Specificity of the Retinal Binding Site of Bacteriorhodopsin: Chemical and Stereochemical Requirements for the Binding of Retinol and Retinal†

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ABSTRACT: The complexes formed from bacterioopsin and various retinyl compounds were analyzed by fluorescence and absorption spectroscopy. The binding of retinol occurs in two steps. In the first reaction the molecule is fixed in the retinal binding site of the protein. In this state, energy transfer from aromatic amino acid residues to the retinyl moiety is observed. *all-trans*-Retinal and the 13-, 11-, and 9-*cis*-retinols are bound in the chromophoric site. In the second reaction the cyclohexene ring and the side chain of the retinyl moiety are forced into a planar conformation. This reaction is mediated by a base (B_1) with a pK of 3.8 and requires the oxygen atom but not the free hydroxyl group of retinol, indicating interaction with a group AH (p$K \geq 10.5$). The ring-chain planarization reaction is blocked for the 9-cis isomer of retinol. Binding studies with bacterioopsin and retinal isomers reveal that, as in the case of the corresponding retinols, B_1 mediates ring-chain planarization in the case of the all-trans, 13-cis, and 11-cis isomers but not with the 9-cis isomer. Reconstitution of the purple complex from the intermediate 430–460-nm chromophore requires the presence of a second base (B_2) with a pK of 4.6. This reaction is exclusive for *all-trans*- and 13-*cis*-retinal.

The purple membrane is a specialized area of the halobacterial cell membrane and contains the retinal–protein complex bacteriorhodopsin as the only protein component. Bacteriorhodopsin has a molecular weight of 26 000 and functions as a photoreceptor. Light absorption by its chromophore initiates a photochemical cycle (for review, see Henderson, 1977; Lozier and Niederberger 1977) which is accompanied by proton translocation into the medium and thereby produces an electrochemical proton gradient across the cell membrane (Michel and Oesterhelt, 1976; Bakker et al., 1976; Belyakova et al., 1975). The energy of the proton gradient is used for various energy-dependent processes inside the halobacterial cell such as photophosphorylation, amino acid transport, and potassium ion uptake (Hartmann and Oesterhelt, 1977; MacDonald and Lanyi, 1977; Wagner and Oesterhelt, 1976; Wagner et al., 1978).

The retinal-containing chromophore of bacteriorhodopsin (purple complex, λ_{max} around 560 nm) mediates the proton translocation process in the purple membrane. The chemical structure of the purple complex and its changes during the photochemical cycle are not yet understood. Feasible experimental approaches toward an understanding of retinal–protein interaction have been illustrated previously by Schreckenbach and Oesterhelt (1977), and Schreckenbach et al. (1977, 1978). This report describes several distinct functional groups in the protein interacting with retinal and retinyl moieties.

Experimental Procedure

Materials. Purple membrane was isolated from R_1M_1 cells as described previously (Oesterhelt and Stoeckenius, 1974). Apomembrane and oxime-free apomembrane were prepared according to Oesterhelt and Schuhmann (1974); product RP_{hv} and protein-bound retinol were prepared according to Schreckenbach et al. (1977).

all-trans-Retinal and 13- and 9-*cis*-retinal were obtained from Sigma; 11-*cis*-retinal was kindly provided by P. Towner. The purity of the retinal isomers was checked by thin-layer chromatography of the corresponding oximes (Oesterhelt et al., 1973). Retinol isomers were prepared by reduction of the corresponding retinals with $NaBH_4$ in 80% ethanol. *retro*-Retinol was synthesized as described by Göβwein (1976). The all-trans isomers of retinyl methyl ether, axerophthene, retinyl acetate, retinoic acid, and retinoic acid methyl ester were a gift of Dr. A. Nürrenbach, BASF. They were checked for purity by thin-layer chromatography in ethyl acetate–*n*-hexane (1:4) and by mass spectrometry. Retinylmethylamine was synthesized from methylamine and retinal according to Akthar et al. (1968). The product was purified by preparative thin-layer chromatography in 1-butanol–acetic acid–chloroform (20:9:1.5) and identified by mass spectrometry. All experiments with retinal, retinol, and their derivatives were performed in dim room light or under red-light illumination using a Kodak Wratten No. 1 filter.

Spectroscopic Methods. Absorption spectra were recorded on an Aminco DW 2 spectrophotometer using a slit width of 3 nm. Corrected fluorescence spectra were taken on a Schoeffel instrument equipped with two GM 250 monochromators for the exciting light. Automatic correction was performed by a RRS 1000 Schoeffel unit using a rhodamin B solution as a fluorescence standard. The membrane suspensions subjected to fluorescence spectroscopy had a maximal absorbance of 0.1 at 280 nm. Mass spectrometry of 10–100-μg samples was performed on a Varian MAT CH-7.

