Protein−β-Ionone Ring Interactions Enhance the Light-Induced Dipole of the Chromophore in Bacteriorhodopsin

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Following light absorption, the retinal chromophore of bacteriorhodospin experiences very large dipolar changes in the vertically excited state. This light-induced dipole is at least 50% larger than that of the retinal chromophore in films or in solution. We have studied the origin of the protein effect by applying second harmonic generation measurements of artificial bacteriorhodopsin pigments derived from synthetic retinal analogues, characterized by a modified polyene chain length and ring−chain conformation. The studies demonstrated a significant influence of a protein domain in the vicinity of the retinal β-ionone. We suggest that tryptophane residues (specifically Trp 138 and 189) enhance the light-induced dipole in bacteriorhodopsin. The data also point to another protein domain (Trp 182) influencing the retinal chromophore. The effect of these tryptophane residues appears not only to stabilize the light-induced charge redistribution but also to enable the migration in the excited state of the positive charge into a region of the protein with a relatively low dielectric constant.

Introduction

Bacteriorhodopsin (bR) is a membrane protein composed of all trans retinal bound via a protonated Schiff base to the ϵ-amino group of lysine 216.[1−3] Following light absorption, bR undergoes a photocycle that consists of various intermediates, which are coupled to the proton pumping activity of the pigment. We have applied second harmonic generation (SHG) to probe the light-induced dipolar changes in the retinal chromophore of bR.[4−6] These studies as well as those that have applied hyper-Rayleigh scattering and two-photon absorption spectroscopy have shown that the chromophore of bR has a very large light-induced dipole, which is at least 50% larger than that of the retinal chromophore in films or in solution.[5,7,8] It was previously suggested that this large induced dipole could trigger protein conformational changes following light absorption.[9−14] Recently it was demonstrated that a light-induced dipole is crucial for initiating the bR photocycle.[15] The object of the present study was to shed light on the possible protein−chromophore interactions that dramatically increase the retinal light-induced dipole.

Chromophore−protein interactions have been studied previously by examining the relative absorption properties of the chromophore in solution relative to the absorption in the protein. bR absorbs at 570 nm, 5100 cm^{-1} red shifted relative to protonated retinal Schiff base in methanol solution. This large red shift was mainly attributed to two factors: (1) weakening of the counterion Schiff base interaction in the protein matrix relative to solution[16−18] and (2) the planar s-trans conformation of the chain/β-ionone ring relative to the twisted s-cis conformation in solution.[19−21] Previous studies in our laboratory attempted to elucidate the effect of these factors on the SHG of the chromophore in the protein. These studies indicated that both of the above factors did not have a major effect on the SHG and could not explain the large induced dipole found in the bR chromophore relative to model retinal protonated Schiff bases.[4−6]

To probe the origins of the protein effect on the large induced dipole of the bR retinylidene chromophore, we have investigated SHG of artificial pigments derived from synthetic retinal analogues, which are characterized by a modified polyene chain length and ring chain conformation relative to the protein environment. The results indicate that the principal effect of the protein in increasing the induced dipole originates from residues that are located in the vicinity of the β-ionone ring of the retinal chromophore.

Materials and Methods

Pigments Preparation. The retinal analogues **2**−**11** were prepared according to previousely described methods.[22−24] The apomembrane was prepared from bacteriorhodopsin by sample irradiation in the presence of hydroxylamine according to established procedure.[25] The artificial pigments were prepared by incubation of the apomembrane with 2.5 equiv of the appropriate retinal analogue at 25° C for 12 h. The samples for SHG measurements were prepared by deposing 10 μL of 1×10^{-4} M of each pigment or the retinal chromophore in ethanol solution (5×10^{-4} M) on a glass slide suspension and drying the film under a stream of nitrogen.

