Photoactivities of the Red-Shifted Azulenic Bacteriorhodopsin Analogues

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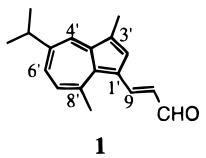
Incorporating red-shifted retinal analogues, such as azulenic retinals, into bacterioopsin provides a method to evaluate the low-energy limit for bacteriorhodopsin (bR) proton-pumping activity. We report results of nanosecond time-resolved studies of three azulenic bR analogues. In contrast to a recent report on an azulenic bR analogue (Druzhko et al., 1996),[4] we conclude that such pigments do not exhibit photocycles (no detectable intermediates) and suspect that the preferred direction of deactivation is internal conversion to the low-lying forbidden S_1 state of the azulene chromophore. We further demonstrated that for slow-binding bR analogues, the samples can easily be contaminated with small amounts of native bR.

Introduction

Of the numerous retinal analogues that have been prepared,[1] several are known to yield bacteriorhodopsin (bR) analogues with absorption maxima red-shifted from that of the parent bR (568 nm).[2] Our interest in these types of analogues is the possible preparation of a series of red-shifted pigments that can be used to establish the threshold energy necessary to initiate the characteristic photoresponses of bR and related photosensitive retinal binding proteins. The azulenic family of retinal analogues that yielded bR analogues with varying degrees of red-shift in the absorption maxima (830 nm being the longest)[3] is of particular interest. For these pigments, it is of interest to examine their photochemical characteristics, for both retention of the characteristic photocycle activities of bR and its possible wavelength dependence.

In a recent paper, Druzhko et al.[4] described their investigation of one of the reported azulenic bR's, the chain-shortened enal analogue **1**, concluding that they had detected photoinduced activities: spectral changes and photocurrent. This conclusion was not in agreement with some preliminary results we had. We have since repeated our work on **1** and studied properties of two additional azulenic retinals and their bR pigment analogues. The results are reported in this paper.



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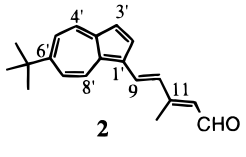

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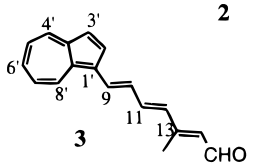

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Materials and Methods

Azulenic Retinal Analogues. Analogues **1** and **3** were reported earlier.[3] Procedures for preparation of **2** and other alkylated azulenic analogues will be disclosed in a separate paper.[5] The final product was purified by preparative HPLC. Some of the characterization data for **2** are listed below. ^{1}H NMR ($CDCl_3$) δ 10.15 (C13−H, d, 8.4 Hz, 1H), 8.44 (C8′−H, d, 10.8 Hz, 1H), 8.21 (C4′−H, d, 10.2 Hz, 1H), 8.14 (C2′−H, d, 4.2 Hz, 1H), 7.7 (C9−H, d, 15.6 Hz, 1H), 7.44 (C7′−H, d, 10.8 Hz, 1H), 7.38 (C5′−H, d, 10.5 Hz, 1H), 6.97 (C10−H, d, 15.6 Hz, 1H), 6.10 (C12−H, d, 8.4 Hz, 1H), 2.48 (C11−CH_3, s, 3H), 1.5 (C6−*tert*-butyl, s, 9H).

Preparation of bR Analogues. Procedures for isolation of bacterioopsin (bO) from wild-type bR and reconstitution with azulenic retinal analogues were essentially the same as those reported,[3] which was based on that described by Oesterhelt.[6] Briefly the membrane-bound bR protein was isolated from whole cell cultures by successive lysing and centrifugation. The bR proteins were suspended in the bleaching buffer[6] and photobleached using light from a 500 W halogen source filtered through glass, 30 cm of water, and an optical filter that allowed only light of >530 nm to pass. Photobleaching was conducted at room temperature. The bleached proteins were washed five times with water and subsequent centrifugation. The bleached proteins were then resuspended in 3 mL of water, to which 20 μL of chromophore/methanol stock solution was added. The solutions were then allowed to incubate at room temperature, or alternatively in a 4 °C cooler when noted, until completion of the reconstitution was detected by UV−vis spectrophotometry. All analogue samples were reconstituted in tandem with a native retinal sample.

