Influence of the Methyl Groups on the Structure, Charge Distribution, and Proton Affinity of the Retinal Schiff Base

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The effect of the methyl substitutions at different carbon atoms of the polyene chain of the retinal Schiff base has been studied on the structure, charge distribution, and proton affinity (PA) in a Schiff base model with the same number of conjugated double bonds (six conjugated double bonds including the Schiff base group). The methyl groups were added to the nitrogen atom and/or different carbon atoms along the main chain, and the results were compared to those of the retinal Schiff base. To study the effect of the polarization functions on hydrogen atoms, all of the structures were studied using 6-31G* and 6-31G** basis sets at the Becke3LYP (B3LYP) level of theory. For each optimized model, the PA was then calculated on the basis of the difference of the total energy of the protonated and unprotonated species. The influence of the zero-point energy corrections on the PA values was also examined at the same level of theory. The results show that, for all species, the application of the larger basis set (6-31G**) does not significantly influence the structures and the derived charges. Utilization of the larger basis set, however, results in PAs which are, by about 2.0 kcal/mol, systematically higher than the corresponding values obtained from the B3LYP/6-31G* level of theory. The consideration of the thermal energy corrections in the PA calculation, on the other hand, systematically shifts the PA, by about 9.0 kcal/mol, to higher values. The effects of the applied basis set and zero-point energy corrections, therefore, can be neglected in the calculation of the relative PA values. The methyl substitutions have substantial effects on the calculated PA of the studied Schiff base structures. On the basis of the calculated PA values of our models, the presence of at least two methyl groups on the terminal carbon atom of the conjugated chain is of great importance in order to obtain a PA value close to the retinal Schiff base. These groups should be considered in the theoretical studies of the pK_a change in this system. The effect of the methyl groups on the overall shape of the chromophore was also found to be significant. These structural features combined with the stabilizing effects of the methyl groups on the positive charge of the main chain potentially influence the isomerization barriers in the retinal Schiff base. Therefore, the location and amount of the twist in the backbone of the chromophore, and consequently the pK_a of the Schiff base group, can be manipulated by the protein environment via steric interactions between the methyl groups and the binding pocket of retinal proteins such as bacteriorhodopsin.

I. Introduction

The transmembrane protein bacteriorhodopsin (bR) present in the outer purple membrane of *Halobacterium salinarium* (formerly *H. halobium*) is one of the simplest known active membrane transport systems. It functions as a light-driven proton pump converting light energy into a proton gradient which is used by the cell as an energy source to activate ATP synthase. Structurally, it folds into seven transmembrane helices, one of them containing the residue Lys_{216} at which the retinal prosthetic group binds via a protonated Schiff base linkage [for reviews see refs 1−5]. The chromophore divides the channel formed by the α-helices of the polypeptide into a cytoplasmic part connecting to the inside of the cell and an extracellular part connecting to the outside.

The general features of the transport mechanism are now understood. The absorption of a photon which starts the bR photocycle induces an excited state of the chromophore followed by the isomerization of the retinal protonated Schiff base around the C_{13}═C_{14} double bond next to the Schiff base group. During the photocycle of bR, the initially protonated retinal Schiff base releases a proton into the extracellular part of the channel and will be reprotonated from a proton source located in the cytoplasmic part. Therefore, a proton is effectively pumped from the inside of the cell to the outside during each cycle.[1−5] There are, however, different proposals regarding when and how the proton starts to move from the retinal Schiff base to the next proton accepting group which is suggested to be the negatively charged carboxylate group of Asp_{85} in the protein backbone.[3] This proton transfer from the protonated Schiff base to the Asp_{85} side chain can be mediated by one or two water molecule(s) present in the vicinity of the chromophore.

The involvement of water molecules in the stability of the protonated Schiff base was suggested by DuPuis et al.,[6] and by Hildebrandt and Stockburger[7] on the basis of the resonance Raman study of dried membranes. The presence of water in the binding site was shown by de Groot et al.[8] on the basis of the ^{15}N NMR studies. Recent crystal structures of bR also demonstrate the presence of a few water molecules in the vicinity of Schiff base group.[9,10] The effect of the water

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molecules on the pK_a of the Schiff base group is demonstrated by pK_a measurement of a series of retinal Schiff base analogues.[11] The possible positions of hydrogen bonded water molecules around the Schiff base group have been theoretically examined.[12,13]

The proton transport mechanism in bR is based on the sequential changes in the pK_a values of the retinal Schiff base and vectorially arranged protonatable groups in the protein. The pK_a change of respective groups in the proton channel, especially the pK_a of the retinal Schiff base, plays a crucial role in the proton-transfer reaction. There are several possible reasons explaining why the pK_a of the Schiff base would be lowered at the beginning of the deprotonation step. Among these are the disruption of the π-system of the retinal Schiff base chain during the trans-to-cis isomerization, which decreases the electron density on the nitrogen atom of the Schiff base group,[14,15] and the conformational changes which modify the electrostatic environment of the retinal Schiff base[16−20] or change the orientation of the hydrogen bonded groups.[21,22]

The decrease in the pK_a of the retinal Schiff base is the first step which may induce the proton transfer. It should be mentioned, however, that the pK_a of the retinal Schiff base will be significantly increased in the protein environment as compared with its isolated form. It is known from experimental data that the pK_a of the protonated retinal Schiff base in methanol/water (1:1) solution is about 7.2[23,24] while the pK_a of the chromophore in bR is shifted to 13.3.[25,26] The protein environment seems to have a very strong effect on the pK_a of the retinal Schiff base. With regard to this, the presence of the negatively charged side chains of Asp_{85} and Asp_{212} in the vicinity of the protonated Schiff base is proposed to have the main influence on the electronic structure and charge distribution of the retinal Schiff base in the bR protein environment.[27]

The dynamics of the excited state of the retinal in bR and the effect of the protein environment on the rate of its photoisomerization have been experimentally investigated.[28−31] The dynamics of the all-trans and the 13-cis conformers of the retinal protonated Schiff base has also been studied recently in different solvents by means of picosecond transient spectroscopy.[32] Ab initio molecular dynamics calculations on the *all-trans*- and the 11-*cis*- retinal structures[33] have been recently reported, and hybrid QM/MM dynamics simulations have been performed in order to understand the photoisomerization process of bR.[34] The dynamic behavior of the retinal Schiff base at different stages of the bR photocycle has been theoretically studied using classical force fields[35−41] or combined classical and quantum chemical approaches where ab initio calculations have been applied to calculate the electronic characteristics of the retinal Schiff base structures obtained from a classical molecular dynamics simulation of bR.[42] The excited-state potential surface of the isomerization, which was studied using semiempirical calculations[34,43,44] has been recently investigated by the application of high level quantum chemical calculations in the study of the photoisomerization dynamics of retinal Schiff base models.[45−47]

Because of the relatively large size of the chromophore, however, it is computationally prohibitive, especially at higher levels of theory, to include the whole retinal molecule in the calculations. For this reason, smaller Schiff base analogues are widely used to model the retinal Schiff base. There is a wide range of model Schiff base structures in the literature. These models have been applied in theoretical and/or experimental studies to investigate the structural and electronic properties of the retinal Schiff base. These models are different from each other with respect to the number of the double bonds conjugated to the Schiff base group (C═N) and/or with consideration of the methyl substitutions on the main polyene chain, as well as the inclusion of the β-ionone ring into the model.

