Photoreaction Dynamics of Red-Shifting Retinal Analogues Reconstituted in Proteorhodopsin

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Supporting Information

ABSTRACT: Microbial rhodopsins constitute a key protein family in optobiotechnological applications such as optogenetics and voltage imaging. Spectral tuning of rhodopsins into the deep-red and near-infrared spectral regions is of great demand in such applications because more bathochromic light into the near-infrared range penetrates deeper in living tissue. Recently, retinal analogues have been successfully used in ion transporting and fluorescent rhodopsins to achieve red-shifted absorption, activity, and emission properties. Understanding their photochemical mechanism is essential for further design of appropriate retinal analogues but is yet only poorly understood for most retinal analogue pigments. Here, we report the photoreaction dynamics of red-shifted analogue pigments of the proton pump proteorhodopsin (PR) containing A2 (all-trans,3,4-dehydroretinal), MOA2 (all-trans-3-methoxy-3,4-dehydroretinal), or DMAR (all-trans-3-dimethylamino-16-nor-1,2,3,4-didehydroretinal), utilizing femto- to submillisecond transient absorption spectroscopy. We found that the A2 analogue photoisomerizes in 1.4, 3.0, and/or 13 ps upon 510 nm light illumination, which is comparable to the native retinal (A1) in PR. On the other hand, the deprotonation of the A2 pigment Schiff base was observed with a dominant time constant of 67 μs, which is significantly slower than the A1 pigment. In the MOA2 pigment, no isomerization or photoproduct formation was achieved in proton pumps, cation channels, and fluorescent microbial rhodopsins using retinal analogues having extended π-electron-conjugated systems.11–16 Importantly, some of these retinal analogues maintain the transport activity of their hosts.

Particularly, the A2 retinal analogue (all-trans,3,4-dehydroretinal), which is the only other naturally occurring retinal analogue and has additional π conjugation between C3 and C4 on the β-ionone ring17–19 (Figure 1), exhibits a high affinity for the opsin protein because of the similar configuration to the native A1 retinal. Accordingly, the A2 analogue has been shown to induce a red shift in channelrhodopsins13 and proton pumping rhodopsins by ~30–40 nm13,14 while nearly completely maintaining their ion transport activity. In addition,
the MOA2 retinal analogue (all-trans-3-methoxy-3,4-dehydroretinal, Figure 1), which has additional π conjugation between C3 and C4 and, in addition, the strong electron-withdrawing methoxy group at position C3 in the β-ionone ring, has been shown to induce very large red shifts (65–80 nm) compared with the native A1 in proton pumping rhodopsins.14 Although the proton pump function in the MOA2 pigment was only clearly detected under white light illumination,14 MOA2 might be an interesting candidate for red-tuning of other rhodopsin proteins. Furthermore, the DMAR retinal analogue (all-trans-3-dimethylamino-16-nor-1,2,3,4-didehydroretinal, Figure 1), which has an aromatized ring moiety with a dimethylamino substituent at C3, is another promising candidate for red-tuning of microbial rhodopsins. For instance, incorporation of DMAR in the proton pump proteorhodopsin (PR) yielded a ~40 nm red shift relative to A1, but only weak proton pump activity was observed.14 On the other hand, in the H134R and H134R/T159C variants of the channelrhodopsin ChR2, which are widely used in optogenetics,20 incorporation of a related retinal analogue, containing an aromatized ring with a dimethylamino group, but lacking the methyl groups on the ring, resulted in a ~40 nm red shift while partially maintaining the transport function.12

Understanding of the photochemical mechanism of rhodopsins containing retinal analogues is essential for further development and design of functional retinal analogue pigments. Typically, in ion-pumping microbial rhodopsins, the photoreaction in the chromophore proceeds in the time range of femtoseconds to milliseconds.1 Hence, observation of this time range is fundamental for understanding the photochemistry. In our previous work, the photochemistry of the 3-methylamino-16-nor-1,2,3,4-didehydroretinal analogue pigment of PR (PR:MMAR) was clarified with femto- to submillisecond transient absorption spectroscopy, showing unusually long-lasting excited states and a very high fluorescence quantum yield.21 However, for other retinal analogue pigments, experimental data are very limited. In this study, we applied femtosecond to submillisecond transient absorption spectroscopy to PR analogue pigments containing A2 (PR:A2), MOA2 (PR:MOA2), or DMAR (PR:DMAR) analogues in order to investigate the excited state and ground-state photoproduct dynamics. Our results reveal significant differences in photodynamic behavior of these three analogue pigments, which show a correlation with their functional activity.

