Eur Biophys J (1989) 16:341–349

European Biophysics Journal

Can normal mode analysis reveal the geometry of the L_{550} chromophore of bacteriorhodopsin?

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Received April 18, 1988/Accepted in revised form September 20, 1988

Abstract. We discuss to what extent recent vibrational spectra of 14-^{2}H, 15-^{2}H and 14,15-^{2}H isotopically labelled L_{550} provide evidence for the occurrence of 13-*cis*, 14-*s*-*trans* or 13-*cis*, 14-*s*-*cis* chromophore structures in bacteriorhodopsin's photocycle. The discussion is based on a quantum chemical (MNDO) vibrational analysis of four molecular fragments as models for the retinal chromophore in bacteriorhodopsin.

Key words: Bacteriorhodopsin, vibrational analysis, retinal

1. Introduction

Bacteriorhodopsin (BR) acts as a light driven proton pump in the cell membrane of *Halobacterium halobium* (Stoeckenius and Bogomolni 1982). The chromophore of BR is retinal, which is bound as a protonated Schiff base (RSBH$^+$) to a lysine residue (see Fig. 1). Time-resolved absorption spectra on light-adapted probes reveal a photocycle which is kinetically coupled to proton transport. In this photocycle spectroscopically distinct intermediates appear which are designated as J_{600}, K_{625}, L_{550}, M_{412} and O_{640}. The absorption maxima of these intermediates, which (in nanometers) are indicated as subscripts, reflect changes in the chromophore's charge environment as well as a deprotonation and subsequent reprotonation of the RSBH$^+$ during the photocycle (Nakanishi et al. 1980; Großjean and Tavan 1988).

It has beeen established that the chromophore undergoes a photoisomerization and a thermal back-reaction which involves an all-*trans* → 13-*cis* isomerization in the photocycle. The aim of research on this protein-chromophore system is to reveal the molecular mechanism of its function, i.e., to reveal how the stereodynamics of retinal during pump activity links to vectorial proton translocation in the protein. An important open question is: which isomerizations participate in retinal's photocycle? It had been suggested, that retinal can work as a proton switch if it undergoes a primary all-*trans* → 13-*cis*, 14-*s*-*cis* photoisomerization, i.e., if an isomerization around the $C_{14}-C_{15}$ bond takes part in the photocycle (Schulten and Tavan 1978; Orlandi and Schulten 1979; Schulten et al. 1984). Such a primary reaction has two effects; (1) it implies a faster primary photoreaction than an all-*trans* → 13-*cis* reaction (Orlandi and Schulten 1979; Schulten et al. 1984) and, thereby, enhances the yield of the primary photochemistry; (2) it enforces a sequential deprotonation-reprotonation of retinal's Schiff base (Schulten and Tavan 1978).

There have been various attempts to prove or disprove the occurrence of 14-*s*-*cis* structures in BR's reaction cycle. These attemtps were based on vibrational, i.e., resonance Raman (RR) and infrared (IR) spectroscopy as well as on a theoretical analysis of vibrational frequencies in terms of molecular geometries. Smith et al. (1986) argued against an involvement of a 14-*s*-*cis* geometry in the early intermediates (K_{625}, L_{550}) of BR's pump cycle. Gerwert and Siebert (1986) as well as two of the authors (Tavan and Schulten 1986) had subsequently put forward arguments in favour of such involvement. These arguments and counterarguments were based on observations and

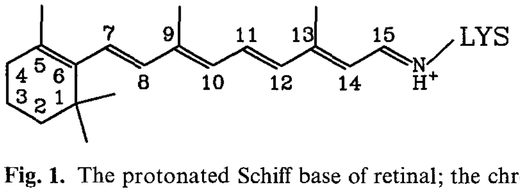

Fig. 1. The protonated Schiff base of retinal; the chromophore is attached through the terminal nitrogen to a lysine residue of BR

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calculations of frequencies of the C_{14}–C_{15} stretch vibration of BR's chromophore.

Recently, Mathies et al. (1988) argued that they had obtained conclusive evidence against participation of 14-*s-cis* structures in the photocycle of BR. The new arguments were based on observations of frequencies of the C_{14}-^2H and C_{15}-^2H in-plane bending vibrations of the chromophore in the L_{550} intermediate of the photocycle and on a vibrational analysis of *butadiene-like* $RSBH^+$ fragments in various isomeric states. These fragments were suggested to suitably model those vibrations of a $RSBH^+$ which are localized in the C_{13}-N moiety (see Fig. 1) of BR's chromophore. According to the vibrational analysis of the fragments, in-plane bending vibrations (rocks) of deuterium atoms should be capable of discriminating between 14-*s-trans* and 14-*s-cis* isomers. We will refer to deuterated rocking vibrations as *n*-D-rocks where *n* indicates the position of that deuterium atom D which has the largest vibrational amplitude in the respective normal mode. In di-deuterated molecules the 14-D- and 15-D-rocks form symmetric (*A*) and antisymmetric (*B*) combinations. Mathies et al. (1988) determined by Wilson FG calculations, that in a 14,15-D *s-trans*-fragment the A_g and B_u frequencies are nearly degenerate at about 970 cm^{-1} whereas in a *s-cis*-fragment the A_1 and B_1 vibrations are split by 156 cm^{-1}. In the *s-cis* geometry the A_1 frequency drops to 859 cm^{-1} and the B_1 combination is located at 1015 cm^{-1}. These computational results were compared with data obtained from resonance Raman experiments on 14-D, 15-D and 14,15-D isotopically labelled L_{550}. In the 14,15-D labelled L_{550} chromophore bands were observed at 952 cm^{-1} and 968 cm^{-1}. The latter band was assigned to the 14,15-D-rock of *A* symmetry. Its frequency is close to frequencies of bands which in mono-deuterated L_{550} were assigned to the 14-D- and 15-D-rock vibrations. Furthermore, no scattering was observed between 800 cm^{-1} and 900 cm^{-1}. Since for a 14-*s-cis* geometry the calculations on the fragments predicted large shifts of D-rock-bands upon di-deuteration, the observation of an essentially invariant D-rock-band in 14,15-D L_{550} appeared to exclude such geometry: *"These data unambiguously demonstrate that L_{550} contains a 14-s-trans chromophore."*

