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Retinal β-Ionone Ring−Salinixanthin Interactions in Xanthorhodopsin: A Study Using Artificial Pigments

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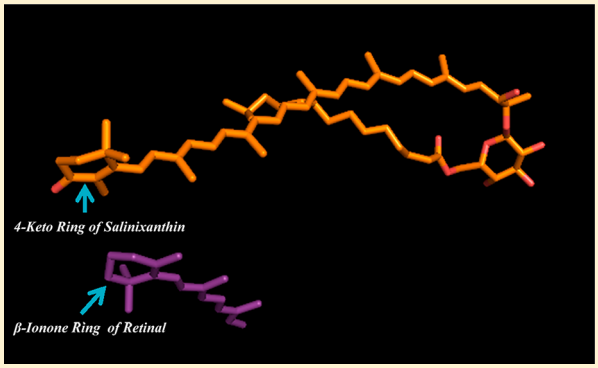
ABSTRACT: Xanthorhodopsin (xR) is a retinal protein that contains, in addition to the retinal chromophore, a carotenoid (salinixanthin) that functions as a light-harvesting antenna [Balashov, S. P., et al. (2005) *Science 309*, 2061−2064]. The center−center distance between the two polyene chains is 12−13 Å, but the distance between the two rings of retinal and salinixanthin is surprisingly small (∼5 Å) with an angle of ∼45° [Luecke, H., et al. (2008) *Proc. Natl. Acad. Sci. U.S.A. 105*, 16561−16565]. We aimed to clarify the role of the β-ionone ring in the binding of retinal to apo-xR, as well as a possible role that the β-ionone ring plays in fixation of the salinixanthin 4-keto ring. The binding of native retinal and series of synthetic retinal analogues modified in the β-ionone ring to apo-xR was monitored by absorption and circular dichroism (CD) spectroscopies. The results indicate that the β-ionone ring modification significantly affected formation of the retinal−protein covalent bond as well as the pigment absorption and CD spectra. It was observed that several retinal analogues, modified in the retinal β-ionone ring, did not bind to apo-xR and did not form the pigment. Also, none of these analogues induced the fixation of the salinixanthin 4-keto ring. In addition, we show that the native retinal within its binding site adopts exclusively the 6-*s*-*trans* ring−chain conformation.



Xanthorhodopsin (xR)[1] is a retinal-based proton pump protein in the cell membranes of the extremely halophilic eubacterium *Salinibacter ruber*.[3] In addition to all-*trans*-retinal that is bound to the protein via a protonated Schiff base (PSB), a carotenoid chromophore is embedded in the protein and functions as a light-harvesting antenna.[1] The chromophore is salinixanthin, a C_{40} carotenoid characterized by a glycoside residue and an acyl tail[4] as well as a conjugated chain that consists of 11 double bonds, and is attached to a cyclohexene ring bearing one double bond and a keto group at the C_4 position (Scheme 1). The absorption spectrum as well as the CD spectrum of the salinixanthin is remarkably affected by the presence of the retinal chromophore,[5] and the absorption spectrum is different from that of the free salinixanthin in solution. The spectrum of bound salinixanthin has a higher extinction coefficient, is more structured, and contains sharp well-resolved absorption maxima at 519, 487, and 456 nm.[1] The CD spectrum is characterized by sharp positive lobes that resemble the absorption maxima of salinixathanin, and a negative band at 530 nm that has a significant contribution from the bound salinixanthin.[5,6] It was suggested that the CD spectrum originates from the asymmetric environment of xR or an asymmetric conformation of the salinixanthin molecule enforced by the protein.[5] Cleavage of the retinal−Lys-240 (the residue homologues to Lys-216 of bR) covalent bond by hydroxylamine leads to a substantial broadening and a slight red shift of the salinixanthin absorption bands. As a consequence, the absorption and CD spectra of the salinixanthin component in the complex become almost identical to that of free salinixanthin in solution.[1,4] The main source of this broadening is derived from the statistical distribution of conformers differing in the torsional angle between the ring and the polyene side chain. The angular twist of the C_6-C_7 bond results in excited state energy changes, and thus, a wide range of transition energies are present in the ensemble.[7,8] This twist is probably responsible for the spectrum broadening of the free salinixanthin in solution and the spectrum of apo-xR.[5] Narrowing of the absorption bands indicates restriction of out-of-plane motions within the conjugated chain of the carotenoid. It was shown that the immobilization of the 4-keto ring in the asymmetric conformation is one of the reasons for narrowing of the UV−vis spectrum of xR[5] as well as for the formation of the CD spectrum of xR.[5] Previous binding studies of apo-xR with retinol and synthetic retinal analogues, which bind considerably slower than the all-*trans*-retinal, indicated that the immediate changes in the salinixanthin absorption[9] and formation of the CD spectrum[6] during binding were due to prepigment formation prior to the formation of the retinal−protein covalent bond.[6,9] Therefore, it was concluded that the tight binding of the carotenoid does not require the formation of the protonated Schiff base.[9]

High-resolution diffraction data[2] revealed that the center−center distance between the two polyene chains is ∼12−13 Å but the distance between the two rings of the retinal and

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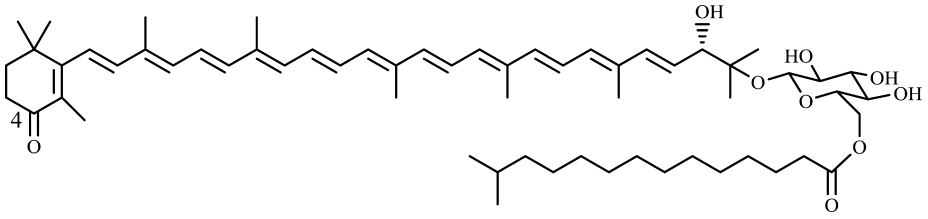
Scheme 1. Salinixanthin Structure



salinixanthin is remarkably small (~5 Å) and the angle formed by the two polyenes is ~45°.[2] The ring moiety of the salinixanthin is rotated relative to the plane of the carotenoid polyene π-system chain and immobilized in its binding site in the vicinity of the retinal β-ionone ring.[2] Recent work of Imasheva et al.[10] demonstrated that the 4-keto group of the salinixanthin ring is critically required for the binding of salinixanthin as well as for the energy transfer to the retinal.

In this work, we have examined the involvement of the retinal β-ionone ring in the binding of the retinal chromophore to the apo membrane of xR using synthetic retinal analogues characterized by a modified β-ionone ring. The results show that modification of the β-ionone ring affected significantly the formation rate of the retinal−protein covalent bond, as well as the pigment UV−vis and CD spectra. It appears that the intact cyclohexenyl ring moiety of the retinal chromophore is crucial for the smooth formation of an xR pigment as well as for the resolved CD spectrum during the early stages of the binding process. The results clearly indicate that the protein−retinal interactions in xR are significantly different from that of bR in the vicinity of the retinal ring. Moreover, we show that the preferable retinal ring−chain conformation in xR during the binding process is 6-*s*-*trans*, in keeping with the conformation suggested by the X-rays studies.[2]

MATERIALS AND METHODS

Sample Preparation. *S. ruber* was grown using slightly modified published methods.[1,3] Sucrose (0.1% per liter) was added to the growing medium according to a previously described procedure.[11] xR membrane samples were prepared using published methods[1]. The membranes were washed with 0.1 M NaCl and then three times with DDW. This treatment partially removed unbound salinixanthin. The process yielded a sample in which the ratio between the absorption at 280 and 568 nm was ~3.

The apoprotein was prepared by incubating the xR membrane with 0.2 M (0.05−0.1 M for artificial pigments) freshly prepared hydroxylamine (pH 7.2) and irradiated for 1.5 h with a Schott 250W cold light source (Carl Zeiss Microscopy, Jena, Germany) equipped with a heat-absorbing filter and an optic fiber (level 4B). The light was filtered through a long pass cutoff filter with a λ of >550 nm (Schott, Mainz, Germany). The samples were dialyzed versus DDW and stored at 4 °C to avoid reconstitution with retinal originating from retinaloxime.