In this laboratory, we have started investigating different structural aspects of the retinal Schiff base chromophore and their roles in the biological activities of the machinery. In our previous report[48] we compared different model Schiff bases with different lengths of the polyene chain. In other words, we studied the effect of the number of the double bonds conjugated to the Schiff base on the electronic configuration of the molecule which, in turn, determines the structure, bond alternation, and proton affinity (PA) of the system. The results of that study[48] clearly confirmed that the approximation of the retinal Schiff base, especially in the protonated form, by shorter polyene models can adversely effect the calculated bond alternation and PA of the molecule. The calculated bond alternation and PA of the molecule influence the conclusions about the rotational barrier[49] and pK_a of the model, respectively. In the same study, we also compared the calculated PA for the planar all-trans conformation of the unsubstituted Schiff base model with six conjugated double bonds (PSB6), the trimethyl-substituted derivative of PSB6 and the complete retinal Schiff base. The comparison of the PA calculated for an unsubstituted conjugated Schiff base with six double bonds (PSB6) and the trimethyl derivative of PSB6 with *N*-methyl-retinal Schiff base show that the PA could be significantly influenced after introduction of the substituents into the main chain of the polyene structure.[48] As compared to the effect of the length of the polyene chain, however, the effect of the methyl groups was found to be less significant with respect to the PA of the molecule.

The stabilizing effect of alkyl substitutions on the positive charge in carbocations is well-known in organic chemistry. On the basis of a Mulliken population analysis, the stabilization effect of the methyl groups on the positive charge located on the substituted carbon atom of the main polyene chain has also been reported for a number of conjugated model Schiff bases,[27] as well as for the retinal Schiff base.[50] This stabilization effect lends higher weights for a number of mesomeric structures which are proposed for the protonated species of the conjugated Schiff base structures.[50]

In one of our recent works,[27] using a methylated model Schiff base for the retinal Schiff base, we also noticed that the positive charge of the protonated species, stabilized by the delocalized π-electronic system of the polyene, could be substantially further stabilized by the methyl substitution.

Furthermore, the opposite effect of methyl groups on the stability of the planar and 90°-rotated transition structures obtained during the isomerization of different bonds in the retinal Schiff base should also be considered, which significantly affects the isomerization barriers especially for the protonated species.[51] On the basis of these observations, we decided to study the effect of the methyl substitutions at different positions of the main chain of the polyene on different structural and electronic properties of the retinal Schiff base. For this reason, the structure, charge distribution, and PA values were calculated for a series of differently substituted model Schiff bases including the complete structure of the retinal Schiff base. In this way, we provide more information about the applicability of different models for addressing different questions about the chromophore.

We applied density functional theory (DFT) to calculate the geometries and therochemical properties of the Schiff base models and to study the effect of the different positions and

numbers of the methyl groups substituted on the main chain, as compared to the retinal Schiff base. As will be discussed later in the present article, DFT calculations have been reported to overestimate the extent of the electronic delocalization in the polarized polyenes. Therefore, the application of the DFT techniques results in the prediction of shorter single bonds and longer double bonds for the polyene Schiff base models. This point has to be kept in mind whenever the absolute values for the bond distances and the PA values are to be considered.

To study the effect of the basis set, the calculations have been performed at two levels, namely B3LYP/6-31G* and B3LYP/6-31G**. For both basis sets, geometries have been fully optimized and the second derivative calculations have been performed. Because of the cost of the computations, especially for the calculation of the second derivatives using the larger basis set, the results of the present study also provide benchmarks for the thermochemical properties of these large molecules. This information may also be used as a measure for the accuracy of the results obtained using more approximated methods. The zero-point energies calculated for each species can be obtained from the tables presented in this paper. Further details are available on request.

2. Computational Details

For the computer graphics and the initial construction of the molecular models we used the InsightII software[52] running on a Silicon Graphics Indigo2 workstation. The model Schiff bases were considered as unprotonated and protonated species, respectively. All ab initio calculations were performed with the GAUSSIAN 94 [53] implementation of DFT on an IBM SP2 cluster. Gradient optimization techniques were employed to optimize the geometries of the molecules at the DFT level, using 6-31G* and 6-31G** basis sets, respectively. The hybrid Becke3LYP (B3LYP) method was used for the DFT calculations.[54,55]

Geometry optimizations were performed without any geometric restrictions using the default GAUSSIAN 94 convergence criteria. All of the stationary points were confirmed to be the minimum by the calculation of the analytical second derivatives. The charges were derived from a Mulliken population analysis, as implemented in the GAUSSIAN 94 program. The charge reported for each fragment of the polyene includes the charges of the main chain atom and the hydrogen(s) and/or methyl group(s) connected to it.

The gas-phase proton affinity (PA) of a compound B can be calculated as the negative standard reaction enthalpy of protonation at 298.15 K:

$$B + H^+ \rightarrow BH^+$$

$$PA = -[E_{DFT}(BH^+) - E_{DFT}(B) + (E_{vib}(BH^+) - E_{vib}(B))] + 5/2\,RT$$

where E_{DFT} will be obtained from the DFT calculations, E_{vib} includes the zero point energy and temperature corrections to the vibrational enthalpy, and 5/2 *RT* includes the translational energy of the proton and the $\Delta(PV)$ term.

DFT has been reported to be very reliable in calculating PA and in reproducing the experimental results within 1−7 kcal/mol.[56] The results obtained with DFT calculations show a significant improvement over the Hartree−Fock results which are off by 1−12 kcal/mol.[56] It has also been reported that MP2 and MP4 results do not significantly improve the calculated values of PA.[56] This is also evident from comparison of the calculated PA values of methylenimine at the MP3/6-31G* (214.2 kcal/mol, ref 57) and B3LYP/6-31G* (216.2 kcal/mol, ref 48) levels of theory.

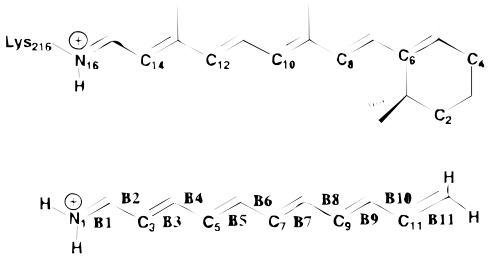

Figure 1. (top) Retinal Schiff base structure in the ground state of the bR photocycle and its conventional numbering scheme. (bottom) Atom and bond numbering used in the text in the case of the all-trans structure of the model Schiff base with six conjugated double bonds (PSB6).