Figure 1. Chemical structures and steady-state absorption spectra. (a) Structures of the native retinal (A1) and retinal analogues (A2, MOA2, DMAR, and MMAR) and (b) the main absorbance band of the corresponding analogue pigments generated with PR, recorded at pH 8. Data are based upon Ganapathy et al. 2017.14

### METHODS

**Protein Purification.** All-trans retinal (hereafter called A1) was obtained from Sigma-Aldrich; all-trans-3,4-dehydroretinal (A2) was a generous gift from Hoffman-LaRoche. All-trans-3-methoxy-3,4-dehydroretinal (MOA2; purity 97.8% according to manufacturer’s certificate of analysis) and all-trans-3-dimethylamino-16-nor-1,2,3,4-didehydroretinal (DMAR; purity 99.9%) were synthesized on order by Buchem, B.V. *Escherichia coli* strain UT5600 transformed with the pKJ900 plasmid was used to express recombinant PR with a C-terminal 6-His tag. The cells were grown in Lysogeny broth medium, harvested, lysed, and regenerated with the appropriate retinal as per protocols described previously.13,14 The lysed cell suspension was incubated in 4% DDM (w/v) overnight at 4 °C and centrifuged to remove the insoluble cellular debris. The His-tagged PRs were purified by immobilized metal affinity chromatography, as reported previously.14 Ni2+-NTA resin (Qiagen, 10 mL) was used per 2000 mL original culture volume. The purified PR was eluted in 5 mL fractions containing 20 mM bis-Tris propane, 500 mM imidazole, 0.5 M NaCl, 0.1% DDM, pH 8 at room temperature. The fractions were combined and concentrated using a 10 kDa cut-off column (Amicon) to OD ∼10 or ∼20 at the absorption maxima for transient absorption spectroscopy and steady-state-stimulated Raman spectroscopy, respectively. Buffer solutions for pH-dependent experiments contained 150 mM NaCl and 2.5% DDM, with 20 mM bis-Tris propane for pH 7.0 or 20 mM Tris–HCl for pH 9.0.

**Steady-State-Stimulated Raman Spectroscopy.** Steady-state-stimulated Raman experiments were performed as reported previously using a home-built-stimulated Raman setup with an 800 nm Raman pump.22–24 The protein samples were filled in a 2 mm pathlength quartz cuvette (100-QS, Hellma Analytics).

**Femto- to Submillisecond Transient Absorption Spectroscopy.** Transient absorption measurements were performed with a home-built femtosecond to submillisecond pump-probe setup as reported previously.25,25,26 The samples were filled in a homemade sample holder that has two 2 mm thick CaF2 plates. The sample thickness was set at 400 μm for transient absorption experiments with an appropriate sample spacer. The sample holder was set on a Lissajous scanner that ensures sample refreshment after each laser shot with a time interval of 60 s between successive exposures to the laser.
pulses.\textsuperscript{37} A CaF\textsubscript{2} plate on a moving stage was used for supercontinuum white light generation, and a selected wavelength region of 400–750 nm was detected by the photodiode array. The polarization between the pump and probe pulses was set at the magic angle (\(\sim 54.7^\circ\)). The time delay varied up to 400 \(\mu\)s at 163 data points with a minimum temporal step of 50 fs. The diameters of the pump and the probe beams at the sample position were \(\sim 200\) and \(\sim 50\) \(\mu\)m (full width at half-maximum), respectively. The central wavelength and the power of the pump beam were set at 510 nm (\(\sim 400\) nJ) and 620 nm (\(\sim 600\) nJ) for PR:A2, at 520 nm (\(\sim 500\) nJ) for PR:MOA2, and at 500 nm (\(\sim 600\) nJ) and 600 nm (\(\sim 600\) nJ) for PR:DMAR. The instrumental response function was \(\sim 100\) fs, as estimated from global analysis.

