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Photochemical Studies of 7-*cis*-Rhodopsin at Low Temperatures. Nature and Properties of the Bathointermediate†

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ABSTRACT: The photoreaction of 7-*cis*-rhodopsin derived from 7-*cis*-retinal and cattle opsin was studied by low-temperature spectrophotometry. Upon irradiation of 7-*cis*-rhodopsin at liquid nitrogen temperature (−190 °C) with blue light, its spectrum shifted to the longer wavelengths, indicating the formation of a bathoproduct. The bathoproduct thus formed was found to be identical with bathorhodopsin formed from rhodopsin in their spectroscopic, photochemical, and thermal properties. Therefore, we believe that the bathoproduct is, in fact, bathorhodopsin. The fact that 7-*cis*-rhodopsin can be readily converted to rhodopsin and to 9-cis-rhodopsin shows that the identical retinal binding site of opsin is involved in the three isomeric rhodopsins. These results appear to be consistent with the notion that the chromophore of bathorhodopsin is a twisted all-trans isomer, which is readily obtainable from the 7-cis, 9-cis, and 11-cis isomers.

In 1963 by irradiating rhodopsin at liquid nitrogen temperature (−190 °C) Yoshizawa & Wald (1963) observed the formation of the red-shifted primary photoproduct, bathorhodopsin (earlier known as prelumirhodopsin). The rigid matrix and low temperature used in their experiment apparently did not have a profound effect on the photochemistry of rhodopsin for it has recently been shown by laser flash photolysis of rhodopsin (Busch et al., 1972; Cone, 1972; Rosenfeld et al., 1972) that the same intermediate was generated immediately after illumination.

In the same paper it was also shown by Yoshizawa & Wald (1963) that an intermediate spectrally identical with bathorhodopsin is also formed by irradiation of 9-*cis*-rhodopsin.[1] It appeared natural to suggest (Yoshizawa & Wald, 1963) that an identical intermediate with the strained all-trans polyene geometry is formed from rhodopsin and 9-*cis*-rhodopsin. However, it has also been suggested (Kropf, 1969) that perhaps two spectrally similar bathoproducts are actually formed which may retain something like the original 11-cis and 9-cis geometrical integrities. This suggestion has perhaps found renewed interest after recent postulation of proton transfer as a primary photochemical event of rhodopsin (Fransen et al., 1976; Peters et al., 1977).

The close absorption maxima of rhodopsin (498 nm) and 9-*cis*-rhodopsin (485 nm) hampered the design of a definitive experiment to prove or disprove Kropf's (1969) original suggestion. However, recently DeGrip et al. (1976) reported the formation of 7-*cis*-rhodopsin from highly hindered 7-*cis*-retinal and cattle opsin. The new pigment analogue has a greatly blue-shifted absorption maximum (λ_{max} = 450 nm) from that of rhodopsin (498 nm). It was thought that batho-7-*cis*-rhodopsin (the bathoproduct from 7-*cis*-rhodopsin), if indeed it existed, would also exhibit a similar blue shift from bathorhodopsin and thus be easily distinguishable from the less hindered isomers (bathorhodopsin and batho-9-*cis*-rhodopsin). Furthermore, it was shown that the final photobleaching product of 7-*cis*-rhodopsin is also *all-trans*-retinal and opsin (DeGrip et al., 1976). It will be of interest to determine whether a bleaching sequence identical with that of rhodopsin is also involved in the new pigment analogue. We have therefore studied the photochemistry of 7-*cis*-rhodopsin at low temperatures.

Materials and Methods

Rod outer segments were isolated from cattle retinas as reported previously (Matsumoto et al., 1978). Opsin was extracted with 1% digitonin buffered in 10 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.0. The synthesis of 7-*cis*-retinal was reported previously (Ramamurthy & Liu, 1975). The conversion of *all-trans*-retinal to 7-*cis*-retinal has also been reported (Denny & Liu, 1977; Maeda et al., 1978b). To prepare 7-*cis*-rhodopsin, equimolar 7-*cis*-retinal in ethanol was added to opsin and then the mixture was incubated at room temperature for 48 h.

