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Reconstitution of Gloeobacter Rhodopsin with Echinenone: Role of the 4-Keto Group[†]

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ABSTRACT: In previous work, we reconstituted salinixanthin, the C_{40}-carotenoid acyl glycoside that serves as a light-harvesting antenna to the light-driven proton pump xanthorhodopsin, into a different protein, gloeobacter rhodopsin expressed in *Escherichia coli*, and demonstrated that it transfers energy to the retinal chromophore [Imasheva, E. S., et al. (2009) *Biochemistry 48*, 10948]. The key to binding of salinixanthin was the accommodation of its ring near the retinal β-ionone ring. Here we examine two questions. Do any of the native *Gloeobacter* carotenoids bind to gloeobacter rhodopsin, and does the 4-keto group of the ring play a role in binding? There is no salinixanthin in *Gloeobacter violaceous*, but a simpler carotenoid, echinenone, also with a 4-keto group but lacking the acyl glycoside, is present in addition to β-carotene and oscillol. We show that β-carotene does not bind to gloeobacter rhodopsin, but its 4-keto derivative, echinenone, does and functions as a light-harvesting antenna. This indicates that the 4-keto group is critical for carotenoid binding. Further evidence of this is the fact that salinixanthol, an analogue of salinixanthin in which the 4-keto group is reduced to hydroxyl, does not bind and is not engaged in energy transfer. According to the crystal structure of xanthorhodopsin, the ring of salinixanthin in the binding site is turned out of the plane of the polyene conjugated chain. A similar conformation is expected for echinenone in the gloeobacter rhodopsin. We suggest that the 4-keto group in salinixanthin and echinenone allows for the twisted conformation of the ring around the C6−C7 bond and probably is engaged in an interaction that locks the carotenoid in the binding site.

Retinal-based light-driven ion pumps, like bacteriorhodopsin of the archaea (*1*, *2*) and proteorhodopsin of the marine bacteria (*3*), are the simplest biological machines that supply cells with energy in the form of electrochemical gradients generated from transmembrane proton transport (*4*−*6*). A single protein with the retinal chromophore attached makes the functional unit. The simplicity of the design when a single molecule of retinal protein performs several functions (light absorption and transformation of energy into an electrochemical gradient) apparently explains that transducers and sensors (*7*) of this type are found in many marine and freshwater bacteria and exist in great varieties as one can judge from the large number of genome sequences in which they have been identified, to date exceeding several thousand, some of them pumps and others sensors (*8*−*12*).

Recently, a proton pump, xanthorhodopsin, bearing in addition to retinal a carotenoid light-harvesting antenna was found in the halophilic eubacterium *Salinibacter ruber* (*13*). The carotenoid, salinixanthin, a C_{40}-carotenoid acyl glycoside (Figure 1) (*14*), is a major component of the total carotenoids in the cell membrane of *S. ruber*. It absorbs light in the blue-green spectral region and when bound to xanthorhodopsin transfers 40−45% of the excited state energy to the retinal (*13*, *15*). The light-harvesting antenna approximately doubles the cross section of light absorption for the retinal protein (*13*, *16*). The energy transfer occurs from the S_2 state of the carotenoid, whose lifetime is 66 ± 4 fs, to the S_1 state of the retinal (*15*, *17*), the lowest singlet excited state of the retinal chromophore (*18*). Reduction of the retinal Schiff base double bond to a single bond causes a shift in retinal absorption to 360 nm that eliminates the transfer of energy to the retinal and results in an increase in the S_2 lifetime to 110 fs (*15*, *17*), consistent with 40% energy transfer. Energy transfer occurs via a Förster-type mechanism (*15*, *17*), but strong coupling of the linear and extended chromophores requires use of sophisticated calculations of the carotenoid−retinal interaction (*19*). The carotenoid is immobilized in a special binding site of xanthorhodopsin with a distance of 11.7 Å between the centers of the conjugated chains of the two chromophores that lie at a 46° angle (*20*). Most of the carotenoid conjugated chain is at the protein−lipid boundary of helix F, but the end 4-keto ring is immersed in the protein near the β-ionone ring of the retinal and twisted, as follows from the CD spectra of the bound carotenoid (*21*, *22*) and the X-ray structure of the complex (*20*). The latter revealed that the 4-keto ring is in the space that in bacteriorhodopsin is occupied by the bulky Trp138 but in xanthorhodopsin is replaced with a Gly. This gave us a clue in our search for other retinal proteins that may bind a carotenoid light-harvesting antenna similar to salinixanthin.

Sequence alignment shows that more than a dozen sequences of retinal proteins, presumably pumps and sensors, from various

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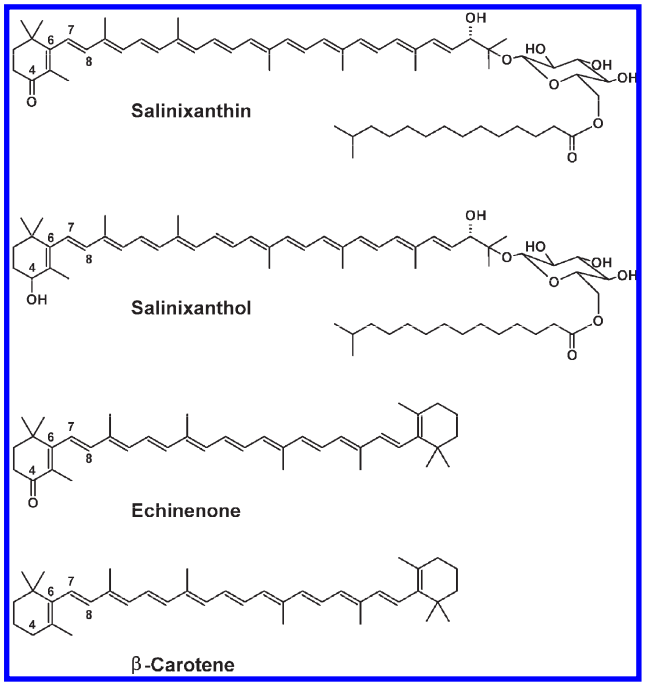

