9,13-*dicis*-Rhodopsin and Its One-Photon–One-Double-Bond Isomerization†

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ABSTRACT: Incubation of purified 9,13-*dicis*-retinal with cattle opsin in 2% digitonin at 20 °C produced two pigments, one unstable (λ_{max} 478 nm) and the other stable (λ_{max} 485 nm) in hydroxylamine. The two pigments exhibited different characteristics. HPLC analysis revealed that the chromophores of these pigments have respectively 9,13-dicis and 9-cis geometries. Under various conditions the amount of 9,13-*dicis*-rhodopsin formed never exceeded 30% of the total pigments. The addition of 9,13-*dicis*-retinal to the ROS suspension containing opsin produced 9-*cis*-rhodopsin in 97% yield. Irradiation of the 9,13-*dicis*-rhodopsin mainly produced 13-*cis*-retinal, while 9-*cis*-rhodopsin produced the all-trans isomer. These results demonstrated that the one-photon–one-double-bond isomerization process took place in 9,13-*dicis*-rhodopsin.

Recently, a large number of retinal analogues and isomers have been synthesized and prepared to respective rhodopsin analogues and isomers, the properties of which led to much new information related to the binding site of opsin and mechanistic details on the photobleaching processes [see recent reviews of Derguini and Nakanishi (1986) and Shichida (1986)]. On the basis of results of new rhodopsin isomers (DeGrip et al., 1976), the concept of longitudinal restriction of the chromophore within the binding site was advanced (Matsumoto & Yoshizawa, 1978). Subsequently, an extension of this concept led to the construction of a two-dimensional projection map for the binding site (Liu et al., 1984). The geometric isomerization mechanism for the primary photochemical process of visual pigments, proposed as a result of early low-temperature spectroscopic studies of rhodopsin and 9-*cis*-rhodopsin (Kito et al., 1961; Yoshizawa & Wald, 1963) was reconfirmed through similar studies of two kinds of rhodopsin analogues having 11-cis-ring-locked analogues: cycloheptatrienylidene (Mao et al., 1981) and cyclopentatrienylidene (Fukada et al., 1984) retinals.

Our interest in the photochemistry of rhodopsin analogues containing the dicis geometry was prompted by two recent postulated models for the photochemical process of the visual chromophore, both involving concerted twist of two bonds: two double bonds in the "Bicycle Pedal" model (Warshel, 1976) and a double bond with an adjacent single bond in the "Hula Twist" model (Liu & Asato, 1985). Since one-photon–two-double-bond isomerization was reported for the 9,13-*dicis*-rhodopsin system (Crouch et al., 1975), we began our study with the more recently available 9,11-*dicis*-retinal (Kini et al., 1980). However, in a preliminary study we found that under a variety of conditions for formation of the pigment a substantial amount of 9-*cis*-rhodopsin was formed concomitantly with 9,11-*dicis*-rhodopsin (Shichida et al., 1987). This observation raised the question of possible isomerization in the 9,13-dicis system. Such a conclusion was in fact reported in an early paper (Hubbard & Wald, 1952), but a subsequent study led to the belief of complete retention of the dicis configuration during pigment formation (Crouch et al., 1975).

Now, we report a detailed reinvestigation of the 9,13-*dicis*-rhodopsin system. The current findings appear to support the earlier claim by Hubbard and Wald that the formation of the 9,13-dicis pigment is accompanied by a substantial amount of isomerization to the 9-cis chromophore. Photochemical properties of these pigments in relation to the postulated one-photon–two-double-bond isomerization process will be discussed.

Materials and Methods

Preparation of Retinal Isomers and Their Oximes. 9,13-*dicis*-Retinal was synthesized according to the established $C_{15} + C_5$ procedure (Liu & Asato, 1982). The dicis isomer was separated from the 9-cis isomer by use of preparative HPLC[1] (column, 25 mm 5μ-Lichrosorb; solvent, 5% diethyl ether in hexane). Other monocis isomers of retinal (9-*cis*, 11-*cis*, and 13-*cis*) were prepared by irradiation of *all-trans*-retinal (Sigma) dissolved in acetonitrile with light from a 2-kW xenon lamp (Ushio Co. Ltd.) for 30 min at 0 °C and isolated by means of HPLC (Ono et al., 1986).