SHG Measurements. The experimental methodology used to obtain the SHG signal has been described elsewhere.[4−6] A titanium sapphire laser (Mira, Coherent Radiation, Santa Clara, CA) pumped by 12 W power of an argon ion laser (Innova 200, Coherent Radiation, Santa Clara, CA) was used as an excitation source. The laser operated in the mode-locked regime and emitted 60 fs pulses at the pulse frequency equal to 76 MHz at a wavelength of 780 nm.

The laser beam was directed to the back port of the optical microscope (Zeiss, Axiovert 135), and a filter was placed in

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TABLE 1: SHG Signal for Different Pigments Relative to bR (I)

pigment[a]	relative SHG signal	absorbance[b] (nm)
I	1 ± 0.06	570
II	0.42 ± 0.02	470
III	0.23 ± 0.01	440
IV	1.87 ± 0.11	560
V	0.61 ± 0.03	620
VI	0.48 ± 0.02	570
VII	0.22 ± 0.01	520
IIX	0.7 ± 0.04	520
IX	0.42 ± 0.02	460
X	0.42 ± 0.02	470
XI	0.7 ± 0.04	530

[a] The samples were prepared by deposing 10 μL of 1×10^{-4} M of each pigment on a glass slide suspension and drying the film under a stream of nitrogen. [b] Absorption maxima of the pigments at pH 7.

TABLE 2: SHG Signals of Different Chromophores Relative to All-Trans Retinal (1)

chromophore	SHG relative signal[a]	absorption[b] (nm)
1	1 ± 0.06	380
2	1 ± 0.06	368
3	0.94 ± 0.05	342
4	1.88 ± 0.01	438
5	2.08 ± 0.12	428
6	1.12 ± 0.07	414
7	0.88 ± 0.04	380
8	1 ± 0.06	380
9	1 ± 0.06	380
10	1 ± 0.06	380
11	1 ± 0.06	380

[a] The samples were prepared by deposing 10 μL of 5×10^{-4} M of each chromophore in EtOH on a glass slide and drying the film under a stream of nitrogen. [b] Absorption maxima of the chromophores in EtOH.

front of this port to cut any SHG signal which might be created in the beam path by the optics before the microscope. The beam was further focused onto the sample with a 50× objective with a numerical aperture of 0.5, giving an average illumination power at the sample of 20−30 mW.

The microscope was also fitted with a trinocular, and one of the ports of the trinocular was equipped with a Nikon PCM 2000 beam scanner unit (Nikon Ltd, The Netherlands). For the experiments in which this unit was employed, the femptosecond pulses were directed through this beam scanner and subsequently through the objective. This allowed for imaging the SHG as a function of time.

Under all conditions the forward propagating SHG light was collected with two focusing lenses and a filter which reduced significantly the fundamental intensity. The SHG light was focused by these lenses onto the entrance slit (2 mm width, corresponding to 16 nm resolution) of a monochromator (Jobin Yvon) and was further detected onto a photomultiplier (Hamamatsu, 931B). A signal from the photomultiplier was processed by an analog processor (Stanford Research Systems). The signal was also monitored with an oscilloscope (Textronix TDS 520).

To verify the signal associated with the SHG, we changed the monochromator settings by a few nanometers around the SHG wavelength (390 nm) and took into account only that fraction of the whole signal which significantly decreased as a result of the monochromator setting change, as would be expected for SHG.

To amplify the signal obtained from the photomultiplier and to increase the electronic sensitivity, we set the analog processor output to give a logarithm of the modulus of the input signal, amplified by a gain factor of 2. Therefore, the observed signal I_{obs} was connected to the real signal I_{real} by the following expression: $I_{obs} = 2 \ln(I_{real})$. Thus, to derive the actual SHG intensity, we calculated $I_{real} = \exp(I_{obs}/2)$, and these are the values recorded in Tables 1 and 2.

