Determination of retinal Schiff base configuration in bacteriorhodopsin
(resonance Raman spectroscopy/retinal isotopic derivatives/proton-pump mechanism/cis-trans isomerization/charge separation)

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ABSTRACT Resonance Raman spectra of the BR548, BR548, K525, and L550 intermediates of bacteriorhodopsin photocycle have been obtained in H2O and 2H2O by using native purple membrane as well as purple membrane regenerated with 14,15-13C2 and 12,14-2H2 isotopic derivatives of retinal. These derivatives were selected to determine the contribution of the C(14)-C(15) stretch to the normal modes in the 1100-
1400-cm-1 fingerprint region and to characterize the coupling of the C(14)-C(15) stretch with the NH rock. Normal mode calculations demonstrate that when the retinal Schiff base is in the cis-N cis configuration, the C(14)-C(15) stretch and the NH rock are strongly coupled, resulting in a large (~50-cm-1) upshift of the C(14)-C(15) stretch upon deuteration of the Schiff base nitrogen. In the C(=N) trans geometry these vibrations are weakly coupled and only a slight (~5-cm-1) upshift of the C(14)-C(15) stretch is predicted upon N-deuteration. In BR548, the insensitivity of the 1201-cm-1 C(14)-C(15) stretch to N-deuteration demonstrates that its retinal C(=N) configuration is trans. The C(14)-C(15) stretch in BR548, however, shifts up from 1167 cm-1 in H2O to 1208 cm-1 in 2H2O, indicating that BR548 contains a N cis chromophore. Thus, the conversion of BR548 to BR548 (dark adaptation) involves isomerization about the C(=N) bond in addition to isomerization about the C(13)=C(14) bond. The insensitivity of the native, [14,15,13C2]-, and [12,14,2H2]K525 and L550 spectra to N-deuteration argues that these intermediates have a C(=N) trans configuration. Thus, the primary photochemical step in bacteriorhodopsin (BR548 K525) involves isomerization about the C(13)=C(14) bond alone. The significance of these results for the mechanism of proton-pumping by bacteriorhodopsin is discussed.

Bacteriorhodopsin functions as a photochemical proton pump in the purple membrane of Halobacterium halobium (1). Absorption of light by bacteriorhodopsin's retinal prosthetic group converts the light-adapted pigment, BR548, to the red-absorbing intermediate, K525, which thermally decays back to BR548 through the intermediates L550, M412, and O404 (2). The initial photocatalytic step involves a trans → cis isomerization about the C(13)=C(14) bond of retinal (3–6), which is followed by deprotonation of the Schiff base nitrogen in the L550 → M412 transition (7). Recently it has been shown that reprotonation of the Schiff base and thermal reisomerization of the C(13)=C(14) bond occur in the conversion of M412 to O404 (8). In the dark, BR548 converts to dark-adapted bacteriorhodopsin, which contains a 60:40 mixture of 13-cis and all-trans protonated Schiff base chromophores denoted BR548 and BR548, respectively. It is generally accepted that chromophore isomerization and Schiff base protonation/deprotonation play an active role in the mechanism of this proton pump. An important element in establishing the orientation and molecular motion of the Schiff base proton in bacteriorhodopsin's photocycle is the configuration of the retinal-lysine Schiff base bond. However, no experimental determination of the C(=N) configuration has yet been made.

Resonance Raman spectroscopy can be used to examine the structure of the protein-bound retinal chromophore in bacteriorhodopsin (9). To interpret these spectra, it is necessary to assign the vibrational lines to specific normal modes and to establish how the vibrations are affected by changes in chromophore geometry. Selective isotopic substitution of the retinal chromophore facilitates the vibrational assignments, while model compounds and normal mode calculations can be used to predict the spectral changes that would result from a specific structural change. This approach has been employed in recent vibrational analyses of all-trans, 13-cis, and 11-cis retinal, which now provide a basis for the interpretation of Raman spectra of protein-bound retinal chromophores (10–12). In this paper, we extend this work by identifying unique vibrational features of C(=N) cis and trans protonated Schiff base chromophores, and then we use these results to examine the C(=N) configuration in BR548, BR548, K525, and L550.

