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Three-layer ONIOM studies of the dark state of Rhodopsin: the protonation state of Glu181

Katherine. F. Hall,^a Thom Vreven,^{b,} Michael J. Frisch,^b Michael J. Bearpark.^a*

Contribution from: ^aDepartment of Chemistry, Imperial College London, London SW7 2AZ, UK; ^bGaussian, Inc., 340 Quinpiac St Bldg 40, Wallingford, CT 06492, USA.

*thom@gaussian.com

Running Title: ONIOM Studies of Rhodopsin.

ABSTRACT

A computational three-layer ONIOM(QM-high:QM-low:MM) hybrid scheme has been applied to analyze the protonation state of the Glu181 amino acid residue in rhodopsin, which is vital to determining the rhodopsin photoactivation mechanism. Due to conflicting evidence from previous studies, it has yet to be conclusively resolved. In this study, we fully optimize dark state rhodopsin model structures differing only at the 181-residue site – protonated and unprotonated Glu181 – and calculate several experimentally observable properties. Comparison of calculated structures, excitation energies, and NMR chemical shifts for the two models with values from the literature allows a reevaluation of previously reported conclusions. A key finding is that the $S_1 \rightarrow S_2$ energy level splitting,

previously used as evidence for a neutral Glu181, is found to be almost identical for the two protonation states. We highlight a need for caution when interpreting experimental data. Small differences in the properties of the two model structures are also identified, which may be useful targets for future high-resolution experimental approaches.

Keywords: Rhodopsin, ONIOM, QM/MM, Protonation State, Computational.

Introduction

Rhodopsin is the photoreceptor pigment involved in monochromatic vision in vertebrates.¹ Until the recently published crystal structure of the human β_2 adrenoreceptor,²⁻⁴ rhodopsin was also the only G-protein coupled receptor (GPCR) for which a high-resolution X-ray structure was available.⁵⁻⁸ In contrast to the majority of GPCRs, the 11-cis retinal ligand in rhodopsin is covalently bound to the protein in the inactive state, acting as an inverse-agonist upon absorption of a photon. This makes rhodopsin an attractive prototype for understanding G-protein activation. As such, it has been the subject of extensive experimental and theoretical research, aimed both at understanding eye function and pathology, and also, importantly, the possible mechanistic extension to GPCRs in general. The pervasive involvement of GPCRs in normal biological processes makes them the target of 40-50% of modern medicinal drugs.^{9, 10}

In the fully dark-adapted (dark) state of rhodopsin, an 11-cis-retinal is attached via a protonated Schiff base (PSB) linkage to the ϵ -amino group of Lys296 in the binding pocket of the seven α -helical trans-membrane opsin protein. The opsin protein environment attenuates the absorption maximum of the retinal PSB, leading to an absorption maximum of 498 nm for the dark state rhodopsin, compared to 610 nm for isolated 11-cis retinal PSB in the gas phase,¹¹ and 450 nm for all-trans retinal PSB in organic solvents¹² (the spectrum of the 11-cis isomer in solution has not been reported). Following photoexcitation of the 11-cis chromophore, isomerization occurs to all-trans, starting a series of conformational changes within the protein. The phototransduction cycle proceeds via a number of photointermediates with distinctive absorption maxima

(figure 1), resulting in the formation of the activated metarhodopsin II intermediate (meta-II).¹³ Meta-II binds and activates the G-protein, transducin, resulting in a signaling cascade and, eventually, the elimination of retinal from the binding pocket (photo-bleaching), prior to reformation of rhodopsin from opsin and all-trans retinal in a multi-step process.¹⁴

Recently reported high resolution X-ray structures of dark-adapted rhodopsin have verified Glu113, situated on helix-III, as the primary counterion to the PSB and led to the characterization of other important binding pocket residues involved in photoactivation.⁵⁻

⁸ In particular, extra-cellular loop II (EII), which connects helices IV and V, and was previously thought to lie on the exterior of the protein, was unexpectedly found to form a plug that penetrates the retinal-binding pocket. EII contains a second carboxylic acid residue, Glu181, which lies just 4.7Å away from C₁₂ of the chromophore. Ser186, and Cys187 on EII, together with two crystallographic water molecules (Wat2a and Wat2b) form a hydrogen-bonded network, which connects Glu181 to Glu113 (figure 2).⁷

To determine a model for the functional roles of these residues, and the H-bonded network, in photoactivation, it is vital to ascertain the protonation states of Glu181, Glu113, and of the retinal Schiff base (SB), both in the dark state, and in each of the photointermediates. There is considerable spectroscopic evidence for a protonated SB, compensated by a charged Glu113 counterion, in the dark state.¹⁵⁻¹⁹ The protonation state of Glu181 in the dark state has not been confirmed, but pH dependence studies of the meta-I intermediate suggest that it is unprotonated, and becomes the primary counterion in this state.²⁰ It is also known that the SB becomes deprotonated in the meta-II active state: This is believed to be an essential requirement for rhodopsin to adopt the active

conformation, which binds transducin.^{21, 22} Based on this, and further evidence described below, there are currently two leading models for the details of the photoactivation mechanism, which differ primarily in the initial protonation state of Glu181:

Counterion-switch model²⁰ – Glu181 is protonated in the dark state and the proton is transferred to Glu113 during the transition from the dark state to the meta-I intermediate. This leads to Glu181 becoming the primary counterion in the meta-I state prior to neutralization of the salt bridge in the active meta-II state. It is proposed that this proton transfer is initiated by a relative motion of helix-III and EII following cis-trans isomerization, and mediated by the H-bonding network connecting the two glutamic acid residues.

Complex counterion model²³ – Both Glu113 and Glu181 are charged (unprotonated) in the dark state. A switch from Glu113 to Glu181 as primary counterion in the transition to the meta-I state therefore does not involve proton transfer, but only a relative motion of EII and helix-III.

The counterion-switch mechanism is supported by apparently converging spectroscopic evidence of a neutral binding pocket in dark state rhodopsin, indicating a protonated Glu181.^{18, 20, 24} Often cited are Birge and co-workers who analyzed the 2-electron UV-visible spectrum of a rhodopsin, containing a locked 11-cis chromophore, with respect to semi-empirical (INDO-PSDCI) calculations on small model chromophore systems differing in numbers and arrangements of counterions. The main conclusion of this study is that the retinal Schiff base is protonated. However, they report a second “somewhat more subtle” observation that, by comparison with their semi-empirical calculations, the magnitude of the splitting between the first and second excited states appears to be most

consistent with a neutral binding pocket containing a single PSB counterion.¹⁸ It should be noted that rigorous calculations were not possible at that time since the X-ray structure was not available. More recent evidence for a neutral Glu181 comes from pre-resonance Raman spectra of the E181Q mutant, which is found to be almost identical to the native pigment.²⁰ While this is suggestive of a neutral Glu181 in the dark state, the clear caveat is the assumption that the protonation state of Glu181 will affect the pre-resonance Raman spectrum. Nevertheless, based on these evidences, and the consistency with an observed deuterium isotope effect in the kinetics of photoactivation,²⁵ the current leading model of photoactivation is the counterion-switch.

