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On the molecular origin of the protein catalysis of the primary process in bacteriorhodopsin photosynthesis: Retinal photoisomerization

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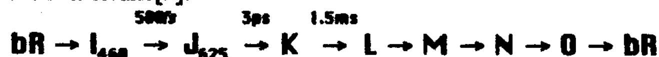
Abstract

Using subpicosecond transient optical absorption spectroscopic techniques, the photoisomerization rates and quantum yields were determined for bacteriorhodopsin, its relevant mutants, its dionized form and at different pH and Cl^- concentrations. It is found that the rate is catalyzed and made highly specific around the C_{13}-C_{14} bond by the presence of negative charges within the retinal cavity (e.g., Asp85 and Asp 212). Any perturbation that genetically removes, acid neutralizes, or changes the geometry of these negative charges is found to decrease the rate of photoisomerization, but does not greatly change its quantum yield. These results are discussed in terms of the changes in the electronic structure of the retinal as well as in the anisotropic charge distribution within the cavity that result from the photoexcitation process. The different potential energy surfaces proposed to explain the dynamics of the photoisomerization process are examined in terms of our observed results.

Introduction

Bacteriorhodopsin (bR) is a light-transducing protein present in the purple membrane of *Halobacterium salinarium*. Its structure and the mechanism of its function are discussed in numberous reviews. [1,2] bR is a potentially important biomaterial for phototonics application in such areas as holographic recording, ultrafast optical devices, neural networks, and associative memory. The free energy retained after absorption of a photon by the retinal chromophore derives the system through the following photochemical cycle and translocates a proton across the membrane[3]:



The quantum yield of the retinal photoisomerization was measured to be around 0.64 using different methods [4-7] and occurs around C_{13}-C_{14} bond exclusively. This high yield and specificity is not observed in the studies on retinal protonated Schiff bases in solutions. [8]

It was shown that the replacement of some amino acid residues has stong effect on the rate of photoisomerization of retinal in bR.[9] We are thus interested in examining the effect of these purturbations on the photoisomerization quantum yield and the energy stored in the K intermediate during the photocycle.

Electron diffraction shows that the protonated Schiff base is located midway between the C and the G helices.[10] It has been demonstrated that Arg82, Tyr185, Asp85, and Asp212 form a special environment around the Schiff base.[1,2,10] The Schiff base counterion is thought to be Asp85 or Asp212 (or both);[2] both exist in the ionized form in bR 568 [11]. Femtosecond optical transient studies with 6 fs pulses suggested that the retinal in bR relaxes in 200 fs from the Franck-Condon state to the excited state potential minimum (I_{460}) which decays within 500 fs to form J625 [12], a 13-*cis* isomer of *all-trans*-retinal as shown by picosecond time resolved Raman [13]. The studies of the time-resolved spectra of retinal in short and wide spectral range [14-16] show that excited state absorption of retinal with a maximum at 460 nm is formed instantaneously with time-

resolution of 50 fs. Stimulated emission located at 890 nm also has an instantaneous rise-time and decays coincidentally with the excited state absorption at 460 nm. These observations suggest that the 90° torsional dynamics in the excited state could not be detected spectroscopically. Fluorescence up-conversion studies show [17] that excited states decay is multiexponential with lifetimes of 90, 600, 10000 fs. The weight of these components differs depending on the monitoring wavelength. The blue side of the fluorescence band has large contribution while in the red part of the band its contribution is minor. However, the rise time of the fluorescence at the red part of the spectra is instantaneous, which eleiminates the assignment of the fast component to torsional dynamics.

Recent molecular stimulation dynamics of retinal in bR performed by Schulten et al. [18] suggest that there is a extremely strong coupling between S_1 and S_2 states of retinal. Within its model the initial excitation into S_1 state crosses within 100 fs to the S_2 state and then crosses to S_0 state at 90 torsional twist.

In this paper, we summarize our experimental work [9,19,20,21] on the effect of different perturbaions on the lifetime of the excited state transient absorption at 460 nm and the quantum yield of formation of the K_{590} intermediate as well as on the energy stored in this intermediate. The perturbations applied are mutagenic replacement of individual charged, H-bonded and geometry controlling amino acid residues, changing the hydrogen ion and chloride ion concentrations. These perturbations have the effect of changing the charge distribution around the retinal system. The general conclusion is that the lifetime of the excited state, which reflects the photoisomerization time, is much more sensitive to these perturbations while the photoisomerization yield of this process is not very sensitive to the retinal environment in bR. Furthermore, it is found that the negative charges within the retinal cavity at the D_{85} (and D_{212})positions seem to catalyze the rate of the photoisomerization process and make it highly specific around C_{13} - C_{14} without changing its yield. These results are discussed in terms of the valence bond description and the different potential energy surfaces proposed for the dynamics of the photoisomerization process.

