Membrane Proteins

DOI: 10.1002/anie.200904031

Cross-Linking of Transmembrane Helices Reveals a Rigid-Body Mechanism in Bacteriorhodopsin Transport**

*Rosana Simón-Vázquez, Tzvetana Lazarova, Alex Perálvarez-Marín, José-Luis Bourdelande, and Esteve Padrós**

In membrane proteins, the mechanisms driving transmembrane (TM) helices through the conformational changes needed to accomplish the translocation of substrates or for intraprotein signal transduction are still a matter of debate.[1–3] Do the helices follow an alternating mechanical rocker-switch mechanism or do they act as soft structures, which would allow a Brownian ratchet mechanism? Are the interruptions or the hinges in the middle of transmembrane helices necessary to allow individual movements of parts of the helices? The activation of the G-protein-coupled receptor (GPCR) subfamily of 7TM helical receptors seems to imply that transmembrane helices VI and VII move apart (cytoplasmic side) or together (extracellular side)[4] following a global toggle-switch mechanism.[5] This mechanism also relies on see-saw movements around proline bends to allow the independent movement of different halves of the TM helices.[6] Bacteriorhodopsin (bR), as a structural homologue of 7TM GPCRs, is a suitable model to further explore these questions. In bR, conformational changes triggered by the absorption of a photon by the retinal chromophore are responsible for the active transport of a proton, through a series of intermediate transport steps named K, L, M, N, and O and known as the photocycle.[7] Although the first half of the photocycle (K–M) involves minor and subtle structural changes, the major conformational changes happen in the second half, in which the proton-transfer events swap from the extracellular to the cytoplasmic domain.

It has been proposed that an outward rotation around the conserved Pro186 of helix F, accompanied by a tilt of helix G, is required for water entry during the M–N intermediate transition. It is believed that this would facilitate the reprotonation of both the Schiff base and Asp96. Conformational changes of the E–F loop during the photocycle have also been described. X-ray diffraction, spin labeling, and fluorescence experiments have provided experimental details of such movements,[8–11] although the functional relevance of these changes has been questioned.[12]

An attractive approach to get insight into the nature of these movements is to restrict the conformational changes occurring between the cytoplasmic ends of helices F and G in the protein. With this purpose, we introduced two cysteines at the strategic positions 166 (end of helix F) and 228 (end of helix G) and induced a disulfide bond in the double mutant E166C/A228C (Figure 1 and the Materials and Methods Section in the Supporting Information). The accessibility of the engineered cysteine residues in the reduced and oxidized forms was checked with the fluorescent probe MIANS (Figure S1 in the Supporting Information).

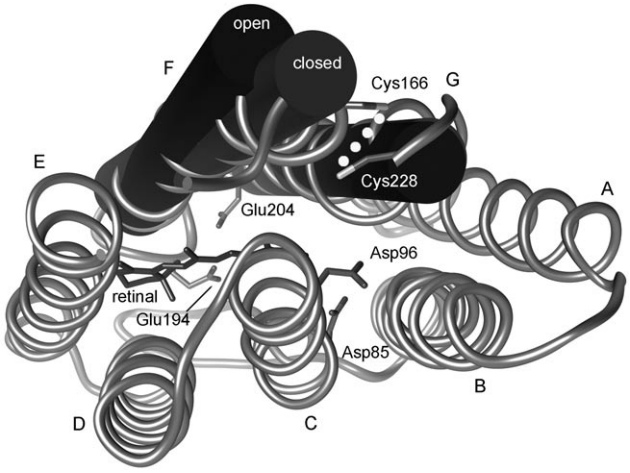

Figure 1. Three-dimensional model of the double E166C/A228C mutant of bacteriorhodopsin projected over the Protein Databank (PDB) file 1c3w coordinates. The cylinders overlapping with helices F and G correspond to the PDB coordinates of the bR open (file 1FBK in black) and closed states (file 1FBB in dark gray), respectively. The disulfide bridge is represented as a white dotted line.

