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On the molecular mechanisms of the Schiff base deprotonation during the bacteriorhodopsin photocycle

(pH dependence/cation dependence/deionized blue/acid blue/proton–cation equilibrium)

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ABSTRACT **Using optical flash photolysis and time-resolved Raman methods, we examined intermediates formed during the photocycle of bacteriorhodopsin (bR), as well as the bR color change, as a function of pH (in the 7.0–1.5 region) and as a function of the number of bound Ca^{2+} ions. It is found that at a pH just below 3 or with less than two bound Ca^{2+} per bR, the deprotonation (the $L_{550} \rightarrow M_{412}$) step ceases, yet the K_{610} and L_{550} analogues are still formed as in native bR. The lack of deprotonation in the photocycle of both acid blue and deionized blue bR and the similarity of their Raman spectra as well as of their K_{610} and L_{550} analogues strongly suggest that both blue samples have nearly the same retinal active site. It is suggested that in both blue species, bound cations are removed via a proton–cation exchange equilibrium, either on the cation exchange column for the deionized sample or in solution for the acid blue sample. The proton–cation exchange equilibrium is found to quantitatively account for the pH dependence of the purple-to-blue color change. The different mechanisms responsible for the large reduction (≈11 units) of the pK_a value of the protonated Schiff base (PSB) during the photocycle are discussed. The absence of the $L_{550} \rightarrow M_{412}$ deprotonation process in both blue species is discussed in terms of the previously proposed cation model for the deprotonation of the PSB during the photocycle of native bR. The extent of the deprotonation and the blue-to-purple color change are found to follow the same dependence on either the pH or the amount of cations added to deionized blue bR. This observed correlation is briefly discussed.**

Bacteriorhodopsin (bR) is the only protein found in the purple membrane of *Halobacterium halobium*, a light-harvesting bacterium. It contains retinal as a chromophore, which is covalently bound via a protonated Schiff base linkage to the ε-amino group of a lysine residue in the protein (1, 2). Upon absorbing a photon, it undergoes a photochemical cycle (3) consisting of at least four intermediates with lifetimes varying from pico- to milliseconds:

$$bR_{570} \underset{\text{psec}}{\overset{h\nu}{\rightleftharpoons}} K_{610} \underset{2\ \mu\text{sec}}{\longrightarrow} L_{550} \underset{40\ \mu\text{sec}}{\overset{-H^+}{\longrightarrow}} M_{412} \underset{\text{msec}}{\overset{+H^+}{\longrightarrow}} O_{640} \rightarrow bR_{570}.$$

The photocycle causes protons to be pumped across the cell membrane to the outside, establishing a pH gradient used by the organism for metabolic processes such as ATP synthesis (4–7). The protons are ejected from the cell at a rate comparable to that for the formation of the M_{412} intermediate (8, 9). A good correlation has been found between the number of protons pumped and the amount of slow decaying (10) form of M_{412}. This intermediate is the only one in which the Schiff base is unprotonated (11–16). Consequently, many studies have inferred that the protonated Schiff base (PSB) deprotonation is closely associated with the proton pump mechanism (17). Preceding M_{412} formation (or PSB deprotonation) is the formation of the early intermediates K_{610} and L_{550}. The retinal in bR_{570} is in the all-trans form, while in K_{610} it has a distorted 13-cis conformation (18–20). In the L_{550} form, the isomerization is complete (18–20).

The pK_a value of the PSB is 13.3 in bR_{570} (21, 22), yet it deprotonates during the cycle, suggesting a large change in its pK_a value. This reduction in the pK_a has been attributed to a change in the electrostatic interaction with the anions (23, 24), isomerization (25), a change in the hydrogen-bonding geometry around the PSB (26, 27), and a cation–PSB electrostatic repulsion during the $L_{550} \rightarrow M_{412}$ step (28). Experimentally, the rate of the $L_{550} \rightarrow M_{412}$ step is found to be determined by the rate of the protein conformational change (28) and not by the rate of the proton dissociation (22). It is also found that the deprotonation of a tyrosine (29, 30) occurs on the $L_{550} \rightarrow M_{412}$ time scale and is closely coupled to the deprotonation of the PSB (28). Furthermore, it is found that neither M_{412} (31, 32) nor tyrosinate (33) is formed if the pH drops below 3.0 or if the protein-bound cations are removed. All of these and other results (28) have been discussed in terms of the cation model (28, 33). In this model, the cation–PSB repulsion and the cation–tyrosinate attraction could lead to a simultaneous reduction of the pK_a value of both the PSB and tyrosine, thus leading to their observed near simultaneous deprotonation. The rate of the process is assumed to be determined by the rate of the protein conformational changes that brings the positively charged group near the PSB and tyrosine.