The equipment used for this study and the general procedure have already been described in detail in the literature (Yoshizawa, 1972). Briefly, 7-*cis*-rhodopsin solubilized in digitonin was mixed with glycerol (1:3 v/v). The mixture was placed in an optical cell (path length = 2 mm) which was fixed

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[1] Since a large number of geometric isomers of rhodopsin are now known to exist, we have decided to abandon the use of common names such as isorhodopsin. In this paper, the term rhodopsin when used carries the implied geometry of 11-cis. A proper prefix will be used in naming an isomer of rhodopsin, e.g., 7-*cis*-rhodopsin or 9-*cis*-rhodopsin, etc.

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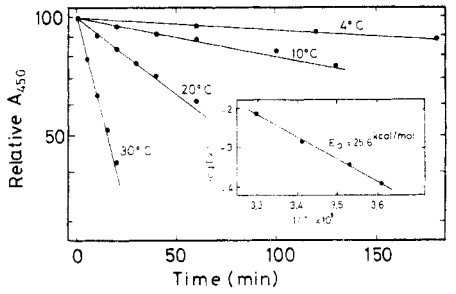

FIGURE 1: Degradation of 7-*cis*-rhodopsin in hydroxylamine. 7-*cis*-Rhodopsin was incubated at 4, 10, 20, and 30 °C in 10 mM Hepes buffer (pH 7.0) containing 0.1 M NH_2OH. The absorbance loss, due to the decomposition of 7-*cis*-rhodopsin by NH_2OH, was monitored at 450 nm. Insert: Arrhenius plot of the degradation reaction.

in a cryostat. A xenon lamp (2 kW, Ushio) was used for irradiating the sample, and the light of desired wavelength was isolated by inserting an interference filter (437 nm, 60.5% transmission, half-bandwidth = 3 nm) or a cutoff filter (Toshiba) in front of the light source. The spectra were recorded on a Shimadzu MPS-5000 spectrophotometer. Temperature was monitored with a copper–constantan thermocouple.

Results

Effect of NH_2OH on 7-cis-Rhodopsin. Since hydroxylamine was found very useful for investigation of the bleaching sequence of visual pigments (Yoshizawa & Wald, 1963), we intended to use the same reagent in studies of 7-*cis*-rhodopsin. However, contrary to the earlier report (DeGrip et al., 1976) that 7-*cis*-rhodopsin is stable in excess NH_2OH, we found that this reagent at a final concentration of 0.1 M decomposes 7-*cis*-rhodopsin, presumably into 7-*cis*-retinal oxime and opsin (Figure 1). The rate of decomposition depended on temperature: the half-life was ~100 min at 20 °C; at 0 °C 7-*cis*-rhodopsin was practically stable. Hence, for all the low-temperature spectroscopic experiments described here, NH_2OH was added to the preparation which was kept below 4 °C.

The second-order reaction constants were calculated from the data in Figure 1. The activation energy was found to be 25.6 kcal/mol (insert, Figure 1).

Photoreaction of 7-cis-Rhodopsin at Liquid Nitrogen Temperature (−190 °C). Upon irradiation of 7-*cis*-rhodopsin with light at 437 nm at −190 °C, a red shift of the absorption maximum was immediately detected (Figure 2a),[2] indicating the formation of a bathoproduct. After 160 min of irradiation, a photostationary state with the absorption maximum at 510 nm (curve 14) was established.

The bathoproduct was then irradiated with light at wavelengths longer than 610 nm (Toshiba Filter No. VR-63). The spectrum shifted to shorter wavelengths with a clear isosbestic point at 513 nm (Figure 2b). Finally, it reached another stationary state with λ_{max} at 502 nm (curve 8, Figure 2b). The changes were found to be completely reversible. Therefore, when the mixture was again irradiated with the 437-nm light, a spectrum identical with that of curve 14 in Figure 2a was again obtained (data not shown). When the photostationary state mixture corresponding to that of curve 8 in Figure 2b was again irradiated with light at wavelengths longer than 530 nm (Toshiba, VO-55), a further blue shift of the spectrum was observed with a well-defined isosbestic point at 503 nm (Figure 2c). The spectrum finally changed to that (curve 7) of a third photostationary state mixture shown in Figure 2c.