3. Results and Discussion

3.1 Model Description. The structure of the all-trans protonated retinal Schiff base which is suggested to be the starting configuration of the chromophore at the beginning of the bR photocycle and its conventional atom numbering are depicted in Figure 1. The structure of the representative model Schiff base which was used in the present study is also presented in Figure 1. The atom numbering (N_1 to C_{12}) and bond numbering (B1 to B11) for the model Schiff base structure start from the nitrogen atom and the Schiff base double bond, respectively, and continue toward the other end of the chain. From now on, this model Schiff base structure, including six conjugated double bonds, will be referred to as PSB6 and SB6, for the protonated and unprotonated species, respectively.

In a previous study,[48] we demonstrated the importance of the number of the conjugated double bonds in the calculation of the PA values. Therefore, since we would like to have an appropriate model for the retinal Schiff base, in the present work we study a model Schiff base which has the same number of the conjugated double bonds (six double bonds including the Schiff base group) as the retinal Schiff base does. The effect of the methyl substitution(s) on the structure and the electronic configuration will then be considered in this model. To facilitate further discussion, the position of the methyl groups on the main chain will be mentioned using the atom numbering scheme of the model Schiff base as shown in Figure 1. For example, *N*,4,8-trimethyl-PSB6 refers to the protonated Schiff base including six conjugated double bonds and being methylated at N_1, C_4, and C_8 atoms (respectively corresponding to the N_{16}, C_{13}, and C_9 atoms in the retinal Schiff base).

All models were considered in the all-trans configuration, the Schiff base group being in the anti form. Resonance Raman spectroscopy studies[58] suggest that, in bR_{568} (the starting configuration of the protein in the photocycle with a maximum absorption at 568 nm), the Schiff base group has the anti C_{15}═N_{16} configuration. That is, the Lys_{216} connects to the chromophore at the anti position of the Schiff base group (C_{15}═N_{16}) and the hydrogen which is located at the syn position will play the role of the leaving proton. For this reason, the anti position of the Schiff base group (C═N) was considered for the unprotonated species as well as for the position of the methyl group in the case of *N*-methylated analogues. It has to be mentioned, however, that syn or anti consideration of the terminal group on the nitrogen atom cannot significantly change the conclusions, and the syn or anti consideration of the Schiff base group results in very slight changes (maximally 1 kcal/mol) in the calculated PA values.[48]

Density functional theory (DFT) has been used in the calculation of the structure[48,50] and the potential energy surface of the isomerization of different bonds for polyene structures.[48,49,59,60] The applicability of DFT in the calculation of the isomerization barriers and the description of the transition states of such rotations has been reported for neutral polyene systems such as β-carotene analogues.[59] It has been reported that the results are in a good agreement with other methodologies applying higher levels of theory with respect to the description of the 90°-rotated species in the double bond rotations as well as the single bond rotations. The results from DFT spin-unrestricted calculations reasonably describe the 90°-rotated structures which will be formed during the rotation of the double bonds.

DFT techniques have also been used for studying the conjugated Schiff base structures, and the ability of DFT calculations in description of the 90°-rotated species are comparable with CAS−SCF level of theory calculations.[48,49,60] However, it seems that when the π-electronic system under study is significantly polarized, DFT calculations result in an overestimated π-electron delocalization effect. The results from the DFT calculations on the retinal (containing a carbonyl group as the terminal group), for example, predict a smaller bond alternation for the planar structure of the molecule[33] as compared to the experimental values.

For the conjugated Schiff base structures, the calculations carried out using a multireference wave function predict a larger bond alternation for both neutral[60] and protonated species,[46,47,60] as compared to the DFT results (see ref 60 for detailed comparison). The difference is more significant for the protonated species. In the B3LYP/6-31G*-optimized structure of a conjugated Schiff base model with four double bonds, for example, the bond distances of B1 (N_1═C_2) and B3 (C_3═C_4) double bonds of the all-trans conformer are 1.323 and 1.385 Å, respectively.[48] The corresponding bond lengths are 1.293 and 1.362 Å, respectively, according to the CAS−SCF calculations applying eight electrons and eight orbitals in the active space.[60] For the single bonds, on the other hand, DFT results in shorter bond distances. The B2 (C_2−C_3) and B4 (C_4−C_5) single bonds, for example, are calculated to be 1.398 and 1.412 Å, respectively, according to the DFT results,[48] whereas the CAS−SCF calculations predict the values of 1.423 and 1.439 Å, respectively, for these bonds.

Because of the less delocalization effect in CAS−SCF calculations, the absolute PAs are predicted to be smaller than the corresponding values in DFT calculations. The PA of an unsubstituted Schiff base model with four conjugated double bonds calculated using CAS−SCF calculations, for example, is 238.41 kcal/mol, whereas according to the DFT results, a value of 251.53 kcal/mol is predicted for the PA of the same molecule. Although, in the present study, we are interested in the comparison of the energies and the PA values of the molecule after the addition of the methyl group(s) to the model, the differences between the DFT results and the results obtained from the application of the multireference wave functions have to be kept in mind whenever the absolute values for the PAs and bond distances are to be considered.

We have investigated several monomethylated (4-methyl, 5-methyl, 7-methyl, 8-methyl, 12-*cis*-methyl and 12-*trans*-methyl) and dimethylated (4,8-dimethyl, and 12,12-dimethyl) analogues of the SB6 and PSB6. Besides, a trimethylated analogue (*N*,4,8-trimethyl), a tetramethylated analogue (4,8,12,12-tetramethyl), and a pentamethylated species (*N*,4,8,12,12-pentamethyl) as well as the *N*-methyl-retinal Schiff base were also studied in both protonated and unprotonated forms. The *N*-methyl-retinal Schiff base was used as a reference to which the structure and PA of the other models were compared. In this way, we explore the effect of the different model simplifications, with regard to the methyl groups, on the studied properties of the model Schiff base in order to find a proper model which can reproduce the results obtained from the study of the entire model of the retinal Schiff base. The positions of the methyl groups on the main chain were selected on the basis of the retinal Schiff base structure. Incorporation of the C_{12} carbon atom into the β-ionone ring of the retinal Schiff base made us examine two different, cis and trans, positions on this carbon with respect to the methyl substitutions. The methyl substitution on the nitrogen atom was also considered to study the effect of the covalent connection to the Lys_{216} residue in the protein environment. Furthermore, we also studied two other positions on the odd-numbered carbon atoms, namely, C_5 and C_7, to compare the results with the models where the even-numbered atoms are substituted.