The retinal analogues were synthesized as described previously.[12−21] Artificial pigments of xR were prepared by incubating the apoprotein (absorbance of 0.2−0.4 OD according to the absorption at 487 nm) overnight with 2 equiv of the synthetic retinal analogue, 50 mM Tris buffer (pH 8.3), 30% sucrose, and 100−200 mM NaCl at 20 °C. The predicted amount of the pigment was estimated on the basis of a bleach process.

UV−Vis Absorbance Measurements. All the UV−visible measurements were taken using an Agilent 4583 diode-array spectrophotometer (Agilent Technologies, Palo Alto, CA) equipped with an Agilent 89090A thermostated cuvette holder. The kinetic measurements were taken every 60 s while each time the interval was increased by 10%. Absorption spectra were corrected for light scattering.

Absorbance Kinetic Calculations. The UV−vis measurements at a specific wavelength were normalized and plotted versus time. The fraction ratio was determined using the bR second-order growth equation:

$$y = a * \exp(-k_1 * x) - (1 - a) * \exp(-k_2 * x) \quad (1)$$

CD Measurements. Circular dichroism spectra were recorded on an Aviv circular dichroism spectrometer as well as on the Chirascan circular dichroism spectrometer (Applied Photophysics). The CD spectra are given in degrees of ellipticity, θ, which is proportional to the difference in absorbance of left and right circularly polarized light [$\theta = 3300°(A_L - A_R)$[22]]. A quartz 1 cm × 1 cm path length cuvette was used. The CD spectra were recorded with a 1 nm bandwidth resolution and in 1 nm steps at 20 °C. The CD spectra were corrected for the baseline distortion by subtracting reference spectra of the corresponding buffer.

CD Kinetic Measurements. CD kinetic measurements were recorded at a single wavelength (480 and 530 nm) every 9 s for 1 h (366 measurements total) The CD measurements at a specific wavelength were normalized and plotted versus time. The fraction ratio was determined using the bR second order growth (1) or decay (2) equations:

$$y = a * \exp(-k_1 * x) + (1 - a) * \exp(-k_2 * x) \quad (2)$$

RESULTS

Effect of the Cyclohexene Ring on the Retinal Binding Process. Steric constraints of retinal protein as well as salinixanthin−retinal interactions in xR were analyzed by studying artificial pigments derived from synthetic retinal analogues. The effects of retinal substitution with a variety of retinal analogues on the absorption maxima are summarized in Table 1. The reconstitution of the synthetic retinal analogues with the apo-membrane of xR was followed by monitoring the absorption and CD spectra simultaneously. It allowed us to clarify whether the binding process is affected by the β-ionone ring structural modifications.

Binding of Linear Retinal (3), 3,4-Dehydroretinal (1), and Phenylretinal (2) to the Apo-Membrane of xR. Linear retinal analogue **3** did not form a pigment following incubation with the apo-membrane of xR. This observation indicates that the β-ionone ring is crucial for the retinal binding process in contrast to bR in which the linear retinal analogue formed a pigment following incubation with the apoprotein of bR.[27] 3,4-Dehydroretinal **1** resembles the retinal structure except with an

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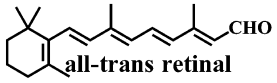
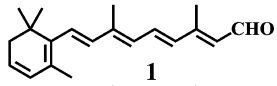
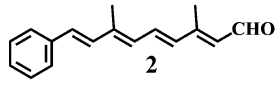
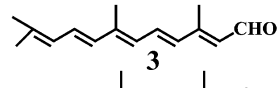
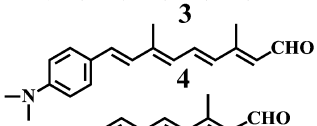
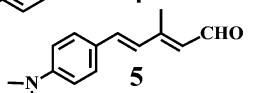
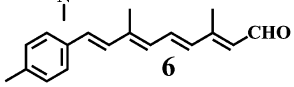
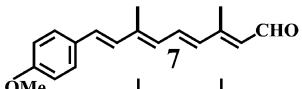
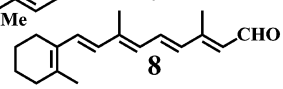
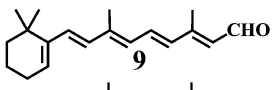
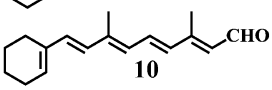
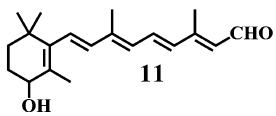
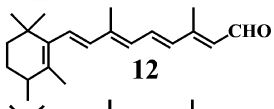
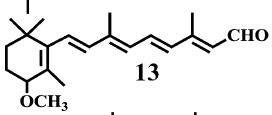
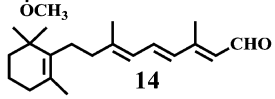
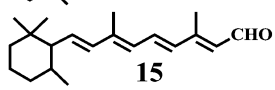
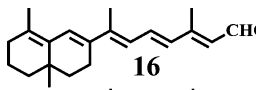
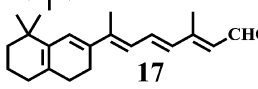
Table 1. Absorption Maxima of Artificial Dark-Adapted Pigments of xR and bR

Chromophore	Absorption Maximum	
	Xanthorhodopsin*	Bacteriorhodopsin
 all-trans retinal	568 nm	558 nm (*21*)
 1	591 nm	590 nm (*23*)
 2	Cannot be detected due to overlap with salinixanthin absorption.	Two pigments: 480 & 520 nm (*24*)
 3	No binding	530 nm (*21*)
 4	No binding	570 nm (*16*)
 5	No binding	580 nm (*16*)
 6	No binding	497 nm (*21*)
 7	No binding	531 nm
 8	567 nm	553 nm (*12*)
 9	570 nm	548 nm (*25*)
 10	No binding	539 nm (*12, 13*)
 11	563 nm	525 nm (*19, 21*)
 12	570 nm	470 nm (*19, 21*)
 13	No binding	480 (data not shown)
 14	430 & 450 nm	442 nm (*21*)
 15	No binding	466 nm (*21*)
 16	575 nm	564 nm (*26*)
 17	No binding	Two pigments: 509 & 596 nm (*26*)

*The absorption maximum of the retinal pigment was obtained from the difference spectrum of the binding process of each analogue. Therefore, the values may result in a slight red shift as the absorption spectrum of the retinal pigment overlaps with the absorption of the carotenoid.

additional double bond in the ring between C_3 and C_4. Incubation of the apo-membrane of xR with 3,4-dehydroretinal **1** led to high yield of pigment that had an absorption maximum at 591 nm[6] (Table 1 and Figure 1B) versus 568 nm for all-*trans*-retinal.[1] The fully resolved CD spectrum of 3,4-dihydro-xR was detected on a longer time scale (40 min), relative to