FIGURE 1: Chemical structures of salinixanthin, salinixanthol, echinenone, and β-carotene.

groups (alpha proteobacteria, actinobacteria, cyanobacteria, flavobacteria, and others) have Gly instead of Trp at this site (*23*). To demonstrate that at least some of them are indeed capable of binding a carotenoid antenna, we reconstituted salinixanthin into gloeobacter rhodopsin, a proton pump from the cyanobacterium *Gloeobacter violaceous* (*24*) expressed in *Escherichia coli*, and showed that it transfers energy to the retinal (*23*). Replacing the Gly with a bulky Trp in the G178W mutant abolished carotenoid reconstitution, thus confirming that accommodation of the ring near the retinal is necessary for the carotenoid binding (*23*).

*G. violaceou*s does not contain salinixanthin, but a simpler carotenoid, echinenone, also with a 4-keto group but lacking the acyl glycoside is present as a minor component in addition to β-carotene and oscillol (*25*).

The questions of whether any of these carotenoids bind to gloeobacter rhodopsin and whether the 4-keto group plays any role in binding arise. We address these questions here by comparing binding of β-carotene and echinenone and testing salinixanthol (Figure 1), an analogue of salinixanthin in which the ring keto group (C=O) is reduced to a hydroxyl group (C−OH). We show that β-carotene and salinixanthol do not bind to gloeobacter rhodopsin, but echinenone does. This finding establishes that a dual-chromophore antenna system will form in *Gloeobacter* cells as in the *in vitro* experiments and emphasizes the importance of the 4-keto group in carotenoid binding.

MATERIALS AND METHODS

Gloeobacter rhodopsin was expressed in *E. coli* as described in ref *24*. The G178W mutant of the protein was produced previously (*23*). Solutions in 0.02% DDM[1] were used in all experiments. Synthetic echinenone was a gift from Roche; β-carotene was from Sigma-Aldrich (>97% pure, catalog no. 22040, Fluka). Absorption spectra were recorded on a Shimadzu UV-1701 spectrophotometer; CD spectra were recorded on a Jasco J-720 spectropolarimeter (*21*), and fluorescence studies were performed on an SLM Aminco fluorometer modified by OLIS as described previously (*15*).

Echinenone and β-carotene were dissolved in acetone. In reconstitution experiments 10 μL of a stock solution was added to 1 mL of solubilized gloeobacter rhodopsin in 0.02% DDM, 25 mM MOPS (pH 7.2), and 0.1 M NaCl. The following extinction coefficients were used in calculating concentrations: gloeobacter rhodopsin, 50000 L mol^{-1} cm^{-1} (*23*); acetone solution of echinenone, 119000 L mol^{-1} cm^{-1}; β-carotene, 139000 L mol^{-1} cm^{-1} (*26*).

Extraction of salinixanthin from cell membranes and reduction of its keto group to C−OH with borohydride to produce salinixanthol were performed following the procedures of Lutnaes et al. (*14*).

RESULTS AND DISCUSSION

Reconstitution with Echinenone. Echinenone in an ethanol solution exhibits a broad absorption band with a maximum at 462 nm and poorly resolved vibronic structure (*26*). Upon addition of echinenone to detergent micelles (0.02% DDM, pH 7.1), this structure becomes even less pronounced and the maximum absorbance decreases. The absorption band shifts to 450 nm, and a broad shoulder appears at 513 nm (spectrum 1 in Figure 2A). Addition of echinenone to gloeobacter rhodopsin solubilized in the same detergent (spectrum 2) at first produced the structureless spectrum 3. However, a long incubation (24 h at room temperature) resulted in dramatic changes in absorption (Figure 2A, spectrum 4). The most noticeable of these is the narrowing of the carotenoid vibronic bands, a feature that indicates specific binding of the carotenoid to the protein (*13*). The difference spectra recorded during reconstitution show that the development of the highly structured spectrum with bands at 486, 458, and 428 nm (Figure 2B) occurs very slowly; the time constant is ~8 h. This is different from reconstitution with salinixanthin in which sharp vibronic bands of the carotenoid appeared with a time constant of 5 min after mixing (*23*).

To check whether binding of echinenone occurs to the same binding site near the retinal chromophore as in xanthorhodopsin, we performed experiments with the G178W mutant of gloeobacter rhodopsin. This mutant was specifically designed for the purpose of filling the space that is occupied by the ring of the carotenoid antenna by the bulky tryptophan (present in bacteriorhodopsin but replaced with glycine in xanthorhodopsin and gloeobacter rhodopsin). Addition of echinenone to this mutant resulted in slow-developing absorption changes that were somewhat similar to those observed for the wild-type rhodopsin (see the comparison in Figure 2C), indicating that binding of echinenone to the protein takes place with kinetics similar to those of the wild type (no such changes observed in the absence of the protein). However, the spectra show one important difference. The sharp bands of bound echinenone at 486, 458, and 428 nm, seen in the difference spectrum for the wild type, are missing in the mutant spectrum. This indicates that the ring does not fit into the space occupied by tryptophan (as expected) and is not immobilized. The lack of ring binding in the mutant has a far-reaching consequence: it eliminates the transfer of energy to the retinal chromophore (see below).