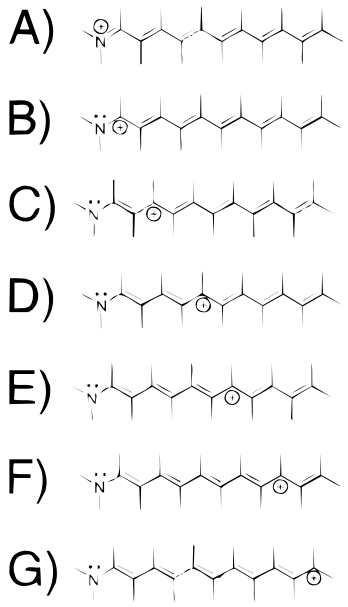

Figure 2. Mesomeric structures for the protonated Schiff base model.

3.2. Resonance Structures and Atomic Charges. The mesomeric structures which can be considered for the protonated Schiff base models are schematically presented in Figure 2. In the first structure (**A** in Figure 2), the positive charge is formally carried by the nitrogen atom and the double bonds are drawn according to the convention which is generally used to describe the retinal Schiff base. Other mesomeric structures (**B**−**G**) can be obtained by shifting the π electrons of the Schiff base double bond (C═N) to the nitrogen atom. This leaves the positive charge to the other part of the molecule, namely, the carbon containing moiety, where, by shifting the other double bonds, the positive charge can formally be assigned to different carbon atoms in the different mesomeric structures (**B**−**G**), as depicted in Figure 2. The examination of the charges obtained for the protonated Schiff base models shows that, apart from the main resonance structure **A**, structure **B** has the largest weight among the mesomeric structures.

For the unprotonated species, similar resonance structures are shown in Figure 3. Because of the appearance of formal positive and negative charges, very small weights for the mesomeric structures **B′**-**G′** are expected in the unprotonated species (Figure 3). In both protonated and neutral cases, however, the largest weight is predicted for the first mesomeric structure (**A** and **A′**

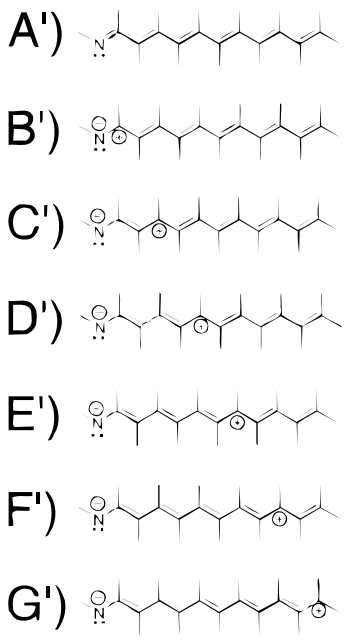

Figure 3. Mesomeric structures for the unprotonated Schiff base model.

for protonated and unprotonated species, respectively). According to the known larger π-electronic delocalization in the protonated species, the weights of other positive mesomeric structures (**B**−**G**) must be considerably larger in the protonated species, as compared to the weight of the corresponding mesomeric structures of the unprotonated species (**B′**−**G′**).

The methyl substitutions increase the weight of those resonance structures where the positive charge is formally assigned to the substituted carbon atom. The examination of the resonance structures shows that, in both protonated and unprotonated species, a positive charge is formally assigned to the even-numbered carbon atoms in different structures. Therefore, we expect that methyl substitutions on the even-numbered carbon atoms have a larger stabilization effect on the positive charge of the main chain.

We start our analysis with the effect of the methyl groups on the atomic charges. These charges are calculated for different molecules and compiled in Tables 1 and 2 for the protonated and unprotonated species, respectively. Comparison of the charges calculated using different basis sets (6-31G* and 6-31G**) revealed that there are only slight differences between the corresponding charges calculated at these two levels of theory. The maximum difference between the charges reported in Tables 1 and 2 and the corresponding values calculated using the smaller basis set amounts to 0.03 e. Therefore, for the sake of space, we report only the atomic charges calculated using the larger basis set.

Comparison of the charges demonstrates that, in both unsubstituted and substituted species, the positive charges assigned to the even-numbered fractions of the molecule are larger than the odd-numbered fractions. Among the even-numbered carbon atoms, however, the last atom in the polyene chain, C_{12}, is not carrying a significant positive charge and therefore, the mesomeric structures **G** and **G′** are predicted to have very small weights among the resonance structures.

The methyl substitutions influence the distribution of the positive charge along the chain. In all cases the methyl groups significantly increase the positive charge which can be assigned locally to the substituted fraction of the main chain (including the substituted carbon atom and hydrogen(s) and/or methyl group(s) connected to it). For example, methyl substitution at C_4 results in more positive charge on the C_4 fraction of the main chain in the 4-methyl-PSB6 (+0.22 e), as compared to the C_4 fraction in the PSB6 model (+0.09 e). The increase of the positive charge in one fraction of the chain causes the increase of the electron density on the neighboring fractions (C_5 and C_3 in this example).

Addition of alkyl groups to the even-numbered carbon atoms results in a larger stabilization of the positive charge on the polyene chain. Because of the large steric interaction, however, simultaneously substituting two very close positions, e.g., C_4 and C_6 or C_6 and C_8, or substitution of a bulky group other than a methyl group may destroy the essentially important planar structure of the polyene which is needed for the optimal delocalization of the π-electronic system. Therefore, according to what nature has decided for the retinal Schiff base chromophore, a maximum number of three such positions, namely, C_4, C_8, and C_{12}, can be simultaneously substituted by methyl groups.

3.3 Geometries. Conjugated Schiff base structures, such as the retinal Schiff base, as well as the other model Schiff bases studied in the present work possess a delocalized π-electronic system. Even in the unprotonated species, because of the larger electronegativity of the nitrogen atom, the conjugated π-electrons are slightly shifted toward the Schiff base group (C═N). In the protonated species, because of the additional effect of the positive proton, this charge shift is much more pronounced. In both protonated and unprotonated species, the charge reorganization will be mainly observed in the Schiff base (C═N) region.

As mentioned before, the extent of the π-electron delocalization, which determines the bond alternation in the main chain of the polyene structure, is found to be differently predicted using different methods.[46−49,60] The examination of the bond distances of the different conventional single and double bonds along the main chain in the protonated species shows that the bond alternation is completely destroyed in the Schiff base region. This is partly because of the overestimation of the delocalization effects in DFT calculations. According to the recent ab initio CAS−SCF calculations on a similar molecule,[46,47] the bond alternation is found to be significantly larger for the polyene Schiff base molecules. The bond alternation of the main chain directly influences the bond order and, consequently, the barrier to the isomerization of different single and double bonds. Therefore, it seems that different methodologies result in different absolute values for the bond distances and the isomerization barriers. In the present study, however, we are mainly interested in the comparison of the different methylated species with the unsubstituted model. Such comparisons are expected to result in similar conclusions in different methodologies. We will examine the lengths of these bonds in different species in order to see if different methyl substitutions may influence the bond alternation of the polyene chain. The bond lengths in the main chain calculated for different species at B3LYP/6-31G** level of theory are compiled in Tables 3 and 4 for the protonated and unprotonated species, respectively.

