Sensitivity of the retinal circular dichroism of bacteriorhodopsin to the mutagenetic single substitution of amino acids: tyrosine

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Bacteriorhodopsin (bR) in the native purple membrane, in wild type expressed in *E. coli* and reconstituted in lipid vesicles, and its constituted mutants with substitutions of Tyr-185 by Phe all are found to have different visible retinal CD spectra. The results strongly suggest that the environment of the retinal in bR determines the sign and heterogeneity of its visible retinal CD spectrum. This supports the recent proposal that the observed biphasic CD spectrum of bR is due to the superposition of the CD spectra having opposite signs of more than one type of bR rather than due to exciton coupling.

Bacteriorhodopsin; Circular dichroism; Exiton interaction; Protein conformation; Purple membrane; Site-specific mutant

1. INTRODUCTION

The retinylidene protein bacteriorhodopsin (bR), the other photosynthetic system in nature besides that of chlorophyll containing pigments, is the sole protein pigment in the purple membrane of *Halobacterium halobium* [1], arranged in clusters of 3 molecules in a two-dimensional hexagonal lattice [2]. Light-adapted bR contains an all-*trans* retinal which is covalently bound via a protonated Schiff base linkage to the protein [2]. Upon the absorption of visible light, bR undergoes through the photochemical cycle consisting of at least 5 intermediates [4], translocating protons from the inside to the outside of the cell [5]. This proton gradient across the membrane is then used to transform ADP into ATP in the final step of the photosynthesis of bR [6].

The CD spectrum of native bR in the visible spectrum region is composed of negative and positive (larger) components of unequal strength [7,8]. This biphasic line shape was attributed to the presence of exciton coupling which splits the degeneracy of the 3 retinal excited states of the trimer into ^1E and ^1A states. The calculation of the exciton interaction [8,9] suggested energy transfer in the tens of femtoseconds. Photoselection studies [10] have not detected energy transport in bR and question about the exciton picture was raised. More recently [11], magnetic circular dichroism showed no signs for an ^1E type state in bR. This, together with the fact that the K_{610} and L_{550} intermediates [12], in which the trimer structure was destroyed by the isomerization of one of the 3 retinals, are found to have CD spectra similar to bR, has again led El-Sayed et al. [11] to raise serious doubts that exciton coupling exists in bR. These authors suggested that the biphasic nature of CD spectra of bR results from the presence of the CD of more than one type of bR with the retinals having different protein environment giving them opposite signs.

A variety of site-directed mutagenesis methods have been used to single-substitute individual amino residue in bO in order to elucidate the mechanism of the proton pump [13–15]. In the present work, we have compared the retinal CD spectrum of bR in the 400–700 nm range with those of wild bR expressed in *E. coli* (ebR) and reconstituted into lipid vesicles and its genetically modified bR in which one of the tyrosines, Tyr-26, Tyr-57, Tyr-64, Tyr-79 or Tyr-185, is substituted by a Phe [13]. In the present work, only the substituted Tyr-185 (Y185F) mutant is found to show large effects on the CD spectrum of the photocycle of bR. The fact that the CD spectra of the 3 species, PMbR, ebR and Y185F, are found to be quite different from each other

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Abbreviations: bR, bacteriorhodopsin; ebR, wild-type bR expressed in *E. coli*; Y185F, mutant bR expressed in *E. coli* in which Tyr-185 is substituted by Phe

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strongly suggests the retinal CD spectrum in bR active site is very much controlled by its protein as well as lipid environment.

2. MATERIALS AND METHODS

The preparation of native bR from *H. halobium* was described previously [16]. The construction of gene coding for site-specific mutants and the preparation of the mutant bacterioopsin apoproteins using a heterologous (*E. coli*) expression system have been described previously [13]. The purified bacterioopsin was regenerated with retinal and reconstituted in vesicles in a 1:1 weight ratio with polar lipids from *H. halobium* [17] using the procedures devised by Popot et al. [18]. The fraction of mutant apoproteins that bound a chromophore was about 50%.

The steady-state absorption spectra were measured with a Hewlett-Packard 8451 diode-array spectrometer or a Cary 219 UV/visible spectrometer and the CD spectra of bR suspensions in water were measured on a JASCO J-500C spectropolarimeter.