In a subsequent paper (Fodor et al. 1988) the observed spectra were shown and additional calculations were presented employing MNDO and Wilson FG methods for a vibrational analysis of larger model molecules which comprise most of the atoms of a $RSBH^+$. The state reversal and increase of splitting between *A* and *B* modes upon *s-trans* → *s-cis* isomerization was fully recovered by the Wilson FG calculations also for the larger molecules (212 cm^{-1}), whereas MNDO calculations predicted a smaller increase of the splitting ($\approx$65 cm^{-1}) and no state reversal (see Fodor et al. (1988) and discussion in section Results of this paper). In addition, the vibrational analysis revealed two further features: in a 13,14-*cis* geometry the 14-D-rock has a lower frequency of about 920 cm^{-1}, compared to about 960 cm^{-1} in the 13-*cis* geometry; in 14,15-D di-deuterated model molecules of 14-*s-cis* geometry D-rocks are found to couple to methyl stretch vibrations located in a range between 821 cm^{-1} and 1080 cm^{-1}. Although marked differences between MNDO and Wilson FG treatments resulted for the larger compounds and although the MNDO vibrational spectra predicted for the large model were drastically different from those determined by Wilson FG for the butadiene-like fragments, the authors essentially retained their arguments against an intermediate 13,14-*s-cis* structure in the photocycle and concluded that *"L_{550} contains a 14-s-trans chromophore."*

In the present paper we study the arguments presented by Mathies et al. (1988) and by Fodor et al. (1988). We show that these arguments, derived on the basis of a vibrational analysis of molecular fragments, do not apply to the chromophore of bacteriorhodopsin, because in the latter additional couplings occur. From our analysis of couplings between deuterated rocking modes and vibrational modes localized in the methyl and lysine moieties of the chromophore we conclude that vibrations observed and interpreted by Mathies et al. (1988) and by Fodor et al. (1988) do not allow one to distinguish between 13-*cis*, 14-*s-trans* and 13-*cis*, 14-*s-cis* geometries.

2. Methods

For a quantum chemical description of vibrational modes of retinal chromophores we employ the MOPAC program package distributed by the Quantum Chemistry Program Exchange of Indiana University/Bloomington (QCPE 455). MOPAC uses the MNDO (*M*odified *N*eglect of *D*iatomic *O*verlap) method (Dewar and Thiel 1977) to calculate the Hartree-Fock ground state of a molecule, taking all valence electrons into account. Technical details concerning the determination of the molecular force field by MOPAC have been described earlier (Tavan and Schulten 1986).

We will list below a compilation of deficiencies of the MNDO method, a knowledge of which is necessary for a proper evaluation of the results:

(1) All force constants, except those for single bond torsional vibrations (see below) are overestimated. To approximately rescale the force matrix one may introduce for each atom type a spectroscopic mass. We

have chosen the spectroscopic masses $m_H=1.094$, $m_C=14.75$ and $m_N=16.92$. This choice results in a reasonable description of most normal modes with the exception, perhaps, of *h*ydrogen *o*ut *o*f *p*lane (hoop) vibrations. In butadiene, for instance, hoop frequencies are calculated $20\,cm^{-1}$ to $75\,cm^{-1}$ above observed values.

(2) Single bond torsional barriers are underestimated (Tavan et al. 1985). As a result, torsional modes around single bonds are predicted at frequencies which are too low (Tavan and Schulten 1986). For example the torsional mode around the central single bond of butadiene is calculated at $40\,cm^{-1}$ and observed at $163\,cm^{-1}$ (Panchenko et al. 1976).

(3) The response of the nitrogen atom to protonation is described in an improper way. This shows up in a comparison between MNDO calculated and experimentally observed frequencies of the $C_{15}=N$ stretch in unprotonated and protonated molecules. Protonation leads to a $30\,cm^{-1}$ upshift of the $C_{15}=N$ stretching vibration, whereas MNDO predicts a downshift. López-Garriga et al. (1986a, b, c) have shown that such an error is a common feature of methods which, like MNDO, neglect the effects of electron correlation beyond the Hartree-Fock level. Owing to this deficiency, the MNDO method also misrepresents other vibrations which are localized at the nitrogen atom, i.e., the N-H-hoop and N-H-rock vibrations.

We consider the following molecules, which for the sake of simplicity, are labelled as if they were part of a retinal chromophore:

(1) Completely and partially deuterated butadiene serves as a check of the MNDO description of deuterated rocking vibrations as well as the vibrational analysis of butadiene-like fragments as presented by Mathies et al. (1988). To decouple the rocks of the central hydrogen atoms from those of the terminal hydrogen atoms we also employ for the latter atoms very large masses $m_H=15.00$. Such large hydrogen masses provide a crude model of massive residues covalently bound to the C_{13} and N atoms of retinal chromophores. The butadiene model with modified hydrogen masses will be referred to as BUTA.

(2) 13-methyl hexatriene has been selected to study the coupling between methyl and deuterated rocking vibrations in the C_{11}-N part of a retinal chromophore. Except for the hydrogen atom, which corresponds to the proton at the Schiff base nitrogen, all hydrogen atoms bound to the terminal carbon atoms of hexatriene chain are treated as in BUTA. This model molecule will be referred to as HEXA.

