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# Retinal−Salinixanthin Interactions in Xanthorodopsin: A Circular Dichroism (CD) Spectroscopy Study with Artificial Pigments[†]

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ABSTRACT: Xanthorhodopsin (xR) is a recently discovered retinal protein that contains, in addition to the retinal chromophore, a carotenoid (salinixanthin) absorbing at 456, 486, and 520 nm, which functions as a light-harvesting antenna. We have studied the interactions between the two chromophores by monitoring the absorbance and circular dichroism (CD) spectroscopies of artificial pigments derived from synthetic retinal analogues characterized by shifted absorption maxima. In addition, we have followed the binding process of the synthetic chromophores to the apomembrane of xR. We have revealed that the CD spectrum of xR originated mainly from the carotenoid chromophore without a significant contribution of the retinal chromophore. Because the binding process rate of these analogues is slower compared to all-*trans* retinal, it was possible to detect and analyze the major alterations in the CD spectrum. It was revealed that the main changes occur as a result of binding site occupation by the retinal chromophore and not because of the formation of the retinal−protein covalent bond.

Xanthorhodopsin (xR)[1] (*1*) is a retinal-based proton pump in the cell membranes of the extremely halophilic eubacterium *Salinibacter ruber* (*2*). In addition to all-*trans* retinal, it contains an additional chromophore salinixanthin, a $C_{40}$ carotenoid bearing 11 double bonds in the conjugated chain, 1 double bond in the ring, and a keto group in the $C_4$ position (Figure 1). On the other end, it has a glycoside and an acyl tail (*3*). This carotenoid functions as a light-harvesting antenna (*4*). Recently, the structure of xR was determined by X-ray diffraction to 1.9 Å resolution (*5*). The absorption spectrum as well as the circular dichroism (CD) spectrum of the pigment is remarkably different from the apoprotein (*6*). Free salinixanthin in solution (without the protein) has a very weak optical activity (*3*); however, following binding to the retinal protein xR, it exhibits sharp CD lobes that arise from the asymmetric environment of xR or an asymmetric conformation of the salinixanthin molecule enforced by the protein (*6*). These lobes disappear following the hydroxylamine reaction with the retinal protonated Schiff base (PSB)−Lys-227 covalent bond (the residue homologous to Lys-216 in bR). Fixation of the ring in an asymmetric conformation, i.e., not in the plane of the conjugated chain, is probably one of the sources of optical activity of bound salinixanthin (*6*).

A most intriguing question is associated with the nature of retinal−salinixanthin interactions, i.e., which interactions enable energy transfer from the salinixanthin to the retinal. These interactions may be reflected in the CD spectrum of the pigment, and therefore, understanding the factors that control the CD spectrum may clarify the nature of the retinal−salinixanthin interactions.

Earlier studies on xR suggested that the CD spectrum of the xR pigment is a combination of the contribution from its two chromophores. The sharp positive maxima at 513, 480, and 455 nm (measured at 0.15% DM) resemble the absorption maxima of salinixathanin itself. The retinal chromophore, which is covalently bound via a PSB bond to Lys-227, contributes mostly to the negative CD band at $\lambda > 550$ nm (*6*). This assumption was based on earlier extensive studies of the CD spectrum of bacteriorhodopsin (bR) (for a review, see ref *7*). The retinal is the only chromophore in bR that absorbs light at 568 nm (LA), and its CD spectrum in the visible region is composed of negative and positive lobes with unequal intensities. Two mechanisms were proposed for the observed biphasic-shaped CD spectrum of bR: (1) excitonic interactions between the retinal chromophores of the bR protein in the trimer structure of the purple membrane and (2) a combination of CD bands with opposite rotational strength because of the heterogenic environment of bR molecules to two possibly close lying long wavelength transitions of the retinal in bR (for a review, see ref *7*).

In this paper, we examine whether the formed PSB covalent bond between the retinal chromophore to Lys-227 is necessary to induce the chiral conformation of salinixanthin or, alternatively, to induce the asymmetric environment of the xR pigment.

To explore the nature of the CD spectrum of the xR pigment and, especially, the nature of the negative lobe at 530 nm, we have prepared a variety of artificial xR pigments having shifted absorption maximum, as was shown previously for bR (*8*−*12*).

Our goal was to follow xR reconstitution processes using CD and absorbance spectroscopies and correlate the changes that take place in its retinal and salinixanthin chromophores. We present direct evidence that the CD spectrum of xR originated from the fixation of salinixanthin in its binding site, without a significant contribution of the retinal chromophore. In addition,

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[1]Abbreviations: bR, bacteriorhodopsin; DM, dodecylmaltoside; PSB, protonated Schiff base; xR, xanthorhodopsin.



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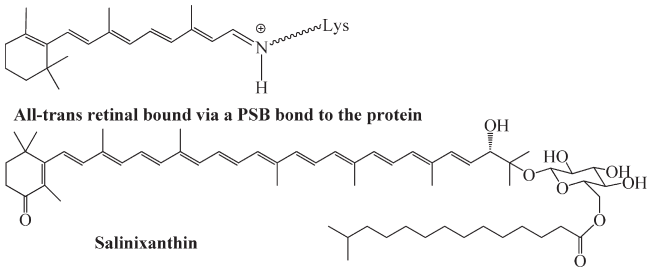



FIGURE 1: Schematic presentation of the two chromophores occupying the binding site of xR. The retinal chromophore is bound to the protein via a covalent bond.

it was found that the pre-pigment species, which probably consists of free retinal occupying the retinal binding site exhibits a CD band around 430 nm, while the retinal chromophore does not exhibit a significant CD band following the formation of the protein−chromophore covalent bond.

Furthermore, it was revealed that the major alterations in the salinixanthin chirality occur as a result of retinal occupying the binding site and not because of the formation of the retinal−protein covalent bond.

## MATERIALS AND METHODS

*Sample Preparation*. Growth of *S. rubber* was carried out according to published methods (*1*, *2*), with the addition of 0.1% sucrose according to ref *13*. xR membrane samples were isolated using published methods (*1*). The membranes were washed with 0.01% dodecylmaltoside (DM) followed by washing 3 times with DDW. This treatment partially removed unbound salinixanthin, proteins, and lipids but did not solubilize the membranes. Following this process, the ratio between the absorption at 280 and 568 nm was ~3.

Apo-protein was prepared by incubation of the wild-type (wt) protein with 0.2 M (0.05−0.1 M in the bleaching process of artificial pigments) freshly prepared hydroxylamine at pH 7.2 and irradiation for 1.5 h, which was carried out with a Schott 250 W 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 $\lambda > 550$ nm (Schott, Mainz, Germany). The samples were thoroughly washed from hydroxylamine by the dialysis procedure versus DDW and were stored at 4 °C to avoid reconstitution with retinal originating from decomposed retinal oxime.

