Synthetic Biology

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Engineering a Chemical Switch into the Light-driven Proton Pump Proteorhodopsin by Cysteine Mutagenesis and Thiol Modification

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Abstract: *For applications in synthetic biology, for example, the bottom-up assembly of biomolecular nanofactories, modules of specific and controllable functionalities are essential. Of fundamental importance in such systems are energizing modules, which are able to establish an electrochemical gradient across a vesicular membrane as an energy source for powering other modules. Light-driven proton pumps like proteorhodopsin (PR) are excellent candidates for efficient energy conversion. We have extended the versatility of PR by implementing an on/off switch based on reversible chemical modification of a site-specifically introduced cysteine residue. The position of this cysteine residue in PR was identified by structure-based cysteine mutagenesis combined with a proton-pumping assay using E. coli cells overexpressing PR and PR proteoliposomes. The identified PR mutant represents the first light-driven proton pump that can be chemically switched on/off depending on the requirements of the molecular system.*

The emerging field of synthetic biology takes advantage of biomolecules and organisms that exist in nature to generate new systems with defined functionalities. This can be approached top-down (introducing new features into living cells) or bottom-up (assembling isolated biomolecules into new functional systems). The potential applications are diverse and cover all colors of biotechnology.[1–5] A simple bottom-up assembled biomolecular nanofactory comprises a nanoscale container, for example, a vesicular structure with functional modules in the membrane and the enclosed compartment. The assembled modules define the functionality and application spectrum of such a system. Modules can be proteins of specific functions found in nature or versions that have been engineered to meet the requirements of the biomolecular nanofactory. Essential components in such systems are energizing modules for the appropriate supply of energy. Proton gradients across biological membranes are commonly used by living cells to power processes such as import, export, and synthesis of molecules. For proton transport and the efficient establishment of a proton gradient across membranes, proton channels[6,7] and light-driven proton pumps[8–12] are good candidates. The latter in particular represent an excellent choice since light energy is easily accessed and supplied to the system. In the past, the light-driven proton pump bacteriorhodopsin from the halophilic archaeon *Halobacterium salinarum*[8,9,11] has been used in liposome- and polymerosome-based nanofactories to power the production of ATP.[13–15] In this work, we chose the green-light-absorbing proton pump proteorhodopsin (PR)[10,12] as energy supply module because its genetic manipulation and overexpression in the bacterium *Escherichia coli* is well established compared to other light-driven proton pumps. PR is a seven-transmembrane α-helix protein carrying a retinal cofactor bound via a lysine Schiff base. Upon light absorption, the all-*trans* retinal photoisomerizes to 13-*cis* retinal, which induces conformational changes and the transfer of a proton from the cytoplasm to the extracellular space. The proton-transfer mechanism of PR has been well characterized,[12,16–18] and the molecular assembly and structure were recently determined by atomic-force microscopy[19] and solution NMR.[20] The availability of functional and structural data for PR paves the way for rational mutagenesis to extend its functionality as a module for applications in synthetic biology. Our approach towards introducing a molecular on/off switch into PR comprised mutagenesis of amino acids that are located near the proton translocation pathway and close to the aqueous solution at the extracellular side into cysteine residues.[21] Subsequent covalent modification of the engineered cysteine residue by a bulky or charged chemical moiety makes interference with the pumping function probable. For the chemical gating of channels and pores, similar approaches have been used in the past.[6,22] The choice of methanethiosulfonates as thiol-modifying reagents[23] opens the possibility of reactivating PR by removing the covalently bound moiety using reducing agents such as β-mercaptoethanol (β-ME).

In the living bacterium, PR pumps protons out of the cell to generate a proton gradient across the cell membrane. As a robust method to measure the pumping activity of PR, we used a micro pH electrode and quantified pH changes in a small volume of unbuffered suspension of PR-overexpressing bacteria (Figure 1a). Water cooling of the system and gentle stirring provide constant experimental conditions. Upon illumination of the *E. coli* cells overexpressing PR, translocation of intracellular protons to the outside solution induces a pH shift that is measured with the micro pH electrode (see Methods in the Supporting Information).