The bond distances calculated using the basis set without the polarization functions on hydrogen atoms (6-31G*) are very close to the corresponding values reported in Tables 3 and 4 and, therefore, will not be reported. The maximum difference in the corresponding bond distances in the main chain between two basis sets amounts to the values of less than 0.001 Å. On the basis of these results and the results reported in the previous section, it can be concluded that the B3LYP/6-31G* level of theory can be used to reproduce structures and charges obtained

TABLE 1: Charges ($\times 10^2$ e) of Different Fractions of the Polyene Chain Calculated for the Protonated Species at B3LYP/6-31G Level of Theory for Unsubstituted (PSB6) and Different Substituted Species[a]**

species	N_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	C_9	C_{10}	C_{11}	C_{12}
PSB6	09	33	02	09	03	08	03	08	04	08	08	07
4-methyl-PSB6	09	32	−03	**22**	−03	07	04	07	04	07	08	07
5-methyl-PSB6	09	34	01	04	**14**	03	03	07	03	07	08	07
7-methyl-PSB6	09	33	01	09	02	04	**15**	02	03	07	08	07
8-methyl-PSB6	09	33	02	08	03	08	−02	**21**	−01	07	08	06
12-*cis*-methyl-PSB6	08	33	01	08	02	07	03	07	03	08	03	**16**
12-*trans*-methyl-PSB6	08	33	01	08	02	07	03	07	03	07	05	**15**
N-methyl-PSB6	**14**	35	−01	08	02	07	03	07	03	07	08	06
4,8-dimethyl-PSB6	09	32	−03	**22**	−02	08	−02	**20**	−01	06	08	05
12,12-dimethyl-PSB6	08	32	01	08	02	07	03	07	03	07	−01	**24**
N,4,8-trimethyl-PSB6	**13**	34	−06	**21**	−03	07	−02	**19**	−01	06	08	04
4,8,12,12-tetramethyl-PSB6	07	31	−04	**21**	−03	07	−03	**20**	−02	07	−01	**22**
N,4,8,12,12-pentamethyl-PSB6	**12**	33	−06	**21**	−03	06	−03	**18**	−02	06	−01	**21**
N-methyl-retinal Schiff base	**11**	33	−07	**20**	−04	06	−04	**18**	−02	−01	14	**11**

[a] Every reported charge is the summation of the atomic charges of the main chain atom and hydrogen(s) and/or methyl group(s) connected to it. The charges of the substituted fractions are shown in bold face.

TABLE 2: Charges ($\times 10^2$ e) of Different Fractions of the Polyene Chain Calculated for the Unprotonated Species at B3LYP/6-31G Level of Theory for Unsubstituted (SB6) and Different Substituted Species[a]**

species	N_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	C_9	C_{10}	C_{11}	C_{12}
SB6	−27	18	03	02	01	01	01	01	01	01	05	−05
4-methyl-SB6	−27	17	−02	**13**	−03	00	01	−00	01	00	05	−05
5-methyl-SB6	−28	18	02	−02	**11**	−05	01	01	00	01	05	−05
7-methyl-SB6	−27	18	03	02	−00	−03	**11**	−05	01	01	05	−05
8-methyl-SB6	−27	18	03	02	01	01	−05	**11**	−03	−00	06	−06
12-*cis*-methyl-SB6	−27	18	03	02	00	01	00	00	00	01	01	**02**
12-*trans*-methyl-SB6	−27	18	03	02	00	01	00	01	−00	00	02	**01**
N-methyl-SB6	**−26**	19	01	02	01	01	00	01	01	01	05	−05
4,8-dimethyl-SB6	−27	17	−02	**13**	−03	00	−05	**11**	−03	−00	06	−06
12,12-dimethyl-SB6	−28	18	03	02	00	01	−00	00	−00	01	−04	**07**
N,4,8-trimethyl-SB6	**−25**	19	−05	**13**	−03	00	−05	**10**	−03	−01	06	−06
4,8,12,12-tetramethy-SB6	−27	17	−03	**13**	−03	00	−05	**10**	−04	−00	−04	**07**
N,4,8,12,12-pentamethyl-SB6	−26	19	−05	**12**	−03	00	−05	**10**	−04	−00	−04	**06**
N-methyl-retinal Schiff base	**−26**	19	−05	**12**	−04	00	−06	**10**	−03	−06	10	**−02**

[a] Every reported charge is the summation of the atomic charges of the main chain atom and hydrogen(s) and/or methyl group(s) connected to it. The charges of the substituted fractions are shown in bold face.

TABLE 3: Bond Distances (in angstroms) of the Protonated Species of Different Schiff Bases Calculated at B3LYP/6-31G Level of Theory for Unsubstituted (PSB6) and Different Substituted Species**

species	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11
PSB6	1.331	1.387	1.396	1.397	1.387	1.406	1.380	1.418	1.369	1.437	1.348
4-methyl-PSB6	1.333	1.386	1.407	1.413	1.384	1.410	1.378	1.419	1.368	1.438	1.348
5-methyl-PSB6	1.331	1.388	1.396	1.407	1.397	1.408	1.380	1.419	1.368	1.437	1.348
7-methyl-PSB6	1.331	1.387	1.396	1.398	1.389	1.418	1.388	1.419	1.369	1.437	1.348
8-methyl-PSB6	1.332	1.386	1.397	1.396	1.391	1.404	1.391	1.434	1.366	1.439	1.347
12-*cis*-methyl-PSB6	1.333	1.386	1.398	1.395	1.391	1.404	1.383	1.414	1.373	1.430	1.359
12-*trans*-methyl-PSB6	1.333	1.385	1.398	1.395	1.391	1.403	1.383	1.413	1.373	1.429	1.356
N-methyl-PSB6	1.326	1.393	1.391	1.401	1.386	1.409	1.378	1.420	1.368	1.438	1.348
4,8-dimethyl-PSB6	1.334	1.385	1.408	1.412	1.387	1.408	1.389	1.436	1.365	1.440	1.347
12,12-dimethyl-PSB6	1.334	1.384	1.400	1.393	1.393	1.401	1.385	1.410	1.377	1.424	1.367
N,4,8-trimethyl-PSB6	1.329	1.391	1.403	1.416	1.384	1.411	1.387	1.437	1.364	1.441	1.347
4,8,12,12-tetramethyl-PSB6	1.338	1.381	1.414	1.405	1.393	1.402	1.396	1.427	1.373	1.428	1.366
N,4,8,12,12-pentamethyl-PSB6	1.332	1.388	1.406	1.411	1.388	1.406	1.392	1.430	1.371	1.430	1.364
N-methyl-retinal Schiff base	1.333	1.387	1.407	1.410	1.389	1.404	1.394	1.431	1.374	1.446	1.379

from the application of the larger basis set (6-31G**) for the conjugated Schiff base structures.

