Resonance Raman Study on Binding of Chloride to the Chromophore of Halorhodopsin†

Akio Maeda,* Tarou Ogurusu, and Tôru Yoshizawa
Department of Biophysics, Faculty of Science, Kyoto University, Sakyo-ku, Kyoto 606, Japan
Teizo Kitagawa*
Institute for Molecular Science, Okazaki National Research Institutes, Okazaki 444, Japan
Received May 23, 1984

ABSTRACT: The resonance Raman spectrum of halorhodopsin, a retinoid protein with light-dependent chloride pumping activity, was observed in the presence and absence of chloride in H_2O and D_2O. The frequency of the in-phase C=C stretching mode was shifted from 1528 to 1530 cm^{-1} upon removal of chloride, in accordance with what was expected from the shift of the wavelength for the absorption maximum from 576 to 567 nm. The chloride effect was also found with the C=NH stretching modes of the protonated Schiff base, which were located at 1635 and 1642 cm^{-1} in the presence and absence of chloride, respectively. However, the corresponding frequencies of the deuterated Schiff base linkage exhibited little dependence on chloride. The N–H bending mode of the protonated Schiff base was assigned to the Raman line at 1352 cm^{-1} since it was apparently replaced by the Raman line at 977 cm^{-1} upon deuteration. This line did not undergo any chloride effect. The Raman spectrum in the 1150–1250-cm^{-1} region was unaffected by chloride and was similar to that of *all-trans*-bacteriorhodopsin. These observations and simple vibrational calculations suggest that chloride is not hydrogen bonded to the Schiff base proton in a trivial manner but that binding of chloride changes a limited number of force constants of the C=NH moiety, presumably through a direct interaction with the π orbital of the Schiff base or indirectly through a local structural change of the protein.

Halorhodopsin (hR)[1] is a light-dependent electrogenic protein present in the membrane of *Halobacterium halobium*, a highly halophilic microorganism [see Stoeckenius & Bogomolni (1982) for a review]. This protein functions as a light-driven chloride pump (Schobert & Lanyi, 1982). It was found that depletion of chloride caused a reversible shift in the visible absorption maximum (λ_{max}) from 576 to 567 nm (Ogurusu et al., 1982), and the titration experiments indicated the presence of a single chloride binding site (Ogurusu et al., 1984). In the photoreaction a product having decreased absorbance at 590 nm but increased absorbance at 500 nm was found only in the presence of chloride or its analogues such as bromide or iodide (Ogurusu et al., 1982; Schobert et al., 1983, Steiner et al., 1984).

Resonance Raman (RR)[1] scattering from retinoid proteins has revealed the vibrational spectra of its chromophore, having brought detailed structural information on retinal skeleton and Schiff base linkage (Callender & Honig, 1977). Accordingly, the RR spectrum of hR is expected to inform more details of the chloride–chromophore interaction mechanism. Here we report a chloride-dependent RR spectral change of hR and its structural implication.

Materials and Methods

hR was purified from a bacteriorhodopsin- (bR-)[1] deficient strain of *Halobacterium halobium* L-33 (Wagner et al., 1981) according to the method described previously (Ogurusu et al., 1984); the lysis of the cell in 1 M NaCl was followed by the collection of the membrane fragments, extraction of hR in Triton X-100 and column chromatography on octyl-Sepharose. The hR thus purified was dialyzed for 16 h against 100 volumes of 10 mM phosphate buffer (pH 6.8) containing either 1 M NaCl or 1 M $NaNO_3$. The external fluid was exchanged once. The preparations with 1 M NaCl and 1 M $NaNO_3$ will be hereafter denoted as hR(Cl) and hR(NO_3), respectively. The deuterated hR was prepared by dialyzing 1 mL of hR(Cl) or hR (NO_3) at 7 °C against 10 mL of the D_2O solution containing either of these salts. During the dialysis for 16 h the external fluid was exchanged once. bR in purple membrane was prepared from *Halobacterium halobium* R_1M_1 by the method of Oesterhelt & Stoeckenius (1974). The RR spectra of bR were measured in 0.2 M KCl and 10 mM phosphate buffer (pH 6.8). Deuteration of purple membranes was carried out through two cycles of centrifugation at 35000*g* followed by suspending the pellets in D_2O solution containing the same salt. Isomer composition of the retinoid chromophore was analyzed after extraction of retinals from the protein (Maeda et al., 1981). The samples of hR and bR were always manipulated in the dark.

