Redetermination of the Quantum Yield of Photoisomerization and Energy Content in the K-Intermediate of Bacteriorhodopsin Photocycle and Its Mutants by the Photoacoustic Technique
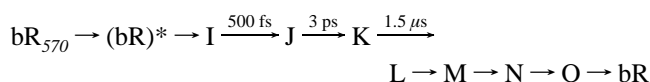
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Data obtained previously in our lab for the quantum yield retinal photoisomerization and the energy content of the K-intermediate formed in the bacteriorhodopsin and its mutants (bR) photocycle are reexamined using time-resolved transient spectroscopy and laser-induced photoacoustic spectroscopy. In the present experiment both nanosecond and subpicosecond laser pulses are used for excitation, with different reference compounds. From these new results it can be concluded that using $CoCl_2$ as a reference compound in the photoacoustic experiment with subpicosecond laser pulses gives a large amount of prompt heat release resulting from multiphoton absorption processes. This results in an overestimated energy content of the K-intermediate of bR and its mutants. Using different reference compounds, the corrected values are 40 ± 10 kJ/mol, which is in agreement with previously reported values. The apparent quantum yield and energy content values (for each of the isomeric compositions) of the mutants D212N, D85N, R82Q, A53G, W182F, V49A, deionized blue bR, and acid purple bR are also recalculated using bR as a reference compound and are similar to those of bR. (Φ is in the range $0.55-0.65$, and E_K is in the range $40-50$ kJ/mol.) The relative insensitivity of the apparent average quantum yield value is discussed.

Introduction

Bacteriorhodopsin (bR) is a light-transducing, retinal-containing, protein present in the purple membrane of *Halobacterium salinarium*. Its structure and the mechanism of its function are discussed in numerous reviews.[1,2] The free energy retained after absorbance of a photon by the retinal chromophore drives the system through the following photochemical cycle and translates a proton against the electrochemical gradient:

$$bR_{570} \rightarrow (bR)^* \rightarrow I \xrightarrow{500 \text{ fs}} J \xrightarrow{3 \text{ ps}} K \xrightarrow{1.5 \text{ } \mu s}$$
$$L \rightarrow M \rightarrow N \rightarrow O \rightarrow bR$$

The ground state of light-adapted bR_{570} containing *all-trans*-retinal absorbs at 568 nm. The first intermediate K, which is the 13-*cis* isomer of retinal, absorbs at 595 nm and strongly overlaps with bR_{570}.[3]

The efficiency of *all-trans* to 13-*cis* photoisomerization of retinal has been the subject of many studies. Different research groups have measured the quantum yield of the retinal photoisomerization in bR and have obtained values that vary from 0.25 to 0.79,[4,5] depending on the experimental conditions used. Recently, a few independent studies have agreed on a value of 0.65 for the quantum yield, which seems to be quite reliable.[5−11] The energy stored in the K-intermediate is also an important parameter of the light-storage efficiency. Very different experimental results have also been obtained for the energy content of the K-intermediate determined by different techniques.[6−10] In addition, measurements of the volume changes for the initial step of isomerization and K → L transition varied considerably when measured by different experimental techniques.

Using low-temperature photocalorimetry measurements, the energy content of K was found by Birge and Cooper[7a] to be about 67 ± 13 kJ/mol, assuming the quantum yield of photoisomerization to be 0.33. Later this value was corrected by the

same authors taking into account the more accurate number for the quantum yield of 0.65 and was found to be equal to 49 ± 17 kJ/mol.[7b] Room-temperature photoacoustic measurements, however, with both nanosecond and femtosecond laser excitation indicate that this energy is much higher, ~ 190 kJ/mol.[6,9]