The retinal analogues were synthesized as described previously (*10*, *14*−*21*). Artificial pigments were prepared by incubating the apoprotein (absorbance of 0.2−0.4 OD according to the absorption at 487 nm) with 2 equiv of the synthetic retinal analogue in 50 mM Tris buffer at pH 8.3, 30% sucrose, and 100−200 mM NaCl at 20 °C, overnight. The amount of the pigment was estimated on the basis of a bleach process using hydroxylamine.

14-Fluoro-xR and 13,14-difluoro-xR were prepared by incubating the apoprotein with 2 equiv of the chromophore in the presence of 20 mM Tris buffer (pH 7 and 6.7, respectively), 30% sucrose, and 1 M NaCl, at 20 °C, for 9 and 29 h, respectively.

Synthetic retinal analogues are designated by numbers, whereas the corresponding pigments are designated by indicating the synthetic analogue and the protein names.

*UV*−*Vis Absorbance Measurements*. All of the absorbance measurements were carried out using a Agilent 4583 diode-array spectrophotometer (Agilent Technologies, Palo Alto, CA) equipped with an Agilent 89090A thermostatted cuvette holder (Agilent Technologies, Palo Alto, CA). Absorption spectra were corrected for light scattering.

*CD Measurements*. CD spectra were recorded on an Aviv CD spectrometer, model 202, as well as on the Chirascan CD spectrometer of Applied Photophysics. The CD spectra are given in degrees of ellipticity, $\theta$, 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 × 1 cm path length cuvette was used, and the measurements were carried out at 22 °C with a 1 nm bandwidth resolution.

## RESULTS

We have studied several artificial pigments aiming at elucidating retinal−carotenoid interactions. The absorption maxima of all artificial xR pigments compared to the corresponding artificial bR pigments, in their dark adapted form, are summarized in Table 1.

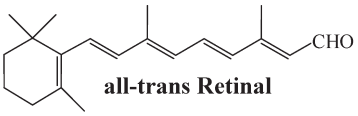
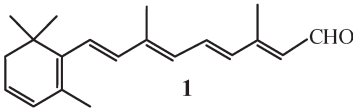
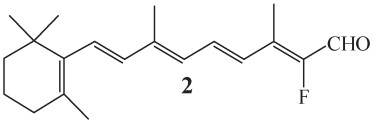
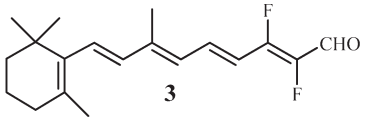
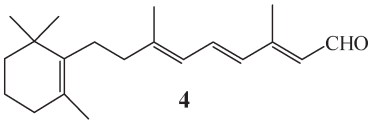
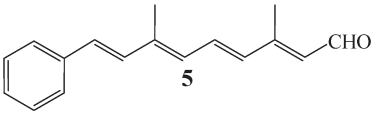
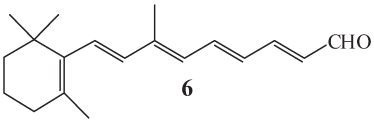
All of the artificial xR pigments were converted to native xR following the addition of stoichiometric amounts of all-*trans* retinal to the artificial pigments. All-*trans* retinal displaces the synthetic retinal analogues by virtue of a stronger association equilibrium constant with the apo-membrane. These results indicate that the retinal analogues and native retinal occupy the same binding site. In addition, all artificial pigments studied were unstable to treatment with hydroxylamine in the dark (50−100 mM, pH 7.2), in contrast to native xR, which undergoes a fast bleaching process only following irradiation.

*CD Spectra of Artificial Pigments. Origin of the 530 nm CD Band*. It has been shown that xR exhibits a CD spectrum with sharp positive bands at 513, 480, and 455 nm (close to the absorption bands) as well as a negative band at 535 nm (*6*).

To clarify whether the negative band originates from the retinal chromophore or from the salinixanthin and to try to understand the nature of retinal−salinixanthin interactions, we used a variety of artificial pigments derived from synthetic retinal analogues that shift the pigment absorption maximum compared to native xR. Reconstitution of the retinal analogues with the apo-membrane of xR allowed us to monitor the absorption as well as the CD spectra of the resulting artificial pigments and to clarify whether the spectroscopic properties of the retinal−salinixanthin complex are influenced by the specific retinal absorption reflected in the CD spectrum. If the CD negative band originates from the retinal chromophore, it is conceivable that the CD band will shift as the retinal absorption maximum is shifted. The effects of the native retinal substitution on the absorption maximum of a variety artificial dark-adapted xR pigments are summarized in Table 1. The binding was followed by monitoring the pigment formation by absorption and CD spectroscopies simultaneously.

It was previously shown that the bR artificial pigment derived from 3,4-dehydro retinal **1**, is red-shifted relative to the native pigment, because of the additional double bond (*8*). Similarly, incubation of 3,4-dehydro retinal with xR apo-membrane produced with high yield an artificial pigment absorbing at 591 nm (Table 1 and panels A and B of Figure 2) versus 565 nm of the native system (*1*). However, the negative band at 530 nm of the CD spectrum did not shift despite the fact that the absorption maximum of the pigment has been shifted to 591 nm (Figure 2C). A shoulder centered at around 585 nm is observed by subtraction

Table 1: Absorption Maxima of Artificial Pigments of xR and bR

| Pigment | Absorption maximum | |
|---|---|---|
| | Xanthorhodopsin | Bacteriorhodopsin[a] |
|  **all-trans Retinal** | 568 nm | 558 nm (*10*) |
|  **1** | 591 nm | 590 nm (*8*) |
|  **2** | 595 nm | 587 nm (*11*) |
|  **3** | 600 nm | 450 & 600 nm (*11*) |
|  **4** | 430 & 450 nm | 442 nm (*10*) |
|  **5** | Overlaps with salinixanthin absorption. | Two pigments: 480 & 520 nm (*12*) |
|  **6** | 566 nm | 565 nm (*9*) |

[a] Data for dark adapted wt and artificial pigments of bR.

the spectrum that was taken following 45 min after the addition of 3,4-dehydro retinal from the spectrum that was taken once the binding process was completed (4 days) (Figure 2D), probably because of the contribution of the retinal chromophore itself. Thus, it is conceivable that, in the native system, this shoulder is part of the negative band, because the retinal chromophore absorbs at 568 nm.