Materials and Methods

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 [22]. Potassium phosphate buffer solutions were used to adjust the pH. For low pH solution the HCl or H_2SO_4 were used. The final potassium phosphate concentration was about 10-30 mM, and should not affect the acoustic properties of water. In the case of low pH solution the ionic strength of the reference compound was adjusted by adding proper amount of NaCl. The blue (deionized) bR was prepared by filtration of wild type bR through the cation exchange (hydrogen form) column. All samples were light adapted.

The laser system is similar to that described previously [19]. Briefly, the laser system consists of a ommercial Coherent Satori dye laser pumped by an Antares mode-locked YAG laser. As a result, 250 fs pulses with a repetition rate of 76 MHz at wavelength between 595 and 605 nm were generated. 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. Amplified pulses with an energy of about 1 mJ and 400 fs pulse duration were obtained.

Transient absorbance

The optical density of the samples used in the transient absorbance measurements was about 1.0 - 1.5 at the excitation wavelength. The detailed set-up for transient absorption measurement was described previously in details [19]. Typical values of the cross- correlation width were 600 fs at 500 nm, 500 fs at 600 nm, 550 fs at 700 nm. The kinetics were analyzed by the least squares method.

Measurements of quantum yield of the K intermediate

In principle, the transient absorption experiment should enable one to determine the quantum yield of photoisomerization to the K intermediate if its absorption is separated from the bleached parent absorption and if one knows the molar extinction of each. Unfortunately, this is not the situation, the two spectra strongly overlap for the bR variants studied here. Several values around the knowm bR quantum yield value were found to deconvolute the spectra reasonably well. In case we have more than one isomer, we can only determine an apparent (average) value for the isomerization yield by this method if one assumes similar absorption of their K intermediate.

Results and Discussion

1. Rate and Specificity of the Photoisomerization Process:

a) Replacement of charged aminoacid residues within Retinal cavity

The transient spectra for the wild type *bR* obtained in our experiment are similar to those measured

previously [16, 24]. The important spectral features are a) the excited state of the retinal has an absorption maximum around 470 nm and decay time of 500 fs, b) the ground state bleaching with a maximum at 570 nm and partial recovery time of 500 fs, c) the first intermediate J with a maximum at 650 nm, formed from the excited state with the time constant of 500 fs, and d) the second ground state intermediate (K) with a maximum at 630 nm is formed from J with a time constant of 3 ps.

The lifetime of the excited state is found to be very sensitive to the charged groups located within the retinal cavity. In the mutants where charged aminoacid residues were replaced by neutral ones D85N, D212N and R82Q [22], and deionized blue bR [13] the lifetime of the excited state becomes considerably longer . The picosecond temporal behavior of the transient absorption spectrum of the D212N mutant is shown in fig. 1.

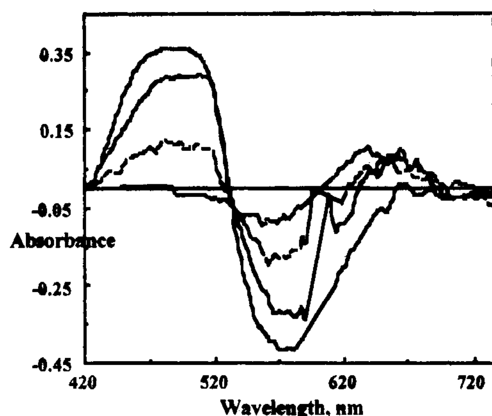


Figure 1. Transient absorption spectra of D212N mutant at pH = 4.5 measured at different delays after the excitation pulse on the picosecond time scale.

The transient absorption spectrum obtained at zero time has positive absorbance from the excited state with a maximum at 470 nm, and negative absorbance originating from the ground state bleaching process with maximum at 570 nm (Fig. 1). The decay of the excited state at 470 nm could be fitted to a double exponential decays with lifetimes of 1.8 and 6.5 ps probably resulting from the two isomers present in the ground state of the D_{212N} mutant at pH 4.8. The amplitude ratio of the two components was found to be equal to 13-*cis* to *all-trans* isomeric ratio, measured previously [25] by chromotrographic method for D_{212N} ground state. The rise of the positive absorbance with a maximum around 660 nm is coincident with the decay of the excited state. This process is attributed to the formation of the J-intermediate. At longer times this absorbance shifts to the blue with the formation of the positive absorbance having a maximum around 630 nm. The last intermediate has spectroscopic properties similar to the K-intermediate of native *bR.* A summary of the dynamics data for bR and its mutants are summarized in Table 1.