Transient absorption studies were conducted using a frequency-doubled, pulsed Nd:YAG laser as the pump source, and an Oriel xenon arc lamp coupled with a monochromator as the probe source. The whole apparatus has been described previously.[7]

Results and Discussion

Our choice to examine first the bR analogue derived from 6-*tert*-butylazulenic dienal, **2**, for photocycle studies is 2-fold. Unlike either analogue **1** or **3**, it reconstitutes to form a pigment analogue at a rapid rate complete within 2 h. Second, its absorption maximum, 675 nm (644 nm for analogue of **3**),[3] is much red-shifted from that of the native bR.

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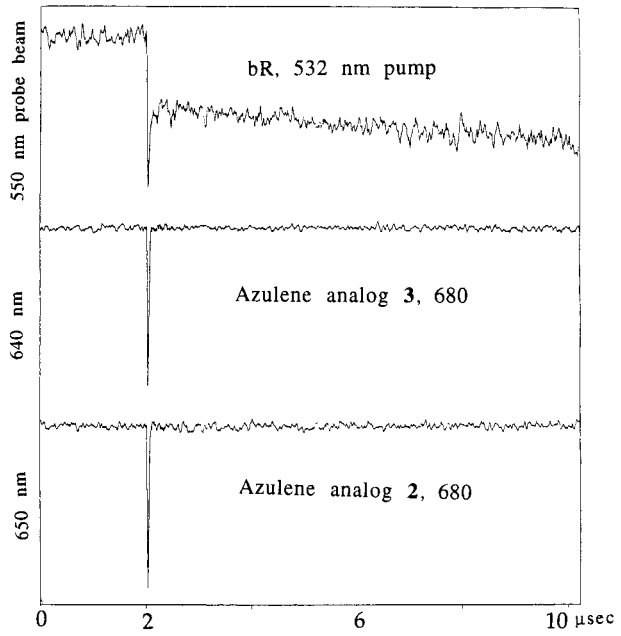

Figure 1. Decay of transient absorption of bR, 10 μs (pump beam, 532, nm; detecting probe beam, 550 nm) and those of analogues **2** and **3** (pump beam, 680 nm; detecting wavelength, 650 nm, **2**; 640 nm, **3**).

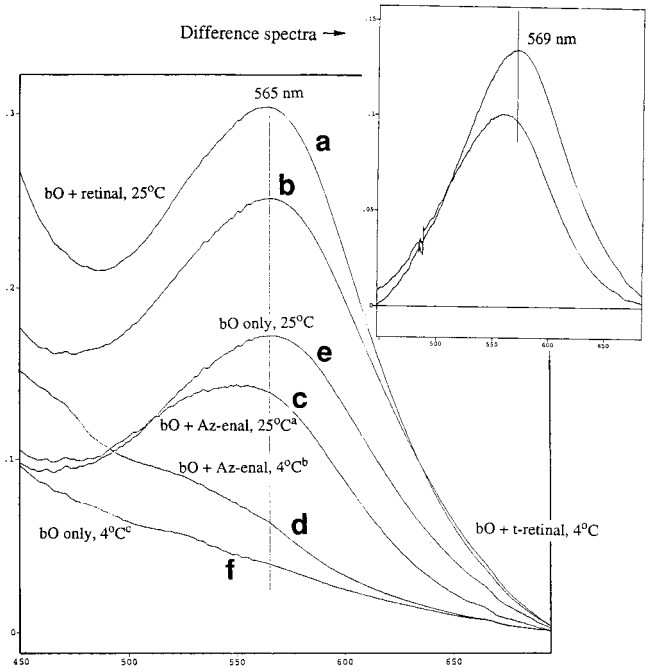

Figure 2. Left: binding interaction between bO and *all-trans*-retinal (curves a and b), analogue **1** (curves c and d), and no analogue added (curves e and f) conducted either at the room temperature, 25 °C (a, c, e), or 4 °C (b, d, f). All spectra recorded 1 month after mixing. Insert right: difference spectra from above. Upper: curve e minus curve f, largely due to slowly regenerated bR. Lower: curve c minus curve f, a mixture of bR and az-bR as shown by the blue-shifted peak from above.

