BLM–Fe(III) complexes and their 1:1 cyanide adducts at 25 °C. The small proton paramagnetic shifts of the BLM–Fe(III) complex (11.6 ppm) and the BLM–Fe(III)–CN_3NH_2 complex (25.1 ppm) indicate low-spin ferric ($S = {}^1/_2$) state. Indeed, the ESR features of these Fe(III) complexes at 77 K are characteristic of a rhombic low-spin type: $g_x = 1.893$, $g_y = 2.185$, and $g_z = 2.431$ for the BLM–Fe(III) complex and $g_x = 1.847$, $g_y = 2.179$, $g_z = 2.540$ for its methylamine adduct.[5] On the other hand, the addition of cyanide ion to the BLM–Fe(III) and BLM–Fe(III)–CH_3NH_2 complexes produced a drastic change in the original ^1H NMR spectra, and new proton peaks appeared in the lower and higher field regions. The magnitude of the chemical shifts (over ±50 ppm) strongly suggests a high-spin ferric type for these cyano complexes.[8] CO, NO, and C_2H_5NC bind to the BLM–Fe(II) complex as a sixth ligand to form low-spin ferrous adduct complexes,[2] and several nitrogenous bases also coordinate to the BLM–Fe(III) complex to give low-spin ferric adducts.[5] If CN ion similarly binds to the vacant sixth coordination site of the BLM–iron complex, which has a rigid square-pyramidal arrangement with the 5–5–5–6 ring member,[5] the cyanide adducts would be expected to have a low-spin iron state.

We have found that the BLM–Fe(II)–O_2 system efficiently produces oxygen radicals such as $O_2^-\cdot$ and ·OH.[9] Indeed, similar ESR spin-trapping experiments using *N-tert*-butyl α-phenyl nitrone have shown that the addition of CO (or C_2H_5NC) strongly interferes with O_2 activation by the BLM–Fe(II) complex, but stoichiometric CN addition slightly increases the production of oxygen radicals in comparison with the original BLM–Fe(II) system.

BLM–iron complexes and hemoproteins apparently display similarities in the binding of oxygen antagonists (CO, NO, and C_2H_5NC) and in external nitrogenous bases, but the interaction of CN ion is remarkably different. In general, cyanide interferes with reaction of heme oxygenases, and CN adducts of ferric hemoproteins are of low-spin type. The present unusual behavior of CN ion toward the BLM–iron complexes appears to be responsible for the cyanide enhancement of the BLM activity against DNA. A detailed investigation of the CN interaction and the complete assignment of the proton signals are now under way.

Acknowledgment. Gratitude is due to Professor Hamao Umezawa for encouragement, Dr. Tomohisa Takita for pertinent advice, and M. Ohara for comments on the manuscript. This study was supported in part by a grant from the Ministry of Education, Science, and Culture, Japan.

(8) When CN ion was added to BLM–Fe(III) and its CH_3NH_2 adduct complexes, the typical low-spin ESR signals disappeared and a new broad ESR absorption near $g = 4$ appeared. However, quantitative consideration of this signal is difficult at present because of its complexity.

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Yukio Sugiura*

Faculty of Pharmaceutical Sciences
Kyoto University, Kyoto 606, Japan

Satoshi Ogawa, Isao Morishima

Department of Hydrocarbon Chemistry
Faculty of Engineering
Kyoto University, Kyoto 606, Japan

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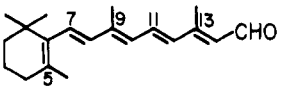
An External Point-Charge Model for Bacteriorhodopsin To Account for Its Purple Color

Sir:

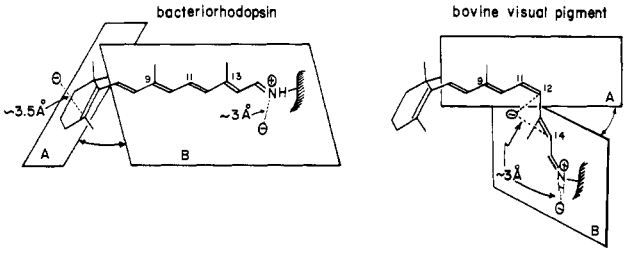
Bacteriorhodopsin is the pigment contained in the purple membrane of *Halobacterium halobium*, a halophilic bacterium, and it functions as a light-driven proton pump.[1] The chromophore is a retinal bound to the ε-amino group of lysine via a protonated Schiff base linkage (SBH^+).[2] *all-trans*-Retinal is the chromophore of the light-adapted form of bacteriorhodopsin, λ_{max} 570 nm, while a 1:1 mixture of *all-trans*- and 13-*cis*-retinals is the chromophore of the dark-adapted form, λ_{max} 560 nm.[3] Since the maximum of the SBH^+, with *n*-$BuNH_2$, in MeOH[4,5] is at 440 nm,

