A Mechanism for Controlling the pK_a of the Retinal Protonated Schiff Base in Retinal Proteins. A Study with Model Compounds

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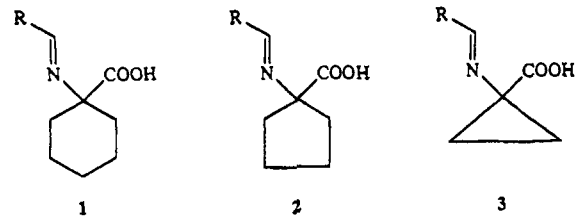
The membrane protein pigments rhodopsins are responsible for a variety of photobiological functions, such as visual transduction (visual rhodopsins, Rh), photosynthesis (bacteriorhodopsin, bR, and halorhodopsin), phototaxis (sensory rhodopsin) and photoisomerization (retinochrome). All of these rhodopsins consist of a similar retinyl polyene (all-trans in bR and 11-cis in Rh), bound to the opsin via a protonated Schiff base with a lysine ϵ-amino group. Light absorption induces a sequence of events which lead to a Schiff base deprotonation process.[1] The latter is crucial for the proton pumping in bR and for activating rhodopsin. Thus, understanding the factors that control the pK_a of the retinal protonated Schiff base in the various pigments and their photochemically induced intermediates is of primary importance. In bR, an apparent pK_a of 13.3 ± 0.3 was observed for the protonated Schiff base,[2] whereas in bovine rhodopsin a recent work pointed to the possibility of apparent pK_a higher than 16.[3]

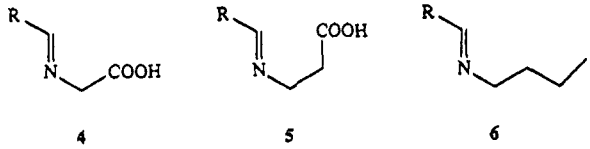
In the present study, we demonstrate that the pK_a of retinal protonated Schiff base can be significantly altered by forming a definite angle between the protonated Schiff base linkage and a carboxylate group which allows for effective interaction with one or more water molecules bridging the two groups.

All-trans retinal was condensed in trifluoroethanol with 1-amino-1-cyclohexane carboxylic acid, 1-amino-1-cyclopentane carboxylic acid, 1-amino-1-cyclopropane carboxylic acid, glycine, β-alanine, and *n*-butylamine to form Schiff bases **1–6**. In methylene chloride, all the Schiff bases exist as the nonprotonated forms, as is evident from their absorption maxima (Table I). We note that the absorption maximum of Schiff base **1** was unusually blue-shifted. A possible explanation is associated with a defined conformation in **1**, which introduces repulsion between the lone pair nitrogen electrons and the carboxyl oxygen, destabilizing mainly the retinal Schiff base excited state.

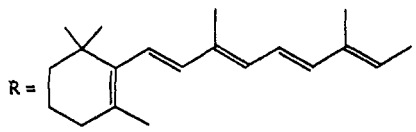
It has been shown that reverse micelles composed from sodium bis(2-ethyl)sulfosuccinate (AOT) dissolve retinal Schiff base in nonpolar solvent, and hydrolysis is prevented even in the presence of water molecules, probably due to the special nature of bound water.[4] Therefore, to evaluate the effect of bound water on the pK_a of protonated retinal Schiff base, we introduced Schiff bases **1–6** into 0.1 M AOT dissolved in methylene chloride. The apparent pK_a of chromophores **1–6** in the reverse micelles using ω([H_2O]:[AOT]) = 5 (in which only bound water exists[5]) was obtained by measuring the absorption maxima of the chromophores, following addition of appropriate buffers. The results are summarized in Table I. The apparent pK_a of compound **1**



1 **2** **3**



4 **5** **6**



is remarkably high (12.3 ± 0.1), approaching the value observed for bR (13.3 ± 0.3). The pK_a is lowered as the ring connecting the carboxyl and the Schiff base groups is altered, and it is influenced as well by the length of the chain connecting the two functional groups, as is evident in compounds **4** and **5**. The important role that the bound water plays and the differences between the pK_a values of the protonated Schiff bases of the various compounds can be judged from the absorption maxima of chromophores **1–6** in ω = 0. Due to the hygroscopic nature of AOT, it contains bound water even without the addition of water. This conclusion is supported by Fischer test,[6] which indicates 0.1% water in AOT, even after vacuum treatment (0.5 mmHg) over P_2O_5. The Schiff base group of compound **1** is completely protonated by the carboxyl group, even in ω = 0, whereas in compounds **2–5** the Schiff base group is only partially protonated. Proton transfer from the carboxyl group to the Schiff base moiety occurs only following addition of water.

The remarkable difference between the apparent pK_a of chromophore **1** relative to chromophores **2–6** is probably due to a specific angle between the protonated Schiff base linkage and the carboxylate group which allows for efficient binding between the ion pair and one or more water molecules, which stabilize the protonated form relative to the nonprotonated one. Interestingly, FTIR measurement of **1** in AOT with ω = 5 reveals a C=N stretching frequency of 1640 cm^{-1} (similar to that of bacteriorhodopsin[7]), which reflects a weak hydrogen bonding with the N—H moiety. Therefore, we propose that in **1** the bridging water molecules form strong hydrogen bonds with the carboxylate and weak hydrogen bonds with the Schiff base moiety. The size of the ring affects the angle between its substituents and their specific conformation. Thus, although we cannot determine the exact angle between the carboxyl group and the Schiff base linkage in the various compounds, it is conceivable that the different ring size in addition to different interactions with the neighboring ring hydrogens impose a different angle and conformation in each compound. In addition, a different strain prevails in the various ring structures and may contribute to the observed alteration in pK_a values. In the retinal-β-alanine complex **5**, the carboxylate group and the protonated Schiff base linkage adopt a better conformation for efficient water bridging than that of glycine complex **4**, due to the longer aliphatic chain connecting