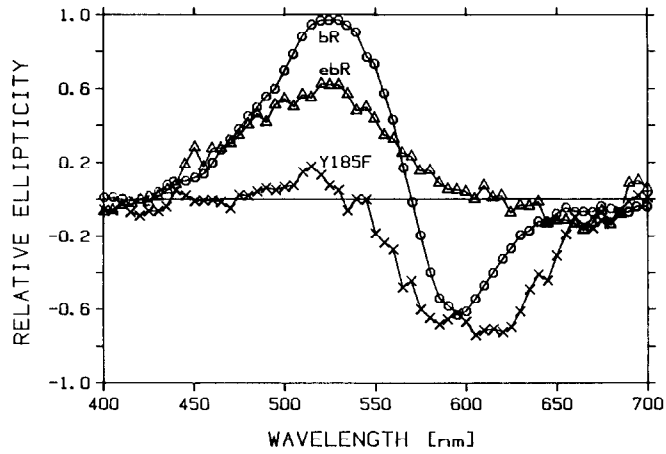

Fig.1. Visible CD spectra of bR (○), ebR (△) and Y185F (×) suspensions in water at pH 6 and room temperature. The relative ellipticity was normalized by the optical density at the wavelength of the retinal absorption maximum.

3. RESULTS AND DISCUSSION

Fig.1 shows that the CD spectra of PMbR, ebR and Y185F are extremely different from each other. While the CD spectrum of native bR shows a distinctive biphasic band shape (with the positive band being larger than the negative one), that of ebR shows a mostly positive band and that of Y185F shows a weak biphasic but more intense negative band. Other bR mutants expressed in *E. coli* in which a single Tyr-26, Tyr-57, Tyr-64 or Tyr-79 is substituted for by Phe show CD spectra very similar to that of ebR.

The CD spectra of ebR look similar to the spectrum of monomeric bR resulting from removing the lipids [7] or increasing the sample temperature [7,19]. ebR and mutant bR are reconstituted in vesicles in a 1:1 weight ratio with polar lipids from *H. halobium* [16]. It was shown [20] that in the reconstituted samples, bR molecules have the P_3 two-dimensional trimer lattice with the same unit cell dimensions as the native purple membrane lattice. The reconstituted bR samples show the CD spectra with positive and negative bands just like PMbR [19]. Furthermore, we have observed that the CD spectrum of 75% delipidated bR from *H. halobium* is very similar to that of PMbR.

The difference in the CD band shape of PMbR and PMbR is of basic interest to the field of genetic engineering. CD is very sensitive to small structural changes. The difference could either suggest the absence of the trimer structure in ebR or a large difference in the environment of the active site around the retinal in the two types of bR. The fact that the absorption maximum in ebR is blue-shifted from that in bR could be explained by either of these possibilities. If the spectral differences result from the lack of formation of the trimer structure in ebR, surely it must result from the difference in the protein structure in ebR and bR. Similarly, if the blue shift in the absorption spectrum and the CD spectral change result from a difference in the retinal microenvironment, it again suggests a difference in the protein microscopic structure in ebR and PMbR.

The difference in the CD between ebR and its genetically modified protein, Y185F, might shed light on the participation of Tyr-185 in the mechanism of the proton pumping in bR as well as the function of bR in general. Such a substitution is known to shift and broaden the visible absorption spectrum [13,15], to reduce the proton pumping efficiency [13], to modify the photocycle kinetics and to make it sensitive to the wavelength of photolysis [15]. All these observations strongly suggest that Tyr-185 is either a part of the active site with a strong coupling to the retinal or else its substitution greatly modifies the active site indirectly. In either case, such a substitution leads to a change in the electronic structure of the active site (i.e. around the retinal). The large change in the CD spectrum could be a result of a change in the retinal environment.

Fig.2 gives the optical absorption and CD spectra of Y185F and the fit of the observed CD spectrum to three CD spectra of different signs. It has already been proposed [15] that the optical spectrum of Y185F represents the absorption of more than one species. The species absorbing at the longest wavelength region is shown not to pump protons nor is it capable of *trans*-to-*cis* isomerization upon dark adaptation [15]. This species seems to have a negative monophasic CD. The two peaks at shorter wavelength are assigned to two species, one with positive CD sign (the short wavelength absorption) and the other with negative CD sign. The CD spectrum of Y185F fits well with the sum of 3 different CD curves and the sum of the absorption of these 3 curves gives the absorption profile and a maximum very similar to those observed for Y185F. It should be pointed out that the CD spectrum of Y185F cannot be fitted to monomer type CD (positive peak)