Figure 3A compares the spectrum of echinenone bound to gloeobacter rhodopsin and its spectrum in ethanol. The clear difference is that bound echinenone exhibits fine structure in its spectrum (maxima at 485 and 458 nm and shoulder at 430 nm). In panel B, the second derivative of the absorption spectra reveals

[1]Abbreviations: DDM, *n*-dodecyl β-D-maltopyranoside; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

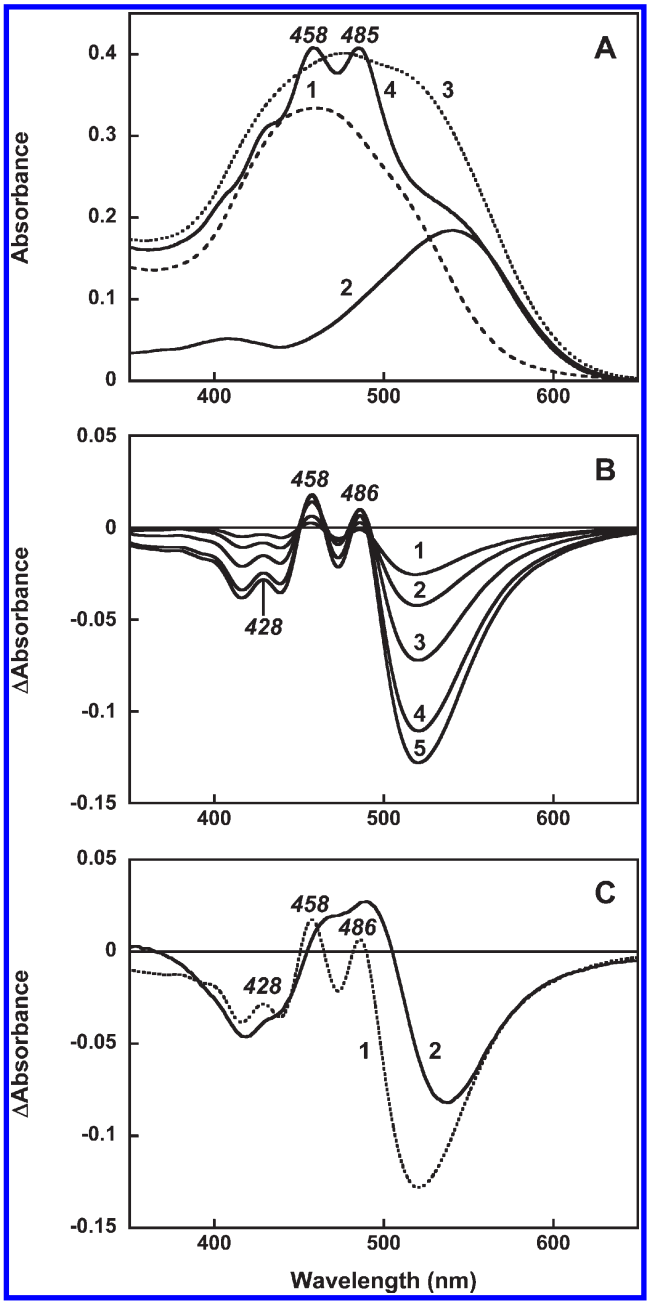

FIGURE 2: Reconstitution of gloeobacter rhodopsin expressed in *E. coli* with echinenone. (A) Absorption spectra in 0.02% DM, 25 mM MOPS (pH 7.2), and 0.1 M NaCl of (1) 4 μM echinenone and (2) 4 μM gloeobacter rhodopsin, (3) the spectrum recorded 3 min after mixing, and (4) the spectrum recorded after incubation for 24 h. (B) Absorption changes after addition of echinenone to gloeobacter rhodopsin and incubation at room temperature for 2, 4, 8, 16, and 24 h (spectra 1−5, respectively). (C) Difference spectra of absorption changes after addition of 4 μM echinenone and incubation for 24 h with (1) wild-type gloeobacter rhodopsin and (2) its G178W mutant.

the overlapping vibronic bands (a sum of the transitions to the C=C and C−C vibrational levels of the S_2 excited state). The greater amplitude and the better resolution of the bands of the carotenoid in the protein are attributed to their decreased width because the amplitude of the second derivative is inversely proportional to the square of the half-width.

The sharper absorption bands of the carotenoid bound to the gloeobacter rhodopsin must originate from restriction of motions in the carotenoid conjugated chain, and especially motions around the C6−C7 bond connecting the ring and the chain, by the