The present work was carried out in an effort to understand the molecular mechanism of the PSB deprotonation (the $L_{550} \rightarrow M_{412}$) process. For this to be accomplished, it is essential to extend the previous work on the effect of adding H^+ and removing inorganic cations (31, 32, 34) on the different photocycle intermediates. This was carried out by studying the dependence of the amount of the deprotonated species formed (M_{412}) during the photocycle in the pH range of 7 down to 1.75 and as a function of the number of protein-bound cations. A titration-like curve was obtained in each case, which shows that a large decrease in the amount of M_{412} occurs at pH ≈2.6 and when the number of protein-bound Ca^{2+} drops below 2 per bR molecule. By using time-resolved resonance Raman techniques, it was further shown that the early intermediates (K_{610} and L_{550}) are formed in the perturbed bR species for which the PSB deprotonation process ceases. These transient Raman and optical absorption results, along with previous optical results (32), show that it is only the PSB deprotonation (the $L_{550} \rightarrow M_{412}$) step that ceases at pH < 2.6 or when the number of bound cations is reduced. The similarity between the Raman spectra of the acid blue and deionized blue bR and that for their photocycle intermediates, as well as their similar absorption spectra (34, 35), suggest that the active sites of both acid and deionized blue

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Abbreviations: bR, bacteriorhodopsin; PSB, protonated Schiff base.

species are very similar. These results are discussed in terms of the cation model (28) for the deprotonation process and a proton-induced cation-displacement equilibrium.

EXPERIMENTAL

Master slants of the ET1-001 strain of *H. halobium* were provided by R. Bogomolni and W. Stoeckenius. The procedure for the growth and purification of the purple membrane was a combination of those outlined by Oesterhelt and Stoeckenius (36) and Becher and Cassim (37).

Time-resolved resonance Raman spectra of bR intermediates were obtained by the method of Terner *et al.* (18). A flowing sample and a tightly focused continuous wave laser beam control the interaction time of the sample with the laser. A fast sample flow using a peristaltic pump through a capillary (or a syringe needle) with a 10-μm laser focus resulted in an interaction time of $\approx$10 μsec and by varying the flow rate and defocusing the laser, longer times were easily obtained. The details of the time resolved Raman experimental set-up were given previously (18).

Laser photolysis experiments were carried out for bR_{570} over the range $1.7 < pH < 7.0$. The purple membrane ($\approx$100 μM) was incorporated in 7.5% polyacrylamide gels (35) to prevent aggregation at $pH < 3.0$. The gel samples were buffered with 0.1 M potassium phosphate and titrated to specific pH values with HCl. Photolysis of the purple membrane was accomplished with a 584-nm beam from a Nd^{3+}:YAG-pumped dye laser (Quanta-Ray, Mountain View, CA) run at a repetition rate of 10 Hz and monitored for constant power. Peak photolysis intensities were kept at $\approx$55 kW/cm^2, and the selected wavelength is at the isosbestic point for the blue–purple bR equilibrium. Thus, the absorption strength at the photolysis wavelength does not change as the pH of the solution changes. The 405-nm line from a Hg arc lamp was used as the probe light and detected by a photomultiplier tube (RCA 1P28A) with a visible cutoff filter. The maximum amount of M_{412} absorbance was measured by using a boxcar averager (PAR Model 162) with two gated integrators (giving I and I_o). The gel samples were cut from one gel preparation so that bR concentration and sample thickness were nearly constant. However, the transient absorbance values were corrected for slight variations in optical density between different gel samples.

For the measurements made on Ca^{2+}-restored bR, samples were prepared from cation exchange deionized bR (suspended in doubly deionized H_2O) by adding aliquots of $CaCl_2$ solution. Transient absorbances were measured in a manner similar to the above, except that a fast shutter (Uniblitz, Vincent Associates) was added to the probe beam path, and a transient digitizer and microcomputer were used for signal averaging as described (38). Absorption spectra were recorded on Hewlett-Packard 8450a and 8451a spectrometers.