Transient absorption studies of the pigments formed by reconstituted analogues **2** and **3** were conducted using the frequency-doubled Nd:YAG laser line at 532 nm (3 mJ/pulse, 7 ns pulse width) for the excitation light source, while monitoring for absorption changes in the sample at several different wavelengths and time scales. As a control, reconstituted native bR was tested at the same wavelengths and time scales. Excitation of native bR with 532 nm light results in the formation of the K-intermediate (~3 ps) followed by conversion to the L-intermediate on a ~2 μs time scale. Our transient absorption data (obtained on a 10 μs time scale) exhibit a decrease in absorbance at 610 nm consistent with the decay of K (absorption maxima at 620 nm) and a simultaneous increase in absorbance at 500 nm (consistent with formation of L). Data obtained at 560 and 400 nm (on a 500 μs time scale) demonstrate the subsequent decay of L and formation of the M-intermediate, respectively. Conversion of M- to the N-intermediate was observed at 400 nm on a 50 ms time scale. However we have been unable to detect any absorption changes in the pigments incorporating either analogue **2** or analogue **3** at any of these wavelengths or on any of these time scales.

The absorption bands of the pigments reconstituted with analogue **2** or **3** are both broad and red-shifted from our 532 nm excitation source. Therefore, we have also employed a Raman shifter cell in order to extend our excitation wavelength to 680 nm. However, we have been unable to detect any absorption changes in these reconstituted pigments using the 680 nm source (Figure 1).

Our data demonstrating the absence of any photocycles for the reconstituted bR pigments formed by analogue **2** or **3** differs from that of Druzhko et al.,[4] who concluded, from a study using absorption difference spectra and photocurrent measurements after steady-state illumination of the reconstituted pigment formed with analogue **1**, the successful detection of photoactivity. The combined results suggested the interesting possibility of wavelength-dependent behavior among this azulenic family of chromophores, with the red-shifted analogues **2** and **3** (675 and 644 nm respectively) not having sufficient energy to initiate the isomerization process while the blue-shifted analogue **1** (520 nm) can initiate the process. We therefore prepared the bR pigment from analogue **1** and subjected the sample to the same transient absorption studies as detailed above. However, as with analogues **2** and **3**, no transient absorption changes were detected.

We then attempted to duplicate the difference absorption spectra studies of Druzhko et al., utilizing steady-state illumination in order to test for the presence of slower photoactivities. First, we found that under the pH 9.5 conditions used by Druzkho et al. for their steady-state measurements, the reconstituted bR formed with analogue **1** was unstable. This is in line with the reported results that most of these slow-forming azulenic pigments are unstable in basic media such as hydroxylamine.[3] The pigment was found to be stable at pH 7; however, no steady-state absorption changes were detected.[8]

We have also observed that **2** and **3** do not undergo photoisomerization when irradiated in solution. This is consistent with an earlier report that an azulenic alkene undergoes one-way cis-to-trans photoisomerization.[9] Thus, our conclusion from the above results is that the azulenic bR analogues, whether red-shifted from the parent bR or not, do not have a normal photocycle as in the native bR. The implication is that they do not undergo photoisomerization. We suspect that these azulenic polyenes have an altered channel of deactivation, i.e., preferring to cascade through the low-lying forbidden S_1 state of the azulene chromophore,[10] which is known to be short-lived[11] and chemically inactive, rather than torsional relaxation of the polyene side chain as in bR. It is interesting to note that the first reported cyanine bR analogue (λ_{max} 662 nm) was reported not to pump protons, where the stiffness of the chromophore was believed to prevent the system from isomerizing to the 13-cis form.[1] And, for the more recently reported series of aromatic merocyanine bR analogues (λ_{max} 610−755 nm), no transients were detected between 100 ns and 10 ms.[2e]