For photoacoustic measurements a value for the change in volume is required, because this process also contributes to the photoacoustic signal. Fortunately, thermal properties of water allow for determination of the volume change contribution. On the basis of a temperature dependence study,[9] the value of volume contraction in the K formation step was found to be 18 A^3/mol. Later, the same group measured the volume expansion of 100 A^3/mol in the K → L transition following retinal isomerization by using the beam-deflection technique.[10] By using the same method as in ref 9, but with more moderate excitation conditions, another group[11] reached the conclusion that the energy storage in the K-intermediate at room temperature is actually about 36 ± 8 kJ/mol, which is close to that for bR at low temperature.[7]

Conclusions made regarding volume changes found previously[11] are opposite to those reported in the work of Schulenberg *et al.*,[9] where they found an initial volume expansion of 2.5 A^3/mol with a subsequent volume contraction of similar magnitude in the K → L step. More important is the fact that neglegible change in volume is found[11] in monomerized bR. This indicates that the changes in volume may be contributed by membrane and/or the conditions of photoexcitation. The value of -18 A^3/mol found in ref 9 may also be overestimated due to assuming a small quantum yield value of 0.09 instead of 0.65. The volume changes contribute no more than $10-12\%$ to the overall photoacoustic signal.[9,11] Thus, it cannot be responsible for the observed low prompt heat value found in the femtosecond experiment.[6,13−15]

Schulenberg and co-workers[6,9] used both 10 ns and 400 fs laser pulses in their study, focused to a $0.2-1.0$ mm spot on the sample, and measured the amplitude of the photoacoustic signal. The time resolution of the experiment was then

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determined by the diameter of the laser spot ($\tau = D/\nu$, where D is the diameter of the laser spot and ν is the speed of sound in water). In addition, $CoCl_2$, which has a low exctinction coefficient at the wavelength of excitation (532 or 595 nm), was used as the calorimetric reference. Because of this low extinction coefficient, high concentrations of the reference compound were required in order to match the absorption of the bR sample (0.2 at the excitation wavelength). The high light intensity used in the case of the 10 ns laser excitation (more than 10^{16} photons/cm^2) can introduce uncertainty in the quantum yield value as a result of multiphoton absorption and the photoequilibrium between the ground state of bR$_{570}$ and the K-intermediate. The correction factor, however, is not easy to measure experimentally. In the case of the short (500 fs) laser pulses the photoequilibrium problem is eliminated, but the possibility of multiphoton excitation becomes more serious.

In our previous work[13] we were interested in determining how the photoisomerization quantum yield and the energy content of the K-intermediate are affected by mutation of bR and whether or not these experimental values are correlated with the large observed changes of the photoisomerization lifetime.[12-15] In rhodopsin, a correlation was found between the photoisomerization rate and its quantum yield (decrease of the quantum yield with increase of the photoisomerization process lifetime).[16] This correlation supports the assumption that the retinal photoisomerization is impulsive, and the coherence of the excitation wavepacket is carried to the photoproducts.

The experimental approach we used previously[13-15] to determine these values was a combination of subpicosecond transient absorption and the photoacoustic technique. Transient absorption was used to determine the quantum yield of photoisomerization, and the photoacoustic signal was used to verify this number and estimate the energy content in the K-intermediate. Our experimental approach for the photoacoustic measurements was analogous to that described by Rohr *et. al.*[6] Our data for the energy content of the K-intermediate were found to be in the range of 190 kJ/mol using $CoCl_2$ as a reference compound. This value is much higher value than that obtained by using the calorimetric method[7] or the method of Zhang *et. al.*,[11] where in addition to different experimental conditions a few different reference compounds were employed (other than $CoCl_2$).

In order to understand the source of the large discrepancy in the value for energy content of K, we reexamined our previous measurements. To do this, we studied the effect of the laser light intensity and used different references in the photoacoustic experiments. From our new results we find that the use of $CoCl_2$ in our previous work leads to multiphoton absorption processes. The use of different reference compounds gives values for the energy stored that are comparable to those previously measured.[7,11] Using bR as a reference compound for the other bR variants gives values for the quantum yield that are similar to those we previously determined.[13] The apparent constancy of the quantum yield values is discussed in more detail.