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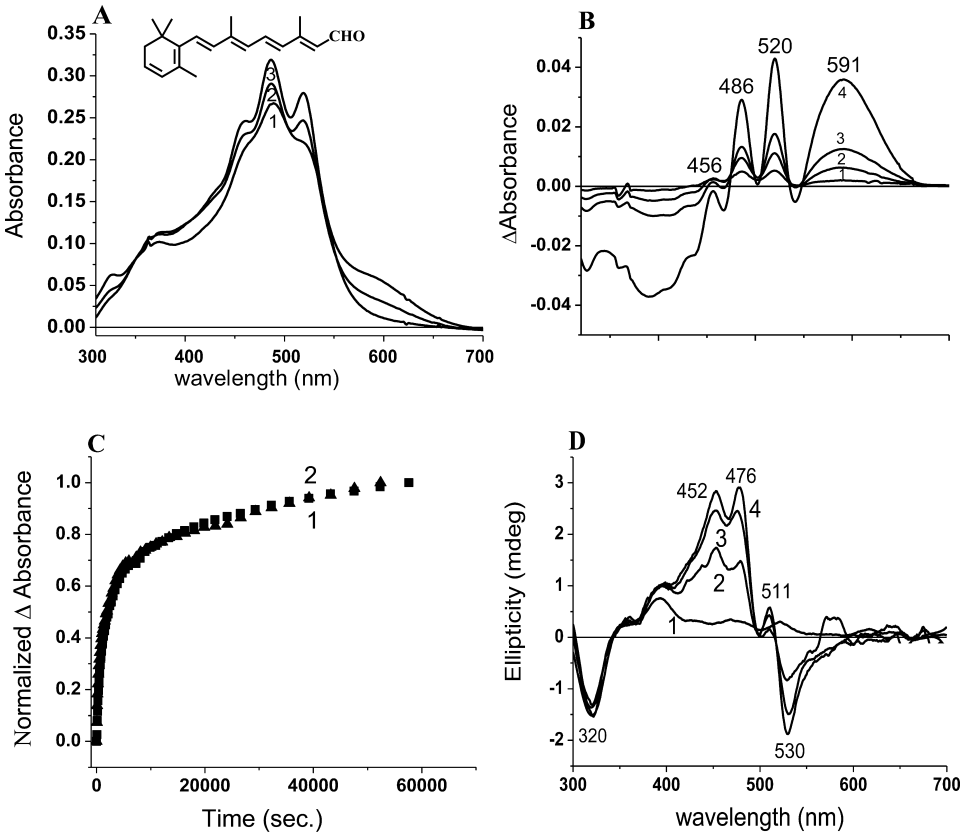

Figure 1. Binding with 3,4-dehydroretinal (**1**). (A) Absorption spectra of binding of 3,4-dehydroretinal to the apo-membrane of xR. Curves 2−3 are spectra taken 40 min, and 24 h after the addition of 3,4-dehydroretinal, respectively. Curve 1 represents the apo-membrane of xR. (B) Difference absorption spectra of binding with 3,4-dehydroretinal. Spectrum taken immediately following addition of 3,4-dehydroretinal was subtracted from spectra taken at specified times. Curves 1−4 are spectra taken 1 min, 5 min, 14 min, and 24 h after the addition of 3,4-dehydroretinal, respectively. (C) Normalized absorbance kinetic traces of the binding of 3,4-dehydroretinal to apo-xR. Spectrum 1 (▲) represents the evolution of the carotenoid at 520 nm and spectrum 2 (■) the formation of the PSB at 600 nm. (D) CD spectra of the binding process at specified times of incubation after addition of 3,4-dehydroretinal to the apo-membrane. Curves 2−4 are spectra taken 12 min, 45 min, and 1.5 h after the addition of 3,4-dehydroretinal, respectively. Curve 1 is a spectrum of the apo-membrane.

that of native pigment formation (9 min[6]). It appears that the additional double bond in the β-ionone ring affects the ring conformation, disturbing the salinixanthin ring fixation process in its binding site. We note that the rate of formation of the artificial pigment (as monitored by absorption spectroscopy) is also slower than the rate of formation of the native pigment. The kinetics of 3,4-dehydro pigment formation (Figure 1C) was detected as the change in absorption at 600 nm. The changes in the carotenoid bands were monitored at 520 nm. In both cases, the kinetics was biphasic and very similar within experimental error (k_1 = 0.0011 s^{-1}, k_2 = 0.00007 s^{-1}, and a = 0.5, and k_1 = 0.0015 s^{-1}, k_2 = 0.00006 s^{-1}, and a = 0.5, respectively). This demonstrates that the processes occur in parallel and are slower than that of all-*trans*-retinal [k_1 = 0.006 s^{-1}, k_2 = 0.0004 s^{-1}, and a = 0.6, and k_1 = 0.0025 s^{-1}, k_2 = 0.0004 s^{-1}, and a = 0.5, respectively (data not shown)].

To shed further light on the role played by the β-ionone ring, we have studied a retinal analogue **2** bearing an aromatic ring substituting for the β-ionone ring. This analogue alters the retinal structure in two ways, (1) the planarity of the ring−polyene conformation and (2) the planarity of the ring itself, altering the chromophore charge distribution. Evidently, a stable pigment was formed following incubation of chromophore **2** with the apo-membrane of xR (as previously described), although the exact absorption of the formed phenyl-xR cannot be determined (Figure 2B), but it is conceivable that the absorption of the formed artificial pigment is covered by the carotenoid absorption (Figure 2B).[6] The rate of formation of the CD spectrum of phenyl-xR was slower during the binding process (Figure 2C) than the rate of the native pigment or pigments that were modified along the retinal polyene.[6] Therefore, we can conclude that replacement of the β-ionone ring with a phenyl ring affects the ring conformation such that it disturbs the salinixanthin fixation process in its binding site.

Substitution of the phenyl moiety with several substituents (**4**−**7**) did not lead to any pigment formation following incubation with apo-membrane even after incubation for 2 days (Table 1). It is important to note that none of these chromophores induced alteration in the carotenoid absorption or the characteristic increase in the resolution of its bands. This observation may suggest that the processes of fixation of the carotenoid and formation of the protonated Schiff base are connected.

Role of the β-Ionone Ring Methyls in the Binding Process. The methyl groups that substitute the retinal β-ionone ring induce steric hindrance, which imposes a twist around the retinal C_6−C_7 bond to relieve the steric interactions. Therefore, deletion of the methyl groups will allow for the ring−chain planar conformation, thereby inducing better

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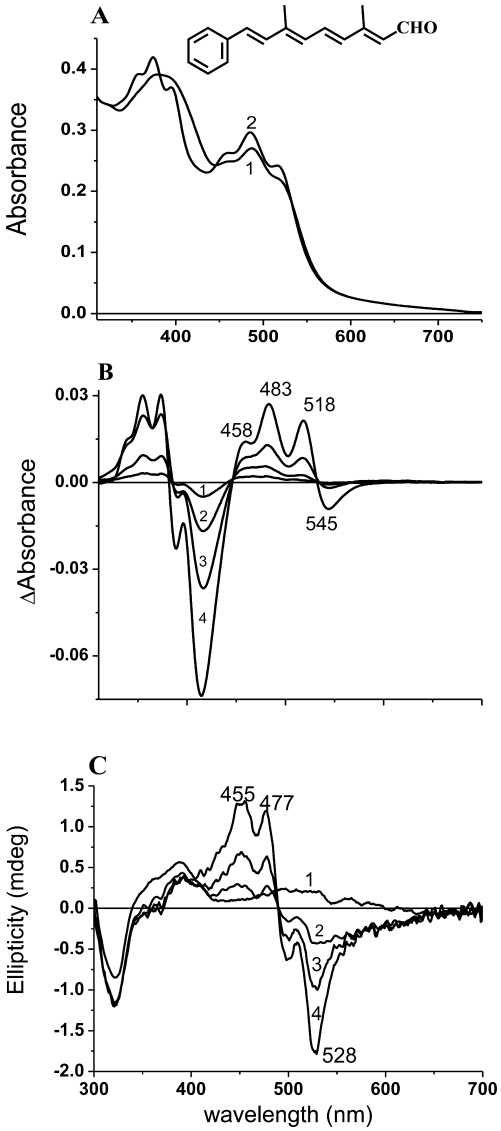

Figure 2. Binding with phenylretinal (**2**). (A) Absorption spectra of the binding of phenylretinal to the apo-membrane of xR. Curves 1 and 2 are spectra taken 1.5 s and 7 h after the addition of phenylretinal, respectively. (B) Difference absorption spectra of the binding with phenylretinal. The spectrum taken immediately following the addition of phenylretinal was subtracted from spectra taken at specified times. Curves 1–4 are spectra taken 1 min, 5 min, 24 min, and 7 h after the addition of phenylretinal, respectively. (C) CD spectra of the binding process at the specified times of incubation after addition of phenylretinal. Curves 2–4 are spectra taken 12 min, 2 h, and 14 h after the addition of phenylretinal, respectively. Curve 1 is a spectrum of the apo-membrane.