Raman spectra were excited by the 514.5-nm line (ca. 25 mW) of an Ar/Kr mixed gas laser (Spectra Physics, Model 165) and measured with a JEOL-400D Raman spectrometer equipped with a cooled RCA 31034a photomultiplier. Frequency calibration of the spectrometer was performed with indene as a standard. All spectra were measured with a spinning cell (diameter = 2 cm, 1800 rpm). For a measurement, 0.4 mL of light-adapted hR or bR with the concentration of OD = 1.2 (580 nm) was used. Temperature of the sample

† This research was supported by Grant-in-Aids for Scientific Research to A.M. (57470188), T.Y. (59440003), and T.K. (58480458) and for Special Project Research on Molecular Mechanism of Bioelectrical Response to A.M. (59123002) and T.K. (59223015) from the Japanese Ministry of Education, Science and Culture. This work was supported by the Joint Studies Program (1983–1984) of the Institute for Molecular Science (Okazaki, Japan).

[1] Abbreviations: bR, bacteriorhodpsin; hR, halorhodopsin; RR, resonance Raman.

0006-2960/85/0424-2517$01.50/0

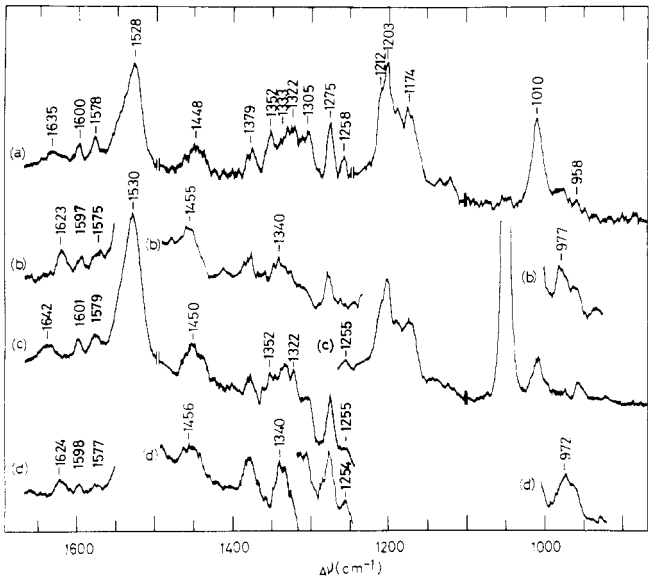

FIGURE 1: RR spectra of hR(Cl) in H_2O (a), hR(Cl) in D_2O (b), hR(NO_3) in H_2O (c), and hR(NO_3) in D_2O (d). Instrumental conditions: slit width 6 cm^{-1}; scan speed 10 cm^{-1}/min, laser 514.5 nm, 25 mW; time constant 4 and 16 s for the 1500–1700- and 900–1500-cm^{-1} regions, respectively. The spectra in the 1500–1700-cm^{-1} region were recorded with the sensitivity of 2500 pulses/s. The scale was expanded 5 times for the 900–1100- and 1250–1500-cm^{-1} regions and 2.5 times for the 1100–1250-cm^{-1} region, respectively.

was kept at 5 °C by flushing cold nitrogen gas against the cell. Visible absorption spectra were always measured after the Raman measurements to confirm no degradation of the protein.

Results

The RR spectra of hR in the presence or absence of chloride in H_2O and D_2O are shown in Figure 1. The most significant spectral change with regard to the chloride effect was noticed in the 1630–1650-cm^{-1} region. The RR lines of hR(Cl) at 1635 cm^{-1} (a) and of hR(NO_3) at 1642 cm^{-1} (c) are shifted to 1623 (b) and 1624 cm^{-1} (d) in D_2O, respectively. These shift patterns strongly suggest that the 1635-cm^{-1} band of hR(Cl) and the 1642-cm^{-1} band of hR(NO_3) are associated with the C=NH stretching mode ($\nu_{C=NH}$) of the protonated Schiff base. In contrast with the frequency of $\nu_{C=NH}$, the frequency of the C=ND stretching mode ($\nu_{C=ND}$) of the deuterated Schiff base was relatively insensitive to chloride.

Two lines at 1600–1601 and 1578–1579 cm^{-1} are insensitive to chloride but slightly shifted upon N-deuteration (2–3 cm^{-1}). Curry et al. (1982) inferred that these two modes of bR_{570} arose from the C(5)=C(6) and the C(7)=C(8) stretching modes. The insensitivity of these modes to chloride in contrast with the clear shift of the $\nu_{C=NH}$ mode suggests that the effect of chloride is considerably localized in the Schiff base moiety.