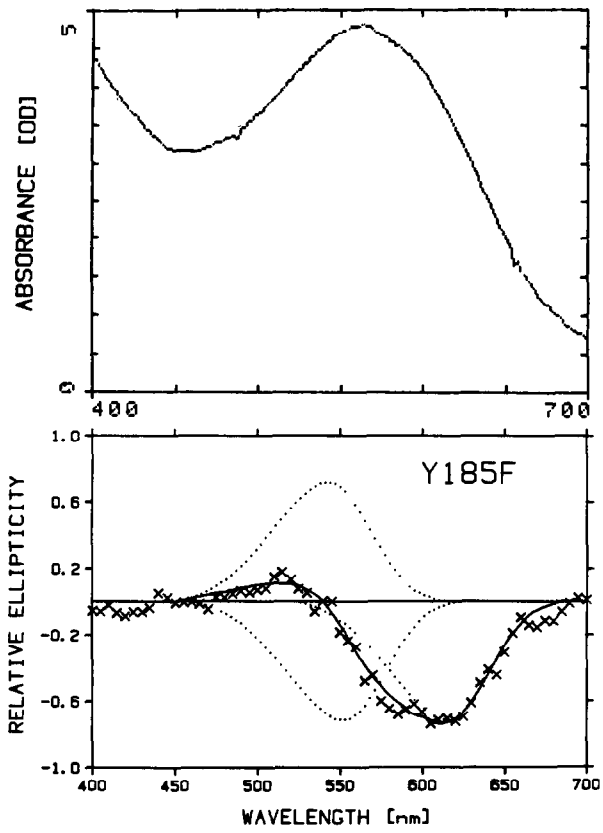

Fig.2. The visible absorption (top) and CD (bottom, ×) spectra of Y185F suspensions in water at pH 6 and room temperature. The solid lines (bottom) are the CD curves fitted by the sum of the dotted 3 different CD curves. The profile of the dotted CD curves is the actual absorption profile of native bR after subtracting the wavelength dependent scattering and reducing the band width by 25%. The peak wavelengths of the three dotted curves are 543, 552 and 615 nm.

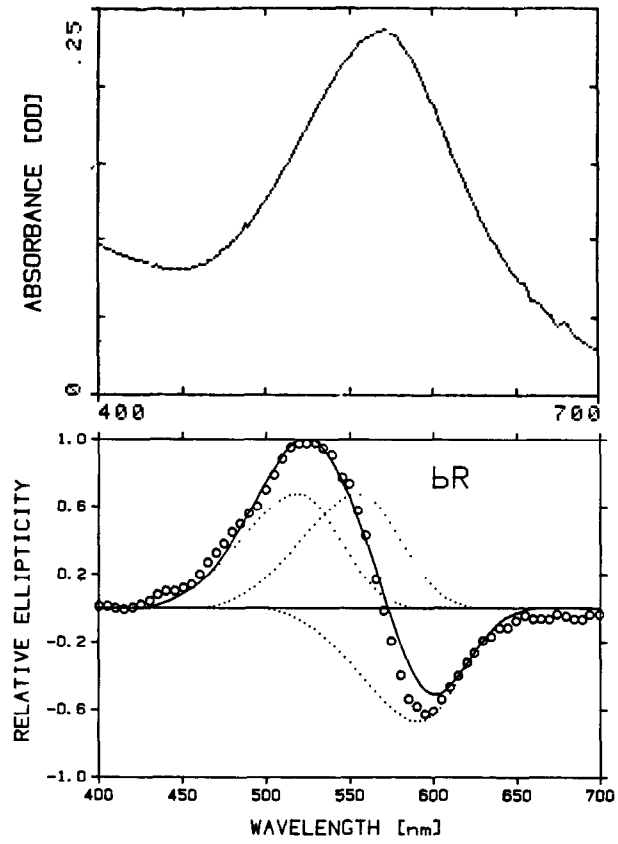

Fig.3. The visible absorption (top) and CD (bottom, ○) spectra of native bR suspensions in water at pH 6 and room temperature. Both of these spectra can be fitted by adding the spectra drawn in dotted lines with maxima at 519, 555 and 591 nm.

and trimer type CD (equal positive and negative peaks). This suggests that the change in the CD spectrum by genetic modification in Y185F is not merely a result of incomplete regeneration by binding to retinal.

The above results lead to the following conclusions: (i) the active site of native bR is different from that in ebR and its Y185F genetically modified mutant reconstituted in lipid vesicles; (ii) the degree of heterogeneity, as determined from CD spectroscopy, changes upon the genetic substitution of Tyr-185 by Phe; (iii) the fact that the substitution of Tyr-185 gives drastic changes in the active site of bR while the substitutions of other Tyr known to affect the proton pumping (although not to the same extent) strongly suggests that Tyr-185 plays an especially important role in determining the environment of the retinal in bR, either directly or indirectly.

The fact that the CD of Y185F is different from ebR and the other Tyr mutants suggests that the protein conformation of the active site not only depends on the lipid composition (and its surface potential) [21] but also on the position of the amino acid being substituted. This is consistent with the proposal [21] that the protein conformation and its changes in bR might be controlled by the electrostatic interaction between the surface charges (greatly determined by the lipid charges, the pH and the ionic strength of the solution) and the internal protein charges and dipoles as well as by the intra-amino acid interaction within the protein itself. The former (surface-protein) interaction depends on the lipid and protein composition and structure while the latter (protein-protein) interaction depends on the relative orientation and the type of the amino acid being substituted.

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