromophore vibrations that change as a result of isomerization and deprotonation during the bR → M photoreaction. The positive (M) peak at 1760 cm^{-1} is known to be due to the COOH group of an aspartic acid residue in the protein (7). When the transient difference spectrum is obtained from a sample in ^2H_2O (Fig. 2*C*), the COO^2H vibration shifts to ≈1750 cm^{-1}, as has been observed previously (1, 4–7). Likewise, the $C{=}NH^+$ stretching vibration of the retinal-protein Schiff's base linkage in the parent bR shifts from 1640 (Fig. 2*B*) to 1625 cm^{-1} (Fig. 2*C*).

These transient IR absorbance changes exhibit appropriate decay kinetics, as shown in Fig. 3. A plot of log(ΔA) vs. *T* (the time interval between the flash and the start of interferogram collection) for the 1526-cm^{-1} peak is nearly linear (Fig. 3 *Inset*). The slope of the plot corresponds to a decay time of ≈6 ms. This agrees well with the time constant of 6.16 ± 0.06 ms for the best single-exponential fit to the visible absorbance decay at 410 nm (typical data shown in Fig. 1*C*), measured using a sample and temperature identical to those employed for the FT-IR measurements. Other major peaks in the bR → M FT-IR difference spectrum also decay with similar kinetics, giving semilogarithmic plots that are nearly parallel to that for 1526 cm^{-1} (Fig. 3 *Inset*). One major peak in the transient FT-IR spectrum with unusual kinetic behavior is at 1557 cm^{-1}. This feature does not decay monotonically; rather, it increases slightly in intensity between the first two time points measured, then decreases. Thus, the ratio ΔA-(1572)/ΔA(1557) changes with *T*, the shoulder at 1572 cm^{-1} being relatively largest at the earliest time point (T = 0.1 ms). This has been observed consistently with a number of samples on different occasions, demonstrating that there are several component vibrations in the broad positive band near 1560 cm^{-1} with different kinetic behaviors.

Mäntele *et al.* have already shown this more directly by using single-wavelength kinetic measurements at room temperature (5). In particular, they found that IR transmission changes at 1555 cm^{-1} had a slower rise time (≈300 μs) than those at 1570 cm^{-1} (≈50 μs). The increase in 1557-cm^{-1} absorbance that we observe between T = 0.1 and 1 ms (Fig. 3) could be the tail end of the 300-μs process observed by Mäntele *et al.* (5). However, unlike our spectra in Figs. 2*B* and 3, their "time-slice" data (5) never show ΔA at 1555 cm^{-1} exceeding ΔA at 1570 cm^{-1}. The reason for this is unclear, although it could possibly be caused by different amounts of water in their samples and in ours (discussed below). Although an intense 1569-cm^{-1} peak has been observed in the resonance Raman spectrum of M, there is no corresponding 1557-cm^{-1} line (20, 21). This, in addition to the kinetic behavior mentioned above, suggests that the 1572-cm^{-1} shoulder in our bR → M IR difference spectrum is due to a chromophore vibration, whereas the 1557-cm^{-1} peak is due to a protein vibration. A similar suggestion was made by Mäntele *et al.* to explain the different kinetic behaviors that they observed at 1555 and 1570 cm^{-1} (5).

Some of the intensity at 1557 cm^{-1} in our bR → M difference spectrum (Fig. 2*B*) appears to shift away when the FT-IR spectrum is measured in ^2H_2O (Fig. 2*C*). The remaining feature near 1557 cm^{-1} exhibits more normal decay behavior—i.e., it decays monotonically and its intensity relative to the 1569- and 1526-cm^{-1} peaks does not change as *T* is varied from 0.1 to 50 ms (data not shown). These observations suggest that there are several vibrations superposed at 1557 cm^{-1} and that the protein vibration with anomalous decay is due to a group with exchangeable proton(s).

The 1557-cm^{-1} peak is furthermore temperature-dependent, as shown by a comparison of bR → M difference spectra

obtained at room temperature and at −20°C (Fig. 4). These higher-resolution spectra show that at room temperature there is both an increased positive peak at 1557 cm^{-1} and an increased negative shoulder at 1538 cm^{-1} (Fig. 4*A*) that appear to be superimposed on the spectral features observed at −20°C (Fig. 4*B*). The frequencies of these positive and negative features are consistent with the idea that they could be due to the amide II vibration(s) of a portion of the protein backbone undergoing a conformational change between bR and its M photoproduct. If this hypothesis is correct, then these spectra demonstrate that this protein conformational change is partially blocked at −20°C. Consistent with the hypothesis of temperature-dependent protein backbone change is the observation of temperature-sensitive features in the amide I spectral region (1650–1700 cm^{-1} in Fig. 4*A*). Peaks in this region of the bR → M difference spectrum are known to be due to protein vibrations based on their insensitivity to isotopic substitutions of the chromophore (6).