However, a more direct probe of the carboxylic acid protonation states is provided by Fourier-transform infra red (FTIR) vibrational spectra, in which the distinctive C=O stretch bands of protonated Glu and Asp residues are visible in the uncrowded region above 1700 cm^{-1} . The case of rhodopsin is complicated by the presence of several carboxylic acid residues, but Ludeke et al²³ have used mutation studies to pick out individual C=O absorptions. These experiments indicate an unprotonated (charged) Glu181 residue in the dark state. Recent molecular dynamics (MD) simulations of the photoisomerization support this conclusion.^{26, 27} Martinez-Mayorga and co-workers performed state-of-the-art $2\text{ }\mu\text{s}$ timescale simulations of rhodopsin models, differing in the initial protonation state of Glu181. They showed that the initially unprotonated Glu181 model was most consistent with experimental evidence, based on the resultant meta-I structures and simulated NMR data. Furthermore, it has been suggested that anomalous NMR spectra at C₁₂ can be accounted for by a charged Glu181 residue.²⁸

There is clearly much contradictory evidence in the literature regarding the protonation state of Glu181 in the dark state. A further source of indirect information may be inferred from studies of other species from the rhodopsin family. Recently, Birge and co-workers²⁹ performed a study of the photoactivation efficiency in two UV pigments: a D108A mutant of *Xenopus* violet cone opsin (VCOP-D108A), and mouse UV pigment (MUV). They found markedly less efficient photoactivation in the former than the latter. Both species contain a Glu176 residue in the retinal binding pocket, which is the homology equivalent of Glu181 in rhodopsin. Semi-empirical (MNDO-PM3) calculations of the relative heats of formation for protonated and unprotonated Glu176 models of the two pigments, suggest that Glu176 is neutral (protonated) in VCOP-D108A and charged (unprotonated) in MUV. The authors therefore suggest that a charged residue at position 176 is important for efficient photoactivation, and, by inference, that the Glu181 residue is deprotonated in rhodopsin. However, it should be noted that, while excited state semi-empirical methods (such as MNDO-SDCI, and INDO-SDCI) have been shown to calculate reliable transition energies and oscillator strengths, PM3 is not a sufficiently accurate ground state method to definitively determine small relative energy differences, especially those due to small structural changes.

Following this discussion, the value of our current study becomes clear. Using a state-of-the-art computational approach, we are able to calculate, for both protonated and unprotonated Glu181 models, several of the experimental properties available in the literature – equilibrium geometry, vertical excitation energy to the first and second excited state, and NMR chemical shifts – to a high accuracy. We use these results to reanalyze and reinterpret the available experimental data. Furthermore, we are able to

identify key small differences in the properties of the two model structures, including differences in C–C bond-length alternation patterns in the retinal chromophore, which may be useful targets for future high-resolution experimental approaches.

We now briefly discuss the requirements of such a study and how our three-layer ONIOM(QM-high:QM-low:MM) approach provides the necessary abilities and accuracies. The problem of determining the protonation state of Glu181 in the dark state involves accurate computation of the ground state equilibrium geometry for the ~40 kDa protein, using a method able to incorporate a number of complex interactions between the chromophore, counterion(s) and other binding pocket residues, as well as water molecules. To usefully compare our data with experiment and draw reasonable conclusions we need also to calculate accurate ground and excited state spectroscopic properties – vertical excitation energies and NMR spectra. To achieve this at acceptable cost we use a combined QM/MM method. A QM/MM computational approach of rhodopsin allows a high level *ab initio* method to be used for the extended π -system of the chromophore and binding pocket residues, while the structure and energetic effects of the entire protein are included through a molecular mechanics calculation. In addition, we may choose a high-level QM method capable of calculating electronically excited states and NMR chemical shifts.

The success of hybrid QM/MM calculations applied to the study of rhodopsins has been widely reported. Wanko et al^{30, 31} have reviewed the various approaches, including both single point and MD calculations, with respect to different types of accuracy, indicating the particular success of hybrid density functional theory (DFT)/Hartree-Fock (HF) functionals such as B3LYP as the QM method, including the use of time-dependent

(TD) B3LYP to calculate excitation energies. The use of MD simulations versus single point calculations must be considered, and involves a trade-off between different types of computational accuracy. For an MD simulation, a more approximate, usually semi-empirical, method is used for each energy calculation (due to the need to calculate many thousands of points), but this reduced accuracy is off-set by an extended sampling of the configuration space. While MD simulations provide a valuable tool for studying protein structure, they are particularly suited to the consideration of kinetic effects, which is not our current focus. Single point QM/MM studies allow a higher-level QM treatment to be used because we perform a single point geometry optimization, requiring far fewer individual energy and gradient calculations than an MD simulation. Previous single point studies of rhodopsin have combined highly correlated ab initio methods, such as CASPT2//CASSCF, with AMBER force fields to calculate accurate excitation energies, map geometrical modes on the excited state,³²⁻³⁴ and ONIOM(B3LYP:AMBER) calculations have been used to simulate NMR data in excellent agreement with experiment.³⁵⁻³⁷ As yet, no single point QM/MM study has focused on the differences in structural or other properties caused by the protonation state of the Glu181 residue.

All previously reported studies use a 2-layer scheme, including only the retinal PSB and (part of) the attached Lysine residue in the QM-layer.³⁰⁻³⁹ (Gascon and Batista³⁶ expanded the QM-region to include several binding pocket residues in order to calculate NMR chemical shifts, but used a reduced QM-region for the geometry optimizations). Since the interaction of the counterion(s) with the retinal PSB determines the crucial energy differences we study here, it is desirable to extend the QM region to include these residues. In our current work we use a three-layer ONIOM(B3LYP:HF:AMBER) scheme

to optimize the dark state structures for the proposed models, initially protonated and unprotonated Glu181, together with the E181Q mutant structure. Our three-layer ONIOM method allows us to expand the QM region, at acceptable cost, without compromising on the level of theory used to model the chromophore. The chromophore and attached lysine are included at the B3LYP (*QM-high*) level, while several residues from the binding pocket, including the counterion(s), the hydrogen-bonding network, and two nearby aromatic residues are included at the HF (*QM-low*) level (figure 2). The remainder of the protein is treated using AMBER (MM). Thus, we incorporate the interaction of retinal with important binding pocket residues, at the ab initio level, offering considerable improvements in accuracy over previous computational studies.