RESULTS AND DISCUSSION

The Lack of Deprotonation in the Two Blue bR Forms: Similar Causes. It has been shown that PSB deprotonation (M_{412} formation) is inhibited when bR is subjected to cation removal and low pH (31–33). The question that immediately arises is whether the formation of the earlier intermediates (K_{610} and L_{550} analogues) is inhibited under these perturbations. Time-resolved Raman spectra of the perturbed bR species indicate the formation of K_{610} and L_{550} analogues, even when deprotonation does not occur. Fig. 1 shows the appearance of the characteristic ethylenic stretch frequencies (39) for K_{610} (1515 cm^{-1}) and L_{550} (1540 cm^{-1} or 1548 cm^{-1}) for neutral, deionized, acid blue, and acid purple bR. Therefore, only deprotonation of the PSB is inhibited in the perturbed species.

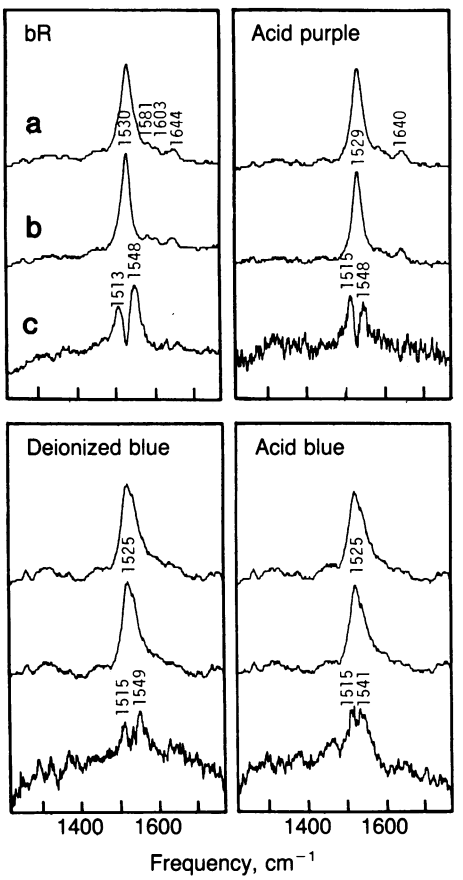

FIG. 1. Time-resolved resonance Raman microbeam flow spectra in the ethylenic stretching region of bR and three of its perturbed species, deionized blue, acid blue, and acid purple, using 514 nm Ar^+ excitation with an $\approx$10-μsec interaction time. Curves: a, high laser power spectra (>200 mW); b, low laser power spectra (<5 mW); c, difference spectra with arbitrary subtraction. The ethylenic stretching frequency in the native bR_{570} photocycle (39) is 1519 cm^{-1} for K_{610}, 1539 cm^{-1} and 1548 cm^{-1} (double peak) for L_{550}, and 1568 cm^{-1} for M_{412}. The difference spectra of the perturbed species show that both the K_{610} and L_{550} analogues are formed within the $\approx$10-μsec interaction time.

Since it has been established that the $L_{550} \rightarrow M_{412}$ step is inhibited under the perturbation of cation removal and low pH, the mechanism of this step can be investigated by examining the details of these two perturbations on the formation of the transient M_{412}. In Fig. 2*a* we present the detailed dependence of the amount of M_{412} formed, as well as the amount of acid blue in equilibrium with bR_{570}, as a function of pH. At a pH below 2.6 $\pm$ 0.4, we observe that the deprotonation of the PBS ceases, which is accompanied by a large change in the amount of blue bR formed. This quantitatively shows that it is the PSB in bR_{570}, and not the PSB in acid blue bR, that leads to the deprotonation step.