The "amide I" (1650–1700 cm^{-1}) and "amide II" (1557 cm^{-1}) temperature-dependent features can also be abolished by decreasing the water content of the sample from 90% by weight (Fig. 5*A*) to 25% (Fig. 5*D*). The change in the 1650- to 1700-cm^{-1} features appears to begin at intermediate degrees of dessication (Fig. 5 *B* and *C*). However, interpretation of these features' behavior is complicated because the 1649- and 1694-cm^{-1} vibrations are known to be highly dichroic, with transition moments oriented nearly perpendicular to the membrane plane (23, 24). Thus, a part of the gradual intensity decrease upon dessication could be due to alignment of purple membrane sheets in a direction parallel to the CaF_2 window surfaces. On the other hand, such an explanation is unlikely for the intensity decreases of the 1557-cm^{-1} (positive) and 1659-cm^{-1} (negative) peaks, since the bR and M vibrations observed at these frequencies have transition dipole moments predominantly parallel with the membrane plane (23, 24).

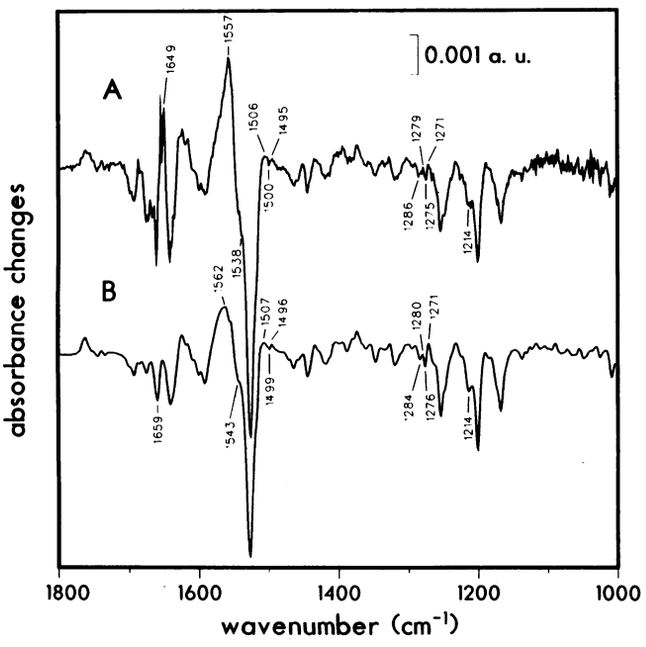

FIG. 4. bR → M difference spectra obtained with rapid-sweep FT-IR technique at room temperature (*A*) and by static FT-IR measurements at −23°C (*B*) (10). For *B*, the resolution was 2 cm^{-1}. For *A*, the spectral resolution was enhanced (to 4 cm^{-1}) by taking interferogram points out to 10 ms following the flash, signal-averaging extensively (50,000 flashes), and employing a deconvolution technique described in the text.