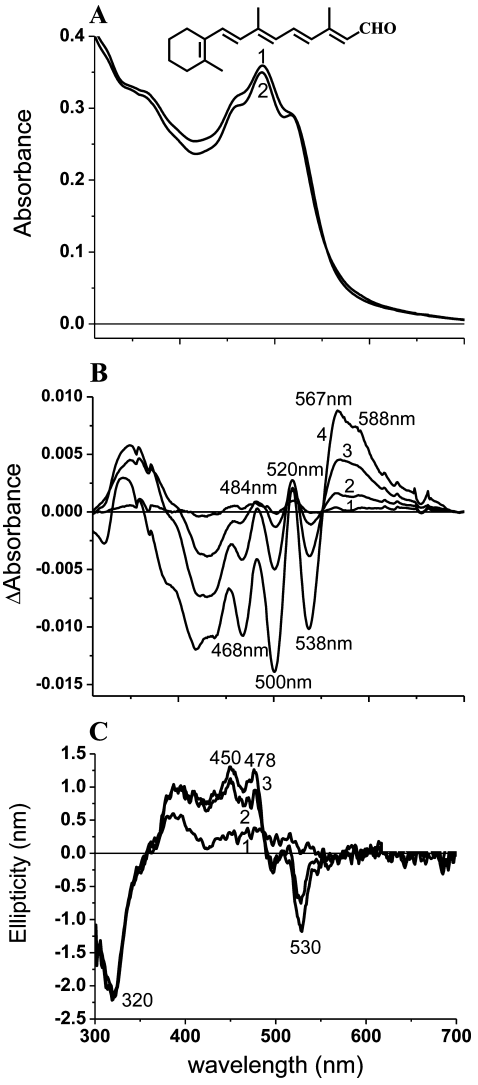

Figure 3. Binding with 1,1-didesmethylretinal (**8**). (A) Absorption spectra monitoring the binding of 1,1-didemethylretinal to the apo-membrane of xR. Curves 1 and 2 are spectra taken 1.5 s and 11 h after the addition of 1,1-didemethylretinal, respectively. (B) Difference absorption spectra of the binding of 1,1-didesmethylretinal. The spectrum that was taken immediately following 1,1-didesmethylretinal addition was subtracted from the spectra taken at specified times. Curves 1–4 are spectra taken 1 min, 14, min, 2 h, and 11 h after the addition of 1,1-didesmethylretinal, respectively. (C) CD spectra of the binding process at the specified times of incubation after the addition of 1,1-didesmethylretinal to the apo-membrane. Curves 2 and 3 are spectra taken 9 min and 15 h after the addition of 1,1-didesmethylretinal, respectively. Curve 1 is a spectrum of the apo-membrane.

conjugation between the C_5–C_6 double bond and the retinal polyene chain.[6,12] To shed light on the role of the methyl groups at positions C_1 and C_5 in the retinal binding process, we have examined the binding of three ring-demethylated retinals (1,1-didemethylretinal **8**, 5-desmethylretinal **9**, and 1,1,5-tridemethylretinal **10**) to the apo-membrane of xR.

Incubation of 1,1-didemethylretinal **8** with the apo-membrane of xR yielded a small amount of pigment with an absorption maximum at 567 nm and a shoulder around 588 nm, which may indicate that a fraction of the retinal analogue adopts a different conformation within the binding site (Table 1 and Figure 3A,B). Interestingly, the intensity of the absorption of salinixanthin does not increase as detected in native xR[1] (Figure 3A). As the 1,1-didemethylretinal pigment forms, the intensity of the salinixanthin chromophore absorption is decreased but the spectrum is more resolved as reflected in the negative bands at 538, 500, and 468 nm that appear in the difference spectra (Figure 3B). In addition, an increase in the intensity of the salinixanthin band at 520 nm was detected. The latter change in the 520 nm band is also observed during the all-*trans*-retinal binding process.[1] The corresponding bR artificial pigment absorbs at 553 nm (Table 1).[12] The rate of formation of the CD spectrum indicates that the fully resolved CD spectrum is formed prior to the

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formation of the protonated Schiff base covalent bond. It is evident that the CD spectrum is formed already 9 min after the addition of the chromophore to the apo-membrane (Figure 3C). Its intensity only slightly increases following formation of the protonated Schiff base as was shown for all-*trans*-retinal,[6] following incubation with the apoprotein of xR.

The absence of 5-CH_3 has a pronounced effect on the rate of formation of bR, as the binding starting with an initial more planar retinal leads to a faster binding process. The light−dark adaptation of 5-desmethyl-bR is very similar to that of unmodified bR as well as the light-driven proton pump action.[6] The binding of apo-xR with 5-desmethylretinal **9** yielded a stable pigment following incubation with the apo-membrane absorbing at ∼570 nm (Figure 4B), very similar to that of the wild type (wt). The UV−vis difference spectrum (Figure 4B) indicates that the formed pigment has a fine structure, suggesting its rigidity within the binding site, although the methyl at position C_5 is missing. We note that the large negative band at 410 nm (Figure 4B) is probably due to the formation of random Schiff bases absorbing at ∼350 nm (in addition to formation of the pigment) and possibly to partial decomposition of the chromophore. Approximately one-fourth of the CD spectrum intensity of 5-desmethyl-xR was detected 9 min after the addition of the chromophore, and the fully resolved CD spectrum was formed after 18 h (Figure 4C), much slower than formation of the CD spectrum following the binding of all-*trans*-retinal and analogues modified along the polyene chain.[6,9] The formed CD spectrum resembles the characteristic CD spectrum of wt xR, although its formation is slower,[6] demonstrating that *β*-ionone ring modification affects the rate of fixation of salinixanthin in its binding site, but the formed pigment has retinal−salinixanthin interactions similar to those of the native pigment.

1,1,5-Tridemethylretinal **10** did not form a pigment following incubation with apo-xR or the characteristic carotenoid resolved bands. These results suggest that at least one of the ring methyls should be present to allow for pigment formation. These results are in contrast to those observed for bR in which the lack of the ring methyls did not prevent pigment formation.[13] This difference between the two pigments may indicate more a confined retinal ring binding site in xR than in bR and may suggest that specific interactions of the retinal ring and the protein are necessary to induce the fixation of the carotenoid chromophore and formation of the protonated Schiff base.

Role of the C_4 Position of the Cyclohexene Ring. We have further studied the effects caused by introducing steric hindrance in the vicinity of the retinal ring. As the C_4 position of bR is sensitive to substitution with bulky groups,[19] it was of our interest to check this position in xR as well. We have prepared modified pigments substituted at the C_4 ring position with substituents bearing different levels of bulkiness. The pigments based on chromophores 4-hydroxyretinal **11** and 4-methylretinal **12** were prepared by incubation of the retinal analogues with the apo-membrane of xR.

Incubation of apo-xR with 4-hydroxyretinal formed a pigment absorbing at 563 nm (Table 1 and Figure 5B). The minor blue shift (3 nm) relative to the native pigment indicates that the chromophore−protein interactions are not disturbed and the retinal analogue adopts a conformation that resembles that of the native retinal. The formation of the retinal pigment that was monitored using UV−vis spectroscopy (Figure 5A,B) was highly similar to the binding of native all-*trans*-retinal.[5] The

