Retinal Isomer Composition in Some Bacteriorhodopsin Mutants under Light and Dark Adaptation Conditions

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Received: February 24, 1995; In Final Form: April 24, 1995[⊗]

The isomeric composition of retinal was measured in a number of bacteriorhodopsin (bR) mutants (D85N, D212N, R82A, Y185F, and D115N) under various conditions, using a rapid retinal extraction technique followed by HPLC analysis. Besides the 13-cis and the all-trans retinal isomers observed in wild type (wt) bR under physiological conditions, the 11-cis and 9-cis retinal isomers were observed in variable but minor amounts in the bR mutants. In addition, the values of the equilibrium constant at two temperatures and the enthalpy change for the all-trans to 13-cis isomerization process in the dark-adapted state of D212N, D85N, deionized blue bR, and wt bR were determined. We find that perturbation of the retinal cavity (pocket) by residue replacement changes the relative thermal stability of the different retinal isomers, allowing for thermal- and/or photoisomerization of the retinal chromophore along C_9-C_{10} and $C_{11}-C_{12}$ bonds to moderately compete with the isomerization around the $C_{13}-C_{14}$ bond. The bR mutants expressed in *Halobacterium salinarium* studied in the present work showed normal 13-cis to all-trans light adaptation, in contrast with abnormal all-trans to 13-cis light adaptation observed for D212E, D212A, and D212N expressed in *Escherichia coli*, suggesting an influence of the purple membrane lattice and/or the lipids on the stability of the different retinal isomers within the protein.

Introduction

Bacteriorhodopsin (bR) is the only protein in the purple membrane of *Halobacterium salinarium*. In the absence of oxygen and under physiological conditions, bR converts solar energy into electrochemical energy be vectorially translocating protons across the membrane. Since its discovery by Oesterhelt and Stoeckenius[1] in 1971 and the demonstration of its role in adenosine-3′-triphosphate (ATP) synthesis by Racker and Stoeckenius,[2] numerous studies have been carried out to unravel its structure and the mechanism of proton transport.[3−7]

The functions of amino acid residues in the protein have been studied by site-directed mutagenesis (SDM), and the important amino acid residues have been identified. Combining SDM results[8−11] with Fourier transform infrared (FTIR)[12−15] spectroscopic studies and electron diffraction results,[16] it was concluded that Arg82, Tyr185, Asp85, and Asp212 form the environment around the Schiff base region. The Schiff base counterion is either Asp85 or Asp212.[17−20] Both are in the ionized (deprotonated) form.[15]

Early work in SDM had been focused on mutants expressed in *Escherichia coli*.[8−11] Since the success of expressing bR in *H. salinarium*,[20,21] more studies have been performed in recent years on mutants expressed in *H. salinarium*. Although the general conclusions obtained from mutants expressed in both *E. coli* and *H. salinarium* were similar regarding several of the important amino acid residues, the detailed results in these two different systems were often different.[20,22−24] Additionally, bR mutants expressed in *H. salinarium* are generally more stable than those expressed in *E. coli*.

The replacement of charged amino acid residues in bR was found to have a strong effect on the photoisomerization (excited-state)[25] and thermal (ground-state) isomerization dynamics.[26−28] A valence-bond model was proposed to explain the effect of amino acid residue replacement on the observed excited-state dynamics. The isomer compositions of the bR mutants used for the excited-state lifetime measurements are yet to be determined.

It was reported that light adaptation in D212E expressed in *E. coli*[29] produced a large amount of retinal isomers besides the 13-cis and the all-trans isomers. Furthermore, abnormal light adaptation was observed in D212E, D212A, and D212N mutants expressed in *E. coli*, in which the absorption maximum was found to blue-shift rather than red-shift upon light adaptation. This blue-shift in the absorption maximum was taken as an indication of retinal conversion from the all-trans isomer to the 13-cis isomer by light. However, the absorption maximum of D212N expressed in *H. salinarium* was found to red-shift upon light adaptation.[20] This red-shift was taken as an indication of normal (13-cis to all-trans) light adaptation process. (In this paper, we consider the all-trans to the 13-cis retinal conversion upon light adaptation as abnormal light adaptation and the 13-cis to all-trans retinal conversion as normal light adaptation.)

