PERSPECTIVE

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Sensory rhodopsin II and bacteriorhodopsin: Light activated helix F movement

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EPR spectroscopy in combination with site directed spin labeling (SDSL) has become a valuable tool for structural investigations as well as for kinetic studies on proteins. This method has been especially useful for membrane proteins in yielding structural and functional data. This information is not easily available from other techniques, like, *e.g.*, X-ray crystallography or electron microscopy. In the first part of this two part review, the topology of the sensory rhodopsin II/transducer complex (NpSRII/NpHtrII) derived from EPR constraints is compared to that obtained from X-ray crystallography. In the second part, the helix F movement observed for both sensory rhodopsin and bacteriorhodopsin is evaluated and discussed in order to establish a common mechanism after photoreceptor activation.

Introduction

Sensory rhodopsin II (SRII or phoborhodopsin) mediates negative phototaxis in halophilic archaea. It is structurally and functionally closely related to the two light driven ion pumps bacteriorhodopsin (BR) and halorhodopsin (HR) (for recent reviews see ref. 1–5). SRII is tightly bound to its cognate transducer protein HtrII (halobacterial transducer of rhodopsins) that consists of an N-terminal domain with two transmembrane helices comprising part of the receptor binding site. The long rod shaped cytoplasmic domain arranged in a four helical bundle harbors the signaling domain involved in the activation of the His-kinase CheA as well as methylation sites implicated in adaptation processes to constant stimuli. Photo-excitation of the receptor leads to a conformational change of the second transmembrane helix (TM2) of the transducer, which is trans-

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mitted all the way down to the tip where CheA binds together with the adaptor protein CheW.

Over the past years, several studies yielded detailed insight into the biophysical properties of NpSRII and its interaction with NpHtrII, thereby leading to the present model for signal generation and transfer.[2,6–18] The first structural information for a sensory rhodopsin was obtained for SRII from *Natronobacterium pharaonis* (NpSRII), showing the strong relation to the ion pumps BR and HR.[19,20] The recently published X-ray crystallographic structure of the complex formed between NpSRII and a C-terminal fragment of its cognate transducer NpHtrII revealed a tight interaction between the transducer transmembrane helices and the receptor helices F and G,[21] a result which was already anticipated from X-ray structural analysis of the receptor.[19,20]

A topological model for the complex was derived earlier using data from electron paramagnetic resonance (EPR) experiments.[17] EPR-spectroscopy in combination with site directed spin labeling (SDSL) is a well suited technique for structural investigations especially for membrane proteins.[22–24] Nitroxide-scanning experiments can provide sequence correlated data about the mobility of the replaced residue as well as the accessibilities of paramagnetic reagents. The latter allows to differentiate between solvent exposed side chains, residues located in the hydrophobic region of a lipid bilayer, and positions which are in the interior of the protein. Additionally, intra- and inter-molecular distances obtained using SDSL can provide long range constraints and relative orientations of secondary structure elements, which permit the construction of topological models of single proteins as well as protein complexes.

In the following the NpSRII/NpHtrII structure derived from EPR data will be compared to that obtained by X-ray crystallography in order to discuss the current state and potentials of EPR spectroscopy in membrane protein structure determination. In a second part, the capability of EPR-spectroscopy in determining dynamic and kinetic parameters of protein conformational changes will be exemplified by describing the results on the helix F movement of both BR and NpSRII.

NpSRII/NpHtrII structure: Comparing the EPR model with the X-ray structure

EPR-model

The SDSL method was applied to construct a topological model of the NpSRII/NpHtrII complex. In this study a C-terminal truncated transducer comprising the first 157 amino acids was used. Both, NpSRII and NpHtrII carried a C-terminal His-tag for purification purposes. The proteins were mutated at the cytoplasmic side of their putative binding region (Fig. 1) and subsequently labeled with the nitroxide spin label MTS ((1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)methanethiosulfonate) yielding the side chain R1. The experiments were carried out in dodecyl maltoside (DDM) in which NpSRII and NpHtrII form a 1 : 1 complex as well as in membranes. Under these latter conditions the receptor transducer complex is present in a 2 : 2 stoichiometry.[18,25]

