Retinal-based Proton Pumping in the Near Infrared

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SUPPORTING INFORMATION

Title: Retinal-based Proton Pumping in the Near Infra-red

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Table S1

Retinal	λ_{max} (nm)			
	[a] DMF	[b] 1% DDM	[c] Oxime DMF	[d] Oxime 1% DDM
A1	382	388	365	368
A2	400	404	375	378
MOA2	434	441	415	402
DMAR	436	433	358	370
MMAR	434	438	358	350

Table S1: λ_{max} values of the retinal analogs and their corresponding oximes: [a] free retinal in DMF solution; [b] free retinal in 1% (w/v) DDM solution (pH 7); [c] retinal oxime in DMF solution; [d] retinal oxime in 1% (w/v) DDM solution (pH 7). Data are the average of at least two measurements, with S.D ≤ 2 nm.

Table S2

Retinal	ε ($M^{-1}cm^{-1}$)				
	[a] DMF	[b] 1% DDM	[c] Oxime 1% DDM	[d] PR:retinal 1% DDM	[e] GR:retinal 1% DDM
A1	45600	39500	52500	54200	55500
A2	44500	38000	44000	46800	49200
MOA2	30400	31500	30400	nd	nd
DMAR	31800	29000	42000	40500	nd
MMAR	28000	31000	36000	35200	34200

Table S2: Molar absorbance (ε) values of the retinal analogs and their corresponding oximes and pigments: [a] free retinal in DMF solution; [b] free retinal in 1% DDM solution; [c] retinal oxime in 1% (w/v) DDM solution; [d] PR containing the various retinal analogs, after solubilization in 1% (w/v) DDM solution; [e] GR containing the various retinal analogs, after solubilization in 1% (w/v) DDM solution. nd: not determined. DDM solution is at pH 7. Data are average of duplicate assays, with S.D ≤ 8%. For the corresponding absorbance maxima see Tables 1 and S1. nd = not determined because of low stability or low pumping activity. The ε values of PR:A2 and GR:A2 were used for PR:MOA2 and GR:MOA2 respectively, while the ε value of PR:DMAR was used for GR:DMAR