Results and Discussion

Reversible Ring–Chain Planarization in the Binding Site. The chromophore of bacteriorhodopsin is bleached irreversibly when exposed to hydroxylamine in the presence of light. Under these conditions, apomembrane is formed which contains the chromophore-free protein, bacterioopsin, and retinal oxime (Oesterhelt and Schuhmann, 1974). Bacterioopsin and retinal together reconstitute the purple complex. This reaction is a valuable tool in the study of retinal–protein interaction because

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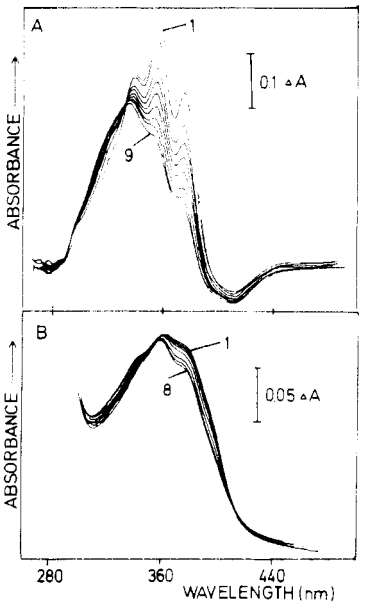

FIGURE 1: pH-dependent conformational changes of retinyl moieties in the binding site. (A) Interconversion of the 357- (spectrum 1, pH 4.70) and 336-nm forms (spectrum 9, pH 3.20) of protein-bound retinol (8 μM). The pH of the sample is (2) 4.20, (3) 3.85, (4) 3.75, (5) 3.65, (6) 3.51, (7) 3.40, and (8) 3.30. (B) Interconversion of the species (spectrum 1, pH 5.80) and acidic species (spectrum 8, pH 3.10) of product RP_{hv} (4 μM), both having their absorption maximum around 360 nm. The pH of the sample is (2) 4.25, (3) 4.00, (4) 3.80, (5) 3.60, (6) 3.40, and (7) 3.20. Both samples were suspended in 50 mM KCl and stirred in 3-mL cuvettes (d = 1 cm) at 2 °C during titration with aliquots of 0.1 N HCl (0.1–0.5 μL). After each addition, the spectra were recorded when the pH had reached a constant new value. The reference cuvette contained an apomembrane suspension (8 μM, A) or water (B). Both transitions were shown to be fully reversible by readjusting the initial pH value and recording the spectrum again.

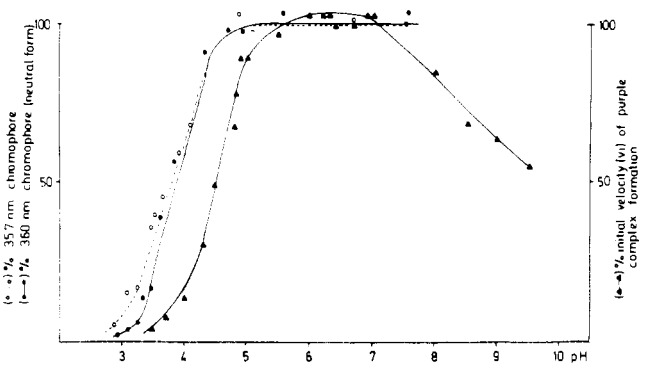

FIGURE 2: pH dependent reactions of retinyl moieties and retinal in the chromophoric site: (○-○) Ring-chain planarization of protein-bound retinol. Experimental conditions as in Figure 1A. Percent 357-nm chromophore was calculated from the ratio $\Delta A_{357nm}/\Delta A_{336nm}$. (●-●) Interconversion of the two retinyl moieties in product RP_{hv}. Experimental conditions as in Figure 1B. Percent 360-nm chromophore (neutral form) was calculated from the ratio $\Delta A_{360nm}/\Delta A_{380nm}$. (▲-▲) pH dependence of the initial velocity (V_i) of purple complex formation during the reconstitution reaction. Apomembrane (7 μM) was suspended in 0.1 M buffer at 2 °C (pH 3.5–4.9, sodium acetate; pH 5.0–7.0, potassium phosphate; pH >7.5, sodium carbonate buffer, all 0.1 M). Reconstitution was started by the addition of retinal (7 μM) in isopropyl alcohol to the sample cuvette; V_i was measured as the initial linear increase in absorbance at 568 nm. Maximal velocity is set as 100%. The reference cuvette contained a buffered apomembrane suspension (7 μM). In all samples, 100% purple complex was reconstituted.

the retinal can be substituted by a suitable analogue which, thus, can be introduced into the chromophoric site.

Using this experimental approach it was shown that retinol competes with retinal for the binding site of bacterioopsin (Schreckenbach et al. 1977). The spectrum of bacterioopsin-bound retinol is shown in Figure 1A, spectrum 1. It exhibits an intense fine structure with a λ_{max} value at 357 nm and, therefore, differs significantly from the absorption spectrum of retinol in solution (λ_{max} around 325 nm). From both the fine structure and λ_{max} value of this absorption band it was deduced that the protein forces the cyclohexene ring and the side chain of the retinyl moiety into a planar conformation (Schreckenbach et al. 1977).

The existence of a pH-dependent equilibrium between the planar and the nonplanar conformations of retinol in the binding site is illustrated in Figure 1A. At neutral pH the equilibrium is dominated by the planar species (spectrum 1), whereas at more acidic pH the nonplanar species is favored (λ_{max} 330 nm, spectrum 9). Evaluation of the pH-dependent spectral changes indicates the participation of a protonable group with a pK of 3.8 (Figure 2, ○-○); ring-chain planarization requires the deprotonated form of this group, assigned as B_1. This experiment does not, however, exclude the possibility of several groups with similar pK values mediating the ring-chain planarization step.

When retinol is bound in the retinal binding site of bacterioopsin, its fluorescence excitation spectrum exhibits an additional band at 280 nm, indicating an energy transfer from the aromatic amino acids of the protein to the retinyl moiety (Schreckenbach et al., 1978). This 280-nm band is observed in the pH range from 7 to 3, indicating that both the planarized and the nonplanarized retinol molecules are located in the chromophoric site. Thus, binding of retinol in the chromophoric site and ring-chain planarization are two distinct steps, as illustrated in Figure 4.