Results and Discussion

In this section we present the geometrical, NMR, and excitation properties on Rhodopsin calculated with the schemes outlined in the Materials and Methods. We discuss each of the properties separately, and compare with experiment, and combine the results in the conclusions.

I. Optimized Geometries

General fit to X-ray structure

As a general accuracy check for the ability of our model to calculate the structure of rhodopsin, we compared the positions of the α -carbons in each of the 348 amino-acid residues for the three optimized structures (protonated and unprotonated Glu181, and E181Q mutant) with the X-ray coordinates. This was done using the Superpose program

in the TINKER package.⁴⁰ Each of the α -carbons of the residues in all of the seven α -helices is displaced less than 1 Å from the X-ray positions (figure 3). The extracellular (E) and cytoplasmic (C) loops are floppy, and therefore highly sensitive to changes in solvation, or the membrane environment. Since our model did not include solvent, or a model for the membrane, large displacements from the X-ray structure are seen for these loops; the notable exception being the E-II loop, which penetrates the protein, coming in close contact with the chromophore, and is therefore geometrically constrained. We conclude that all of our structures are in good agreement with the X-ray coordinates for all of the residues in the internal protein, close to the chromophore, which is our region of interest.

Structure of the binding pocket and H-bonding network

The focus of our study is to examine the protonation state of the Glu181 residue. The retinal-binding pocket is therefore the most important region for our purposes. Figure 4 shows the displacement from the X-ray coordinates of the α -carbons in the 27 residues within 4.5 Å distance from the retinal molecule (as identified by Palczewski).⁵

All of the calculated alpha-carbon coordinates are within 1 Å of the X-ray coordinates, which is well within the 2.2 Å resolution of the 1U19 X-ray structure.⁸ The binding pocket has the lowest B-factor in the protein, and the coordinates can perhaps be considered to be more accurate than the 2.2 Å resolution, but this argument is offset by the fact that the chromophore, and its surrounding region, is the least well suited to the CNS refinement algorithms used.⁴¹ To get a rough estimate of the error associated with the refinement of the position of binding pocket residues from an X-ray data set, we

compared the coordinates for the α -carbons in the binding pocket for two published PDB structures, refined from the same data set, 1F88⁵ and 1HZX⁶. We found an RMS deviation between the two structures of 0.22 Å. We therefore consider deviations from the crystal structure larger than 0.22 Å to be significant.

With the exception of a few points, the charged Glu181 model gives a slightly better fit to the crystal structure coordinates, with an RMS deviation of 0.42 Å, compared with 0.43 Å for the neutral Glu181 structure. However, we note the limitations in this analysis due to the resolution of the experimental data, the restrictions of the CNS refinement procedure, and the fact that only the α -carbon positions are considered.

There is significant deviation from the 1U19 crystal structure in the position of the α -carbon of the Glu113 counterion residue for all three optimized structures. To fully analyze the significance of this result we will next examine in greater detail the geometry of the retinal PSB and the positions of the counterion(s) and H-bonding network. Chart 1 shows a schematic view of some important binding pocket residues, involved in H-bonding with the retinal PSB and counterion(s), with important bonds labeled. Table 1 gives the corresponding values for the three optimized rhodopsin structures (charged and neutral Glu181, and E181Q mutant) and the X-ray structure, of key bond distances and dihedral angle within the H-bonding network involving the PSB, Glu113, Ser186, Cys187, Glu181, and the two water molecules, Wat2a and Wat2b.

For the bond distances and angle in table 1, both the charged and neutral Glu181 optimized model structures are in reasonable agreement with the X-ray structure. One notable difference, which is consistent with previous computational³⁷ and NMR^{42, 43} structural studies, is the shorter distance **A** between the PSB nitrogen and the closest

oxygen atom of the Glu113 counterion: 2.89 and 2.95 Å, respectively for the neutral and charged Glu181 structures, compared to 3.45 Å in the X-ray structure. As expected both neutral and charged Glu181 model structures optimized to a minimum with one oxygen atom of Glu113 pointing towards the PSB, and the other at a considerably larger distance (4.42 and 4.34 Å, respectively), this is in agreement with recent MD simulations.⁸ By contrast, the X-ray structure has both oxygen atoms almost equidistant from the PSB nitrogen (3.45 and 3.71 Å). Some NMR studies have predicted values larger than 4 Å for this distance.⁴⁴⁻⁴⁶ The reasons for the difficulty in conclusive experimental determination of this distance may lie in the complex charge delocalization effects surrounding the salt bridge, and also the possible existence of dynamic equilibria. There is evidence to support the involvement of an extended H-bonding network involving both Glu113 and Glu181, acting to delocalize the negative charge of the Glu113 counterion,^{47, 48} which may account for miscalculations based on NMR and X-ray data. The separation distance from the Glu113 counterion has been shown in simulations to have significant effect on the protonation state of the Schiff base^{49, 50}, indicating the need for a more accurate determination of this distance.

As might be expected, the neutral and charged Glu181, and E181Q mutant structures exhibit significant differences from one another in the binding pocket region surrounding the Glu181 residue. In the neutral (protonated) Glu181 structure, Wat2a is within hydrogen bonding distance of oxygen atoms of Glu181, Cys187 and Ser186, with O-O distances of 2.53, 2.91, and 2.74 Å, respectively. This is in agreement with the crystal structure. However, for the charged (unprotonated) Glu181 structure, Wat2a may only H-bond with two of the three residues (Glu181 and Ser186, with O-O distances of 2.76 and

2.96 Å, respectively), causing a relative motion of the side chain of Cys-187 away from Wat2a, resulting in an O–O distance of 4.23 Å, and increasing the N–C₂–C₁–O dihedral angle in Cys187, from –26.3° in the neutral Glu181 structure to –65.8° in the charged Glu181 structure. Interestingly, where these significant differences in H-bonding occur between the two optimized structures, the corresponding values for the crystal structure are often intermediate between the two (table 1, bond lengths G, H, and I, and dihedral angle). One could speculate a dynamic equilibrium situation in the real crystal.

To conclude this discussion, we note that there are several small differences between the two optimized structures (charged and neutral Glu181). By comparison with the X-ray structure we can infer the existence of dynamic equilibria in the real structure. However, lack of a sufficiently high resolution experimental structure, makes any conclusions tentative at best.