Figure S1

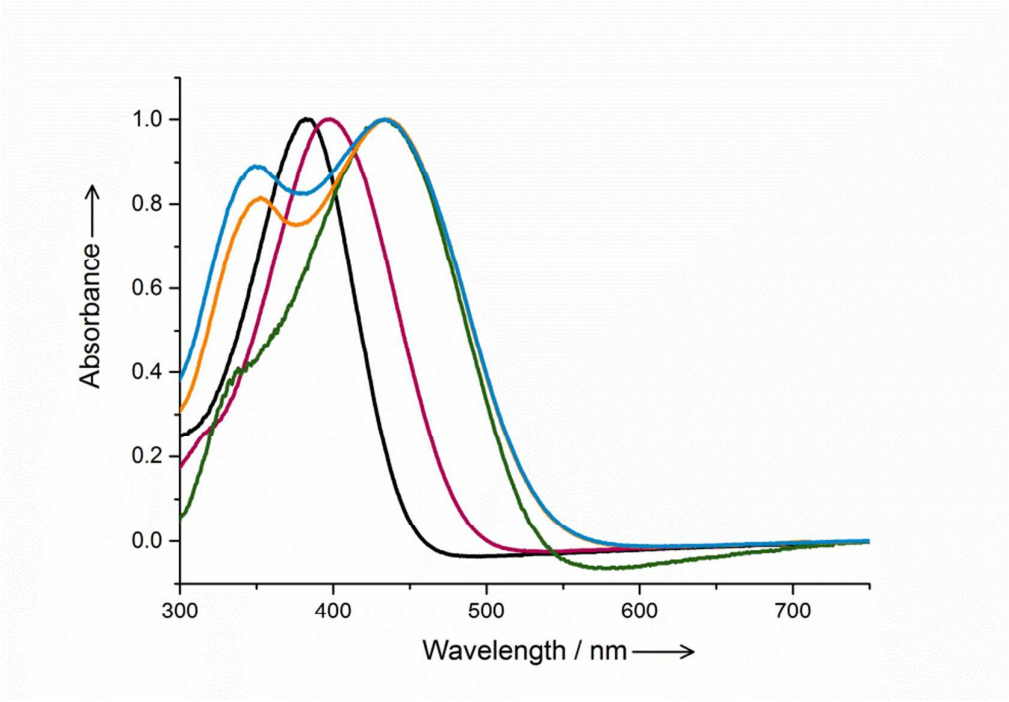

Fig S1: Normalized absorbance spectra of retinal analogs in DMF solution. A1 (black), A2 (pink) MOA2 (green), DMAR (orange), MMAR (blue). Baseline drift is a spectrophotometer artefact.

Figure S2

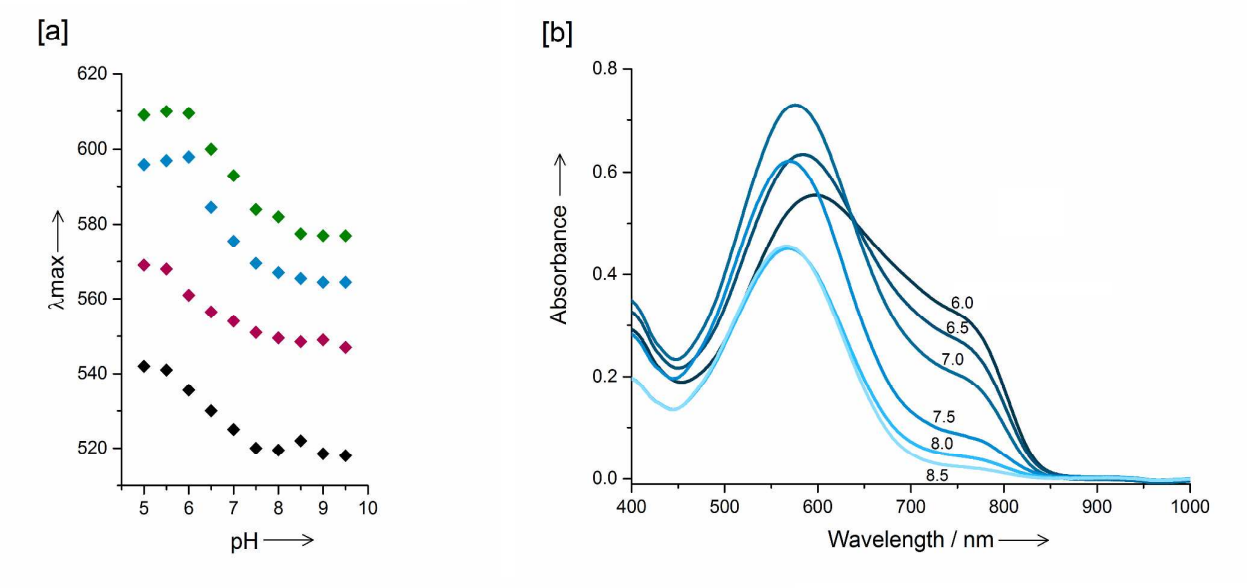

Fig S2: Spectral properties of PR pigments depend on pH [a] Protonation of the counterion D97 in PR red-shifts the absorbance band by 20-30 nm in all pigments tested. PR containing A1 (black), A2 (pink), MOA2 (green) and MMAR (blue). For all pigments the pKa of this transition lies between 6.5 and 7.0. [b] Absorbance spectra of His-tag purified PR:MMAR in DDM solution at pH 6, 6.5, 7, 7.5, 8, 8.5. pH values are indicated next to the corresponding spectrum in the graph.