(3) Model compounds for $RSBH^+$ in BR, which comprise most of the atoms of the complete chromophore, have been chosen to study the interaction of deuterated rocking vibrations with lysine modes. The models differ from the real chromophore in three points:

(a) The β-ionone ring is omitted and the ring carbon atoms C_1 and C_4 are replaced by hydrogen atoms with masses $m_H=15$. We denote that modified polyene part of retinal covering the atoms between C_5 and C_{15} by "RET".

(b) Lysine chain and nitrogen are modelled by a residue X. We have chosen residues $X=NH^+CH_2CH_3$ and $X=NH^+CH_3$. The compounds including these residues are referred to as RET-X.

(c) We account for the influence of the protein environment by placing a single Cl^- counterion at a distance *d* from the proton of the Schiff base (see Fig. 2). This model charge environment deviates from the one employed by Tavan and Schulten (1986), where a second negative ion had been placed near C_5. A recent analysis of the low lying $\pi-\pi^*$ excitations in BR has shown that a single counterion at varying distances suffices to explain the absorption spectra of intermediates in the photocycle of BR (Großjean and Tavan 1988). In accordance with that analysis we describe the L_{550} chromophore by a counterion distance of $d=3$ Å.

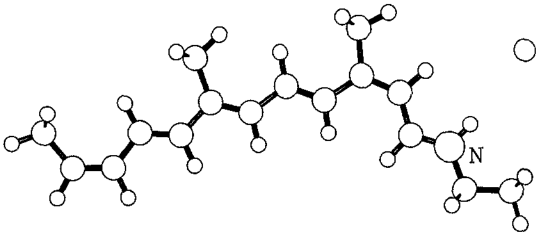

Fig. 2. Geometry of the 13-*cis* RET-X model $RSBH^+$ with a Cl^- placed $d=3$ Å distant from the proton at the nitrogen ($X=NH^+CH_2CH_3$)

13-*cis* RET-X compounds have been chosen planar, whereas for 13,14-*cis* isomers distorted ($X=NH^+CH_2CH_3$) as well as planar geometries have been assumed. In planar models all bond lengths, bond angles and dihedral angles, except those of the C−C bonds, were optimized. The geometry of the distorted 13,14-*cis* model compound was calculated in two steps. In a first step we calculated the geometry of the RET-$NH^+CH_2CH_3$ cation optimizing all nuclear degrees of freedom. We obtained dihedral angles $\alpha(C_{12}-C_{13})=27°$ and $\alpha(C_{14}-C_{15})=17°$. In a second step we added a counterion at a distance of 3 Å, fixed all single bond dihedral angles and optimized the remaining coordinates. That procedure resulted in a dihedral angle $\alpha(C_{13}=C_{14})=11°$.

3. Results

In this section we investigate, based on the calculations described in Sect. 2, to what extent the coupling between 14-D- and 15-D-rocking vibrations in di-deuterated retinal chromophores provides a criterion to distinguish between 14-*s*-*trans* and 14-*s*-*cis* geometries. Using butadiene and BUTA as models we first study the coupling pattern presented by Mathies et al. (1988). We then consider model molecules, which more completely represent retinal chromophores. We will establish two results:

(1) butadiene is not a suitable model for the chromophore of BR;

(2) mono- and di-deuterated 14-D- and 15-D-rocking vibrations do not allow one to distinguish between 14-*s*-*trans* and 14-*s*-*cis* geometries of L_{550}.

3.1 Coupled 14,15-D-rock vibrations in butadiene and BUTA

Figure 3 shows calculated 14-D-, 15-D- and 14,15-D-rocking virbration frequencies for 14-*s*-*trans* and 14-*s*-*cis* BUTA. 14-*s*-*trans* and 14-*s*-*cis* frequencies of mono-deuterio rocks are close, 988 cm^{-1} and 1001 cm^{-1}, respectively. 14,15-di-deuteration yields A and B rocking combinations. In the 14-*s*-*trans* geometry the frequencies of these vibrations are located at 973 cm^{-1} (A_g) and 995 cm^{-1} (B_u) such that they are split by 22 cm^{-1}. In the 14-*s*-*cis* geometry A and B vibrational energies show an enhanced splitting. We calculate frequencies of 902 cm^{-1} (A_1) and 1051 cm^{-1} (B_1). Whereas mono- and di-deuterio rock frequencies in the *s*-*trans* geometry are not more than 15 cm^{-1} apart, we calculate in the *s*-*cis* geometry shifts between 49 cm^{-1} (B_1) and 99 cm^{-1} (A_1). Between 830 cm^{-1} and 870 cm^{-1} MNDO predicts additional vibrations with 14-D- and 15-D-rocking character, which are present in both isomers but do not exhibit geometry-specific effects with respect to di-deuteration. Therefore, we have omitted these vibrations in Fig. 3.

Concerning the enhanced splitting of A and B modes upon *s*-*trans* → *s*-*cis* isomerization our results agree with those presented by Mathies et al. (1988) and Fodor et al. (1988), confirming, that in a butadiene-like fragment coupled 14,15-D-rock vibrations should qualify as markers to distinguish between *s*-*trans* and *s*-*cis* geometries. There is, however, an aspect of our results which is at variance with those presented by Fodor et al. (1988). We consistently find in butadiene-type *s*-*trans* fragments the A_g mode at *lower* frequencies than the B_u mode. To check whether this finding is a particular property of the BUTA model or if it is a general property of *s*-*trans* deuterated butadiene-like fragments we have calculated a series of such fragments including also cationic Schiff bases. In all investigated models the A_g mode was found below the B_u mode.