Table 1
The estimated average quantum yield and the measured average lifetime of photoisomerization and the rise time of the K intermediate for wild type bR, mutants with charged aminoacid residues replaced by neutral ones (D85N, R82Q, D212N), deionized blue bR and mutantsA53G, V49A, and W182F.

Sample			
	Φ_{iso}	τ_{iso}, ps	τ_K, ps
wild type bR	0.58	0.5	3.0
D212N, pH=4.4	**0.65**	**3.0**	**5.0**
D85N, pH=4.5	**0.7**	**5.3**	**4.0**
Blue bR	**0.65**	**9.5**	**5.0**
R82Q, pH=4.5	**0.6**	**3.2**	**5.0**
V49A	0.5	1.0	4.0
A53G	0.63	0.5	3.0
W182F	0.6	0.8	5.0

τ_{iSO} *for multiexponential processes was calculated as* $\tau_{aver} = \Sigma A_i \tau_i$, *where* A_i *is relative amplitude of the component with lifetime* τ_i

The average lifetime of the excited state is very sensitive to the bR perturbation if negatively charged residues are replaced by neutral ones (D_{85N}, D_{212N}) or if they are neutralized (blue bR). At pH 4.5, replacement of the positively charged Arginine 82 by neutral residue (e.g. in R_{82Q}) is known to lead to the neutralization of the negatively charged Asp85.

b) Changing the geometry of retinal binding cavity.

The replacement of amino acid residues V49, W 172. and A53 affects the rate of the Schiff base deprotonation at the L→M transition and the rate of Schiff base protonation at the M→N step in the photocycle [26, 27]. This indicates that the geometrical relationship between the protonated Schiff base (the proton donor) and D85 (the proton acceptor) is changed [26, 27]. The transient absorption spectroscopy kinetic data in Table 1 show that the excited state lifetime of retinal becomes slightly longer for V49A and W172F, but almost the same in A53G [26]. This suggests that the residues that affect the relative distribution of the charged residues (in particular D_{85}) around the retinal by changing its configuration can have indirect effect on the photoisomerization lifetime.

From the above, it seems that the negative charges on D_{85} and D_{212} are important for the rapid retinal photoisomerization. In further support of this conclusion is the fact that the long lifetime observed for D85lq or for acid blue bR is reduced upon the addition of 1M NaCl solution [28]. It is known that the Cl^- ion resides within the retinal cavity from the observed recovery of the proton pump photocycle of bR that was inhibited in D_{85N} or D_{212N} bR mutants [29]. The negative charge of the Cl^- ion seems to compensate for that of the carboxylate groups lost in these bR variants.

2. Changes in the Cavity Charge Distribution and Retinal Electronic Structure Upon Excitation:

The closest distance between the negatively charged oxygen of Asp 85 or Asp 212 and C_{13} of the retinal is ~4.5 A° and ~5A° respectively [10]. In the ground state, the attraction between the negative charge on the oxygen of Asp85 or Asp212 and the positive charge on the nitrogen of the protonated Schiff base stabilizes the valence bond structure in which the C13 - C14 bond is a double bond. The valence bond description of the excited state of the retinal electronic system attached to a protonated Schiff base is a linear combination of structures in which each structure has the positive charge localized on different odd numbered carbon atom along the polyene retinal chain[9]. If the positive charge is on C_{13}, the C_{13}-C_{14} double bond becomes single bond in the excited state and thus reduces the barrier to rotation (and thus to photoisomerization) around this bond. Having the negatively charged oxygen of Asp85 very near C_{13} stabilizes this structure and make photoisomerization around this bond more rapid than any of the other bonds. This could explain the electronic origin of the protein catalysis and specificity of the retinal photoisomerization in bR. Once the C_{13}-C_{14} bond becomes single and the barrier to rotation around this bond is removed, anisotropic forces are needed to rotate the C_{12}=C_{13} and C_{14}=C_{15} double bonds around the C_{13}=C_{14} single bond (in paddle type motion) to transform the all-trans into the 13-Cis configuration.. These anisotropic forces arise from the nonsymmetric charge distribution of the residues around the retinal and is triggered by the large change in the retinal dipole moment resulting from the displacement of the positive charge from the nitrogen to C_{13} within the nonsymmetric cavity charge distribution upon photoexcitation.