Retinal oximes of each isomer were used as the standard for identification of the chromophore extracted from each pigment. They were prepared as follows: One-tenth volume of neutralized hydroxylamine solution (1 M, pH 7.0) was added to an ethanol solution of each isomer for formation of its retinal oxime, followed by drying the solution under a nitrogen stream and extracting the oxime by hexane. Each retinal isomer and its oxime were stored at −80 °C until use.

Preparation of Cattle Opsin. Rod outer segments (ROS) of cattle retina were prepared by use of conventional sucrose stepwise flotation method (Shichida et al., 1987a). The ROS thus obtained was suspended in 10 mM HEPES buffer containing 10 mM hydroxylamine (pH 7.0) and irradiated with

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[1] Abbreviation: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; ROS, rod outer segment.

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orange light (>520 nm) for bleaching of rhodopsin to retinal oxime and opsin. After five washings with 10 mM HEPES buffer by centrifugation, the ROS was lyophilized followed by treatment of light petroleum ether for removing the retinal oxime. The ROS thus obtained was used for the extraction of opsin by 2% digitonin dissolved in 10 mM HEPES buffer (pH 7.0).

Pigment Formation. The formation of 9,13-*dicis*-rhodopsin was first attempted by incubating ROS suspension containing opsin with 2 times molar excess of 9,13-*dicis*-retinal according to methods described by Crouch et al. (1975). The pigments thus produced were constituted mainly of 9-*cis*-rhodopsin and a negligible amount (less than 2%) of 9,13-*dicis*-rhodopsin according to results of chromophore extraction experiments (see below). In an attempt to increase the yield of 9,13-*dicis*-rhodopsin, we varied the amount (0.2–5 times excess) of 9,13-*dicis*-retinal added to the ROS suspension but could not prepare any sample containing mainly 9,13-*dicis*-rhodopsin. In our hands the best sample containing about 3% of 9,13-*dicis*-rhodopsin (the remainder being the 9-*cis*-rhodopsin) was obtained when 0.2 molar equivalent of 9,13-*dicis*-retinal was added to the ROS suspension. On the other hand, reaction of opsin extracted with 2% digitonin from the ROS suspension with 9,13-*dicis*-retinal was found to give relatively larger amounts (up to 30%) of 9,13-*dicis*-rhodopsin. Hence, the latter procedure was followed in most of this study.

9,13-*dicis*-Retinal in ethanol was added in the digitonin extract containing opsin and then incubated at 20 °C for about 20 h. The amount of 9,13-*dicis*-retinal added to the opsin solution was about 2.5 times smaller in molar concentration than that of opsin so as to ensure complete reaction of the retinal.

Extraction of the Chromophores from the Pigments. The chromophores of the pigments formed by incubation of 9,13-*dicis*-retinal and cattle opsin was extracted by a method slightly modified from that in the literature (Suzuki & Makino-Tasaka, 1983). The sample (maximal absorbance about 0.2) in 1 mL of digitonin extract containing 100 mM of neutralized hydroxylamine was mixed with equal volumes of CH_2Cl_2 and methanol. After vigorous trituration by use of ULTRA-TURRAX (Janke & Kunkel), 5 mL of hexane was added, followed by retrituration. The hexane layer containing the chromophore was transferred into a test tube, dried over anhydrous Na_2SO_4, and evaporated under N_2 stream. The residue was dissolved in 200 μL of hexane, 7 μL of which was analyzed by HPLC [Shimadzu–Du Pont LC-1 system equipped with a Zorbax SIL column (4.0 × 150 mm)] for isomeric composition. The solvent used was a mixture composed of benzene, diethyl ether, and 2-propanol (95:4.4:0.6 v/v) with a flow rate of 1 mL/min. A Shimadzu SPD-1 detector was employed with the detecting beam set at 360 nm. Each retinal oxime peak was assigned by comparison of retention times with those of authentic samples prepared as described above. All the samples were handled under dim red light and ice-chilled conditions.

In order to check the effect of digitonin on the thermal isomerization of the chromophore during the extraction procedure, the pigment formed by incubation of 9,13-*dicis*-retinal with opsin in ROS suspension was divided into two parts. One part was extracted with 2% digitonin, and the other part remained untreated. Then both parts were brought to the chromophore extraction, resulting in indistinguishable isomer patterns in the chromatograms. The facts indicated that digitonin had no effect on the thermal isomerization of the chromophores.

all-trans-Retinal oxime was not thermally isomerized to the 13-cis isomer under our experimental conditions.