The reconstitution of the receptor with its transducer into polar lipids isolated from purple membrane resulted in a strong immobilization of the nitroxide side chains of the transducer compared to the DDM-solubilised samples. For V78R1 and L82R1 the line widths were significantly larger than those for the other positions, indicating dipolar interactions between the spin labels of two adjacent TM2 helices. Measurements at low temperatures proved a considerable line broadening due to dipolar interaction for the spectra of V78R1 and L82R1. The inter-spin distances, obtained by fitting of EPR spectra,[26] were determined to be less than 12 Å. These results were explained by two TM2 helices of the 2 : 2 complex in close contact with

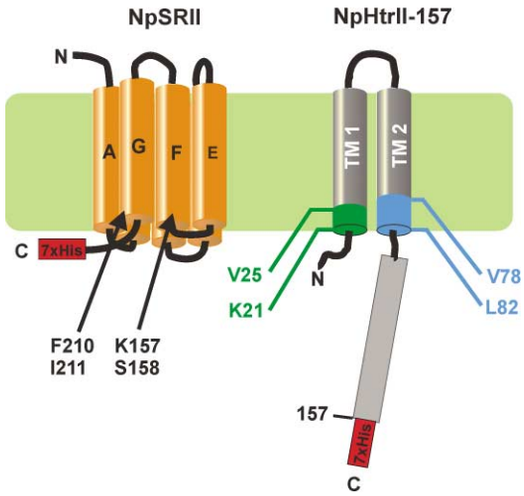

Fig. 1 Single site mutations in NpSRII-His and NpHtrII-157-His as discussed in the text.[17]

residues V78 and L82 located at the interface. More recent studies using gene fusion analysis on the SRI/HtrI system of *H. salinarum* demonstrated the same stoichiometry for this complex,[27] indicating that this arrangement might be a general feature of archaeal photo-receptor/ transducer complexes. Contrary to the close interaction between the two TM2 helices TM1 is further apart from its partner as only the spectrum of M22R1 displays a weak spin–spin interaction.

To determine the topology of the NpSRII/NpHtrII reconstituted complex, distance measurements between TM1 or TM2 and helix F or G were carried out. The positions were chosen at the cytoplasmic boundary of TM1 (21 to 25), TM2 (80, 81), helix F (157, 158), and helix G (210, 211). The inter-spin distances were used as constraints for modeling the topological arrangement of the NpHtrII/NpSRII complex. The strongest interactions, which correspond to the closest distance (<12 Å), were observed between positions 25^{TM1}–210^{G}, 81^{TM2}–210^{G} and 81^{TM2}–211^{G} indicating that both TM1 and TM2 are close to helix G. Medium distances (12–15 Å) were determined for 80^{TM2}–210^{G}, 80^{TM2}–211^{G}, 80^{TM2}–158^{F}, and 81^{TM2}–158^{F} (see Fig. 2a for residue positions). It was concluded that TM2 has to be located between the C-terminal helices F and G as depicted in Fig. 2a. The relative orientation of TM1 in the model complex was based on distance measurements between positions 21–25 on this transducer-helix and residues 158 and 210 on the receptor.

The topological model derived from this data is in line with the previous observation that in the presence of the NpHtrII-157 the accessibility of S158R1 towards a water soluble quencher (chromium oxalate) as well as the oxygen accessibility of F210R1 is abolished. The results point to a quaternary complex with two NpHtrII-157 and NpSRII molecules each, forming a structure with an apparent two-fold symmetry (Fig. 2a). Since the structure of NpSRII was not known at the time of these investigations, the BR structure was used as a template.[28] The transducer helices were modeled as canonical α-helices.

X-ray structure

The crystallization of the receptor/transducer-complex was carried out using a further shortened transducer comprising only residues 1–114.[21] This fragment was shown to contain the complete receptor binding domain.[11] The crystals were grown using the lipidic cubic phase crystallization method.[29] The structure was solved to 1.94 Å resolution. The asymmetric unit contains one dimer with a crystallographic two-fold rotation axis located in the middle of the four helical bundle formed by TM1, TM2, TM1′ and TM2′. Helices F and G of the receptor are in contact with the transducer-helices (Fig. 2b). It is note-