The in-phase C=C stretching mode ($\nu_{C=C}$) characteristic of retinoid chromophore is seen at 1528 cm^{-1} for hR(Cl) (a) and 1530 cm^{-1} for hR(NO_3) (c). Each exhibited an asymmetric feature toward higher frequency, indicating the presence of a weak satellite band at ~1545 cm^{-1}. This may suggest a formation of the small amount of photointermediates in a photo steady state produced by illumination of laser light for about 0.1 ms in every 33 ms. The frequency shift of the $\nu_{C=C}$ line from 1528 to 1530 cm^{-1} upon removal of chloride is evident and is consistent with the shift of λ_{max} from 576 to 567 nm when an empirical linear relation between the frequencies of $\nu_{C=C}$ and λ_{max} values of various retinoid proteins (Heyde et al., 1971; Aton et al. 1977) is referred. Neither of $\nu_{C=C}$ features of hR(Cl) or hR(NO_3) was affected by deuteration (not shown in the figure).

Table I: Retinal Isomer Composition of hR under Various Conditions

sample	retinal isomer composition: 13-cis (%)	retinal isomer composition: all-trans (%)
(a) kept in the dark in 1 M NaCl	30 ± 0	70 ± 0
(b) kept in the dark in 1 M $NaNO_3$	31 ± 2	69 ± 2
(c) irradiated with red light (>590 nm) in 1 M NaCl at 0 °C	79 ± 3	21 ± 3
(d) irradiated with 510-nm light in 1 M NaCl at 0 °C	28 ± 2	72 ± 2

In the 1250–1500-cm^{-1} region, the disappearance of the 1352-cm^{-1} line of hR(Cl) (a) or hR(NO_3) (c) upon deuteration (parts b and d, respectively) is noted. Accordingly, they are assignable to the in-plane N–H bending mode (φ_{N-H}) as first proposed by Massig et al. for bR (1982). While the RR lines at 1379, 1305, and 1275 cm^{-1} are little influenced by deuterium substitution, an upward shift of a broad line at ~1450 cm^{-1} upon deuteration was repeatedly observed. Therefore, this line may not be solely attributed to methyl vibrations contrary to the previous suggestions for bR (Curry et al., 1982). No clear effect of chloride was seen with these RR lines. The 1322-cm^{-1} line is reduced in relative intensity upon deuteration but remains unaffected upon substitution of chloride with nitrate. The 1258-cm^{-1} line of hR(Cl) is appreciably diminished upon deuteration or depletion of chloride. The corresponding RR lines were located in three or four separate experiments at 1259 ± 2, 1255 ± 1, and 1254 ± 1 for hR(Cl) in D_2O, hR(NO_3) in H_2O, and hR(NO_3) in D_2O, respectively, though with weak intensities. The 1255-cm^{-1} line of bR, which also diminishes its intensity upon N-deuteration (Massig et al., 1982), is reported to be sensitive to C(15)-deuteration (Braiman & Mathies, 1980). These lines probably correspond to a mode involving the C(15)–H in-plane bending mode.

The RR lines in the 1100–1250-cm^{-1} region are sensitive to isomerization of retinal skeleton. In fact, it is reported that three RR lines of bR_{570} with all-trans chromophore at 1212, 1202, and 1171 cm^{-1} are replaced by two lines at 1202 and 1183 cm^{-1} for bR_{550} with 13-cis chromophore (Stockburger et al., 1979; Terner et al., 1979b). The RR spectrum of bR_{570} (not shown) observed under the same instrumental conditions as those used for hR was in agreement with those reported (Stockburger et al., 1979; Terner et al., 1979b; Massig et al., 1982) and resembled the RR spectra of hR(Cl) and hR(NO_3). Therefore, the presence of the all-trans chromophore is most likely.

It is conspicuous in the 900–1100-cm^{-1} region that a new Raman line appears upon deuteration; 977 cm^{-1} for hR(Cl) (Figure 1b) and 972 cm^{-1} for hR(NO_3) (Figure 1d). In the case of bR, a new line appeared at 975 cm^{-1} in addition to the well-resolved two lines at 959 and 985 cm^{-1} which are present commonly in H_2O and D_2O (not shown). Therefore, the new line is most likely to be assigned to a mode primarily associated with the N–D in-plane bending mode (φ_{N-D}) of the deuterated Schiff base, as previously suggested by Massig et al. (1982). The Raman line at 1010 cm^{-1} remained unchanged by either D_2O substitution or replacement of chloride with nitrate. This line was first assigned to the C–CH_3 stretching mode (Rimai et al., 1971) but later reassigned to the C–CH_3 in-phase rocking mode (Curry et al., 1982; Saito & Tasumi, 1983).