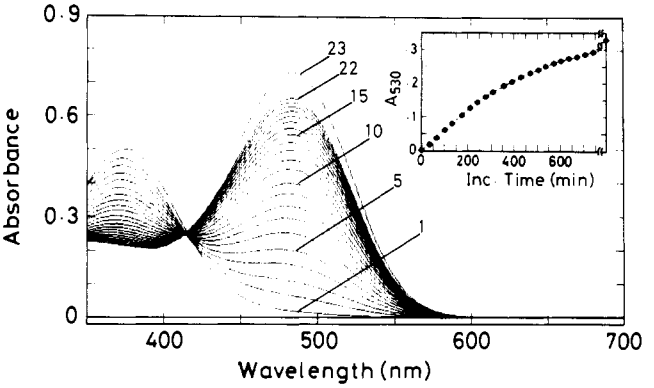

FIGURE 1: (a) Formation of 9,13-*dicis*-rhodopsin by incubation of chromatographically purified 9,13-*dicis*-retinal and cattle opsin at 20 °C. 9,13-*dicis*-Retinal in ethanol was added to a 2% digitonin extract of cattle opsin in 10 mM HEPES buffer (pH 7.0). The absorption spectra were recorded successively at intervals of from 32 to 36 min (curves 1–22). Curve 23 was a difference absorption spectrum of the sample after 20 h of incubation before and after irradiation with orange light (>520 nm). (Insert) Increase in absorbance at 530 nm of the sample plotted against incubation time.

Spectroscopy and Actinic Light. Absorption spectra were recorded on a Hitachi-330 spectrophotometer interfaced with a NEC PC-9801F computer and the CD spectra on a Jasco J-20 spectropolarimeter. A tungsten–halogen lamp (1 kW, Sanko), was used as a light source of actinic light, in conjunction with a glass cutoff filter (Toshiba V052 or V059). A glass optical cell filled with water (light path, 6 cm) was placed as a heat shield between the filter and the light source.

RESULTS

Spectral changes during the course of reaction of 9,13-*dicis*-retinal with cattle opsin (2.5 times in excess) are shown in Figure 1. In the insert is shown the change of absorbance at 530 nm as the reaction proceeded. Difference absorption spectra were calculated from these spectra. Interestingly, subtracting curve 1 from curve 3 in Figure 1 gave a spectrum with an absorption maximum at slightly shorter wavelength than that calculated by subtracting curve 4 from curve 6, while the latter was indistinguishable from that calculated by subtracting curve 19 from curve 22. These observations suggested that at least two pigments, different in absorption maxima from each other, were formed during incubation of 9,13-*dicis*-retinal with cattle opsin.

Figure 2a shows thermal decomposition of the pigment after addition of neutralized hydroxylamine to give a final concentration of 100 mM and photobleaching by irradiation with an orange light (>520 nm). The first-order kinetics displayed by the thermal decomposition process (Figure 2b) should most likely be due to one pigment. After complete decomposition of the fraction of the unstable pigment, the residual pigment was bleached with the orange light (curve 15 in Figure 2a) until completion. The photobleaching process also followed the first-order kinetics (Figure 2c), indicating that the hydroxylamine-stable fraction also contained only one pigment. On the other hand, a distinctly different spectrum was obtained (curve 16 in Figure 2a) when the irradiation was carried out immediately after addition of the neutralized hydroxylamine. This difference indicated that the photobleaching product of the hydroxylamine-stable pigment was different in absorption spectrum and hence in structure from that of the thermally decomposed product.

Figure 3 shows the chromatograms of the composition of isomeric chromophores extracted from the pigments formed