When purple membrane is treated with sodium borohydride in the presence of light, the chromophore reduction product RP_{hv} is obtained (Peters et al., 1976; Schreckenbach et al., 1977). In product RP_{hv} the retinyl moiety is in a planar ring-chain conformation. However, product RP_{hv} is different from protein-bound retinol in two respects: firstly, the retinyl moiety is covalently bound to the ϵ-amino group of a lysine residue (Bridgen and Walker, 1976; Schreckenbach and Oesterhelt, 1977); secondly, the membrane structure of product RP_{hv} is crystalline. In contrast, apomembrane does not show a crystalline lattice even in the presence of retinol (R. Henderson, personal communication).

Product RP_{hv} retains its fine structured absorption band and its 360-nm λ_{max} value between pH 5.8 and 3.1 but small reversible changes of intensity at 377 nm occur (Figure 1B). The plot of these changes vs. pH (Figure 2, ●-●) reveals a pK of 3.8 and suggests an influence of group B_1 on the spectral changes observed. In contrast to bacterioopsin-bound retinol, the protonation of B_1 does not lead to the relaxation of the planar ring-chain conformation of the retinyl moiety. This could be caused by either the covalent attachment of the retinyl moiety to the protein or the crystalline state of the membrane. In order to decide between these two alternatives, the titration shown in Figure 1B was repeated in the presence of 1% (w/v) Triton X-100, which is known to destroy the crystalline lattice of the membrane (Henderson, 1977; Heyn et al., 1975). Under these conditions, the spectra were identical to those obtained from bacterioopsin-bound retinol in the absence of detergent (Figure 1A). In summary, it seems justified to conclude that the covalent linkage of the retinyl moiety to the protein is a prerequisite for the crystallinity of product RP_{hv}. Furthermore, the crystalline state prevents the relaxation of the planar ring-chain conformation of the retinyl moiety upon protonation of B_1, as depicted in Figure 4.

TABLE I: Reactions of Retinyl Compounds with Bacterioopsin.[a]

retinyl compd	bound in the chromophore site	rel fluorescence intensity in EtOH	rel fluorescence intensity in the binding site	ring-chain conformation	inhibition of reconstitution
retinylmethylamine	−	1	1	nonplanar	−
axerophthene	+	1	1	nonplanar	−
retinyl acetate	+	1	2	nonplanar	−
retinyl methyl ether	+	1	5	partly planar	−
retinol	+	1	7	planar	+
retro-retinol	+	1	7	planar	+

[a] Inhibition of reconstitution was assayed according to Schreckenbach et al. (1978); the minus symbol indicates that no inhibition was found under the experimental conditions described for retinol and *retro*-retinol.

Binding Studies with Analogues of Retinol. The chemical nature of the group B_1 is unknown. In order to elucidate its mode of action in the ring-chain planarization reaction, we performed binding studies with bacterioopsin and analogues of retinol in which the hydroxyl group was modified. Absorption and fluorescence spectroscopy revealed that the interaction of bacterioopsin with retinyl compounds could be classified into three groups by the following criteria (Schreckenbach et al., 1978): (1) Retinyl compounds which do not fit into the retinal binding site have a normal retinyl absorption spectrum (λ_{max} 325 nm) and have no 280-nm band in their fluorescence excitation spectrum; (2) retinyl compounds within the binding site having no planar ring-chain conformation exhibit a normal retinyl absorption spectrum and show a 280-nm excitation band; (3) retinyl compounds which occur in a planar conformation have a three-peaked absorption band and a 280-nm band in their excitation spectrum.

Retinyl methyl ether and axerophthene are two suitable analogues of retinol with which to investigate the interaction of its hydroxyl group with bacterioopsin. Retinyl methyl ether contains no free hydroxyl group and, therefore, could only accept a hydrogen bond from the protein. Axerophthene, on the other hand, does not contain oxygen and under no circumstances could it form a hydrogen bond with the protein. The absorption spectra of *all-trans*-retinol (1), *all-trans*-retinyl methyl ether (2), and *all-trans*-axerophthene (3) in the presence of equimolar amounts of bacterioopsin are shown in Figure 3A. In contrast to the three distinct maxima in the spectrum of retinol, only shoulders at 357 and 376 nm are seen in the case of retinyl methyl ether (λ_{max} 336 nm), whereas axerophthene has a normal retinyl spectrum (λ_{max} 332 nm). The fine structure of the absorption bands clearly indicates that retinyl methyl ether occurs in an equilibrium between planar and nonplanar species, whereas axerophthene is not planarized at all.

The fluorescence excitation spectra of the three retinyl-protein complexes are shown in Figure 3B; a 280-nm excitation band is common to all three spectra, indicating that the three retinyl compounds are bound within the chromophoric site of bacterioopsin. By comparison of Figure 3A and B it can be seen that the shape of the absorption spectrum of protein-bound retinol corresponds to its excitation spectrum. This is not found in the case of retinyl methyl ether. In this case, the λ_{max} value of the absorption band is 336 nm, whereas excitation is maximal at 352 nm. This phenomenon is explained by the observation that planarized retinyl moieties in the binding site of bacterioopsin show a more intense fluorescence than molecules which are bound in a nonplanar ring-chain conformation (Table I). Thus, in the spectra of retinyl methyl ether the small number of planarized molecules contributes little to the absorption band, since the molar absorption of this species does not differ significantly from that of other retinyl compounds ($\epsilon_{RP_{hv}} \simeq 50\,000$; Peters et al., 1976). In the fluorescence spectrum, however, the planarized molecules dominate.