\textbf{Global Analysis Methodology.} Global analysis was performed for the transient absorption spectra using the Glotaran program.\textsuperscript{28} With global analysis, all wavelengths/wavenumbers were analyzed simultaneously with a set of common time constants.\textsuperscript{55} A kinetic model was applied consisting of sequentially interconverting, evolution-associated difference spectra (EADS), that is, \(1 \rightarrow 2 \rightarrow 3 \rightarrow \ldots\), in which the arrows indicate successive monoexponential decays of a time constant, which can be regarded as the lifetime of each EADS.\textsuperscript{59} The first EADS corresponds to the difference spectrum at time zero. The first EADS evolves into the second EADS with time constant \(\tau_1\), which in turn evolves into the third EADS with time constant \(\tau_2\), and so forth. The procedure clearly visualizes the evolution of the intermediate states of the protein.\textsuperscript{23,25,30,31} Decay-associated difference spectra (DADS) indicate the spectral changes with parallel decay channels and independent decay time constants. Intuitively, the DADS provide the difference spectra between the subsequent EADS, provided that the time constants are sufficiently widely separated. It is important to note that the parallel and the sequential analysis are mathematically equivalent and yield identical time constants.\textsuperscript{29,32} The standard errors in the time constants were less than 5%.

\section*{RESULTS AND DISCUSSION}

\textbf{Photochemistry of PR:A2.} First, we investigate the ground-state retinal configuration of the A2 chromophore in PR. As seen in Figure 1, PR:A2 shows two main absorption bands at \(\sim 552\) and \(\sim 515\) nm, while the native pigment (PR:A1) has a single band at \(\sim 520\) nm. This observation implies that PR:A2 might have heterogeneous ground-state conformers. Therefore, preresonance-watermarked stimulated Raman spectroscopy with an 800 nm Raman pump\textsuperscript{21} was applied to PR:A2 at a physiological pH (7.0) to investigate the ground-state characteristics of the chromophore (Figure 2, magenta line). Note that our previous pH titration study showed that the pKa of PR:A2 is \(\sim 6.2\) in DDM solution,\textsuperscript{14} thus, mostly PR:A2 is in the alkaline form. The Raman spectrum of PR:A2 is quite similar to that of PR:A1,\textsuperscript{33,34} except for a strong additional band at 1512 cm\(^{-1}\) in DDM solution,\textsuperscript{14} which is characteristic for the 13-cis configuration,\textsuperscript{37} also agree with an all-trans configuration of the A2 chromophore. Although a minor amount of 13-cis species may be present, represented by a small 1185 cm\(^{-1}\) peak,\textsuperscript{37,38} there is general consensus that in the ground state, PR:A1 contains an all-trans chromophore,\textsuperscript{33,36,38,39} and we conclude that the same is true for PR:A2. In the C=C stretching region of PR:A2, two large peaks at 1539 and 1512 cm\(^{-1}\) are present. This could reflect ground-state heterogeneity in PR:A2, which for instance could express itself in the pluriform absorption spectrum (cf. Figure 1b). It should be noted, however, that the 1512 cm\(^{-1}\) band relatively poorly matches the corresponding absorption maximum at 552 nm, when compared to the established relation between these quantities.\textsuperscript{40–43}

Next, the photochemistry of PR:A2 was investigated at pH 7.0 by applying femto-to submillisecond transient absorption spectroscopy with 510 nm excitation. The transient absorption spectra were globally fitted with eight time components: 170 fs, 1.4 ps, 3.0 ps, 13 ps, 1.3 ns, 1.7 \(\mu\)s, 67 \(\mu\)s, and infinite. The EADS and DADS are shown in Figure 3a,b, and selected time traces are shown in Figure S1a. The first EADS (gray line, Figure 3a,b) evolves to the second EADS (red line) in 170 fs; observable are positive excited state absorption (ESA) at \(\sim 610\) nm (strong) and \(\sim 500\) nm (weak), with negative stimulated emission (SE) at \(\gg 700\) nm, which confirms that the molecules are in the excited state. Notably, the red-shifted ESA was not seen in PR:A1,\textsuperscript{44,45} but a similar red-shifted ESA was reported in channelrhodopsins (ChR2\textsuperscript{46,47} and CIC\textsubscript{2}\textsuperscript{25}). The negative ground-state bleach (GSB) signal is probably strongly overlapped with the ESA, thus not visible in the red EADS. In 1.4 ps, development to the third EADS (blue in Figure 3a,b) was observed, involving a large decay in the SE and ESA signals, while a small SE component still remained. The negative GSB signal at \(\sim 550\) nm became visible on the third EADS likely because the strong ESA decayed partially in 1.4 ps. After 3.0 and 13 ps evolution, the fifth EADS appears (magenta in Figure 3a), which no longer showed a SE band, indicating that the excited states were no longer populated after the 3.0 and 13 ps decay. Hence, it can be considered that the fifth EADS (magenta), which has an absorption peak at \(\sim 610\) nm, shows the first K-like photoproduct state. The ESA at \(\sim 620\) nm strongly overlaps with the K-like absorption at \(\sim 610\) nm. Thus, from the current transient absorption data, it
is difficult to decide which one of the time components (1.4, 3.0, and/or 13 ps) is involved in the all-trans to 13-cis isomerization in PR:A2, and it also is difficult to identify the J−K state transition which is assigned to vibrational cooling of the first photoproduct state. Using single-pulse or multipulse spectroscopy, it was reported that at acidic to neutral pH, multiphasic isomerization in PR:A1 proceeds with three components (0.2−0.7, 6−10, and 20−30 ps).44,45,48,49 Therefore, it seems like the photoisomerization of A2 in PR proceeds in a time frame similar to A1.

