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BACTERIORHODOPSIN PROTON PUMPING MECHANISM: INSIGHTS FROM COMPUTER SIMULATIONS

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Bacteriorhodopsin is a model system for cellular proton pumps. To understand how bacteriorhodopsin works, we performed classical mechanical, quantum mechanical, and combined quantum mechanical/molecular mechanical computations. These computations provided detailed insight into the energetics and directionality of the proton-pumping reaction cycle. Active-site water molecules have a significant effect on the energetics of the proton transfer path, and can even determine the pathway followed by the proton.

Ana-Nicoleta Bondar obtained her B.S. in Physics from the "Alexandru Ioan Cuza" University of Iaşi, Faculty of Physics in 1997, and then continued at the Faculty of Physics of the same University with advanced post-graduate studies in Nonlinearity and self-organization within complex systems (1997-1999). In 2004, A.-N. B obtained her doctoral degree with magna cum laudae from the University of Heidelberg, Germany, with research performed in the period 2000-2004 at the University of Heidelberg, Institute for Scientific Computing, and the German Cancer Research Center Heidelberg (DKFZ). She continued her research in computational biophysics at the University of Heidelberg, Institute of Scientific Computing as a post-doctoral fellow (2004-2006). In 2006 she moved to the University of California at Irvine, School of Medicine, to work on computational biophysics of membrane proteins and lipid/protein interactions.

INTRODUCTION

Proton-pumping proteins are key components of biological cells. These proteins are embedded in the lipid membranes surrounding the cells or various cell organelles, where they pump protons across the membranes. The functioning of proton pumps is essential for important cellular processes such as coupled transport of molecules across cell membranes, or regulation of the pH of cell organelles.[1,2] Due to their roles in cell physiology, proton pumps are valuable candidates for drug design.[2,3]

Bacteriorhodopsin, a light-driven proton pump from the archaeon *Halobacterium Salinarium*, has been studied in great detail and is a model system for understanding proton pumps (for reviews see, *e.g.*, Refs. 4-9). The retinal chromophore is covalently attached via a protonated Schiff base to Lys216, a protein amino-acid residue located approximately at the middle of the protein. In the resting state (bR) retinal is all-trans, and the Schiff base hydrogen bonds to a water molecule (w402) that also connects to Asp85 and Asp212 (Fig. 1). Absorption of light triggers isomerization of the retinal to 13-cis, followed by the passage of the protein through a sequence of spectroscopically distinct intermediate states denoted as K, L, M, N, and O, associated with five consecutive proton transfer steps. The net effect of one reaction cycle

is the pumping of one proton from the cytoplasmic to the extracellular side of the membrane.

In the first proton-transfer step, a proton is transferred from the retinal Schiff base to Asp85 during the L-to-M transition on the ~10μs timescale. Crystallographic models of L indicate similar protein conformations, but differ significantly in details of the geometry of the active site (Fig. 1).[10-14] The conflicting information provided by the crystal structures contributed to the controversies over the mechanism of the first proton transfer step.