Results and Discussion

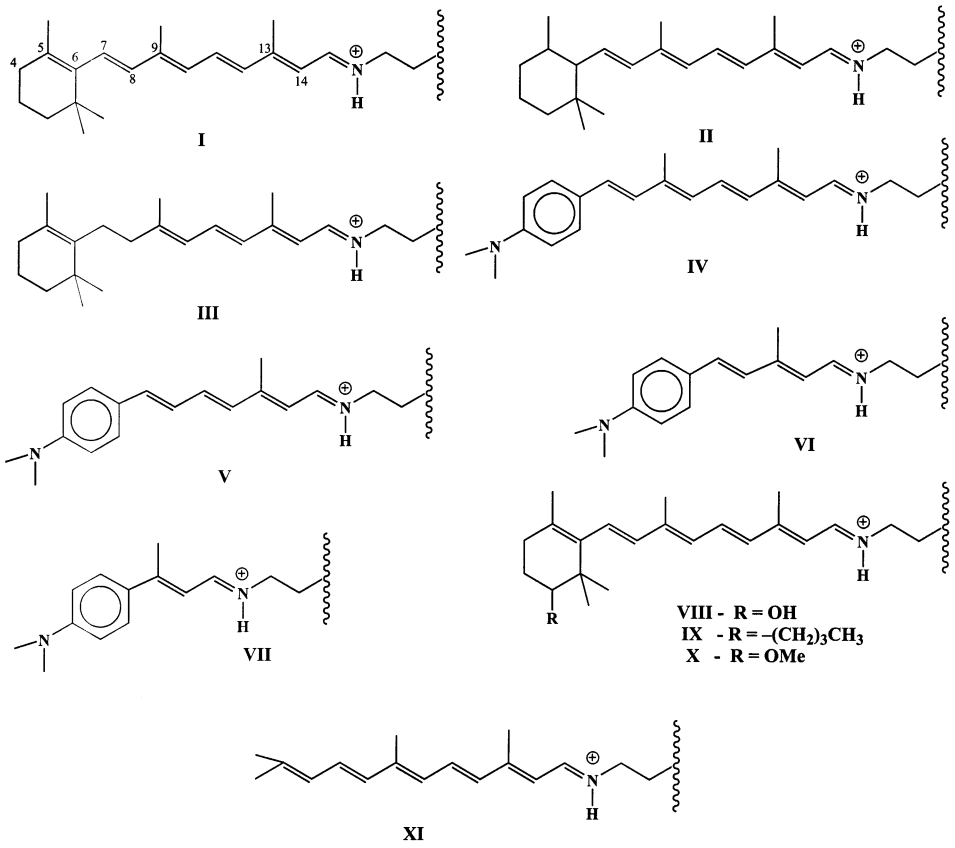
Similar to previous studies, films of bR and its artificial pigments were investigated.[4−6] We have found that, at a suspension concentration of 1.6×10^{-4} M, bR films spontaneously form organized membrane fragment films on a microscope cover slip which exhibit a SHG signal. Interestingly, placing a drop of the suspension on a glass cover slip produced a weak SHG (even in the form of a suspension), which grows as the films dry. Even following a dilution of 150 times, a signal was still detected but with a much weaker intensity. We cannot exclude the possibility in these measurements that the signal in suspension occurred due to a thin initial film that was adsorbed to the surface of the glass substrate on which the suspension was placed. We have found that the extent of the SHG was not altered by increasing the optical density (OD) of the film above 0.1. This was indicated by attaining a characteristic SHG signal which was varied by ±7% by increasing the OD of the film by as much as 10 times. The SHG can be detected only as a result of orientation of the fragments in the film. Therefore, the constant SHG indicates that the extent of orientation of the fragments in films of OD above 0.1 does not increase.