Structure of the retinal protonated Schiff base

One of the key features, which we seek to accurately model with any computational method, are the bond lengths, including single/double bond length alternation (BLA), and dihedral angles for the retinal PSB. The BLA is an indication of the degree of positive charge (de)localization. Due to the difference in charge stabilization between the ground and excited state electronic wave functions, the BLA has a large effect on the absorption maximum of the dark state species.³⁰ The dihedral angles, in particular the relative orientation of the β -ionone ring and the degree of negative pre-twist in the C₁₁=C₁₂ double bond involved in isomerization, are largely a consequence of steric interactions with the protein binding pocket and also have important effects on the absorption energy.

Before proceeding, it is again important to discuss the limitations of the experimental procedures used to determine the retinal Schiff base geometry, with which we will make comparison. It is well known that the refinement procedures commonly employed in X-ray diffraction studies are not always suited to reproducing the structures of prosthetic groups, including structures like the delocalized π -systems in the retinal PSB. There are large differences in the refined structures of the retinal PSB for the two published crystal structures, 1U19⁸ and 1L9H⁷. The resolutions (2.2 and 2.6Å, respectively) are not sufficient to model individual bond lengths for the chromophore. Okada et al⁸ employed an SCCDFTB/CHARMM MD simulation to equilibrate the chromophore geometry in the binding pocket, starting from different published PDB structures. Whilst the calculated structures were all in extremely good agreement, they reported significant differences between those and the published crystal structures. Carravetta et al⁵¹ have also calculated the chromophore bond lengths from double-quantum (DQ) NMR experiments. Figure 5 shows the bond lengths for the retinal PSB, optimized with ONIOM for the neutral, and charged Glu181 models, the QM/MM MD results of Okada, the 1U19 crystal structure, and the correlated DQ NMR results.

For the retinal PSB C-terminal region, there is good qualitative agreement for the BLA between the three calculated, and two experimentally determined structures. However, from C₁₂ to the N⁺-terminus, significant qualitative and quantitative differences occur. This is as expected, because the N⁺-terminal region is the most difficult to model, and measure, due to the subtle electronic effects surrounding the positively charged protonation site, including the interaction with the counterion.

The MD simulation and ONIOM optimization of neutral Glu181 both started from the same crystal structure, with a neutral binding pocket, and there is good agreement between them. Indeed we find an RMS bond length deviation below 0.01 Å between the two structures: the only significant difference occurring at the C₁₄–C₁₅ bond, where the MD simulation predicts a significantly longer bond length. Both our models (neutral and charged Glu181) predict considerable double bond character for C₁₄–C₁₅ in agreement with NMR results,⁴⁴⁻⁴⁶ and computational studies.⁵² However, pre-resonance Raman experiments⁵³⁻⁵⁵ predict single bond character for the C₁₄–C₁₅ bond in agreement with the recent DQ NMR result.

The pronounced reduction in BLA around C₁₃, C₁₄, and C₁₅ in both charged and neutral Glu181 structures suggests a polaronic positive charge defect due to the delocalization of the positive charge from the protonated PSB into the extended π -network, as predicted by NMR experiments.⁴⁴ For the neutral Glu181 model the main defect is localized about the C₁₄ position: the C₁₃=C₁₄ and C₁₄–C₁₅ bond lengths are similar (1.408 and 1.387 Å respectively). For the charged Glu181 model the defect occurs earlier, at the C₁₃ position: the C₁₂–C₁₃ and C₁₃=C₁₄ bond lengths are similar (1.405 and 1.421 Å, respectively), indicating more pronounced delocalization of the PSB positive charge into the polyene chain, probably due to the stabilization of positive charge in the vicinity of the second Glu181 counterion.

Figure 5 shows also the calculated bond lengths from a recent DQ NMR study.⁵¹ In contrast to previous NMR predictions, and X-ray data, these results show a strong BLA reduction in the vicinity of C₁₂–C₁₃, while the alternation recovers at the end of the chain, so that the C₁₄–C₁₅ bond (1.428 ± 0.026 Å) is longer than the C₁₂–C₁₃ (1.410 ± 0.027 Å)

bond. The results of this study have been used to support a hypothesis that the polar residues on EII, together with Wat2a, assist the rapid selective photoisomerization of the retinal PSB by stabilizing a partial positive charge in the center of the polyene chain. However, the resolution of the experimental data (error bars on figure 5) is not sufficient to conclusively support this. Further, a recent paper disputes the original findings on the basis of new C-13–C-13 J-couplings, which do not support significant effects on the BLA from the protein environment.⁵⁶ Nevertheless, it is worth noting that our calculated bond lengths for the neutral Glu181 model show a similar trend, which may be consistent with the same hypothesis, but slightly shifted along the polyene chain with respect to the DQ NMR results: a small BLA reduction is seen around the C₁₀–C₁₁ position, in good agreement with MD simulations.⁸ A similar feature is not observed for the charged Glu181 model.

Due to the extraordinary similarity between the neutral and charged Glu181 structures and the limited resolution of the X-ray and NMR studies, it is difficult to assign either model as a better fit to experiment. However, the neutral Glu181 model reproduces the localized BLA reduction in the center of the polyene chain, predicted from DQ NMR results, whereas the charged Glu181 model does not. We also observe opposite trends for the neutral and charged Glu181 models for the adjacent bond lengths, C₁₂–C₁₃ and C₁₃=C₁₄, due to the different positions of the polaronic defects in the two models. These differences in BLA behavior for the two models might be a productive area for future higher-resolution experimental studies.

Pre-twist of the $C_{11}=C_{12}$ bond

The $C_{10}-C_{11}-C_{12}-C_{13}$ dihedral angle defines the degree of pre-twist towards the trans conformation in the $C_{11}=C_{12}$ double bond, which is critical for rhodopsin function. Different X-ray structures predict very different pre-twist angles, ranging from 0.0° for the 1L9H structure to -40.8° for the most recent, and highest resolution, 1U19 structure. All three of our model structures optimized to dihedral angles with significant negative pre-twist, with the neutral and charged Glu181 structures giving angles of -17.1° and -26.2° , respectively, and the E181Q mutant optimizing to -16.1° . All three angles are well within the range of experimentally calculated values and the neutral Glu181 result is in good agreement with the calculated structure of Gascon and Batista (-11.0°) and the MD simulation of Okada et al ($-17.7 \pm 9.1^\circ$), which both used a neutral Glu181 model.

II. Vertical Excitation Energies

Accurate calculation of vertical excitation energies for rhodopsin is difficult because of sensitivity to small structural changes of the retinal PSB, and subtle charge effects in the binding pocket. There have been many computational investigations aimed at determining the chemical origin of the opsin shift (the difference in excitation energy between the retinal PSB in solution, and in the protein environment) and the spectral tuning within the family of rhodopsin proteins. Remarkably, different rhodopsins absorb over the full visual spectrum in the red, green and blue color pigments in humans, despite having the same chromophore and only minor changes in protein sequence.