14-Fluoro retinal **2** represents an example of a chromophore that bears an electron-withdrawing group and red shifts the absorption because of ground-state destabilization (*23*). This analogue produced a red-shifted xR pigment, having an absorption maximum at 595 nm (Table 1 and panels A and B of Figure 3). The CD spectra indicate that the negative band at 530 nm did not shift (Figure 3C), which supports our previous conclusions based on 3,4-dehydro-xR pigment results, assigning this band mainly to the carotenoid chromophore. In addition to the main 527 nm band, a shoulder centered at ca. 600 nm is clearly observed (Figure 3C). This shoulder is probably originated from the retinal analogue chromophore contribution, which is red-shifted relative to the native pigment. The positive bands at 449 and 477 nm are similar to the native pigment exhibiting somewhat different intensity.

Similarly, binding of the apo-membrane with 13,14-difluoro retinal **3** formed a stable pigment following incubation for 28 h in the dark, yielding absorption maximum at 600 nm (Table 1 and panels A and B of Figure 4). The resolved CD spectrum of 13,14-diF-xR shows high resemblance to the CD spectrum of 14-F-xR. The negative band at 527 nm was not red-shifted despite pigment formation at 600 nm, and a red-shifted shoulder appeared at around 600 nm.

The binding process of both 14-F retinal **2** and 13,14-diF retinal **3** monitored by absorbance difference spectra clearly exhibited at least two separate phases as detected previously for 13-desmethyl-xR (*24*). The absorption difference spectra indicated that the PSB linkage between the retinal chromophore and the protein did not form during the first phase of the process. This phase was associated with a change in the fine structure of the salinixanthin chromophore as well as in its intensity, which was detected before PSB formation (Figures 3B and 4B). The carotenoid absorption change was accompanied by a formation of a band around 450 nm (pronounced especially in the 13,14-diF retinal binding process, Figure 4B), because of the formation of a red-shifted pre-pigment (*24*). In the second phase of the binding process, the PSB is formed, as evident by a formation of the

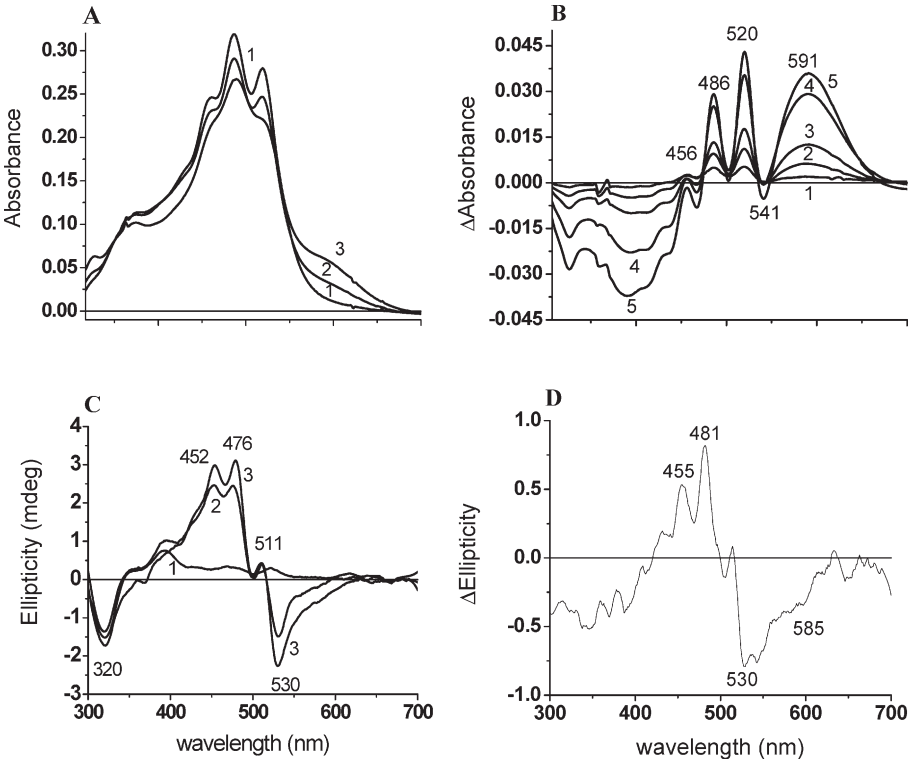



FIGURE 2: Binding process of 3,4-dehydro retinal (**1**). (A) Absorption spectra of the 3,4-dehydro retinal binding process to the apo-membrane of xR. Curves 2 and 3 are spectra taken 40 min and 24 h after the addition of chromophore **1**, respectively. Curve 1 represents the spectrum of the apo-membrane. (B) Difference absorption spectra of the binding of 3,4-dehydro retinal. The spectrum that was taken immediately following 3,4-dehydro retinal addition was subtracted from spectra that were taken at specified times. Curves 1−5 are spectra taken: 62 s, 5 min, 14 min, 41 min, and 24 h after the addition of 3,4-dehydro retinal, respectively. (C) CD spectra of the binding process at specified times of incubation after the addition of 3,4-dehydro retinal to the apo-membrane. Curves 2 and 3 are spectra taken 45 min and 4 days after the addition of 3,4-dehydro retinal, respectively. Curve 1 is a spectrum of the apo-membrane. (D) Difference CD spectrum of the binding with 3,4-dehydro retinal. The spectrum taken 45 min following chromophore addition was subtracted from the spectrum taken 4 days after chromophore addition.

pigment absorption at 600 nm, accompanied by a change in the ratio between the bands of salinixanthin (484 and 520 nm). The band at 484 nm looses intensity as the binding proceeds, and the ratio between 484 and 520 nm bands resembles the ratio which characterizes the binding of all-*trans* retinal (*1*). The CD spectra, which monitor the two phases of the binding process will be discussed below.

The absorption maximum of the pigment can be significantly blue-shifted by employing the artificial pigment 7,8-dihydro-xR, derived from 7,8-dihydro retinal **4**. It is evident that two pigments are formed, probably because of two possible conformations within the binding site of the retinal chromophore (430 and 450 nm, Figure 5B). The CD spectrum indicates that the negative band appears at 528 nm (Figure 5C), supporting the previously derived conclusion that the main component of this band is not associated with the retinal chromophore but rather the carotenoid chromophore.

The binding process of phenyl-retinal **5** with the apo-membrane of bR produces two pigments with absorption maxima at 480 and 520 nm (Table 1) (*12*). Thus, assuming that phenyl-xR absorbs in this range as well, the absorption maximum of the pigment cannot be detected, because the salinixanthin and the phenyl-xR absorptions should overlap. Although we could not detect the formation of the phenyl-xR pigment during the process of pigment formation, it is conceivable that the phenyl-retinal binds covalently and forms the PSB with the lysine xR binding site because of several observations: (1) gain of the characteristic fine structure by salinixanthin (Figure 6A), (2) a positive change in the intensity of salinixanthin (Figure 6B), and (3) addition of hydroxyl-amine to the pigment in the dark causing a bleach process, which was accompanied by the characteristic reduction in the salinixanthin bands (*1*), and appearance of a species with a fine-structured absorption spectrum at $\lambda_{max}$ = 374 nm, indicating the formation of phenyl-retinal oxime (Figure 6C).