EXPERIMENTAL

Purple membrane was prepared from H. halobium cultures according to published procedures (13). Bacterio-opsin was isolated from a retinol-deficient strain of H. halobium (IW5) and regenerated with isotopic derivatives as described by Smith et al. (8). The syntheses of [14,15,13C2]- and [12,14,2H2]retinal have recently been described (14).

The Raman apparatus and general data collection procedures have been described in ref. 8. Whenever the Raman lines overlapped significantly, the indicated frequencies were determined by fitting to a sum of Lorentzian peaks convoluted with a triangular slit function.

Raman spectra of BR548 were obtained by using a 30-mW 514-nm probe laser beam cylindrically focused on a light-adapted purple membrane sample flowing at 300 cm/s (13). The photoalteration parameter F was less than 0.1 (15). Raman spectra of BR548, the 13-cis component of dark-adapted bacteriorhodopsin (BR548), were obtained by subtraction of a light-adapted BR548 spectrum from the spectrum of BR548 (8). The low-photoalteration probe excitation at 514 nm (<5 mW) was cylindrically focused on a flowing sample (300 cm/s). K525 Raman spectra were obtained at 77 K by using the spinning cell procedure of Braun and Mathies (3, 16) with a 350-mW 514-nm pump beam (F = 1.0) and a 200-mW 752-nm probe beam (F = 0.1).

L550 Raman spectra were obtained by using the dual-beam flow apparatus previously described (8, 13). The flow rate was 450 cm/s, and the pump and probe beams were cylindri-

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cally focused on the flow capillary producing a pump-probe time delay of 17 μs (beam separation = 76 μm). Probe excitation of 5–10 mW at 514 nm yields a photobleaching parameter for both BR<sub>98</sub> and L<sub>550</sub> of less than 0.1. Pump excitation of 75 mW at 568 nm was chosen to minimize photobleaching of the L<sub>550</sub> component (F = 0.5 based on ε = 40,000 M<sup>-1</sup> cm<sup>-1</sup> (2) and assuming a quantum yield of unity) while still producing a useful concentration of this intermediate.

Normal mode calculations were performed by using the Wilson FG method (10–12). The geometry for a C=NH<sub>trans</sub> retinal protonated Schiff base was determined with QCFF-π calculations (17) and used to define the positions of the atoms in the protonated Schiff base fragments shown in Fig. 1; a 180° rigid rotation about the C=N bond generated the cis geometry. Force constants developed by Curry (12) were modified to approximate the observed protonated Schiff base frequencies (see Fig. 1 legend).

RESULTS

Vibrational Coupling in C=N cis and C=N trans Chromophores. The vibrations of the C<sub>9</sub>H<sub>2</sub>—C<sub>11</sub>H—C<sub>12</sub>H<sub>2</sub> fragment in retinal serve as a useful model for the C<sub>14</sub>H—C<sub>15</sub>H—NH<sub>2</sub> fragment in the protonated Schiff bases. Vibrational analysis of all-trans and 11-cis retinal demonstrates that there is much stronger coupling of the C<sub>12</sub>H<sub>2</sub> rock with the C<sub>10</sub>—C<sub>11</sub> single bond stretch when the C<sub>11</sub>—C<sub>12</sub> bond is in a cis geometry. For example, deuteration of C<sub>12</sub> in 11-cis retinal results in a 79-cm<sup>-1</sup> increase of the C<sub>10</sub>—C<sub>11</sub> stretch frequency, whereas in all-trans-[12-<sup>2</sup>H]retinal only a minor shift of 1 cm<sup>-1</sup> is observed (12). This difference in rock—stretch coupling between the cis and trans configurations suggests that a similar pattern of coupling should exist between the C<sub>14</sub>—C<sub>15</sub> stretch and the NH rock.