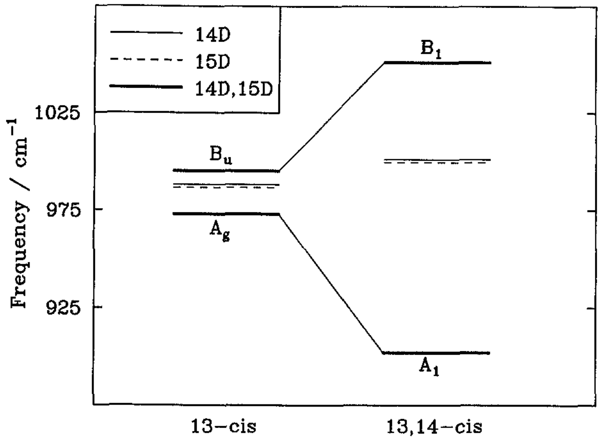

Fig. 3. Results of MNDO calculations on frequencies of 14-D-, 15-D- and 14,15-D-rock vibrations of *s*-*trans*- and *s*-*cis*-BUTA. Since BUTA serves as a model for the terminal $C_{13}-N$ part of a retinal chromophore, the *s*-*trans* geometry is denoted as 13-*cis* and the *s*-*cis* geometry as 13,14-*cis*. 14-D- and 15-D-rock frequencies are degenerate. Yet we have plotted these frequencies slightly separated from each other

In fully deuterated butadiene, for instance, we have calculated for these modes frequencies of 924 cm^{-1} (A_g) and 973 cm^{-1} (B_u). These results agree reasonably well with the experimental values of 919 cm^{-1} (A_g) and 1009 cm^{-1} (B_u) (Panchenko et al. 1976). We note that in this *s*-*trans* compound the splitting between A_g and B_u modes is large, measuring 90 cm^{-1}. Upon *s*-*cis* isomerization the splitting is calculated to increase by only about 30 cm^{-1}.

To give a second example, in *s*-*trans* butadiene, deuterated at the central positions, the A_g-mode is calculated at 931 cm^{-1} and observed at 942 cm^{-1} (Panchenko 1975). The A_g frequency is predicted 30 cm^{-1} below the center of two modes of B_u symmetry and D-rock character since in that molecule the B_u-D-rock combination is accidentally degenerate with a B_u scissoring mode of the terminal hydrogen atoms. In unlabelled butadiene the latter mode is observed at 991 cm^{-1} (Panchenko et al. 1976) and predicted by MNDO at 979 cm^{-1}. The accidental degeneracy leads to a large splitting resulting in two B_u combination modes calculated at 871 cm^{-1} and 1059 cm^{-1}, respectively. Unfortunately, we found no experimental data for comparison with these B_u modes.

In most cases the frequency ordering with A_g-mode above B_u-mode applies also to undeuterated rocking modes. An exception is 1,1,4,4-D-butadiene. In agreement with experimental data MNDO predicts the A_g-mode (1288 cm^{-1}) in this compound above the B_u mode (1281 cm^{-1}). The corresponding spectroscopic figures are 1298 cm^{-1} (A_g) and 1274 cm^{-1} (B_u) (Furukawa et al. 1983).

The excellent agreement of MNDO predictions with observations on di-deuterated rocking modes demonstrates that MNDO vibrational analysis provides a valid description of such modes. For butadiene the computational results as well as the experimental data suggest that a considerable splitting between di-deuterated A_g and B_u combination modes should exist in *s-trans* conformations and that the lower of the two modes should be of A_g symmetry. Since conclusions drawn from Wilson FG calculations point to the opposite direction (Fodor et al. 1988) we are tempted to speculate that the empirical force field employed in these calculations might be not very reliable.

3.2 Coupled 14,15-D-rock vibrations in HEXA

A butadiene-like fragment exhibites couplings between 14-D- and 15-D-rocking vibrations, however it lacks the methyl group bound to C_{13} and, therefore, does not involve vibrational couplings to modes localized in that group. To examine such couplings we have investigated 14-D, 15-D and 14,15-D deuteration effects in the methylated model compound HEXA. Figure 4 presents for 14-D, 15-D and 14,15-D isotopically labelled HEXA the frequencies of deuterated rocking vibrations. The corresponding 14,15-D-rocking vibrational modes are depicted in Fig. 5. The frequencies of mono-deuterated rocks for the 13-*cis* geometry are found at 964 cm^{-1} (14-D) and 954 cm^{-1} (15-D) and for the 13,14-*cis* geometry at 941 cm^{-1} (14-D) and 933 cm^{-1} (15-D). Di-deuterated-rocking vibrations in 13-*cis* HEXA are calculated at 947 cm^{-1} (A_g) and 967 cm^{-1} (B_u) and in 13,14-*cis* HEXA at 915 cm^{-1} (A_1) and 966 cm^{-1} (B_1).

The frequency pattern presented in Fig. 4 is different from that shown in Fig. 3 for BUTA. Apart from a general shift of the whole pattern to lower frequencies, the major difference is a diminished splitting between the di-deuterated A and B rocks in the 13,14-*cis* geometry. A further difference is the missing up-shift of the B_1 rock with respect to its B_u analogue. A third difference is a frequency splitting of the mono-deuterio rocking vibrations. A fourth difference is the down-shift of mono-deuterio rocks upon *s-trans* → *s-cis* isomerization.