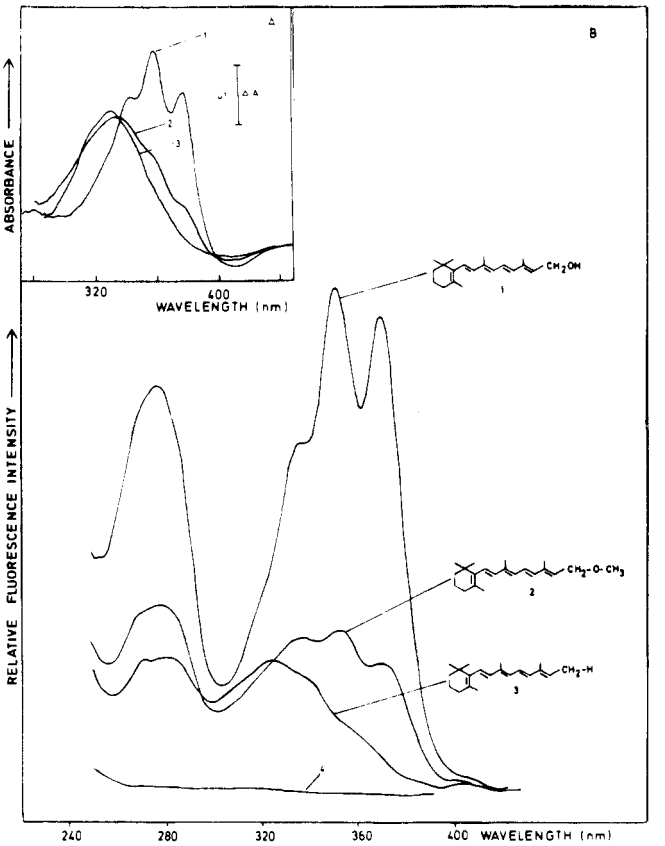

FIGURE 3: Conformation of retinol analogues in the binding site. (A) Absorption spectra of protein-bound *all-trans*-retinol (1), *all-trans*-retinyl methyl ether (2), and *all-trans*-axerophthene (3) (all samples 6 μM) in aqueous suspension. (B) Corrected fluorescence spectra of the samples from A and of an apomembrane suspension (4). Emission was recorded at 470 nm, the concentration of all samples was 0.8 μM.

The spectrum of protein-bound retinyl methyl ether reversibly loses its residual fine structure upon acidification as already shown for retinol, indicating that the group B_1 is required for planarization of this molecule.

From the spectra in Figure 3 and the data in Table I, several conclusions can be drawn. Retinol and its analogues retinyl methyl ether and axerophthene are fixed in the chromophoric site. This indicates that the binding step requires only the retinyl moiety of the molecule. The ring-chain planarization reaction, however, requires the presence of a specific functional