Table I. Absorption Maxima (nm) of Chromophores and Pigments[a]



chromophore	all-trans	5,6-2H	7,8-2H	9,10-2H	11,12-2H
aldehyde[b]	381	370	338	278	236
SBH^+ [c]	440	425[d]	385	322	270
calcd	*460*	*441*	*398*	*342*	
bacteriorhodopsins	560	476	400	325	*f*
calcd	*576*[e]	*485*[e]	*415*[e]	*347*[e]	
opsin shift (cm^{-1})[g]	4870	2500	1000	300	
calcd	*4400*	*2100*	*1000*	*420*	
bovine rhodopsins[h]	485[d]	460[d]	420[d]	345	315
opsin shift (cm^{-1})[g]	2100	1800	1700	2100	5300

[a] The trans isomers were incubated with bacterioopsin in the dark, 1 h, room temperature, 67 mM pH 7.0 phosphate buffer. [b] In MeOH. The maxima of the aldehyde-containing all-trans chromophores are listed. [c] Protonated Schiff base with *n*-$BuNH_2$, in MeOH; see ref 5. [d] Values are for the 9-cis isomers. Values for the rhodopsins are for the 9-cis isomers because the 9-*cis*-retinals are more readily synthesized then the 11-cis isomers. For 9,10- and 11,12-dihydro chromophores the values are for the "all-trans" isomers; here flexibility of the single bond allows the all-trans isomer to form pigments (see ref 6). [e] Calculated shifts for model shown in Figure 1a. See ref 8 for details of calculation. [f] Could not be measured due to overlap with protein absorption. [g] See text. [h] Data from ref 6; measured in 0.5% digitonin, phosphate buffer, pH 7.0.



a b

Figure 1. Models for the wavelength-determining electrostatic interactions in the chromophore binding site of (a) bacteriorhodopsin and (b) bovine rhodopsin. The ring-chain angle was *arbitrarily* set at 45° for both rhodopsin and bacteriorhodopsin; this is the value derived from NMR solution studies[10] and theoretical calculations.[10] The existence of a counterion near the protonated nitrogen is assumed. A second negative charge is located in the case of bacteriorhodopsin (Figure 1a) ~3.5 Å above C-5. The position of the charge depicted in Figure 1a, however, is only one example of a location consistent with the experimental results; other locations of the negative charge near the β-ionone ring are also possible. For bovine rhodopsin (Figure 1b) the negative charge is placed ~3 Å from C-12 and C-14.

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it follows that the environment of the protein binding site induces a considerable bathochromic shift in the maximum of the chromophore. The extent of this shift is a measure of the influence of the binding site; hence we propose to call this shift, i.e., λ_{max} of SBH^+ (in cm^{-1}) minus λ_{max} of pigment (in cm^{-1}), the "*opsin shift*" (see Table I).

In the following we show that the large opsin shift for bacteriorhodopsin is due to electrostatic interactions between the protonated chromophore and charged or polar groups located on the protein near the β-ionone ring of retinal (Figure 1a). The method is similar to that employed earlier[6,7] to account for the variation in the maxima of visual pigments. Namely, a series of synthetic dihydroretinals were bound to the apoprotein, and the maxima of these pigment analogues were compared to those of the respective SBH^+; interpretation of these opsin shifts has led to the model depicted in Figure 1a.

The fact that synthetic dihydro chromophores[6] occupied the same binding site as that of the natural chromophore is indicated by the following experiments. Incubation of the dihydro chromophore with an aqueous suspension of the apoprotein in the dark, 1 h, at room temperature, was followed by a 1.5-h incubation with natural *all-trans*-retinal. Under these conditions only ca. 5% of natural bacteriorhodopsin was formed, as judged from the intensities of the different absorption maxima. When the sequence was reversed and the opsin was incubated first with *all-trans*-retinal and then with the artificial chromophores, only ca. 5% of the bacteriorhodopsin analogues were formed. Furthermore, the light-adapted form of 7,8-dihydrobacteriorhodopsin was also prepared by leaving the dark-adapted form (λ_{max} 400 nm) in room light for 3 h. This light-adapted species absorbed around 410 nm or at ca. 10-nm longer wavelength than the dark-adapted form. Since this shift compares well with the 10-nm difference between the dark- and light-adapted forms of natural bacteriorhodopsin (see above), it provides further evidence that the chromophores bind to the same site.