Figure S3

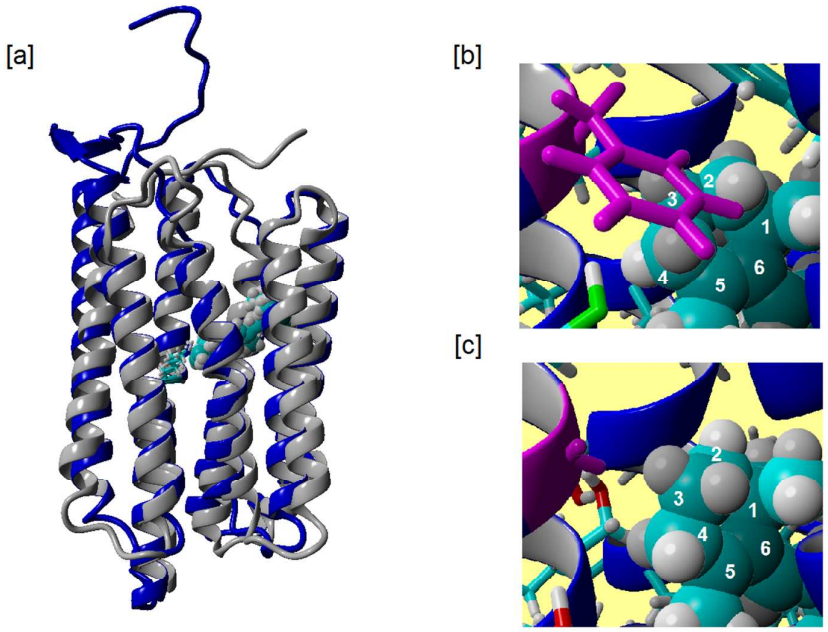

Fig S3: [a] Side view of overlay of Homology models for PR (grey) and GR (blue). [b] Binding pocket view of PR with Phe152 highlighted in magenta [c] Binding pocket view of GR with a G178 at an equivalent position highlighted in magenta. Retinal is represented in cyan as a space filled residue. The ring carbons are numbered [b-c]. For details regarding the construction of the homology models, please refer to Ganapathy et al.[16]

Experimental Section

Materials

Escherichia coli strain UT5600 and the pKJ900 plasmids encoding PR or GR with a C-terminal 6-His tag were a generous gift from Dr. K. Jung, University of Seoul, South Korea. All-*trans* retinal A1 was obtained from Sigma-Aldrich. all-*trans*-3,4-dehydroretinal (A2) was a generous gift from from Hoffman-LaRoche. all-*trans*-3-methoxy-3,4-dehydroretinal (MOA2; purity 97.8% according to the manufacturer's certificate of analysis), all-*trans*-3-dimethylamino-16-nor-1,2,3,4-didehydroretinal (DMAR; purity 99.9%) and all-*trans*-3-methylamino-16-nor-1,2,3,4-didehydroretinal (MMAR; purity > 99.9%) were synthesized on order by Buchem, B.V. Sources of special chemicals include: isopropyl β-D-1-thiogalactopyranoside (IPTG, Promega), 1-n-dodecyl-β-D maltopyranoside (DDM, Anatrace), benzonase (Merck Millipore), lysozyme (Sigma), Ni^{2+}-NTA columns (ThermoFischer-Scientific), EDTA-free protease inhibitor tablets (Roche), restriction enzymes (ThermoFischer-Scientific), ampicillin (Sigma), valinomycin (Sigma), protein ladder (ThermoFischer-Scientific), *Pfu* DNA polymerase (ThermoFischer-Scientific). All other chemicals were of analytical grade.

Site-directed mutagenesis:

Site directed mutagenesis was performed on the PR gene using mis-match PCR as described previously for PR D212N, F234S[1]. In brief, the pKJ900 plasmid containing PR/GR was linearized by restriction with Esp3I and subjected to mis-match PCR using overlapping primers containing the corresponding mutation sites for the T101A (PR) and the F260S (GR) mutations. 25 cycles of PCR were run at 95°C for 30 seconds, 55°C for 30 seconds and 68°C for one minute. The mutant gene was further amplified using outside vector primers with the same PCR program. The amplified mutant gene and vector were restricted at their HindIII and XbaI site and run on an agarose gel with 0.5 µg/ml ethidium bromide. The bands corresponding to the restricted mutant gene and the empty vector were cut out, extracted using a Qiagen gel extraction kit and ligated overnight at 4°C. The ligated plasmid was then transformed into *E. coli* UT5600 and plated on LB agar containing ampicillin. The successful clones were verified using colony PCR and sequencing.