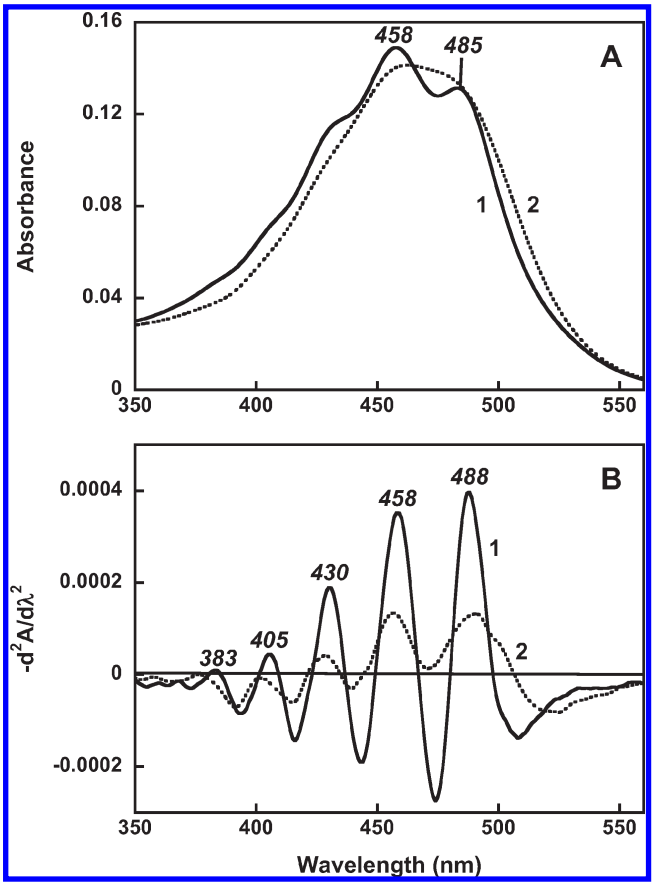

FIGURE 3: (A) Absorption spectra of echinenone (1) bound to the protein (obtained as a difference between spectra 4 and 2 of Figure 2A) and (2) in ethanol, normalized at a maximum of 485 nm. (B) Second derivatives of the spectra shown in panel A ($dA^2/d\lambda^2$), multiplied by −1.

protein, known to affect the spectral resolution (*27*, *28*). In the crystal structure of xanthorhodopsin, the ring of salinixanthin is rotated from the plane of conjugation and immobilized (*20*). If similar changes occur in echinenone, one can expect a large Cotton effect in the CD spectrum, from the asymmetric conformation of the bound carotenoid and its interaction with retinal.

The CD spectra of gloeobacter rhodopsin, echinenone in ethanol, and gloeobacter rhodopsin reconstituted with echinenone are shown in Figure 4A. Free echinenone lacks chiral centers (*26*); in ethanol, it shows only a small Cotton effect (spectrum 1). Gloeobacter rhodopsin shows bilobe bands (positive with a maximum at 567 nm, negative with a minimum at 506 nm, and crossover at 536 nm) from the retinal chromophore (spectrum 2), and two bands in the UV region at 298 and 264 nm, all of relatively low intensity. However, after reconstitution with echinenone, a much greater amplitude of the CD spectrum is observed, with maxima at 477, 452 (main), and 430 nm and a UV band at 257 nm (spectrum 3). A similar increase in the intensity of the CD signal had been observed upon reconstitution of gloeobacter rhodopsin with salinixanthin (*23*). It is interesting to compare the two CD spectra, scaled to the same concentration of gloeobacter rhodopsin and shown in Figure 4B. The overall similarity of the shapes is obvious, in spite of the differences in the position of the bands. The maxima in the CD spectrum of the protein with echinenone at 452 and 477 nm are blue-shifted from similar maxima at 480 and 512 nm in the protein reconstituted with salinixanthin by 28 and 35 nm, respectively. This corresponds to similar shifts in the absorption

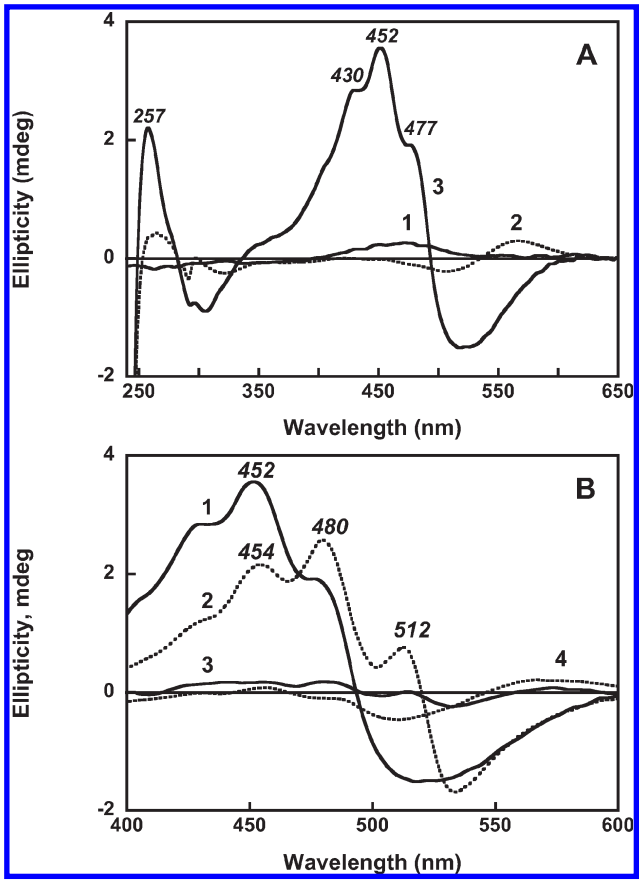

FIGURE 4: (A) Circular dichroism spectra of (1) echinenone in ethanol, (2) 4 μM gloeobacter rhodopsin in 0.02% DDM, 25 mM MOPS, and 0.1 NaCl (pH 7.2), and (3) gloeobacter rhodopsin after reconstitution with echinenone. (B) Comparison of the CD spectra of gloeobacter rhodopsin reconstituted with (1) echinenone, (2) salinixanthin, (3) salinixanthol, and (4) β-carotene. The spectra were scaled to the same amount of rhodopsin (4 μM). Spectrum 2 was adapted from ref *23*.