To obtain a crude measure of the coupling strength between 14-D- and 15-D-rocks in di-deuterated molecules we consider a two by two coupling matrix. Taking squares of mono-deuterated rocking vibration frequencies as diagonal elements of that matrix and squares of di-deuterated rocking vibration frequencies as eigenvalues, we obtain for the coupling strengths Δ_i ($i = t$ or c to denote 14-*s-trans* or 14-*s-cis* geometries), $\Delta_t = 147\ \text{cm}^{-1}$ for *trans* BUTA and $\Delta_c = 381\ \text{cm}^{-1}$ for *cis* BUTA. For HEXA, we obtain $\Delta_t = 129\ \text{cm}^{-1}$ in the 13-*cis* and $\Delta_c = 218\ \text{cm}^{-1}$ in the 13,14-*cis* geometry. This analysis proves that the presence of the C_{13} methyl group diminished strongly ($\Delta_c = 381\ \text{cm}^{-1} \rightarrow \Delta_c = 218\ \text{cm}^{-1}$) the coupling of A and B rocking vibrations in a 13,14-*cis* geometry, although it is still large and present.

What causes the decrease of effective coupling strength between the two rocking modes? Inspection of the corresponding mono- and di-deuterated modes reveals that two types of methyl group vibrations contribute significant amplitudes (cf. Fig. 5). In di-deuterated molecules of either 13-*cis* and 13,14-*cis* geometry, for instance, the B combination is strongly coupled to a higher frequency methyl-hydrogen rocking mode (M). This coupling explains why in either geometry the B mode experiences large spectral downshifts as compared to the BUTA model. Similar effects are found for the other D-rocking modes. The A combinations are weakly coupled to a lower frequency

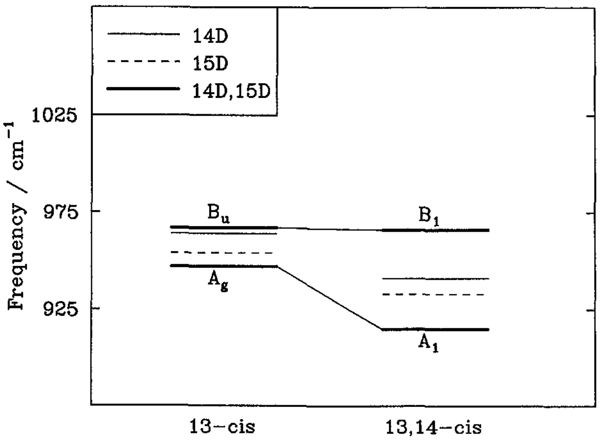

Fig. 4. 14-D-, 15-D- and 14,15-D-rock frequencies of 13-*cis* and 13,14-*cis* HEXA. The numbering of atoms has been chosen as to match the $C_{11}-N$ part of a corresponding $RSBH^+$

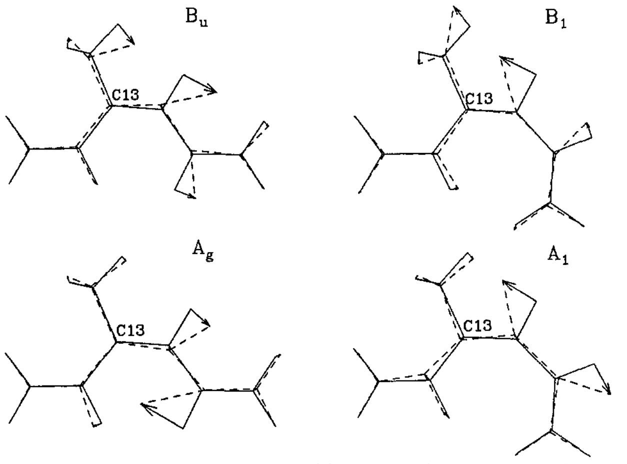

Fig. 5. B_u, A_g, B_1 and A_1 rocking vibrations in HEXA; the position of that carbon atom which corresponds to atom C_{13} of a retinal chromophore is indicated

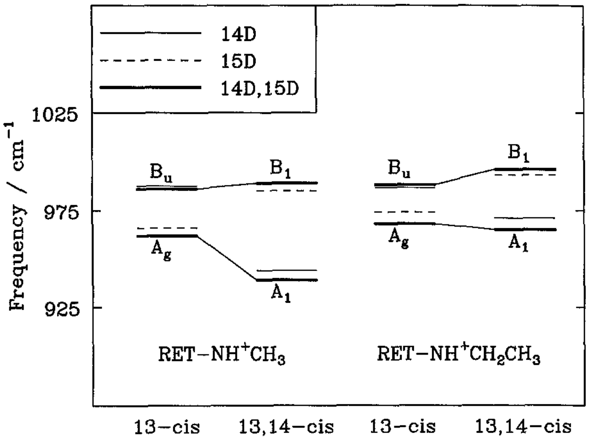

Fig. 6. 14-D-, 15-D- and 14,15-D-rock frequencies of planar 13-*cis* and 13,14-*cis* RET-NH$^+$CH$_3$ and RETNH$^+$CH$_2$CH$_3$ model compounds

13-methyl stretch. In mono-deuterated compounds methyl group modes mix with D-rocks as well. Vice versa, 14-D- and 15-D-rocking modes are present in the respective methyl group vibrations, which, in the 14-*s-trans* geometry, are calculated to lie in the frequency range between 854 cm^{-1} and 1031 cm^{-1}. In the 14-*s-cis* geometry these vibrations are computed between 822 cm^{-1} to 1027 cm^{-1}. Di-deuteration causes frequency shifts of about 10 cm^{-1} in the 14-*s-trans* and of about 20 cm^{-1} in the 14-*s-cis* geometry.

The example presented above demonstrates that strong couplings to modes localized at a neighbouring methyl group drastically change the frequency patterns of deuterated rocks. Such couplings are *completely* missing in butadiene-like fragments. Hence, butadiene-like fragments do not provide satisfactory models to investigate 14-D, 15-D and 14,15-D rock-rock couplings in a retinal chromophore.