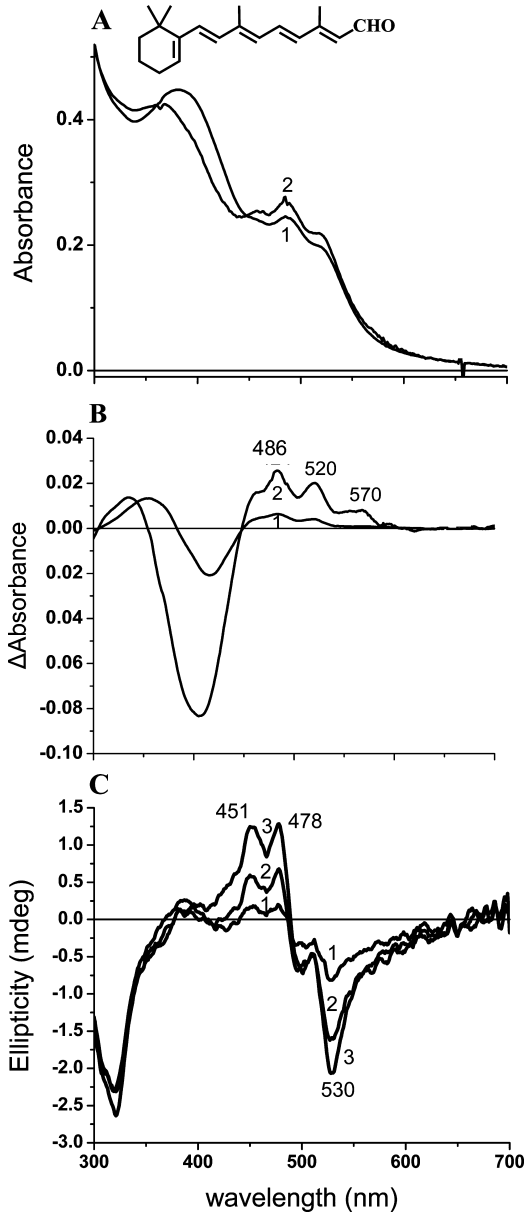

Figure 4. Binding of 5-desmethylretinal (**9**). (A) Absorption spectra of binding of 5-desmethylretinal to the apo-membrane of xR. Curves 1 and 2 are spectra taken 1.5 s and 16 h after the addition of 5-desmethylretinal, respectively. (B) Difference absorption spectra of binding with 5-demethylretinal. The spectrum that was taken immediately following 5-desmethylretinal addition was subtracted from the spectra taken at specified times. Curves 1 and 2 are spectra taken 4 min and 16 h after the addition of 5-desmethylretinal, respectively. (C) CD spectra of the binding process at the specified times of incubation after the addition of 5-desmethylretinal to the apo-membrane. Curves 1−3 are spectra taken 9 min, 2 h, and 18 h after the addition of 5-desmethylretinal, respectively. Curve 1 represents the spectrum of the apo-membrane.

formed CD spectrum also indicates that the conformation of 4-hydroxy-xR is not disturbed, exhibiting a CD spectrum similar to that of native xR (Figure 5C). In contrast, formation of the corresponding bR pigments is associated with a substantial blue-shifted absorption (525 nm), indicating perturbation of the retinal−protein interactions in the binding site.[19] The similarity of the 4-hydroxy-xR and native pigment absorptions is in contrast to the rate of formation of the CD spectra, as well

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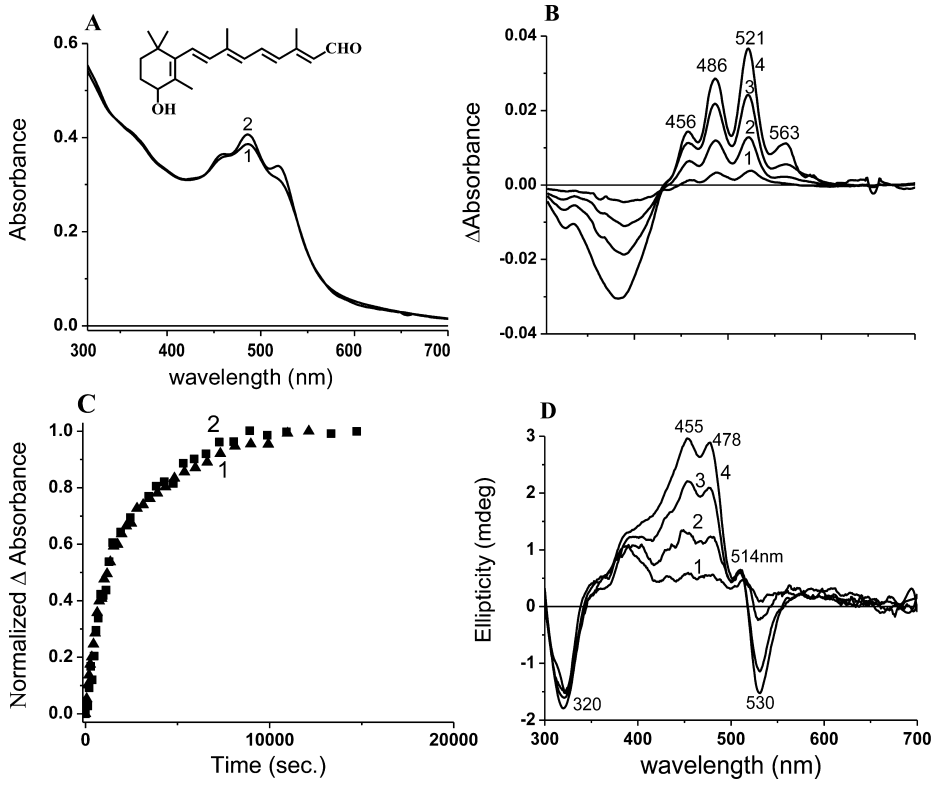

Figure 5. Binding of 4-hydroxyretinal (**11**). (A) Absorption spectra of the binding of 4-hydroxyretinal to the apo-membrane of xR. Curve 1 represents the spectrum of the apo-membrane, and curve 2 is a spectrum that was taken 16 h after the addition of 4-hydroxyretinal. (B) Difference absorption spectra of binding with 4-hydroxyretinal. The spectrum that was taken immediately following 4-hydroxyretinal addition was subtracted from spectra taken at specified times. Curves 1–4 are spectra taken 5 min, 28 min, 2.5 h, and 11 h after the addition of 4-hydroxyretinal, respectively. (C) Normalized absorbance kinetic traces of the binding of 4-hydroxyretinal to apo-xR. Spectrum 1 (▲) represents the evolution of the carotenoid at 520 nm and spectrum 2 (■) the formation of the PSB at 563 nm. (D) CD spectra of the binding process at specified times of incubation after the addition of 4-hydroxyretinal to the apo-membrane. Curves 2–4 are spectra taken 30 min, 4 h, and 13 h after the addition of 4-hydroxyretinal, respectively. Curve 1 represents the spectrum of the apo-membrane.

as the rate of formation of the carotenoid UV–vis vibrational bands, monitored during the binding process; panels B and D of Figure 5 show that the rate of formation of the CD spectrum and UV–vis bands of the carotenoid during the binding process is slower than that of the native pigment or artificial pigments modified along the polyene chain.[6,9] Apparently, β-ionone ring substitution at its C_4 position affects the retinal–protein interactions such that the formation of the CD spectrum and the retinal–protein covalent bond is disturbed. The kinetics of 4-OH pigment formation (Figure 5C) was monitored by the absorption change at 563 nm. The changes in the carotenoid bands were detected by the absorption changes at 520 nm. In both cases, the changes were biphasic with very similar k_1, k_2, and a values (k_1 = 0.0011 s^{-1}, k_2 = 0.0003 s^{-1}, and a = 0.45, and k_1 = 0.0014 s^{-1}, k_2 = 0.0002 s^{-1}, and a = 0.41, respectively), demonstrating that the processes occur in parallel. Thus, while the formation of the artificial retinal pigments resembles the formation of the native xR, the rate of carotenoid fixation is affected by the retinal ring substitution. Therefore, we suggest that the intact structure of the retinal ring moiety is crucial for attaining the twisted conformation of the carotenoid ring in its binding site,[5] while addition of steric interactions decreases the rate of fixation.

4-Methylretinal formed a pigment with the apoprotein of xR, absorbing at 570 nm (Table 1 and Figure 6A,B), in contrast to the bR pigment derived from 4-methylretinal, which exhibits a shoulder around 550 nm in the spectrum in addition to the main absorption band at 470 nm.[19,21] The two bands were attributed to a fraction of the chromophore existing in a different conformation in the binding site. It can be concluded that the steric constraints for accommodating bulky residues at the retinal C_4 position in the xR binding site are much smaller than those detected in bR. The CD spectrum obtained for 4-methyl-xR resembles the characteristic native pigment CD spectrum (Figure 6C). In contrast, we were not able to bind 4-methoxyretinal **13**, which bears a bulkier group at position 4 even after incubation for 2 days. In addition, incubation of this chromophore with apo-xR did not induce the formation of the resolved carotenoid bands. Thus, it is evident that in xR the ability of the apo-membrane to bind retinal analogues and to form the protonated Schiff base linkage is correlated with the bulkiness of the C_4 substituent.