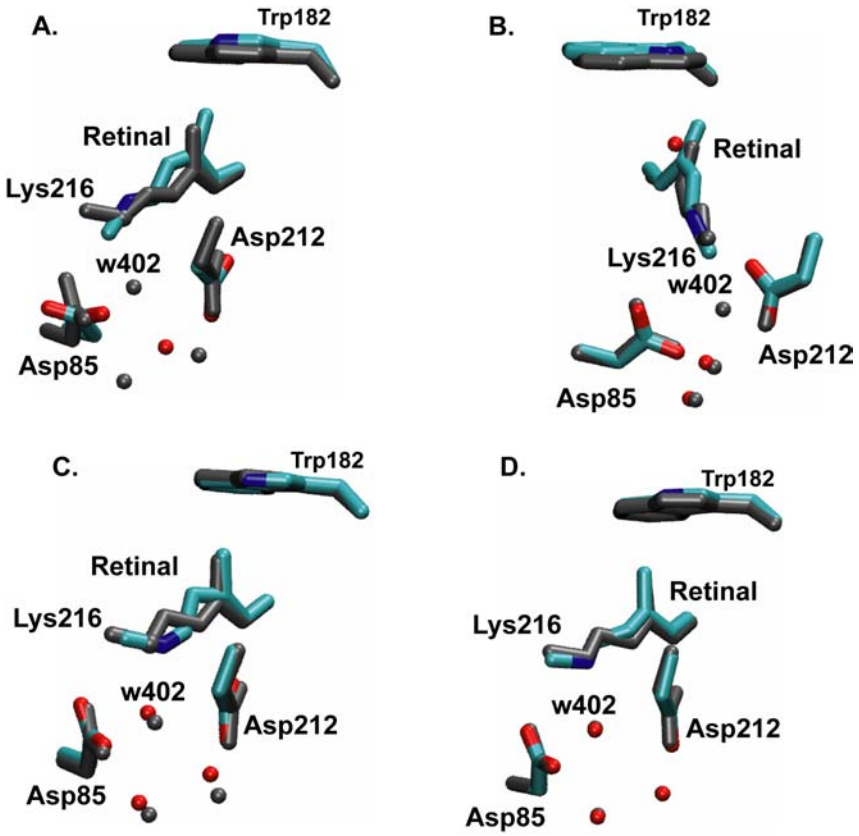

Fig. 1 – Geometry of the active site in crystal structures of the pre-proton transfer state L relative to the bR resting state. The structural coordinates were taken from Refs. 13 (panel A), 14&39 (panel B), 10 (C), and 11 (D). For simplicity, only the C_{11}-C_{15} segment of the retinal and the C_ε and N_z atoms of Lys216 are shown. The bR resting state is depicted in gray.

The structural models of L indicating a cytoplasmic orientation of the retinal Schiff base (Fig. 1A,B)[12-14] are distinguished by the configuration of the water molecules in the retinal binding pocket (Fig. 1A,B). Ref. 13 (Fig. 1A) indicates only one water molecule in the active site; in constrast, the crystal structure from Ref. 14 contains coordinates for three water molecules (Fig. 1B). Classical molecular dynamics (MD) simulations starting from a cytoplasmic-oriented L-state model with one water molecule in the active site (Fig. 1A) indicated the transient formation of hydrogen bonds between the Schiff base and Asp85, or between the Schiff base and Thr89.[13] The observation of these transient hydrogen bonds led to the suggestion that proton transfer from the Schiff base to Asp85 could occur directly, or via Thr89.[13] The presence of a water molecule hydrogen-bonding to the cytoplasmic-oriented retinal Schiff base from Ref. 14 (Fig. 1B) inspired a different model for how the Schiff base deprotonation occurs: The cytoplasmic water molecule would be the bR-state w402 that has translocated during the K-to-L transition.[14] Proton transfer from the Schiff base to Asp85, and the further displacement of w402 towards the cytoplasmic side in M, would make bacteriorhodopsin an inward water pump/outward proton pump.[14,15] In the case of an extracellular orientation of the retinal Schiff base in L (Fig. 1B, C), it was suggested that proton transfer to Asp85 occurs via w402.[10,11]

A key open question regarding the L-state structure is to what extent the geometry indicated by the crystal structures can be reconciled with the solid-state NMR data. Solid-state NMR data has indicated that in L there is a strong interaction between the retinal Schiff base and its counterion – one of the negatively charged Asp85/Asp212, or a highly polarized water molecule.[16] These observations from NMR have raised the question of whether bacteriorhodopsin may act as an inward hydroxyl pump.[5,16]

To understand the mechanism by which bacteriorhodopsin pump protons it is necessary to know the rate-limiting barriers and the reaction energies of the possible retinal deprotonation pathways. Computer simulations are a valuable here because they can provide information at the atomic level of detail about the structure and energetics at the transition state(s), which give the reaction mechanism, and are difficult to characterize experimentally. We have carried out extensive computer simulations using combined Quantum Mechanical/Molecular Mechanical (QM/MM) and QM approaches.[17-23]