3.3 Couplings of 14,15-D-rocking vibrations in RET-X compounds

In the preceding section we have shown that methyl modes tend to mix to deuterated rocking vibrations such that the simple coupling pattern between 14-D- and 15-D-rocking vibrations determined in butadiene-like fragments (Mathies et al. 1988) is destroyed. In this paragraph we examine to what extent the results presented by Fodor et al. (1988), which refer to larger model compounds, provide evidence for a 13-*cis*, 14-*s-trans* geometry of L_{550}. We will show that modes, which were not considered by Fodor et al. (1988) and which are localized in the lysine part of the chromophore, couple to 14-D-, 15-D- and 14,15-D-rocks and render a geometry assignment on account of isotope-dependent frequency shifts difficult.

As model compounds we employ planar 13,14-*cis*, 13-*cis* RET-NH$^+$CH$_3$ and RET-NH$^+$CH$_2$CH$_3$ as well as distorted 13,14-*cis* RET-NH$^+$CH$_2$CH$_3$. Results for the planar RET-X model compounds are presented in Fig. 6. We first discuss results obtained if the lysine chain is modelled by a simple methyl residue. In that case frequencies of mono-deuterated rocking vibrations are calculated at 987 cm^{-1} (14-D) and 966 cm^{-1} (15-D) for the 13-*cis* and at 944 cm^{-1} (14-D) and 985 cm^{-1} (15-D) for the 13,14-*cis* geometry. Di-deuterated rocking vibrations in 13-*cis* RET-NH$^+$CH$_3$ are computed at 962 cm^{-1} (A_g) and 986 cm^{-1} (B_u) and in the 13,14-*cis* isomer at 939 cm^{-1} (A_1) and 989 cm^{-1} (B_1).

In the di-deuterio compound the B rock and the 13-methyl-hydrogen-rock M are coupled to form $B+M$ and $B-M$ combinations. Figure 6 shows only the frequencies of the lower-lying $B+M$ combinations which, for the sake of simplicity, are denoted as B modes. The higher frequency $B-M$ combinations are calculated at 1034 cm^{-1} for the 13-*cis* geometry and at 1068 cm^{-1} for the 13,14-*cis* geometry. In the latter geometry, amplitudes of a further mode, localized in the methyl model of the lysine moiety, are present in the $B-M$ vibration. The 34 cm^{-1} difference between the frequencies of the $B-M$ normal mode in 14-*s-trans* and 14-*s-cis* geometries does not discriminate these isomeric states as it vanishes if the lysine chain is modelled by an ethyl group. For RET-NH$^+$CH$_2$CH$_3$ this mode in either geometry is calculated at almost the same frequency of about 1034 cm^{-1}. Therefore, we have omitted the high frequency $B-M$ combination in Fig. 6.

To obtain a measure of the interaction between 14-D- and 15-D-rocks we have calculated the two-mode coupling-strength defined above employing frequencies of A modes and of $B+M$ combinations. For planar RET-NH$^+$CH$_3$ the coupling strengths are smaller than in HEXA measuring $\Delta_t = 106$ cm^{-1} and $\Delta_c = 166$ cm^{-1}, respectively. The difference between Δ_t and Δ_c becomes very small in planar RET-NH$^+$CH$_2$CH$_3$ in which we obtain $\Delta_t = 118$ cm^{-1} and $\Delta_c = 146$ cm^{-1}. Thus, in contrast to the case of butadiene-like fragments, splitting of di-deuterated rocking vibrations does not provide a criterion for discriminating between *s-cis* and *s-trans* geometries of retinal chromophores.

As compared to HEXA, the mono-deuterated rocks in RET-NH$^+$CH$_3$ exhibit a different frequency spacing and in the 13,14-*cis* geometry a reversed energy order. In that geometry the 14-D-rock is calculated at a much lower frequency than in the 14-*s-trans* geometry. To obtain insight to what extent the low frequency of the 14-D-rock in 13,14-*cis* RET-NH$^+$CH$_3$ relates to geometry, we have studied the coupling of lysine modes to the 14-D-rock. Replacing the methyl group attached to the nitrogen by an ethyl residue the mono-deuterated rocks are found at 971 cm^{-1} (14-D) and 993 cm^{-1} (15-D) in a planar 13,14-*cis* geometry (see Fig. 6). For the distorted 13,14-*cis* compound 14-D-rock character is found in normal vibrations at 952 cm^{-1} and 922 cm^{-1}. The latter vibration also exhibits 9-methyl-stretch character. The 15-D-rock is found at 981 cm^{-1}. Corresponding values for the 13-*cis* model are 988 cm^{-1} (14-D) and 974 cm^{-1} (15-D). These results show that absolute and relative energies of the mono-deuterated rocking vibrations depend both on the choice of **X** and on the choice of the torsional state of the molecule. The dependence on the choice of **X** is more pronounced in the 13,14-*cis* geometry, i.e., the frequency splitting of mono-deuterated rocks varies in planar 13,14-*cis* compounds from 41 cm^{-1} ($X = NH^+CH_3$) to 22 cm^{-1} ($X = NH^+CH_2CH_3$) and from 21 cm^{-1} ($X = NH^+CH_3$) to 14 cm^{-1} ($X = NH^+CH_2CH_3$) in a 13-*cis* model compound. The mixing of D-rocks with modes localized in the methyl and ethyl models of the lysine group in BR, which is the cause of the **X**-dependence of these modes, and the unknown conformation of that group leads to a considerable uncertainty in theoretical predictions. According to the numbers given above that uncertainty is at least of the order of 20 cm^{-1}.