The CD spectrum of phenyl-xR (Figure 6D) showed only a slight shift of the negative band toward 529 nm (Figure 6D), indicating that this CD band mainly originated from the salinixanthin chromophore as a result of binding site occupation by the retinal analogue.

*530 nm Transition in the Absrbance Spectrum*. Because no transition around 530 nm could be detected in the absorbance spectrum of xR as well as in the absorbance spectrum of its apo-membrane, we have studied the absorption difference spectra of the binding process for the artificial pigments 3,4-dehydro-xR, 14-F-xR, 13,14-diF-xR, 7,8-dehydro-xR, and phenyl-xR.

A band at 546 nm was previously detected in the difference absorption spectrum that monitored the binding process of the retinal chromophore to the apo-membrane of xR. It was suggested that the rigid fixation of salinixanthin is the cause for this band, which is due to a decrease in the absorption bandwidth of salinixanthin (*24*). We have detected this negative band around 545 nm, in the absorption difference spectra that monitored the binding process of each of the synthetic analogues. For 3,4-dehydro-xR, this transition was detected at 541 nm (Figure 2B); for 14-F-xR, this transition was detected at 543 nm (Figure 3B); for 13,14-diF-xR, this transition was detected at 545 nm (Figure 4B); for 7,8-dehydro-xR, this transition was detected at 542 nm; and for phenyl-xR, this transition was detected

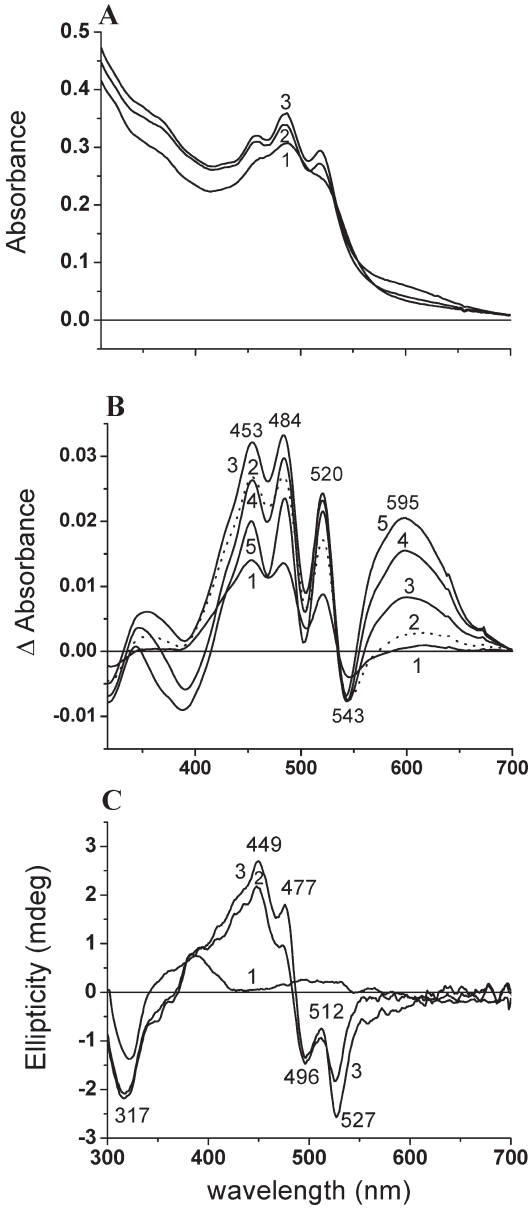



FIGURE 3: Binding process of 14-F retinal (**2**). (A) Absorption spectra of various stages of 14-F retinal binding to the apo-membrane of xR. Curves 2 and 3 are spectra taken 2 and 28 h after the addition of 14-F retinal, respectively. Curve 1 is a spectrum of the apo-membrane. (B) Difference absorption spectra of the binding process of 14-fluoro retinal. The spectrum that was taken immediately following 14-F retinal addition was subtracted from spectra taken at specified times. Curves 1−5 are spectra taken 32 s, 7 min, 80 min, 3 h, and 9 h after the addition of 14-F retinal, respectively. (C) CD spectra of the binding process at specified times of incubation after the addition of 14-F retinal. Curves 2 and 3 are spectra taken 10 min and 10 h after the addition of 14-F retinal, respectively. Curve 1 represents the spectrum of the apo-membrane.

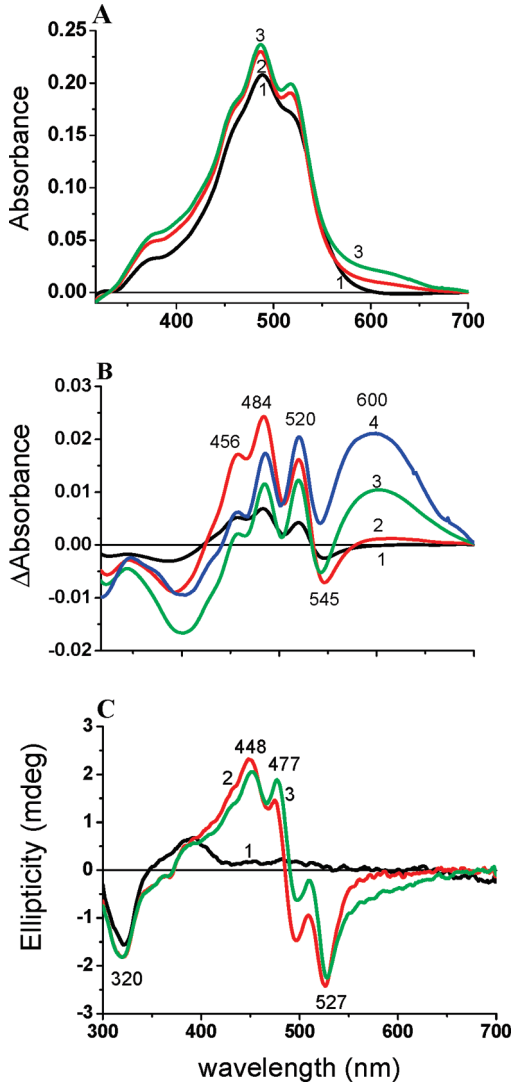



FIGURE 4: Binding process of 13,14-diF retinal (**3**). (A) Absorption spectra of the binding of 13,14-diF retinal to the apo-membrane of xR. Curves 2 and 3 are spectra taken 6 and 29 h after the addition of 13,14-diF retinal, respectively. Curve 1 represents the spectrum of the apo-membrane. (B) Difference absorption spectra of several stages of the 13,14-diF retinal binding process. The spectrum that was taken immediately following 13,14-diF retinal addition was subtracted from spectra taken at specified times. Curves 1−4 are spectra taken 80 s, 23 min, 375 min, and 29 h after the addition of 13,14-diF retinal, respectively. (C) CD spectra of the binding process at the specified times of incubation after the addition of 13,14-diF retinal. Curves 2 and 3 are spectra taken 19 min and 23 h after the addition of 13,14-diF retinal, respectively. Curve 1 is a spectrum of the apo-membrane.