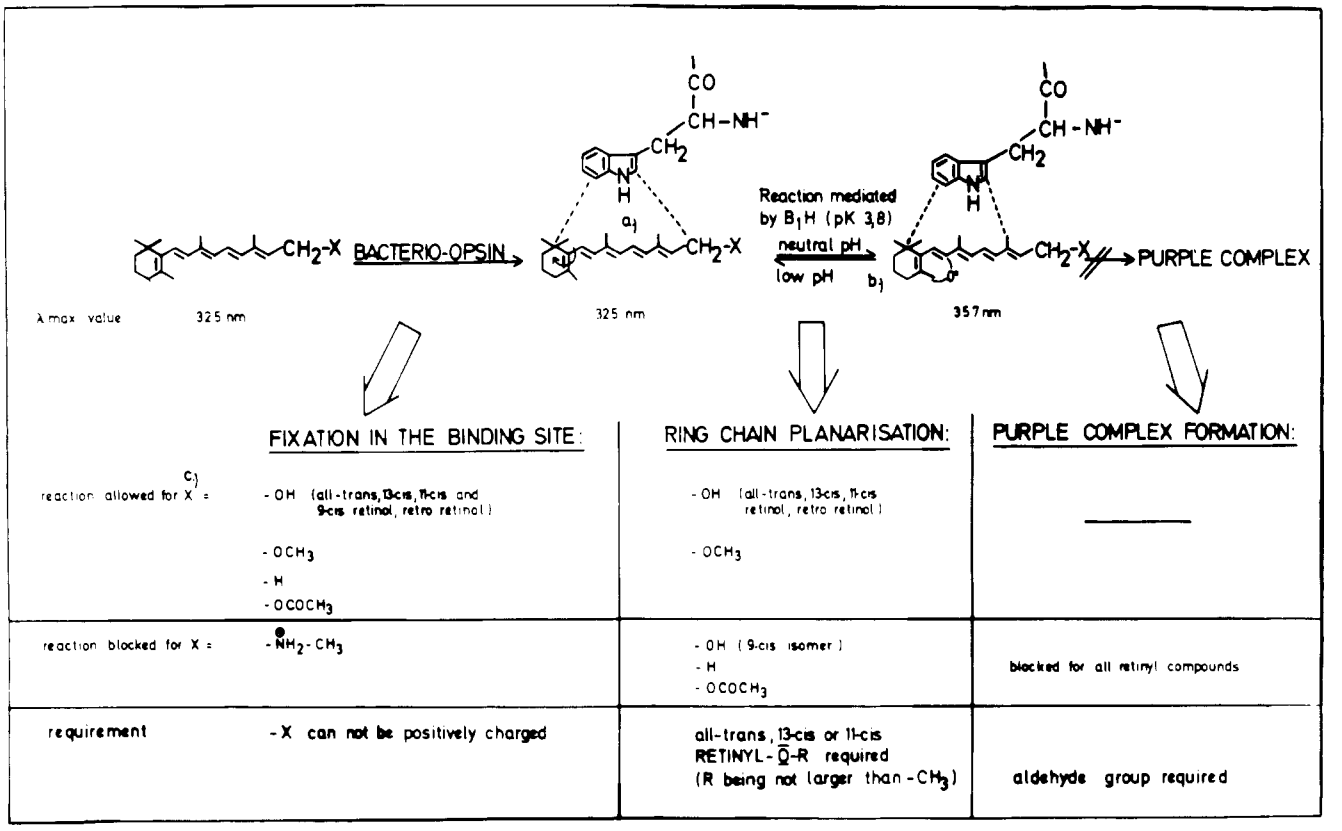

FIGURE 4: Interaction of retinyl compounds with bacterioopsin. (a) The scheme indicates energy transfer from tryptophan residues of the protein to the retinyl moiety (Schreckenbach et al., 1978). (b) Relaxation of the planar conformation at low pH is not observed when the membrane is in a crystalline state. (c) The retinyl compounds listed are retinol (X = -OH), retinyl methyl ether (-OCH_3), axerophthene (-H), retinyl acetate (-$OCOCH_3$), and retinylmethylamine (-$^+NH_2CH_3$).

group adjacent to the C_{15} atom of the retinyl compound (Figure 4). This functional group can be either a hydroxyl or a methoxyl group, indicating that the oxygen atom with its free electron pairs is required. The planar conformation of retinyl methyl ether is observed only to a limited extent. This might be caused through steric hinderance of the methyl group in the binding site. Axerophthene, on the other hand, is smaller than retinol, and hence steric hindrance is not possible, but is not planarized due to the absence of an oxygen atom in the molecule. The reconstitution of the purple complex is inhibited by retinol and *retro*-retinol and is not inhibited by retinyl methyl ether and axerophthene (Table I). The former two molecules are fully planarized in the binding site and thereby compete effectively with retinal during purple complex formation (Schreckenbach et al., 1978).

As stated in the preceding section, a group B_1 is required for ring–chain planarization. This group in its deprotonated form could interact with the hydroxyl of retinol via a hydrogen bond but not with the oxygen in retinyl methyl ether. Interaction with the pair of free electrons of oxygen can only be performed by a proton-donating group. We, therefore, assume that another group assigned as AH forms a hydrogen bond to the hydroxyl and methoxyl groups of retinol and retinyl methyl ether, respectively, but not with the methyl group of axerophthene. The pK of AH has to be greater than 10.5 because protein-bound retinol does not change its absorption spectrum in the pH range from 5 to 10.5.

Further evidence for the existence of a functional group AH could not be obtained using the retinol analogues retinyl acetate or retinylmethylamine, which both have a free electron pair. Retinyl acetate, although fitting into the binding site, is not planarized, as shown by its absorption and fluorescence characteristics; this no doubt is due to steric hinderance by the -$OCOCH_3$ group. Retinylmethylamine does not enter the binding site of bacterioopsin at all, since no 280-nm excitation band is observed in the fluorescence spectrum. This compound, however, is a secondary amine and is positively charged at neutral pH, which may explain why the molecule is not taken up into the chromophoric site.

The absorption spectra of membrane-bound retinoic acid, retinoic acid ethyl ester, and retinal oxime indicate that none of these compounds are planarized by the protein. Whether these molecules are held in the binding site at all was not checked because they show only very weak fluorescence. It should be mentioned, however, that other workers have postulated from the CD spectra of membrane-bound retinal oxime that this molecule is localized in the binding site of bacterioopsin (Becher and Cassim, 1977).

Binding Studies with Retinol Isomers. The results presented in the preceding section have illustrated that the oxygen atom of *all-trans*-retinol is required for the ring–chain planarization reaction. The sterical requirements for this reaction were investigated using stereoisomers of retinol. The absorption spectra of protein-bound *all-trans*-, 13-*cis*-, 11-*cis*-, and 9-*cis*-retinol are shown in Figure 5A. The absorption bands of the 13- and 11-cis isomers are due to an equilibrium between planar and nonplanar molecules within the binding site, whereas 9-*cis*-retinol is not planarized at all. This interpretation of the absorption bands is confirmed by the fluorescence spectra shown in Figure 5 B.

The fine structure of protein-bound 13- and 11-*cis*-retinol is reversibly abolished when the pH is adjusted to 3. Thus, as in the case of the all-trans isomer, the presence of group B_1 is required for planarization of the molecules in the binding site.