Experimental Section

Bacteriorhodopsin containing cells were grown from the master slants of *Halobacterium salinarium* ET1-001 strain, kindly provided by Professor Bogomolni at UC Santa Cruz. The purple membrane was isolated and purified as described previously in ref 19. Potassium phosphate buffer solutions were used to adjust the pH of bR and reference dyes. The final potassium phosphate concentration was about 10 mM (pH = 6.5) and did not affect the acoustic properties of water. The blue (deionized) bbR was prepared by running wild type bR through

a cation-exchange (hydrogen form) column. All samples were light adapted for at least 30 min. The $CoCl_2$, Evans Blue, Malachite Green, and Congo Red were obtained from Aldrich and used as received. All solutions were made fresh before measurements. Sample degradation was checked by monitoring the absorption spectrum of each solution before and after measurements and was found to be negligible. All dye solutions (except $CoCl_2$) used for bR measurements were prepared in phosphate buffer. For measurements of bbR deionized water was used as a solvent for all reference compounds.

The laser photoacoustic setup used was analogous to that described previously.[13] The sample cuvette was attached to the surface of the transient transducer (Panametrics, V101-RM or V104-RM). The surface of the transducer was parallel to the direction of beam propagation. In order to improve the signal-to-noise ratio, all photoacoustic signals were amplified and stored in a transient digitizer (LeCroy 9450). Solutions of $CoCl_2$, Congo Red, Malachite Green, Evans Blue, and Amigo Black were used as reference compounds. In this study we applied the technique similar to ref 11. The laser beam used was unfocused and had the diameter of 1 cm. A MOPO (optical parametric oscillator, Spectra Physics) was used as a laser source and provided a good quality beam that assured the uniform distribution of excitation on the sample. The signal was then processed on a PC computer in order to deconvolute fast and slow components of the heat release. The suspension of the sample was stirred after every 100 shots. For the variable temperature measurements the Peltier elements, with an external temperature-stabilized water cooling system, were used. The temperature fluctuation around the target temperature during experiments was 1 deg. The optical densities of all samples were adjusted to 2.0 at the excitation wavelength. The laser system used for short-pulse experiments was described previously.[12] This laser system consisted of a commercial Coherent Satori dye laser pumped by an Antares mode-locked YAG laser. Pulses of 250 fs were generated with a repetition rate of 80 MHz, at wavelengths between 595 and 605 nm. The output of the dye laser was amplified by a regenerative amplifier (Quantel, RGA 60), in a dye amplifier (Quantel, PTA 60), at 10 Hz. An amplified pulse with an energy of 1 mJ, and 400 fs pulse duration, was obtained.

Results

If the energy gap between K and L is estimated to be small, which is reasonable for wild type bR,[13] the energy balance equation can be written as

$$1 - \alpha \sim \Phi E_K / E_{exc} \tag{1}$$

where E_{exc} is the energy absorbed by the sample, Φ is the quantum yield of photoisomerization, E_K is the energy of the K-intermediate, and α is the ratio of the amount of the prompt heat released by the sample after photoexcitation to that of reference compound.

As explained earlier, the α value can be determined as a ratio of the amplitudes of the acoustic signals for the sample and reference compound (as in refs 6 and 9) or alternatively using a deconvolution procedure from the sample and the reference (as in ref 11).