Binding with Dihydroretinal Analogues. Dihydroretinal analogues may shed light on specific retinal regions that are associated with the protein–retinal interactions because they can isolate the β-ionone ring and part of the polyene chain, without changing significantly the overall structure of the retinal.

Incubation of 7,8-dihydroretinal **14** with the apo-membrane of xR formed two stable pigments absorbing at 430 and 450 nm, as previously described.[6] The difference UV–vis spectra of this binding process are different compared to the difference spectra obtained during the binding with native all-*trans*-retinal.

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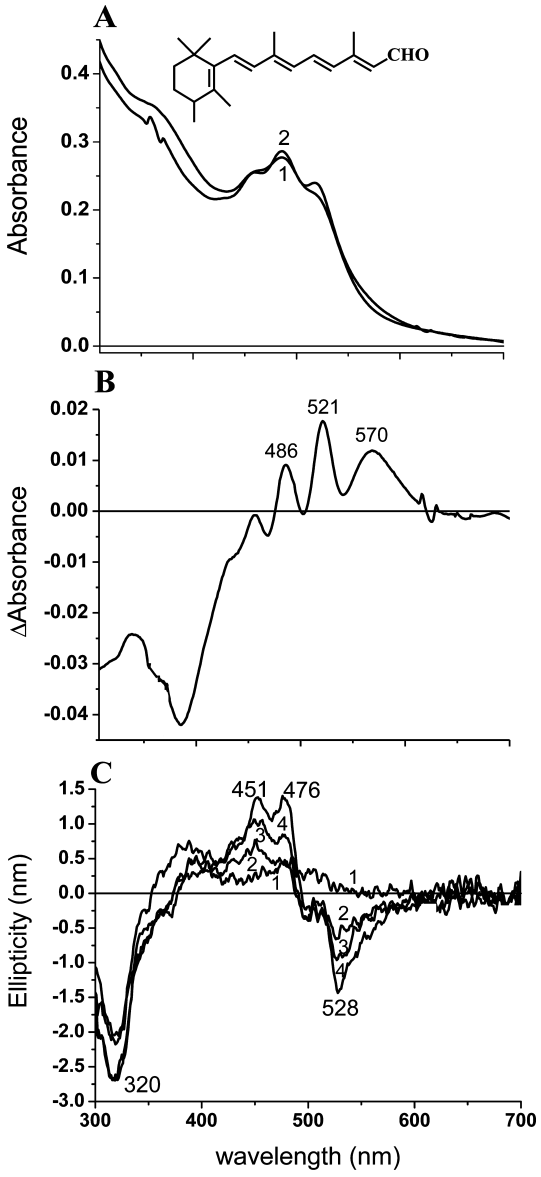

Figure 6. Binding of 4-methylretinal (**12**). (A) Absorption spectra of the binding of 4-methylretinal to the apo-membrane of xR. Curves 1 and 2 are spectra taken 1 s and 13 h after the addition of 4-methylretinal, respectively. (B) Difference absorption spectra monitoring the binding of 4-methylretinal. The spectrum that was taken immediately following 4-methylretinal addition was subtracted from the spectrum taken after incubation for 13 h. (C) CD spectra of the binding process at the specified time of incubation after the addition of 4-methylretinal to the apo-membrane. Curves 2−4 are spectra taken 9 min, 20 min, and 13 h after the addition of 4-methylretinal, respectively. Curve 1 is a spectrum of the apo-membrane.

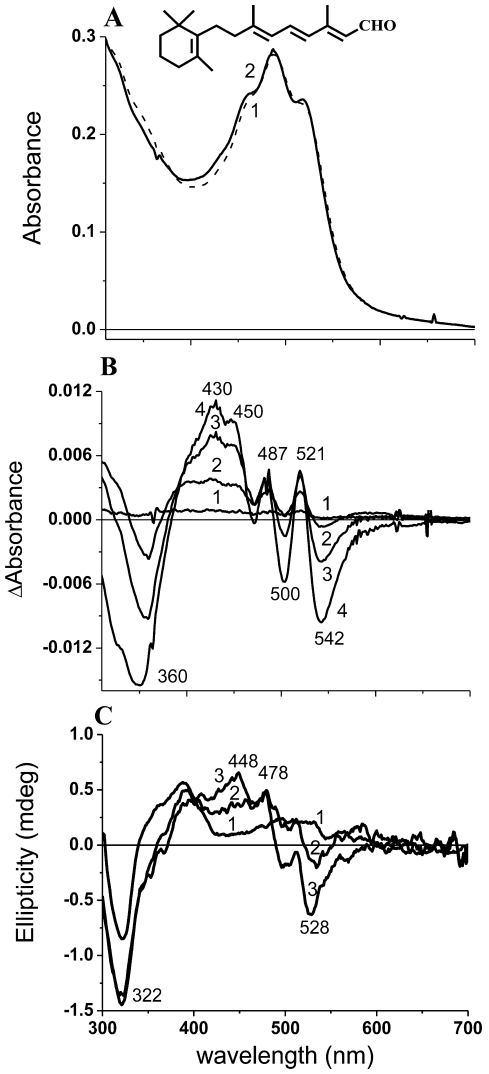

Figure 7. Binding of 7,8-dihydroretinal (**14**). (A) Absorption spectra of the binding of 7,8-dihydroretinal to the apo-membrane of xR. Curves 1 and 2 are spectra taken 2 min and 14 h after the addition of 7,8-dihydroretinal, respectively. (B) Difference absorption spectra of the binding with 7,8-dihydroretinal. The spectrum taken immediately following 7,8-dihydroretinal addition was subtracted from spectra taken at specified times. Curves 1−4 are spectra taken 2 min, 26 min, 8 h, and 14 h after the addition of 7,8-dihydroretinal, respectively. (C) CD spectra of the binding process at specified times of incubation after the addition of 7,8-dihydroretinal. Curves 2 and 3 are spectra taken 12 min and 18 h after the addition of 7,8-dihydrioretinal, respectively. Curve 1 is a spectrum of the apo-membrane.

This demonstrates that the interactions with salinixanthin were altered in 7,8-dihydro-xR (Figure 7A,B).

In addition, the rate of formation of the CD spectrum for 7,8-dihydro-xR is much slower than that of the native system, and the intensity of the resolved spectrum is low as can be seen in Figure 7B. We suggest that saturation of the 7,8-double bond induces an altered ring−polyene−chain torsional angle, causing a considerable perturbation in the ring region. This may lead to a different and slower reorganization of the β-ionone ring within the binding site, thereby decreasing the rate of fixation of salinixanthin in the binding site.

We next tried to form an xR artificial pigment derived from 5,6-dihydroretinal **15**, which is similar in all other respects to retinal except that the retinal β-ionone ring double bond is saturated. However, retinal analogue **15** did not form a pigment following incubation with the apo-membrane of xR, nor it did not induce the characteristic resolved fine structure of the carotenoid chromophore. It may indicate that perturbation of the β-ionone ring due to the absence of the double bond induces a modified ring conformation, thereby affecting the surrounding protein residues. This result is in contrast to that of bR, which regenerates 5,6-dihydro-bR pigment, indicating that the C_5−C_6 retinal double bond is not required for pigment formation in bR.[28] Moreover, 5,6-dihydro-bR exhibits a photocycle and an ability to pump protons, suggesting that

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the presence of the double bond is not essential to the functionality of the bR pigment.

Conformation of the β-Ionone Ring within the Binding Site. It was reported that the conformation about the C_6-C_7 single bond plays an important role in determining the spectroscopic properties of bR.[26] Therefore, we aimed to clarify the ring−polyene−chain conformation in xR. To try to determine whether the conformation of the β-ionone ring chain is 6-*s*-*cis* or 6-*s*-*trans*, we tried to form an xR artificial pigment derived from locked 6-*s*-*trans*-retinal **16** as well as locked 6-*s*-*cis*-retinal **17**.