To determine the origin of this difference in rock—stretch coupling and its applicability to the C≡N case, normal mode calculations were performed on the six-atom protonated Schiff base fragments shown in Fig. 1. The C<sub>14</sub>—C<sub>15</sub> stretch is calculated to shift up by 5 cm<sup>-1</sup> in the C=N trans fragment and by 62 cm<sup>-1</sup> in the C=N cis fragment, consistent with N-deuteration. This difference in coupling between cis and trans is insensitive to changes in the force field. It is preserved even when all force constants are set to zero except the C—C and C≡N stretching and CNH bending constants, demonstrating that the effect originates from predominantly kinetic interactions among only three basis coordinates. This effect can be rationalized by realizing that the NH rock and the C<sub>14</sub>—C<sub>15</sub> stretch couple kinetically to form symmetric and antisymmetric combinations in the normal modes and that only the symmetric combination can interact strongly with the higher-frequency (≈1640 cm<sup>-1</sup>) C≡N stretch. In the C=N trans configuration (see Fig. 1), the symmetric combination is the higher-frequency mode (the "NH rock" at 1352 cm<sup>-1</sup>), while the lower frequency "C<sub>14</sub>—C<sub>15</sub> stretch" at 1197 cm<sup>-1</sup> has little interaction with the C≡N stretch. When the nitrogen is deuterated, moving the rock to 964 cm<sup>-1</sup>, the C<sub>14</sub>—C<sub>15</sub> stretch at 1202 cm<sup>-1</sup> becomes the higher-frequency symmetric combination, and its increased coupling with the C≡N stretch prevents it from shifting up. The situation is reversed in the C≡N cis configuration, in which the symmetric combination of the NH rock and C<sub>14</sub>—C<sub>15</sub> stretch is the lower-frequency mode. When the nitrogen is protonated, the symmetric combination is the "stretch," which is pushed down to 1144 cm<sup>-1</sup> by its interaction with the C≡N stretch (Fig. 1). Upon N-deuteration, the C<sub>14</sub>—C<sub>15</sub> stretch becomes the antisymmetric higher-frequency combination, and the reduction in coupling with the C≡N stretch allows it to shift up to 1206 cm<sup>-1</sup>. To summarize, in the C=N trans configuration, coupling with the C≡N stretch always tends to push the NH (or NH<sub>2</sub>) rock and the C<sub>14</sub>—C<sub>15</sub> stretch closer in frequency, making them appear less coupled; in the C≡N cis configuration it tends to push them apart, making them appear more coupled.

Preliminary calculations on the complete retinal protonated Schiff bases with force constants adjusted to fit isotopic data for BR<sub>98</sub> and BR<sub>546</sub> (unpublished) verify that the above results apply to the entire molecule as well, with one modification. In the full chromophore, coupling of the C<sub>14</sub>—C<sub>15</sub> stretch with other single-bond stretches may delocalize the C<sub>14</sub>—C<sub>15</sub> stretching character over several normal modes. If this occurs, the large isotopic shift expected upon N-deuteration of a C≡N cis chromophore will be distributed among these modes. The calculations indicate that the mixing of the stretches is sensitive to the C≡N—C<sub>14</sub> configuration and the details of the force field; however, the sum of the shifts in all of the single-bond stretches upon N-deuteration is consistently large (40–50 cm<sup>-1</sup>) when the C≡N bond is cis and small (5–12 cm<sup>-1</sup>) when it is trans.