Experimental results are summarized in Table I and are compared with the results for bovine rhodopsin and its dihydro analogues.[6] *In the case of visual pigments the largest opsin shift, 5300 cm^{-1}, was encountered in the shortest chromophore, 11,12-dihydroretinal, which contains only two double bonds (an enal). The situation in the purple membrane is completely reversed; the largest opsin shift, 4870 cm^{-1}, is encountered in the longest chromophore.* Bacteriorhodopsin (dark-adapted species) absorbs at longer wavelength than bovine rhodopsin (560 vs. 500 nm). However, bacterial pigments derived from shorter chromophores absorb at shorter wavelength than the corresponding bovine pigment. The λ_{max} of the 9,10-dihydroretinal-derived bacteriorhodopsin is thus seen to be blue shifted from the bovine pigment; in addition the SBH^+ maximum (322 nm) and pigment maximum (325 nm) are nearly identical.

Thus a large opsin shift of 4870 cm^{-1} is obtained only in the case of the natural chromophore with six double bonds. As the saturated bond is moved toward the terminus of the side chain, i.e., from 5,6-dihydro- to 7,8-dihydro- to 9,10-dihydrobacteriorhodopsins, the opsin shifts decrease. These results lend themselves to a simple interpretation since the magnitude of the shifts induced by external charges would depend on their proximity to the conjugated π-electron system. Thus if a charge were present in the vicinity of the β-ionone ring, this would have a maximal effect on the natural chromophore with six double bonds and the effect would successively decrease as the location of the double bond which was saturated approaches the side-chain terminus.

It is well-known on both experimental and theoretical ground[8] that the Schiff base nitrogen becomes more basic in the excited state. That is, negative charge accumulates in the vicinity of the Schiff base while positive charge migrates into the polyene chain toward the β-ionone ring. For this reason a negative charge located near the ring or a positive charge located near the Schiff base could both, in principle, produce large spectroscopic red shifts. In the case of bacteriorhodopsin, however, it is clear that a negative charge must be involved since a positive charge near the nitrogen would shift the spectra of all the bacteriorhodopsin analogues of Table I. Since large shifts are encountered for only the longer chromophores while the shifts for shorter analogues progressively decrease, the data require, as discussed above, an interaction localized near the ring end of the chromophore. In this region, only a negative charge can produce red shifts.

Using a PPP scheme[8b,c] which was parametrized to yield good agreement with model polyene spectra and which includes the effects of external charges, we carrried out π-electron calculations to see whether our experimental results could be accounted for with a simple model. Table I contains the calculated transition energies for various SBH^+ with a counterion separated 3 Å from the terminal N, the counterion being introduced to represent the environment of the SBH^+ molecules in solution[4,8b] (also see below). For the model compounds the calculated shifts resulting from shortening the π system are in good agreement with experiment.

As discussed above, the bacteriorhodopsin analogue data suggest that a negative charge is located somewhere near the β-ionone ring. This charge must be closer to the 5,6 double bond than to the 7,8 double bond since the opsin shift for bacteriorhodopsin is twice that of its 5,6-dihydro analogue. However, even given this constraint, there still remain a number of positions for a charge in the vicinity of the β-ionone ring that are possible. One such location is shown in Figure 1a. Transition energies were calculated for the various chromophores interacting with this additional negative charge in the bacteriorhodopsin binding site (as in Figure 1a) to account for the further red shifts encountered in the pigments relative to the SBH^+ species in solution. The agreement between theoretical and experimental results (Table I) support the conclusion that a *charged or dipolar group near the β-ionone ring is responsible* for the opsin shifts of natural and artificial bacteriorhodopsins; namely, the origin of the color of the purple membrane can be accounted for by the external point-charge model shown in Figure 1a.

The point charge near the β-ionone ring could in principle be a dipolar amino acid (such as a tryptophan) positioned so that the negative electron density is near the β-ionone ring. However, the magnitude of the spectral shift in bacteriorhodopsin is accounted for by a negatively charged amino acid that is a member of a salt bridge oriented in such a manner that the positive end is distal from the ring. On the basis of the recently proposed three-dimensional model of bacteriorhodopsin,[9] Asp-96 is a likely candidate. In addition to the charged group near the β-ionone ring, we have assumed, as in our previous study of bovine rhodopsin,[7] the existence of a counterion located ca. 3 Å from the Schiff base nitrogen. Displacement of the counterion to longer distances could, in principle, lead to large wavelength shifts[4a,8b] but it was argued on energetic ground that a charge separation of this magnitude was unlikely in a stable pigment. It is clear from Table I that the source of the wavelength shift in bacteriorhodopsin cannot involve the immediate environment of the Schiff base since, if this were the case, the absorption maxima of *all* the dihydro pigments would be considerably shifted relative to the solution values of the corresponding SBH^+, which is not the case.