The detailed effect of the removal of cations (in this case Ca^{2+}) from bR is shown in Fig. 2*b*. Again we see that inhibition of transient M_{412} formation (i.e., of deprotonation) is directly related to the formation of deionized blue bR. We find that $\approx$2 Ca^{2+} ions per bR molecule are needed to regenerate the purple color and to restore the deprotonation (M_{412} formation) process. Due to the large protein–cation association constant for Ca^{2+} (31, 34) and the existence of at least two binding sites (34), the amount of Ca^{2+} added to deionized bR can be used as a measure of the number of bound cations for values of <2 Ca^{2+} ions per bR molecule. The apparent requirement of 2 bound cations for deprotonation of the PSB and regeneration of the purple color could either mean that the 2 cations have a similar high association

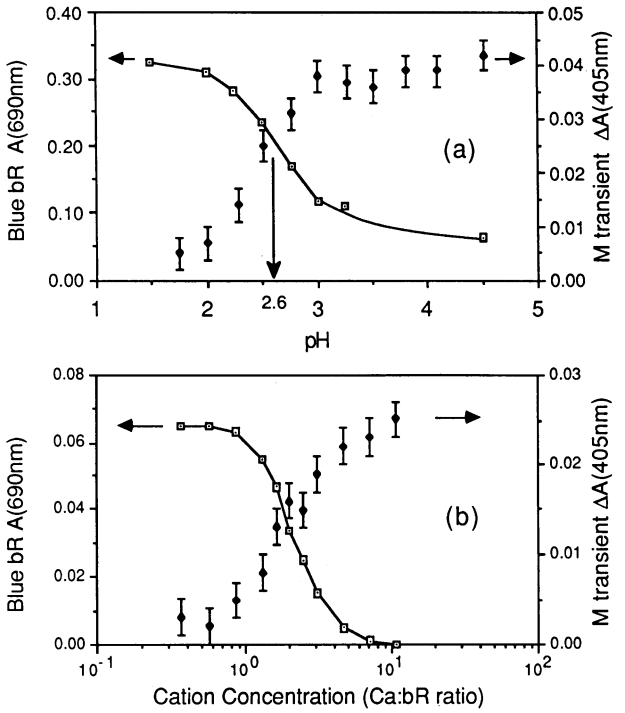

FIG. 2. The dependence of the extent of the deprotonation of the PSB during the photocycle (solid symbols) and the extent of the purple-to-blue bR conversion (open squares) on (*a*) the hydrogen ion concentration and (*b*) the calcium ion concentration added to deionized bR. The absorbance at 690 nm is far removed from the purple bR absorption band and was used as a measure of the blue bR concentration. From these results, we conclude that the removal of cations or the addition of hydrogen ions eliminates the deprotonation process and changes the purple color of bR to blue. This, together with the observed close similarity of the Raman spectra of K_{610} and L_{550} of either acid blue (pH less than ≈2.6) or deionized blue bR suggest that both blue samples have the same retinal active site, which is perturbed by the removal of the cations. Representative error bars are shown for the transient M_{412} amplitude, whereas the 690-nm absorbance values are more precise.

constant (and one or both are effective) or else only the cation with the smallest binding constant is effective in producing the observed perturbations.

The above results show that removing the cations from bR or adding large amounts of H^+ create the blue form of bR, which, while it contains a protonated Schiff base, does not lead to proton dissociation. The fact that the resonance Raman spectra of both unphotolyzed blue bR species (40–42, 50) and their photocycle intermediates (K_{610} and L_{550}) (43) show a great resemblance, as well as their similar absorption spectra (34, 35), suggests strongly that we are dealing with the same species (or two species with very similar retinal active sites). This can be understood if one believes that in both blue bR forms, protons have displaced bound cations. In preparing our deionized blue sample, the protons were introduced from the cation exchange column, while for the acid blue sample, the protein bound cations were displaced by the addition of excess protons to the bR_{570} solution. This strongly suggests that the inhibition of the deprotonation process in both blue bR forms results from the absence of bound cations and might provide support for the cation model (28). In this model, the electrostatic repulsion between a protein-bound cation and the positively charged nitrogen of the PSB leads to the needed reduction of its pK_a value to allow for its deprotonation. It has recently been shown (44) that indeed positive charges located near the PSB in model compounds do decrease their pK_a values. Furthermore, lowering the pK_a of a substituted ammonium ion in a reporter group at the active site of acetoacetate decarboxylase has been discussed in terms of electrostatic repulsion with positively charged groups (45).