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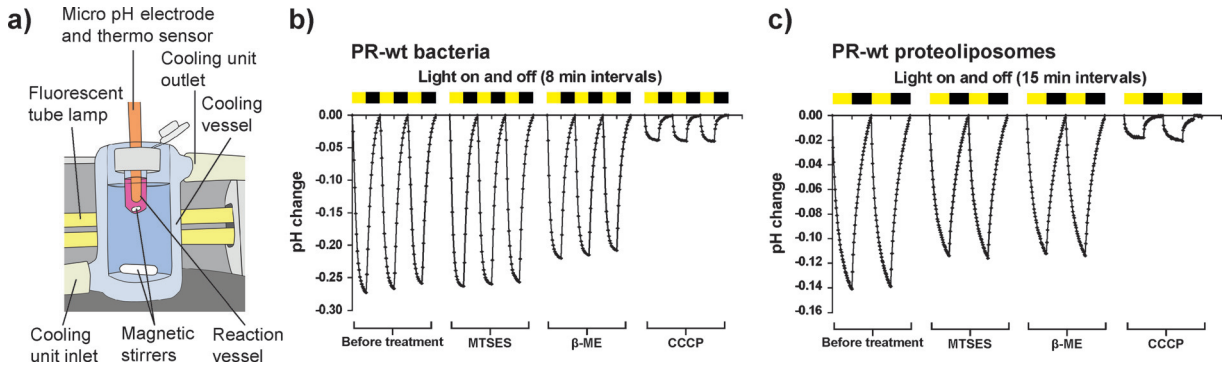

Figure 1. a) Experimental set up. b, c) Photoactivity measurement of *E. coli* cells overexpressing PR-wt (b) and PR-wt proteoliposomes (c). Yellow and black bars indicate light/dark cycles (interval time indicated) in which the acidification occurs and recedes. The first three sets of experiments in panels (b) and (c) show the same sample before treatment, after 8 mM MTSES (sodium (2-sulfonatoethyl) methanethiosulfonate), and after 75 mM β-ME (β-mercaptoethanol) treatment, respectively. A control treated with the proton ionophore CCCP (carbonyl cyanide 3-chlorophenylhydrazone; 150 μM for bacteria and 50 μM for proteoliposomes) is shown for comparison.

Figure 1b shows a representative experiment with wild-type PR (PR-wt): within 8 min of light exposure, the pH in the solution dropped by about 0.25 (see the Supporting Information for raw data) and was regenerated within 8 min of dark incubation. This effect is fully reproducible over numerous light/dark cycles. For the chemical modification of PR, we selected the membrane-impermeable thiol-modifying agent sodium (2-sulfonatoethyl) methanethiosulfonate (MTSES). After treatment of the previously measured sample with MTSES, the measurement was repeated and the results indicated similar proton-pumping activity (Figure 1b). This result implies that MTSES does not inhibit the function of overexpressed PR-wt in *E. coli* cells. Subsequent treatment of the sample with the reducing agent β-ME slightly reduced the signal produced by PR-wt (Figure 1b). A possible explanation for the slight drop in activity after β-ME treatment is the modification of other proteins of *E. coli*, or the partial lysis or loss of cells during the modification and washing steps. Addition of the proton ionophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP) abolished the light-induced pH change almost to background levels (Figure 1b; see also Table S1 in Supporting Information for background levels), thus clearly demonstrating that the measured signal originates from a proton gradient across the cell membrane. In contrast to bacteria, functional studies using proteoliposomes have the great advantage that the system is clearly defined as consisting of membrane protein and lipid only. Therefore, possible interference in the measured signal from other cellular components can be excluded. For reconstitution, membranes isolated from PR-overexpressing *E. coli* cells were solubilized in n-octyl-β-D-glycopyranoside (OG) and the protein was purified by nickel-affinity chromatography (see Methods in the Supporting Information). The quality of the purified protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and UV/Vis spectroscopy (see Figure S1 and Table S1 in the Supporting Information). In SDS-PAGE, PR migrated as a double band around 24 kDa and was highly pure (Figure S1a). The two bands represent full-length PR and N-terminally truncated PR lacking the signal peptide, as described previously.[16] PR-