According to the DADS in Figure 3b (bottom panel), in 1.3 ns, the absorbance of K at 610 nm is decaying with a concomitant rise around 500 nm. The ∼500 nm absorbance band can be assigned to an L-like intermediate. The K-like absorption largely persists after the 1.3 ns signal development, implying that a K/L equilibrium is formed in 1.3 ns. In 1.7 and 67 μs, an absorbance band around 420 nm appears, which is assigned to an M-like intermediate, in which the RSB is deprotonated. It may be concluded that proton transfer from the RSB of A2 proceeds with 1.7 and 67 μs time components. From the DADS in Figure 3b (bottom), the rise of the M-like intermediate appears to be ∼5-fold larger in amplitude in 67 μs than in 1.7 μs, which implies that the 67 μs component is dominant for the proton transfer process. The K-intermediate absorption still persists after 67 μs, indicating that a K/L/M equilibrium, also observed in the chimeric channelrhodopsin C1C2,25 is formed in 67 μs. For PR:A1, it was reported that at acidic to neutral pH, the M-like intermediate is formed with components of 2−4 μs and several tens of μs.45,50 Hence, the dominant M formation in PR:A2 (67 μs) seems to be somewhat slower than in PR:A1, while the minor M formation proceeds at almost the same rate in both pigments.

Because our steady-state absorption and Raman results suggest ground-state heterogeneity of PR:A2, we also applied 620 nm excitation to predominantly excite the ∼550 nm absorbing species. We observed a different set of ESA species peaking at ∼500, 600, and 670 nm (Figure 3c,d, top) but a similar set of photoproducts (Figure 3d,c, bottom). Selected time traces are shown in Figure S1b. Significantly, the blue-shifted ESA was also seen in the native PR:A144 and other microbial rhodopsins.23,30,51−53 The GSB includes a peak at ∼520 nm too, implying that a substantial part of the ∼500 nm species was also excited by the 620 nm pump. Likely, the ESA at ∼600 and ∼670 nm upon 620 nm excitation results from the ESA as was shown above for 510 nm excitation (Figure 3a,b). The signal development of the photoproducts was almost identical for 620 and 510 nm excitations, but the signal intensity was significantly larger with 510 nm excitation. The molar absorption coefficient of PR:A2 at 510 nm is about 5.6-fold higher than at 620 nm (Figure 1), the excitation power at 620 nm was 1.5 times that at 510 nm (see the Methods section), and with the same power, the photon flux at 620 nm is higher by a factor of 1.2. Therefore, we estimate that the ratio of the number of excited molecules is about 3.1:1 for 510 and 620 nm excitation, respectively. However, the M-intermediate population was about 4.8-fold stronger with 510 nm excitation (Figure 3b,d, bottom and Figure S1). Hence, this would indicate that 510 nm light can trigger the photocycle somewhat more efficiently than 620 nm light, implying that the hypsochromic ground-state species is somewhat more dominant in producing a functional photo-
cycle in PR:A2. On the other hand, higher production of the M-intermediate with blue light under acidic conditions was also reported for PR:A150 and hence could simply be the result of populating higher energy vibronic states on the excited state surface. For PR:A1, it has been reported that the general scheme of the photocycle is similar at pH 7 and pH 9, be it that the kinetics and quantities of the photoproducts may vary.33,44,48

In Figure 3e, the photocycle of PR:A2 upon 510 nm excitation at pH 7.0 is proposed. Obviously and not surprisingly, the photocycle bears great similarity to that of PR:A1.44,48−50