at 545 nm. For all of the pigments, the shift of the negative band at 546 nm was minor, indicating that it does not change even when different synthetic analogues are incorporated into the apo-membrane. Similarly, the major transitions of salinixanthin at 456, 486, and 520 nm are not affected as well. It is conceivable that the main negative CD band at 530 nm corresponds to the 546 nm vibronic transition in the absorption spectrum of xR. Thus, because the negative band at 530 nm is not affected by the specific absorption of the artificial pigment, it can be concluded that the negative band at 530 nm originates mainly from the salinixanthin chromphore. The retinal chromophore contributes only partially to this band, as demonstrated by the spectra of 3,4-dehydro-xR, 13,14-diF-xR, and 14-F-xR.

*Alterations of the CD Spectrum During the Binding Process*. It was revealed that the major changes detected in the salinixanthin absorption spectrum are due to binding site occupation by the ligand (all-*trans* retinal or its synthetic analogues), whereas the formation of the PSB induces significantly smaller changes (*24*). Thus, to shed further light on the reconstitution process, we followed the CD spectral changes during the retinal binding process.

First, we have followed the CD and absorption spectral changes induced by the binding process of all-*trans* retinal

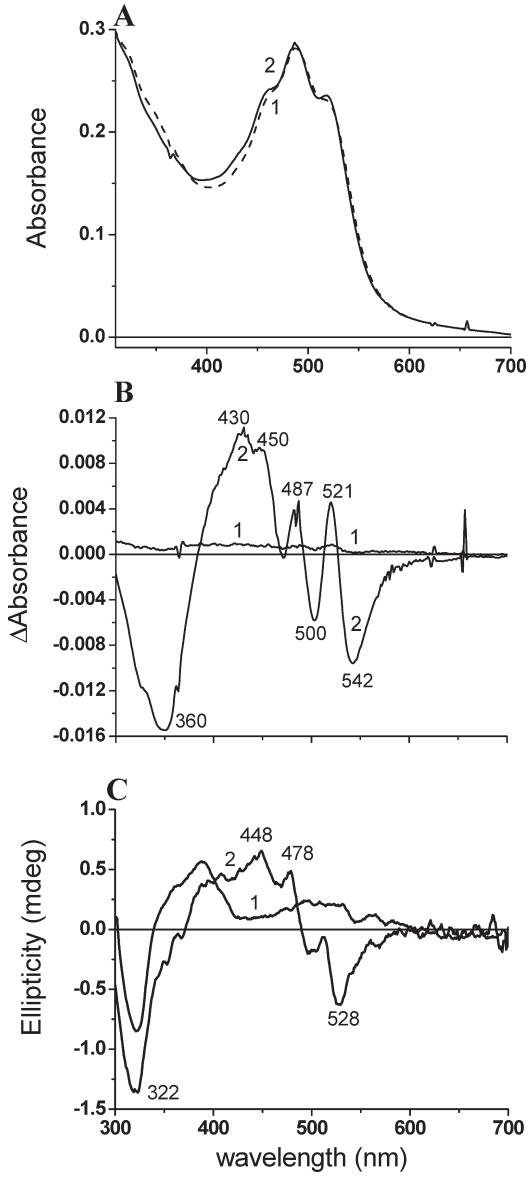



FIGURE 5: Binding process of 7,8-diH retinal (**4**). (A) Absorption spectra of the binding of 7,8-diH retinal to the apo-membrane of xR. Curves 1 and 2 are spectra taken 2 min and 14 h after the addition of 7,8-diH retinal, respectively. (B) Difference absorption spectra of the binding with 7,8-diH retinal. The spectrum taken immediately following 7,8-diH retinal addition was subtracted from spectra taken at specified times. Curves 1 and 2 are spectra taken 2 min and 14 h after the addition of 7,8-diH retinal, respectively. (C) CD spectra of the binding process at specified times of incubation after the addition of 7,8-diH retinal. Curves 2 is spectrum taken 18 h after the addition of 7,8-diH retinal. Curve 1 is a spectrum of the apo-membrane.

(panels A and B of Figure 7). At 10 min after chromophore addition, the CD spectrum was more intense around 450 nm than that detected following completion of the binding process, suggesting the formation of the pre-pigment. We note that at this stage of the binding process (10 min), a fraction of the PSB is already formed.

To further clarify the origin of the CD spectrum, we followed the CD changes caused by binding the synthetic analogues, 13-desmethyl retinal, 14-F retinal, and 13,14-diF retinal. Binding of 13-desmethyl retinal **6** with the apo-membrane indicated that the formation of the pigment band is much slower compared to that of all-*trans* retinal and the characteristic sharpening of the salinixanthin bands was initiated long before the retinal PSB is formed. The salinixanthin change is probably triggered by the occupation of the retinal binding site by the retinal chromophore (*24*). The resolved CD spectrum of 13-desmethyl-xR was detected already following the first 10 min of the binding process (Figure 8B), as in native xR (*6*) (Figure 7B). These observations suggest that removal of the methyl group at $C_{13}$ does not substantially affect the rate of CD spectrum formation (Figure 8B). Interestingly, most of the CD spectrum was detected while the PSB was formed much later (Figure 8A). Thus, the PSB is not required for salinixanthin fixation in its binding site, which probably induces its CD spectrum. In addition, it is clearly observed that, already after 10 min of the binding process, a CD band at about 440 nm appears, which decays as the PSB is formed. This band can be attributed to the pre-pigment species, which was also detected in the absorbance spectrum (*24*).

Further clarification of the changes occurring because of the formation of the PSB linkage could be obtained by subtraction of the spectrum that was taken 30 min after the addition of 13-desmethyl retinal from that which was taken after binding completion (10 h) (Figure 8C). It is clearly observed that the pre-pigment band at about 430 nm disappeared, while a positive band is obtained at 487 nm and a negative band at 543 nm was increased. It was previously shown that the pre-pigment absorbs around 450 nm (*24*), and therefore, it is conceivable that the intense ellipticity around 430 nm is originated from the pre-pigment formation. Furthermore, it is possible that the negative band at 543 nm (Figure 8C) is originated from the contribution of the retinal PSB that was formed. The formation of the positive band at 487 nm during the formation of the retinal−protein covalent bond indicates that the carotenoid chromophore experiences further alterations during the PSB formation.