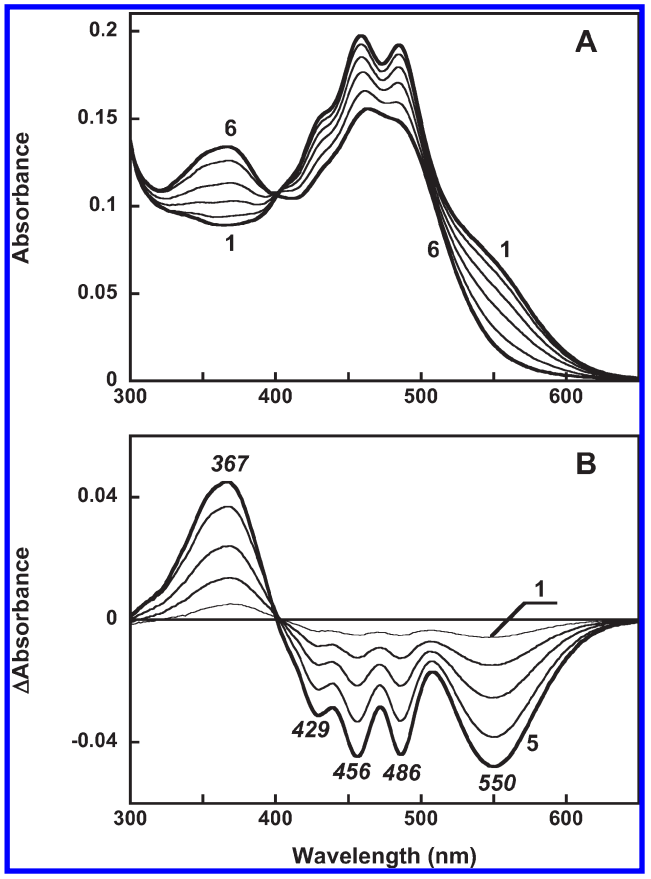

FIGURE 5: Absorption changes accompanying hydrolysis of the retinal Schiff base with 0.2 M hydroxylamine (pH 7.2). (A) Spectra of (1) gloeobacter rhodopsin reconstituted with echinenone immediately after addition of 0.2 M hydroxylamine and (2−6) the mixture 10, 30, 60, 120, and 360 min later, respectively. (B) Difference "spectrum *i* minus spectrum 1".

maxima of the vibronic bands from 0 to 1 and 0 to 0 transitions in the two spectra (458 vs 486 nm and 484 vs 521 nm). The correlation indicates that as in the case of xanthorhodopsin (*21*), the structure of the CD spectra originates from the bands of bound carotenoid, from its asymmetric conformation and its steric and electronic interaction with the retinal. In the UV, gloeobacter rhodopsin with bound echinenone exhibits an intense CD band at 257 nm that was not observed upon reconstitution with salinixanthin.

Further evidence of the interaction of echinenone with retinal was obtained in experiments with removal of retinal from the pigment by hydrolyzing the retinal Schiff base with hydroxylamine (Figure 5A). Besides the disappearance of the retinal chromophore band at 540 nm (550 nm in the difference spectrum) and the appearance of the retinal oxime band at 367 nm, prominent negative peaks from the decrease in extinction and broadening of carotenoid bands are present in the difference spectrum, at 486, 456, and 429 nm (Figure 5B). This indicates that removal of the retinal strongly affects the environment of the carotenoid, presumably decreasing the number of conformational restraints on its ring that strongly affects the resolution of its vibronic bands.

The most important evidence of the functional interaction between the echinenone and retinal chromophores was obtained from the excitation spectrum for retinal fluorescence emission. These measurements were performed at two pH values, pH 7.2 and 4.2. At pH 4.2, the quantum yield of retinal emission is ~6 times higher than at pH 7.2 because the fraction of rhodopsin with a protonated counterion is greater at this pH. As in bacteriorhodopsin (*29*, *30*) and xanthorhodopsin (*15*), the latter has a much higher quantum yield of fluorescence than the protein with a deprotonated counterion (*23*). This helps to minimize the contribution from other fluorescing impurities in the sample that are not pH-dependent. The excitation spectrum of retinal chromophore emission of gloeobacter rhodopsin exhibits a single broad band with a maximum at 563 nm (Figure 6A). Additional bands at 488, 458, and 430 nm are present in the excitation spectrum of the protein reconstituted with echinenone. The data clearly show that light absorbed by echinenone bound to the protein is transferred to the retinal chromophore, and hence, the bound echinenone functions as a light-harvesting antenna. The quantum efficiency is at least 30%. No energy transfer takes place when echinenone is added to the G178W mutant, as the lack of the carotenoid bands in the spectrum taken 28 h after addition of echinenone indicates (Figure 6B). Echinenone binds much slower than salinixanthin (time constant of 8 h vs 14 min when the latter is added as a solution in ethanol), indicating that the acyl glycoside moiety and/or the 2′-hydroxy group of the latter facilitates binding of the carotenoid to the protein.

Attempt To Reconstitute Gloeobacter Rhodopsin with Salinixanthol. Unlike salinixanthin and echinenone, salinixanthol, in which the 4-keto group in the ring is reduced to the C−OH group, exhibits a well-structured spectrum even in a solvent (*14*).