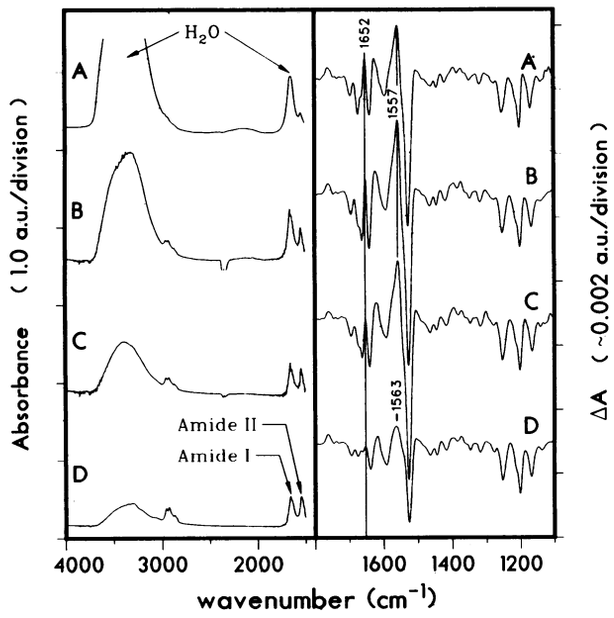

FIG. 5. Effect of purple membrane water content on the bR → M difference spectrum. On the left-hand side are unsubtracted spectra of samples that differed in water content. Water content of the samples was estimated to be (by weight) 90% (*A*), 70% (*B*), 50% (*C*), and 25% (*D*). These crude estimates are based on approximate values of $\varepsilon_{3500}(H_2O) = 100\ M^{-1}\cdot cm^{-1}$ (22) and $\varepsilon_{1545}(bR) = 100{,}000\ M^{-1}\cdot cm^{-1}$ (unpublished results). (The anomalous negative peak near 2400 cm^{-1} in *B* and *C* is due to an incorrectly subtracted CO_2 absorption band.) On the right-hand side are corresponding bR → M difference spectra obtained by the 5-ms rapid-sweep technique. For *D*, modification in this technique was required because the sample was so dry that M did not decay fully between laser flashes.

In any case, at a level of dessication where M decay is slowed, permitting it to be observed under steady-state illumination (Fig. 5*D*), the 1652- and 1557-cm^{-1} intensities decrease to the same level observed with the sample at −20°C (Fig. 4*B*). The similar ability of freezing or drying to reduce amide I and amide II features of the bR → M difference spectrum suggests that the protein conformational change responsible for these features can occur fully only in the presence of a liquid water environment.§ Based on changes in the activation enthalpy of M → bR decay upon drying or freezing, Váró and Keszthelyi (26) also inferred the presence of a protein conformational change requiring bulk liquid water.

Even taking into account incomplete (≈30%) conversion to M under our experimental conditions, the magnitude of the transient, water-dependent amide I and amide II spectral features in Fig. 5 represents <1% of background protein absorbance. This is comparable to the ≈1% change in the amide I and II bands observed for rhodopsin bleaching intermediates at room temperature (2) and could be accounted for by a localized secondary structure rearrangement of just a few of bR's 248 residues. The wet purple membrane samples in the current studies were not well-oriented and the

§An alternative explanation for the difference between the transient bR → M spectrum from wet samples and static spectra from frozen or dried samples could be that photon absorption by bR photointermediates leads to accumulation of a different M-like species. However, by treating wet purple membrane films with La^{3+} at high pH it is possible to slow down the photocycle enough to generate large steady-state M concentrations at room temperature, and the static bR → M difference spectrum measured under these conditions corresponds more closely to our transient FT-IR spectrum of a wet sample than to static FT-IR spectra of dried or frozen bR (25).

f/1 measuring beam did not have a well-defined plane of polarization; therefore, it is difficult to use our data to state a limit on the possible tilt of the transmembrane helices upon M formation. Still, the sizes of the amide I and II features in our spectra are inconsistent with any large-scale global changes in protein structure, unless [as Draheim and Cassim proposed (27)] they could be accomplished with very minor secondary structure alterations.

Other than the amide I and amide II features, the additional details observed in the resolution-enhanced spectrum of Fig. 4*A* are strikingly similar to features observed previously in the low-temperature static bR → M spectrum (Fig. 4*B*). In particular, we observe very similar patterns in the ≈1280- and ≈1500-cm^{-1} regions, which are characteristic frequency regions for the C—O^- stretching and aromatic C=C stretching vibrations of tyrosinate anions, respectively (8–10). Peaks in these regions in the low-temperature bR → M difference spectrum have been attributed to protonation changes of three distinct tyrosine residues, based on spectral shifts observed with bR samples containing isotopically substituted (8–10) or chemically modified (28) tyrosines. The similar appearance of these spectral features in Fig. 4 *A* and *B* suggests that these same tyrosine protonation changes also occur transiently when M is formed at room temperature—i.e., under conditions where bR is known to have proton-pumping activity. Our spectra are consistent with a net tyrosine deprotonation during the bR → M reaction, as originally suggested by transient UV absorption measurements (29–33). These results furthermore support models involving tyrosines as transient carriers of pumped protons (34, 35).

In conclusion, we have demonstrated that the rapid-sweep FT-IR technique is sensitive enough to measure 7-ms transient changes of individual residues in a 26,000-dalton membrane protein (bR). Each of the transient FT-IR difference spectra presented in Figs. 2–5 was obtained with only 10 pmol of bR in the measuring beam. Furthermore, excellent spectra (e.g., Fig. 5*A*) were obtained even when such samples also contained up to 90% water by weight. These results illustrate the potential power of this technique for studying fast biochemical processes under nearly physiological conditions.

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