The isomer composition of the retinoid chromophore was determined with HPLC after extraction of retinal from the

Table II: Summary on the Frequencies of the Various Modes Related to the Schiff Base (cm^{-1})

	chloride	nitrate
C=NH stretching	1635 ± 1 (11)[a]	1642 ± 2 (8)
C=ND stretching	1623 ± 1 (8)	1624 ± 1 (8)
C=N–H in-plane bending[b]	1352 ± 1 (3)	1353 ± 1 (3)
C=N–D in-plane bending	977 ± 1 (5)	972 ± 2 (4)
C=C in-phase stretching	1528 ± 0 (7)	1530 ± 1 (9)

[a] The number in parentheses is that of the data used for summarizing the results. [b] The assignment by Massig et al. (1982).

protein and is summarized in Table I. For unirradiated hR(Cl) (a) and hR(NO_3) (b), about 70% of the chromophore was recovered as *all-trans*-retinal but the remaining 30% as the 13-cis form. Irradiation of hR(Cl) with red light (>590 nm), which resulted in the accumulation of hR_{410} (Steiner & Oesterhelt, 1983; Taylor et al., 1983; Ogurusu et al., 1984), increased the content of the 13-cis isomer to 80% (c). However, irradiation at 520 nm yielded no changes in the isomer composition (d) as well as in the absorption spectra (not shown). This is consistent with the fact that the present spectra did not exhibit a trace of Raman line around 1565 cm^{-1} where the species with λ_{max} at 410 nm should give a strong $\nu_{C=C}$ line on the basis of the $\lambda_{max} - \nu_{C=C}$ relation (Aton et al., 1977).

Discussion

The present spectra have clarified for the first time that the chromophore of hR possesses the all-trans protonated Schiff base irrespective of the presence or absence of chloride. The main subject to be discussed is structural implication of the chloride effect on the RR spectra of hR. For the convenience of discussion, the frequencies of key Raman lines of hR(Cl) and hR(NO_3) are summarized in Table II. The $\nu_{C=NH}$ frequencies increased by 7 cm^{-1} upon removal of chloride, whereas the $\nu_{C=ND}$ frequencies remained unchanged. Frequencies of all other RR lines are practically unaffected by chloride. Therefore, the interaction of chloride with the chromophore is presumed to be localized to the Schiff base.

Previously, Argade & Rothschild (1983) proposed a view for various photointermediates of bR that the $\nu_{C=NH}$ frequencies are also related to the λ_{max} as was observed with the $\nu_{C=C}$ frequencies. However, in applying this idea to the present case, there is serious difficulty about the insensitivity of the $\nu_{C=ND}$ frequencies to λ_{max} under the conditions that $\nu_{C=C}$ of the deuterated compounds exhibit the λ_{max} dependence similar to $\nu_{C=C}$ of the normal compounds. Furthermore, 7 cm^{-1} of the frequency shift in $\nu_{C=NH}$ between hR(Cl) and hR(NO_3) in comparison with 2 cm^{-1} of the difference in $\nu_{C=C}$ seems too large to be explained in terms of the delocalization effect of π-electrons.

It is known that the $\nu_{C=NH}$ frequencies of *N*-retinylidenebutylamine undergo a significant solvent effect: 1622 cm^{-1} in chloroform, 1636 cm^{-1} in trifluoroethanol, and 1650 cm^{-1} in trifluoroacetic acid (Sugihara et al., 1982). The hydrochloride form of the model Schiff base in ethanol gave the highest value (1662 cm^{-1}) (Braiman & Mathies, 1980). The deuterated form of these compounds gave the $\nu_{C=ND}$ in the frequency region between 1622 and 1630 cm^{-1}. A broader frequency distribution in $\nu_{C=NH}$ than in $\nu_{C=ND}$ for various intermediates of bR was interpreted in terms of different abilities to form hydrogen bond (Terner et al., 1979a).