The spectral changes shown in parts b and c of Figure 2 are reminiscent of the curves recorded in the study of interconversions between bathorhodopsin and rhodopsin and between rhodopsin and 9-*cis*-rhodopsin (Yoshizawa & Wald, 1963). In fact, the isosbestic points (513 and 503 nm) in these two figures are identical with those observed in the conversion of bathorhodopsin to rhodopsin and that of rhodopsin to isorhodopsin.

While the interconversion of photostationary mixtures shown in parts b and c of Figure 2 was found to be completely reversible (the composition depending on the excitation wavelength), these mixtures could not be reverted back to the 7-cis isomer. In Figure 2d are shown results of irradiation of the photoproducts from 7-*cis*-rhodopsin (curve 7, Figure 2c) with light absorbed strongly only by the products (light at wavelengths longer than 500 nm). There were no indications of any increase of absorption at 450 nm that could be attributed to the formation of 7-*cis*-rhodopsin. Similar photochemical reactions were also observed in a 7-*cis*-rhodopsin preparation without NH_2OH.

Absorption Spectrum of the Bathoproduct from 7-cis-Rhodopsin. In principle, the absorption spectrum of the bathoproduct can be obtained from the spectra before and after warming of an irradiated sample, such as that of curve 14 in Figure 2a. This was done by Yoshizawa & Wald (1963) in their original paper on bathorhodopsin, but only after having taken special precautions to remove errors introduced during thawing and refreezing of the sample. A much simpler and more accurate method for obtaining at least a partial spectrum of the bathoproduct is also available that does not involve warming of the samples. This entails measuring the difference spectrum between a sample containing the bathoproduct and other rhodopsin isomers and the same sample after irradiation with light absorbed only by the bathoproduct. The spectra shown in Figure 2b satisfy our need. Figure 3 shows the difference spectrum between curve 1 (before irradiation) and curve 3 (after irradiation for 15 s) of Figure 2b (open circles). For comparison, the same difference spectrum obtained by Yoshizawa & Wald (1963) in their study of rhodopsin is also shown (solid circles). The two spectra are virtually identical.

That the photostationary state mixture after irradiation (curve 8, Figure 2b) contained only rhodopsin and its 9-cis isomer was shown in the following manner. The mixture was warmed to room temperature and then irradiated with light at wavelengths longer than 560 nm, primarily absorbed by rhodopsin. Since it is known that the photosensitivity of photobleaching of rhodopsin is much higher than that of 9-*cis*-rhodopsin (Hubbard & Kropf, 1958) under such selective bleaching conditions (Yoshizawa & Wald, 1964; Kawamura et al., 1978), only rhodopsin was expected to be destroyed during the initial stages of irradiation. Results of such a study are shown in Figure 4. The difference spectrum between curves 1 and 2 is identical with the absorption spectrum of rhodopsin (insert). Toward the end of the reaction, only 9-*cis*-rhodopsin was expected to remain. Indeed the difference spectrum between curves 6 and 14 is essentially the absorption spectrum of 9-*cis*-rhodopsin (insert).

Thermal Bleaching Sequence of the Bathoproduct from 7-cis-Rhodopsin upon Warming. The sample containing the bathoproduct from 7-*cis*-rhodopsin was warmed up gradually from −190 °C. As expected, its absorption spectrum changed during the warming process. For the purpose of recording the spectra at different stages, the process was interrupted periodically and the sample recooled to −190 °C. Eventually the

[2] The apparent shoulder near 550 nm of curve 1 is due to the base line of the spectrophotometer. After adjustment the shoulder was absent.