It is not yet known whether the crucial positive charge in the cation model is itself an inorganic cation or whether the inorganic bound cations, because of their control of the protein conformation, assist in bringing an organic positively charged amino acid near the PSB during the $L_{550} \rightarrow M_{412}$ process. The regeneration of the purple color from the deionized blue species (by adding cations) seems not to be highly cation specific. This might support the second possibility—i.e., that the presence of inorganic cations may affect protein surface charges such as to allow protein conformations that make the approach of an organic cation to the PSB possible during the $L_{550} \rightarrow M_{412}$ process. It is also possible that the inorganic cation(s) on the surface could create a positive field within the near surface interior of the protein in which the PSB finds itself during the $L_{550} \rightarrow M_{412}$ process.

The Large PSB pK_a Change During the Photocycle. It is important to conclude from Fig. 2*a* that the pK_a of the PSB in the M_{412} precursor is ≤ 2.6. This represents a decrease in the pK_a value of the PSB by nearly 11 units during the $bR_{570} \rightarrow K_{610} \rightarrow L_{550} \rightarrow M_{412}$ transformation. This large change in the pK_a is partially explained by the relatively high value for the PSB in bR_{570}, as compared to its value in model compounds (pK_a, ≈7) (46, 47). This is a result (23, 24) of the electrostatic attractive interaction between the positively charged PSB and the nearby anion [the counter ion proposed previously in discussing the point charge model of the opsin shift (48)]. Upon isomerization, the PSB moves away from this region of low potential energy. Thus, upon changing to K_{610}, a decrease in the pK_a value of the PSB could take place on two accounts. The first is the decrease in the attractive electrostatic interaction (23, 24) between the PSB and the anion. The second is a result of the effect of the isomerization process itself on the energy of the retinal system (25). A change in the hydrogen-bonding geometry (26) around the PSB (27) or the H-bond strength (44) could also lead to further reduction in its pK_a value. During the $K_{610} \rightarrow L_{550}$ transformation, the retinal system and its environment change further and this could lead to further reduction in the pK_a of the PSB. This could be due to completing the isomerization process, further reduction in the electrostatic coupling, and/or more changes in the H-bonding strength and geometry around the PSB. During the $L_{550} \rightarrow M_{412}$ transformation, protein conformation changes could occur and lead to further changes in the nature of the electrostatic coupling between the protein and the PSB. It is during this process that a protein-bound (inorganic or organic) cation (28) may find itself in near proximity to the positively charged PSB. The electrostatic repulsion between this cation and the PSB could lead to the final decrease in the pK_a value of the PSB (to ≤ 2.6) and, thus, to deprotonation.

The Deprotonation and Color Change of bR. The results in Fig. 2 indicate that the color of the starting species and the deprotonation step of the photocycle are closely related and are affected in a similar way by the same perturbations. The sudden change in the concentration of the blue bR form or in the concentration of transient M_{412} resulting from either the addition of protons or the removal of the cations occur at the same critical concentrations of H^+ or Ca^{2+}. It is thus tempting to question whether the cation (or the mechanism) that is involved in the deprotonation process also plays a role in controlling the color of bR. NMR chemical shift studies (49) have recently suggested the presence of a positive charge near the β-ionone ring close to C_7 of the retinal system. Could it be that this is the same cation that, upon protein conformation changes in the $L_{550} \rightarrow M_{412}$ transformation, finds itself near the PSB thus leading to its deprotonation? At this time, this is just a speculation and more results are required to test

this proposal. The only possible support this proposal now has is the fact that if the cation is removed from C_7 it is expected that the retinal absorption in bR shifts to lower energy, as observed on going from bR_{570} to deionized blue bR. However, protein conformation changes (34) resulting from removing inorganic cations could also change the distances between the retinal and the counter anions, either near the β-ionone ring or the PSB, in such a manner to lead to an increased opsin shift (24). An experimental test of whether the cation near the C_7 can be removed by deionizing bR would be to compare the anomalous NMR downfield shift reported (49) for the ^{13}C-labeled C_7 position of the bR_{570} retinal with that of deionized blue bR. If the anomalous shift remains as that for bR_{570}, or possibly moves to a different cation, then the positive charge that controls the color near C_7 is an organic bound cation. If, on the other hand, the anomaly disappears, then the effect could be due either to the removal of an interacting inorganic cation or to the movement of an organic cation upon protein conformational changes (34) that occur with the removal of the inorganic cations.