Although different methods are used, the α value obtained in refs 6 and 9 is not significantly different from that obtained in ref 11 (0.8 vs 0.9). The value of the photoisomerization quantum yield, however, is much lower in refs 6 and 9 than that used in ref 11 (0.65 vs 0.1), due to the correction for the photoequilibrium between bR$_{570}$ and K$_{595}$ in the nanosecond

K-Intermediate of Bacteriorhodopsin

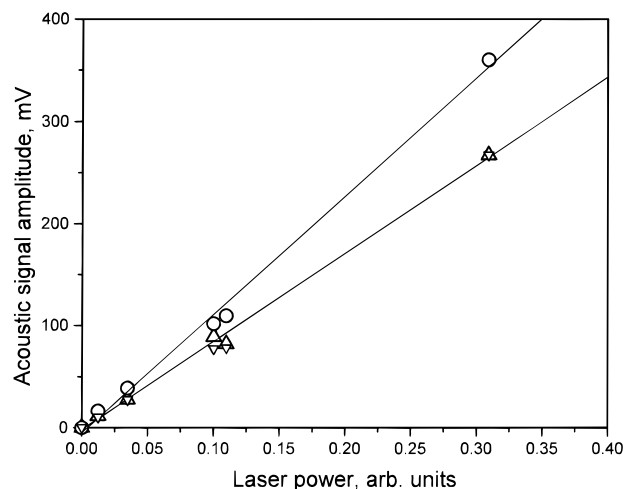*J. Phys. Chem. B, Vol. 101, No. 33, 1997* **6631**

Figure 1. Amplitude of the acoustic signal from different reference compounds as function of laser power with 530 nm, 10 ns excitation source in the low power laser intensity region. The optical density of all samples is adjusted to 2 at 530 nm by changing the sample concentration. The laser spot diameter was equal to 1 cm, and the maximum laser energy used was 1 mJ (0.3 mJ/cm^2). Open circles measurements are for CoCl$_2$, open up-triangles for Evans Blue, and open down-triangles for Malachite Green. The fits are linear based on least-squares method, which give a ratio for the slopes for CoCl$_2$ to either Evans Blue or Malachite Green to be 1.15 ± 0.05.

Figure 2. Dependence of the amplitude of the photoacoustic signal from CoCl$_2$ (solid circles) and Evans Blue (open circles) on laser power in the high laser intensity region. Optical density of each sample was adjusted to be 2.0 at 530 nm. Inset shows the same measurements, at the lower energy region. The fit for Evans blue (open circles) is linear and for CoCl$_2$ (closed circles) is a parabolic dependence. (The energy used in ref 11 is typically 0.3 mJ/cm^2 and in ref 9 20 mJ/cm^2.)

experiment.[6,9] When the 400 fs pulse is used,[6] the equilibration between the bR and K-intermediate does not occur (the quantum yield should be around 0.65), but the α value measured in ref 6 and in refs 13−15 is close to 0.5, which is much less than that measured by Zhang *et al.*[11]

The photoacoustic signal of bR at room temperature contains a contribution from the volume changes in the protein and must be considered in order to obtain the real amount of the prompt heat released during the photoisomerization process. Carrying out the experiment at different temperatures assists in evaluating the amount released due to the fact that at 4 °C the thermal contribution to the acoustic signal in aqueous solution will be *zero* (this is the "magic temperature").

The use of the short laser pulses (400 fs) as excitation source may result in multiphoton excitation of the bR, but the excited state of bR does not absorb significantly at 595 nm (excitation wavelength for 400 fs laser pulse).[17,18] In addition, if the excited state of bR absorbs a second photon, bR should produce a larger amount of prompt heat than the reference (α > 1). Experimentally, however, α is ~0.5, so multiphoton absorption by retinal cannot be the source of the error.

There can be at least two others sources contributing to the observed difference between our value[13−15] of energy stored and those of Zhang et al.[11] First, the concentration of CoCl$_2$ used in our measurements was high in comparison with that of bR. This makes the thermal properties of water at room temperature different for the two samples, which would change the amplitude of the acoustic signal. Second, multiphoton excitation of CoCl$_2$ could be another source of the error when the subpicosecond excitation source is used. Different reference compounds and different levels of excitation are used in order to evaluate the relative importance of these two sources of error.