As demonstrated above, neither the coupling of 14-D- and 15-D-rocks nor the absolute positions of mono-deuterated rocks allow a decision as to whether a $RSBH^+$ has a 14-*s-trans* or 14-*s-cis* geometry. Now we want to investigate, to what extent other vibrations, which are influenced by 14-D, 15-D and 14,15-D deuteration, may provide such a geometry test. In mono- and di-deuterated RET-X compounds rock character is found in two further normal vibrations, which are calculated in a range between 830 cm^{-1} and 930 cm^{-1}. In both geometries these vibrations exhibit methyl-stretch character. They are, according to their main stretching amplitudes, designated as 9-methyl- and 13-methyl-stretch vibrations.

The 9-methyl-stretch is found at about 930 cm^{-1} in the 13-*cis* and at about 920 cm^{-1} in the planar 13,14-*cis* geometry for 14-D, 15-D, and 14,15-D labelled RET-X compounds. In 13,14-*cis* RET-X it is coupled with either the 14-D-rock or the di-deuterio rock of A symmetry to a degree which is sensitive to the torsional state of the molecule. In 13-*cis* RET-X it exhibits 14-D-rock character in either 14-D or 14,15-D deuterated compounds. In view of the small frequency difference between *s-trans* and *s-cis* geometries and of the variability of the 14-D-rock frequency caused by coupling to lysine modes we consider the spectral position of the 9-methyl-stretch to be irrelevant for the current discussion.

The 13-methyl-stretch might provide a better geometry test as it exhibits a geometry dependence of spectral position and pronounced isotope shifts in 13,14-*cis* isomers whereas such shifts are smaller in a 13-*cis* geometry. In unlabelled 13-*cis* compounds the 13-methyl-stretch is calculated at 905 cm^{-1}. Mono- and di-deuteration cause downshifts of at most 15 cm^{-1}. In unlabelled planar 13,14-*cis* RET-NH^+CH_3 its frequency is calculated at 861 cm^{-1}. 15-D and 14,15-D mono- and di-deuteration lead to frequency downshifts of about 25–30 cm^{-1}. In non-planar compounds the pattern of frequency shifts is similar except for slight changes of absolute numbers. However, with increasing torsions around the $C_{14}-C_{15}$ bond the 13-methyl-stretch, instead of coupling to a 15-D-rock, gains 15-D-hoop character. In view of the uncertainties associated with the MNDO description of hoop frequencies (see Methods) and in view of the unknown degree of distortion of a 13,14-*cis* retinal chromophore such coupling might reduce the significance of the computational results presented here for the 13-methyl-stretch.

4. Discussion

4.1 Summary of results on rock vibrations

Recent arguments (Mathies et al. 1988; Fodor et al. 1988) concerning the possibility of determining the isomeric state of BR's chromophore in the L_{550} intermediate from vibrational spectra using a 14-D, 15-D- and 14,15-D-labelling technique have been studied. According to these arguments in 14-*s-trans* chromophores 14-D-rock frequencies are expected in a frequency range between 960 cm^{-1} and 980 cm^{-1}. A_g and B_u di-deuterated rocking combination modes should exhibit a small frequency splitting with A_g *above* B_u. In 14-*s-cis* chromophores 14-D-rock frequencies are expected 30 cm^{-1} to 60 cm^{-1} below the corresponding spectral positions in *s-trans*. Di-deuterio compounds should exhibit a large frequency splitting of A_1 and B_1 combination modes with A_1 *below* B_1. Concerning these arguments the results of our MNDO vibrational analysis can be summarized as follows:

(1) In butadiene-like fragments modelling the C_{13}-N moiety of retinal chromophores the 14,15-D-rocking vibrations show increased frequency splittings upon *s-trans* → *s-cis* isomerization and, therefore, can serve as a tool to discriminate *s-trans* and *s-cis* geometries of such fragments. In *s-trans* fragments the A_g combination is predicted at a *lower* frequency than the B_u combination, a spectral order which agrees with observations on deuterated butadiene.

(2) Deuterated rocks strongly interact with modes localized at neighbouring methyl groups. Using a

methylated hexatriene-like model of the $C_{11}-N$ moiety of retinal chromophores we have demonstrated that couplings of C_{13}-methyl modes to 14-D-, 15-D- and 14,15-D-rocking vibrations can drastically change spectral positions and mutual coupling patterns of the latter modes rendering predictions derived from butadiene-like fragments invalid. In particular, the large frequency splitting between 14,15-D-rock combination modes predicted for *s-cis* butadiene-like fragments becomes strongly reduced upon coupling with methyl modes.

(3) In a retinal chromophore 14-D-, 15-D- and 14,15-D-rocks not only couple to vibrations localized in the 9- and 13-methyl groups but also interact with modes localized in the lysine residue. Modelling the lysine chain by methyl and ethyl groups we have shown for 14-*s-cis* retinal model chromophores that a butadiene-like splitting pattern is completely absent in the frequencies of di-deuterated rocks. The coupling to lysine modes changes both absolute frequencies and frequency splittings of mono-deuterio rocks. Owing to the unknown conformation of the lysine residue reliable predictions of these frequencies are hampered.

The MNDO result that couplings to methyl and lysine modes can destroy the splitting pattern of di-deuterated rocks determined in butadiene-like fragments contradicts the conclusions of Mathies et al. (1988) which were entirely based on a vibrational analysis of such fragments. In particular the result shows that in *s-trans and s-cis* chromophores mono- *and* di-deuterio rock frequencies can be expected in the same spectral region around 960 cm^{-1} whereas for *s-cis* di-deuterio compounds that was not to be expected according to the arguments of Mathies et al. (1988). Therefore, we consider the data on 14,15-D rocking vibrations in BR's L_{550} intermediate to be compatible also with a 14-*s-cis* geometry.

