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Asp_{83}, Glu_{113} and Glu_{134} are not specifically involved in Schiff base protonation or wavelength regulation in bovine rhodopsin

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Site-specific mutagenesis was employed to investigate the proposed contribution of proton-donating residues (Glu, Asp) in the membrane domains of bovine rhodopsin to protonation of the Schiff base-linking protein and chromophore or to wavelength modulation of this visual pigment. Three point-mutations were introduced to replace the highly conserved residues Asp_{83} by Asn ($D_{83}N$), Glu_{113} by Gln ($E_{113}Q$) or Glu_{134} by Asp ($E_{134}D$), respectively. All 3 substitutions had only marginal effects on the spectral properties of the final pigment (≤ 3 nm blue-shift relative to native rhodopsin). Hence, none of these residues by itself is specifically involved in Schiff base protonation or wavelength modulation of bovine rhodopsin.

Amino acid substitution; Baculovirus; Heterologous expression; Rhodopsin; Spectral property; Site selective mutagenesis

1. INTRODUCTION

Rhodopsin is the visual receptor protein of the rod photoreceptor cell in the vertebrate retina. Upon light absorption it triggers the conversion of light energy (photons) into electrochemical potential gradients in the rod cell. Rhodopsin's absorbance band in the visible spectrum (λ_{max} = 498 nm) dictates the spectral sensitivity of the rod photoreceptor cell. This absorbance band originates in a chromophore, 11-Z-retinal, which is covalently linked to lysine-296 via a protonated Schiff base [1]. Upon illumination, this chromophore isomerises to the all-E form, which triggers a series of protein conformational changes finally resulting in the release of the all-E-retinal. Rhodopsin and its fellow visual pigments are members of a well-established family of membrane receptor proteins that use G-proteins as signal mediators and which show a high degree of structural similarity [2]. Bovine opsin, the apoprotein of rhodopsin, consists of a single 348 amino acid long polypeptide chain [1,3,4]. The characteristic alternation of polar and apolar segments suggests 7 putative transmembrane domains interconnected by 6 hydrophilic stretches.

In order to investigate the working mechanism of rhodopsin, including wavelength regulation, signal expression, G-protein binding and desensitization, on a molecular level, selective labeling and/or mutagenesis is a very powerful tool. A prerequisite for this is a suitable in vitro biosynthesis system [5-8]. We recently succeeded in producing bovine opsin in vitro using the baculovirus-based expression system [8], where heterologous DNA is integrated into the viral genome under transcriptional control of the polyhedrin promoter (for review, see [9,10]). Here, we use this system to present a first global investigation of one very intriguing property of visual pigments: their wavelength regulation. On theoretical grounds and conservational patterns it has been argued that single proton-donating residues (Glu or Asp) in the membrane domain of the protein are responsible for protonation of the Schiff base and additional shifts of the absorbance band (wavelength modulation). In this report we demonstrate that none of the 3 highly conserved Glu or Asp residues in the membrane domain of bovine rhodopsin (Asp_{83}, Glu_{113}, Glu_{134}) is specifically involved in these processes. This indicates that the mechanism underlying wavelength regulation in visual pigments is much more complex than hitherto expected.

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Abbreviations: AcNPV, *Autographa californica* nuclear polyhedrosis virus; bp, base pair; dpi, days post-infection; MOI, multiplicity of infection; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanosulfonate; DTE, 1,4-dithioerythritol; EDTA, (ethylene-1,2-diamine)*N*-tetraacetic acid; Con A, concanavalin A; G-protein, GTP-binding protein or transducin; RT, room temperature; v-ops, opsin produced in vitro by recombinant baculovirus; v-rho, rhodopsin obtained by incubation of v-ops with 11-Z-retinal

2. MATERIALS AND METHODS

2.1. *Materials*

Restriction enzymes were purchased from BRL (Bethesda, MD, USA). Con A-Sepharose was obtained from Pharmacia (Uppsala, Sweden), CHAPS from Sigma (St. Louis, MO, USA) and immunoglobulins from Dakopatts (Glostrup, Denmark). Oligonucleotides