Experimental Determination of C≡N Configuration. On the basis of our calculations, the ideal way to study the C≡N configuration in bacteriorhodopsin is to assign the C<sub>14</sub>—C<sub>15</sub> single-bond stretch and then determine the magnitude of the coupling of this mode with the NH rock. Assignment of the C<sub>14</sub>—C<sub>15</sub> stretch is most easily accomplished by selecting labeling with carbon-13. Although C<sub>14</sub>—C<sub>15</sub> stretch character is often distributed in a number of normal modes, it is convenient to describe the fingerprint vibration most sensitive to <sup>13</sup>C substitution at C<sub>14</sub> and C<sub>15</sub> as the C<sub>14</sub>—C<sub>15</sub> stretch. The coupling of the C<sub>14</sub>—C<sub>15</sub> stretch to the NH rock is determined by examining the Schiff base proton for a deuteron. BR<sub>546</sub>. Spectrum A in Fig. 2 is the Raman spectrum of native BR<sub>546</sub>. The fingerprint region from 1100 to 1400 cm<sup>-1</sup> consists of mixed C—C stretches and CCH in-plane rockers, which are nearly unchanged when the sample is suspended in <sup>2</sup>H<sub>2</sub>O (Fig. 2, spectrum B). The 16 cm<sup>-1</sup> drop of the C≡N
stretch from 1640 to 1624 cm$^{-1}$ and the appearance of the N$^2$$H$ in-plane rock at 976 cm$^{-1}$ confirm that the chromophore is N-deuterated. In $[14,15,13C_2]$BR568, the intense 1201 cm$^{-1}$ line has shifted down to 1177 cm$^{-1}$ (Fig. 2, spectrum C). This establishes this vibration as an essentially localized C$_{14}$-C$_{15}$ stretch. Suspension of $[14,15,13C_2]$BR568 in $H_2O$ shifts the C$_{14}$-C$_{15}$ stretch up by only 4 cm$^{-1}$ (Fig. 2, spectrum D). Thus, the C$_{14}$-C$_{15}$ stretch is only weakly coupled to the NH rock. This behavior is characteristic of a chromophore with a C$=$N cis configuration. This result is supported by spectra of $[12,14,2H_2]$BR568. Deuteration at C$_{12}$ and C$_{14}$ strongly redistributes the single-bond stretch character among the fingerprint vibrations (Fig. 2, spectrum E). The insensitivity of the fingerprint region of this spectrum to $H_2O$ suspension (Fig. 2, spectrum F) is consistent with weak coupling of the NH rock with the C$_{14}$-C$_{15}$ stretch.

$BR548$. In contrast to BR568, the fingerprint region of the BR548 spectrum is strongly affected by suspension in $H_2O$. Comparison of spectra A ($H_2O$) and B ($H_2$O) in Fig. 3 shows that the intense 1183 cm$^{-1}$ line has shifted down 4 cm$^{-1}$ to 1179 cm$^{-1}$, the 1167 cm$^{-1}$ line has apparently disappeared, and a new line appears at 1208 cm$^{-1}$. The 1167 cm$^{-1}$ line in the $H_2O$ spectrum can be assigned to the C$_{14}$-C$_{15}$ stretch on the basis of its shift to 1148 cm$^{-1}$ in $[14,15,13C_2]$BR548 (Fig. 3, spectrum C). The 1148 cm$^{-1}$ line disappears upon suspension of the $^{13}$C-labeled sample in $H_2O$ (Fig. 3, spectrum D), consistent with the idea that the C$_{14}$-C$_{15}$ stretch has shifted up underneath the lines at 1181 and 1200 cm$^{-1}$. This interpretation argues that we should assign the 1208 cm$^{-1}$ line in NH BR548 as the C$_{14}$-C$_{15}$ stretch, which has shifted up from 1167 cm$^{-1}$. Further support for this assignment comes from Raman spectra of $[12,14,2H_2]$ and $[12,14,2H_2]$BR548 (Fig. 3, spectra E and F). The C$_{14}$-C$_{15}$ stretch in $[12,14,2H_2]$BR548 shifts from 1167 to 1159 cm$^{-1}$, consistent with its behavior in 13-cis-[12,14-2H$_2$]retinal (11). Comparison of spectra E and F in Fig. 3 shows that N-deuteration causes a shift of the 1159 cm$^{-1}$ line up to 1197 cm$^{-1}$. The magnitude of this shift (38 cm$^{-1}$) is very similar to that inferred for the C$_{14}$-C$_{15}$ stretch upon N-deuteration in native (41 cm$^{-1}$) and in $[14,15,13C_2]$BR548 ($\approx$ 33 cm$^{-1}$). Thus, the C$_{14}$-C$_{15}$ stretch in BR548 is strongly coupled to the NH rock. This is characteristic of a C$=$N cis Schiff base.