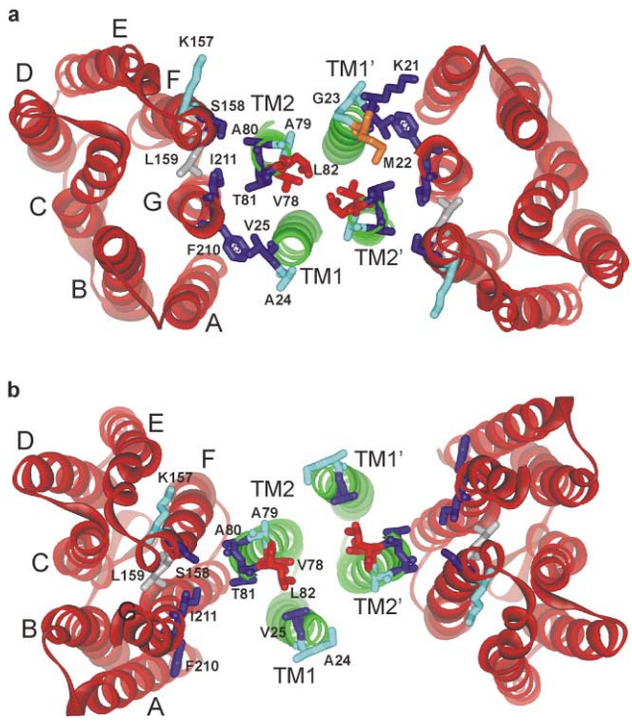

Fig. 2 (a) EPR model of the receptor/transducer complex transmembrane region[17] *vs.* (b) crystal structure.[21] View from the cytoplasmic side. Receptor helices are shown as red, transducer helices as green ribbons. Side chains replaced by Cys and subsequently labeled in the EPR study are shown in stick representation, colored according to the strength of the observed dipolar broadening of the EPR spectra (strong interaction, blue and red; weak interaction, cyan and orange).

worthy that with the exception of Tyr199 the structure of NpSRII is indistinguishable from that in the complex.[19,20]

Direct comparison of the EPR-model with the crystal structure (see Fig. 2a and b) emphasizes the consistency of the SDSL model with that of the X-ray structure, especially, if the general topology, the stoichiometry, and the location and relative orientation of TM2 are considered. Notably, most of the side-chain orientations within the complex coincide quite well between X-ray structure and SDSL model, although in the latter model the BR instead of the NpSRII structure had to be taken (see above). A slight difference between the models is recognized concerning the distance of TM1 from the receptor, which is longer than predicted in the EPR model.

Light induced conformational changes: comparison of NpSRII with BR

Sensory rhodopsin II

On light excitation the NpSRII receptor reverts to the long-lived intermediate M with a deprotonated Schiff base. This intermediate has been proposed to represent the signaling state.[30] To elucidate light induced conformational changes of the receptor as a possible key step in the signal transfer mechanism, EPR difference spectra[31] between photo-activated states and the initial state were determined for NpSRII variants with spin labels bound to the cytoplasmic ends of helices F and G.[16,17] These spectra revealed considerable light-induced changes of the motional restrictions of only those spin label side chains, which are located between helices G and F of NpSRII. The shape of the difference spectra was found to be in agreement with the assumption of a transient mobilization of nitroxide side chains at positions 158, 159 and 211. Since the nitroxide side chains at G helix positions 212 and 213, which are oriented towards helices B and C, did not show any considerable mobility change during the NpSRII photocycle, a movement of helix G is unlikely. Hence it was concluded that the required transient increase of the accessible space for the reorientational motion of the spin label side chains at positions 158, 159 and 211 had to result from an outward movement of helix F. This conclusion was further supported by the observation that in the presence of the transducer the transient difference spectrum determined for side chain position 158 is inverted: the accessible space for the reorientational motion of the nitroxide was decreased by the outward movement of helix F towards TM2 of the transducer. The kinetics of this conformational change has been followed at fixed ***B***-field values where the difference spectrum shows local extremes. The time courses of the mobility changes yield clear evidence that the conformational change occurs with an apparent half time of 3 ms during the M state, which can be correlated to the M_1 to M_2 transition.[32] A recent FTIR study confirmed that protein conformational changes take place during the M_1 to M_2 transition.[9] The outward movement remains unchanged for about two orders of magnitude in time and reverts during the recovery of the initial state. These findings are in line with FTIR studies, which show that conformational changes occurring during the M state persist until the reformation of the parent state.[6,10,33]