Figure 1 shows the photoacoustic signal from different reference compounds as a function of laser power at low excitation level. In this case the maximum laser power used is about ~0.3 mJ/ cm^2. Due to the high ionic concentration, the CoCl$_2$ solution has a slightly higher acoustic signal than that of Malachite Green, Evans Blue, and the other dyes. The difference between these dyes, however, is less than 10−15%
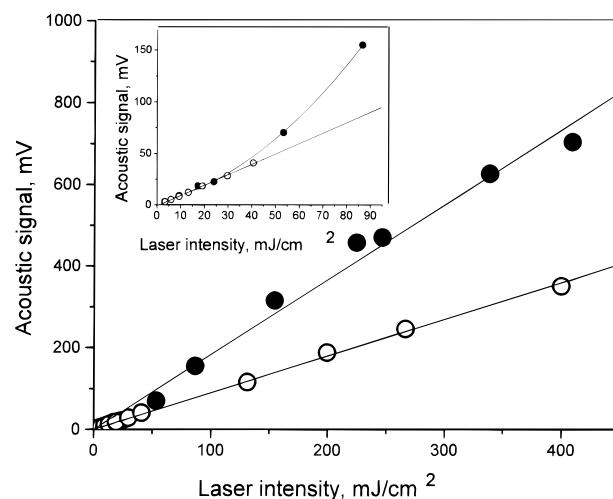
at room temperature. This, therefore, cannot account for the large difference between the value of prompt heat release and the energy content determined in our previous measurements,[13−15] and those in refs 6 and 9.

Figure 2 shows the acoustic signal dependence from CoCl$_2$ and Evans Blue solutions at higher laser power of the 10 ns laser. The power density was increased by both increasing the laser power and focusing the beam into a smaller than 1 cm spot on the sample. As one can see, there is a significant deviation from linear dependence. At moderate laser power (~90 mJ/ cm^2, inset to Figure 2) there is a quadratic power dependence, which suggests a two-photon absorption process. At higher laser powers (~500 mJ/cm^2) the dependence changes from quadratic to a lower value, suggesting a saturation effect. The slope for the CoCl$_2$ sample is different from the other reference compounds by a factor of ~2. The other reference compounds do not exhibit any deviation from linear dependence. A saturation effect could only be observed only at very large laser intensities. This suggests that our previous values for the fast heat release were underestimated, mainly due to the nonlinear absorption in the CoCl$_2$ reference compound. The effect of using a high concentration of CoCl$_2$ gives an error of about 10−15%, but its contribution to the overall error is less than that from nonlinear excitation, in particular, when subpicosecond laser pulses were used.

We repeated our previously reported[13−15] measurements for bR and bbR using Evans Blue, Malachite Green, Condo Red, and Amigo Black as reference compounds. As in the case of the 10 ns excitation, the α value was found to be 0.98 ± 0.05. Variable temperature experiments with bR showed that volume changes give a positive contribution (~10%) to the prompt heat at room temperature. This is similar to the results found in ref 11. We also obtained similar results for bbR. This indicates that 1 − α = 0.11 ± 0.05, and with Φ = 0.65 ± 0.05, E_K for bR is found to be 42 ± 15 kJ/mol, which agrees with the data in ref 11.

In order to show that the excitation energy in this experiment was too low to excite the K$_{595}$ intermediate, we varied the excitation wavelength of bR from 530 to 630 nm. We did not find any wavelength effect on α, within the accuracy of our measurements.

Similar results for the α values are obtained with 500 fs laser excitation and under tight focusing conditions (setup similar to that used in refs 13−15) but using different reference compounds. In the case of 500 fs laser pulses and energies of up to 50 μJ/pulse (∼5 mJ/cm²), the photon flux becomes at least 1 order of magnitude higher than that for the highest laser intensity used with the 10 ns laser pulse in present study. When Evans Blue or Malachite Green is used as a reference compound, the α value is found to be 0.95 ± 0.1 (without volume contribution correction) while for CoCl₂ a lower value of 0.5 ± 0.05 is found. The observed difference in the α value for these reference compounds accounts for the difference in the energy content of the K-intermediate determined by us previously[13−15] and those by Zhang *et al.*[11] We thus conclude that the main source of error in our previous measurements was the nonlinear excitation of CoCl₂. Due to the high laser intensity, the relatively long excited state lifetime of CoCl₂ (and probably high excited state absorption cross section) an additional absorption from the excited state becomes highly probable.