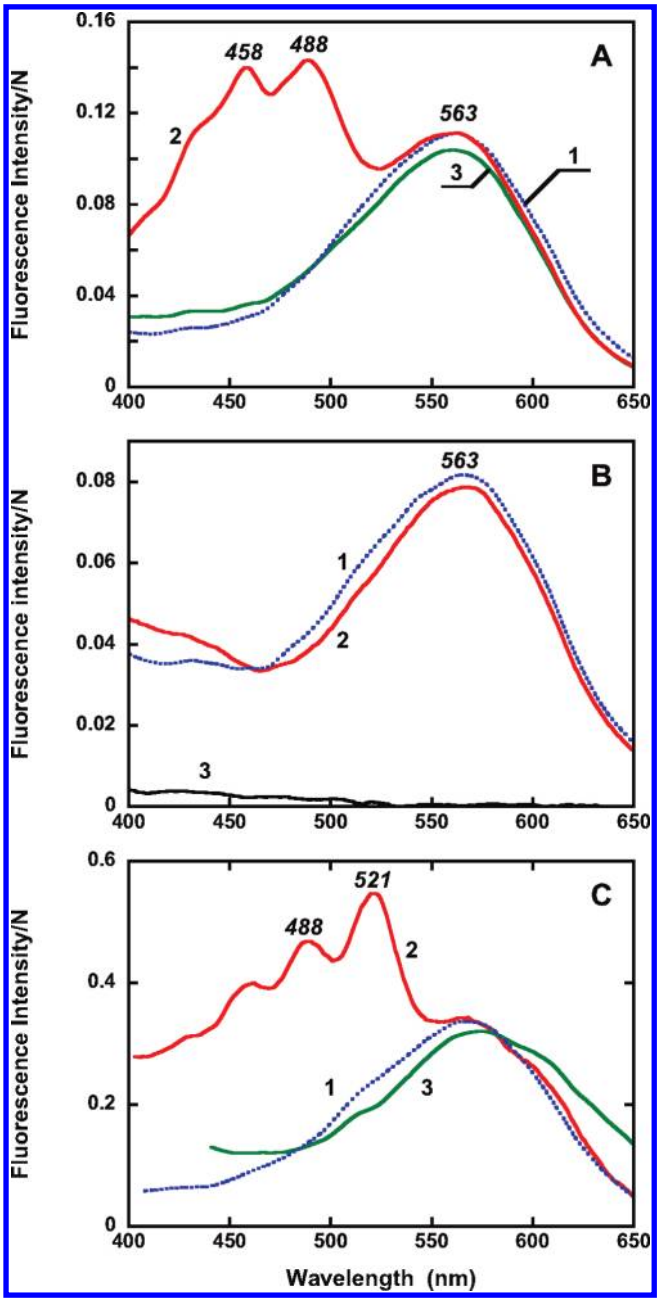

FIGURE 6: (A) Excitation spectra for fluorescence emission of the retinal chromophore at 720 nm for (1) gloeobacter rhodopsin, (2) gloeobacter rhodopsin reconstituted with echinenone, and (3) gloeobacter rhodopsin with β-carotene added. The carotenoid/retinal ratio was 1/1; the concentration of rhodopsin was ~4 μM (pH 4.2). The absorbance of the samples in the maximum is between 0.1 and 0.2. (B) Excitation spectra for fluorescence emission of the retinal chromophore at 720 nm for (1) the G178W mutant (pH 4.2), (2) the G178W mutant after reconstitution with echinenone (28 h after addition), and (3) echinenone in 0.02% DDM (same concentration as for spectrum 2). (C) Excitation spectra for fluorescence emission of the retinal chromophore at 720 nm for (1) gloeobacter rhodopsin, (2) gloeobacter rhodopsin after reconstitution with salinixanthin, and (3) gloeobacter rhodopsin after being mixed with salinixanthol. The bandwidth of the excitation beam was 8 nm.

Thus, although addition of salinixanthol to gloeobacter rhodopsin also results in a well-resolved spectrum (data not shown), this cannot be viewed as a sign of binding. Notably, no time-dependent changes were observed upon addition of the analogue. Moreover, the mixture did not show any significant CD bands (Figure 4B, spectrum 3). The weak bands at 514 and 480 nm are near the

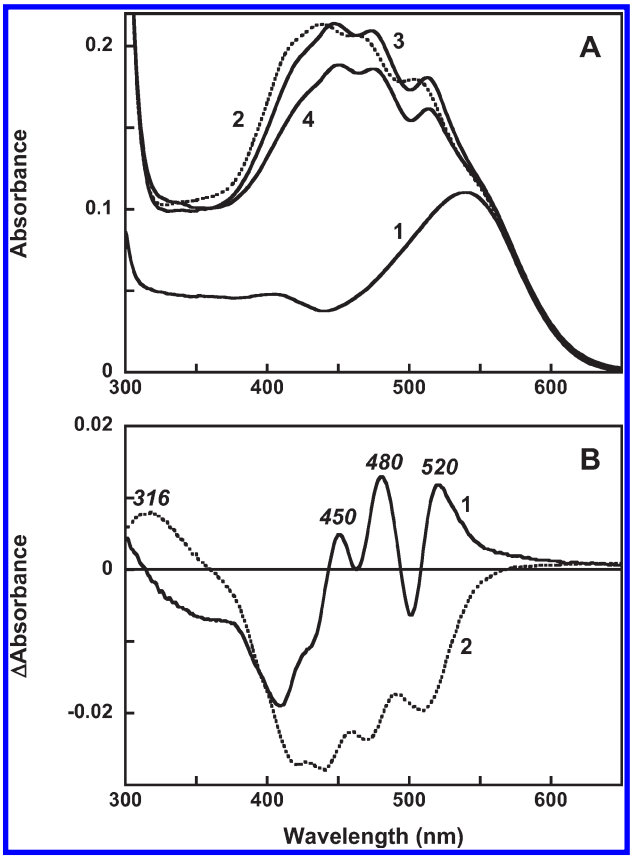

FIGURE 7: (A) Absorption spectra of (1) 2 μM gloeobacter rhodopsin in 0.02% DDM (pH 7.2) and 0.1 M NaCl, (2) 2 μM gloeobacter rhodopsin with 2 μM β-carotene added, and the mixture after (3) 1 and (4) 5 h. (B) Difference spectra: (1) 3 minus 2 and (2) 4 minus 3.