The resolved CD spectrum of 13,14-diF-xR (Figure 4C) was detected already after 10 min of the retinal analogue addition, prior to the formation of the PSB, similar to 13-desmethyl-xR. The intriguing feature of this process is associated with the observation that the resolved CD spectrum that was obtained already 19 min after chromophore addition was even more intense than the spectrum that was obtained 23 h after the addition of the chromophore. As the process was initiated, the dominant band was at 448 nm, but later, this band lost some of its intensity, while the band at 477 nm was increased. These changes in the CD spectra correspond to the changes in the absorbance spectra (Figure 4B).

Further clarification of these changes could be obtained by subtraction of the CD spectrum that was taken 19 min after 13,14-diF retinal addition to the apo-membrane from the spectrum that was taken following completion of the binding process (23 h) (Figure 9A). It is clearly observed that the pre-pigment band at about 441 nm disappeared, while the positive bands were obtained at 489 and 519 nm, exactly as the absorption bands. Moreover, a broad negative band around 574 nm was increased, indicating a possible contribution of the retinal PSB chromophore itself to the CD spectrum. The absorption maximum of 13,14-diF-xR was shifted to 600 nm because of the withdrawing electron capability of the fluoro substituents. The above-described observations demonstrate the formation of a pre-pigment species that has a CD band, as detected as well for 13-desmethyl-xR. The subtraction of the absorbance spectrum that was taken 23 min after the addition of 13,14-diF retinal from the spectrum that was taken following completion of the binding process (29 h) provided further evidence for the formation of the pre-pigment

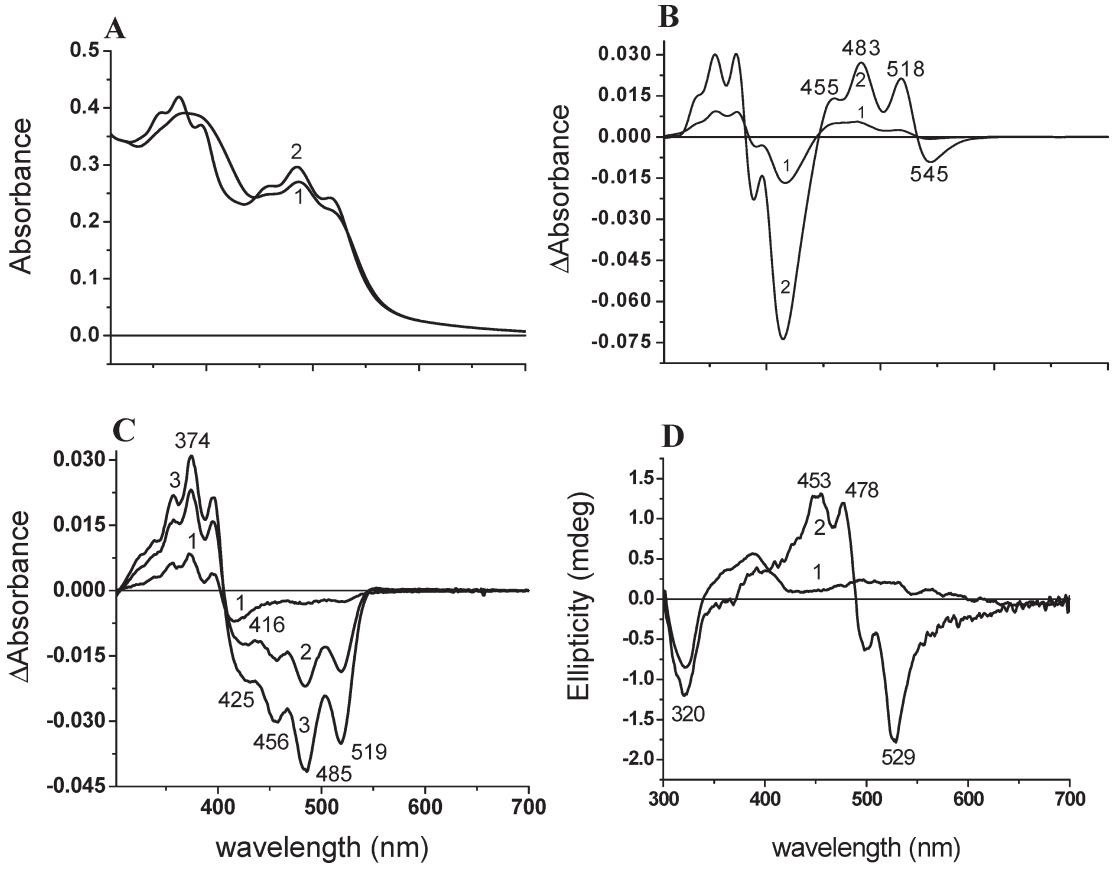



FIGURE 6: Binding of phenyl-retinal analogue (**5**) to the apo-membrane. (A) Absorption spectra of phenyl-retinal binding to the apo-membrane of xR. Curves 1 and 2 are spectra taken 1.5 s and 6.5 h after the addition of phenyl-retinal, respectively. (B) Difference absorption spectra of the phenyl-retinal binding process. The spectrum taken immediately following phenyl-retinal addition was subtracted from spectra taken at specified times. Curves 1 and 2 are spectra taken 5 min and 6.5 h after the addition of phenyl-retinal, respectively. (C) Difference absorption spectra of the phenyl-xR bleaching process with $NH_2OH$ in the dark. The spectrum taken immediately following $NH_2OH$ addition was subtracted from spectra taken at specified times. Curves 1−3 are spectra taken 62 s, 22 min, and 57 min after the addition of hydroxylamine, respectively. (D) CD spectra of the binding process with phenyl-retinal. Curve 2 is the spectrum taken 18 h after the addition of phenyl-retinal. Curve 1 represents the spectrum of the apo-membrane.

around 460 nm (Figure 9B), which supports our assumption that the CD band at 440 nm originated from the pre-pigment.

Similar to 13-desmethyl retinal and 13,14-diF retinal, binding of 14-F retinal to the apo-membrane induced the formation of the CD spectrum prior to the formation of the chromophore−protein covalent bond (PSB). To detect the rate of formation of the CD bands, we have carried out kinetic measurements with CD spectroscopy of 14-F retinal binding to the apo-membrane of xR at 452 nm as well as at 530 nm, in comparison to the absorbance kinetic measurements at 599 nm (Figure 10). It is evident that the main fraction of the CD band at 452 nm and 530 nm is formed at about 300 s, whereas the major fraction of the pigment (detected at 599 nm of the absorption spectrum) is formed significantly slower.