To further study this phenomenon of orientation saturation, we have obtained images using a beam scanning SH microscope. For films with an OD below 0.1 which were prepared from a suspension with a concentration of 1.6×10^{-6} M, no SHG is observed. With a more concentrated suspension (8×10^{-6} M), there is already enough orientation such that an image of the dried film produced from this more concentrated solution is readily obtained. Furthermore, doubling of the concentration of the suspension does not alter the intensity of the SHG from the film obtained from these higher concentration suspensions. An analysis of the average value variations of the pixels in such images indicated an error of ±6% in the intensity of the images of dried films obtained from concentrations above 8×10^{-6} M. Similar behavior is seen in the artificial pigments, where once again there is no increase in the average SHG of a film that has an OD above 0.1. Such artificial pigment films below an OD of 0.1 show increasing SHG until such an OD of 0.1 is reached. In all the measurements reported in this paper, we have worked well above this plateau, so that complications due to concentration dependent effects are minimized.

In addition to the above, retinal analogue films were studied which were prepared by spin coating from an ethanol solution by standard methods.[4−6] To locate the protein domain that has the largest effect on the SHG, we have investigated artificial pigments derived from retinal analogues characterized by different polyene lengths. As depicted in Chart 1, pigment **II** (6,7-dihydro-bR) lacks the double bond in the β-ionone ring, and as shown in Table 1, there is a significant decrease in the SHG relative to that of native bR. Pigment **III** (7,8-dihydro-bR) breaks the conjugation at the 7,8 position and is characterized by a shorter conjugated polyene. This results in a further decrease in the SH signal relative to that of pigment **II**. Both of these alterations in the polyene chain cause a significant unexpected decreases in the SH signal.

To further study the polyene chain length effect, we have examined pigments **IV** to **VII** characterized by an aromatic core substituting the retinal β-ionone ring with different polyene lengths[26] (Table 1). The observed trends for this sequence of

CHART 1



retinals studied as pigments or free retinal show significant differences. For example, pigment **IV**, which has a polyene length similar to that of native retinal, exhibits a SHG which is considerably larger than that of pigment **V.** Similar dramatic changes are seen when pigment **IV** is compared to pigment **VII**. Significant changes are also seen by comparing pigment **VI** to pigment **VII**. However, a comparison of pigment **V** with pigment **VI** does not follow this trend of SH reduction either in this series of pigments or in the sequence of retinal analogues absorbed on a glass surface (Table 2). In the sequence of retinal analogues both in this series of aromatic molecules and in the dihydro sequence, a reduction of one double bond from the full polyene chain length does not cause significant SH alterations (compare in Table 2 aromatic molecules **4** and **5** and the dihydro retinals **2** and **3**). A further shortening by one double bond in the aromatic retinal analogues reduces the SH signal by roughly a half (Table 2, molecules **5** and **6**).

A further parameter that has been studied in this sequence of experiments is the effect of ring−chain planarity. The retinal chromophore adopts in the bR binding site an s-trans ring−chain planar conformation. It has been previously demonstrated[22] that substitution at the C(4) position of the ring causes steric hindrance, which causes a twist in the C(6)−C(7) single bond, perturbing the conjugation of the C(5)−C(6) double bond with the rest of the polyene chain. The conformation of such twisted chromophores in terms of their s-cis or s-trans character cannot be predicted; however, the main important conclusion is that the chromophores are highly distorted. This is indicated by a blue shift in the absorption maxima. The induced steric hindrance can also be used to probe the effect on the SHG as a result of altering the position of the β-ionone ring. To observe this effect, we have compared pigments **VIII**−**X** to native bR. Specifically, pigment **VIII** is characterized by a relatively small C(6)−C(7) single bond twist reflected in a relatively small blue shift (relative to bR) in the absorption maximum. The SHG also exhibits a small reduction due to this steric perturbation (Table 1). On the other hand, pigments **IX** and **X** are characterized by significant steric perturbations reflected in a large blue shift and SH signal decrease relative to the case of native bR **(I)**. Pigment **XI** sheds further light on the polyene planarity issue. It has been suggested that the blue shift in this pigment relative to bR is due to a twist of the single bond connecting the terminal double bonds.[27] This is also effective in reducing the SHG of this pigment. As shown previously,[28] this is in contrast to the cases of similar retinal molecules that in solution show opposite phenomena in both the absorption and SHG as compared to the cases of these molecules covalently bound to the protein.