Cell transformation:

A single colony of *E. coli* UT5600 was inoculated into 25 ml of LB medium and allowed to grow at 37°C for 4-6 hours. The cells were made competent using calcium chloride according to standard protocols. 40 ng of plasmid DNA, was added to 200 µl of competent cells and incubated on ice for 30 minutes. The mixture was heat shocked for 2 min at 42°C. The cells

were allowed to recover after addition of 1 ml of SOC medium for half an hour at 37°C, to express ampicillin resistance. The cells were then plated onto LB-agar plates containing ampicillin (50 µg/ml) and incubated overnight at 37°C.

Bacterial cell culturing:

The cells were grown in LB medium with ampicillin selection at 30°C in an orbital shaker at 180 rpm. Overnight cultures were grown from frozen glycerol stocks of transformed cells, which were diluted 1:100 to get the working culture. At a cell density corresponding to an OD_{600} of 0.3-0.4, expression of the proteorhodopsin apoprotein (opsin) was induced by the addition of IPTG to a final concentration of 1 mM. The cells were allowed to grow for a further 24 hours at 30°C, and then harvested.

Regeneration of proteo-opsin with retinal:

Retinal stocks were stored at -80 ºC in hexane. At the time of use, the required aliquot of stock solution was evaporated and the residue re-dissolved in dimethylformamide (DMF) to obtain a concentration of 1 mM. Absorbance spectra of the retinal analogs in DMF solution are presented in Figure S1. This solution was then added to a crude cellular lysate or to isolated membrane vesicles containing the opsin to achieve a final retinal concentration of 10-20 µM, and incubated under dim light for up to 60 min at room temperature (RT) or, if necessary, subsequently overnight at 4 ºC.

Preparation and analysis of membrane vesicles:

The cells were harvested by centrifugation (3,200xg, 20 min, RT), and the pellet was resuspended in an ice-cold solution of 50 mM Tris-HCl, 150 mM NaCl, pH 7 (10 ml per 50 ml of culture). The suspended cells were lysed by sonication at 4°C using a Sonics vibra-cell sonicator (10 min, 4 s pulses, 5 s pauses, 25% amplitude) and centrifuged to remove insoluble material and cellular debris (4,000xg, 15 min, 4°C). The resulting supernatant with membrane vesicles was incubated with the selected retinal for one hour at RT and the vesicles were then pelleted by high-speed centrifugation (147,000xg, 1 h, 4°C). The pellet was resuspended in 50 mM Tris-HCl, 150 mM NaCl, pH 7 (4 ml per 50 ml culture). Absorbance spectra of this membrane vesicle suspension was measured as described below. For solubilisation of membrane proteins, DDM was added to a final concentration of 2% (w/v), and the suspension was incubated with shaking for an hour at 4°C, followed by overnight incubation at 4°C. The insoluble material was removed by centrifugation (16,000xg, 20 min, 4°C). The supernatant was used for spectral analysis.

Proteorhodopsin purification:

The cell pellet was resuspended in ice-cold lysis buffer (5 ml/100 ml culture volume) containing 20 mM Tris, 50 mM NaCl, 20 mM imidazole, 0.1% DDM, pH 7 supplemented with an EDTA-free protease inhibitor tablet, benzonase (4 units/100 ml culture) and lysozyme (4 mg/100 ml culture). The suspension was sonicated at 4°C and centrifuged to remove cellular debris as described in the previous section. At this stage, the crude mixture was incubated with the selected retinal for 1 hour at RT. DDM was then added to a final concentration of 1.5% (w/v) and the sample was kept rotating overnight at 4°C. The insoluble material was removed by centrifugation (10,000xg, 30 min, 4°C) and the resulting supernatant was utilized as a crude extract. For purification of the his-tagged proteorhodopsins, immobilized-metal affinity chromatography (IMAC) was exploited using 0.4 ml Ni^{2+}-NTA resin per 100 ml original culture volume. The resin was contained in a spin column and first equilibrated with buffer A (20 mM bis-tris propane, 0.5 M NaCl, 0.1% DDM, pH 8) containing 20 mM imidazole. The crude extract was then allowed to equilibrate with the column for 2 hours at 4°C. The column was washed 5 times with 5 column volumes of buffer A containing 50 mM imidazole at 4°C Finally, strongly bound protein was eluted using buffer A containing 500 mM imidazole and 0.1% DDM at RT. Fractions of 0.3 ml were collected. Fractions containing the purified proteorhodopsin were combined and analyzed by spectroscopy and SDS-PAGE.

