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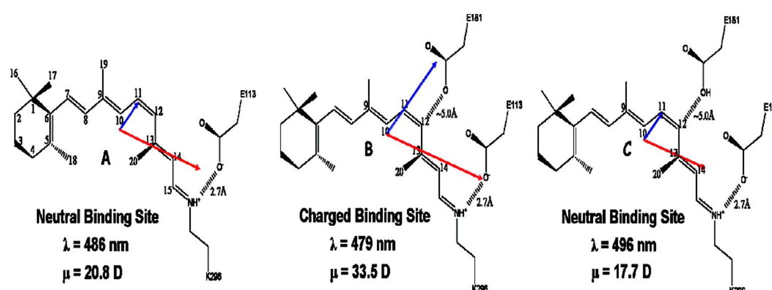

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Glutamic Acid 181 Is Uncharged in Dark-Adapted Visual Rhodopsin

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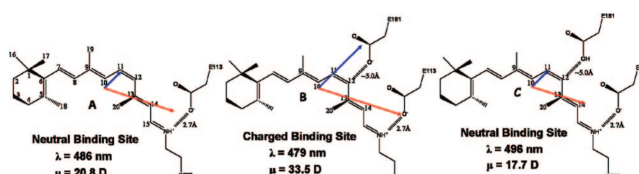
A general feature of the retinal proteins, whether of animal or archeal origin, is the binding of the chromophore to the ϵ-amino group of a Lys side chain of the protein. In the vertebrate visual protein rhodopsin (Rh) the chromophore is 11-*cis*-retinal which is bound as a protonated Schiff base (**pSb11**) to K296 inside the protein binding pocket. The chromophore absorbs at 498 nm as a consequence of a robust electrostatic interaction with a negatively charged glutamate counterion (E113). The counterion balances the positive charge of the chromophore *via* a strong H-bonded network involving the Schiff base nitrogen atom which has been found to remain intact in bathorhodopsin, the first 45 nm red-shifted photointermediate of the visual cycle.[1−5]

In contrast, X-ray crystallographic and NMR evidence established the existence of a counterion complex in archeal rhodopsins (bR, hR, sRI, and sRII), which encompasses, in addition to H-binding water molecules, several ionized side chains.[6] Consequently the binding pocket in these species is negatively charged. A second ionisable glutamate, E181 which was found close to E113 and to the isomerizing C11═C12 double bond in vertebrate rhodopsin, has stimulated the curiosity of researchers who envisage a charged binding pocket similar to archeal rhodopsin. Interestingly, the counterion of invertebrate rhodopsins was found to be E181.[7] Exceptions are the long-wavelength visual pigments like iodopsin and green and red cone pigments that have His which with a nearby Lys can serve as a Cl^- binding site.[8]

In vertebrate rhodopsin, the ionization state of E181 has not been established unequivocally. Two photon spectroscopy studies,[9] 3-D crystal structure,[3,4] and site-specific mutagenesis studies involving preresonance Raman vibrational spectra of the unphotolyzed E181Q mutant found E181 to be uncharged (protonated) in the dark state[10] paving the way for earlier QM/MM studies on visual rhodopsin to assume E181 to be neutral.[11] The findings based on helix translocation resulted in a counterion-switch mechanism, where E181 being uncharged or protonated in rhodopsin can transfer a proton via a H-bonded network to the primary counterion, E113, during the formation of Meta I. The photobleaching sequence of the UV pigments has also evolved to include a counterion-switch mechanism providing support to this theory.[12] Arguments in favor of a deprotonated side chain close to the C12─C13 bond have been put forward by NMR[13−15] and FTIR[16] studies, with molecular dynamics simulations,[17−19] and have only raised the ongoing debate about the protonation state of E181.

Nearly 30 years ago the "external point charge model" was proposed by Nakanishi and co-workers to rationalize the spectral shifts in natural rhodopsins due to point charges in the protein binding pocket and their location relative to the chromophore chain.[20] Although the validity of that model and the identity of the external point charges with respect to their protein counterpart were unknown until the tertiary structures were clarified, it remains a generally accepted model for further theoretical studies.