The differences between MNDO results *(1)-(3)* and conclusions drawn from the more recent vibrational calculations of Fodor et al. (1988) using Wilson FG and MNDO methods have two sources. First, these conclusions rely strongly on the results of Wilson FG calculations employing empirical force fields. Such calculations, in contrast to MNDO (Fodor et al. 1988 and this work), tend to reproduce butadiene-type rock-rock coupling patterns even if larger compounds are considered. For instance, in a 13-*cis*, 14-*s-trans* retinal model chromophore the A_g combination is predicted *above* the B_u combination and a reversal of spectral order as well as a large (183 cm^{-1}) splitting is calculated upon *s-cis* isomerization (Fodor et al. 1988). Second, the MNDO calculations of chromophore models presented by Fodor et al. (1988) did not provide the possibility for studying couplings to lysine modes. Therefore, the strong coupling between deuterated rocks and these modes could not be determined. Concerning the reliability of the Wilson FG model we have presented arguments stressing our doubts. As most important in that respect we consider the fact, that Wilson FG calculations do not even reproduce the observed order of di-deuterated combination modes in *s-trans* butadiene-like fragments. Furthermore, the methyl-rock couplings predicted by MNDO are not accounted for by the empirical force field employed in the Wilson FG method. Although MNDO vibrational calculations also have certain deficiencies (see Methods) comparisons with well-studied small molecules have demonstrated that calculated spectra are not completely unreasonable. Thus, we believe that the couplings of methyl and lysine modes to deuterated rocks predicted by MNDO should exist in a retinal chromophore. Then results *(1)-(3)* demonstrate that the consequences of such couplings render an analysis of vibrational spectra of 14-D, 15-D and 14,15-D labelled chromophores which *excludes* an 14-*s-cis* geometry of the L_{550} intermediate invalid.

4.2 The $C_{14}-C_{15}$ stretching vibration in L_{550}

In conclusion we like to comment on the significance of the $C_{14}-C_{15}$ stretching vibration for an assignment of chromophore geometries to the primary intermediates of the BR photocycle. Based on calculations of small chromophore fragments this mode was suggested by Smith et al. (1986) to allow a decision as to whether the intermediates K_{625} and L_{550} assume a 14-*s-trans* or a 14-*s-cis* geometry. The authors determined for the fragments a 100 cm^{-1} frequency decrease of the $C_{14}-C_{15}$ stretch going from a 14-*s-trans* to a 14-*s-cis* geometry. Since in K_{625} that vibration is observed at about 1190 cm^{-1} which is close to its position in the 14-*s-trans* chromophore BR_{568}, Smith et al. (1986) suggested a 14-*s-trans* geometry for K_{625}. In the resonance Raman spectrum of L_{550} they assigned the $C_{14}-C_{15}$ stretch to a shoulder at 1172 cm^{-1}. Because this value is only slightly below the $C_{14}-C_{15}$ stretch frequency (1175 cm^{-1}) of a 13-*cis*,14-*s-trans* $RSBH^+$ in solution, they concluded that L_{550} assumes a 14-*s-trans* geometry as well. Subsequent FTIR experiments of Gerwert and Siebert (1986) showed a clear band of predominant $C_{14}-C_{15}$ stretch character at 1155 cm^{-1}. It has been shown (Tavan and Schulten 1986) that for this vibration a pronounced dependence on charge environment exists in the 13,14-*cis* geometry and that this dependence can explain the observed frequencies of the $C_{14}-C_{15}$ stretch in K_{625} and L_{550} in a consistent way within the picture of a 14-*s-cis* geometry. Fodor et al. (1988) have corrected the previous assignment of the $C_{14}-C_{15}$ stretch in L_{550} to a spectral feature at 1165 cm^{-1}. Furthermore, they have attributed the frequency decrease of this stretching vibration in L_{550} as compared to a 13-*cis* model $RSBH^+$ in solution to an altered coupling of C−C stretching modes in the former chromophore. Certainly, as we have shown above for the 14,15-D-rocks, couplings of modes localized in the $C_{13}-N$ moiety to modes localized in neighbouring parts of the chromophore can be important and have to be analysed. Particularly, since possible couplings of C−C stretches to lysine modes have not been systematically explored, the previous study of the $C_{14}-C_{15}$ stretch by Tavan and Schulten (1986) may not represent the final proof for a 14-*s-cis* geometry in K_{625} and L_{550}. However, it has to be demonstrated by appropriate model calculations that such couplings can actually induce sufficiently large changes into the spectral ordering of C−C stretching vibrations as to render arguments and conclusions of Tavan and Schulten (1986) invalid. From the calculations on the model compounds investigated in this paper we did not find indications that this might be the case. Therefore, we still consider the arguments presented by Tavan and Schulten (1986) and Gerwert and Siebert (1986) to provide strong support for a 13-*cis*, 14-*s-cis* primary reaction.

We have some doubts that the question of the chromophore geometries in K_{625} and L_{550} can be finally resolved by comparisons of vibrational spectra with results of model calculations. For that purpose we consider the available force fields obtained from quantum chemical or empirical procedures to be not sufficiently accurate. Furthermore, to the extent to which a coupling to lysine modes is important for retinal modes, calculations become intractable since the flexible lysine chain may assume a very large number of different conformations. Therefore, more direct experimental methods are required to determine the chromophore geometries in the primary intermediates. In that respect experiments on the linear dichroism of infra-red bands of BR intermediates appear to be most promising since such measurements, in addition to frequencies, also render directions of vibrational dipoles. Such experiments are currently being persued by F. Siebert (personal communication) and by J. Breton and E. Nabedryk (personal communication).

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