bands of salinixanthin and might be caused by the presence of < 10% salinixanthin, or alternatively by a small fraction of bound salinixanthol. Most importantly, the excitation spectrum for the emission at 720 nm (close to the maximum of retinal chromophores emission) did not show any bands from salinixanthol absorption (Figure 6C). This is clear evidence that no transfer of energy from salinixanthol to the retinal chromophore takes place and that a seemingly minor modification of the carotenoid by reduction of its 4-keto group to 4-hydroxy strongly interferes with the binding of the carotenoid, for steric or other reasons.

Reconstitution with β-Carotene. The question of whether the 4-keto group will affect binding arises. Addition of β-carotene dissolved in acetone to 0.02% DDM results in a spectrum that shows some vibronic structure, but it is less pronounced than in organic solvent. With time, the pigment undergoes changes that result in a decrease in the absorption maximum and formation of a red-shifted product with a broad band within 1 h (data not shown). These changes are most likely from aggregation of the carotenoid. However, if β-carotene is added when gloeobacter rhodopsin is present, then a red shift of absorption maxima still occurs but no decrease in extinction takes place during the first hour (Figure 7). These changes indicate that some kind of binding of β-carotene to the protein takes place. The changes occur with a time constant of 10 min and saturate after an hour. Additional incubation results in the slow decay (bleaching) of the carotenoid component. The CD spectrum exhibited only a weak band with a maximum at 456 nm (spectrum 4 in Figure 4B). The overall amplitude of the spectrum is ~1 order of magnitude lower than when gloeobacter rhodopsin was reconstituted with echinenone.

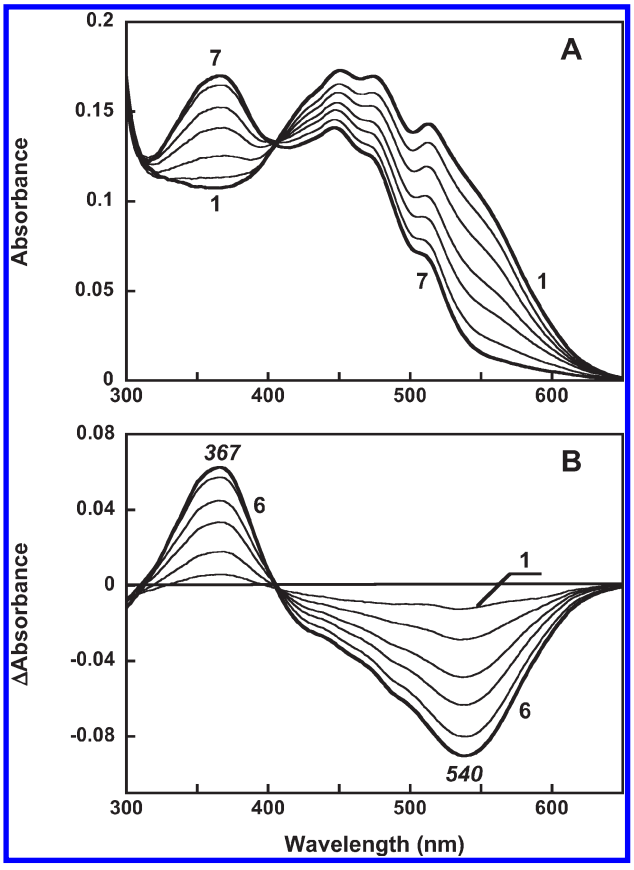

FIGURE 8: Reaction of gloeobacter rhodopsin, with β-carotene added, with 0.2 M hydroxylamine (pH 7.2) in the dark. (A) Absorption spectra (1) immediately after addition of hydroxylamine and (2−7) measured 10, 30, 60, 90, 150, and 240 min after addition, respectively. (B) Absorption changes "spectrum *i* minus spectrum 1", where spectrum *i* is spectrum 2 through 7 in panel A (spectra 1−6, respectively).

The small Cotton effect indicates that the conformation of β-carotene is not substantially distorted and no strong interaction with retinal takes place that could produce changes seen when gloeobacter rhodopsin was reconstituted with echinenone and salinixanthin.

To determine whether there is interaction of bound β-carotene with the retinal chromophore, we cleaved the Schiff base with hydroxylamine (Figure 8A). The difference spectra did not show a contribution from the carotenoid (Figure 8B). This indicates that unlike echinenone, β-carotene does not interact sterically with the retinal chromophore.

Finally, the excitation spectrum of the retinal chromophore emission for gloeobacter rhodopsin with β-carotene added does not exhibit noticeable carotenoid bands (Figure 6A, spectrum 3). This indicates that no transfer of energy from β-carotene to the retinal chromophore takes place. It correlates with the lack of CD bands and steric interaction with retinal and suggests that this carotenoid does not bind or binds but not to a specific site that would be suitable for efficient energy transfer. β-Carotene acts as an antenna in a number of chlorophyll-binding photosynthetic proteins (*31*−*33*), but its inability to bind to a specific site in gloeobacter rhodopsin precludes it from light harvesting in that protein. Echinenone is not a common light-harvesting carotenoid (*33*), but as our data indicate, it can perform this function in gloeobacter rhodopsin.