In both protonated and unprotonated species, the methyl substitution causes some changes in the bond distances for the main chain of the polyene. These changes are, however, more pronounced for the protonated species. The most significant change, in both protonated and unprotonated species, can be seen for the bonds connecting the substituted atom to the neighboring atoms in the main chain. Substitution on a carbon atom in the main chain by a methyl group results in the elongation of the bond distances on both sides of the carbon atom. This may be partly related to the stabilization effect of the methyl group on the positive charges located on the respective atom which results in higher weights for some of the mesomeric structures (Figures 2 and 3), especially for the protonated species. However, this effect turned out to be remarkably localized to the substituted component of the chain and not all of the bond distances along the polyene chain were significantly influenced by a methyl substitution in one part of the chain. Therefore, probably the steric effects of the substituted methyl group play a more important role with regard to the observed elongation of the bond distances.

The bond angles and dihedrals are also important structural aspects of the conjugated Schiff base structures. The planarity of the main chain of the polyene is necessary for the maximum conjugation of the double bonds. Especially in the protonated

TABLE 4: Bond Distances (Å) of the Unprotonated Species of Different Schiff Bases Calculated Using B3LYP/6-31G Level of Theory for Unsubstituted (SB6) and Different Substituted Species**

species	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11
SB6	1.284	1.452	1.356	1.437	1.361	1.434	1.361	1.436	1.357	1.446	1.344
4-methyl-SB6	1.286	1.452	1.363	1.452	1.359	1.437	1.360	1.437	1.357	1.446	1.344
5-methyl-SB6	1.284	1.453	1.355	1.450	1.368	1.434	1.362	1.436	1.358	1.446	1.344
7-methyl-SB6	1.284	1.452	1.355	1.439	1.360	1.448	1.369	1.437	1.359	1.446	1.344
8-methyl-SB6	1.284	1.452	1.356	1.437	1.362	1.434	1.369	1.450	1.356	1.448	1.344
12-*cis*-methyl-SB6	1.248	1.452	1.356	1.437	1.361	1.434	1.361	1.435	1.359	1.445	1.350
12-*trans*-methyl-SB6	1.284	1.452	1.356	1.437	1.361	1.434	1.361	1.436	1.358	1.444	1.347
N-methyl-SB6	1.280	1.450	1.356	1.437	1.361	1.434	1.361	1.436	1.357	1.446	1.344
4,8-dimethyl-SB6	1.286	1.452	1.363	1.451	1.360	1.437	1.368	1.451	1.356	1.448	1.344
12,12-dimethyl-SB6	1.284	1.452	1.356	1.436	1.361	1.433	1.362	1.435	1.360	1.443	1.355
N,4,8-trimethyl-SB6	1.283	1.450	1.364	1.451	1.361	1.437	1.368	1.450	1.356	1.448	1.344
4,8,12,12-tetramethy-SB6	1.287	1.451	1.364	1.451	1.361	1.436	1.369	1.449	1.358	1.445	1.354
N,4,8,12,12-pentamethyl-SB6	1.283	1.449	1.364	1.451	1.361	1.436	1.369	1.449	1.358	1.445	1.354
N-methyl-retinal Schiff base	1.283	1.450	1.364	1.450	1.361	1.436	1.369	1.453	1.359	1.466	1.367

species, this is essential for the compensating effect of the conjugated double bonds on the positive charge of the Schiff base group (C═N) and, subsequently, on the PA of the Schiff base.[48] Any strong steric interaction between the substituted groups in the retinal Schiff base (for example steric interaction between the substituted methyl groups and the adjacent hydrogen atoms) or between the retinal Schiff base and the environment may induce a twist in the planar structure of the chromophore, which, in turn, significantly influences the pK_a of the Schiff base.[14,48,49]

In none of the models studied, methyl groups could induce a twisted conformation in the structure of the polyene. Within the applied theoretical model, the conjugated Schiff base model maintained its planar structure, in all cases, after introduction of the methyl groups and the steric interactions of the methyl groups with the adjacent hydrogen atoms were, to some extent, compensated by the change in the valence angles of the main chain. This effect resulted in a banana shaped structure of the backbone of the molecule.

Crystallographic studies have shown that the retinal molecule, as well as the protonated retinylidene Schiff base molecule, adopts a banana-shaped structure.[61−63] This can be explained, on one hand, by the large steric interaction of the substituted methyl groups on the main chain of the polyene and, on the other hand, by the significant barriers to the rotation of the conventional single bonds[49] which could compensate for such steric interactions. Therefore, since the polyene cannot easily rotate around the single bonds, the methyl steric interactions have to be partly compensated by the adoption of the known banana shaped backbone and not a twisted structure. In our study, substitution of a methyl group on the C_n atom of the main chain caused a significant decrease of the valence angle at the C_n atom (<CCC in the main chain) and an increase of the valence angles at C_{n+1} and C_{n-1} atoms. In the case of the complete retinal Schiff base, the large β-ionone ring substitution additionally influences the valence angles and dihedrals at the terminal region of the polyene structure.

Almost all of the dihedral angles of the main chain were calculated to be 180°±1° in both protonated and unprotonated species of all models. The only exception to this general rule was the model for the complete retinal Schiff base structure. In both protonated and unprotonated species of the retinal Schiff base, we observed slight deviations from the planar structure for the B9, B10, and B11 (1.5°, 11.5° and 4.5° in unprotonated species and 1.5, 9.0, and 7.5 in protonated species, respectively). These bonds are located very close to the β-ionone ring, where large steric interactions are present. Because of the single bond nature of B10, as compared to the double bond natures of B9 and B11, the amount of twist is predictably larger for this bond in both the protonated and unprotonated species of the retinal Schiff base. In the protonated retinal Schiff base, however, because of the more double-bond character, B10 demonstrates less deviation from the planar structure. B9 and B11, on the other hand, have less double-bond character in the protonated species and show, therefore, slightly more deviations from planarity.