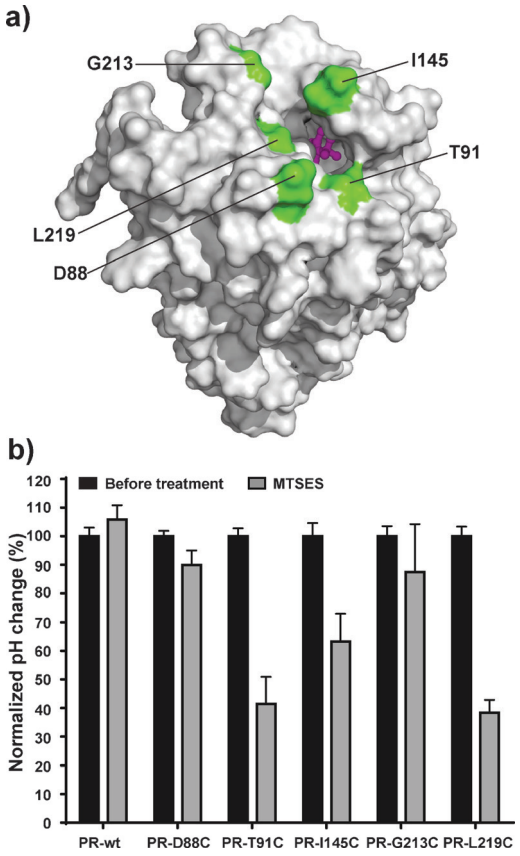

Figure 2. First screening round for the PR cysteine mutants. a) A space-filling model of the PR surface viewed from the extracellular side (PDB ID: 2L6X[20]). Identified amino acid residues for cysteine mutagenesis that are accessible to the aqueous solution and the retinal cofactor are indicated in green and magenta, respectively. b) Photoactivity measurements with *E. coli* cells overexpressing PR-wt and PR mutants. The signal height before treatment is normalized to 100% and compared to the activity after MTSES (sodium (2-sulfonatoethyl) methanethiosulfonate) treatment. Bars represent averages of 9 peaks from 3 independent experiments. Error bars show the standard deviation.

wt was reconstituted into OG-destabilized preformed 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) liposomes (see Methods in the Supporting Information). Upon illumination, acidification of the outside medium was observed, thus indicating that PR had been successfully reconstituted, was functional, and had the same orientation as in cell membranes of PR-overexpressing *E. coli* cells. In proteoliposomes, the total pH change observed upon illumination was lower and was established more slowly compared to in bacteria (Figure 1b,c). Similar to PR overexpressed in bacteria, the effects of MTSES and β-ME on the pH change in PR-wt proteoliposomes were negligible. These screening conditions are thus suitable for detecting the effects of MTSES and β-ME on the function of PR cysteine mutants. In a first screening round, amino acid residues near the proton translocation pathway on the extracellular surface of PR (Figure 2a) were selected by using the available solution NMR structure as template.[20] The initial positions tested were T91C and L219C, which are at the extracellular end of the transmembrane α-helices C and G, and D88C, I145C, and G213C, which are in the loops B-C, D-E, and F-G (Figure 2a). Our activity assay indicated most prominently reduced proton-pumping activity for the mutants PR-T91C and PR-L219C after MTSES treatment (Figure 2b). Because the photoactivity of T91C was only half of that of PR-L219C, PR-T91C was not further considered. In a second screening round, we tested the neighboring residues of L219C on helix G by generating the PR mutants N218C, N220C, and L221C and assessing their photoactivity (Figure 3a; also see Figure S1 and Table S1). From the four mutants, only L219C and N220C showed substantially reduced proton-pumping activity after MTSES modification in bacteria and proteoliposomes, with N220C exhibiting the most prominent effect (Figure 3b,c). The regeneration of the function upon reduction with β-ME was successful for both mutants in proteoliposomes and for N220C in *E. coli* cells as well. Based on these results, we identified PR-N220C as a light-driven proton pump that can be reversibly switched on/off through chemical modification. From a practical point of view, initial screening for mutants with novel characteristics can be performed by using PR-overexpressing *E. coli* cells, thus avoiding the laborious and time-consuming generation of proteoliposomes. Nevertheless, results from the clearly defined proteoliposome system are most reliable for the functional characterization of the best screening hit(s). Next, we evaluated the robustness of the introduced chemical switch by performing several sequential on/off switching cycles with PR-N220C proteoliposomes and measuring the light-induced pH changes (Figure S2a). A decrease in the pH change was observed after each switching cycle and this was attributed to loss of proteoliposomes during centrifugation, for example, in the washing steps (Figure S2b). In summary, chemical on/off switching of the engineered PR version appears to be robust and reproducible. The observed decrease in pH change over several on/off switching cycles results mainly from loss of PR-N220C proteoliposomes during centrifugation.