The finding that neither β-carotene nor salinixanthol binds to the site at the retinal in gloeobacter rhodopsin is consistent with our observation that salinixanthol does not bind to xanthorhodopsin. It suggests that the 4-keto group plays a key role in binding. Salinixanthin binds to gloeobacter rhodopsin ~30-fold faster than echinenone. If one assumes that configuration of the conjugated chains is similar in both carotenoids, then this indicates that the glycoside, the acyl tail, and the 2′-hydroxy group, missing in echinenone, strongly accelerate binding of salinixanthin to gloeobacter rhodopsin but are not as crucial as the 4-keto group, the lack of which completely eliminates binding. Further experiments with carotenoid analogues are needed to dissect the roles of each of these additional groups in salinixanthin. The polar 2′-hydroxy group and glycoside would certainly increase the solubility of the carotenoid and facilitate its delivery to the protein in an aqueous environment and proper orientation, with the polar glycoside being close to the cytoplasmic surface. In xanthorhodopsin, the glycoside is hydrogen bonded to the side chains of Arg184 and Asn191 (*20*). The latter residue is conserved in gloeobacter rhodopsin.

According to the crystal structure of xanthorhodopsin, the oxygen of the 4-keto group is not involved in hydrogen bonding. The existence of such a bond could work as a "lock" (*34*) and would explain its role in binding. An example is the orange carotenoid protein (OCP), which binds 3′-hydroxyechinone (*35*). The 4-keto group is hydrogen bonded to the side chains of two residues, Trp and Tyr. It is critical for function (*36*) and affects the spectroscopic properties of the carotenoprotein (*37*).

The lack of hydrogen bonding in xanthorhodopsin implies that another mechanism by which the 4-keto group could be involved in carotenoid binding exists. Two mechanisms involving the 4-keto group might be considered. The first is based on the crystal structure of xanthorhodopsin (*20*) that shows that the cyclohexanone ring of salinixanthin is rotated 82° from the plane of the polyene conjugated chain. The high degree of conservation of residues involved in carotenoid binding and the fact that gloeobacter rhodopsin binds salinixanthin (*23*) and that binding of echinenone and salinixanthin is abolished by the G178W mutation suggest similar binding sites and conformations of the bound carotenoid in the two proteins. Thus, to fit the binding site, the ring should be able to undergo a large turn around the C6−C7 bond. The presence of the 4-keto group in the ring apparently facilitates this turn because it allows for a broader distribution of dihedral angles around this bond. The evidence of this is the broad absorption spectra of carotenoids with conjugated carbonyl that show poor vibronic structure even in nonpolar solvents (*28*, *38*, *39*). Under these conditions, the interaction of the solvent with the carbonyl oxygen is minimal, and broadening is most likely from multiple conformers differing in the dihedral angle between the ring and H8 on the polyene chain. This ability to undergo larger turns around the C6−C7 bond may help to fit the ring into the binding site. Carotenoids with cyclohexene or cyclohexanone β-rings have a 6-s-*cis* conformation in the crystal structures and also in solution (*40*). This has been shown for β-carotene (*41*), and 4-keto carotenoids, canthaxanthin (*42*, *43*) and astaxanthin (*43*). The β-rings are turned 43° to 50° out of the polyene conjugation plane around the C6−C7 single bond, because of steric conflict between the methyl groups of the ring and the hydrogen atoms bonded to the carbon atoms of the chain. In carotenoproteins, the conformation might be very different. Thus, astaxanthin noncovalently bound to β-crustacyanin of lobster shell is in the s-*trans* conformation (*44*). The ring and the 4-keto group are strongly hydrogen bonded in this protein, which is different from salinixanthin in xanthorhodopsin where the ring is in a twisted s-*cis* conformation and nonbound.

The ability of the 4-keto carotenoids to undergo large turns around the C6−C7 bond may be rationalized by the presence and polarization of the 4-keto group, which produces a partial positive charge on C4 and, through conjugation, on C6. This will facilitate rotation (turning) of the ring around this bond and fitting the ring into its binding site. This twisted conformation of the ring apparently cannot be achieved in β-carotene and salinixanthol, and that could interfere with the binding and immobilization of the polyene chains in the protein. Thus, the twisted ring might work as a lock (or at least a key factor) for optimal positioning of the carotenoid on the protein.

An additional factor explaining the role of the 4-keto group in binding is based on the observation that in the structure of xanthorhodopsin obtained after MD simulation and QM/MM optimization the carotenoid ring is rotated not by 82.8° but by 40.4° (*19*). In this conformation, a non-hydrogen bonding interaction between the carotenoid carbonyl and the Met233 sulfur atom becomes feasible. Such interaction between carbonyl groups and methionine has been observed in a number of proteins, with an average O−S distance of 3.6 Å (*45*). In xanthorhodopsin and gloeobacter rhodopsin, this interaction might serve the purpose of "locking" the carotenoid into the binding site when it enters it.

In conclusion, we show that gloeobacter rhodopsin can be reconstituted with echinenone, a carotenoid present in the native organism *G. violaceus*. Bound echinenone functions as a light-harvesting antenna. In contrast, β-carotene and salinixanthol do not bind in a specific way that would lead to energy transfer, indicating that the 4-keto group in echinenone and salinixanthin is important for binding. The lack of this group in β-carotene and its modification to a hydroxyl group in salinixanthol eliminate functional interaction with the retinal chromophore.

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