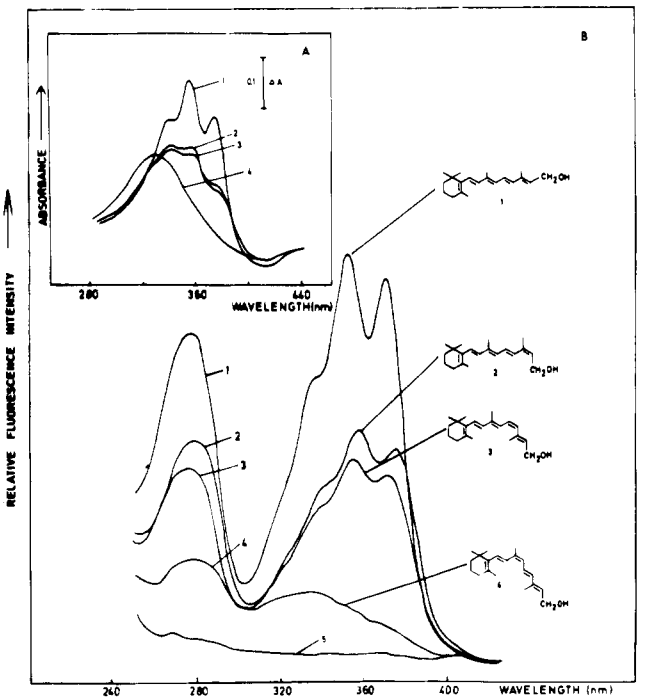

FIGURE 5: Conformation of retinol isomers in the binding site. (A) Absorption spectra of protein-bound *all-trans*-retinol (1), 13-*cis*-retinol (2), 11-*cis*-retinol (3), and 9-*cis*-retinol (4) (all samples 6 μM) in aqueous suspension. (B) Corrected fluorescence spectra of the samples from A and of an apomembrane suspension (5). Emission was recorded at 470 nm; the concentration of all samples was 0.8 μM. Shoulders at 357 and 374 nm in spectrum 4 are due to the isomerization of 9-*cis*-retinol by the measuring light.

It is further concluded that the oxygen adjacent to the C_{15} atom of the retinyl moiety is required in a certain spatial position in order to allow the conformational change to take place (Figure 4).

Retinal Isomers in the Binding Site; pH Dependence of Purple Complex Formation. The purple complex of bacteriorhodopsin contains *all-trans*-retinal when light adapted and an equilibrium mixture of the 13-cis and all-trans isomers after dark adaptation (Oesterhelt et al., 1973). This means that bacteriorhodopsin is able to accommodate these two geometrically different compounds in its binding site and that it is capable of facilitating their thermal isomerization.

It has been shown earlier that the reconstitution reaction of the purple complex from apomembrane and retinal (λ_{max} 380 nm) occurs via at least two spectroscopically distinct intermediates according to the equation:

$$\text{retinal} + \text{bacterioopsin} \xrightarrow{a} \text{400-nm chromophore}$$
$$\xrightarrow{b} \text{430–460 nm chromophore} \xrightarrow{c} \text{purple complex}$$

(Schreckenbach et al., 1977). Reaction a has been interpreted as the ring–chain planarization of the retinal molecule; the reactions b and c, however, are not understood in chemical terms. The 400-nm chromophore contains free retinal which can be reduced in the binding site by sodium borohydride (Schreckenbach et al., 1977). The 430–460-nm chromophore cannot be reduced directly. Treatment with 80% ethanol saturated with sodium borohydride yields free retinol, whereas under identical conditions the purple complex yields retinyl protein. Thus, the 430–460-nm chromophore is likely to contain retinal noncovalently bound in the chromophoric site.

The absorption spectra of the chromophores formed from bacterioopsin with *all-trans*-, 13-*cis*-, 11-*cis*-, or 9-*cis*-retinal are shown in Figure 6. *all-trans*-Retinal and 13-*cis*-retinal are able to form purple complex, whereas for 11-*cis*-retinal, reaction c is blocked. This isomer only forms an 11-*cis*-analogue of the 430–460-nm chromophore, as depicted in Figure 7 (Schreckenbach et al., 1977). 9-*cis*-retinal does not change its absorption characteristics upon binding to bacterioopsin.