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Table 1

Sequences of the oligonucleotides that were used to substitute carboxyl residues in the 2nd and 3rd transmembrane domain of bovine opsin, and the endonucleases for which simultaneously restriction sequences were created

Oligonucleotide	Endonuclease	AA substitution	Abbreviation
5′-atgaagaggttggccacggcc-3′	*Bal*I	Asp(83)Asn	$D_{83}N$
5′-aagccctgcaggttg-3′	*Pst*I	Glu(113)Gln	$E_{113}Q$
5′-ccacgtaccgatcgatggcca-3′	*Pvu*I	Glu(134)Asp	$E_{134}D$

were synthesized on a cyclone DNA synthesizer (Milligen, Bedford, MA, USA).

2.2. *Cell culture and infection*

Baculovirus propagation, transfections, biosynthesis of recombinant proteins and insect cell (IPLB Sf9) culture were performed as described [9]. Cells were cultured in Costar culture flasks (1×10^6 cells/ml) for the isolation of recombinant AcNPV. Suspension cultures of Sf9 cells were used for the in vitro production of recombinant opsin.

2.3. *Preparation of recombinant virus and site-directed mutagenesis*

A 1.3 kb *Sma*I fragment of the opsin cDNA [4] was subcloned after the addition of *Bam*HI-linker DNAs into the *Bam*HI site of M13mp18 (5-50/2). The *Bam*HI fragment was subsequently transferred to the AcNPV transfer vector pAcRP23 [8]. Co-transfection with viral DNA produced recombinant AcNPV [11]. Recombinant AcNPV was isolated as described previously [8].

Site-directed mutagenesis was performed using the approach developed by Kunkel [12] incorporated in the MutaGene kit (BioRad, Richmond, CA, USA). The sequences of the oligonucleotides used to introduce the desired base substitutions are shown in table 1. In order to allow rapid isolation of clones with the desired point-mutation present, the sequences were altered in such a way so as not only to change the codon sequence of the bovine opsin cDNA as desired but also to create the recognition sequence of a restriction enzyme. Plaques were picked and analysed for the presence of one additional restriction site at the correct position. Mutant opsin cDNA with the desired restriction site was isolated from 5-50/2 and transferred to pAcRP23. Recombinant AcNPV expressing the mutant opsins (AcNPVRP23 · $opsE_{134}D$, AcNPVRP23 · $opsD_{83}N$ and AcNPVRP23 · $opsE_{113}Q$) respectively were isolated as described [8].

2.4. *Production and partial purification of recombinant rhodopsin (v-rho)*

Spinner culture flasks (200 ml) were used to grow about 10^8 IPLB-Sf9 cells. Cells were infected with recombinant virus (MOI 10) and at 3 dpi the infected cells were removed from the culture flasks and collected by centrifugation (4000 × *g*, 60 min, 4°C). Cell pellets were resuspended in 5 ml buffer solution (buffer A: 20 mM PIPES, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM EDTA and 5 mM DTE, pH 6.8). All subsequent manipulations were performed under dimred light ($\lambda > 650$ nm). The resulting cell suspension was incubated for 2 h at RT in the presence of 400 nmol of 11-Z-retinaldehyde added as a solution in 0.4 ml 0.5% CHAPS. The regenerated v-ops (v-rho) was subsequently solubilized by adding 0.6 ml of buffer A containing 10% CHAPS and incubating for 16 h at 4°C. Cellular debris was removed by centrifugation (4000 × *g*, 60 min, 4°C) and the supernatant was used for further purification of v-rho over Con A-Sepharose. Hereto, 0.75 ml bed-volume of Con A-Sepharose is washed with several vols of buffer A (containing 1% CHAPS). After application to the column of a v-rho sample derived from 10^8 cells (flow rate: 1 ml/h), unbound material is eluted (4 ml/h) with 24 vols of buffer A containing 20 mM nonylglucose [13] and 50 mM NH_2OH (to convert all free and randomly bound retinal into the free oxime). Subsequently buffer A plus 20 mM nonylglucose and 200 mM α-methylmannose was used to elute bound glycoproteins (1 ml/h). Wild-type and mutant v-rhos eluted in the latter group. Absorbance spectra of the v-rho containing fractions were taken on a Pye Unicam PU 8800 spectrophotometer [14]. Difference spectra, obtained by subtracting the spectrum of the sample after illumination from the spectrum of the non-illuminated sample, were used to determine absorbance maxima with an average accuracy ± 3 nm.