The difference between the $\nu_{C=NH}$ and $\nu_{C=ND}$ frequencies is presumably attributed to the vibrational coupling of the N–H bending mode with the C=N stretching mode as pointed out by Aton et al. (1980). Importance of such coupling was also stressed previously for the higher frequency shift of the C=NH stretching mode of flavin upon N–deuteration (Kitagawa et al., 1979). For this kind of discussion, the location of the N–H bending mode is substantial. The N–H bending mode of 1-methyluracil has been identified at 1417 cm^{-1} (Miles et al., 1973); also, this mode of a monosubstituted amide compound with cis conformation was theorteically calculated at 1445 cm^{-1} while that of the trans conformation is seen around 1550 cm^{-1} (Miyazawa, 1960). Massig et al. (1982) assigned the N–H bending mode of bR to the 1350-cm^{-1} band. The overall spectral feature of hR is very similar to that of bR. The RR line at 1352 cm^{-1} of hR(Cl) is likely due to the N–H bending mode, since this is the only RR line that completely disappears upon N-deuteration.

The 1332- and 1258-cm^{-1} bands of hR(Cl) do not completely disappear the intensities upon N-deuteration. Therefore, the possibility that these lines involve primarily the N–H bending mode is extremely low. At present we cannot rule out the possibility that the N–H bending mode is located at higher frequencies than the 1352-cm^{-1} line, but it is not observed in our present spectrum. In this case the 1352-cm^{-1} line would be one of the C–H in-plane bending modes, which is significantly coupled with the N–H bending mode, and be expected to exhibit an appreciable frequency shift when the N–H bending mode undergoes a frequency shift.

It seems extremely important to elucidate a probable factor which the $\nu_{C=NH}$ frequency depends on. Accordingly, we carried out normal coordinate calculations for a simplified model of the protonated Schiff base, which was illustrated in the inset of Figure 2. The harmonic potential function represented by eq 1 was assumed for the in-plane vibrations:

$$2V = K_1(\Delta r_1)^2 + K_2(\Delta r_2)^2 + K_3(\Delta r_3)^2 + H_1(\Delta\alpha_1)^2 + H_2(\Delta\alpha_2)^2 + P(\Delta\alpha_1)(\Delta r_2) \quad (1)$$

where Δr_i and $\Delta\alpha_i$ are the stretching and bending coordinates, respectively, which are specified also in the inset of Figure 2. Atomic arrangements and force constants were assumed to be always the same between the hydrogen and deuterium compounds.

We are curious to know whether a reasonable manipulation of the force constants related with the strength of the hydrogen bond (K_3 and H_2) can reproduce the observed difference between hR(Cl) and hR(NO_3). Hence, first the dependence of the C=NH stretching (ν_2) and N–H bending (ν_3) frequencies on the hydrogen-bond stretching force constant (K_3) was examined, and is illustrated in Figure 2a, where the corresponding frequencies for the deuterated Schiff base are represented by broken lines. Both ν_2 and ν_3 slightly increase as the hydrogen bond becomes stronger, and the sensitivity of ν_2 is rather higher than that of ν_3. However, for producing the 6-cm^{-1} shift in the C=NH stretching mode, it requires to assume K_3 = 1.2 mdyn/A, with which the N–H stretching frequency should be shifted by 270 cm^{-1}. The point to be noted is that $\nu_{C=NH}$ and $\nu_{C=ND}$ exhibit a similar amount of frequency shift upon a change of the stretching force constant of the hydrogen bond. In this regard, the different response of these two modes of hR to chloride cannot be reproduced by manipulation of only K_3, although a change of the hydrogen-bond strength in a trivial sense should result in a variation of K_3.

Figure 2b depicts the dependence of the ν_2 and ν_3 frequencies upon H_2, the bending force constant of the hydrogen bond. As intuitively expected, an increase of H_2 causes an increase of the restraining force against the movement of hydrogen atom toward perpendicular to the N–H···X linkage and thus gives rise to the effect similar to what would take place upon an increase of the N–H bending force constant (H_1). Accordingly, ν_3 is extremely sensitive to the bending force constant