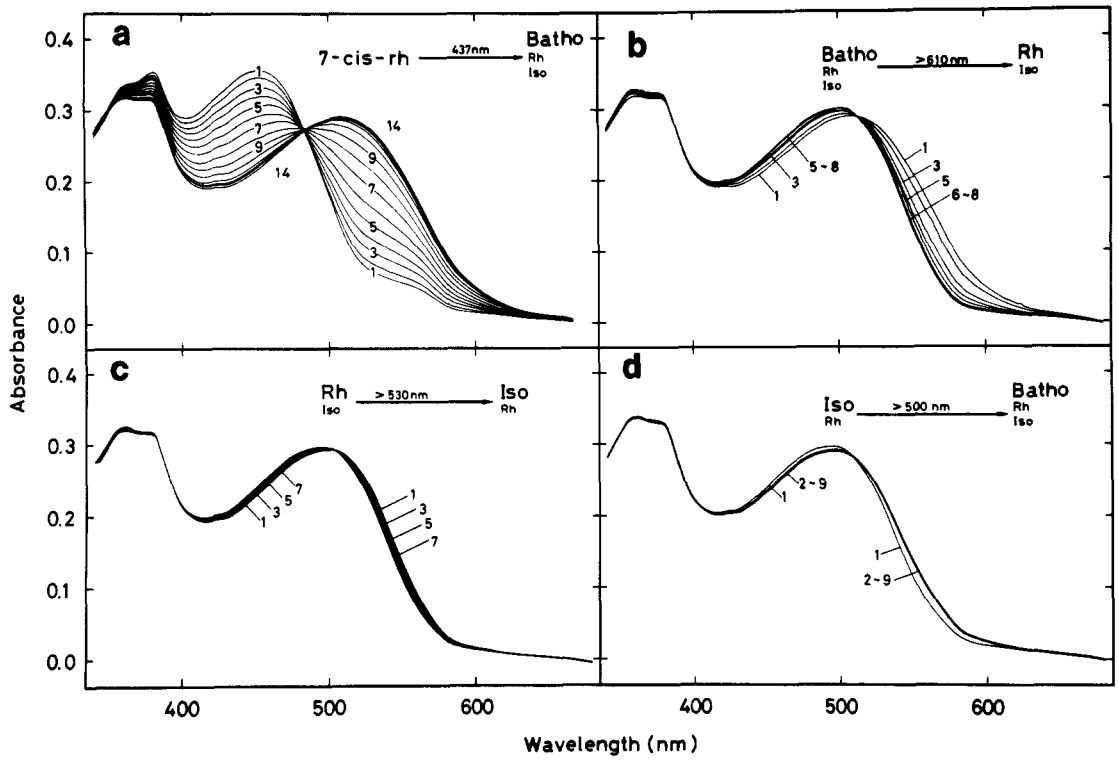

FIGURE 2: Photoreaction of 7-*cis*-rhodopsin at liquid nitrogen temperature (−190 °C). (a) 7-*cis*-Rhodopsin (curve 1) was irradiated at 437 nm for a total of 5, 15, 30, 60, 120, 240, 480, 960, 1920, 3840, 5760, 7860, and 9600 s (curves 2–14). The last curve represents a photostationary state mixture. (b) Curve 14 of (a) was redrawn as curve 1. The preparation was irradiated at wavelengths longer than 610 nm for a total of 5, 15, 30, 60, 120, 240, and 480 s (curves 2–8). The last curve represents another photostationary state mixture. (c) Curve 8 of (b) was redrawn as curve 1. The preparation was irradiated at wavelengths longer than 530 nm for a total of 5, 15, 30, 60, 120, and 240 s (curves 2–7). (d) Curve 7 of (c) was redrawn as curve 1. The preparation was irradiated at wavelengths longer than 500 nm for a total of 5, 15, 30, 60, 120, 240, 480, and 960 s (curves 2–9).

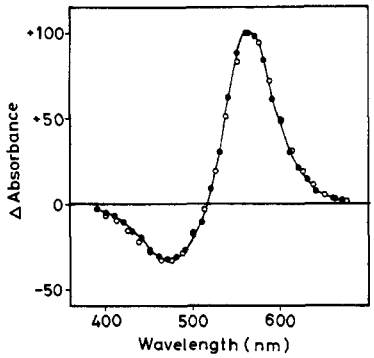

FIGURE 3: Difference spectrum between the bathoproduct and rhodopsin which were generated from 7-*cis*-rhodopsin. The difference spectrum between curves 1 and 3 in Figure 2b (●) is shown together with the difference spectrum between rhodopsin and bathorhodopsin (○) [from curves 1 and 3 at the bottom of Figure 4 in Yoshizawa & Wald (1963)].