$K_{625}$. Fig. 4 presents Raman spectra of $K_{625}$ at 77 K. The fingerprint region of native $K_{625}$ is dominated by a single line at 1195 cm$^{-1}$ that has a low-frequency shoulder at 1187 cm$^{-1}$. Deuteration of the Schiff base nitrogen (Fig. 4, spectrum B) results in only a slight (4 cm$^{-1}$) shift of the 1187 cm$^{-1}$ shoulder to 1183 cm$^{-1}$. A normal mode involving the C$_{14}$-C$_{15}$ stretch is most clearly identified as a weak shoulder that appears at 1170 cm$^{-1}$ in $[14,15,13C_2]$K$625$ (Fig. 4, spectrum C). In $[14,15,13C_2, N$-2H$_2$]K$625$ (Fig. 4, spectrum D) this line shifts up by only 2 cm$^{-1}$ and is thus weakly coupled to the NH rock.

Since the appearance and slight shift of the 1170 cm$^{-1}$ shoulder in the 14,15-$^{13}C_2$ spectra are subtle features, spectra of $K_{625}$ in $H_2O$ and $H_2$O were also obtained by using purple membrane regenerated with $[12,14,2H_2]$retinal (Fig. 4, spectra E and F). This derivative of retinal has the advantage of spreading out fingerprint intensity into lines at 1175, 1188, 1206, and 1227 cm$^{-1}$, allowing a more critical comparison of the spectral shifts that occur when the Schiff base nitrogen is deuterated. Comparison of the fingerprint regions of spectra E and F in Fig. 4 shows very little change. This indicates that the C$=$N configuration of $K_{625}$ is trans. However, the strength of this conclusion is weaker than that for BR548 and BR568 because an isolated C$_{14}$-C$_{15}$ stretch has not been identified in the K$625$ spectra.

$L_{550}$. Fig. 5 presents Raman spectra of L$550$. The fingerprint region of native L$550$ is dominated by a single line at 1191 cm$^{-1}$ with weak shoulders at 1172 and 1159 cm$^{-1}$. Deuteration of the Schiff base nitrogen leaves the fingerprint region largely unchanged (Fig. 5, spectrum B). The 1172 cm$^{-1}$ line contains significant C$_{14}$-C$_{15}$ stretch character on the basis of its 24 cm$^{-1}$ downshift to 1148 cm$^{-1}$ in $[14,15,13C_2]L_{550}$ (Fig. 5, spectrum C). The frequency and the $^{13}$C-induced shift of the C$_{14}$-C$_{15}$ stretch in L$550$ are very similar to those observed in BR$548$. However, unlike BR$548$, the
C14–C15 stretch frequency is essentially unchanged when the Schiff base nitrogen is deuterated, shifting up only 2 cm\(^{-1}\) to 1150 cm\(^{-1}\) in spectrum D of Fig. 5. Raman spectra of L550 regenerated with [12,14-\(^2\)H]retinal were also obtained in \(^1\)H\(_2\)O and \(^2\)H\(_2\)O (Fig. 5, spectra E and F). The deuteration-induced shifts in the fingerprint region are insignificant, indicating that the C=N configuration in L550 is trans.