Although the steric repulsion of the methyl groups will be compensated to some extent by the adoption of a banana shaped structure in the retinal Schiff base, the introduced structural strains result in an elevation of the energy of the planar form, relative to the twisted structures of the polyene. Consequently, the effect of the methyl groups can also be considered as a way to reduce the barrier against rotation of different bonds in the chromophore. Furthermore, the importance of the methyl groups with regard to stabilization of the positive charge located on the even-numbered carbon atoms should also be considered in the transition state of the rotation of different bonds. The methyl groups have significant effects on the stability of these transition structures by the stabilization of the positive charge which will be increased on the carbon atoms in the 90° rotated species of the double-bond rotations.[49,51] Therefore, the methyl groups can significantly decrease the energy gap between the planar structures (cis or trans) and the 90° rotated transition states of the rotation of different bonds, thus decreasing the isomerization barriers. In the retinal Schiff base, these isomerization processes are of great importance with respect to the photoisomerization events as well as to the ground-state rotation of double bonds. These isomerization processes constitute, for example, critical steps in the bR photocycle[1−5] and for the retinal photoisomerization in rhodopsin.[64] The importance of the methyl groups has been discussed in relation to the experiments studying the excited-state potential energy surface of isomerization in different isomers of the retinal.[65,66]

The presence of the methyl groups may be an interesting point of discussion concerning the interaction of the chromophore with the protein environment. Due to the substantial steric interactions between the binding pocket and the chromophore, it is quite possible that the chromophore adopts a twisted structure. As compared to the isolated system, the location and extent of this twist, which can influence the pK_a of the chromophore, can be controlled by the methyl substitutions, especially at atoms C_9 and C_{13} of the retinal Schiff base (corresponding to atoms C_4 and C_8 in our model). This is also important for the coupling of the conformational changes of the protein and the chromophore. The location of the methyl groups on the polyene chain is of utmost importance in determining the overall shape of the retinal ligands.[67] These structural effects, added to the dominant steric and electronic restrictions of the binding

TABLE 5: Proton Affinities (kcal/mole) Calculated at the B3LYP Level of Theory Using Different Basis Sets for Unsubstituted (PSB6) and Different Substituted Species as Well as for the Retinal Schiff Base (with and without Including Zero-Point Energies, zpe)

species	6-31G*	6-31G* (zpe)	6-31G**	6-31G** (zpe)
PSB6	260.61	251.64	262.66	253.69
4-methyl-PSB6	261.44	252.60	263.54	254.70
5-methyl-PSB6	261.03	252.11	263.10	254.18
7-methyl-PSB6	261.32	252.40	263.39	254.47
8-methyl-PSB6	261.39	252.51	263.49	254.61
12-*cis*-methyl-PSB6	262.91	254.04	265.00	256.14
12-*trans*-methyl-PSB6	263.41	254.52	265.51	256.63
N-methyl-PSB6	260.46	251.03	262.52	253.10
4,8-dimethyl-PSB6	262.13	253.38	264.29	255.53
12,12-dimethyl-PSB6	264.92	256.08	267.09	258.24
N,4,8-trimethyl-PSB6	261.76	252.50	263.92	254.66
4,8,12,12-tetramethyl-PSB6	266.08	257.46	268.33	259.71
N,4,8,12,12-pentamethyl-PSB6	265.31	256.14	267.54	258.38
N-methyl-retinal Schiff base	266.45	257.26	268.72	259.52

pocket,[28,29] would explain the discrimination exhibited by the protein binding site for different analogues during incubation studies.[67] These effects can also influence the rate of the photoisomerization and dynamics of the ground and excited states of the retinal Schiff base.[28−31]

3.4 Proton Affinity. One of the main objectives of the present study was to determine the effect of the different methyl substitutions on the PA of the Schiff base model. In this way, we may estimate the effect of the methyl groups on the pK_a of the retinal Schiff base. The PA values calculated for different Schiff base models in the present study are compiled in Table 5. The PA values have been calculated on the basis of the difference of the electronic energies of protonated and unprotonated species, respectively, using the B3LYP functional and two different basis sets. To examine if and to what extent the inclusion of the zero point energies may influence the conclusions, the PA values have been calculated with and without inclusion of the zero point corrections to the energy, respectively. The evaluation of the zero point corrections requires the calculation of the second derivatives of the energy with respect to the coordinates which is usually expensive and, for large systems, such as the complete retinal Schiff base, computationally prohibitive. With this regard, the presented results of this work may provide benchmark calculations for the whole set of Schiff base models studied.

It is generally accepted that polarization functions on hydrogen atoms are necessary to describe the relative stability of unprotonated and protonated species. To examine the effect of the applied basis set, we performed the geometry optimization and the frequency calculation for all of the Schiff base models with both the 6-31G* and 6-31G** basis sets, respectively. As can be seen in Table 5, the application of the larger basis set, 6-31G**, increases the calculated PA values by 2.05−2.25 kcal/mol, in all cases. A very similar systematic shift of PA calculated using different basis sets has also been reported in a previous study.[48]

Thermodynamic corrections, on the other hand, introduce a constant decrease of 8.60−9.45 kcal/mol to the PA values (Table 5). Although utilization of polarization functions on hydrogen atoms and/or inclusion of zero point energy corrections can significantly influence the absolute PA values, the PA changes are constant (Table 5) and, therefore, can be safely neglected in comparative studies. For this reason and because of the cost of the calculations when using the larger basis set, especially with respect to the calculation of the second derivatives of the energy, relative PA values can be estimated on the basis the total energy differences calculated at the B3LYP/6-31G* level of theory. The same conclusions have been drawn in a previous study calculating PA values for a set of conjugated Schiff base models with different lengths of the polyene chain.[48]

As it can be observed in Table 5, in all cases substitution of a methyl group on the main chain results in an increase of the calculated PA. This can be related to the hyperconjugation effect of the methyl group which stabilizes the positive charge of the protonated species. For the methyl substitution on the nitrogen atom, however, an opposite effect can be observed. In this case, after the addition of a methyl group on the nitrogen atom, the PA value decreases. This behavior can be explained by a larger weight of the resonance structure **A** for the protonated species (Figure 2), after addition of a methyl group to the nitrogen atom, which increases the localization of positive charge on the proton-carrying fraction (nitrogen atom and its substitutions). Comparison of the charges on the nitrogen atom in *N*-methyl-PSB6 species (+0.14e) and unsubstituted PSB6 (+0.09e) clearly confirms this point. This effect is, however, smaller than the increasing effect of methyl groups on the PA, when substituted on carbon atoms. The PA of *N*-methyl-PSB6 is about 253.10 kcal/mol which is only 0.6 kcal/mol less than the corresponding value of 253.69 kcal/mol for PSB6.