Locked 6-*s*-*trans*-retinal binds efficiently to apo-bR to form a bR artificial pigment absorbing at 564 nm. It exhibits a light−dark adaptation process and pumps protons like the native system.[26] Locked 6-*s*-*trans*-retinal incubated with apo-xR, forms a stable pigment with a λ_{max} of 575 nm (Table 1 and Figure 8A,B) suggesting that the protein−chromophore interactions in this case are similar to those existing in the native pigment. As monitored by UV−vis spectroscopy, the pigment was formed with a normal rate, and its formation was detected after incubation for 1 min with locked 6-*s*-*trans*-retinal (Figure 8B). The characteristic rate of change in formation in the UV−vis spectrum of salinixanthin chromophore were also very similar to the rate detected during the binding process with native all-*trans*-retinal.[6] The CD spectra that were recorded during the binding process support as well the conclusion that protein−chromophore interactions in the locked 6-*s*-*trans*-retinal are similar to those existing in the native pigment. We detected a fully resolved CD spectrum already 9 min after the addition of the chromophore to the apo-membrane of xR (Figure 8C), as for the binding with all-*trans*-retinal.[6] To detect the rate of formation of the CD lobes following binding of locked 6-*s*-*trans*-retinal to the apo-membrane, we conducted time-resolved CD measurements at 480 and 530 nm, and compared the results to the rate of binding of all-*trans*-retinal to the apo-membrane (Figure 9). The results indicate that the CD band formation rate during the binding of locked 6-*s*-*trans*-retinal to the xR apo-membrane is not significantly different from the rate detected for the binding of all-*trans*-retinal (Table 2), suggesting that the preferred conformation of the retinal chromophore in the xR binding site is 6-*s*-*trans*, in keeping with the conformation suggested by the X-ray studies.[2] In contrast, the locked 6-*s*-*cis*-retinal analogue did not form a pigment following incubation with the apo-membrane of xR, supporting our suggestion that the conformation of the ring chain is 6-*s*-*trans*. In analogy, the reaction of locked 6-*s*-*cis*-retinal with apo-bR is much more complicated compared to its reaction with locked 6-*s*-*trans*-retinal. Two pigments are formed with two maxima at 509 and 596 nm in a ratio of 2:3.[26] The results described above indicate that the orientation of the retinal chromophore in the binding site of xR is exclusively 6-*s*-*trans* and the requirement of this specific conformation is even more demanding than for bR.

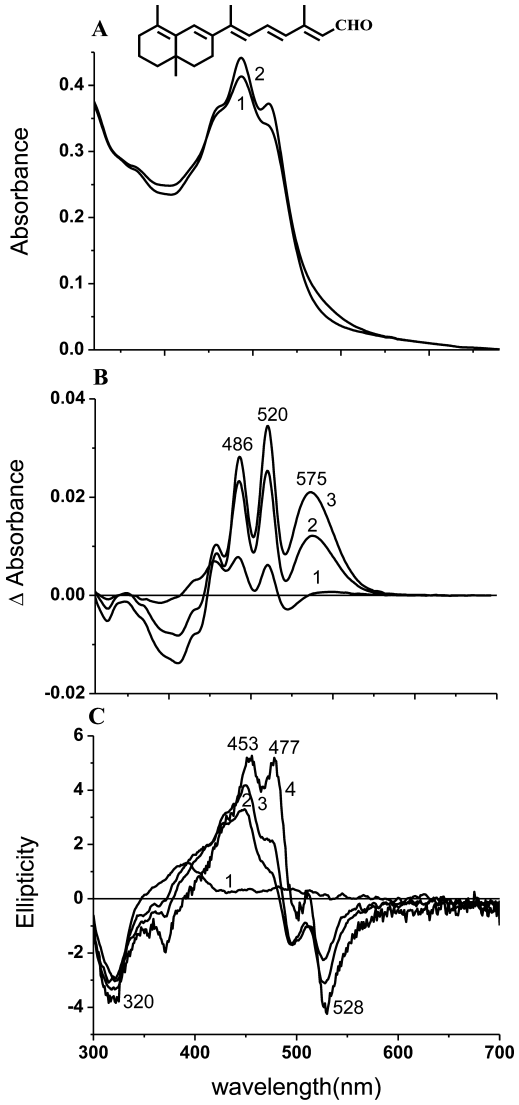

Figure 8. Binding of locked *s*-*trans*-retinal (**16**). (A) Absorption spectra of the binding of locked *s*-*trans*-retinal to the apo-membrane of xR. Curve 1 is the spectrum of the apo-membrane and curve 2 the spectrum that was taken 12 h after the addition of locked *s*-*trans*-retinal. (B) Difference absorption spectra of the binding with locked *s*-*trans*-retinal. The spectrum taken immediately following the addition of locked *s*-*trans*-retinal was subtracted from spectra taken at specified times. Curves 1−3 are spectra taken 1 min, 30 min, and 12 h after the addition of locked *s*-*trans*-retinal, respectively. (C) CD spectra of the binding process at specified times of incubation after the addition of locked *s*-*trans*-retinal. Curves 2−4 are spectra taken 9 min, 2.5 h, and 24 h after the addition of locked *s*-*trans*-retinal, respectively. Curve 1 is a spectrum of the apo-membrane.

DISCUSSION

Because both rings of the retinal and of salinixanthin are located in the proximity of each other,[2] and the salinixanthin 4-keto group plays a crucial role in its binding,[10,29] we aimed to clarify the role of the β-ionone ring in the retinal binding process, as well as its possible effect on the fixation of the salinixanthin 4-keto ring, which leads to the CD spectrum as well as structured UV−vis band formation, as was suggested previously.[5,9] Our results show that substitutions of the β-ionone ring significantly affect the binding of the retinal to the apo-membrane of xR. To further pinpoint the role of the β-ionone ring, we have checked an analogue (linear retinal **3**) that completely lacks the β-ionone ring. As this analogue did not bind to apo-xR or form the carotenoid resolved absorption bands, it is evident that the retinal β-ionone ring has a specific binding site, and its presence is crucial for the formation of the retinal−protein covalent bond as well as fixation of the salinixanthin. In addition, we have checked the role of the ring methyls groups on the β-ionone ring. We found that the presence of a methyl group at position C_5 or the methyls at C_1 is crucial for the ring orientation and locking the retinal chromophore in a conformation that allows

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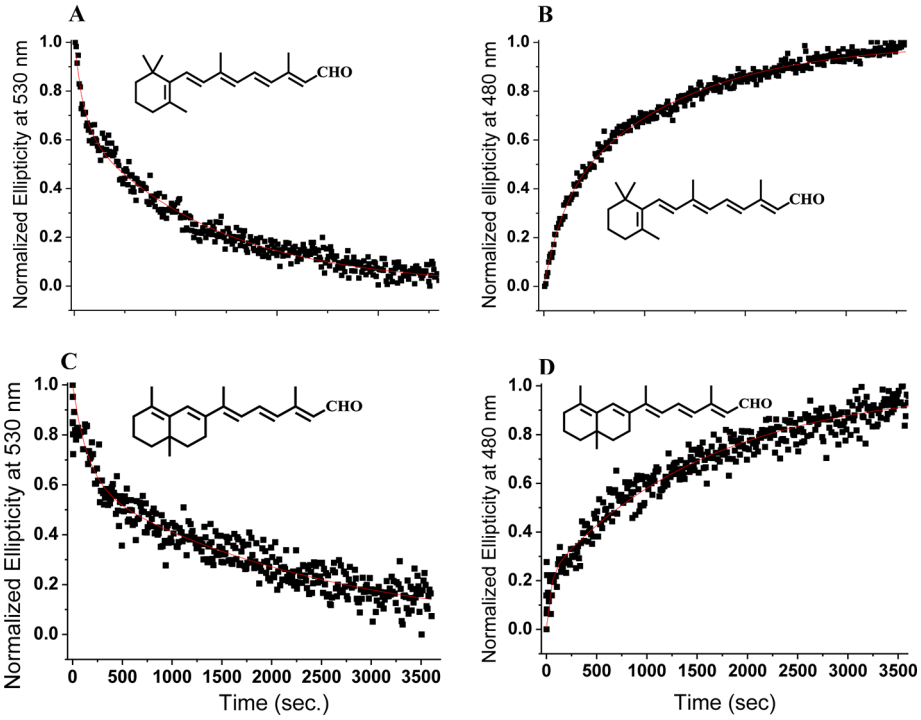