Bacteriorhodopsin

The light induced isomerization of the retinal and rearrangements of the protein region and internal water molecules in its vicinity lead to the release of a proton from the Schiff base to the extracellular medium during the L to M transition of BR. A detailed analysis of the electron crystallographic data taken at various times after illumination shows a single large conformational change that is roughly coincident with formation of the M intermediate.[34] Upon comparison of projection difference maps, Subramaniam and Henderson[35] concluded that the structure of illuminated wild-type BR is identical to that of the D96G, F171C, F219L triple mutant. This mutant was then used to obtain a detailed picture of the state characterized by an open cytoplasmic channel in BR. According to these results an outward movement of helix F is evident starting from position 182, whereas the changes in helix G are manifest starting from position 222. The top cytoplasmic end of helix F is displaced by ≈3.5 Å away from the centre of the protein, whereas helix G is displaced by ≈2 Å towards a position between helix F and the centre of the protein and also becomes more ordered at its carboxy terminus.[35] The large tilt of helix F leads to a significant rearrangement of the EF loop, and to minor rearrangements of the uppermost residues in helix E. Other studies, aimed at determination of the structure of the open cytoplasmic channel conformation, are based on analysis of X-ray diffraction patterns of three-dimensional crystals of either wild-type BR[36] or the D96N mutant.[37] The conclusions of the X-ray crystallographic analyses disagree with each other and with the findings of the electron diffraction studies regarding the nature of the main conformational change. Formation of the M intermediate was found to be associated with increased structural disorder in the crystal of the D96N mutant. Coordinates for the cytoplasmic sections of helices E and F, and the EF loop, residues 154 to 175, could not be determined.[37] Similarly, residues 223 to 228 in helix G could not be resolved. These regions correspond to the sites where the electron diffraction studies reveal the protein conformational changes. Coordinates determined from illuminated crystals of wild-type BR with an estimated occupancy of 35% of the M_2 intermediate show also rearrangements in the vicinity of helices F and G.[36] However, these coordinates report a smaller extent and different direction of the helix F movement. Hence, the main structural change seems to be inhibited or muted in the three-dimensional crystals.

During the subsequent transition from M_2 to N the Schiff base is reprotonated with a proton released from Asp96. The

protein conformation of both intermediates, M_2 and N, were found to be very similar.[34] The subsequent reprotonation of Asp96 from the cytoplasm during the transition from N to O is accompanied by the recovery of the initial state conformation.

A systematic investigation of a series of BR variants with spin label side chains introduced along the E-F loop and into the cytoplasmic ends of helices E and F, where only poorly resolved X-ray crystallographic data have been available, revealed a light induced transient increase of the motional freedom for the nitroxide side chains located between helices B, C and F. This result can be explained by a transient outward movement of helix F during the photocycle.[38] The extent of the observed helix movement was determined from inter-spin distances of stabilized intermediates. Cooling the BR sample to 220 K under illumination accumulates the M intermediate to approximately 75%.[39] Inter-spin distance changes determined between spin labels bound to helices C and E, positions 101 and 159, C and F, positions 101 and 168, and C and G, positions 100 and 226, revealed that the cytoplasmic end of helix F moves outwardly by about 1 to 2 Å. This transient helix tilt is accompanied by an inward movement of helix G of similar extent towards helix C. Thus, regarding the movement of helix F, the conformational change observed in NpSRII reveals considerable similarities to that observed for BR in purple membrane. However, no significant movement of helix G could be observed in NpSRII so far. It is interesting to note that the initial state structure of crystallized NpSRII shows the cytoplasmic end of helix G already shifted towards helices B and C, similar to what is proposed for helix G of M_2 in BR (see Fig. 3).