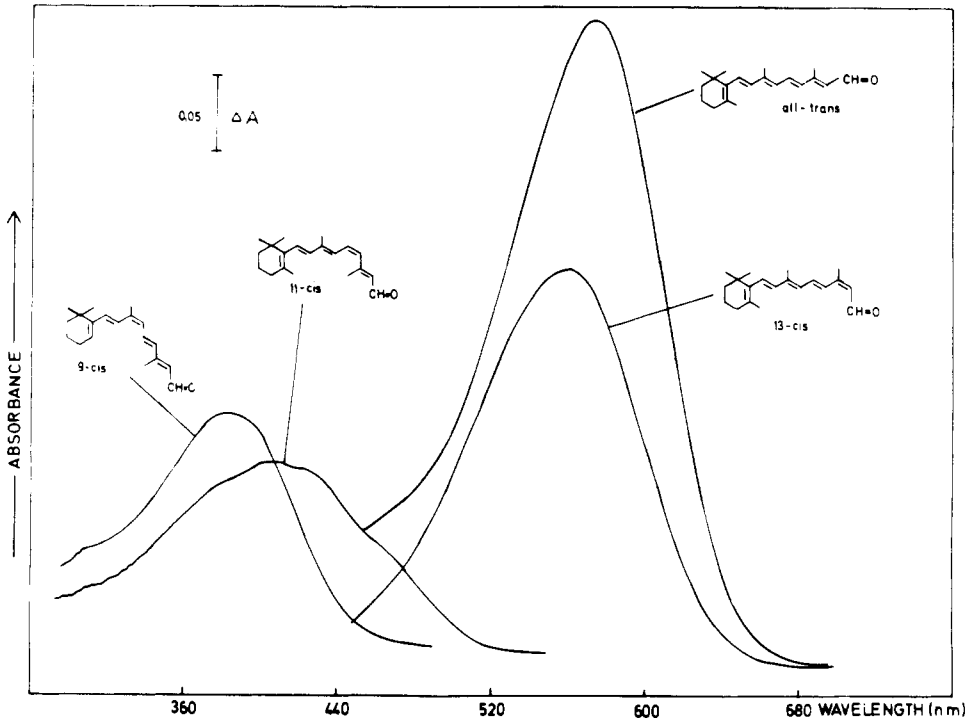

FIGURE 6: Different reactivity of retinal isomers in the binding site. Absorption spectra of the chromophores formed from *all-trans*-, 13-*cis*-, 11-*cis*-, and 9-*cis*-retinal and bacterioopsin. Six nanomoles of retinal isomer was added to an apomembrane suspension (7.5 μM bacterioopsin) in 0.1 M KPO_4 buffer (pH 6.5) at 0 °C. Until complete reaction and during recording of the spectra, the samples were kept in the dark at 0–2 °C in order to prevent thermal isomerization.

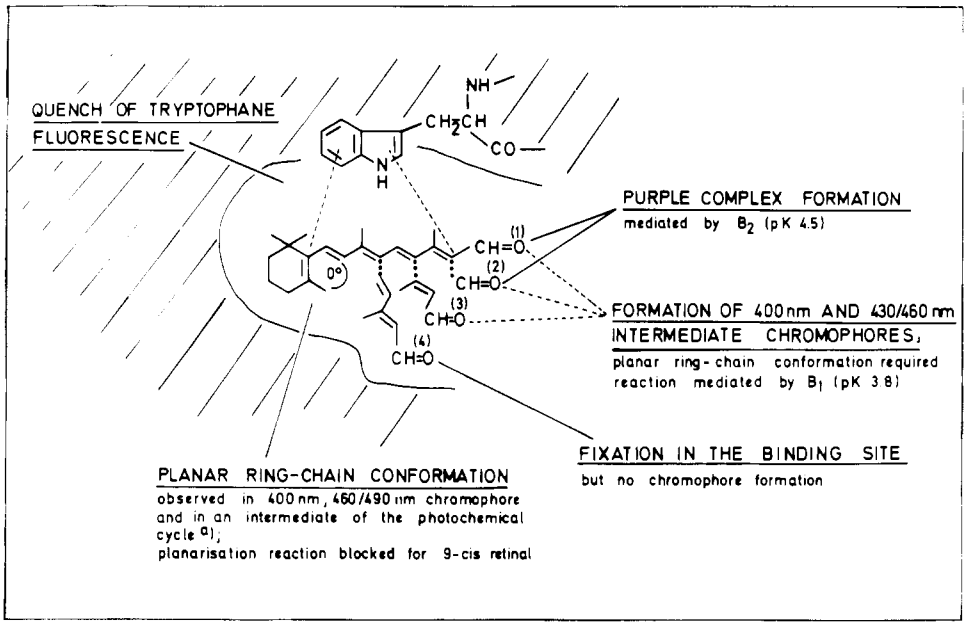

FIGURE 7: Properties of the retinal binding site. Different reactivity of *all-trans*- (1), 13-*cis*- (2), 11-*cis*- (3), and 9-*cis*-retinal (4). Footnote *a*: according to Schreckenbach et al. (1977).

The reactivity of the retinal isomers can be correlated with the behavior of the corresponding retinols. With both aldehydes and alcohols, the ring–chain planarization reaction is blocked for the 9-cis isomer and allowed for the all-trans, 13-cis, and 11-cis isomers. The planarization step is apparently a necessary prerequisite for steps b and c, since 9-*cis*-retinal, although being fixed in the binding site, does not form a 430–460-nm chromophore. Retinols and retinals interact with tryptophan residues of the protein; when *all-trans*-retinal is added to an apomembrane suspension, a rapid quenching of the tryptophan fluorescence of bacterioopsin is observed. Quenching is complete before the 400-nm intermediate has formed and is also observed in the purple complex (unpublished results). Quantitative evaluation of this effect reveals that optimal quenching is achieved when one retinal molecule, regardless of its configuration, is bound per protein molecule, indicating that only specifically bound retinal interacts with the tryptophan residues of bacterioopsin.

In the reaction sequence leading to the formation of purple complex, the last step (c) is rate limiting in the pH range from 3.5 to 9, which results in an accumulation of the 430–460-nm chromophore (Schreckenbach et al., 1977). The initial velocity of purple complex formation decreases significantly with decreasing pH, indicating a strong pH dependence of reaction c (Figure 2, ▲-▲). The involvement of a group with a pK of 4.5 assigned as B_2 is indicated. The unprotonated form of B_2 is required for the formation of the purple complex from the 430–460-nm intermediate.

One could speculate that B_2 is a lysine residue which reacts with the carbonyl moiety of retinal. The low pK value could then be explained by assuming an extreme hydrophobic environment of its ϵ-amino group. In this case, B_2 must be localized close to the carbonyl group of *all-trans*- and 13-*cis*-retinal (Figure 7).