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Our "two-charge" model shown in Figure 1a[10] suggests that it should be possible to titrate specific residues in bacteriorhodopsin and obtain large spectral shifts. The long-wavelength form of bacteriorhodopsin (λ_{max} 605 nm) observed at pH <2.8[1,2a] has been attributed to the titration of a charged amino acid near the Schiff base terminus.[11a,b] This presumably corresponds to the counterion. The 565-nm species formed at pH <0.8 could result from the titration of a second group[11a] which we have placed near the β-ionone ring. However, since bound anions have also been shown to induce wavelength shifts,[11b] the factor involved in the formation of the 565-nm species is still an open question.

It should be noted that the model carrying a negative charge in the vicinity of the β-ionone ring (Figure 1a) is in contrast to the model derived previously for bovine rhodopsin, where the important wavelength-determining interactions were located near the 11,12 double bond of the polyene side chain[7] (Figure 1b). We showed that simple synthetic compounds could simulate the spectral shifts observed in the binding site of bovine rhodopsin;[12] synthetic models, designed to simulate the bacteriorhodopsin binding site, are currently being prepared to check the external point-charge model for bacteriorhodopsin.

The influence of the opsin binding site on the absorption maximum of SBH^+, i.e., the opsin shift, is a central problem in the rhodopsin field since it accounts for the wide variance in λ_{max} encountered in the various pigments, including the visual cone pigments responsible for color perception. Thus numerous models have been proposed to account for the opsin shift. However, we point out that a model which rationalizes the opsin shifts of natural systems is far from satisfying the necessary conditions. The model should also account for the data derived from various synthetic retinals, e.g., data which led to the proposal of models 1a (Table I) and 1b.[13]

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Koji Nakanishi,* Valeria Balogh-Nair
Maria Arnaboldi, Kazuo Tsujimoto
Department of Chemistry
Columbia University
New York, New York 10027

Barry Honig*
Department of Biophysics
University of Illinois
Urbana, Illinois 61801

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Opsin Shifts in Bovine Rhodopsin and Bacteriorhodopsin. Comparison of Two External Point-Charge Models

Sir:

We proposed the external point-charge model I (Figure 1)[1-3] to account for the variance in the absorption maxima of various visual pigments; more recently,[4] we proposed model II (Figure 1) to explain the purple color (λ_{max} 570 nm) of bacteriorhodopsin (bR), the pigment present in purple membrane.[5] Note that the two models have contrasting charge distributions; i.e., *the second charge is near C-12/C-14 in visual pigment I, whereas it is near the ring in bacteriorhodopsin II.*

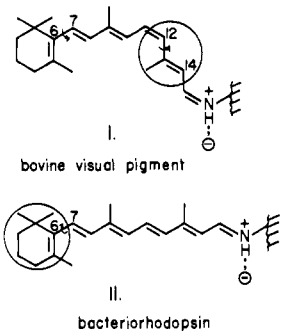

Figure 1. External point-charge models in the binding sites for bovine visual pigment (I) and bacteriorhodopsin (II). The existence of a counterion near the protonated nitrogen is assumed. The second negative charge shown by the circle could be a member of a charge pair in a salt bridge or one end of a neutral dipole. In I, it is located near C-12/C-14 whereas in II, it is near the ring. The small curved arrows indicate that the bonds are twisted.

The retinal chromophore is bound to the apoprotein lysine residue through a protonated Schiff base (SBH^+) in these pigments.[6] The charges shown by circles in Figure 1 do not exist in solutions of SBH^+ (formed from *n*-butylamine) and are present only in the binding sites. Hence the difference in the maxima between the *n*-butylamine SBH^+ and pigments can be regarded as a measure of the influence of the binding site and we have proposed to call it the "opsin shift".[4] The contrasting external point-charge distributions seen in models I and II should manifest themselves in the opsin shifts of pigments derived from appropriately tailored retinals; investigation of such data should in turn provide us with a means to check the validity of the two models. Namely, we may expect aromatic retinal **1** with an altered ring structure not to exert large effects on the λ_{max} of rhodopsin but instead affect that of bR; the opposite tendency is predicted for bromoretinals **4** and **5** having altered side chains. In the following we report the results of such observations.

Retinal Synthesis. Benzaldehyde and *p*-(dimethylamino)-benzaldehyde were converted into phenyl dienals **6** and **7** through conventional[7] Emmons reaction with ethyl phosphonosenecioate, Dibal reduction, MnO_2 oxidation, and flash chromatographic separations[8] of isomers. A second series of the same C-5 elongation process gave the all-trans, 13-cis, 9-cis, and 9,13-dicis isomers[9] of aromatic retinals exemplified by **1–3**.[10]

The synthesis of 13-bromoretinal **4** was carried out by condensation of Wittig salt **8**[11] with aldehyde **9** (Scheme I), which

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