Photochemistry of PR:MOA2. The ground-state Raman spectrum of PR:MOA2 at pH 9.0, where the counterion Asp97 is fully deprotonated,14 is shown in Figure 2 (green line). Again, C–C stretching peaks were observed at 1172, 1180 (weak), 1200, 1236, and 1254 cm⁻¹, but two C==NH stretching peaks were seen at 1640 and 1654 cm⁻¹. The most straightforward explanation is that the chromophore of PR:MOA2 adopts heterogeneous all-trans and 13-cis formers in the dark state.35,37 The stronger peak at 1118 cm⁻¹ would also support the presence of a 13-cis chromophore. On the other hand, no significant peaks of isolated hydrogen out-of-plane modes were observed in the 800−900 cm⁻¹ region, which would reflect structurally distorted 13-cis conformers as observed in microbial rhodopsins containing 13-cis chromophores.37,56 The two strong C=C stretching peaks at 1504 and 1538 cm⁻¹ further suggest that heterogeneous species exist in the dark state of MOA2, which could also explain the broad absorbance peak (Figure 1).

The photochemistry of PR:MOA2 was studied with 520 nm excitation at pH 9.0. Remarkably, only femtosecond and picosecond components were required in the global analysis, yielding time components of 120 fs, 2.0, and 4.2 ps (Figure 4a,b). In 120 fs, a slight rise in a signal at ∼610 nm was observed. In the 2.0 and 4.2 ps components (red and blue lines in Figure 4a, respectively), strong positive signals were seen, but no negative peaks were detected. The multiple positive peaks are assigned to ESA bands with maxima at ∼520, ∼560, ∼610, and ∼700 nm. Probably, the GSB and SE signals...
strongly overlap with the ESA, explaining the absence of clear negative signals in the red and blue spectra in Figure 4a. Concomitantly, with the decay of the 4.2 ps component, all A components decayed completely (Figures 4a,b and S2), implying that all excited molecules returned to the initial ground state in 4.2 ps. This observation indicates that under the applied conditions, PR:MOA2 does not generate detectable photoproducts, that is, photoisomerization does not seem to occur. Furthermore, the absence of M-like absorption in the near-UV region suggests that proton transfer from the protonated RSB in PR:MOA2 does not occur either. This would explain why hardly any pump activity is obtained upon illumination with small bandwidth light-emitting diode sources.14 However, continuous white light illumination does produce detectable proton pump activity.14 These observations are not easily reconciled. Our ground-state Raman data indicate heterogeneity in the Schiff base region, possibly due to the presence of all-trans as well as 13-cis conformers in the chromophore. We cannot exclude that this complex system reacts differently to continuous white light illumination than to femtosecond laser light excitation at 520 nm. In Figure 4c, we present the photocycle of PR:MOA2 as deduced from our 520 nm laser light excitation at 520 nm. In Figure 4c, we present the photocycle of PR:MOA2 as deduced from our 520 nm excitation data.

**Photochemistry of PR:DMAR.** The photochemistry of PR:DMAR was studied at pH 9.0 with 500 and 600 nm excitations. In the previous steady-state preresonance Raman study of PR:MMAR,21 the data support an all-trans configuration for the chromophore. Considering the strong structural similarity of DMAR and MMAR, we tentatively assume that this is also the case for PR:DMAR. The fs−μs transient absorption data upon 500 nm excitation were globally fitted with five components: 230 fs, 3.4, 57, 170 ps, and 1.7 μs (Figure 5a). The negative signals at ∼480 nm seen in all transient absorption spectra are assigned to GSB. The ESA on the first four components is strongly overlapped with GSB and likely SE. The second EADS (red line, Figure 5a) has a negative signal at >700 nm, which is assigned to SE. Hence, the excited state was still populated after the first 220 fs decay. The third and fourth EADS have no or only a slight negative signal at >700 nm. This observation may indicate that the excited state was depopulated in 3.4 and/or 57 ps. Alternatively, the negative SE signals were not apparent because of the strong overlap with ESA, and the excited state might still exist after 3.4 and 57 ps. In the fifth EADS (magenta line in the inset, Figure 5a), a slight positive peak is observed at ∼640 nm, which is probably derived from an isomerized K-like photoproduct. Thus, the ground-state photoproduct is formed after 170 ps at the latest. In 1.7 μs, all transient signals disappeared without detectable formation of an M-like state, which would result from deprotonation of the protonated RSB and appearance of near-UV absorption (Figures 5a and S3). Perhaps, the initial ground state was repopulated in 1.7 μs with thermal reisomerization, or, more likely, considering that PR:DMAR shows weak proton pumping activity,14 an M-state was formed in 1.7 μs but not detectable in our transient absorption measurements.