COMPUTER SIMULATIONS OF BACTERIORHODOPSIN

Combined QM/MM methods allow us to investigate the retinal configurational changes and proton transfer reactions in the complex environment of bacteriorhodopsin. In the QM/MM approach, the groups participating in the chemical reaction are treated at the QM level, whereas the remaining protein environment is described with classical molecular mechanics.[24-26] The QM method used must be accurate in describing the structures and energetics associated with the reaction of interest, while computationally efficient. Based on a set of test QM and QM/MM test calculations[18,23] we chose the approximate Density Functional Theory (DFT) method Self-Consistent Charge Density Functional Tight Binding (SCC-DFTB).[27] SCC-DFTB with a specific parametrization of the Schiff base nitrogen atom agrees to within 1 kcal/mol with B3LYP/6-31G**, and within 4 kcal/mol with MP2/6-311+G** in describing the relative proton affinity of retinal Schiff base and acetate models in the gas phase.[23] SCC-DFTB is also in reasonable agreement with B3LYP calculations in describing the geometry and torsional values of retinal models.[28] For proton transfer in proteins, SCC-DFTB was found to agree to within 2-4 kcal/mol (rms values) with B3LYP/6-31+G** in the case of triose phosphate isomerase,[29] and within ~2 kcal/mol with B3LYP/6-31G** (rms values) for the energetics of low-energy proton transfer paths in bacteriorhodopsin.[17,18]

The QM region included the retinal chain, the side-chains of Lys216, Asp85, Thr89, and Asp212, and one to four water molecules. We used hydrogen link atoms[25] attached to C_β of Ly216, and to C_α in the case of Asp85, Thr89, and Asp212. The QM/MM implementation of the SCC-DFTB method is described in Ref. 29. We used a hybrid representation for the protein atoms, with parameter set 22[30] for the aromatic side chains, and parameter set 19[31] for the remaining protein amino-acid residues. We used the TIP3P model[32] for the MM water molecules.

Proton transfer in bacteriorhodopsin involves degrees of freedom of the retinal chain, protein groups, and water molecules. This makes very difficult the *a priori* choice of a given reaction coordinate.[17,18,23] To calculate pathways for proton transfer and retinal configurational changes we employed the Conjugate Peak Refinement (CPR) method[33] as implemented in the CHARMM software.[34] The CPR algorithm uses energy-optimized reactant and product state structures to build and optimize a minimum-energy path along which all energy maxima are first-order saddle points that give the transition states of the reaction.

GEOMETRY OF THE RETINAL SCHIFF BASE

To be compatible with proton pumping, the rate-limiting barriers for proton transfer and retinal configurational changes computed from a putative L-state model must be consistent with the known kinetics of the reaction cycle. Following deprotonation, the retinal Schiff base must be accessible to the proton coming from the cytoplasmic Asp96. For the reaction cycle to be productive, the rate-limiting barrier for the K-to-bR back isomerization must be significantly higher than the barriers separating K from the M intermediate.[20] Indeed, although twisting lowers significantly the cis-trans isomerization barrier, the barrier for the unproductive cis-trans back isomerization of the K-state retinal is 5-6 kcal/mol larger than the barrier for the photocycle to proceed with the first proton-transfer step.[20]

We performed a detailed analysis of the compatibility with an active proton-pumping cycle of conformers with retinal in the 13-cis, 14-trans,

15-anti, 13-cis,14-cis, 15-anti, and 13-cis, 14-trans, 15-syn configurations.[18,22] The computations demonstrated that the 13-cis, 15-anti retinal is compatible with an active proton pumping in the both *cis* and *trans* configurations of the C_{14}-C_{15} bond.[18] However, rather than proceeding with proton transfer, 14-cis retinal is more likely to convert to the cytoplasmic-oriented 13-cis, 14-trans, 15-anti.[18]