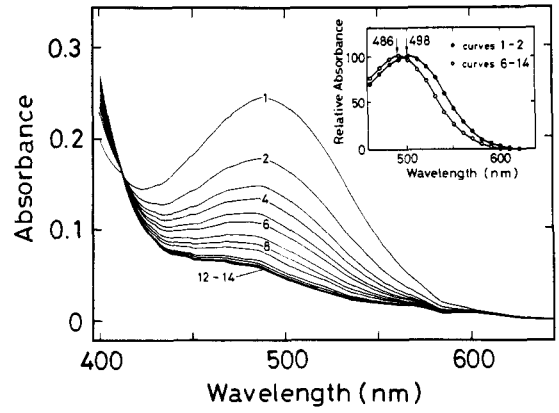

FIGURE 4: Photobleaching of photoproducts formed from 7-*cis* rhodopsin on irradiation at wavelengths longer than 610 nm. A mixture equivalent to that of curve 8 in Figure 2b was warmed up to room temperature (curve 1) and successively irradiated at wavelengths longer than 560 nm for a total of 5, 10, 15, 25, 35, 55, 95, 175, 335, 655, 1295, 2575, and 5135 s (curves 2–14). In the inserted figure are shown the difference spectra between curves 1 and 2 and between curves 6 and 14.

sample reached room temperature. In Figure 5, the absorption maxima of the sample, which contained different intermediates during different stages of warming, are plotted against the temperature (solid circles). The transition temperatures of one intermediate to another are evident. These transition temperatures, as well as the absorption maxima, appear to coincide exactly with the data obtained by warming a sample of bathorhodopsin obtained from rhodopsin (open circles). The only difference is the slight shift of the absorption maxima of the samples before warming. This slight difference probably is due to a small amount of unchanged 7-*cis*-rhodopsin.

Photosensitivity of 7-cis-Rhodopsin Relative to Its Isomers. By following the rates of disappearance of pigments in samples containing 7-*cis*-rhodopsin or mixtures of rhodopsin and 9-*cis*-rhodopsin, we obtained qualitative information relating the photosensitivity (relative quantum yield) of the 7-cis pigment in bathoproduct formation to those of the other isomers. A single sample of 7-*cis*-rhodopsin was used for the entire experiment. The sample was irradiated at −190 °C with 437-nm light, and the progress of the reaction was followed by the increase of absorbance at 580 nm corresponding to the appearance of the bathoproduct (similar to the experiment shown in Figure 2a). Upon completion of irradiation, the sample was converted to a mixture containing 75% rhodopsin and 25% 9-*cis*-rhodopsin [the percentages were estimated according to

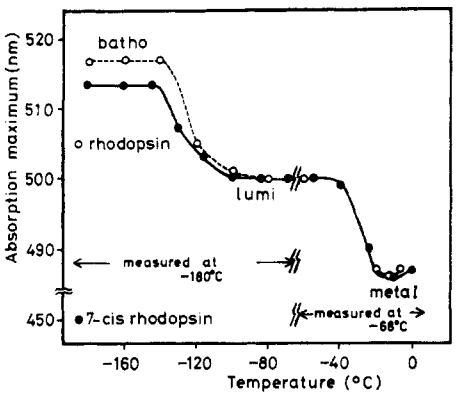

FIGURE 5: Thermal degradation of the bathoproduct from 7-*cis*-rhodopsin. The photostationary state mixture (corresponding to curve 14 in Figure 2a) was warmed briefly to a temperature indicated on the abscissa and then recooled to −190 or −68 °C to record the spectrum. The λ_{max} of the spectrum was plotted against the temperature to which the sample was warmed [(●) from 7-*cis*-rhodopsin; (○) from rhodopsin, data of Yoshizawa & Wald (1963)].