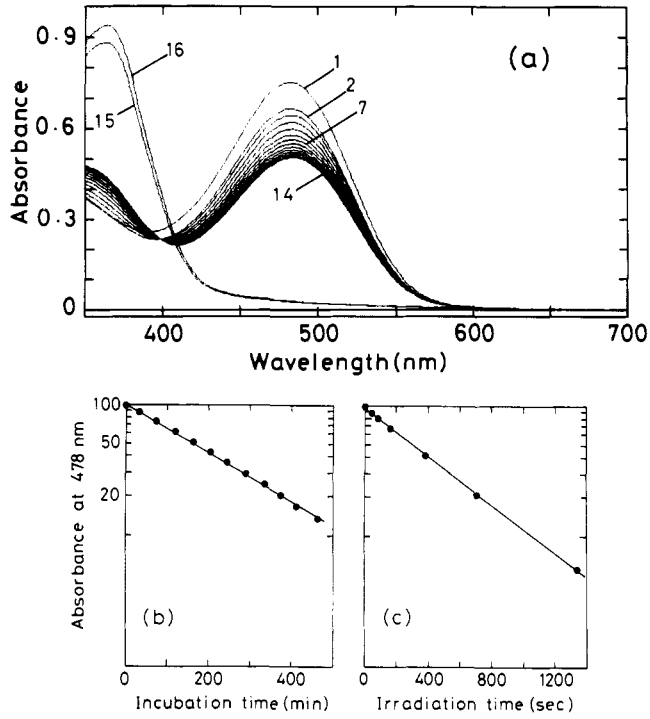

FIGURE 2: (a) Effect of hydroxylamine and subsequent irradiation with orange light on the pigments produced from 9,13-*dicis*-retinal and cattle opsin. Curve 1 shows the absorption spectrum of the pigment produced from 9,13-*dicis*-retinal and cattle opsin. After addition of neutralized hydroxylamine (1 M) at a final concentration of 100 mM (curve 2), the spectrum of the sample was successively measured at intervals of about 40 min (curves 3–14). The sample was then irradiated with light >520 nm (curve 15). Curve 16 shows the absorption spectrum of the same sample irradiated with the orange light just after addition of hydroxylamine. (b) Decrease in absorbance at 478 nm of the absorption spectrum of the sample during the incubation in 100 mM hydroxylamine plotted against incubation time. (c) Decrease in absorbance at 478 nm of the absorption spectrum of the sample after incubation in 100 mM hydroxylamine plotted against irradiation time with light >570 nm.

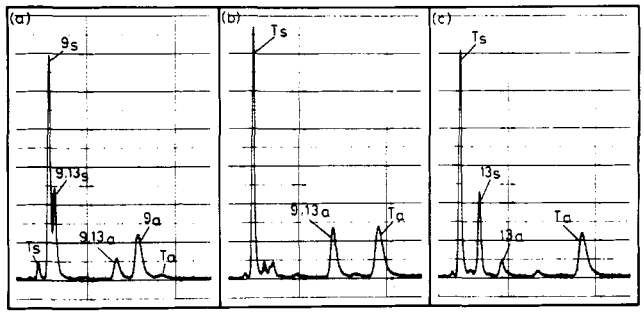

FIGURE 3: HPLC patterns of the chromophore extracted from the pigments produced from 9,13-*dicis*-retinal and cattle opsin: (a) original pigment; (b) irradiated after incubation for about 9.5 h with 100 mM hydroxylamine; (c) irradiated immediately after addition of 100 mM hydroxylamine.

from 9,13-*dicis*-retinal with cattle opsin. The HPLC pattern of the chromophore extracted from the original pigment (corresponding to curve 2 in Figure 2a) revealed roughly a 2:1 ratio of the 9-cis isomer to the 9,13-dicis isomer (Figure 3a, Table I), indicating that the 9-cis pigment was concomitantly formed with the 9,13-dicis pigment. The presence of small peaks corresponding to *all-trans*-retinal oxime, which should not originate from the pigment, could be due to thermal isomerization of 9,13-*dicis*-retinal via the 9-cis form during incubation.

The HPLC pattern of the chromophore extracted from the sample irradiated after incubation in the presence of the 100 mM hydroxylamine (corresponding to curve 15 in Figure 2a)

Table I: Molar Composition of Isomers of Retinal Oxime Extracted from the Pigment Produced by Incubation of 9,13-*dicis*-Retinal with Cattle Opsin

	molar % of isomers			
	9,10-dicis	9-cis	all-trans	13-cis
original pigments	28.8	67.2	4.0	
incubation in the presence of NH_2OH followed by irradiation	27.4	3.9	65.9	2.9
irradiation immediately after addition of NH_2OH	3.0	1.4	70.3	25.4