## DISCUSSION

It is well-established that the retinal binding process to the apo-membrane of bR, which yields the bR pigment, involves several stages, including the formation of a pre-pigment species (*25*). The pre-pigment, which actually may consist of several subspecies, has a wide absorbance band around 440 nm (*25*). It was shown that the covalent chromophore−protein bond is not yet established (*26*). The decay of the latter pre-pigment to the characteristic 568 nm bR band is associated with the formation of the retinal−protein covalent bond, and it is inhibited at low humidity (*27*). The retinal itself occupies its binding site in a millisecond process, as evident by a tryptophane emission quenching process (*28*). The carotenoid chromophore of xR has a very weak CD spectrum in solution (without the protein), as was previously demonstrated (*3*). Its relatively strong CD in the protein environment is due to the interaction with the protein chiral environment or a specific asymmetric conformation adopted by the carotenoid enforced by the protein matrix. The carotenoid−retinal interactions are crucial for achieving the carotenoid CD, as was shown previously (*6*). However, our present work demonstrates that these specific interactions do not require the formation of the retinal−protein PSB linkage and most of the CD spectrum is obtained before the retinal−protein covalent bond is formed. The distinct fine structure in the absorption spectrum of the carotenoid is induced as well before the formation of the PSB (*24*). As was suggested previously, it is possible that fixation of the carotenoid ring is required for obtaining the CD spectrum of the carotenoid (*6*). Our present results derived from studies with artificial pigments demonstrate that the carotenoid chromophore gains its chirality (at 20 °C) in about 300 s following the addition of the retinal chromophore to the apo-membrane of xR. Although it is currently not clear if the retinal occupies the binding site much faster, it is conceivable that indeed the retinal chromophore penetrates the binding site before

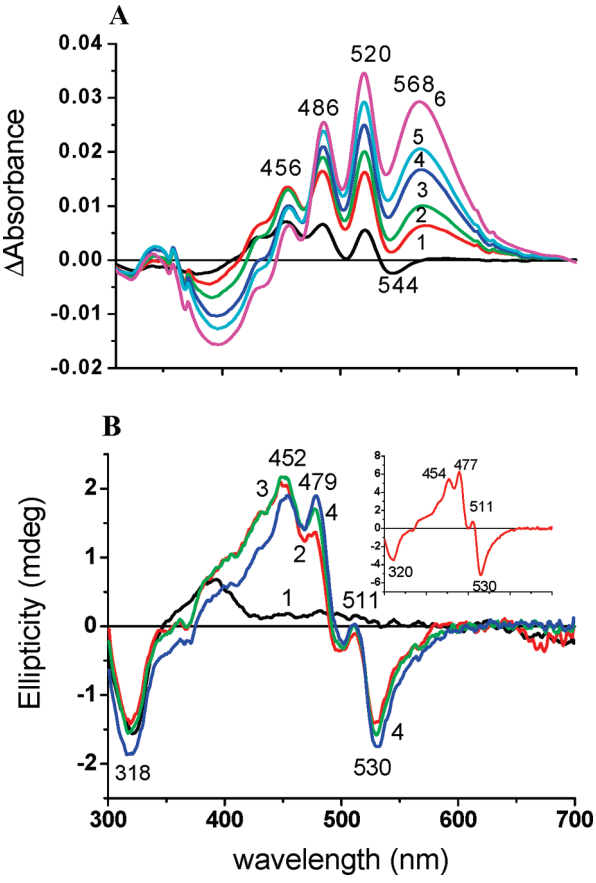



FIGURE 7: Binding of all-*trans* retinal to the apo-membrane. (A) Difference absorption spectra of the binding process with all-*trans* retinal. The spectrum taken immediately following all-*trans* retinal addition was subtracted from spectra taken at specified times. Curves 1−6 are spectra taken 62 s, 6 min, 14 min, 51 min, 150 min, and 13 h after the addition of retinal, respectively. (B) CD spectrum of the binding process at the specified times of incubation after the addition of all-*trans* retinal to the apo-membrane. Curves 2−4 are spectra taken 10 min, 30 min, and 3.5 h after the addition of retinal. Curve 1 is a spectrum of the apo-membrane. (Inset) CD spectrum of native xR.

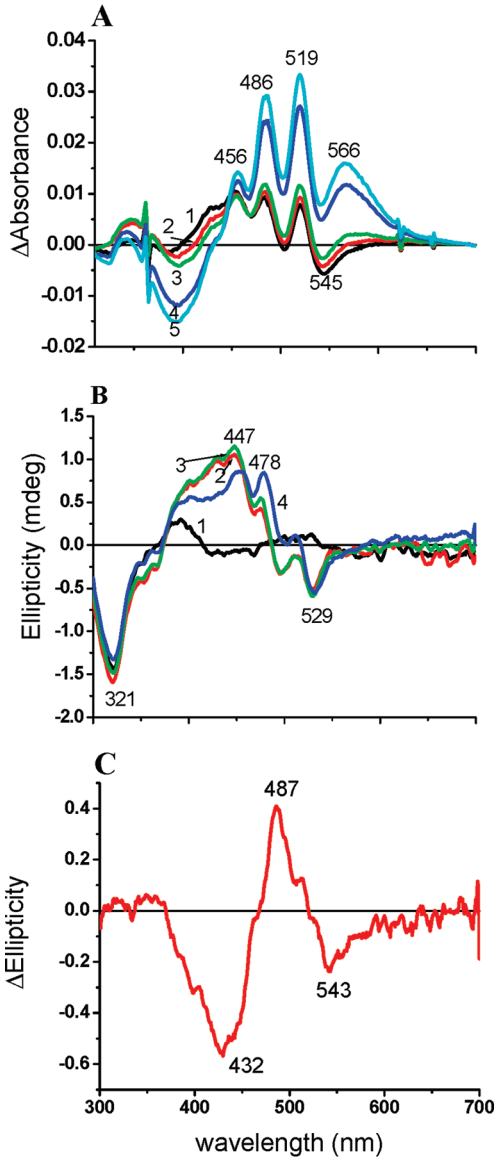



FIGURE 8: Binding of 13-desMe retinal to the apo-membrane. (A) Difference absorption spectra of the binding with 13-desMe retinal. The spectrum taken immediately following chromophore addition was subtracted from spectra taken at specified times. Curves 1−5 are spectra taken 6 min, 32 min, 74 min, 8 h, and 13.5 h after the addition of retinal, respectively. (B) CD spectra of the binding process in the specified times of incubation after the addition of 13-desMe retinal. Curves 2−4 are spectra taken 10 min, 30 min, and 10 h after the addition of 13-desMe retinal. Curve 1 is a spectrum of the apo-membrane. (C) Difference CD spectrum. The spectrum taken 30 min following chromophore addition was subtracted from the spectrum taken after 10 h.

the CD spectrum of the carotenoid chromophore is gained. Therefore, we propose that the occupation of the binding site by the retinal triggers a protein conformation alteration that induces fixation of the carotenoid. The formation of the PSB induces relatively small alterations in the carotenoid CD spectrum (especially around the 490 nm band), as evident in the binding process of 13-desMe (**6**) and 13,14 di-F retinal (**3**) (Figures 8 and 9), which indicates alteration in the carotenoid fine structure as a result of the retinal−protein covalent bond formation. This change may point to an additional carotenoid conformation change.