Comparison of the position of the methylation in different monomethylated species shows that methylation on the odd-numbered carbon atoms, C_5 and C_7, has a smaller influence with regard to increasing the PA, as compared to the effect of the methyl substitution on even-numbered atoms (C_4, C_8, and C_{12}). Among the even-numbered atoms, substitutions on C_{12} demonstrate a significantly larger effect. Addition of a methyl group on C_{12}, at cis or trans position, results in an increase of about 2.45−2.94 kcal/mol in PA, whereas C_4 and C_8 methylation increases the PA only by 0.99 and 0.92 kcal/mol, respectively. It can be inferred that the substitution at the terminal part of the main chain (C_{12} in our model) has the most pronounced effect with regard to the PA enhancement. In the retinal Schiff base chromophore, this part of the conjugated chain is a segment of the β-ionone ring and can be considered as being largely substituted. Comparison of the doubly substituted species also confirms the larger effect of the substitution at the C_{12} atom. For example, 12,12-dimethyl-PSB6 has a PA of 258.24 kcal/mol, whereas the PA of 4,8-dimethyl-PSB6 is found to be 255.53 kcal/mol. The PA calculated for the 12,12-dimethyl-PSB6 is only 1.28 kcal/mol different from the PA of the retinal Schiff base.

In the protein environment, the steric effects of the methyl groups seem to have more importance. The steric interaction, in this case, can be considered not only with the neighboring hydrogen atoms in the chromophore itself, but also with the amino acid residues in the binding pocket. The steric interaction of the binding pocket with the retinal Schiff base may influence the structure of the chromophore. There are experimental and theoretical studies which propose a twisted structure of the retinal Schiff base in the bR protein environment. The biphasic nature of the CD spectrum of the retinal Schiff base in bR has been explained on the basis of adoption of a twisted structure by the chromophore.[68] In a very recent paper, more evidence for the nonplanarity of the retinal Schiff base structure in the protein environment have been reported after the analysis of the optical rotation of the second harmonic radiation from retinal in bR monomers in Langmuir−Blodgett film.[69] Polarized infrared spectroscopy studies also suggest the existence of distortions around different dihedral angles along the retinal

chain.[70,71] It has been reported that the protonated retinal Schiff base chromophore is able to perform ground-state rotation around the C_{13}═C_{14} (B3 in our model) double bond in solution and in the protein environment. This ground-state isomerization event happens during the last step of the bR photocycle as well as in the dark adaptation of the pigment and requires a low barrier to the rotation of the B3 double bond. Accordingly, because of the low barrier against the rotation of the B3 double bond, it is quite possible that the retinal structure acquires a twisted structure at the C_{13}═C_{14} double bond in the protein environment. It has been suggested that this twist may have a significant effect on the control of the pK_a of the retinal Schiff base in bR.[49] With this respect, the potential importance of the methyl substitution at C_{13} can be discussed with regard to its steric interaction with the protein environment.

Comparison of the PA calculated for the retinal Schiff base and that of the PSB6 shows that complete consideration of the chromophore structure results in a significantly higher PA value. The PA of the retinal Schiff base is calculated to be 259.52 kcal/mol at the B3LYP/6-31G** level of theory including zero-point energy corrections, which is 5.83 kcal/mol larger than the corresponding value of 253.69 kcal/mol the for the PSB6 model. This difference in PA corresponds to a pK_a difference of about 4.2 pH units. Therefore, in the quantitative analysis of the PA or in the study of the potential energy surface of the proton transfer process, the inclusion of the methyl groups in the applied model is important. In other words, neglecting the methyl substitutions may result in different relative stabilities for the hydrogen bonded counterparts, thus influencing the potential energy surface of the proton transfer between them. For example, for the simulation of the proton transfer between a Schiff base model and a model for the aspartate group, as proposed for the binding pocket of the bR, the application of an unsubstituted model resulted in a different potential for proton transfer from the Schiff base group to the negatively charged aspartate group. As a matter of fact, setting up a suitable and stable model for this ion pair (a model of retinal Schiff base and a model of an aspartate group) was one of the motivations for the present study. With this regard, we would like to stress that consideration of the dielectric effect of the media is of utmost importance.[16−20]

With regard to the closeness of the PA of the model to the PA of retinal Schiff base, it is expected that the pentamethylated species demonstrate the closest value to the retinal Schiff base. However, among the studied models, the tetramethylated species demonstrates the closest PA value to that of the retinal Schiff base. The PA of 4,8,12,12-tetramethyl-PSB6 is calculated to be 259.71 kcal/mol which is only about 0.2 kcal/mol different (higher) from the corresponding value for the retinal Schiff base (259.52 kcal/mol). This observation can be explained by the following reason. In the retinal Schiff base, it is expected that the complete β-ionone ring has a slightly larger stabilization effect of the positive charge of the main chain than only two methyl substitutions on C_{12} which are considered in the tetramethylated and pentamethylated species. This should result in a slightly higher PA for the retinal Schiff base. However, for the tetramethylated species, the lack of any substitution on the nitrogen atom compensates for such differences and, therefore, the PA of the tetramethylated species is even slightly higher than retinal Schiff base.

Conclusions

The effects of the methyl groups on structure, charge distribution, and proton affinity of the retinal Schiff base have been studied at the B3LYP/6-31G* and B3LYP/6-31G** levels of theory. The results show that, for all of the Schiff base models studied, the application of the smaller basis set (6-31G*) results in the structures and charges which are very close to the corresponding ones obtained from utilization of the larger basis set (6-31G**). With regard to the proton affinity, the application of different basis sets results in a systematic shift in the PA. This can be safely neglected while examining relative PA values. We have also studied the effect of the inclusion of the zero-point energies, calculated at the same level of theory, on the PA of all models. The results show that the zero-point energy corrections to the total energy cause a systematic decrease in the PA. Therefore, in comparative studies, the PA values calculated at B3LYP/6-31G* level of theory can be used without inclusion of the zero point energies.

Methyl substitutions increase the PA of the Schiff base models. This effect is more pronounced for the substitutions on the even-numbered carbon atoms of the main chain and is found to be maximum for the substitutions on the terminal atom of the conjugated chain (C_{12}). With this regard, to calculate the PA of the retinal Schiff base and/or to address the problem of proton transfer potential surfaces, the presence of at least two methyl groups, substituted on the terminal carbon atom of the conjugated double bonds, is of essential importance for the applied model.

The effects of the methyl substitutions on the structure of the retinal Schiff base are also significant. In this case the substitutions on C_4 and C_8 are of great importance. The methyl groups influence the overall shape of the chromophore. Furthermore, these groups have a significant stabilization effect on the positive charge of the main chain. The positive charge which is distributed along the whole conjugated chain, will be localized on the carbon atoms during the rotation of the double bonds. Therefore, the methyl groups potentially decrease the barrier to the isomerization of different bonds in the retinal Schiff base. The importance of the methyl groups in the determination of the location and extent of the twist in the main chain of the retinal Schiff base should also be mentioned. These factors significantly influence the pK_a of the retinal Schiff base. Accordingly, the protein environment can use the steric interactions with the methyl groups in order to fine-tune these twists and, therefore, influence the pK_a of the chromophore. In conclusion, the results show that the methyl groups are very important structural aspects of the retinal Schiff base chromophore and have essential influences on different biological roles of this small machinery.

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