In view of the above results, we suggest that a major effect of the protein environment on the SHG is in the vicinity of the retinal β−ionone ring. We arrive at this conclusion as a result of our observation of a dramatic decrease in the SHG following shortening of the polyene chain, for example, the elimination of the 5,6 or 7,8 double bonds (Table 1, pigments **II** and **III** as compared to native bR **(I)**).

Additional experimental support, for a protein effect around the β-ionone ring, can be derived from the data observed for the artificial pigments in which the ring adopts non-native conformations and displays consistent reductions in the second harmonic signal (Table 1, pigments **VII**−**X** as compared to native bR **(I)**). It should be noted that resonance effects can modulate the SH signal. However, in terms of the results we have obtained, it would be expected that blue shifting the absorption maxima of the artificial pigments would result in more rather than less resonance enhancement. This was the opposite of what was observed, and thus, our conclusions below are a conservative estimate of these effects.

Further support for the above conclusion comes from the results on the aromatic artificial pigments. A reduction in the

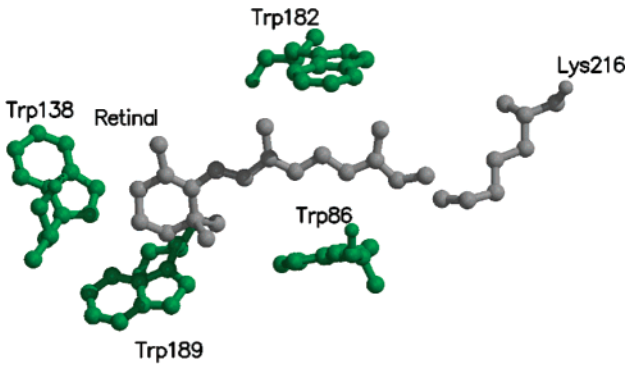


Figure 1. Tryptophane residues in the immediate environment of the retinal chromophore in bR, adapted from ref 29.

polyene chain length by one double bond relative to native bR leads to a large decrease in SHG, similarly to the dihydro case (Table 1, pigment **IV** as compared to pigment **V**).

All of these experimental observations are consistent with the presence of a protein entity in the vicinity of the β-ionone ring that significantly affects the SHG. It should be noted that the absorption maxima of artificial pigments vary widely and are generally blue shifted relative to the case of native bR. Such a blue shift would in fact increase the resonance contribution to the SHG. Thus, it would be expected, simply on the basis of a resonance effect, that where we see a decrease in signal there would be, from a resonance point of view, a signal that would be higher. Therefore, from resonance effects alone, the results would have been expected to be opposite to what was actually observed. In essence, neglecting in our analysis the effect of resonance enhancement of the SH signal is a conservative analysis of the data.

It is known from previous studies that the protein enhances the induced dipole and the associated nonlinear second order polarizability, which directly affects the intensity of the SHG of the retinal chromophore.[5,7,8] The bR X-ray structure indicates that the β-ionone ring is tightly coupled with two tryptophan residues (Trp138 and 189, Figure 1).[29]

Previous solution studies in our laboratory using hyper-Rayleigh scattering of retinal chromophores have shown that a polarizable environment is able to significantly enhance the nonlinear second order polarizability.[30] In these studies a variety of solvents were investigated including hexane, acetonitrile, chloroform, dichloromethane, ethanol, and benzene. The object of this investigation was to study the effect on the nonlinear second order polarizability of the dipole of a solvent such as acetonitrile, the hydrogen bonding character of a solvent such as ethanol, and the polarizability of a solvent such as benzene when compared to the case of hexane, which exhibits none of these characteristics. These studies showed that polarity could increase the nonlinear second order polarizability by about 80% (comparing hexane to acetonitrile). Most interestingly, comparing hexane to benzene, which have similar dipole moments, that is, zero, the nonlinear second order polarizability was almost doubled in benzene. This effect is even bigger than what is seen by the protein effect in bR.