The retinal chromophore in the pre-pigment species has a CD band around 440 nm, as clearly observed especially in the binding process of 13-desMe retinal (Figure 8). This band is probably originated from the chiral environment of the protein. A similar behavior was detected during the binding process of retinal to the apo-membrane of bR and supports the assumption that, in the pre-pigment of xR, the retinal occupies the binding site.

Of special interest is the origin of the 530 nm negative band in the CD spectrum. The present studies using artificial pigments clearly indicate that a major fraction of this band originates from the carotenoid chromophore. This conclusion lends support to the fact that this band persists in pigments in which the absorption maximum of the retinal chromophore is red-shifted or significantly blue-shifted relative to the native pigment. In the red-shifted pigments, a shoulder to the main band at 530 nm probably originates from the retinal chromophore. Because the "fixation" process of the carotenoid in the protein is triggered by the binding site occupation by the retinal chromophore, the question arises if retinal analogues can induce this effect as well. The recent X-ray studies of xR indicate that the two chromophores are very close to each other and especially the retinal ring and that the carotenoid are in a very close proximity (*5*). Therefore, the specific structure of the retinal may play an important role in controlling the specific conformation of the carotenoid.

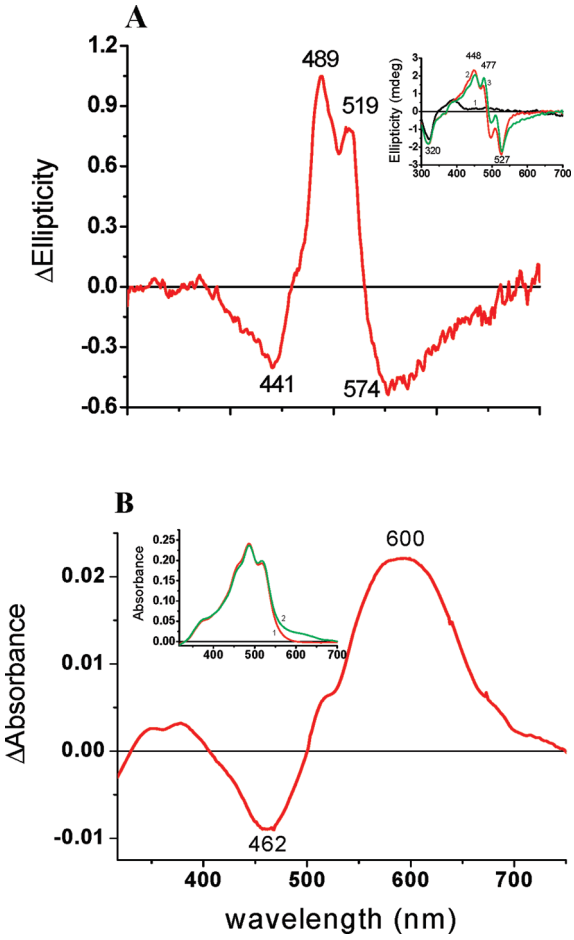



FIGURE 9: Difference spectra of the 13,14-diF retinal (**3**) binding process to the apo-membrane of xR. (A) Difference CD spectrum. The spectrum that was taken 19 min following chromophore addition was subtracted from the spectrum taken after 23 h. (Inset) CD spectra 19 min and 23 h following 13,14-diF retinal addition. (B) Difference absorbance spectrum. The spectrum taken 23 min following chromophore addition was subtracted from the spectrum taken after 29 h. (Inset) Absorbance spectra 23 min and 29 h following 13,14-diF retinal addition.

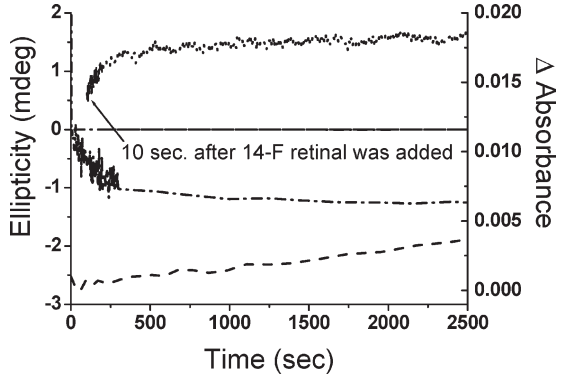



FIGURE 10: Kinetic traces showing the evolution of the 452 and 530 nm CD bands and 599 nm UV−vis band in the binding of 14-F retinal (**2**). The ellipticity of the apo-membrane at 452 nm is negligible (data not shown). CD measurements at 452 nm (· · ·) and 530 nm (· - ·). UV−vis measurements at 599 nm (- - -).

Alterations of the retinal structure in the vicinity of the PSB, such as 14-F (**2**), 13,14-diF (**3**) and 13-desMe (**6**) retinals, do not prevent carotenoid "fixation", as evident by their CD spectra. Substitution of the retinal ring by the phenyl ring still does not prevent the formation of the CD spectrum despite the fact that the aromatic core lacks the retinal ring methyls, and in addition, the aromatic ring is flat in contrast to the cyclohexene ring of the retinal chromophore. Further studies with retinal analogues may shed more light on the effect of the retinal structure on the retinal−carotenoid interactions.

The CD spectrum of bR in the visible region is composed of negative and positive lobes with unequal intensity. One possibility to explain this CD spectrum is based on excitonic interactions between the retinal chromophores of the bR protein in the trimer structure of the purple membrane. It is not known if xR adopts a trimer structure or exists as as aggregated form and whether the two lobes appearing in the CD spectrum are the result of the excitonic interaction between carotenoid chromophores located in close protein molecules. Especially, the CD spectra of 14-F retinal, 13,14-diF retinal, and aromatic retinal pigments (Figures 3, 4, and 6) exhibit almost symmetrical negative and positive lobes, which can originate from excitonic interaction. However, at this stage, the CD can also be explained by a combination of CD bands with opposite rotational strength of two close lying long wavelength transitions of the carotenoid.

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