Figure 2 (left and middle panels) presents the results of flash-photolysis experiments monitoring the photocycle; the M intermediate (at 410 nm), bR depletion and recovery (at

[*] Dr. R. Simón-Vázquez, Dr. T. Lazarova, Dr. A. Perálvarez-Marín,[+] Dr. E. Padrós
Unitat de Biofísica, Departament de Bioquímica i de Biologia Molecular and Centre d'Estudis en Biofísica, Facultat de Medicina Universitat Autònoma de Barcelona, 08193 Bellaterra (Spain)
E-mail: esteve.padros@uab.cat
Dr. J.-L. Bourdelande
Departament de Química, Universitat Autònoma de Barcelona 08193 Bellaterra (Spain)
[+] Current address:, Brigham and Women's Hospital, Francis St. 75 Boston, MA 02115 (USA)

[**] We thank Dr. Víctor Lórenz-Fonfría and Dr. Pere Garriga for helpful discussions, Dr. Enric Querol for advice at the beginning of this work, Jordi Hernando for help in the flash photolysis experiments, Elodia Serrano and Neus Ontiveros for excellent technical assistance, and Dr. Mathias Lübben (Ruhr-Universität Bochum) for providing the pEF191 vector and the MPK409 strain. This work was supported by a Direcció General de Recerca fellowship (2007PIV10038) to T.L. and by a Dirección General de Investigación grant (BFU2006-04656/BMC).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200904031.

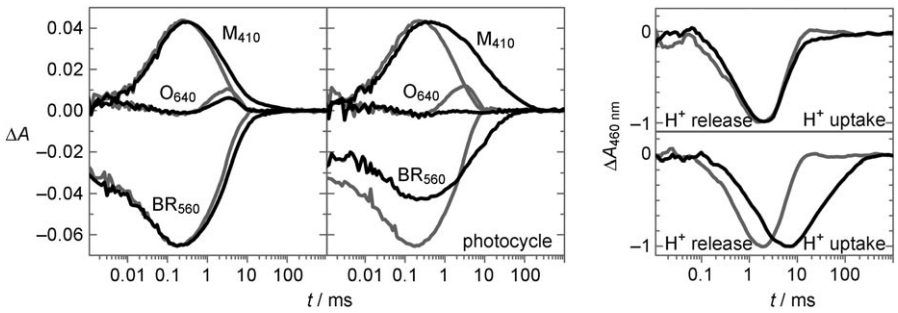

Figure 2. Left and middle panels: Photocycle flash-photolysis transient changes for the double E166C/A228C mutant corresponding to the M (410 nm) and O (640 nm) intermediates and the bR recovery (560 nm). The black line corresponds to the double mutant without (left panel) and with (middle panel) the disulfide bond. The traces corresponding to WT bR under the same reducing/oxidizing conditions as those for the mutant are overlaid in gray. Right panel: Pyranine proton uptake and release as a measure of bR proton transport. The double mutant without (upper panel) and with (lower panel) the disulfide bond (black line) is plotted against the WT bR under the same reducing/oxidizing conditions (gray line). Purple membrane patches were at a concentration of 15 μM, in 150 mM KCl at pH 7.0. The concentration of pyranine was 50 μM.

560 nm), and the O intermediate (at 640 nm) at pH 7.0 are represented. The reduced form of the mutant (without the disulfide bond; Figure 2, left panel) showed a behavior nearly identical to that of the wild type (WT; in gray). In contrast, the disulfide-cross-linked form of the E166C/A228C mutant showed a slightly delayed rise in the M kinetics, a severely delayed M decay, and the absence of the O intermediate (Figure 2, middle panel). The kinetics of the proton release and uptake were assayed by monitoring the bulk pH changes by using pyranine dye in suspensions of membrane fragments (Figure 2, right panel). Time-resolved traces of the pyranine absorption changes after the laser flash indicated that the kinetics of the pH changes for the double mutant with reduced cysteines were similar to those of the WT, whereas the cross-linked protein presented clearly delayed proton release and uptake (Figure 2, right panel). Therefore, the immobilization of the cytoplasmic ends of helices F and G also affected the extracellular side to some extent. The efficiency of proton transport was estimated by measuring the light-induced pH changes of bR reconstituted into liposomes. The reduced double mutant had a transport efficiency that was slightly decreased relative to that of the WT (about 17% less efficient), whereas the transport efficiency of the oxidized mutant was severely affected (about 54% less efficient; Figure S2 in the Supporting Information).