The Proton–Cation Displacement Equilibrium of bR. The similarity between the spectral properties of acid and deionized blue bR (31, 34, 40, 50) and their photocycle intermediates suggests that we are dealing with the same species (bR without cations) prepared by either adding excess H^+ or removing cations. This could best be explained if one proposes the existence of a possible equilibrium in which high hydrogen ion concentration displaces protein-bound cations, thus producing (acid/deionized) blue bR. Below, a model is presented in which the purple-to-blue color change is related to the hydrogen ion–cation displacement reaction as follows:

$$bR_{570} + nH^+ \rightleftharpoons \text{blue bR} + Ca^{2+}, \qquad [1a]$$

for which

$$K_n = \frac{[\text{blue bR}][Ca^{2+}]}{[bR_{570}][H^+]^n}, \qquad [1b]$$

where K_n is the equilibrium constant for the displacement of 1 cation by n hydrogen ions. A purple-to-blue bR equilibrium, which did not include the hydrogen ion concentration, was presented by Chang *et al.* (31) and was used to obtain an "effective cation dissociation constant" $K_d \approx 10^{-8}$ M at near neutral pH. This quantity is not a "constant" due to the fact that it will change with pH and defines the binding of cations only at that pH (51). The above equilibrium should not be

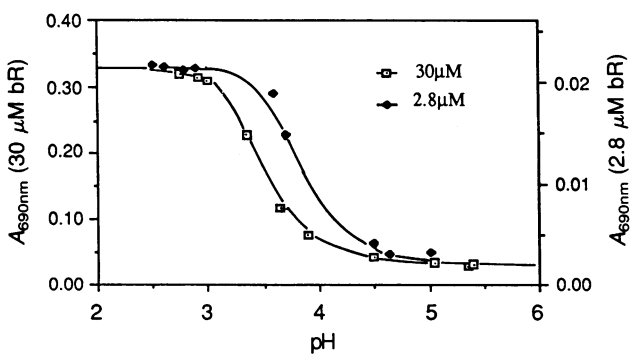

FIG. 3. The observed (points) and calculated (solid curves) dependence of the amounts of blue bR (relative to the initial bR_{570} concentration, C_0) on the pH of the solution at two initial bR_{570} concentrations (30 and 2.8 μM). The solid curve is calculated by using Eq. 1 with $K_{n=3} \approx 10^7$ M^{-2}, where $n = 3$ is known to be the number of protons released upon binding a Ca^{2+} ion to deionized bR (34). It is seen that both the shape of the curves and the bR concentration dependence support the proposal of a proton–cation displacement equilibrium.

expected to apply to drastic conditions such as extremely high hydrogen ion and cation concentrations since another form of purple bR can exist under such severe circumstances.

It has been shown (34) that up to three protons are released upon binding a calcium ion to bR. Consistent with this result is the pH and bR concentration dependence of purple (bR_{570}) and blue bR, shown in Fig. 3, which yields a best fit to Eq. 1 using $n = 3$ and an equilibrium constant $K_{n=3} \approx 10^7$ M^{-2}. If we start with native bR solution with no excess Ca^{2+} and lower its pH, according to Eq. 1 the free cation concentration in the solution is equal to the concentration of acid blue bR—i.e., $[Ca^{2+}]$ = [blue bR]. From Eq. 1, the critical pH for the color change in the absence of additional cations can be obtained by simply finding the pH at which the blue and purple bR concentrations are equal—i.e., [blue bR] = $[bR_{570}]$. Using C_0, the initial concentration of bR_{570}, as the total bR concentration in equilibrium (i.e., C_0 = [blue bR] + $[bR_{570}]$), it is easily seen from Eq. 1 that $[H^+]_{crit} = (C_0/2K_n)^{1/n}$. From this result, it is obvious that at higher bR concentrations a lower pH is needed to produce the purple-to-blue color change. This was observed previously (52, 53). Eq. 1 is also in agreement with our observation that a lower pH is needed to produce the purple-to-blue color change when excess cations are present. This, as well as the good fit of Eq. 1 with the observed pH and bR concentration dependence, might support the presence of this equilibrium and its possible importance in controlling both the color changes and the deprotonation of the PSB in bR and its photocycle.

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