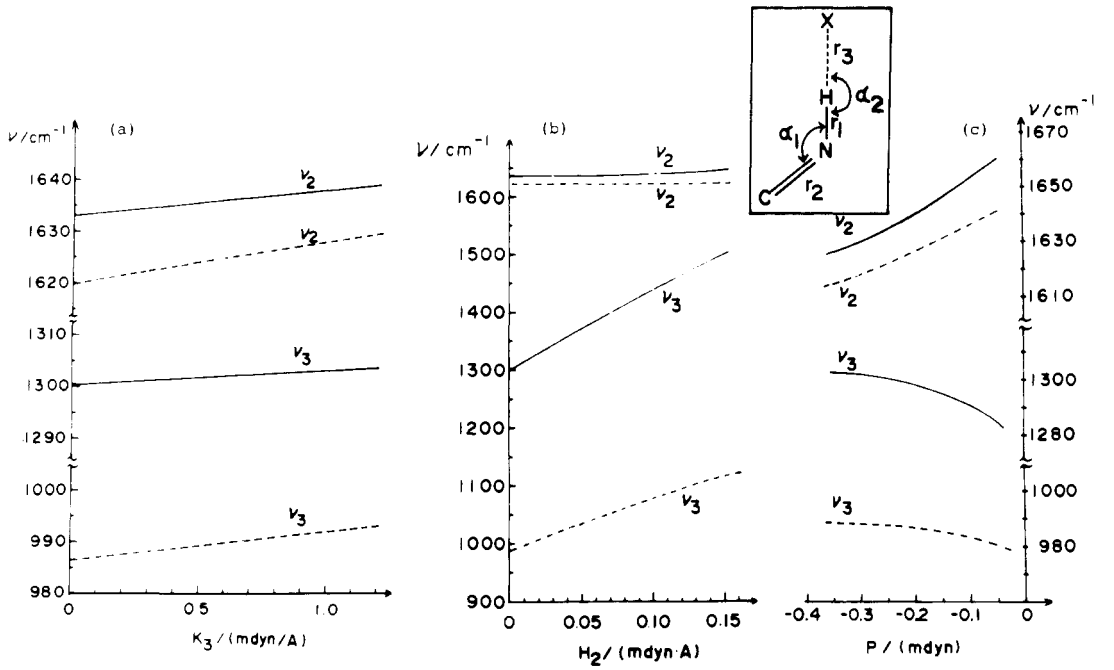

FIGURE 2: Dependence of the C=NH stretching (ν_2) and the N-H in-plane bending (ν_3) frequencies on a specific force constant. (a) Dependence on the stretching force constant of the hydrogen bond (K_1 = 6.3, K_2 = 10.3, H_1 = 0.85, H_2 = 0, and P = −0.25). (b) Dependence on the bending force constant of the hydrogen bond (K_1 = 6.3, K_2 = 10.3, K_3 = 0.3, H_1 = 0.85, and P = −0.25). (c) Dependence on the stretching-bending coupling constant (K_1 = 6.3, K_2 = 10.3, K_3 = 0.3, H_1 = 0.85, and H_2 = 0) (K in mdyn/A, H in mdyn·A, and P in mdyn). Solid lines and broken lines stand for the hydrogen and deuterium compounds, respectively. The inset illustrates the model assumed for the calculations and also specifies the internal coordinates. X denotes chloride ior The equilibrium geometry assumed was as follows: r_1 = 1.0, r_2 = 1.33, r_3 = 1.7 (A), α_1 = 120° and α_2 = 180°.

Table III: Calculated Frequencies for the Schiff Base C=NH(D) Stretching and N-H(D) Bending Vibrations (cm^{-1})

	set I[a]	set II[b]
C=NH stretching	1635	1643
C=ND stretching	1623	1624
N-H in-plane bending	1301	1285
N-D in-plane bending	988	981

[a] Set I: K_1 = 6.3, K_2 = 10.30, K_3 = 0.3, H_1 = 0.85, H_2 = 0, and P = −0.25. [b] Set II: K_1 = 6.3, K_2 = 10.08, K_3 = 0.3, H_1 = 0.85, H_2 = 0, and P = −0.05. K_i is in mdyn/A, H_i is in mdyn·A, and P is in mdyn.

of the hydrogen bond, but ν_2 is not. This trend is just opposite to what was observed.

Figure 2c illustrates the dependence of the ν_2 and ν_3 frequencies upon P, the coupling constant between the N-H bending and C=NH stretching vibrations. This term arises from the nonbonded repulsive interaction between the Schiff base proton and two carbon atoms attached to nitrogen when the Urey-Bradley force field was assumed for a rigorous model. It is manifest that both $\nu_{C=NH}$ and $\nu_{C=ND}$ change similarly. It is again contrary to the observations. Although Kakitani et al. (1983) stressed the importance of the coupling between the $\nu_{C=NH}$ and φ_{N-H} coordinates and adjusted the $\nu_{C=NH}$ freqeuncy by varying the N-H bending frequency, they are not concerned with the change of the observed N-H bending frequency. Since the RR spectra of hR(Cl) and hR(NO_3) in the 1200-1400-cm^{-1} region are quite alike, a change of diagonal term of φ_{N-H} proposed by Kakitani et al. cannot be applied to the present observation.