To analyze whether or not the conformational changes during the photocycle were affected by the constraints imposed by the disulfide bond linking helices F and G, we obtained infrared difference spectra under continuous illumination (Figure 3). Under conditions in which the WT produces the N intermediate (gray in Figure 3, spectra 1 and 2), the double mutant with reduced cysteines showed an N-like spectrum, whereas the disulfide-cross-linked E166C/A228C mutant showed a distorted M-like state (black in Figure 3, spectrum 2). At pH 7.0 and 15°C, neither the WT nor the reduced mutant showed any difference spectrum, because their fast photocycle prevents any intermediate accumulation under continuous illumination. However, the oxidized E166C/A228C mutant showed a difference spectrum with an overall shape resembling that for the M intermediate (Figure 3, spectrum 3) and including the protonated Asp85 peak at 1761 cm^{-1}. Even at alkaline pH values and 15°C (conditions that favor the N form in the WT), the M intermediate was trapped in the oxidized E166C/A228C mutant (data not shown).

The results prove that restriction of mobility of the cytoplasmic ends of helices F and G deactivates photocycle events occurring after the M intermediate. The deprotonation of Asp96 does not occur, the Schiff base is reprotonated from the bulk, and the protein goes back to its resting state directly from an M-like state, in a slow relaxation process. This behavior

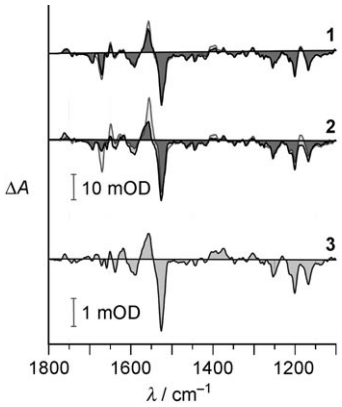

Figure 3. FTIR spectra of hydrated films of the double E166C/A228C mutant (in black) under conditions favorable for the N intermediate in WT bR (in 150 mM KCl at pH 10.0, 4°C, and 92% humidity; in gray). Spectrum 1: the double mutant without the disulfide bond; spectrum 2: the double mutant with the disulfide bond; spectrum 3: the double mutant with the disulfide bond in 150 mM KCl at pH 7.0, 15°C, and 92% humidity. These conditions did not yield any intermediate for WT bR or for the double mutant E166C/A228C without the disulfide bond.

is similar to that described for the purple membrane at a low hydration level, which is known to inhibit protein dynamics.[13] It is therefore evident that movements of the cytoplasmic side of helices F and G are a prerequisite to the events that take place in the second part of the bR photocycle: reprotonation of the Schiff base from Asp96 and reprotonation of Asp96 from the bulk. Interestingly, proton release on the extracellular side[14] also shows slower kinetics and is therefore decoupled, to some extent, from the M intermediate. Clearly, the proton-release process requires appropriate conformational changes on the extracellular side of helices F and G, where the main residues involved in the proton-release mechanism (Glu194 and Glu204) are located.

Taken together, our observations demonstrate that a relaxation/accommodation movement of bR helices F and G

is necessary to permit 1) sequential reprotonation of the Schiff base and of Asp96 and 2) synchronization of the cytoplasmic and extracellular events occurring in the photocycle. A Brownian ratchet mechanism can therefore be discarded in bR transport. Rather, we can recognize two different kinds of movement: Pro186 in helix F allows opposite movements in the cytoplasmic and extracellular halves, as in a toggle-switch mechanism,[15,16] whereas helix G should behave as a rigid body to permit the propagation of movements from one side of the membrane to the other. This type of mechanism presumably needs a sort of pivot or anchor besides the proline bends; this may consist of specific interactions at a location near the center of the helices, like the Asp115–Thr90 interhelical hydrogen bond present in bR.[17] It is likely that this behavior is common not only to the family of 7TM proteins, like GPCRs[4] or sensory rhodopsin II,[18] but also in other receptors and membrane transporters. In these, the existence of broken helices reflects the necessity of the movement of one part of the helix being independent from the movements in the other part of an otherwise rigid helix. A similar approach to that used here could be applied to membrane transporters to reveal whether TM helices behave as rigid bodies, as can be deduced from polarized FTIR experiments demonstrating changes in helix tilting for the melibiose permease,[19] or if they follow a Brownian ratchet mechanism.[20]

In conclusion, immobilization of the cytoplasmic ends of helices F and G of bacteriorhodopsin by disulfide cross-linking between engineered cysteines leads to a defective protein. The photocycle of this modified protein is blocked, proton transport is severely decreased, and proton release and uptake kinetics are delayed. The results demonstrate that rigid-body coordinated movements of transmembrane helices F and G are required for an efficient proton-transport mechanism, which provides evidence for a toggle-switch behavior.