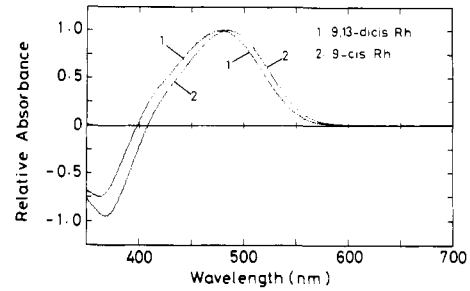

FIGURE 4: Difference absorption spectra of 9,13-*dicis*- and 9-*cis*-rhodopsins. The difference absorption spectrum of 9,13-*dicis*-rhodopsin (1) was obtained by subtracting curve 14 (9,13-*dicis*-retinal oxime) from curve 2 (9,13-*dicis*-rhodopsin) in Figure 2a. The difference absorption spectrum of 9-*cis*-rhodopsin (2) was obtained by substracting curve 15 (*all-trans*-retinal oxime) from curve 14 (9-*cis*-rhodopsin) in Figure 2a. These difference spectra were normalized at absorption maxima.

is shown in Figure 3b. The major peaks in the figure were identified as syn and anti forms of *all-trans*-retinal oxime and only the anti form of 9,13-*dicis*-retinal oxime. The presence of the latter shows conclusively that the chromophore of the hydroxylamine-unstable pigment intercepted directly by hydroxylamine before the irradiation must have retained the 9,13-dicis geometry while the chromophore of the hydroxylamine-stable pigment in a 9-*cis* form was converted to the all-trans form upon irradiation as established earlier (Yoshizawa & Wald, 1963).

Figure 3c shows an HPLC pattern of the chromophore extracted from the sample irradiated immediately after addition of 100 mM hydroxylamine (corresponding to curve 16 in Figure 2a). In addition to the expected peaks from the all-trans isomer, the syn and anti forms of 13-*cis*-retinal oxime were irrefutably detected. Therefore, irradiation of 9,13-*dicis*-rhodopsin must have produced primarily the 13-cis chromophore rather than the all-trans isomer.

Pursuing the latter conclusion, we calculated the isomeric compositions as revealed in the chromatograms in Figure 3. The molar extinction coefficients at 360 nm of the syn forms of *all-trans*- and all *monocis*-retinal oximes and those of anti forms of *all-trans*- and 13-*cis*-retinal oximes were reported (Groenendijk et al., 1979; Tsukida et al., 1985; Hamanaka et al., 1986). We have now determined the molar extinction coefficients of the syn and anti forms of 9,13-*dicis*-retinal oxime and of the anti form of 9-*cis*-retinal oxime at 360 nm to be 37 600, 31 000, and 30 600, respectively, by first separating each form by preparative HPLC. The result (Table I) showed that almost all the chromophore of 9,13-dicis pigment was converted to 13-*cis*-retinal oxime.

Having established that hydroxylamine-unstable and -stable pigments are respectively 9,13-*dicis*- and 9-*cis*-rhodopsins, we now show the difference absorption spectra of the 9,13-*dicis*-rhodopsin and 9-*cis*-rhodopsin (Figure 4) from the appropriate curves in Figure 2a. Since 9,13-*dicis*- and *all-trans*-retinal oximes do not absorb at wavelengths longer than 420 nm, the difference absorption spectra in this region must

be due to the absolute absorption spectra of 9,13-*dicis*- and 9-*cis*-rhodopsins.

The absorption maximum of 9,13-*dicis*-rhodopsin was located at 478 nm and that of 9-*cis*-rhodopsin at 485 nm. It should be noted that the absorption spectrum of 9-*cis*-rhodopsin produced by incubation of 9,13-*dicis*-retinal with cattle opsin was identical with that of 9-*cis*-rhodopsin produced directly from incubation of 9-*cis*-retinal with cattle opsin.

The molar extinction coefficient of 9,13-*dicis*-rhodopsin was estimated to be 36 000 by comparing the ratio of absorbance and content between 9,13-*dicis*- and 9-*cis*-rhodopsins in the sample, assuming that the molar extinction coefficient of 9-*cis*-rhodopsin is 43 000 (Hubbard et al., 1971).