The important features deduced form this simple calculations are as follows: (1) So far as the $\nu_{C=NH}$ and $\nu_{C=ND}$ lines change dissimilarly as in the case of hR, a change of the C=N stretching force constant or a change of hydrogen-bond strength is unlikely. (2) So far as the N-H bending frequency exhibits little shift, a change of the $\nu_{C=NH}$ frequency is difficult to explain in terms of the coupling between the N-H bending and C=NH stretching modes. However, combination of changes in two or more force constants, which would be often not unique, may give rise to the values relatively close to the observed values. A trial is shown in Table III, which indicates two sets of the force constants which are different with regard to only K_3 and P. This may give an idea of the difference between the presence and absence of chloride.

The essential point of the present calculations was unaltered by more refined treatments which incorporate all freedom of in-plane vibrations of retinylidene chromophore (T. Kitagawa et al., unpublished results). However, it should be noted that the N-H and N-D bending modes are considerably but differently mixed with other vibrations. Therefore, the description that the N-H bending mode was shifted from 1352 to 977 cm^{-1} upon N-deuteration might be also misleading. The sensitivity of the 1322- and 1258-cm^{-1} lines to N-deuteration may be due to an involvement of the N-H bending mode in these lines. Even if this is the case, the discussion above described would be unaltered, because these frequencies show no change upon removal of chloride.

In conclusion, the chloride effect on the RR spectra of hR cannot be interpreted by an idea that chloride is hydrogen bonded to the Schiff base proton in a trivial sense. Chloride bound may induce a local change of the force constants of the C=NH moiety through a weak direct interaction with a π orbital of nitrogen or, more likely, through a secondary interaction with protein residues that are primarily altered by chloride. Some lysine residue was found to be responsible for the chloride binding site with bR (Maeda et al., 1982) or with hemoglobin (Perutz et al., 1980). The binding site should be further studied with hR.

Registry No. Cl, 16887-00-6; NO_3, 14797-55-8; *all-trans*-retinal, 116-31-4.

REFERENCES

Argade, P. V., & Rothschild, K. J. (1983) *Biochemistry 22*, 3460-3466.