Scheme 1. CASPT2 Calculated $S_0 \rightarrow S_1$ Absorption Maxima and Dipole Moments of Protonated 11-*cis*-Retinal Schiff Base (pSb11) in the Presence of Different Glutamate Residues[a]



[a] **A**, pSb11+E113⁻; **B**, pSb11+E113⁻+E181⁻; **C**, pSb11+E113⁻+E181. The dipole moment vectors are shown as blue (S_0) and red (S_1) arrows. The distances shown are based on the theoretically refined 2.2 Å crystal structure of rhodopsin from ref 4.

Using external point charges from an ab initio calculation of the complete rhodopsin binding pocket as a template, we have performed a high-level quantum chemical study of the chromophore to investigate how the protonation state of E181 affects the electronic properties of rhodopsin. Based on an analysis of static (dipole moments) and dynamic (transition probabilities) data of the chromophore we conclude that E181 is uncharged in the dark state of rhodopsin. Calculations pinpoint distinct property changes which might be susceptible to experimental verification.

The chromophore binding pocket is prepared based on the 2.2-Å crystal structure of rhodopsin (PDB:1U19),[4] and chain-B was chosen for our studies. The optimized structures are obtained by the combined QM/MM methodology, where SCC-DFTB[21] is used for the description of the QM and CHARMM force field for the MM regions.[22] The MM point charges for the rhodopsin environment (28 amino acids and 2 H_2O molecules) have been calculated using the sophisticated Natural Population Analysis (NPA) method[23] with B3LYP/6-31G** wave function. Ground- and excited-state energies were calculated by the CASSCF/CASPT2 method as provided by the MOLCAS set of routines.[24] Six-root state-averaged wave functions were expanded in an atomic natural orbital (ANO) basis set.[25] We have used the contraction of the present work C,N,O[4s3p1d]/H[2s] before in the studies of retinal model chromophores.[22,26] It was found to yield highly accurate excited-state energies, the average deviation being 0.05 eV. The active space is (12,12); i.e., all pseudo π-electrons and valence pseudo π-orbitals were considered. Second-order corrections to the CASSCF energies were obtained with CASPT2. All core orbitals were kept frozen during calculations, and the level shift to avoid the effect of intruder state was set uniformly to 0.3 au. For the oscillator strengths, CASPT2 corrected state energies were combined with transition dipole moments calculated by the CAS state interaction method with an error limit of ± 2.0 D.

Three different chromophore−counterion complex models were prepared: namely **A** consisting of pSb11 and the primary counterion (E113⁻); **B** consisting of **A** plus a charged E181; **C** consisting of **A** plus a neutral E181. In Table 1, the calculated ground- and excited-state energies, oscillator strengths, and dipole moments are listed for the complex in Scheme 1. The results for the **A**, **B**, **C** models are from pure high level quantum-mechanical calculations devoid of any protein matrix. The protein matrix in the form of NPA point charges is mounted onto **A** giving the **wild-type**, while

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Table 1. Calculated Energies and Dipole Moments μ of Retinal Chromophore Models[a,f]

model[a]	state	CASPT2[b,c]	μ	configuration[d]
A[e]	S_0	−1060.0803	10.22	$(6a)^2(7a)^0$ *65*
	S_1	58.8 (**486**) *0.82*	20.79	$(6a)^1(7a)^1$ *62*
	S_2	67.6 (423) *0.00*	10.03	$(6a)^0(7a)^2$ *29*
B	S_0	−1248.9914	27.42	$(6a)^2(7a)^0$ *66*
	S_1	59.7 (**479**) *0.86*	33.55	$(6a)^1(7a)^1$ *61*
	S_2	67.8 (422) *0.00*	27.49	$(6a)^0(7a)^2$ *29*
C	S_0	−1249.4122	7.41	$(6a)^2(7a)^0$ *65*
	S_1	57.6 (**496**) *0.81*	17.71	$(6a)^1(7a)^1$ *62*
	S_2	67.3 (425) *0.00*	7.24	$(6a)^0(7a)^2$ *29*
Wild-Type[e]	S_0	−1060.7835	9.64	$(6a)^2(7a)^0$ *65*
	S_1	57.0 (**502**) *0.79*	21.59	$(6a)^1(7a)^1$ *62*
	S_2	66.6 (429) *0.00*	9.64	$(6a)^0(7a)^2$ *29*
E181QMutant	S_0	−1060.7818	9.54	$(6a)^2(7a)^0$ *65*
	S_1	56.5 (**506**) *0.79*	21.33	$(6a)^1(7a)^1$ *62*
	S_2	66.5 (430) *0.00*	9.51	$(6a)^0(7a)^2$ *29*

[a] See text for abbreviations. [b] S_0 energies in au. [c] S_1 and S_2 energies relative to S_0 in kcal·mol^{-1}. [d] Only π-type MO's are counted; weights (*in italics*) are in %. [e] Reference 22. [f] In bold, wavelength (in nm), and in italics oscillator strength (*f*) of the allowed optical transition.

