PROLONGED LIFETIME OF THE 410-nm INTERMEDIATE OF BACTERIORHODOPSIN IN THE PRESENCE OF GUANIDINE HYDROCHLORIDE

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SUMMARY

Lifetime of the 410-nm intermediate of bacteriorhodopsin was markedly prolonged by the addition of high concentration of guanidine hydrochloride. Circular dichroism spectra at low temperature in the presence of guanidine hydrochloride indicated that the secondary protein structure in the 410-nm intermediate did not change from that in the purple complex but that the state of the aromatic amino acid residues, as well as the retinal chromophore, significantly differed one another.

Bacteriorhodopsin is a sole protein contained in purple patches of the plasma membrane of *Halobacterium halobium* (1), functioning as a light-driven proton pump (2,3). Isolated bacteriorhodopsin shows absorption maximum around 560 nm (the purple complex) and undergoes photochemical reaction cycle upon illumination (4). Several intermediates of the cycle have been identified but the steady-state concentrations under illumination are very low, since the photoequilibrium favors the formation of the purple complex. Therefore the inhibitor that inhibits or slows down the reaction of specific step in the cycle is useful in studying the structure and function of the intermediates. Oesterhelt and Hess reported that the intermediate, which had the absorption maximum around 410 nm (the 410-nm intermediate), was accumulated under illumination when the purple membrane was suspended in 4 M NaCl

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solution saturated with diethyl ether (5). This communication describes another inhibitor, high concentration of guanidine hydrochloride (Guan-HCl), and circular dichroism (CD) of the 410-nm intermediate.

MATERIALS AND METHODS

Cells and purple membranes were obtained as described (6). The solution of purple membranes in Guan-HCl was prepared by adding solid Guan-HCl to the concentrated suspension of purple membranes in a graduated glass tube. After buffer and KCl solution were added, the volume was adjusted by water. The resulting solution was sonicated for 10 sec to homogenize the fragments of purple membranes. This solution was stable for months at 4° or at room temperature.

Absorption spectra were recorded with a double-beam spectrophotometer (Shimazu UV-200) with scanning speed 200 nm per min from 700 nm to 300 nm. Decay of the 410-nm intermediate in the dark was measured with a single-beam spectrophotometer attached with an actinic light source, 650 W quartz halogen lamp filtered through a 10 cm water layer and a cut-off filter (Toshiba,VO-55). The illumination (5 sec) was started and terminated with a shutter (COPAL,No.0). The changes of transmittance at 410 nm after closing the shutter were memorized by a transient recorder (Datalab,Model DL 905) and recorded on a chart recorder.

CD spectra were measured in a Jasco J-20 recording spectrometer with a quartz cuvette placed in a low temperature cell holder. The sample was cooled slowly under illumination by dry ice-aceton (-86°) and then measured in the dark. The absorption maximum around 560 nm had completely disappeared throughout measurement and the 410-nm intermediate did not transform back to the purple complex for overnight in the dark at this temperature. The sample for the purple complex was cooled without illumination and the other conditions were the same as those of the illuminated one.

RESULTS AND DISCUSSION

Bacteriorhodopsin was found to be capable of photochemical reaction in 8 M Guan-HCl solution. However, we noticed that purple membrane solution containing 8 M Guan-HCl changed the color from purple to yellow under illumination by actinic light. When the sample was returned to the dark, purple color reappeared spontaneously. Fig.1 shows a time series of the regeneration of the purple complex in the dark. Upon turning

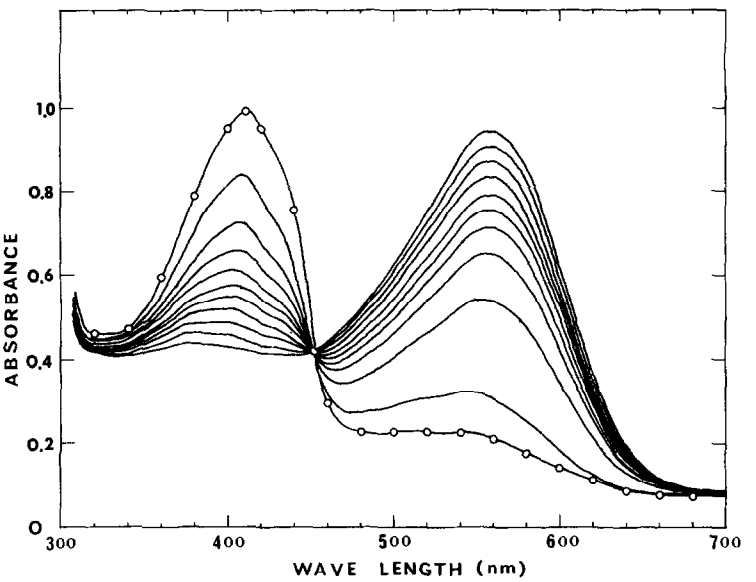

Fig.1 Time series of spectra taken after turning off the actinic light. The purple membrane solution contained 8 M Guan-HCl, 0.2M KCl, 0.05M glycine and 0.5mg protein per ml of purple membrane. The pH was adjusted to 9.5 by 1N KOH solution. The time of starting and terminating the scanning (700nm-300nm) of each spectrum was in min : (1) 0-2 (2) 3-5 (3) 6-8 (4) 9-11 (5) 12-14 (6) 15-17 (7) 21-23 (8) 30-32 (9) 50-52 (10) overnight. The spectrum of zero time (—o—) was obtained by the measurement of absorbance at each wave length just after turning off the actinic light. Temperature in the cuvette was 12°.

off the light, the absorption spectrum indicated the presence of the sole component with a maximum around 410 nm, which disappeared in times simultaneously with the regeneration of the purple complex. The half life of the absorption maximum at 410 nm was 5 min under the conditions used. An isosbestic point was observed at 452 nm. The transition from the 410-nm intermediate to the purple complex was not a simple first-order process and more than 50 min was necessary for complete regeneration of the purple complex. The ratio of the absorption increase at 560 nm and the corresponding decrease at 410 nm was obtained to be 1.3.