In the present paper, we report on the isomeric composition of the retinal chromophore in a number of bR mutants expressed in *H. salinarium* at various pH and two different temperatures. Our results show that 13-cis to all-trans retinal conversion takes place in bR mutants expressed in *H. salinarium* upon light adaptation, in agreement with previous optical absorption studies in which the absorption spectrum was red-shifted upon the light adaptation process.[20] A minor but significant amount of the retinal is observed in other isomeric forms that are not seen in the wild type. Their relative amounts (relative stability) and the enthalpy change of the all-trans to 13-cis isomerization process are sensitive to the residue being replaced, pH, and the temperature.

[⊗] Abstract published in *Advance ACS Abstracts*, June 1, 1995.

0022-3654/95/2099-10052$09.00/0

Experimental Section

bR mutants were obtained according to the procedures reported before.[20,21] The purified mutant samples were suspended in doubly deionized water. The pH of the sample was adjusted by the use of potassium phosphate buffer solutions. The final phosphate buffer concentration was about 100 mM.

The procedure of retinal extraction and HPLC analysis was similar to the one reported elsewhere.[30] The HPLC system was a Waters 625 LC system combined with a Waters 486 tunable absorbance detector. Only one silicon (Rainin) normal phase column (4.6 mm × 250 mm) was used instead of two columns used in ref 18. (Note: Millipore silicon normal phase columns converted all-trans retinal into 13-cis retinal, making it difficult to study the content of retinal isomers.) The solvent was 5% ethyl acetate and 95% HPLC grade hexane (Fisher). Both solvents were filtered before use. The peak for each isomer was integrated, and the area was used for the retinal isomer composition analysis. The detection wavelength was 365 nm, and retinal isomer (13-cis, 9-cis, and all-trans) standards were used for calibration to take care of the differences in their absorption coefficients. The absorption coefficient of the 11-cis retinal was obtained from a previous publication.[31]

Dark adaptation was performed at room temperature for at least 24 h to ensure completion of thermal equilibrium. Light adaptation was performed by using a projector light with a cutoff filter (to pass $\lambda > 520$ nm light) for 10 min. The light- or dark-adapted bR mutant sample was first reacted with precooled ethanol (0 °C) for 2 min, and the retinal isomers were extracted with precooled hexane (0 °C) for 2 min. About 30−40% of retinal was extracted from bR under these conditions. The mixture was centrifuged for 1 min, and the hexane phase was removed carefully for immediate HPLC analysis. The isomeric composition from different extractions typically varied a few percent.

In the temperature dependence studies, the samples used for the 35 and 20 °C were prepared under identical conditions. They were then dark-adapted either in a 35 ± 0.3 °C temperature-regulated water bath or in a dark drawer at room temperature (20 ± 1 °C) for 16−24 h. The procedure of HPLC is the same as described above.

Results and Discussion

The results of retinal isomer composition of a number of bR mutants are summarized in Table 1. The amount of the 13-cis retinal in most mutants expressed in *H. salinarium* studied here was decreased, and the amount of the all-trans retinal was increased under light adaptation compared to the dark-adapted state. Only in the blue forms, such as D85N at pH 4.2 and R82A at pH 4.2, where D85 is mostly protonated,[32] was the amount of the all-trans retinal decreased slightly. In these cases, some of the 13-cis and the all-trans retinal chromophores were converted into the 11-cis and the 9-cis retinal chromophores.

Under dark adaptation condition, D212N has the most abundant 11-cis and 9-cis retinal chromophores. This suggests that the replacement of Asp212 by Asn has the strongest effect of the thermal stability of different retinal chromophores.