E181Q is a mutant binding pocket model derived from the wild-type. CASPT2 calculations are performed at a huge computational cost of 12−14 GB main memory, since we use the ANO basis sets which are constructed to optimally treat correlation and polarization effects and should be large enough to describe the electronic structure with sufficient accuracy.

Table 1 reveals the ground state is mainly closed shell in all cases, with 65% contribution from configuration $(6a)^2(7a)^0$, referred to as S_0 state. The excited state with an oscillator strength (f→0.8) always involves the HOMO to LUMO configuration $(6a)^1(7a)^1$, referred to as S_1 state, with the weight of 62%. The next higher state S_2 is mostly doubly excited HOMO to LUMO $(6a)^0(7a)^2$. The perturbational treatment lowers the energy of this state by, on average, 10 kcal·mol^{-1}. We have already discussed the spectroscopic properties of the distorted bare chromophore in vacuo compared to that of the chromophore−counterion pair models in our previous work.[22] Thus we focus on the calculations involving different chromophore/counterion (**A**, **B**, **C**) complexes. Negatively charged groups in the vicinity of N16 will destabilize the S_1 state resulting in an absorption maximum of 486 nm for **A**; i.e., they will cause a strong shift of the absorbance toward higher energy.

Introduction of charged E181 results in a secondary counterion (**B**) situated close to the C11═C12 bond (~5.0 Å). The extent of its interaction on the chromophore is very limited (−7 nm blue shift) compared to that of the primary counterion (**A**). This is reasonable considering that E181 is closer to the N-terminus than to the ionone terminus and thus induces effects similar (in sign) to those of E113. However, when E181 is uncharged (**C**), the absorbance is shifted by +10 nm resulting in 496 nm. Note in both cases the magnitude of the shift on either side remains within ±10 nm, validating the findings from DFT calculations[4] and $^{13}C-^{13}C$ *J*-coupling measurements[27] on the absence of polar perturbation on the chromophore electronic structure especially in the vicinity of the isomerization site. That the blue shift of −7 nm is so much less compared to that from the primary counterion is due to the fact that the large electronic change from the free chromophore to the counterion can be accomplished only once.

The key to unlocking the puzzle behind the protonation state of E181 lays in the orientation of the ground (S_0) and the excited state (S_1) electric dipole moments (Scheme 1). Contrary to general convention, the arrowhead points to the center of the negative charge with the origin fixed in the center of the nuclear framework. The moment decreases by almost 50% in the case of neutral binding site models (**A** and **C**) with values 20.7 and 17.7 D for the S_1 state compared to that of the S_0 state (10.2 and 7.4 D), a relative difference of ~10.0 D. In the case of **B**, as the chromophore becomes electronically excited a relative difference of only ~6.0 D is seen between S_0 and S_1 (27.4 vs 33.5 D). As the protein matrix is introduced (**wild-type**), the effect of polarization of the environment causes an increase in the relative difference of the dipole moment to 11.9 D (9.6 vs 21.5 D) in agreement with the 12.0 ± 2.0 D observed by Mathies et al. for pSb11/Cl$^-$.[28] We have also calculated the λ_{max} of the **mutant** model where E181 is replaced by a glutamine (**E181Q**) and the whole binding pocket is reoptimized fixing only the peptide backbone in space. The resulting chromophore structure and its electronic spectrum (506 nm, 11.8 D) in the mutant binding pocket is similar to the **wild-type** chromophore[10,29] (502 nm), as the calculated absorption spectra is +4 nm red-shifted compared to the +10 nm originally observed.[7,10] Therefore we rule out the presence of a charged E181 in the dark state and that the binding pocket of visual rhodopsin essentially remains electrostatically neutral.[9] In conclusion a model for resolving the protonation state of E181 is presented with the role of the orientation of dipole moments being brought to the forefront.

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