This report shows, for the first time, that several functional groups of bacterioopsin interact either directly or indirectly with the retinal molecule during those steps which lead to the reformation of the purple complex. The chemical nature of these groups, as well as their localization relative to the retinal molecule, is not known. We have now started to investigate the structure-function relationship of retinal and the protein by studying the interaction of retinal with chemically modified bacterioopsin. Preliminary experiments have shown that the spectroscopic techniques described here provide an experimental basis for the identification of the functional amino acid residues involved in purple complex formation.

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Characterization of the Pyridoxal Phosphate Site in Glycogen Phosphorylase *b* from Rabbit Muscle†

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ABSTRACT: The cofactor site in glycogen phosphorylase *b* from rabbit muscle (EC 2.4.1.1) has been characterized by circular dichroism and electronic absorption studies on the reconstitution of the apoenzyme with pyridoxal 5′-phosphate (natural cofactor) and its analogues modified at the 5′ position, i.e., 5′-sulfate, 5′-phosphate monomethyl ester, 5′-phosphate monobenzyl ester, 5′-deoxy-5′-sulfonate, 5′-diphosphate, and 5′-diphosphate β-monophenyl ester, and P^1,P^2-bis(5′-pyridoxal) diphosphate (bis-PLP). All the cofactor analogues bind to the apoenzyme in the same (or similar) binding mode as pyridoxal 5′-phosphate, though these reconstituted enzymes show no enzyme activity. The asymmetric environment of the pyridoxal bound at the cofactor site is not altered by introducing a bulky substituent to the 5′ position. The size of the 5′ substituent of pyridoxal seems not to impose any restriction on the rate of reconstitution. Therefore, the 5′-phosphate locus of the cofactor site is a wide space or of flexible structure enough to adapt a large group therein. The rate of reconstitution markedly increases with the increments in the number of anions at the 5′ position of pyridoxal. A cationic group or groups interacting with the phosphate group should be present at the cofactor binding site. Two ionizable groups with p*K*s of 6.0 and 8.0 affect the rate of reconstitution, and are assigned to the 5′-phosphate group of the cofactor and to the ε-amino group of Lys-679 (the original cofactor-binding residue) in apophosphorylase, respectively. The optical properties of the bis-PLP cross-linking two ε-amino groups of Lys-573 and Lys-679 indicate that the environment around Lys-573 is similar to 70–80% dioxane in water, while around Lys-679 to more than 95% dioxane. The presence of the hydrophobic region adjacent to the 5′-phosphate locus of the cofactor site is also suggested by the finding that the cofactor derivatives having a benzene ring on the 5′-phosphate group can rapidly bind to the apoenzyme.

Pyridoxal 5′-phosphate (PLP[1]) is the cofactor present in all known α-glucan phosphorylases (1,4-α-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1). Removal of the cofactor from rabbit muscle phosphorylase produces the inactive apoenzyme which, in turn, restores its original activity on incubation with PLP (see Fischer et al., 1970; Graves & Wang, 1972). The cofactor binds to Lys-679 in rabbit muscle phosphorylase (Titani et al., 1977). The amino acid sequence around the lysine residue linking to the cofactor is highly conserved in phosphorylases from rabbit muscle, yeast, and potato tubers (Lerch & Fischer, 1975; Nakano et al., 1978), while these phosphorylases have different regulatory properties. The results of reconstitution studies using various PLP analogues have demonstrated the importance of the 5′-phosphate group of PLP in phosphorylase action (Shaltiel et al., 1969b; Pfeuffer et al., 1972a,b; Vidgoff et al., 1974; Parrish et al., 1977). The results of recent X-ray crystallographic studies on rabbit muscle phosphorylase *a* have shown that PLP is buried inside the protomer and its 5′-phosphate group is located adjacent to the substrate site (Sygusch et al., 1977). Close proximity of the two sites has also been suggested from pyrophosphate inhibition of PL-reconstituted phosphorylase *b* (Parrish et al., 1977). These findings lead one to speculate that the cofactor, especially its 5′-phosphate group, participates in catalysis, while no definitive evidence for its catalytic role has so far been obtained.

Although it has been shown from spectroscopic and fluorescence studies that the pyridoxal moiety of the PLP in phosphorylase is in a highly hydrophobic environment (Shaltiel & Cortijo, 1970; Johnson et al., 1970), detailed characterization of the cofactor site, e.g., what kind of amino acid residues are present, and whether the 5′-phosphate moiety of the cofactor is also in a hydrophobic environment or not, is lacking. We have previously been using circular dichroism (CD) to study the interaction of the allosteric activator (5′-AMP) and the cofactor with rabbit muscle phosphorylase, and found that

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[1] Abbreviations used: CD, circular dichroism; UV, ultraviolet; EDTA, ethylenediaminetetraacetic acid; PLP, pyridoxal 5′-phosphate; SA, salicylaldehyde; PL, pyridoxal; PLSN, 5′-deoxypyridoxal-5′-sulfonate; PLS, pyridoxal 5′-sulfate; PLP-Me, pyridoxal 5′-phosphate monomethyl ester; PLP-Bz, pyridoxal 5′-phosphate monobenzyl ester; PLDP, pyridoxal 5′-diphosphate; PLDP-Phe, pyridoxal 5′-diphosphate β-monophenyl ester; bis-PLP, P^1,P^2-bis(5′-pyridoxal) diphosphate; 3-*O*-MePLP, 3-*O*-methylpyridoxal 5′-phosphate.

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