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Table I. Absorption Maxima and Apparent pK_a Values

compd	λ_{max} (nm) CH_2Cl_2	AOT ($\omega = 0$)	AOT ($\omega = 5$)[a]	apparent pK_a[b]
1	340	440	450	12.3 ± 0.1
2	386[c]	400	448	9.5 ± 0.1
3	386[c]	405	450	8.0 ± 0.1
4	380[c]	402	444	8.5 ± 0.1
5	375[c]	402	442	10.0 ± 0.1
6	360	360	440	7.5 ± 0.1

[a] Absorption of the protonated Schiff base species. [b] pK_a of the protonated Schiff base species measured in reverse micelles with $\omega = 5$. [c] The absorption is concentration dependent and was measured at 0.5×10^{-4} M concentration of chromophore.

the two groups in **5**. This conclusion is derived from the higher pK_a of **5** (10 ± 0.1 vs 8.5 ± 0.1). Removal of the carboxyl group lowers the apparent pK_a of the protonated retinal Schiff base close to its value in methanol–water solution, as observed in compound **6** (7.5 ± 0.1). Further insight into the exact factors that control the pK_a in these compounds should be gained by future studies.

Various models have been suggested to account for the high apparent pK_a (13.3 ± 0.3) observed in bR. A relatively polar protein environment surrounding the protonated Schiff base was suggested by Warshel et al.[8] A different approach attributed the change in the pK_a during the photocycle to a twist around the C_{14}-C_{15} single bond.[9] Other mechanisms attributed the stabilization to effective hydrogen bonding of protein residues and/or bound water mainly with the negatively charged counterion[10] and destabilization following light absorption due to charge separation.[11] The involvement of water in stabilizing the protonated Schiff base was suggested by DuPuis et al.,[12] by Hildebrandt and Stockburger[13] on the basis of resonance Raman study of dried membranes, and the presence of water in the binding site by de Groot et al. on the basis of ^{15}N NMR studies.[14] The importance of the relative orientation of the protonated Schiff base and its counterion for proton transfer was suggested by Scheiner and Hildebrandt.[15] Based on the present model studies, we suggest that the apparent pK_a of the retinal protonated Schiff base in bR and in other retinal proteins can be significantly raised by a proper orientation between the Schiff base linkage and a carboxyl group, which allows for water molecules to adopt a definite structure and to form a bridge between the proton donor and acceptor groups, thereby stabilizing the ion pair. We note that recent neutron diffraction studies indicated that bound water is located in the interior of bacteriorhodopsin.[16] Following light absorption, the conformation of the Schiff base relative to the carboxyl group is altered, resulting in an inadequate angle between the two groups to form a stable structure with water molecules. The destruction of the stable structure and the efficient ion pair stabilization by the water molecules will cause a proton transfer from the Schiff base to the carboxyl group and will lead to formation of the M_{412} intermediate, which consists of retinal Schiff base. The retinal Schiff base in M_{412} might be further stabilized by hydrogen bonding with water molecules. FTIR studies and an electron density map established the presence of Asp 85 and Asp 212 in the Schiff base vicinity of bR, and it has been suggested that Asp 85 serves as the primary counterion to the retinal Schiff base.[17,18] Substitution of Asp 85 by a neutral residue alters the apparent pK_a of the protonated Schiff base and reduces it to 8.5.[19] This result is in keeping with a water bridge between Asp 85 and the Schiff base linkage. (Further stabilization of Asp 85 negative charge by electrostatic interaction with Arg 82 and by hydrogen bonding with protein residues is possible.) Removal of this group leaves Asp 212 as the primary counterion. It is possible that Asp 212 is strongly hydrogen-bonded to aromatic residues, preventing an efficient water bridge between Asp 212 and the protonated Schiff base linkage. Therefore, the pK_a in Asp 85 mutant is lower than that of the native system. Mutation of Asp 85 to glutamic acid modified the pK_a of the protonated Schiff base as well,[20] possibly due to the longer carbon chain which changed the orientation of the carboxyl group and the Schiff base linkage. In this respect, we note that in bovine rhodopsin, which has a very high protonated Schiff base pK_a,[3] substitution of Glu 113 with glutamine modified the Schiff base apparent pK_a to 6.5.[21] This result is in keeping, as well, with a very well defined structure including the protonated Schiff base, water molecules, and carboxylate ion prevailing in bovine rhodopsin, which is destroyed by the carboxylate mutation, leaving the retinal Schiff base as a regular Schiff base similar to chromophore **6** in AOT.

Finally, the possibility of modifying the pK_a of the protonated Schiff base and controlling the proton transfer from the carboxyl group to the Schiff base by a defined structure of the donor–acceptor and water molecules offers a possible mechanism for proton transfer from various carboxyl groups in the protein to the retinal Schiff base. It has been suggested that Asp 96 is the proton donor to the retinal Schiff base, following M formation.[22] Thus, we suggest that following M intermediate formation, the Schiff base and Asp 96 adopt a conformation that allows water to efficiently bridge the two groups and to facilitate proton transfer by stabilizing the ion pairs.

In summary, we suggest that a specific orientation between the Schiff base and different carboxyl groups, which allows water molecules to form a defined structure and to bridge the groups, is formed in the bR ground state and in the various photochemically induced intermediates. These structures stabilize different ion pairs and induce a proton transfer to the Schiff base from a specific carboxyl group. Our studies are in agreement with an earlier suggestion that the angle between the donor and acceptor groups plays a role in proton transfer.[15]

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