Received: July 21, 2009
Published online: October 6, 2009

Keywords: bacteriorhodopsin · helical structures · membrane proteins · proton transport · structural biology

[1] G. Diallinas, *Science* **2008**, *322*, 1644.
[2] O. Boudker, R. M. Ryan, D. Yernool, K. Shimamoto, E. Gouaux, *Nature* **2007**, *445*, 387.
[3] S. Weyand, T. Shimamura, S. Yajima, S. Suzuki, O. Mirza, K. Krusong, E. P. Carpenter, N. G. Rutherford, J. M. Hadden, J. O'Reilly, P. Ma, M. Saidijam, S. G. Patching, R. J. Hope, H. T. Norbertczak, P. C. Roach, S. Iwata, P. J. Henderson, A. D. Cameron, *Science* **2008**, *322*, 709.
[4] S. Ahuja, V. Hornak, E. C. Yan, N. Syrett, J. A. Goncalves, A. Hirshfeld, M. Ziliox, T. P. Sakmar, M. Sheves, P. J. Reeves, S. O. Smith, M. Eilers, *Nat. Struct. Mol. Biol.* **2009**, *16*, 168.
[5] T. W. Schwartz, T. M. Frimurer, B. Holst, M. M. Rosenkilde, C. E. Elling, *Annu. Rev. Pharmacol. Toxicol.* **2006**, *46*, 481.
[6] C. E. Elling, T. M. Frimurer, L. O. Gerlach, R. Jorgensen, B. Holst, T. W. Schwartz, *J. Biol. Chem.* **2006**, *281*, 17337.
[7] J. K. Lanyi, *Biochim. Biophys. Acta Bioenerg.* **2006**, *1757*, 1012.
[8] N. Radzwill, K. Gerwert, H. J. Steinhoff, *Biophys. J.* **2001**, *80*, 2856.
[9] T. Rink, M. Pfeiffer, D. Oesterhelt, K. Gerwert, H. J. Steinhoff, *Biophys. J.* **2000**, *78*, 1519.
[10] D. Chen, J. M. Wang, J. K. Lanyi, *J. Mol. Biol.* **2007**, *366*, 790.
[11] L. S. Brown, G. Varo, R. Needleman, J. K. Lanyi, *Biophys. J.* **1995**, *69*, 2103.
[12] J. Tittor, S. Paula, S. Subramaniam, J. Heberle, R. Henderson, D. Oesterhelt, *J. Mol. Biol.* **2002**, *319*, 555.
[13] G. Varo, J. K. Lanyi, *Biophys. J.* **1991**, *59*, 313.
[14] F. Garczarek, K. Gerwert, *Nature* **2006**, *439*, 109.
[15] A. Perálvarez-Marín, J. L. Bourdelande, E. Querol, E. Padrós, *Mol. Membr. Biol.* **2006**, *23*, 127.
[16] A. Perálvarez-Marín, V. A. Lórenz-Fonfría, R. Simón-Vázquez, M. Gomariz, I. Meseguer, E. Querol, E. Padrós, *Biophys. J.* **2008**, *95*, 4384.
[17] A. Perálvarez-Marín, V. A. Lórenz-Fonfría, J. L. Bourdelande, E. Querol, H. Kandori, E. Padrós, *J. Mol. Biol.* **2007**, *368*, 666.
[18] V. I. Gordeliy, J. Labahn, R. Moukhametzianov, R. Efremov, J. Granzin, R. Schlesinger, G. Buldt, T. Savopol, A. J. Scheidig, J. P. Klare, M. Engelhard, *Nature* **2002**, *419*, 484.
[19] N. Dave, V.-L. Lórenz-Fonfría, G. Leblanc, E. Padrós, *Biophys. J.* **2008**, *94*, 3659.
[20] R. J. Naftalin, N. Green, P. Cunningham, *Biophys. J.* **2007**, *92*, 3474.