In view of the broad absorbance band, fs−μs transient absorption spectra were also recorded with 600 nm excitation and again were globally fitted in five components: 60 fs, 710 fs, 43 ps, 210 ps, and 380 ns (Figure S4). Immediately after photoabsorption, the excited state was populated (gray line, Figure S4), which develops to the second EADS (red line) in 60 fs. Significantly, in the second EADS, broad negative signals are seen at >∼620 nm, which are assigned to a mixture of GSB and SE. Moreover, in contrast to the 500 nm excitation data, no negative signal was observed below ∼480 nm. This observation indicates that some extent, different ground state components of PR:DMAR were excited, by the 500 and 600 nm excitation. In the third and fourth EADS (blue and green lines, respectively, Figure S4), spectral features similar to the second EADS were observed, while the >700 nm negative SE signal was still present. Furthermore, as in the transient absorption spectra obtained upon 500 nm excitation, only a tiny photoproduct at ∼640 nm was detected upon 600 nm excitation (magenta line, Figure S4a, inset), without detectable formation of an M-intermediate. Hence, globally, the photodynamics are quite similar upon 500 or 600 nm excitation. In Figure 5b, the photocycle of PR:DMAR is shown as we deduce from excitation at 500 nm.

It is interesting to compare the photochemistry of PR:DMAR with that of PR:MMAR, reported previously,21 because DMAR and MMAR have very similar chemical structures, except that in MMAR, the aromatic amino substituent only contains a single methyl group.14 In PR:MMAR, three excited state decays were observed in ∼3, 30, and ∼300 ps, and we proposed that the photoisomerization proceeds within ∼30 ps.21 Even though it is difficult to derive this from only the present data set, we propose that upon excitation of PR:DMAR at 500 nm, the excited state decay proceeds in 3.4, 57, and 170 ps (Figure S5a) in view of the similar DADS and time constants in PR:MMAR. In addition, the peak position and the peak width of the K-like intermediate of PR:DMAR (magenta inset, Figure 5a) are quite comparable with the photoproduct detected in PR:MMAR.21 Considering the ratio of the peak intensities of the biggest EADS (red line in Figure S5a, top) and the photoproduct state (magenta line, Figure S5a, inset), it can be suggested that the efficiency of photoproduct formation in PR:DMAR is approximately half of that in PR:MMAR. Likely, this small photoproduct yield of PR:DMAR explains the low proton pumping activity of PR:DMAR, which is less than half of that of PR:MMAR.14

**Comparison of Native A1 Retinal with Reconstituted Retinal Analogues in PR.** At last, we briefly overview the photochemistry of PR:A2, PR:MOA2, and PR:DMAR, that we reported in this manuscript, together with the previously published PR:A1 photochemistry.

PR:A1: A natural light-driven proton pump, characterized by rapid and efficient trans–cis isomerization of its chromophore, recruited from retinal A1, subsequent rapid proton transfer from the protonated Schiff base to initiate proton transport, little energy loss by fluorescence, and a quite good pumping rate.

PR:A2: The additional double bond in the retinal moiety leads to a less flexible ring structure but effectuates a significant red shift relative to A1 (∼30 nm) thanks to the elongated conjugated system. This analogue maintains rapid and efficient trans–cis isomerization, but the proton transfer seems to proceed slightly slower, possibly due to slight rearrangements in the binding site structure to optimally accommodate this analogue. Nevertheless, the pumping rate is not strongly affected, and the action spectrum is also red-shifted.14

PR:MOA2: This analogue with a monomethyloxy substituent at the ring-C3 position fits much better than DMAR14 and evokes a very large red shift (∼65 nm), probably thanks to the elongated conjugated system in combination with the...
methylxylo substituent, that will increase the charge delocalization in the system and may induce some resonance effects. In spite of this, no photoproducts or proton transfer are observed and instead very rapid decay of the excited states to the ground state occurs. The ground state also seems to contain some 13-cis chromophore, which may complicate the photodynamics and may be correlated with the observation that only under white light illumination, very low proton pump activity is observed without a clear red shift in the action spectrum.14