The effect of the protonation state of Glu181 on the excitation energy has been less well studied. A study by Birge and co-workers,¹⁸ using 2-photon spectroscopy on a

locked 11-cis rhodopsin model, is frequently cited as evidence for a neutral Glu181 in the retinal-binding pocket, since semi-empirical calculations on model chromophores carried out by the same group indicate a much larger S_1 – S_2 energy splitting for a two counterion model. A more recent study by Schreiber et al.⁵⁷ concludes that the effect on the vertical excitation energies of the protonation state of Glu181 is negligible. However, these calculations were performed on small model systems, and furthermore did not calculate the excitation energy for an unprotonated Glu181 in addition to the Glu113 counterion, making meaningful comparison difficult.

In this work we calculate three-layer ONIOM excitation energies according to the scheme set out in the computational details section. Table 2 compares the $S_0 \rightarrow S_1$ excitation energies for our three model structures, with the experimental value,⁵⁸ and that calculated by Olivucci and co-workers³² using a CASPT2//CASSCF/6-31G*:Amber approach with the chromophore and partial Lys296 residue in the QM layer.

Our calculated $S_0 \rightarrow S_1$ excitation energies for the neutral and charged Glu181 systems and the E181Q mutant are all slightly lower than the experimental value, deviating by 2.6, 1.2 and 2.6 kcal/mol, respectively. Despite the charge differences, the values for neutral and charged Glu181 systems are quite similar, and both in good agreement with the experimental value. In fact all three calculated values have the same or smaller deviation from the experimental value than that for the quoted CASPT2//CASSCF result (2.6 kcal/mol). We note that our value for the charged Glu181 system gives slightly better agreement with experiment than the neutral Glu181 calculation.

We have also calculated the $S_0 \rightarrow S_2$ excitation energy. Our calculations show that neutral and charged Glu181 (one and two counterion) systems give almost identical

vertical excitation energies from S_0 to S_1 and S_2 , contradicting the analysis of Birge et al that the protonation state would markedly affect the S_1 – S_2 splitting. Our calculated values for the S_1 – S_2 splitting (18.2, and 16.8 kcal/mol, for neutral and charged Glu181, respectively) are in poor agreement with the experimental value (5.7 kcal/mol), but similar to the splitting calculated with CASPT2//CASSCF (27 kcal/mol). The poor agreement with the 2-photon spectroscopy result may be due to the restrained chromophore used in their study. We note that our ONIOM $S_0 \rightarrow S_2$ excitation energies are in fairly poor agreement with the CASPT2//CASSCF value.

TD-DFT has been shown to have difficulty describing doubly excited states.⁵⁹ According to Sekharan et al⁶⁰ the S_2 state has significant double excitation character (homo)⁰(lumo)², which means it will not be well described by TD-DFT. However, this description of S_2 may not be appropriate, originating from a simple analogy to polyenes and the reversal of the S_{single} covalent and S_{double} ionic states upon protonation of the Schiff base.^{18, 61} Olivucci et al³² propose, on the basis of CASPT2//CASSCF calculations on retinal models, that for the isolated PSB the S_1 state has a strong S_{double} (ionic) character, and S_2 a strong S_{single} (covalent) character, but that this situation is reversed by the introduction of the Glu113 counterion. Further, the introduction of the binding pocket charges using QM/MM (using AMBER to describe the binding pocket residues), gives an intermediate situation with both states having some degree of mixed character. Therefore, we might expect TD-DFT to give an equally good approximation to the energies of the S_1 and S_2 states.

In conclusion, our three-layer ONIOM model is able to accurately reproduce the experimental $S_0 \rightarrow S_1$ excitation energy, but not the $S_0 \rightarrow S_2$ excitation energy. This is not

unexpected because other computational methods, including CASPT2//CASSCF,³² also have difficulty reproducing the experimental energy of the second excited state. Furthermore, the $S_0 \rightarrow S_1$ and $S_0 \rightarrow S_2$ excitation energies are very similar for all three calculated structures (neutral and charged Glu81 and E181Q mutant). Therefore, we are unable to distinguish the protonation state of Glu181 in this way.

III. NMR Simulations

In the absence of sufficient accuracy in the crystal structure analysis, NMR spectroscopy has been widely used to study the orientation and conformation of the retinal chromophore in the binding pocket.^{19, 42-46, 51} Recently, Gascon et al showed that QM/MM calculations in conjunction with the GIAO method are capable of simulating NMR data in very good agreement with experiment.³⁶ Previous studies have indicated a large effect on chemical shifts due to the position of the counterion,⁶² so one might expect a large effect of introducing a second charged species into the binding pocket.

Proton NMR spectrum

Figures 6 and 7 show the proton and C-13 NMR spectra, respectively, for the three rhodopsin model systems. All three models give good agreement with experimental data for the H^1 NMR (figure 6). The minimized RMS deviation from the experimental results, for the neutral Glu181 and E181Q mutant respectively, are 0.51 and 0.52 ppm, both of which are within the approximate experimental error of ± 0.5 ppm.⁴⁴ The charged Glu181 structure gives a slightly worse fit to the experimental data, with an RMS deviation of 0.59 ppm from the experimental results. There is a particularly large deviation for the

protons on C₁₁, which is in close proximity to Glu181, and has particular significance as the C₁₁=C₁₂ bond is the site of isomerization, following photoexcitation. A second significant difference between the neutral and charged Glu181 structures occurs for the protons on C₁₅. We expect this to be the most difficult region to model as it is next to the positive charge of the PSB and in close proximity to the Glu113 counterion. Indeed, both models give significant deviation from experiment at this position, as do previous calculated NMR results from 2-layer ONIOM calculations by Gascon and Batista.³⁶

All three models also differ significantly in the proton spectra from the experimental result for C₁₇, which is in close proximity to Phe293 and His211. Aromatic residues are expected to affect chemical shifts due to charge delocalization effects, and inclusion of these effects at the ab initio level may be important. However, recalculation of the proton NMR spectra with Phe293 included in the medium layer had little effect on chemical shift at position C₁₇. Histidine residues are potentially positively charged (protonated) at biological pH, however N-15 NMR studies indicate that His211 is neutral (protonated at the δ -N) in dark state rhodopsin.⁶³ In order to test the effect of the protonation state of His211 on the calculated NMR spectra, we reoptimised the full protein structure with His211 protonated and recalculated the NMR spectra. However, there was very minimal effect on either the overall fit to experimental spectra, or the fit to the proton chemical shift at the C₁₇ position. The reason for the poor fit at C₁₇ is therefore not clear.