The retinal composition of the D212N mutant in this study was very different from that reported for D212E expressed in *E. coli*.[29] In D212N expressed in *H. salinarium*, about 25% retinal was in the all-trans form in the dark-adapted state, while in D212E expressed in *E. coli* about 90% of the retinal was this isomer. Although the retinal isomer composition for D212N expressed in *E. coli* was not reported,[29] the authors did observe a blue-shift in the absorption maximum for D212N upon light adaptation, even greater than that observed for D212E. It was concluded that in D212E, D212N, and D212A expressed in *E. coli* abnormal light adaptation takes place. Our HPLC results show that normal light adaptation (conversion from the 13-cis retinal isomer to the all-trans retinal isomer upon light adaptation) takes place in D212N expressed in *H. salinarium*, consistent with a recent report on the red-shift of its absorption maximum upon light adaptation.[20] On the basis of these observations, we conclude that the origin of the abnormal light adaptation is not due to the replacement of Asp212, but due to having expressed the protein in *E. coli*. These proteins have been examined in liposomes rather than in the crystalline array of the purple membrane. Thus, immobilization in the purple membrane lattice or the lipids also plays an important role in the stability of different retinal isomers in bR.

TABLE 1: Retinal Isomeric Composition of bR Mutants under Different Conditions

sample	pH	13-cis (%)	11-cis (%)	9-cis (%)	all-trans (%)
			A.Dark-Adapted		
wt bR	6.2	64	0	0	36
D85N	4.2	33	0	4	63
D85N	6.0	35	0	2	63
D212N	4.2	60	8	3	29
D212N	9.6	63	10	2	25
R82A	4.2	50	4	1	45
Y185F	9.6	77	3	1	19
Y185F	4.2	71	3	1	25
D115N	4.2	62	0	0	38
D115N	9.6	54	2	4	40
			B: Light-Adapted		
wt bR	6.2	6	0	0	94
D85N	4.2	31	4	9	56
D212N	4.2	34	12	7	47
D212N	9.6	31	18	11	40
R82A	4.2	50	6	3	41
Y185F	9.6	39	10	4	47
Y185F	4.2	29	6	4	61
D115N	4.2	13	1	7	79
D115N	9.6	8	2	5	85

In wt bR the spectral shift in λ_{max} near neutral pH is a good indication of isomer composition. A 10 nm red-shift in wt bR near neutral pH indicates 100% conversion of the 13-cis retinal chromophore to the all-trans retinal chromophore. However, in bR mutants an 8−10 nm shift in λ_{max} upon light adaptation does not necessarily mean 100% conversion of the 13-cis retinal chromophore to the all-trans retinal chromophore. λ_{max} of D212N at pH 9 is 584 nm in the light-adapted form and is shifted to 576 nm when dark-adapted. The 8 nm red-shift in the absorption maximum upon light adaptation is comparable to that observed in wt bR. However, the retinal chromophore composition in D212N at pH 9.6 is 31% 13-cis, 18% 11-cis, 11% 9-cis, and 40% all-trans when it is light-adapted compared to 63% 13-cis, 10% 11-cis, 2% 9-cis, and 25% all-trans retinal when it is dark-adapted. The amount of the 13-cis retinal isomer in D212N is very similar to that of the wt bR in the dark-adapted state, but it is very different from that of the wt bR in the light-adapted state. Thus, in bR mutants the shift in λ_{max} upon light adaptation alone is not a measure of chromophore composition. (The rate of dark adaptation of the light-adapted D212N sample is about 25 min at room temperature. Both the optical and the HPLC measurements were performed within a few minutes after the light adaptation process.) This is not difficult to rationalize since the 13-cis and the all-trans retinal isomers might have different absorption maxima and molar extinction coefficients in bR mutants than in wt bR, as a result of the changes in the cavity charge distribution around the retinal which must play an important factor in controlling the extinction coefficients of retinal isomers. Furthermore, the existence of the 9-cis and 11-

cis isomers in both the dark- and the light-adapted states of D212N makes it harder to deconvolute the absorption spectra of the light- and dark-adapted states into the absorption spectrum of the individual retinal isomers.