PR:DMAR: Because of the bulky dimethylamino substituent at the ring-C3 position, this analogue does not fit very well in the PR binding site14 and will require more substantial rearrangements. Nevertheless, it induces a quite significant red shift (~40 nm), most likely because thanks to the substituent, it has access to resonance structures with enhanced electron delocalization, similar to MMAR.21,57 However, the rate and extent of photoisomerization are strongly reduced, and proton transfer is slowed down. This agrees with the low proton pumping rate determined experimentally.14 Nevertheless, the action spectrum is also red-shifted.14

From this overview, we may cautiously conclude that for maintaining substantial proton pumping, rearrangements in the binding site should be avoided as much as possible, unless structural modifications in the analogue can be properly offset by protein mutations. Modifications in the ring that effectively communicate with the conjugated system will be most effective in inducing red shifts in the absorbance band, but their size is an important factor, a condition which, however, probably will vary for different rhodopsins. Again, offsetting this by protein mutation should be an option. Finally, it seems that amino substituents are most effective in combining red-shifting properties with maintaining activity, probably because resonance with the Schiff base yields optimal electron delocalization and molecular orbital hybridization, which even can be further enhanced by protein mutation.14 MMAR already combines these positive elements to some extent,14,21,47 but further optimization in combination with protein modification is required, in particular to enhance pump activity. The strongly negative effect of the methoxy substituent on the pump activity is clearly due to nearly complete blockage of photoproduct formation. Possibly, electronic effects of the methoxy group affect the excited states, or it indirectly affects the conformation of the counterions—water—Schiff base complex by rearranging the protein structure around the ring. A recent theoretical study indicates that substituents on the conjugated chain may affect the mixing and lifetime of excited states and the speed of photoisomerization.58 This may relate to the negative effects of the methoxy and (di)methylamino substituents on the photoisomerization efficiency in PR:MOA2, PR:DMAR, and PR:MMAR, respectively, the stronger electronegative methoxy group exerting the most inhibitory effect. Further biophysical and theoretical studies should be able to analyze these systems in more detail.

## CONCLUSIONS

Insights into the photodynamics of microbial rhodopsins containing retinal analogues that red-shift their absorbance band is very limited; however, it is of high importance for further developments in fields such as optogenetics and fluorescent voltage sensors. In this study, we present a comparison of the photochemistry of three red-shifted analogues of the proton pump PR using femto- to submilli-

second transient absorption spectroscopy. The PR analogue pigments contain the retinal analogues A2, MOA2, and DMAR, respectively, as their chromophore. This red-shifts the absorbance band by about 30, 60, and 40 nm, respectively, relative to the native retinal A1.14 In PR:A2, the photoisomerization proceeds with 1.4, 3.0, and/or 13 ps time constants upon 510 nm excitation at pH 7.0, which is comparable to that in native PR:A1. The additional C3—C4 double bond in A2 (Figure 1) apparently does not strongly influence the photodynamics. Moreover, M-intermediate formation, reflecting deprotonation of the protonated Schiff base linkage to the protein, predominantly occurred with the 67 μs constant, which is significantly slower than that of PR:A1. Notably, green (510 nm) and red (620 nm) excitation resulted in different excited state properties, but the photoproduct spectra were very similar. We infer that 510 nm photons can trigger the photocycle more efficiently than 620 nm photons. On the other hand, photoisomerization was not detectable in PR:MOA2. All excited molecules relaxed to the initial ground state with time constants of 2.0 and 4.2 ps, without detectable photoproduct formation. Finally, the photoproduct was detected in PR:DMAR but only in small quantities. The excited state transition kinetics obtained with PR:DMAR (3.4, 57 and 170 ps) were similar to those in PR:MMAR, reported before.21 Moreover, it is suggested that different ground-state species were excited at 500 and 600 nm excitations, implying that ground-state heterogeneity exists in PR:DMAR at pH 9.0. The transient absorption signals completely decayed in 1.7 μs without detectable formation of an M-like state in PR:DMAR, probably implying that the M-state signal was too weak to be detected in our measurements. Overall, the photodynamical signature of these PR analogue pigments correlates well with their functional activity as proton pumps.

## ASSOCIATED CONTENT

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpcb.9b01136.

Additional femto- to submillisecond transient absorption data with selected time traces (PDF)

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### Notes

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