C-13 NMR spectrum

Again, all three models are in reasonable qualitative agreement with experimental data (figure 7), with RMS errors of 6.29, 7.51 and 5.51 ppm, for the neutral, charged and

E181Q mutant, respectively. The C-13 spectrum is a worse overall fit than the proton spectrum, as the approximate experimental error for the C-13 NMR experiment is ± 1 ppm.⁴⁴ Again, we observe a slightly worse fit for the charged Glu181 structure. As with the proton NMR, we identify a downfield shift at C₁₁, and an upfield shift at C₁₅, in the spectrum of the charged Glu-181 compared with experiment, and the calculated values for neutral Glu181 and E181Q.

We note that the chemical shifts for the carbons C₈ to C₁₃ calculated with a charged Glu181 are slightly deshielded compared with the neutral and E181Q mutant calculations (mean chemical shift of 146 ppm for charged Glu181, compared to 143 ppm for neutral Glu181). This is in qualitative agreement with semi-empirical calculations by Han et al,²⁸ which show that placing a negative charge close to C₁₂ causes a net downfield shift for the carbons C₈ to C₁₃.

To conclude, we find that our three-layer ONIOM approach is capable of reproducing experimental proton NMR results to a high degree of accuracy, and C-13 NMR results to an acceptable degree of accuracy. We observe that the neutral Glu181 structure is most consistent with the experimental NMR spectra.

IV. Conclusions

The goal of our study was to generate high-level computational results for two models of the photoreceptor protein rhodopsin, differing in the protonation state of the Glu181 amino acid residue. We have compared these results with data from the literature to review the previously made conclusions regarding the protonation state of Glu181, which has important implications for the photoactivation mechanism.

We have demonstrated the ability of our three-layer ONIOM(QM-high:QM-low:MM) computational approach to calculate several experimental properties – equilibrium geometry, vertical excitation energy to the first and second excited state, and NMR chemical shifts – in good agreement with those values given in the literature. Moreover, we were able to show the remarkably small differences in all calculated properties caused by the introduction of a second charged residue (deprotonation of Glu181) into the retinal-binding pocket. With this knowledge, we were able to reanalyze and reinterpret the available experimental data.

A key finding of our work is that the calculated values for the $S_0 \rightarrow S_1$ and $S_0 \rightarrow S_2$ vertical excitation energies for both neutral and charged Glu181 models are almost identical for the two model structures. This result contradicts previous semi-empirical vertical excitation energy calculations performed on simplistic retinal PSB models. In the light of our new, more accurate results, we conclude that it is not possible to draw conclusions regarding the charge of residues in the retinal-binding pocket based on measurements of the first and second vertical excitation energies, as has previously been suggested.¹⁸

Based on comparison of the calculated geometries and NMR spectra for the retinal PSB, for the neutral and charged Glu181 models, with experimental results, the results for the protonated (uncharged) Glu181 model are slightly more consistent with experimental data than the unprotonated (charged) Glu181 model. The neutral Glu181 structure reproduces more accurately the bond length alternations (BLA) of the retinal chromophore when compared with a recent high resolution NMR structure.⁵¹ In particular, a reduction in BLA around the $C_{11}=C_{12}$ bond, indicating a localization of

positive charge, which is thought to be important for efficient selective isomerization, is reproduced by the neutral Glu181 model, but not by the charged Glu181 model. Furthermore, calculated proton and carbon-13 NMR spectra for the neutral Glu181 structure provide a better fit to the experimental results than the charged Glu181 structure, particularly for the retinal C₁₂ and attached hydrogens.

The main conclusion of our study is that there is a clear need for caution when attempting to interpret experimental data on the basis of chemical arguments or computational models that may not be adequate for the purpose. Our results indicate that the effect of changing the protonation state of Glu181 on the physical properties of the retinal PSB chromophore in rhodopsin are very minor. These differences are often below the resolution of current experimental techniques.

In terms of suggestions for future work, the highlighted differences in BLA patterns for the retinal chromophore in our two calculated models, together with key small differences in the bond lengths and angles in the binding pocket region, may provide useful targets for future high-resolution experimental approaches. In the recent literature, vibrational spectra provide important experimental evidence in support of one or the other protonation state of Glu181 in rhodopsin. The similarity between the pre-resonance Raman spectra of native rhodopsin and the E181Q mutant supports a protonated Glu181,²⁰ while the lack of a protonated C=O stretch band for Glu181 in the dark state, inferred from FTIR difference spectra,²³ supports an unprotonated Glu181. Computational calculation of the vibrational spectra of our three model structures (neutral and charged Glu181 and E181Q mutant) would enable comparison with experimental spectra, in particular the frequencies of the individual C=O stretches, which is likely to

aid in reaching a more definitive conclusion regarding this important topic. However, the analytical calculation of the full vibrational spectra at the present level of theory is currently not within reach, due to the coupling between each point charge and the QM wave functions. Full evaluation using numerical differentiation of the gradients is prohibitively expensive for a system this size, while the calculation of reduced spectra (either numerically or analytically) may not be sufficiently accurate due to the neglect of coupling.

Material and Methods

I. Structural Model

We constructed a starting structural model based on chain A of the recent X-ray crystallography structure reported by Okada and co-workers (PDB code: 1U19).⁸ The carbohydrate chains, and Zn^{2+} and Hg^{2+} ions, which reside on the outer surface of the protein, and are artifacts of the crystallization process, were removed and only the 37 crystallographic water molecules in close proximity to chain A were included. Hydrogen atoms were inserted using the GAUSSVIEW program.⁹ The protonation of all titratable groups is standard, with the exception of those in the retinal-binding pocket, which is neutral in the case of the neutral Glu181 and E181Q mutant structures, and with a charge of $-1e$ for the charged Glu181 structure. Amino acids Glu122, Asp83, and His211 are assumed to be neutral as indicated by FTIR⁶⁴ and NMR⁶³ experiments. The Schiff-base linkage between Lys296 and retinal bears a net positive charge compensated by the Glu113 counterion. The N (Met1) and C (Ala348) termini are attached to acetyl (COCH_3)

and methylamine (NHCH₃) capping groups respectively. Thus, in the case of the neutral Glu181 structure, the model contains 5636 atoms in total and an overall charge of +4e.