3. RESULTS AND DISCUSSION

3.1. *In vitro biosynthesis of bovine opsin and mutants*

Production of the described mutant opsins gave similar results as wild-type opsin [8] with respect to yield and regeneration capacity (30–60%). Expression levels obtained in the recombinant baculovirus system (1.0–1.5% of total protein) allow fast and reliable spectral analysis of rhodopsin mutants.

3.2. *Targets for site-directed mutagenesis and the isolation of mutant opsin cDNAs*

As targets for mutagenesis we selected three Asp/Glu residues in the membrane domain opsin which are highly conserved in the family of visual pigments as far as sequences are known [15]: Asp_{83} is conserved in all visual pigments except for the human blue cone pigment, Glu_{113} is conserved in all vertebrate pigments and the Glu_{134}–Arg_{135} sequence is conserved in all vertebrate pigments and replaced by Asp-Arg in invertebrate pigments. In order to keep dimensional changes as small as possible, Asp_{83} and Glu_{113} were replaced by the corresponding amides Asn and Gln, which do no longer possess any proton-donating potential. Glu_{134} was replaced by Asp, which is about 0.15 nm shorter. The latter substitution maintains the ion pair 134–135 and was therefore preferred over $Glu_{134} \rightarrow$ Gln, since perturbation of the ion pair might lead to larger structural perturbations. These mutant opsin cDNAs could be rapidly isolated as mutagenesis was used to simultaneously introduce an additional recognition sequence for the endonucleases *Bal*I, *Pst*I and *Pvu*I, respectively (table 1), which allowed us to screen a large number of clones for the presence of the desired point-mutation. This is illustrated in fig.1 for the opsin cDNA in which a point-mutation was selected, which at the protein level replaced glutamic acid (134) by aspartic acid while at the DNA level the recognition sequence for the endonu-