Figure 9. Kinetic traces showing the evolution of the 480 and 530 nm CD bands during the binding of all-*trans*-retinal and locked 6-*s*-*trans*-retinal (**16**). The kinetic measurements were fit to a second-order bR decay or growth equation. The rate of each fraction and the *a* values are listed in Table 2. (A) Formation of the (−) lobe at 530 nm during the binding of all-*trans*-retinal. (B) Formation of the (+) lobe at 480 nm during the binding of all-*trans*-retinal. (C) Formation of the (−) lobe at 530 nm during the binding of locked 6-*s*-*trans*-retinal. (D) Formation of the (+) lobe at 480 nm during the binding of locked 6-*s*-*trans*-retinal.

Table 2. Fractions of the CD Bands Detected by the Kinetic Traces of 480 and 530 nm during the Binding of all-*trans*-Retinal and 6-*s*-*trans*-Locked Retinal (16)

CD lobes	k_1	k_2	*a*
530 nm, all-*trans*-retinal	0.01	0.0008	0.3
480 nm, all-*trans*-retinal	0.005	0.0008	0.3
530 nm, 6-*s*-*trans*-locked retinal	0.007	0.0004	0.4
480 nm, 6-*s*-*trans*-locked retinal	0.02	0.0006	0.3

formation of the chromophore−protein covalent bond. In analogy to bR, the methyl at position 1 or 5 is probably required for specific retinal−protein interactions that induce a ring−chain retinal planar conformation within its binding site.[13,25] The phenylretinal that does not bear any bulky groups and has a planar conformation is able to form a covalent bond with Lys-240, even though it lacks the crucial methyl group at position C_5. It is evident that its ability to form a stable pigment does not correlate with its relatively small size in the ring area, as the linear analogue that lacks the ring did not form a pigment. Thus, it can be suggested that the phenylretinal analogue is able to lock itself in the specific binding site. This ability can possibly be attributed to the planar conformation of the phenyl ring.

The protein surrounding the retinal position C_4 seems to be crowded as there is no binding of retinal analogues bearing substituents bulkier than a methyl group at C_4. In addition, it is evident that the immediate protein surrounding the C_4 position is different from that of bR,[21] as can be deduced from the binding of 4-hydroxy- and 4-methylretinal analogues. It appears that the artificial pigments derived from 4-hydroxy- and 4-methylretinal do not exhibit any significant perturbation and exhibit spectroscopic properties similar to those of the native pigment, in contrast to bR.[21] In addition, we have shown that the preferable ring−chain conformation of native retinal within its binding site, during the binding process, is probably exclusively 6-*s*-*trans* as the formed locked 6-*s*-*trans*-xR pigment exhibits a strong resemblance to wt xR in its absorption and CD spectrum. Additional support for this assumption is gained by the failure to obtain an artificial xR pigment derived from locked 6-*s*-*cis*-retinal. These findings are consistent with the retinal conformation suggested by the X-ray studies[2] and the 6-*s*-*trans* conformation, found in bR and other related proteins,[26,30−33] which also affects the absorption maxima of the pigments.

It was previously shown that the formation of the protonated Schiff base is not required for the fixation of the carotenoid chromophore or formation of the characteristic vibronic bands in the UV−vis spectrum as well as formation of the CD spectrum.[6,9] However, a major fraction of the retinal synthetic analogues modified in the β-ionone ring region did not form the chromophore−protein covalent bond but also did not form the characteristic CD spectrum or the carotenoid sharp vibration bands in the UV−vis spectrum. Therefore, it is possible that these processes are connected. In addition, modifications in the β-ionone ring moiety that did not prevent formation of retinal pigments and the characteristic CD and UV−vis spectra still affect the rate of formation of the vibronic carotenoid structure and pigment. For example, addition of a single double bond between positions C_3 and C_4 (analogue **1**) or substituting the retinal ring at its C_4 position with a hydroxyl group decreased the rate of carotenoid fixation during the binding process, relative to those of the all-*trans*-retinal and synthetic retinal analogues that were not modified in the

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polyene portion.[6,9] Interestingly, once the rate of fixation of the carotenoid chromophore is considerably decreased (relative to that of native retinal), this rate is still very similar to the rate of formation of the protonated Schiff base, which might support the suggestion that the two processes are connected. Therefore, it can be suggested that carotenoid fixation is necessary for pigment formation. This suggestion is based on two observations. (1) Retinal analogues that did not induce the fixation process did not lead to pigment formation. (2) Once the carotenoid fixation rate is considerably decreased, this rate matches the protonated Schiff base formation rate. This suggestion needs to be studied further.

Several factors may affect covalent bond formation. Accessibility of the retinal aldehyde moiety for interaction with Lys-240 may require a specific protein conformation, and in addition, appropriate pK_a tuning of the lysine amino group is a prerequisite for nucleophilic attack of the amino group on the retinal aldehyde.[34] Therefore, it is possible that carotenoid ring fixation induces an allosteric effect that may affect the Lys- 240 region and network of hydrogen bonding, to allow for covalent bond formation.

The crystal structure of xR indicates that the salinixanthin ring is immobilized by several amino acids as well as by the β-ionone ring of the retinal that is within 5 Å of the 4-keto ring.[2] Recently, salinixanthin was reconstituted into a protein homologous to xR (*Gloeobacter* rhodopsin).[29] It was shown that substitution of a Trp residue in the vicinity of the retinal β-ionone ring with a glycine is a prerequisite for the binding of salinixanthin. Evidently, the bulky Trp-138 that is located in bR in the vicinity of the retinal ring is replaced in xR with Gly, and the formed space is occupied by the 4-keto ring of salinixanthin. Apparently, the key factor for the binding of salinixanthin[5,9,10] is associated with accommodation of its 4-keto group in the vicinity of the retinal β-ionone ring. Moreover, it was shown that carotenoids that lack the 4-keto ring do not bind to *Gloeobacter* rhodopsin.[29] Imasheva et al. also found that reduction of the salinixanthin carbonyl C=O group suppresses the binding of salinixanthin to the protein and eliminates the transfer of energy to the retinal, demonstrating the crucial role of the carbonyl moiety.[10] These findings are in keeping with our suggestion that the β-ionone ring plays an important role not only in the orientation and binding of the retinal but also in the fixation of the 4-keto ring of salinixanthin within its binding site and formation of the characteristic vibronic bands. Because of the proximity of the two rings, the β-ionone of the retinal and 4-keto of salinixanthin, we suggest that fixation of salininixanthin occurs in the region of the 4-keto ring, leading to a chiral conformation of salinixanthin. Modifications of the retinal ring moiety may lead to a slower reorganization of β-ionone ring in the binding site and apparently to slower fixation of the salinixanthin 4-keto ring. Previous results of binding of retinal to the apo-membrane of xR[6,9] and our conclusions identifying a crucial role for the β-ionone ring in controlling the retinal binding process can be summarized as follows. (1) The retinal chromophore occupies the binding site of the apo-membrane of xR. (2) The retinal undergoes reorganization associated with fixation of the retinal β-ionone in the *6-s-trans* conformation. (3) This reorganization leads to the salinixanthin ring fixation reflected in the vibronic transitions of salinixanthin, and formation of the CD spectrum, as was shown previously.[6,9] (4) The fixation of the salinixanthin ring may allow for the formation of the covalent bond between the retinal and Lys-240 (i.e., formation of the retinal pigment).

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

xR, xanthorhodopsin; bR, bacteriorhodopsin; PSB, protonated Schiff base; CD, circular dichroism.

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