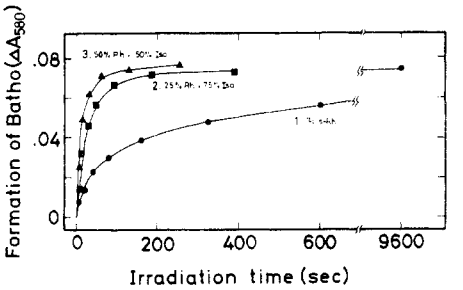

FIGURE 6: Relative photosensitivities of 7-*cis*-rhodopsin and rhodopsin and 9-*cis*-rhodopsin. Curve 1 (●): 7-*cis*-rhodopsin was successively irradiated at 437 nm at −190 °C with the progress of reaction monitored at 580 nm. Curve 2 (■): a mixture of rhodopsin and 9-*cis*-rhodopsin (1:3), obtained after irradiation of the final mixture of curve 1 with wavelengths longer than 610 nm for 480 s, was irradiated at 437 nm and the reaction followed at 580 nm. Curve 3 (▲): a mixture of rhodopsin and 9-*cis*-rhodopsin (1:1), obtained by irradiating the final mixture from curve 2 successively with wavelengths longer than 610 nm (480 s) and wavelengths longer than 530 nm (240 s), was irradiated at 437 nm and the reaction followed at 580 nm.

Kawamura's method (Kawamura et al., 1978)] by irradiation with light at wavelengths longer than 610 nm for 480 s (similar to the experiment in Figure 2b). With this mixture, the rate of formation of bathorhodopsin upon irradiation with 437-nm light was monitored at 580 nm. Finally, the sample was driven to a different (50:50) mixture of rhodopsin and 9-*cis*-rhodopsin by successive irradiations with lights at wavelengths longer than 610 nm for 480 s and then at wavelengths longer than 530 nm for 240 s (similar to the experiment in Figure 2c). Again, the rate of formation of bathorhodopsin from this third mixture as a result of irradiation at 437 nm was monitored at 580 nm. In Figure 6 are shown three curves corresponding to the progress of bathorhodopsin formation from the three mixtures. In spite of the much stronger absorbance of the 7-*cis*-rhodopsin at 437 nm than rhodopsin and 9-*cis*-rhodopsin, the plots clearly show that the bathoproduct from 7-*cis*-rhodopsin was formed at a much slower rate, indicating a much lower quantum yield for that reaction. The faster rate of bathorhodopsin formation from the third mixture compared with the second is consistent with the fact that the quantum yield of photobleaching of rhodopsin is higher than that of 9-*cis*-rhodopsin (Rosenfeld et al., 1977; Hubbard & Kropf, 1958).

Discussion

The foregoing results show that (1) the bathoproduct from 7-*cis*-rhodopsin has identical absorption properties with bathorhodopsin (Figure 3) and (2) the thermal bleaching properties (number of intermediates and transition temperatures between intermediates) of the bathoproduct from 7-*cis*-rhodpsin are identical with those of bathorhodopsin (Figure 5). These results appear to be compelling evidence that the bathoproduct from 7-*cis*-rhodopsin is in fact bathorhodopsin. Our results (parts a–d of Figure 2) further show that the bathorhodopsin formed from 7-*cis*-rhodopsin is readily convertible to rhodopsin and 9-*cis*-rhodopsin at liquid nitrogen temperature. This result clearly demonstrates that the same retinal binding site of opsin is involved in the three isomeric pigment analogues.

The low-temperature spectroscopic and photochemical results obtained in this work and those reported earlier for rhodopsin and 9-*cis*-rhodopsin do not unequivocally establish the structure of the chromophore of bathorhodopsin. However, we believe that the present results are consistent with the idea that bathorhodopsin is the strained all-trans isomer of rhodopsin (Yoshizawa & Wald, 1963). First, we failed to detect the formation of a blue-shifted bathorhodopsin even during early stages of irradiation of 7-*cis*-rhodopsin. Therefore, there is no evidence to suggest the presence of a primary photoproduct which still retains the severely hindered 7-cis geometry. Second, it is necessary to account for the fact that bathorhodopsin, the common primary photoproduct from rhodopsin and its 9-cis and 7-cis isomers, is apparently formed with ease from all three isomeric rhodopsins at low temperatures. The molecule that requires the least reorganization of structure from all three isomeric pigments should be the corresponding all-trans isomer.