Aton, B., Doukas, A. G., Callender, R. H., Becher, B., & Ebrey, T. G. (1977) *Biochemistry 16*, 2995–2999.
Aton, B., Doukas, A. G., Narva, D., Callender, R. H., Dinur, U., & Honig, B. (1980) *Biophys. J. 29*, 79–94.
Braiman, M., & Mathies, R. (1980) *Biochemistry 19*, 5421–5428.
Callender, R. H., & Honig, B. (1977) *Annu. Rev. Biophys. Bioeng. 6*, 33–55.
Curry, B., Broek, A., Lugtenburg, J., & Mathies, R. (1982) *J. Am. Chem. Soc. 104*, 5274–5286.
Heyde, M. E., Gill, D., Kilponen, R. G., & Rimai, L. (1971) *J. Am. Chem. Soc. 93*, 6776–6780.
Kakitani, H., Kakitani, T., Rodman, H., Honig, B., & Callender, R. (1983) *J. Phys. Chem. 87*, 3620–3628.
Kitagawa, T., Nishina, Y., Kyogoku, Y., Yamano, T., Ohishi, N., Takai-Suzuku, A., & Yagi, K. (1979) *Biochemistry 18*, 1804–1808.
Maeda, A., Iwasa, T., & Yoshizawa, T. (1981) *Photochem. Photobiol. 33*, 559–565.
Maeda, A., Takeuchi, Y., & Yoshizawa, T. (1982) *Biochemistry 21*, 4479–4483.
Massig, G., Stockburger, M., Gärtner, W., Oesterhelt, D., & Towner, P. (1982) *J. Raman Spectrosc. 12*, 287–294.
Miles, H. T., Lewis, T. P., Becker, E. D., & Frazier, J. (1973) *J. Biol. Chem. 248*, 1115–1117.
Miyazawa, T. (1960) *J. Mol. Spectrosc. 4*, 155–167.
Oesterhelt, D., & Stoeckenius, W. (1974) *Methods Enzymol. 31*, 667–678.
Ogurusu, T., Maeda, A., Sasaki, N., & Yoshizawa, T. (1982) *Biochim. Biophys. Acta 682*, 446–451.
Ogurusu, T., Maeda, A., & Yoshizawa, T. (1984) *J. Biochem. (Tokyo) 95*, 1073–1082.
Perutz, M. F., Kilmartin, J. V., Nishikura, K., Fogg, J. H., Butler, P. J. G., & Rollema, H. S. (1980) *J. Mol. Biol. 138*, 649–670.
Rimai, L., Gill, D., & Parsons, J. L. (1971) *J. Am. Chem. Soc. 93*, 1353–1357.
Saito, S., & Tasumi, M. (1983) *J. Raman Spectrosc. 14*, 236–245.
Schobert, B., & Lanyi, J. K. (1982) *J. Biol. Chem. 257*, 10306–10313.
Schobert, B., Lanyi, J. K., & Cragoe, E. J., Jr. (1983) *J. Biol. Chem. 258*, 15158–15164.
Steiner, M., & Oesterhelt, D. (1983) *EMBO J. 2*, 1379–1385.
Steiner, M., Oesterhelt, D., Ariki, M., & Lanyi, J. K. (1984) *J. Biol. Chem. 259*, 2179–2184.
Stockburger, M., Klusmann, W., Gattermann, H., Massig, G., & Peters, R. (1979) *Biochemistry 18*, 4886–4900.
Stoeckenius, W., & Bogomolni, R. A. (1982) *Annu. Rev. Biochem. 52*, 587–616.
Sugihara, T., Kitagawa, T., & Blout, E. R. (1982) *Siebutsu Butsuri 22*, S152.
Taylor, M. E., Bogomolni, R. A., & Weber, H. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6172–6176.
Terner, J., Hsieh, C.-L., Burns, A. R., & El-Sayed, M. A. (1979a) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3046–3050.
Terner, J., Hsieh, C.-L., & El-Sayed, M. A. ((1979b) *Biophys. J. 26*, 527–541.
Wagner, G., Oesterhelt, D., Krippahl, G., & Lanyi, J. K. (1981) *FEBS Lett. 131*, 341–345.

Proton and Phosphorus Nuclear Magnetic Resonance Studies of an Oligothymidylate Covalently Linked to an Acridine Derivative and of Its Binding to Complementary Sequences

G. Lancelot,* U. Asseline, and N. T. Thuong
Centre de Biophysique Moléculaire, 45045 Orléans Cedex, France
C. Hélène
Laboratoire de Biophysique, INSERM U.201, CNRS ERA 951, Muséum National d'Histoire Naturelle, 75005 Paris, France
Received May 21, 1984; Revised Manuscript Received August 23, 1984

ABSTRACT: An oligodeoxynucleotide containing four thymines and covalently attached to an acridine derivative through its 3′-phosphate [$(Tp)_4(CH_2)_5Acr$] was synthesized. Its conformation in solution was investigated by proton magnetic resonance. Both intramolecular interactions between the acridine dye and thymines and intermolecular interactions were demonstrated. Both proton and phosphorus magnetic resonances were used to study the specific interaction of $(Tp)_4(CH_2)_5Acr$ with poly(rA) and $(Ap)_3A$. The results were compared to those obtained when the acridine-containing substituent was replaced by an ethyl group attached to the 3′-phosphate of the oligothymidylate. The acridine dye strongly stabilized the complexes formed with both poly(rA) and $(Ap)_3A$. Upfield shifts of both adenine and acridine proton resonances were observed in the complexes. These results were ascribed to an intercalation of the acridine ring between A·T base pairs of the duplex structure formed by the oligothymidylate with its complementary oligoadenylate sequence. An analysis of proton and phosphorus chemical shifts as well as measurements of T_1 relaxation times at different temperatures allowed us to propose several structures for the complexes formed by $(Tp)_4(CH_2)_5Acr$ with its complementary sequence.

The control of gene expression in both procaryotes and eucaryotes requires molecules that bind selectively to specific nucleic acid sequences during either transcription of DNA or translation of messenger RNAs. These processes are usually regulated by specific nucleic acid binding proteins. The interactions between functional groups in protein–nucleic acid complexes have been recently reviewed (Hélène & Lancelot, 1982). In some cases, however, regulation can be achieved by a nucleic acid fragment that is complementary (at least in part) to the control sequence (Mizuno et al., 1984).

0006-2960/85/0424-2521$01.50/0