3. Quantum Yield Results:

A summary of the quantum yield results on some of the mutants and blue bR is given in the second column of Table 1. The important conclusion is that while the lifetime of the excited state is sensitive to the retinal environmental changes, the quantum yield is not. The same conclusion is reached if the lifetime and quantum yield are measured as a function of the pH . The excited state lifetime is sensitive to the pH while the average isomerization quantum yield does not change greatly. It should be mentioned that these are average quantum yield values. It is known that the isomeric composition changes with pH around pH = 5.5 [25]. However, the large change in the excited state lifetimes occurs at lower pH. At neither pH does the quantum yield change. In the measurement of the quantum yield of photoisomerization in Table 1, it is assumed that both all trans and 13-Cis isomers have similar values of quantum yields. But even if one assumes extreme values for both isomers, the value of the quantum yield of the all trans would change between 0.6 and 0.3. This is not as large a change as the change in the lifetimes which could be from 0.5 ps to 10 ps. Thus the conclusion that the excited state lifetime is much more sensitive to environmental changes than the photoisomerization quantum yield is probably correct.

4. The Dynamics of the Primary Process:

The previously accepted picture of the dynamical was that the rise time of the absorption at 460 nm was believed to be 200 fs and was assigned to the time it takes the retinal excited state to change to the 90° configuration [12] which is the equilibrium configuration of the excited state. The decay to the ground state and photoisomerization was believed to occur from this 90° configuration in 500 fs.

There are, however, reports that show that the rise time of the absorption at 460 nm as well as the stimulated fluorescence actually is very short [14,16], and is almost instantaneous. Below, we shall attempt to describe our results in terms of both of these observation.

A. *Absorption at 460 nm is from retinal excited state at 90° configuration:*
In order to explain our results, we proposed that if this model is correct, the minimum of the excited state surface should be shallow at the 90° configuration and symmetric with respect to the maximum of the barrier to thermal isomerization. With this surface, the excited state lifetime would depend on the energy separation between the excited and ground state surfaces at 90°. Thus as the barrier to thermal isomerization decreases by environmental changes, the excited state lifetime increases. Thus, the removal of the negative charges from near the positively charged nitrogen of the protonated Schiff base, the easier the positive charge is able to delocalize over the retinal structure in the ground state, allowing the C_{13}-C_{14} to be partially single bond and thus decreasing the barrier to thermal isomerization. This would also allow for less localization of the positive charge on C_{13} in the excited state and decrease the specificity as well as the rate of photoisomerization around C_{13}-C_{14}. The lower sensitivity of the quantum yield to environmental changes is attributed to a statistical crossing from the shallow minimum of the excited state surface which is symmetrically disposed with respect to the top of the ground state barrier at 90°. This makes the branching ratio (quantum yield) to remain insensitive to environmental changes.

B. *Absorption at 460 nm is from retinal excited state at 0° Configuration*
If indeed the 460 nm absorption is from the excited state at its Franck Condon 0° configuration (all-trans retinal), one has to assume that retinal remains in this state for 500 fs then somehow changes to 90° and rapidly can either isomerize to 13 Cis or return to the ground state of all trans retinal. The 500 fs is thus the time that the rate limiting process takes for the overall photoisomerization process. The constancy of the quantum yield might be explained as in Case A, as long as the rapid photoisomerization is rapid compared with respect to 500 fs but not so rapid as to be impulsive.

The lifetime of the 460 nm absorption might thus be determined by the time it takes the excited state to twist to 90°. Schulten et al [19] in a recent paper assumes the presence of two excited states. The lifetime might be determined by the time S_1 crosses to S_2 which thus rapidly isomerizes. We could think of another process that might take 500 fs. The absorption process leads to an excited state in the all-trans configuration (the Franck Condon Configuration) in which some of the double bonds become single and visa versa, e.g. C_{13}-C_{14} becomes single as a result of electronic excitation. Anisotropic forces need to be applied in order to rotate around C_{13}-C_{14} in the photoisomerization process. This process takes time and should depend on the anisotropic electrostatic forces between the asymmetric charge distribution of the charged residues within the retinal cavity and the dipole moment induced by the displacement of the positive charge from nitrogen of the protonated Schiff base to C_{13} resulting from the absorption process. Removing the negative charges from the cavity is expected to reduce the electrostatic coupling between the charged cavity and the retinal system. This leads to reduced rate of the tortional motion around C_{13}- C_{14} bond and thus to reduced rate of the process that changes the all-trans configuration (0°) into the 90° configuration. Since this is rate limiting in this model, this leads to reduction in the rate of the overall photoisomerization process.

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