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Bacteriorhodopsin: A Spectroscopic Intermediate with Two Conformations and Three Relay Events Is Voltage Sensitive

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Bacteriorhodopsin in the purple membrane of halobacteria is a prominent prototype of an integral membrane protein, especially of the family of seven membrane-spanning α-helical proteins. At present, this light-driven proton pump is one of the best characterized active ion-translocating proteins. Soon, it will be the first membrane protein whose vectorial transport mechanism is understood at the molecular and even atomic level. To unravel the mechanism of a catalytic protein, such as bacteriorhodopsin, the link between the structure, its dynamics, and the function has to be established. The concerted application of both powerful biophysical methods and tailor-made bacteriorhodopsin samples shall make it possible to reach this goal. The report by Nagel and co-workers (1997) in this issue is a marvelous example of that; indeed, it is a successful way to approach the goal.

Tracking the individual steps of charge translocation through bacteriorhodopsin obviously requires knowledge of its structure. Fortunately, by high-resolution electron crystallography on natural, two-dimensional purple membrane crystals (Kimura et al., 1997) as well as by x-ray diffraction on three-dimensional (3D) crystals (Pebay-Peyroula et al., 1997) the 3D structure of bacteriorhodopsin has been recently determined at a resolution of 3.0 and 2.5 Å, respectively, supporting the pioneering work of R. Henderson and co-workers. Soon, we can expect a bacteriorhodopsin structure at 2.0 Å. However, to pinpoint the elementary steps of proton translocation, the bacteriorhodopsin structure of the ground state is not sufficient. The structure of the photocycle intermediates, at least of the functionally relevant intermediates is also required. Although controversial at the beginning, the light-induced appearance of tertiary structural changes in the protein, predominantly at helix F and G, has now been established by various investigations using electron crystallography and neutron and x-ray diffraction. The photocycle and the proton pumping cycle of bacteriorhodopsin are accompanied and most probably propelled by structural changes in both the chromophore, the protonated Schiff base of retinal, and the protein (Hauss et al., 1994). Recent structural studies on wild type and mutant bacteriorhodopsin have revealed that the tertiary structural changes in the protein moiety occur in the M intermediate and persist also in the N intermediate. The onset of these tertiary structural changes is correlated with the transition between two structurally distinct substates of the spectroscopic intermediate M, designated M_1 and M_2 (Sass et al., 1997; Nagel et al., 1997). These large changes in the structure take place after the transfer of the proton from the Schiff base to the amino acid Asp-85 and after the release of the proton to the extracellular surface. This indicates that these structural changes follow a redistribution of charges. (It should be noted that a different view has been published in some Fourier transform infrared studies, i.e., the onset of the largest structural changes, as solely defined by difference bands in the amide I and II regions, in the transition from M to N.)

How are these conformational changes in bacteriorhodopsin coupled to the vectorial, electrogenic proton transfer from the cytoplasmic side to the extracellular side? The kinetics, location, and stoichiometry of proton transfer reactions, such as proton ejection to and migration along the membrane surface as well as into the aqueous water phase, have been successfully determined by the application of laser absorption spectroscopy with pH probes either covalently bound to bacteriorhodopsin or residing in the bulk (Heberle et al., 1994). In contrast, charge translocation through the core of the protein is best studied by electrical measurements. During the past 25 years, several approaches to monitor light-induced charge translocation across the purple membrane have been applied, which are marked by their extreme sensitivity and often by their high time resolution. However, the interpretation of the data of most studies dealing with the light-induced charge translocation across bacteriorhodopsin and of all studies examined the voltage dependence was severely hampered by the undefined and non-unidirectional orientation of the current generator bacteriorhodopsin. In this respect, the study by Nagel et al. (1997) is unique. For the first time, the voltage dependence of charge translocation by the proton pump could be unequivocally determined, i.e., under well defined voltage clamp conditions, by expressing bacteriorhodopsin from *Halobacterium salinarium* in the plasma membrane of oocytes from *Xenopus laevis* and by measuring the current voltage behavior of this unidirectionally oriented bacteriorhodopsin population. According to the interpretation of the voltage dependence of the pump current and blue light quenching effect, the externally applied electrical field changes the ratio of the M_1 and M_2 states. This should also occur during light-induced generation of an electrochemical gradient in the halobacteria. The observation that negative po-

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tentials arrest the protein in M_1 is of functional relevance, yet it could also be an experimental tool to study this substate, e.g., by diffraction techniques or Fourier transform infrared spectroscopy. The voltage dependence of the ratio between M_1 and M_2 is a strong hint for the functional importance of these two M substates in controlling the vectoriality of the proton pump. During formation of M_1, a proton is transferred from the Schiff base to Asp-85 and at the same time, one proton is ejected to the extracellular surface of bacteriorhodopsin. In the M_2 to N transition, the Schiff base becomes reprotonated, but via the cytoplasmic side, receiving its proton from Asp-96, whose pK is lowered. Thus, the spectroscopic intermediate M is, in fact, composed of two structural different substates that differ in the accessibility of the Schiff base for protons and its pK (intermediate M_1: Schiff base accessible to the extracellular proton pathway, pK shifted from originally >13 to <4; M_2: Schiff base accessible to the cytoplasmic proton pathway, pK >10). The important finding by Nagel et al. (1997) is that the electrical potential regulates the ratio between M_1 and M_2 in this key transition, which might be the sole or at least a "molecular switch" in the pumping mechanism (another molecular switch could be the observed positional change of the retinal polyene chain close to the Schiff base; Hauss et al., 1994). The electrical measurements on membranes with perfectly oriented bacteriorhodopsin molecules can bridge the gap between proton translocation steps through this pump and the light-triggered tertiary structural changes. This experimental system would be the perfect one to unravel the elementary steps of proton translocation if spectroscopic and structural investigations would be feasible. However, because the density of bacteriorhodopsin expressed in the plasma membrane is relatively low, not even the photocyle can be studied at present. Presumably bacteriorhodopsin is in the monomeric state and highly mobile as opposed to the trimeric state in the crystalline purple membrane. This might be even advantageous for data interpretation, as the bacteriorhodopsin monomer is the functional unit for proton pumping.

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