CD spectra of 9,13-*dicis*- and 9-*cis*-rhodopsins in the sample were also calculated from the respective CD spectra of the sample corresponding to curves 2, 14, and 15 in Figure 2a. CD maxima at the α-band of 9,13-*dicis*-rhodopsin and 9-*cis*-rhodopsin were located at 465 and 475 nm, respectively. Molar ellipticity at the α-band of 9,13-*dicis*-rhodopsin was about 1.4 times larger than that of 9-*cis*-rhodopsin, suggesting that the chromophore of 9,13-*dicis*-rhodopsin was more twisted at the center of the chromophore than that of 9-*cis*-rhodopsin. Molar ellipticity at the β-band of 9,13-*dicis*-rhodopsin was 0.2–0.4 times smaller than that of 9-*cis*-rhodopsin. CD maximum at the β-band of 9,13-*dicis*-rhodopsin could not be estimated because of the small ellipticity and the limited amount of 9,13-*dicis*-rhodopsin.

When an excess amount (about 5 times higher in molar concentration) of 9,13-*dicis*-retinal was incubated with opsin in digitonin extract, a smaller amount (15%) of 9,13-*dicis*-rhodopsin was produced than that (28.8%) from lesser amount of 9,13-*dicis*-retinal. Experiments carried out at two different temperatures (20 and 0 °C) revealed an absence of temperature dependence for formation of the 9,13-*dicis*-rhodopsin.

Discussion

The major points of interest from the current reinvestigation of 9,13-*dicis*-rhodopsin are the following. First, 9,13-*dicis*-retinal did not give pigment with complete retention of configuration. In fact, in our hands, the 9-*cis*-rhodopsin was found to be invariably the major product pigment. This is not in agreement with the results reported by Crouch et al. (1975), where complete retention of configuration was claimed. While the conditions used in this study (in digitonin) are different from those employed by Crouch et al. (in ROS suspension or with Ammonyx-LO), we found that only in digitonin could moderate amounts of 9,13-*dicis*-rhodopsin be obtained. The formation of 9,13-*dicis*-rhodopsin was not possible in Ammonyx-LO, and in ROS suspension the amount of the 9,13-dicis pigment never exceeded 3%.

Second, in agreement with Crouch et al., we found that 9,13-*dicis*-rhodopsin exhibited spectral characteristics different from those of 9-*cis*-rhodopsin. However, for 9,13-*dicis*-rhodopsin we observed an absorption maximum at 478 nm (20 °C in 2% digitonin), which is 7 nm shorter in wavelength than that of 9-*cis*-rhodopsin (485 nm) instead of the reported 481 nm (in 2% Ammonyx-LO) and 2 nm shorter in wavelength than that of 9-*cis*-rhodopsin (483 nm) in 2% Ammonyx-LO. Also, we found that 9,13-*dicis*-rhodopsin displayed a large CD at the α-band (maximum at 465 nm), 1.4 times larger than that of 9-*cis*-rhodopsin. This is also different from that described by Crouch et al., who claimed to have detected similar CD maxima for the two pigments.

Third, we have established that photoisomerization of 9,13-*dicis*-rhodopsin is stereospecific, giving only the 13-cis isomer. Clearly, it is a one-photon–one-double-bond isomerization process. The regioselectivity of the process (i.e., at the 9,10-bond instead of at the 13,14-bond) is striking. Probably this result reflects the highly confined nature of the binding site near the iminium center of the chromophore in rhodopsin. The current results do not support the claim of a one-photon–two-double-bond isomerization reported for this system (Crouch et al., 1975).

A related issue on the confined binding site is probably the observation of stereospecific formation of the *anti*-oxime during reaction of hydroxylamine with the unstable 9,13-*dicis*-rhodopsin. This result is uniquely different from all other chromophore extraction work, where stable pigments require prior denaturation of the protein. We suspect this unique stereochemical result is due to the partially protected environment of the unstable 9,13-*dicis*-rhodopsin with the consequence that a protein residue(s) directs effectively the approach of hydroxylamine to the imino group in a stereospecific manner, giving exclusively the anti isomer. Hence, it is not surprising that when denaturation preceded chromophore extraction, 9,13-*dicis*-rhodopsin also gave a mixture of *syn*- and *anti*-oximes.

Instead of the earlier picture of a stable 9,13-*dicis*-rhodopsin, the current results are more consistent with the following molecular pathways during incubation of 9,13-*dicis*-retinal with opsin. The *dicis*-retinal reacts with opsin at a slow rate comparable to its isomerization to the more stable 9-*cis*-retinal. The latter, in turn, reacts at a much faster rate with opsin, giving a relatively higher proportion of 9-*cis*-rhodopsin. Hence, the employment of a large excess of the *dicis*-retinal during incubation with opsin only enhances the isomerization rate, resulting in the formation of a larger amount of 9-*cis*-rhodopsin.