In summary, three sources of uncertainty in determining the proper value for the quantum yield and energy content E_K can be concluded. The first source of error is the high concentration of the reference compound which may affect the thermal properties of water. The contribution of this effect is shown to be less than 10−15% when using CoCl₂. The second source of error is the uncertainty in the volume changes of the sample. This factor is found to vary from study to study, but never exceeds 12%[7,11,14] of the amplitude of the photoacoustic signal at room temperature. These two factors combined together, however, cannot explain the differences between the prompt heat obtained in the nanosecond and femtosecond experiments. We conclude, therefore, that the third factor, which is the excited state absorption of CoCl₂, must be the largest source of error in the previous determination of the underestimated α value (by ∼50%).

It is necessary to evaluate how these changes affect the quantum yield data for the different mutants. After repeating the experiments for bbR with the new photoacoustic setup, within the error of our measurements, we find that the photoacoustic signal from bbR is identical to that of bR, with matched absorption at the excitation wavelength. from these measurements the α value at room temperature is found to be 0.95 ± 0.05, and the contribution of volume changes is also close to that of bR, 10% of that at room temperature. This means that $1 - \alpha \sim 0.14 \pm 0.05$.

In order to correct the parameters for the mutants from the values reported previously,[13−15] bR was used as a reference compound instead of CoCl₂. This eliminates the sources of error due to differences in concentration and nonlinear absorption of the substances (all bR variants have similar absorption coefficients). The new data obtained with bR as a reference compound for the different mutants are listed in Table 1. It is assumed that the contribution of the volume changes to the observed signal is the same.

Because eq 1 relates two unknown variables (the quantum yield Φ and the energy content, E_K), there is some uncertainty in the determination of these two parameters from eq 1. The quantum yield of the mutant, however, can be found from

$$\Phi_m = \Phi_{bR}(1 - \alpha_m)/(1 - \alpha_{bR})E_{KbR}/E_{Km} \qquad (2)$$

where Φ_m and Φ_{bR} are the photoisomerization quantum yields of the mutant and bR, respectively; α_m and α_{bR} are the corresponding amounts of the prompt heat; E_{KbR} and E_{Km} are

TABLE 1: Prompt Heat α, Quantum Yield of Photoisomerization Φ, and Energy Content in the K-Intermediate E_K for bR and Its Mutants

	α	Φ	E_K (kJ/mol)	τ_{ave}[a] (ps)
bR	1.0 ± 0.1	0.65 ± 0.1	41 ± 15	0.5
D212N	0.80 ± 0.1	0.6 ± 0.15	50 ± 30	3.0
R82Q	0.86 ± 0.1	0.6 ± 0.10	50 ± 25	3.2
D85N	0.80 ± 0.1	0.6 ± 0.2	50 ± 30	5.3
bbR	1.05 ± 0.1	0.6 ± 0.2	40 ± 30	9.5
V49A	1.1 ± 0.1	0.6 ± 0.1	50 ± 20	1.0
A53G	1.0 ± 0.1	0.55 ± 0.15	50 ± 25	0.45
W182F	0.9 ± 0.1	0.6 ± 0.15	50 ± 25	0.8

[a] Taken from ref 14.

the corresponding energy contents of the K-intermediate for bR and mutant, respectively.