We can now consider it as established that the 7-cis, 9-cis, and 11-cis isomers of rhodopsin must all involve the identical structure of the retinal binding site. Whether the 7,9-dicis, 7,13-dicis, 9,13-dicis, and 7,9,13-tricis isomers of rhodopsin (DeGrip et al., 1976; Crouch et al., 1975) involve the same binding site will be examined in the future, though there is now no evidence to suggest the contrary. That *all-trans*-rhodopsin (bathorohodopsin) is thermally unstable agrees with the fact that no stable pigment analogues are generated by the interaction of opsin with *all-trans*-retinal. All of these observations, as recently discussed by Matsumoto & Yoshizawa (1978), suggest that there is a longitudinal requirement of the binding site of opsin.

That 7-*cis*-rhodopsin cannot be re-formed by irradiation of rhodopsin and 9-*cis*-rhodopsin at −190 °C is interesting, particularly in view of the fact that at a higher temperature (−75 °C) such reversion is apparently possible (Maeda et al., 1978a). One possible explanation for the lack of reversion at −190 °C is the irreversible transformation of shape of the strained retinal binding site in 7-*cis*-rhodopsin upon irradiation. In fact, the slow rate of pigment formation during incubation of opsin and 7-*cis*-retinal is believed to be due to the need for opsin to reorganize itself conformationally in order to accommodate the structually different isomer (DeGrip et al., 1976). The resultant conformation is probably a strained one, contributing to the instability of the pigment in NH_2OH.

Finally, a few words of comment about the size and shape of the binding site of opsin appear appropriate. From the above discussion, it is clear that we take the view that the space available in the binding site is quite limited. That it can accommodate retinal isomers and analogues of different geometry is entirely due to the capability of the lipoprotein to undergo conformational reorganization. Therefore, there is

no exact, well-defined shape of the space constituting the binding site of the lipoprotein. It varies, taking the final shape that is compatible with the geometry of the retinal analogue. We believe that the view that the binding site has a large static empty space sufficient to accommodate all the different retinal analogues (further imposing some longitudinal restrictions) is not consistent with the experimental results reported here. For example, it cannot explain the varying degree of stability of the rhodopsin analogue in hydroxylamine, nor can it account for the failure to reverse the photoisomerization process from rhodopsin and 9-*cis*-rhodopsin back to 7-*cis*-rhodopsin.

Acknowledgments

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Effect of Hydrogen Bonding on Electronic Spectra and Reactivity of Flavins†

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ABSTRACT: Riboflavin tetrabutyrate undergoes characteristic spectral changes, in both the first and second absorption band regions, upon hydrogen bonding with trichloroacetic acid or trifluoroacetic acid. On the basis of the calculated electron densities, hydrogen bonding at the heteroatoms of the isoalloxazine nucleus is considered to occur with increasing concentrations of the proton donor, first at N(1), then at O(12), O(14), and N(3)H, and finally at N(5). The idea that the major effect of the hydrogen bonding at the N(1), N(3)H, and oxygen atoms of the flavin nucleus is to facilitate the electrophilicity of the N(5) position, which was predicted by molecular orbital calculations, was supported by the observation that the hydrogen-bonded flavin in its triplet state abstracts hydrogen from the donor *N*-benzyl-*N*,*N*′-dimethylethylenediamine at a faster rate than do the non-hydrogen-bonded species in CCl_4. The implications of the present study in the spectroscopic and catalytic properties of flavoproteins are briefly discussed.

The effect of hydrogen bonding on the electronic spectra of flavins has been studied by Yagi & Matsuoka (1956) and Kotaki et al. (1970). The effect of hydrogen bonding on the electronic structure and spectra of the flavin nucleus has also been described in terms of the self-consistent field molecular orbital method (Nishimoto et al., 1978). These studies suggested that the spectral characteristics of the flavin chromophore in *Clostridium* MP and *Desulfovibrio vulgaris* flavodoxins (Mayhew & Ludwig, 1975) can be at least partly accounted for in terms of hydrogen bonding, the occurrence of which in these proteins was verified by X-ray crystallography (Ludwig et al., 1976; Watenpaugh et al., 1976).

In the present work, we performed an experimental study using model systems in order to ascertain the possible consequence of hydrogen bonding for the spectral characteristics and catalytic reactivity of flavins. For the assessment of the catalytic reactivity of the flavin nucleus as an acceptor of electrons and nucleophiles, we adopted the flavin photoreactivity on the basis of our analysis of the flavin's N(5) elec-

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