Ready isomerization of 9,13-*dicis*-retinal under the incubation condition was demonstrated in the following experiment. 9,13-*dicis*-Retinal was incubated with thermally denatured opsin in a 2% digitonin solution in a ratio equivalent to that described in Figure 1. Retinal was then extracted periodically and its isomer composition analyzed by HPLC. Within 2 h, the amount of 9-*cis*-retinal was found to reach a maximum of 74%, which diminished subsequently due to isomerization to the all-trans isomer.

Finally, we might add that the current method for product analysis, whether of the pigments or their photoproducts, does not allow detection of structures involving rotation of a single bond or the imino double bond in the polyene chromophore. Such structural features are relevant for consideration of the theoretical models requiring two bond rotations for the primary photochemical process. Future FTIR or resonance Raman studies on this and related systems might provide additional structural data useful for elucidation of the specific nature of the isomerization process.

Acknowledgments

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Registry No. 9,13-*dicis*-Retinal, 23790-80-9; *syn*-9,13-*dicis*-retinal oxime, 115073-51-3; *anti*-9,13-*dicis*-retinal oxime, 115073-50-2; *anti*-9-*cis*-retinal oxime, 72689-82-8.

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Involvement of Tryptophan Residues at the Coenzyme A Binding Site of Carbon Monoxide Dehydrogenase from *Clostridium thermoaceticum*†

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ABSTRACT: Carbon monoxide dehydrogenase (CODH) from *Clostridium thermoaceticum* plays a central role in the newly discovered acetyl-CoA pathway [Wood, H. G., Ragsdale, S. W., & Pezacka, E. (1986) *FEMS Microbiol. Rev. 39*, 345–362]. The enzyme catalyzes the formation of acetyl-CoA from methyl, carbonyl, and CoA groups, and it has specific binding sites for these moieties. In this study, we have determined the role of tryptophans at these subsites. *N*-Bromosuccinimide (NBS) oxidation of the exposed and reactive tryptophans (5 out of a total of ~20) of CODH at pH 5.5 results in the partial inactivation of the exchange reaction (~50%) involving carbon monoxide and the carbonyl group of the acetyl-CoA. Also, about 70% of the acetyl-CoA synthesis was abolished as a result of NBS modification. The presence of CoA (10 μM) produced complete protection against the partial inhibition of the exchange activity and the overall synthesis of acetyl-CoA caused by NBS. Additionally, none of the exposed tryptophans of CODH was modified in the presence of CoA. Ligands such as the methyl or the carbonyl groups did not afford protection against these inactivations or the modification of the exposed tryptophans. A significant fraction of the accessible fluorescence of CODH was shielded in the presence of CoA against acrylamide quenching. On the basis of these observations, it appears that certain tryptophans are involved at or near the CoA binding site of CODH.

Carbon monoxide dehydrogenase (CODH)[1] from *Clostridium thermoaceticum* catalyzes the final steps of the newly proposed acetyl-CoA pathway (Wood et al., 1986a–c). Several of the enzymes involved in this complex pathway have been purified and characterized (Drake et al., 1981; Hu et al., 1984; Ragsdale & Wood, 1985; Pezacka & Wood, 1986). CODH is an $\alpha_3\beta_3$ metalloenzyme and has a molecular weight of 440 000 (Ragsdale et al., 1983a,b). Ragsdale and Wood (1985) have shown that CODH per se catalyzes exchange of ^{12}CO with [1-^{14}C]acetyl-CoA ($CH_3{}^{14}COSCoA + {}^{12}CO \leftrightarrow CH_3{}^{12}COSCoA + {}^{14}CO$). This exchange involves cleavage of both the C–C and C–S bonds of the acetyl-CoA, resynthesis of the acetyl-CoA, and binding of the CH_3, CO, and CoA groups to CODH. On the basis of this exchange reaction, they have proposed that CODH catalyzes the final steps of the

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[1] Abbreviations: CODH, carbon monoxide dehydrogenase; DTT, dithiothreitol; NBS, *N*-bromosuccinimide; CoA, coenzyme A; Trp, tryptophan; ESR, electron spin resonance; CD, circular dichroism; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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