II. The ONIOM method

In this work, we use QM/MM within the ONIOM framework, developed by Morokuma and co-workers.⁶⁵⁻⁶⁸ ONIOM allows division of the full system into up to three (or more, in principle) layers and uses an extrapolation to calculate the total energy. In our current work we use a three-layer scheme with electronic embedding.^{69, 70}

$$E^{\text{ONIOM(QM-high:QM-low:MM)-EE}} = E_{\text{real}}^{\text{MM}} + E_{\text{int-model}}^{\text{v, QM-low}} - E_{\text{int-model}}^{\text{v, MM}} + E_{\text{model}}^{\text{v, QM-high}} - E_{\text{model}}^{\text{v, QM-low}}, \quad (1)$$

where *real* refers to the full system, treated with molecular mechanics (MM), *int-model* indicates an intermediate model system (binding pocket residues and chromophore) which is treated with an intermediate (*QM-low*) computational level, and *model* refers to the chemically most important (chromophore) region, treated with a higher level QM method (*QM-high*). ONIOM uses link atoms to saturate the dangling bonds, which together with the QM-high and QM-low regions form the model and intermediate systems, respectively. Because all the terms in eq. 1 represent chemically realistic systems, the ONIOM scheme can be used to combine QM methods with QM methods, as well as QM methods with MM methods. This is in contrast to most other hybrid schemes, which are cast as a summation instead of extrapolation, and can only combine QM methods with MM methods. In eq. 1, both MM calculations include the model region of the system (the chromophore in this case), and need parameters for the bonding contributions such as stretches, angles, and dihedral angles. However, as these contributions are identical in the two MM calculations, and $E_{\text{real}}^{\text{MM}}$ and $E_{\text{int-model}}^{\text{v, MM}}$ enter eq. 1 with opposite sign, they cancel exactly in the overall ONIOM energy. Thus the bonding

parameters for the model region do not affect the final energy, and can be set to zero when not readily available.[†] Note that this exact model region cancellation does not occur between two QM terms in the ONIOM expression; unlike MM methods, a low-level QM method in ONIOM must be able to correctly describe the processes in the model region at least at a qualitative level. The difference is part of the ONIOM extrapolation.

From the ONIOM extrapolation expression it also follows that, for each boundary, the interaction between the two subsystems is implicitly included at the lower of the two theoretical levels. Therefore, the interaction between the two QM layers in eq. 1 is included at the QM-low level of theory, allowing for description of electronic effects between the two layers, and making this a particularly powerful method. In the standard ONIOM scheme, the interaction between the MM layer and QM layer(s) is included entirely using Molecular Mechanics terms, and is therefore referred to as *Mechanical* or *Classical Embedding*. In order to evaluate also the electrostatic interaction between the MM and both QM layers at the QM level, we use an *Electronic Embedding* scheme, where the environment charges from the MM layer are included in the calculations of the QM layers, such that all of the model and int-model calculations are embedded in the field v , comprising the charges of the atoms in the MM layer.^{69, 70} The ONIOM formulation in eq. 1 reflects the use of electronic embedding by the superscripts v .

Partial charges close to the QM region are scaled to avoid over-polarization of the wave function. In this study, we used the default scaling scheme implemented in GAUSSIAN, which sets to zero the charges for atoms separated by less than three bonds from the QM layer. This does not account for charges positioned close in space, but not covalently bonded, to the QM region. However, we note that since the same charges are present in

both the $E_{\text{int-model}}^{v,\text{QM-low}}$ and $E_{\text{int-model}}^{v,\text{MM}}$ terms, any charge interactions which are overcounted or undercounted in the $E_{\text{int-model}}^{v,\text{QM-low}}$ term will be balanced in the $E_{\text{int-model}}^{v,\text{MM}}$ term, and the same holds for the calculations on the model system.

In three-layer ONIOM calculations as presented here, we have two choices for the electronic embedding scheme. In *EE1*, the MM charges from the intermediate layer are zeroed in the calculations on the small model system. In *EE2*, we retain those charges. We used *EE1* for the ground-state calculations in this work, and *EE2* for the excitation energies. The motivation will be discussed in the relevant sections below.

ONIOM layers

Figure 2 shows the residues in the QM-high layer as a ball and bond diagram, and the residues in the QM-low layer as a tube diagram. The important electronic effects for photoactivation occur in the conjugated π -system of the chromophore. Therefore, the retinal PSB and the attached Lys296 residue are included in the QM-high layer: We use the B3LYP density functional and a 6-31G* basis set. This level of theory recovers important electron correlation energy and has been shown to be successful in modeling the chromophore in previous QM/MM studies.^{30, 31, 35-37}

Introducing an intermediate (QM-low) layer containing several residues from the retinal-binding pocket then extends the QM region. Glu181, together with the Glu113 counterion, the residues and solvent molecules involved in the hydrogen bonding network which connects these two residues – Ser186, Cys187, Gly188, Wat2a, Wat2b, Wat3 – and two nearby aromatic residues – Trp265 and Tyr268 – were included in the intermediate layer. The aromatic residues are included because they have been found to

affect the proton NMR spectrum;³⁶ Trp265 is also involved in the interaction with the C₂₀ methyl group, which leads to the significant twist around C₁₁–C₁₂ and the orientation of the β -ionone ring, and Tyr268 is in H-bonding proximity to Glu181. We used HF theory and a smaller 6-31G basis set as the QM-low method. The remaining protein is described using the AMBER force field.⁷¹

All calculations, including NMR and excitations, were performed using ONIOM with electronic embedding according to equation (1), implemented in a development version of GAUSSIAN.^{72,73}

III. Geometry optimizations

Geometry optimizations were carried out using the default *macro-micro* scheme^{74, 75} implemented in GAUSSIAN, with gradients calculated as described in reference 65. For a two-layer ONIOM geometry optimization this involves alternating a second-order optimization of the QM region using internal coordinates (*macro-iterations*), and a first-order optimization of the MM region using Cartesian coordinates (*micro-iterations*) until self-consistency is achieved. The standard macro-micro scheme applies only to QM/MM with mechanical embedding, but has been modified for electronic embedding potentials.⁷⁴

The electronic embedding scheme used for the geometry optimizations (EE1) does not include the partial charges from the intermediate layer in the model system calculations (this is the default in Gaussian). All the electronic interactions between the QM-low and QM-high layers are then described at the QM-low level of theory. For equilibrium ground state structures as obtained here, HF is expected to adequately include these interactions.

IV. Excitation Energies

The ONIOM excitation energies are extrapolated from separate calculations on the QM-high and QM-low subsystems at the TD-B3LYP and TD-HF levels. We discuss the accuracy of the TD-B3LYP method for calculating the vertical excitation energies to the S_1 and S_2 states in the relevant results section.

Similar to equation (1), the excitation energy expression is given by:

$$\Delta E^{\text{ONIOM(QM-high:QM-low:MM)-EE}} = \Delta E_{\text{int-model}}^{\text{v,QM-low}} + \Delta E_{\text{model}}^{\text{v,QM-high}} - \Delta E_{\text{model}}^{\text{v,QM-low}}, \quad (2)$$

where $\Delta E_{\text{system}}^{\text{v,QM-level}}$ is defined as the contribution to the total excitation from a sub-calculation, $E_{\text{system}}^{\text{v,QM-level}} - E_{\text{system}}^{\text{v,QM-level}}$. The molecular mechanics terms do not enter expression (2), since they cannot describe electronic excitations. The QM terms, however, are affected by the molecular mechanics region through the potential from the partial charges.