From our spectroscopic studies we can obtain the quantum yield values by deconvoluting the absorption spectrum to the difference of the absorption of the ground state and the K-intermediate, when the K-intermediate spectrum is known from the previously published global analysis treatment.[20−23] This kind of analysis was shown to give value for bR[24] similar to that obtained by independent techniques.[4−10] On the basis of this analysis,[20−23] we can determine the quantum yield values for D212N, D85N, R82Q, and V49A, which are shown in Table 1. These numbers are very similar to those of bR and extend from 0.55 to 0.7. This means that, according to eq 2, the energy content in the K-intermediate of studied mutants is also very close to that of bR (Table 1).

The largest uncertainty in our measurements is found for deionized blue bR, A53G, W182F, and acid purple bR, since the spectrum of the K-intermediate is not known for these samples. In the case of A53G, and W182F, we estimate the quantum yield values to be 0.55 ± 0.15 and 0.65 ± 0.15, respectively. In the case of deionized blue bR we can assume that the spectrum of the K-intermediate is similar to that of D85N. This gives a quantum yield value for deionized blue bR equal to 0.6 ± 0.2. The low transient signal for the K-type intermediate of D85N, R82Q, and deionized blue bR leads to the large error bars for the value of quantum yield of the photoisomerization for these compounds.

It is difficult to test for correlation between the photoisomerization yield (and the K-intermediate energy content) of bR with its variants studied in this work. This is because while the retinal in bR is in the *all-trans* configuration, retinal in the variants is a mixture of *all-trans* and *13-cis* configurations.[25] In these variants, the rate of dark adaptation is so fast we cannot light adapt these samples to obtain only the *all-trans* retinal isomers as we do in bR. Barring sample heterogenity, this may be the reason for the observed two components in the excited state decay of all bR variants. If so, then the correlation we observed between the values of amplitudes of the excited state decay and the isomeric composition (determined by chromatographic methods[25]) for the different mutants suggests[13−15] that the fast experimentally determined components in each case are due to the decay of the *all-trans* isomer, and the slow component is due to the *13-cis* isomer. Using the relative amplitude of each isomer and the corresponding lifetime, an average lifetime is calculated for each of the bR variants. The results are listed in Table 1. These values can be compared with the apparent quantum yield, which is also an average quantity. It is clear that there is no correlation between the average quantum yield of photoisomerization and the corresponding average lifetimes. The ratio of the rate of photoisomerization of bR to that of bbR, for example, is equal to ∼20, while the corresponding ratio of the quantum yields of photoisomerization is near unity.

It is clear from Table 1 that the photoisomerization rate is sensitive to the change in the protein environment of the retinal. The apparent quantum yield seems to be insensitive to environmental changes. This apparent constancy, however, can be due to the cancellation effect of the parameters that determine the observed quantum yield. Determination of the quantum yield for the mixture of two isomers can be expressed as

$$\Phi_{total} = \Phi_1/(1 + N_2\sigma_2(l)/N_1\sigma_1(\lambda)) + \Phi_2/(1 + N_1\sigma_1(\lambda)/N_2\sigma_2(\lambda)) \quad (3)$$

where Φ_i, N_i, and $\sigma_i(\lambda)$ are the yield, the concentration, and the absorption cross section (at the wavelength of the excitation λ) of isomer i. The interesting limits are clear from eq 3. If $N_1\sigma_1(\lambda) = N_2\sigma_2(\lambda)$, $\Phi_{total} = (\Phi_1 + \Phi_2)/2$ is an average of the two isomers. If $\Phi_1 = \Phi_2$, then $\Phi_{total} = \Phi_1 = \Phi_2$. To observe a constant Φ_{total}, it is possible that Φ_1 and Φ_2, N_1 and N_2, and $\sigma_1(\lambda)$ and $\sigma_2(\lambda)$ can change for the different variants as to make the observed Φ_{total} insensitive to changes in the protein environment. Since it is unlikely that cancellation occurs at different excitation wavelength, experiments are now underway in which the wavelength dependence of Φ_{total} will be examined for each variant.

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