Thus, it is plausible to suggest that the presence of these polarizable tryptophan residues is the likely cause of the significant protein effect on the SHG of retinal.

Previous studies have shown that aromatic residues such as tryptophan can permit positive charge to enter protein regions where the dielectric constant is low.[31] Upon excitation of the retinal chromophore, there is a major redistribution of positive charge from the protonated retinal Schiff base region to the β-ionone ring. In a protein one could expect microdielectric environments that result from, for example, charge heterogeneity or heterogeneity of the polarizable groups discussed in this study. Such microenvironments in proteins are difficult to probe with the necessary nanometric resolution. One monitor of polarizability of an environment is the index of refraction of the medium. Such an approach was applied for bR[32] to obtain information of the average dielectric constant around the chromophore, and it has revealed a refractive index of 1.53. These measurements would not be sensitive to specific regions around the chromophore to understand in molecular detail dielectric inhomogeneity. It is reasonable to assume that, despite average dielectric constant measurements within the bR absorption, the region around the β-ionone ring and, for that matter, a part of the polyene chain could be a relatively low dielectric medium. Therefore, in the protein one could argue that the positive charge redistribution upon vertical excitation could be significantly retarded by the low dielectric nature of the protein medium surrounding the β-ionone ring. Thus, Trp 138 and 189, by their ability to stabilize positive charge, could not only compensate for the nature of the surrounding protein medium but also enhance the ability of positive charge to redistribute in the excited state through the entire polyene chain. Therefore, when the polyene chain length of a chromophore is shortened and the effect of Trp 138 and 189 is reduced, then positive charge redistribution in the excited state is perturbed and the reduction in the SHG corresponds to not only a reduction in the chain length but also the removal of an enabling chemical environment that is the cause for maintaining and enhancing the SHG relative to the case of free retinal.

The data also point to another domain of influence in the protein on the SHG of the chromophore. This is concluded by comparing the SHG of pigment **VI** with the SHG of pigment **V**. In pigment **VI** the conjugated chain length has been reduced by one double bond relative to that of pigment **V**, which is reflected in a decreased SHG in the free chromophore. However, this same effect is not observed in the protein matrix, suggesting that the protein matrix in the region of carbon 9 also has an enhancing effect on the SHG. This could result from Trp 182. We note that previous authors[33,34] have addressed, through calculations, the problem of other tryptophan and tyrosine residues acting as polarizable groups around the chromophore and causing bathochromic shifts in the absorption. These authors suggested that Trp 86, 182, and 185 could have such effects. Further calculations on Trp 138 and 189 highlighted in this paper may be illuminating.

In summary, this study has demonstrated a significant protein domain around the β-ionone ring plausibly associated with tryptophanes, which enhances the SHG and the induced dipole of the retinal chromophore observed in bR. The studies may also clarify an important question related to color regulation in retinal proteins. For regulating the absorption, one requires an alteration between the ground and the excited states. The first step in this alteration is a redistribution of charge. For such delocalization in the vertically excited state, we have suggested in this paper that polarizable groups such as tryptophan have a crucial effect. Therefore, one factor that has to be important in inducing a large red shift in the absorption must be the presence of such polarizable groups. An initial suggestion for the involvement of polarizable groups as modulators of chromophore absorption was by Peter Leermakers[35] and more recently by several other groups.[33,34,36] It was proposed that such groups would stabilize the vertically excited state of the retinal and thus alter the energy of this excited state. Our study certainly

indicates the important effect that such groups play in stabilizing what we now know as significant charge redistribution of the retinal chromophore in this state, which is a prerequisite for inducing a red shift in the absorption. However, our study does not clarify whether this stabilization necessarily affects significantly the energetics of the excited state.

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