For these calculations, we use electronic embedding scheme EE2, in which the model system is evaluated in the potential from the partial charges from both the real and intermediate layers. In this way the charge redistribution upon excitation in the small model interacts directly with the charge distribution from the intermediate layer. In the EE1 scheme, this interaction would only be described through the calculation on the intermediate model. Preliminary calculations showed that EE1 is not an appropriate approximation in this case, presumably because the charge redistribution at the TD-HF level of theory is not similar enough to that at the TD-B3LYP level of theory. We therefore chose to use the full charge cloud embedding scheme (EE2) in the three-layer calculations. This is similar to the QM:QM electronic embedding scheme presented in reference 76, although in the present study we use static charges for the embedding

V. NMR chemical shifts

We calculate the proton and carbon-13 NMR chemical shifts for the retinal chromophore. NMR spectra were calculated using the Gauge Invariant Atomic Orbital (GIAO) method. The calculated NMR shifts were fitted to experimental results by minimization of the root of the mean square (RMS) deviations.

These calculations are integrated over the two QM-layers, and in the potential field v as used in equations (1) and (2).⁷⁷ Separate calculations of the NMR chemical shifts are performed for each QM-subsystem (the model and int-model systems). Because we are interested only in calculating the isotropic NMR chemical shielding, it is not necessary to transform the shielding tensors to a common coordinate system (the traces of the individual shielding tensors are invariant to orthogonal transformation). The ONIOM NMR chemical shift is then calculated according to equation (3).

$$\sigma_{ij}^{N(\text{ONIOM}(\text{QM-high:QM-low:MM})-EE)} = \sigma_{ij,\text{int-model}}^{N(v,\text{QM-low})} + \sigma_{ij,\text{model}}^{N(v,\text{QM-high})} - \sigma_{ij,\text{model}}^{N(v,\text{QM-low})} \quad (3)$$

We used the same electronic embedding scheme (EE1) as used for the geometry optimizations. However, we note that the difference between chemical shifts calculated with EE1 and EE2 are negligible. This is as expected because NMR calculations concern only the ground state, and do not involve the charge redistribution as in the excitation energies.

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Figure Legends

Figure 1: The vertical excitation of 11-cis retinal PSB in the dark state of rhodopsin, and the resulting phototransduction cycle; displaying major photointermediates and their corresponding absorption energies.¹³

Figure 2: Layer scheme used for ONIOM calculations. Atoms in the *model* system are shown as ball and bond, while atoms only in the *int-model* layer are shown as tubes. The remaining protein, included in the *real* system in the calculations, is not shown in the figure. Hydrogen bonds are shown in green.

Figure 3: Displacement from the 1U19 X-ray structure positions of the α -carbons of all 348 amino acid residues in the three ONIOM optimized rhodopsin structures: neutral (blue), and charged (red) Glu181, and E181Q (green).

Figure 4: Displacement from the 1U19 X-ray structure positions of the α -carbons of the 27 amino acid residues within 4.5 Å of the retinal PSB, in the three ONIOM optimized rhodopsin structures: neutral (blue), and charged (red) Glu181, and E181Q (green).

Figure 5: C–C bond lengths for the retinal PSB in rhodopsin, optimized with ONIOM for the neutral (blue diamonds) and charged (red squares) Glu181 models, the QM/MM MD results of Okada⁸ (green triangles), the 1U19 crystal structure (purple crosses), and the correlated DQ NMR results⁵¹ (orange circles, including error bars).

Figure 6: Calculated proton NMR spectra for the retinal PSB in rhodopsin using ONIOM, for neutral (blue diamonds) and charged (red squares) Glu181 models, and the E181Q mutant (green crosses), and comparison to the experimental spectra (black stars).⁴⁴

Figure 7: Calculated C-13 NMR spectra for the retinal PSB in rhodopsin using ONIOM, for neutral (blue diamonds) and charged (red squares) Glu181 models, and the E181Q mutant (green crosses), and comparison to the experimental spectra (black stars).⁴⁴

Table 1: Bond distances (Å) and dihedral angle (degrees) in the retinal-binding pocket.

Labels refer to chart 1.

Internuclear distance	X-ray	Neutral Glu-181	Charged Glu-181	E181Q mutant
A : O ₂ (E113)–N(PSB)	3.45	2.89	2.95	2.85
B : O ₃ (E113)–N(PSB)	3.71	4.42	4.34	4.33
C : O ₂ (E181) ^a –C ₁₂ (RET)	4.83	5.02	5.50	5.16
D : O ₂ (E113)–Wat2b	2.88	2.88	2.84	2.88
E : O ₁ (E113)–Wat2b	2.94	3.01	3.03	3.01
F : O ₂ (E181) ^a –Wat2a	2.82	2.53	2.76	2.71
G : O(C187)–Wat2a	3.25	2.91	4.23	3.00
H : O ₂ (S186)–Wat2a	2.87	2.74	2.96	2.74
I : O ₂ (S186)–O(C187)	5.45	4.79	6.20	4.97
J : N(E181)–O ₁ (S186)	2.84	3.10	3.02	3.15
K : N(C187)–O ₃ (E113)	2.93	2.80	2.89	2.82
∠N–C ₂ –C ₁ –O (C187)	–46.3	–26.3	–65.8	–30.0

^aor equivalent N atom for E181Q mutant.

Table 2: Vertical excitation energies (kcal/mol) to the first and second electronic excited states.

Method	Structure	$\Delta E(S_0 \rightarrow S_1)$	$\Delta E(S_0 \rightarrow S_2)$	$\Delta E(S_1 \rightarrow S_2)$
ONIOM-EE(B3LYP/6-31G*: HF/6-31G: Amber)	Neutral Glu181	54.8	73.0	18.2
	Charged Glu181	56.2	73.0	16.8
	E181Q mutant	54.8	73.6	18.8
^a QM/MM(CASPT2//CASSCF /6-31G*: Amber)	Neutral Glu181	60	87	27
Experiments	^b Native bovine rhodopsin	57.4		
	^c E181Q mutant	56.3/56.6		
	^d Rhodopsin with locked 11-cis retinal	58.3	64.0	5.7

^aAndruniw et al³²; ^bPan et al⁵⁸; ^cYan et al²⁴ determined in the absence (56.3 kcal/mol) or presence (56.6 kcal/mol) of 200 mM NaCl; ^dBirge et al¹⁸.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Chart 1