Spectroscopy of membrane vesicles:

The spectral properties of the pigments were measured in intact membrane vesicle suspensions using the end-on spectrophotometer SPECORD 210 PLUS. The end-window configuration of the photomultiplier and close distance to the cuvette strongly reduces the contribution of light scattering by the vesicles to the measurements. To isolate the major absorbance band of the proteorhodopsin out of the composite spectrum of membrane vesicles, hydroxylamine was added from a 1 M stock solution, pH 7, to a final concentration of 50 mM, followed by illumination with white light for 10 minutes. Hydroxylamine attacks the Schiff base and releases the retinal from the opsin binding pocket as retinaloxime. A difference spectrum then reveals the major absorbance band of the proteorhodopsin present. The same protocol was repeated upon solubilization of the membrane vesicles with DDM.

Spectroscopy of purified protein:

The spectral properties of all solubilized and purified samples in DDM solution were measured using a Shimadzu UV-Vis spectrophotometer (UV-1601). In order to test the pH-dependence of the main absorbance band of the PR analog pigments [2,3], the purified

proteins were analysed at different pH values by diluting the samples 1:1 with buffers containing either 100 mM bis-tris-propane at pH 9, 8.5, 8, 7.5, 7, 6.5, 6 or 100 mM MES at pH 5.5 and 5 (Fig S4). Absorbance maxima were determined using the internal peak-pick function of the software UVProbe. The purified analog pigments of PR and GR in DDM solution were further bleached with hydroxylamine as described above. The absorbance band of the analog oximes were measured, and were used to calculate the molar absorbance values of the analog species of wild type PR and GR (see below).

Molar absorbance of retinal analogs and corresponding pigments:

The λ_{max} (nm) and molar absorbance ε ($M^{-1}.cm^{-1}$) values of A1, A2, MOA2, DMAR and MMAR and their corresponding retinal oximes are listed in Tables S1 and S2. In order to calculate the ε values, a stock solution was made containing 1 mg of the crystalline powder dissolved in 1.0 ml of DMF. The absorbance of the stock solution was measured using appropriate dilutions. From the known concentration of the stock, the ε of the analogs in DMF could be calculated using Beer-Lambert's law. An aliquot of this stock was diluted 1:100 with 10 mM Tris pH 7, 1% DDM solution to determine the corresponding ε of the analogs in 1% (w/v) DDM (Table S2). Hydroxylamine was then added to a final concentration of 50 mM and the solution was illuminated as described above. The absorbance spectra of the resulting retinal oximes were measured and used to calculate their ε values. These measurements were done in duplicate, resulting in a S.D. ≤ 8%. The ε of the oximes of A1 and A2 were previously calculated to be 51,700 (λ_{max} 367 nm) and 45,400 (λ_{max} 386 nm) $M^{-1}.cm^{-1}$ respectively in 1% (w/v) digitonin solution [4-6]. From our measurements, we calculated ε values of 52,500 (λ_{max} 368 nm) and 44,000 (λ_{max} 385 nm) $M^{-1}.cm^{-1}$ for A1 and A2 oximes in 1% DDM, respectively, in good agreement with the literature values.