From Table 1, it can be seen that different amounts of 9-cis and/or 11-cis retinal isomers exist in the dark-adapted bR mutant samples. This suggests that all of the amino acid substitutions/replacements change the protein structure within the retinal cavity to some extent and stabilize retinal isomers other than the 13-cis and all-trans observed in wt bR. This suggests an enhanced thermal stability of the 9-cis and 11-cis retinal chromophores or destabilization of the 13-cis and all-trans isomers in these bR mutants. In wt bR 9-cis retinal was found only after illumination of deionized samples at acid pH, i.e. in the blue membrane.[33,34] Both 9-cis and 11-cis retinal isomers were observed in wt bR at acid pH under long illumination conditions.[35] Recently, it was reported that in the light-adapted R82Q mutant the amount of 13-cis retinal was high.[36] The amount of 9-cis and 11-cis retinal isomers observed in bR mutants depends on the amino acid residue replaced by mutation, the pH, and the temperature. The enhanced amount of 9-cis and 11-cis retinal chromophores observed in bR mutants under light adaptation can result from thermal- or photoreactions of intermediate states during light adaptation or from direct photoisomerization along the C_9-C_{10} and $C_{11}-C_{12}$ bonds in all-trans or 13-cis retinal. More work is needed to differentiate between these mechanisms. It is possible that the observed 9-cis and 11-cis retinal chromophores in bR mutants upon light adaptation are due to direct photoisomerization of the all-trans or 13-cis retinal chromophore upon the slight modification of the retinal cavity after mutation. If this is the case, our results suggest that amino acid replacement changes the photoisomerization specificity. Techniques such as time-resolved Raman spectroscopy are needed to confirm the direct all-trans or 13-cis to 9-cis and 11-cis photoisomerization. This work is under way in our group.

From our results, it can be seen that the smallest effect of mutation on the isomer composition was observed in D115N. The strongest effect was observed in D212N but not in D85N. This is in support of previous studies on the binding of Ca^{2+} to deionized bR mutants. Replacement of Asp212 was found to affect the affinity constant most.[37] Therefore, replacement of Asp212 has a strong influence on the ground state of the retinal (as well as some on the excited state) and its protein environment. Asp85 has a strong effect on the energy separation between the excited-state and the ground-state potential surfaces of the retinal chromophore within the protein cavity since a larger color change was observed in D85N than in D212N.[20,29]

Table 2 shows the retinal composition of a few mutants at 20 and 35 °C and at different pH. The pH was chosen carefully based on preliminary dark adaptation kinetics studies of these bR samples to ensure that no serious aggregation occurs at 35 °C. In addition, D212N changes color on going from pH > 8 to pH < 6.[20] It is of interest to compare the blue and purple forms of D212N. Since the all-trans and the 13-cis isomers are the dominant species for all, we shall use these data to extract some thermodynamic quantities.

Let us consider the following thermal isomerization reaction:

$$\text{all-trans retinal} \rightleftharpoons \text{13-cis retinal}$$

The equilibrium constant is defined as K = [13-cis retinal]/[all-trans retinal]. The enthalpy change of the above reaction was calculated by using LN $[K(T_2)/K(T_1)] = -(\Delta H/R)\,[1/T_2 - 1/T_1]$ and is listed in the last column of Table 2.

The important observations for the dark-adapted samples in Table 2 can be summarized as follows: (1) both wt bR at pH 6.0 and deionized bR at pH ~ 4 show a $\Delta H = 0$, as was concluded previously.[30] This means that the enthalpy of formation of all-trans retinal within the protein cavity is similar to that of the 13-cis retinal in wt bR and in the deionized form of the corresponding bR opsin. Since the internal energies of the isolated retinal isomers are different for the two isomers, their intermolecular interaction energies with the cavity must be different for the two isomers. (2) The situation is different for D85N and D212N mutants. Not only does isomerization change the energetics but the energy change is also very sensitive to pH. Thus while the enthalpy of formation of the 13-cis retinal in D85N is less than that of the all-trans retinal by 10 kJ/mol at pH = 8, it is higher by 45 kJ at pH = 6. For retinal in D212N, the retinal enthalpy of formation decreases by 14 kJ/mol when the all-trans is transformed to its 13-cis isomer at pH = 8, but the isomerization to 13-cis seems to decrease the retinal energy by 24 kJ/mol at pH = 6. Thus, the protein environment in the blue form of D212N (at pH > 8) is very different from that in the purple form of D212N (at pH < 6). In both D85N and D212N mutants at pH 6 and 8, the protein around the retinal chromophore could change more upon isomerization, giving different charge distributions of the other amino acids that could change their ionization states, thus leading to a wide range of changes in $\Delta H_{\text{isomerization}}$.