Consistent with experiments indicating a 15-anti configuration of the light-adapted 13-cis photocycle intermediates,[35] the calculations[18] indicated that the 15-syn conformer[10] is unlikely to participate in proton transfer. Proton transfer from the 15-syn retinal to Asp85 involves crossing over rate-limiting energy barriers of 6-7 kcal/mol, and the reaction energy is ~3 kcal/mol.[18] Importantly, following deprotonation retinal remained 15-syn with the Schiff base oriented towards the extracellular side. The high energy barrier for rotating around the C_{15}=N of the deprotonated retinal indicated that reorientation of the deprotonated 15-syn Schiff base towards the cytoplasmic side (to receive a proton from Asp96) is not kinetically feasible.[18,22] This unproductive reaction is avoided due to the high energetic cost associated with the formation of 15-syn retinal states prior to proton transfer.[18,22]

The energy barriers against retinal configurational changes are significantly influenced by twisting of the retinal.[20] For a planar retinal optimized in the gas phase, the barrier for torsion around the C_{13}=C_{14} bond is ~28 kcal/mol at the SCC-DFTB level,[20] and ~30 kcal/mol with B3LYP/6-31G*.[36] In the protein environment, the rate-limiting barrier for the K-to-bR isomerization from 13-cis to all-trans is ~11 kcal/mol, and is due almost entirely to the internal energy of the retinal.[20] The substantial decrease of the cis-trans isomerization barrier upon twisting of the retinal chain suggests that transient twisting of the retinal may be involved in lowering the energetic cost for the cis-trans thermal isomerization during the N-to-O transition.[20]

Twisting of the retinal chain can also affect the proton affinity of retinal Schiff base models.[23,36,37] Twisting around single bonds reduces the proton affinity relative to the planar retinal, whereas twisting around double bonds has the opposite effect.[36] The combined effect of twisting around single and double bonds in the C_{12} ··· N segment of the retinal is approximately additive.[23] For example, twisting by 20^0 the C_{13}=C_{14}, C_{14}-C_{15}, and C_{15}=N bonds increases the intrinsic proton affinity by 3 kcal/mol, which is very close to the value of 2.3 kcal/mol obtained by summing up the individual effects of twisting by 20^0 each of these three bonds.[23] The combined effect of moderate twisting of single and double bonds in the Schiff base segment is rather small.[23]

PROTON TRANSFER FROM THE RETINAL SCHIFF BASE TO ASP85

We computed pathways for proton transfer from the retinal Schiff base to Asp85 by using reactant and product states models modeled using 6 different crystal structures,[10,12,38,40-42] and we investigated various geometries of the active site and intermediate proton carriers.[17-19,21] The calculations demonstrated that in the presence of w402 the rate-limiting barriers of three pathways with very different sequences of motions are in the range of 11.5 – 13.6 kcal/mol[17] (in good agreement with the experimental enthalpy of activation of ~13 kcal/mol[43]). These three different pathways are direct transfer from the Schiff base to Asp85, or concerted proton transfer via Thr89 or, on the Asp212 side of the retinal, via Asp212 and water w402.[17] The transfer on the Asp212 side of the retinal involves the passage through an intermediate state in which the 13-cis, 14-trans, 15-anti retinal has an extracellular orientation towards Asp212.

A detailed decomposition of the energy profiles associated with proton transfer paths computed for different starting geometries revealed that the energy profiles are dominated by opposite-sign contributions of the interaction between the active-site QM groups, which largely favor proton transfer, and between the active-site QM groups and the surrounding protein environment, which oppose proton transfer.[18] The interaction between the protonated Schiff base of the 13-cis retinal and the negatively charged Asp85 largely favors proton transfer regardless of the relative orientation of the Schiff base and Asp85.[18] The electrostatic conflict between the active site region and the rest of the protein appears to be relieved in a late-M structure[41] in which the non-bonded interactions between the QM active site groups and the protein environment favor the deprotonated retinal state.[18,23]