The molar absorbance values of PR:A1 and GR:A1 in detergent solution were estimated before. Our value for GR:A1 (55,500 ± 3,500 $M^{-1}.cm^{-1}$) is not significantly different from the reported value of 50,000 [7]. Our value for PR:A1 (54,200 ± 4,000 $M^{-1}.cm^{-1}$) is however significantly larger than the earlier reported value of 44,000 [8]. Considering the reported molar absorbance values for bacteriorhodopsin (54,000 [9] and 63,000 [10]), we decided to keep to our own, higher, values for both PR and GR.

Starvation of cells:

Overnight cultures were diluted 1:100 times to get a working culture of 25 ml. At a cell density corresponding to an OD_{600} of 0.3-0.4, production of the proteorhodopsin was induced by the addition of IPTG to a final concentration of 1 mM and of the selected retinal to a final concentration of 20 µM. The cultures were allowed to grow for a further 24 hours at

30°C in the dark, and then harvested. The cells were washed twice in starvation buffer (SB) containing 250 mM KCl, 10 mM NaCl, 10 mM $MgSO_4$, 100 µM $CaCl_2$, 10 mM Tris-HCl pH 7 and were starved by incubation with continuous mixing for 4 days at RT. The cells were washed another 3 times and resuspended in 4 ml SB supplemented with 40 µM final concentration of valinomycin. The cell suspension was incubated for 30 min in the dark at RT with continuous mixing.

Proton pumping assay:

The cell suspension was illuminated using the following light sources and photon flux values: white light (DLED9-T, DEDOLIGHT; 800 $\mu E.m^{-2}.s^{-1}$); 617 nm LED (M617L3, Thorlabs; 1500 $\mu E.m^{-2}.s^{-1}$); 660 nm LED (M660L4, Thorlabs; 800 $\mu E.m^{-2}.s^{-1}$); 730 nm LED (M730L4, Thorlabs; 150 $\mu E.m^{-2}.s^{-1}$). Light-induced pH changes were measured with a pH microelectrode (SenTix MIC, WTW) and the readout was monitored by a pH meter (Inolab pH 7310, WTW) fed into a computer. The following light regime was used: 1 min dark, 1 min light, 2 min dark, 1 min light, 2 min dark. For the 730 nm illumination of all species, and 660 nm illumination for PR-DNFS, a longer 1.5 min illumination period was used, along with a 2 x concentration of the cell suspension (50 ml cells in 4 ml starvation buffer). Pumping rates were calculated for 4 ml of the cell suspension using two independent trials. A calibration curve was measured using 0.1M HCl and 0.1M oxalic acid. Pumping rates were calculated as protons/sec from the initial rate of the light-induced pH change, if required corrected for baseline drift in controls (starved cells without expression of proteo-opsin or without retinal). Molecular pumping rates (protons/sec/molecule) could subsequently be calculated after assay of the proteorhodopsin level (see below).

Determination of proteorhodopsin levels for the proton pumping assay:

The above cell suspension from the proton pumping assay was rinsed with SB and the pellet was resuspended in 10 ml buffer B (50mM Tris-Cl, 150 mM NaCl, pH 7). The cell suspension was sonicated as mentioned above and the membrane vesicles and cell debris were pelleted together (147,000xg, 4°C, 1 h). The pellet was resuspended in 4 ml of buffer B. An aliquot of this membrane vesicle suspension was used to extract the main absorbance band of the analog pigments upon bleaching with hydroxylamine, as described above. This method was useful to estimate the concentration of analog pigments which were not sufficiently stable enough in DDM, particularly GR:DMAR, GR:MMAR, GR-FS:MMAR and PR-TA:MMAR. To another aliquot of the membrane vesicle suspension, DDM was added to a final concentration of 2.5% and incubated at RT with mixing overnight. Under these conditions maximal extraction of all stable pigment species was achieved without significant losses. The following day, the insoluble material was removed by centrifugation (16,000xg, 4°C, 20 min).

The supernatant was used to measure an absorbance spectrum before and after bleaching with hydroxylamine as described above. The optical density value at the absorbance maximum was used to calculate the original proteorhodopsin level in the cell suspension, using the calculated pigment molar absorbance values (Table S2). Hereby we assumed that the molar absorbance of the mutants is not significantly different from the parent pigments.

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