In summary, the frequencies of the fingerprint vibrations of BR\(_{568}\) are very sensitive to N-deuteration, while those of BR\(_{566}, K_{625},\) and L\(_{550}\) are not. We conclude that the configuration of the C=N bond in BR\(_{568}, K_{625},\) and L\(_{550}\) is trans and that the configuration of the C=N bond in BR\(_{548}\) is cis (Fig. 6). These BR\(_{568}\) and BR\(_{548}\) results are in agreement with recent solid-state \(^1\)C NMR studies on dark-adapted bacteriorhodopsin (18). Dark adaptation, then, involves a concerted thermal isomerization about both the C\(_{13}=\)C\(_{14}\) and C=N double bonds. This result is in agreement with the earlier proposal by Orlandi and Schulten (19). In contrast, the formation of K\(_{625}\) is a photochemical process and only the C\(_{13}=\)C\(_{14}\) bond undergoes isomerization. MINDO calculations on retinal protonated Schiff bases (19) are consistent with our observation that C=N isomerization does not accompany photochemical C\(_{13}=\)C\(_{14}\) isomerization. The clear demonstration that L\(_{550}\) has a C=N trans configuration indirectly supports our K\(_{625}\) results since L\(_{550}\) is produced via direct structural relaxation of the K chromophore. The C=N configuration in the O\(_{560}\) intermediate can now be determined by examining previous resonance Raman results on native O\(_{640}\) in \(^1\)H\(_2\)O and \(^2\)H\(_2\)O (8). By comparison with BR\(_{568}\), the C\(_{14}=\)C\(_{15}\) stretch in O\(_{640}\) can best be assigned to the 1198-cm\(^{-1}\) line, which shifts up by only 2 cm\(^{-1}\) upon N-deuteration (8). This argues that O\(_{640}\) also has a C=N trans configuration.

DISCUSSION

Now that the configuration of the C=N bond in the proton-pumping photocycle is established, it is possible to discuss more explicit models for the orientation and motion of the Schiff base proton. When both the C\(_{13}=\)C\(_{14}\) and C=N bonds isomerize in the formation of BR\(_{548}\), the Schiff base proton maintains the same orientation it has in BR\(_{568}\) (Fig. 6), the chromophore undergoing a “bicycle pedal” motion (20) that minimizes chromophore displacement. However, if C=N isomerization does not accompany C\(_{13}=\)C\(_{14}\) isomerization, as in the BR\(_{568}\)-to-K\(_{625}\) transition, the Schiff base proton is directed away from its original position and translated into a different protein environment. This picture is
consistent with models in which the Schiff base proton is hydrogen bonded with a protein counterion in BR\textsubscript{568} and BR\textsubscript{548} but displaced from that counterion in the photoconversion to the red-shifted K\textsubscript{625} intermediate (21, 22). Another important consequence of the concerted C==N and C\textsubscript{13}=C\textsubscript{14} isomerization that forms BR\textsubscript{568} is that the bend introduced in the all-trans chromophore by C\textsubscript{13}=C\textsubscript{14} isomerization is relieved by C==N isomerization (see Fig. 6). However, when C\textsubscript{13}=C\textsubscript{14} isomerization occurs alone, as in the BR\textsubscript{568} \rightarrow K\textsubscript{625} conversion, distortion of the skeletal backbone of the retinal chromophore must result since large motions of the bulky ionone ring and covalently attached lysine group are restricted by the protein. The observation of intense hydrogen-out-of-plane wagging vibrations in the K Raman spectrum (800–960 cm\textsuperscript{-1}) provide direct evidence that the chromophore in K\textsubscript{625} is conformationally distorted (Fig. 4, spectrum A, and ref. 3).

In bacteriorhodopsin the Schiff base bond links the retinal chromophore to lysine-216. If the chromophore in BR\textsubscript{568} is oriented with the Schiff base proton pointing toward the cell exterior, it might hydrogen bond with aspartate-212, which may also serve as the protein counterion (for the amino acid sequence and one possible secondary structure see ref. 23). In such a model, photochemical isomerization to form K\textsubscript{625} would result in reorientation of the Schiff base proton toward the cell interior. This suggests that the Schiff base proton is initially displaced in a direction opposite to that of proton pumping. Alternatively, if the Schiff base proton is oriented toward the cell interior in BR\textsubscript{568}, photochemical isomerization would displace it in the direction of proton pumping. This motion would place the Schiff base proton near aspartate-212 which may then serve as the proton acceptor in the L\textsubscript{550}-to-M\textsubscript{412} transition. Protonation of a carboxylate residue in the L\textsubscript{550}-to-M\textsubscript{412} step has been suggested by infrared results (24, 25). Further Raman, Fourier transform infrared, and solid-state NMR experiments with isotopic retinal derivatives should help to discriminate between these competing models and provide a more precise structural picture of the proton-pumping mechanism in bacteriorhodopsin.

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