For a minimal gas-phase proton transfer model consisting of 13-cis, 14-trans, 15-anti retinal and Asp85, the neutral state of the retinal was favored

by ~17 kcal/mol.[18] The active-site groups Thr89, Asp212, and w402, stabilize the protonated state of the retinal Schiff base.[18,44] A similar role of an active-site threonine residue was found in investigations of the visual rhodopsin.[45] Computations in which we assessed the influence of the Thr89:Asp85 distance on the energetics of the back proton-transfer from Asp85 to the retinal Schiff base indicated that breaking of the hydrogen bond between Thr89 and the protonated Asp85 may be important for supressing the back proton transfer from Asp85 to the Schiff base.[23]

ROLE OF PROTEIN FLEXIBILITY AND WATER MOLECULES IN PROTON TRANSFER

Experiments do not indicate any significant structural changes of the protein in the early intermediates of the reaction cycle.[46] Nevertheless, flexibility of the protein is critical for lowering the energetic cost of proton transfer.[17,18,22] Of particular importance is flexibility of helix C[6,13] containing the proton acceptor group Asp85: allowing flexibility of helix C, Trp182, w401, and w406, decreased the rate-limiting barrier for direct proton transfer by ~6 kcal/mol relative to the case where the reaction is computed in a rigid MM protein environment.[22] The important role of local flexibility is also illustrated by observation that proton transfer is faster in the Asp85Glu mutant than in the wild type.[6,47-51] Little structural rearrangement is required for direct proton transfer in the Asp85Glu mutant, and the calculated rate-limiting energy barrier is ~5 kcal/mol lower than in the wild-type.[22]

Water molecules are critical for the functioning of bacteriorhodopsin and other retinal proteins.[52-54] Changes in the number and location of internal water molecules accompany the passage of bacteriorhodopsin through the photocycle intermediate states.[14,54] Water molecules can have a significant effect on the energetics of proton transfer,[17,18,23,55-57] can participate as intermediate carriers for the proton,[17,58] and are involved in the storage of the proton at the extracellular proton release group.[52]

QM/MMM and QM computations of proton transfer reaction pathways allowed us to dissect the role of the active-site water molecules on the energetics of the retinal Schiff base proton transfer reactions. The computations indicated that absence of w402 reduces the energy barrier for direct proton transfer from 12.4 kcal/mol (in the presence of w402) to 6.3 kcal/mol.[17] The enhanced flexibility of Asp85 when not hydrogen bonded to w402 contributes to the smaller energy barrier. When w402 is absent, but a water molecule is present on the cytoplasmic side of the Schiff base (as in Fig. 1B), proton transfer occurs via the cytoplasmic water molecule with an energy barrier of 7-10 kcal/mol; because the cytoplasmic water molecule (Fig. 1B) stabilizes the cytoplasmic-oriented retinal, proton transfer no longer occurs via Asp212.[59]

Water molecules may also be important for suppressing a back proton transfer from Asp85 to the retinal Schiff base. In gas-phase models, the absence of water molecules w401, w402, or w406 leads to an increase of the Asp85 proton affinity (relative to the Asp85/w401/w402/w406 model) by 1.9, 7,7, and 6.7 kcal/mol, respectively.[23] In the protein environment, absence of water molecules w401 and/or w402 increases the reaction energy for the back-proton transfer from Asp85 to the retinal Schiff base. That is, relocation of water molecules hydrogen-bonding to Asp85 can stabilize the neutral (M-like) states, decreasing the likelihood of a back proton transfer to the Schiff base, and thus contributing to the directionality of the reaction cycle.[23]

CONCLUSIONS

We used QM and QM/MM methods to assess the retinal states that may accumulate during the first proton transfer step, the compatibility of putative active site geometries with an overall productive reaction cycle, and the mechanism of the retinal deprotonation step. Flexibility of the protein and of the retinal chain is required for proton transfer, and needs to be taken into consideration when discussing the proton-transfer mechanism. Relocation of water molecules and changes in the hydrogen bonding at the active site are important structural events that may contribute to suppressing the back proton transfer from Asp85 to the retinal Schiff base. Further computations are necessary to better understand the role of specific water molecules, and whether bacteriorhodopsin could act as an inward hydroxyl pump.[16]

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