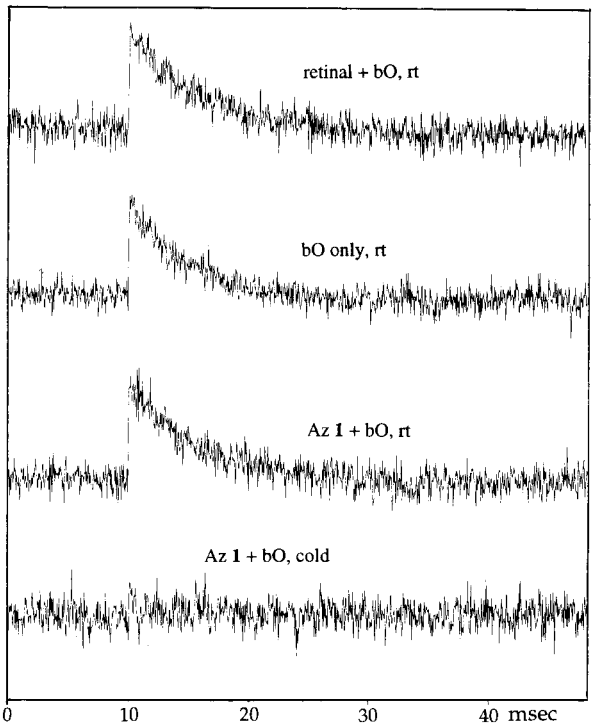

Figure 3. Decay of transients (pump beam, 532 nm; detecting beam, 410 nm, 50 ms) from bR, bO, az-bR (**1**), (from top to bottom) samples a, e, c above and of "cold" bR, sample f.

In the parent azulene, the allowed transition to the S_2 state ($\epsilon \sim 4000$) centers at 347 nm, while the transition to the well-resolved forbidden S_1 state ($\epsilon \sim 300$) centers at 578 nm.[10] For azulenes appended with an unsaturated side chain on C-1 of the C_5-ring, the position of the S_2 band is highly sensitive to the length of the conjugated side chain, e.g., shifting from 436 to 465 to 484 nm for the enal, dienal, and trienal guaiazulenic analogues.[3] On the other hand, the S_1 band appears to be insensitive to the unsaturated side chain.[3b] The position of the S_1 band in the bR pigment analogue, however, is unclear, being buried underneath the enhanced and further red-shifted S_2 band. Nevertheless, it is safe to assume that closing the S_2-S_1 gap will no doubt enhance internal conversion from S_2 to the short-lived S_1 state.[11]

It is difficult to speculate on the reason(s) for the different conclusion on photoactivities of analogue **1** in the reported paper[4] especially when the work was conducted under different conditions (steady-state illumination followed by spectral detection at a time delayed in excess of seconds). However, we note that the reported photoactivities were similar to those of the control bR sample, albeit at reduced sensitivity. This leads to the worry of a possible involvement of a residual amount of bR, an experimental complication that can easily arise during preparation of bO, especially for slow-forming pigments.

To examine such a possibility, we repeated the reconstitution studies of analogue **1** at two different temperatures. The reconstitution of analogue **1** was conducted alongside reconstitution of native retinal, and a sample of bO without the addition of any chromophores at room temperature and at 4 °C. In Figure 2 are shown the spectra of the reconstituted pigments. The samples reconstituted at room temperature were found to have the same transient decay characteristics (Figure 3). At the lower temperature, hydrolysis of the retinal oxime was suppressed or retarded, and this accounts for the differences in the two "bR" spectra. Druzhko et al. conducted their analogue reconstitution at 10 °C, which could have allowed the hydrolysis of residual retinal oxime and subsequent reconstitution of the native bR pigment to compete effectively with reconstitution of analogue **1** during the 1 month incubation period.

One might point out that to avoid possible contamination of bR in studies of low-level photoactivities of slow-forming pigments, the use of a "white membrane" for pigment reconstitution as recently done for the equally slow-forming merocyanines,[2e] is advisable.

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