Table I
Effect of pH on The Half Life of Decay of The 410-nm Intermediate at 20° [a].

Guan-HCl (M)	pH	Half Life (sec)
7	4.68	0.08
7	7.95	1.60
7	9.80	27
0	7.40	0.011

[a] The purple membrane suspension (0.29mg protein per ml) contained 0.2M KCl, 50mM Tris and with or without 7M Guan-HCl. The pH was adjusted by 1N KOH or 1N HCl.

The half life of the 410-nm intermediate,in the dark, in the presence of Guan-HCl was much longer at alkaline pH than at acidic pH (Table I). The half life of the 410-nm intermediate increased about 340 times with increasing pH from 4.68 to 9.8.

The 410-nm intermediate existed stably for hours in alkaline solution containing Guan-HCl at low temperature. Under such conditions, it was possible to observe the CD spectrum of the 410-nm intermediate in the dark. As shown in Fig.2, it is evident from the CD spectrum of the 410-nm intermediate that ; *(i)* the optical activities around 550 nm and 600 nm were almost completely lost, *(ii)* a new positive band appeared around 400 nm, *(iii)* a considerable change of the spectrum occured in the near-ultraviolet region (250 nm-310 nm), *(iv)* there was no detectable change in the far-ultraviolet region (210 nm-250 nm) compared to that of the purple complex. Therefore it is suggested that α-helix

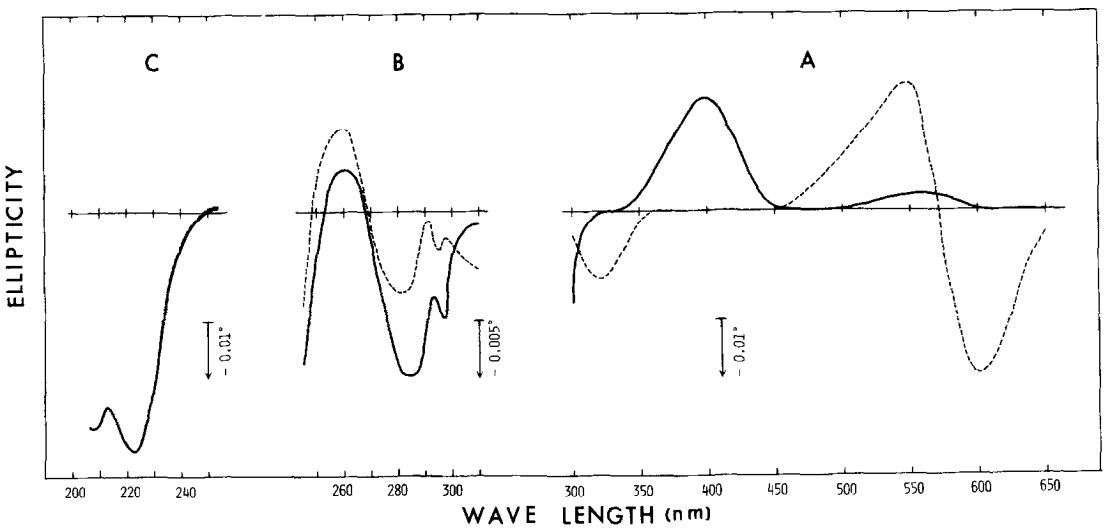

Fig.2 The CD spectra of the purple complex and the 410-nm intermediate. The purple membranes were suspended in 5.4 M Guan-HCl and 33% (w/v) of glycerol. The pH was adjusted to 8.6 by 1N KOH solution. The cuvette was slowly cooled by dry ice-aceton (-86°) in the illuminating light (——) or in the dark (----). Protein concentrations and light path lengths of cuvettes were : (A) 1mg per ml,1cm light path (650nm-300nm) (B) 0.35mg per ml,1cm light path (310nm-250nm) (C) 0.2mg per ml, 1mm light path (250nm-210nm).

content of bacteriorhodopsin remains constant in both the purple complex and the 410-nm intermediate, whereas marked conformational changes occur in both side chains of the aromatic amino acids and the retinal chromophore. Our results of CD spectra did not coincide with those of Heyn *et al.*(6), in which an exiton type band appeared around 410 nm under photostationary conditions at -30°. The difference between them may be due to the different conditions employed. Further characterization of the 410-nm intermediate in the presence of Guan-HCl is in progress.

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REFERENCES

1. Oesterhelt,D. and Stoeckenius,W. (1971) *Nat.New Biol.* 233,149-152
2. Oesterhelt,D. and Stoeckenius,W. (1973) *Proc.Natl.Acad. Sci.U.S.A.* 70,2853-2857
3. Kagawa,Y.,Ohno,K.,Takeuchi,Y. and Sone,N. (1977) *Fed. Proceedings* 36, in press.
4. Kung,M.C.,Devault,D.,Hess,B. and Oesterhelt,D. (1975) *Biophys.J.* 15,907-911
5. Oesterhelt,D. and Hess,B. (1973) *Eur.J.Biochem.* 37, 316-326
6. Yoshida,M.,Sone,N.,Hirata,H.,Kagawa,Y.,Takeuchi,Y. and Ohno,K. (1975) *Biochem.Biophys.Res.Commun.* 67,1295-1300
7. Heyn,M.P.,Bauer,P.-J. and Dencher,N.A. (1975) *Biochem. Biophys.Res.Commun.* 67,897-903