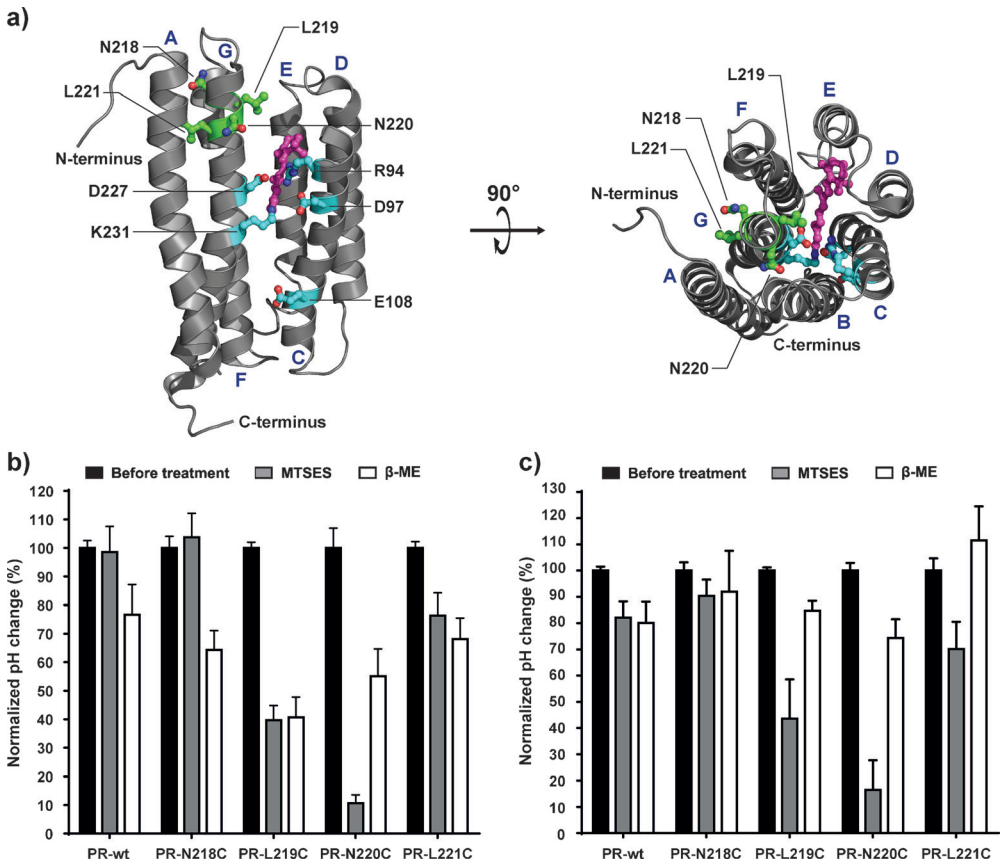

Figure 3. Second screening round for the PR cysteine mutants. a) A ribbon model of the PR structure (PDB ID: 2L6X):[20] side view (extracellular side up) and top view from the extracellular side, with amino acid residues for cysteine mutagenesis indicated in green. Also indicated are residues involved in proton translocation (cyan) and the retinal cofactor (magenta). Oxygen and nitrogen atoms in the depicted side chains are colored in red and blue, respectively. In the side view, helix B was omitted for an unobstructed view of relevant residues. b,c) Photoactivity measurements with *E. coli* cells overexpressing PR-wt and PR mutants (b) and proteoliposomes containing PR-wt and PR mutants (c). The signal height before treatment is normalized to 100% and compared to the activity after MTSES (sodium (2-sulfonatoethyl) methanethiosulfonate) and subsequent β-ME (β-mercaptoethanol) treatment. Bars represent averages of 9–17 peaks from 3–6 independent experiments (b) and 6–8 peaks from 3–4 independent experiments (c). Error bars show the standard deviation.

The availability of controllable modules with different functions for the bottom-up assembly of molecular factories is essential. Light-driven proton pumps as energy-converting modules are of particular interest in synthetic biology because of the easy accessibility of light energy (e.g., solar energy) and their high efficiency for establishing proton gradients across membranes. Such gradients can then be used to power proton-driven modules of molecular factories, for example, proton-driven transporters. In this study, we extended the versatility of PR by implementing a chemical on/off switch. The identified PR mutant N220C represents the first light-driven proton pump and energizing module that can be activated and deactivated chemically to meet the requirements of the molecular system.

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