TABLE 2: Temperature Dependence of the Isomeric Composition, the Equilibrium Constant, and the Enthalpy Change (ΔH) for the All-Trans to 13-cis Retinal Thermal Isomerization Reaction of Dark-Adapted Samples of Wild Type Bacteriorhodopsin and Some of Its Mutants

sample	pH	temp (°C)	13-cis	11-cis	9-cis	all-trans	K	ΔH (kJ/mol)
D85N	8.0	20	72	0	1	27	2.7	−10
		35	67	0	2	31	2.2	
D85N	6.0	20	27	0	1	72	0.37	45
		35	47	0	2	51	0.92	
D212	8.0	20	60	9	10	21	2.8	−14
		35	59	1	12	28	2.1	
D212N	6.0	20	70	3	2	25	2.8	−25
		35	61	0	4	35	1.7	
wt bR	6.0	20	61	0	0	39	1.6	3
		35	63	0	0	37	1.7	
blue bR (deionized)	3.9	20	51	0	1	48	1.1	~0
		35	51	0	1	48	1.1	

From our results, it is clear that the isomer composition in the bR mutant is influenced by the structure of the protein (which depends on the presence of charged amino acid residues nearby) around the retinal chromophore, the pH of the solution (which could change the protein structure and the charge distribution within the protein), and the temperature.

The replacement of Asp85 and Asp212 by neutral amino acid residues was found to inhibit proton pumping. This could be a result of (1) a change in the isomeric form of retinal, (2) a drastic decrease in the quantum yield of retinal photoisomerization, (3) a decrease in the free energy stored in the K intermediate upon photoisomerization (which is used to translocate protons across the membrane), or (4) an increase in the free energy required to pump the protons across the protein membrane. The present work certainly eliminates the first as a possibility since while the all-trans form of retinal has been reduced by 50% in D85N, the proton pumping efficiency was reduced to a much greater extent (it completely stops in D85N). Studies in our group are presently being carried out to examine some of the remaining possibilities.

Recently, a simple valence-bond molecular model was proposed based on the observed excited-state lifetimes in some important bR mutants.[25] According to this model, any perturbation to the charge distribution in the vicinity of the retinal

chromophore will change the excited-state structure of the retinal and thus the rate of photoisomerization around different carbon–carbon bonds and the isomerization specificity. The extent of the perturbation on the retinal chromophore of a specific amino acid mutation depends on the nature of the residue to be replaced, its distances from the different carbon atoms of the retinal chromophore, and the local structure of water molecules around the amino acid residue. From previous studies, it is concluded that Asp85 is an important component of the counterion.[17–20] A recent study showed that the hydrogen bonding in D85N is as strong as in wt bR.[38] Therefore, the most important effect of replacing Asp85 in D85N is the loss of electronic coupling between the retinal chromophore and the amino acid residue, but not the loss in the hydrogen-bonding strength. From our results, it is concluded that Asp212 is more important in controlling the ground-state chromophore configuration than Asp85. It is very likely that in the D212N mutant the strength of the H-bonding is significantly reduced, leading to most dramatic changes in the thermal stability of the different retinal isomers. More careful studies are needed to fully characterize the function of Asp212 in bR.

In summary, bR mutants expressed in *H. salinarium* showed the normal direction for light adaptation as measured by the 13-cis and the all-trans isomer composition of retinal obtained by extraction and HPLC analysis. However, upon light adaptation, the amount of 9-cis and 11-cis retinal chromophores also increased in the bR mutants. The amount of the 9-cis and 11-cis retinal chromophore in these bR mutants was sensitive to the nature of the amino acid residue being replaced by mutation, the pH of the solution, and the temperature. Both changing of the pH and the replacement of charged residues change the charge distribution around the retinal. Neutral and non-hydrogen-bonding residue replacement has a reduced effect that results from small indirect changes in the distances between the charged residues and the retinal chromophore due to changes in the steric factors.

Acknowledgment. We would like to thank the Department of Energy for financial support (under Grants DE-FG03-88ER-13828 at Georgia Institute of Technology and DE-FGOER 13525 at UCI). L.S. would like to thank Dr. B. E. Ruiz Silva for assistance in using the HPLC instrument. We wish to thank Professor Robin L. Garrell for allowing us to use her HPLC apparatus.

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JP950537C