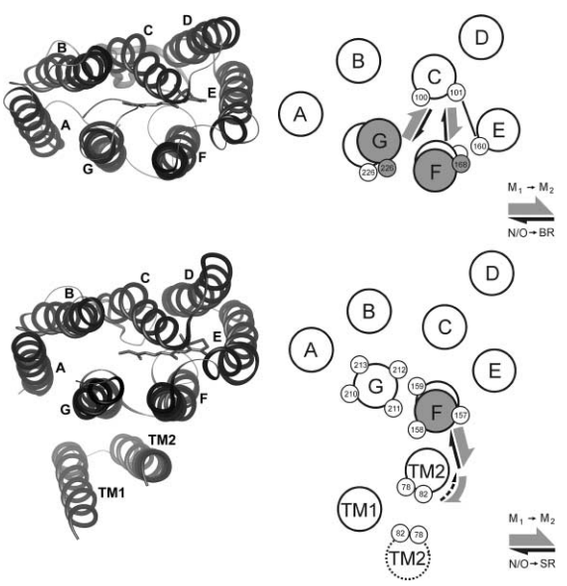

Fig. 3 View onto the cytoplasmic side of BR[28] (top) and the asymmetric unit of the sensory rhodopsin/transducer complex, NpSRII/NpHtrII[21] (bottom). The schemes on the right depict the cytoplasmic end of the transmembrane helices constituting the complex (circles) and the conformational changes during the M_1 to M_2 transition for samples in purple membrane as determined by site directed spin labeling and EPR spectroscopy (grey and black arrows). Inter-spin distance data reveal an outward movement of the BR helix F by at least 0.1 nm accompanied by an inward shift of the cytoplasmic terminus of helix G by 0.2 nm. The transient increase of the side chain mobility at positions 158, 159 and 211 indicate a similar outward movement of helix F in NpSRII. The lack of any considerable conformational changes in the vicinity of positions 212 and 213 makes large shifts of helix G unlikely. Measurements of inter-spin distance and distance changes between V78R1 and V78R1′, and between L82R1 and L82R1′ in TM2 and TM2′ (dotted), provide strong evidence that the tilt of helix F leads to a 20–30° rotation of TM2.

Time constants of the rise and decay of the BR conformational change were determined from a superposition of exponentials fitted to the experimental EPR data. A comparison with the respective time constants for the photocycle intermediates of BR extracted from optical and FTIR spectroscopy have shown that the observed conformational change occurs in phase or prior to the reprotonation of the Schiff base depending on the nitroxide location.[39] The trigger for this conformational change must therefore be independent from the reprotonation of the Schiff base. In conclusion, the extent and direction of the helix movements determined by EPR spectroscopy in BR samples in purple membrane are in agreement with the results of electron crystallographic data. In addition, time resolved EPR data provided direct evidence for the occurrence of the conformational change during intermediate M, most probably during the M_1 to M_2 transition, quite similar to the observations on NpSRII.

Transfer of the signal from NpSRII to NpHtrII

The tilt of helix F of the NpSRII receptor is transferred to the transducer as was shown by comparing the inter-residual distance changes observed between transducer/ transducer as well as receptor/ transducer during the photocycle.[17] Low temperature experiments (T = 170 K) with the receptor trapped in the M intermediate revealed decreases and increases of the dipolar interaction strengths between the two TM2 of the transducer dimer and between TM2 and helix F of NpSRII. Residues S158R1/K157R1 of helix F approached TM2-residues A80R1/T81R1 in the M state. Simultaneously, the strong dipolar interaction between the two V78R1 in neighboring TM2 helices was reduced significantly whereas the distance between the two L82R1 sites only one helical turn away did not change. This result clearly revealed a rearrangement of the two TM2 helices (see Fig. 3). From various possible mechanisms proposed to explain the signal transfer in chemo-receptors,[40,41] a clockwise rotation of TM2 by approximately 20–30° was found to be consistent with the experimental data.[17] The room temperature EPR signal changes recorded for V78R1 were compared with that of L159R1 which corresponds to an amino acid position on helix F oriented towards the inside of the receptor and with optical traces monitoring the depletion and reformation of the NpSRII parent state. The kinetic difference spectra recorded at room temperature represent features of a transient increase of the inter-spin distance for V78R1 and a transient mobilization for L159R1. Thus, the spectra detect events occurring at the level of the retinal chromophore, of helix F and of TM2, which allow to follow the signal transfer in the sequence retinal⟶helix F⟶TM2 and *vice versa*. From comparison to the optical traces, it is clear that the transducer signaling state is formed simultaneously with the receptor helix F movement, parallel the M_1 to M_2 transition.[42] With the reformation of the parent state, the reaction characteristic of the receptor seems to be decoupled from those of the transducer. The resetting movement of helix F into the original position seems to precede the recovery of TM2 position, the latter one being delayed by approximately 200 ms.

Concluding remarks

In recent years EPR spectroscopy has been successfully applied to biological questions. In combination with site directed spin labeling its potentials for providing dynamic and kinetic data as well as structural information are especially useful for the elucidation of structure and function of membrane proteins. This class of proteins is notoriously difficult to crystallize and therefore structural information is as yet scarcely available. The possibility to obtain dynamic and kinetic data on membrane processes will become decisive for the analysis of transmembrane signaling of eukaryotic and bacterial receptors.

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