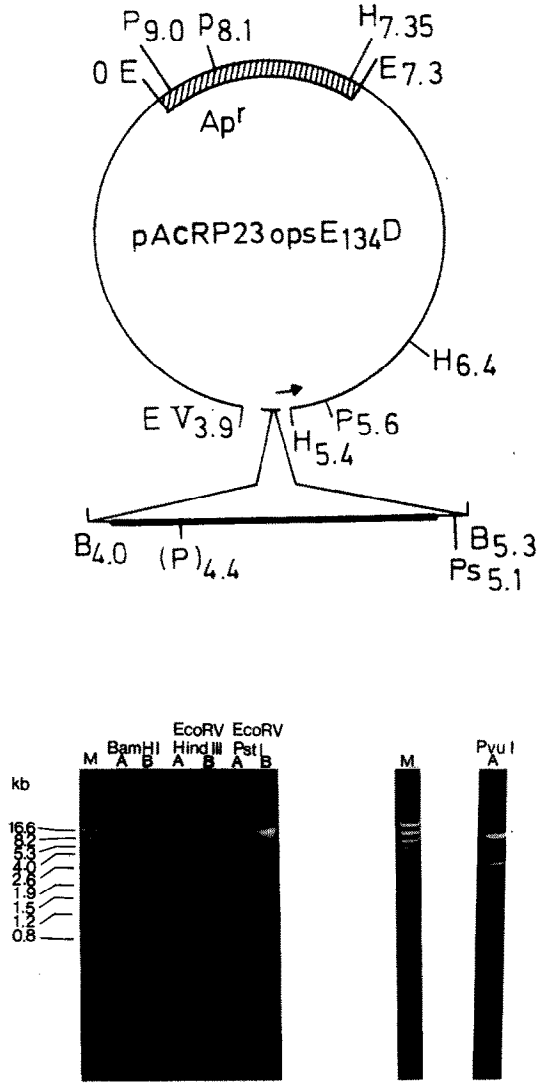

Fig.1. (*Upper panel*) Schematic diagram of the transfer vector pAcRP23·opsE_{134}D. An unique *Bam*HI site (B) is located following position +1 with respect to the translation initiation codon ATG of the polyhedrin gene. Symbols: (▨) pUC8; (▬) opsin gene coding sequence; (→) 3′-end of the polyhedrin gene; Ap^r ampicillin-resistance gene. Restriction sites: H for *Hin*dIII, EV for *Eco*RV, E for *Eco*RI, Ps for *Pst*I, P for *Pvu*I and (P) for *Pvu*I introduced by site selective mutagenesis. Subscript numbers represent distances in kb. (*Lower panel*) A typical example of restriction fragment analysis used for the isolation of mutant opsin cDNA in which site-specific mutagenesis simultaneously created the recognition sequence for an endonuclease. This example represent E_{134}D from table 1. (Lane A) Bovine opsin cDNA cloned into the unique *Bam*HI site of the baculovirus transfer vector pAcRP23 (pAcRP23·ops5). (Lane B) Bovine opsin cDNA carrying the desired point-mutation cloned into the same transfer vector (pAcRP23·opsE_{134}D). Restriction analysis with *Eco*RV/*Hin*dIII and *Eco*RV/*Pst*I were performed to determine the orientation and the copy number of the inserted DNA fragment. Restriction analysis with *Pvu*I exposes the additional restriction site in the mutant cDNA.

clease *Pvu*I was created. Restriction analysis shows that both wild-type pAcRP23·ops5 (lane A) and mutant pAcRP23·opsE_{134}D (lane B) contain one copy (*Hin*dIII/*Eco*RV, 1.5 kb fragment) of the bovine opsin cDNA (*Bam*HI, 1.3 kb fragment) in the correct orientation (*Eco*RV/*Pst*I, 1.2 kb fragment). The difference between both clones is illustrated by using the endonuclease *Pvu*I. The largest fragment (5.7 kb) of wild-type opsin (lane A) is further cleaved in the mutant (lane B) yielding an additional small restriction fragment (1.2 kb). The presence of this point-mutation was further confirmed by sequence analysis according to [16]. Clones carrying the substitutions Asp (83) Asn or Glu (113) Gln were isolated in a similar way.

3.3. *Spectral properties of bovine rhodopsins carrying the specified amino acid substitutions*

In order to study the spectral properties of mutant rhodopsins the synthesized v-ops should be able to recombine in vitro with 11-Z-retinal to generate sufficient amounts of v-rho to record absorbance spectra before and after illumination. Recombinant baculovirus-infected insect cells at 3–4 dpi were incubated in darkness with a 30–40-fold molar excess of 11-Z-retinal and the resulting v-rho was purified 10-fold via Con A-affinity chromatography. V-rho elutes in the Con A-binding fraction and is sufficiently concentrated to record absorbance spectra. The difference spectrum obtained from wild-type v-rho has its λ_{max} at 498 nm, which is identical to that of native rhodopsin (fig.2A, table 2).

The substitution of aspartic acid (83) by asparagine does barely affect the position and shape of the absorbance band in the visible spectrum (fig.2B, table 2). This invalidates previous postulations that this single residue protonates the Schiff base [15,17,18]. Glutamic acid (134) is also strongly conserved during visual pigment evolution [15], but the substitution of this residue by aspartic acid had no influence at all on the position of the absorbance band (fig.2C, table 2). It is conceivable that Glu_{134} does not contribute to the chromophoric pocket and rather is located at the membrane interface [3,19,20]. The last candidate, glutamic acid (113) was shown to be involved in ligand binding in an equivalent position in the β-adrenergic receptor [21]. As the β-adrenergic receptor and rhodopsin share both structural [2] and functional [22,23] homology and ligand binding by this receptor also appears to require a negative counter-ion, it is conceivable that the postulated role of Asp_{83} is in fact played by Glu_{113}. To thest this hypothesis we replaced Glu_{113} by glutamine. Again, this substitution only had a minor effect on the position of the absorbance band (fig.2D, table 2). It is evident, that none of these 3 residues can solely be responsible for Schiff-base protonation and/or wavelength modulation in bovine rhodopsin. Substitution of Asp_{83} or Glu_{113} leads to small shifts in the absorbance maximum, so these residues might contribute to some kind of network wavelength modulation mechanism, as discussed below.

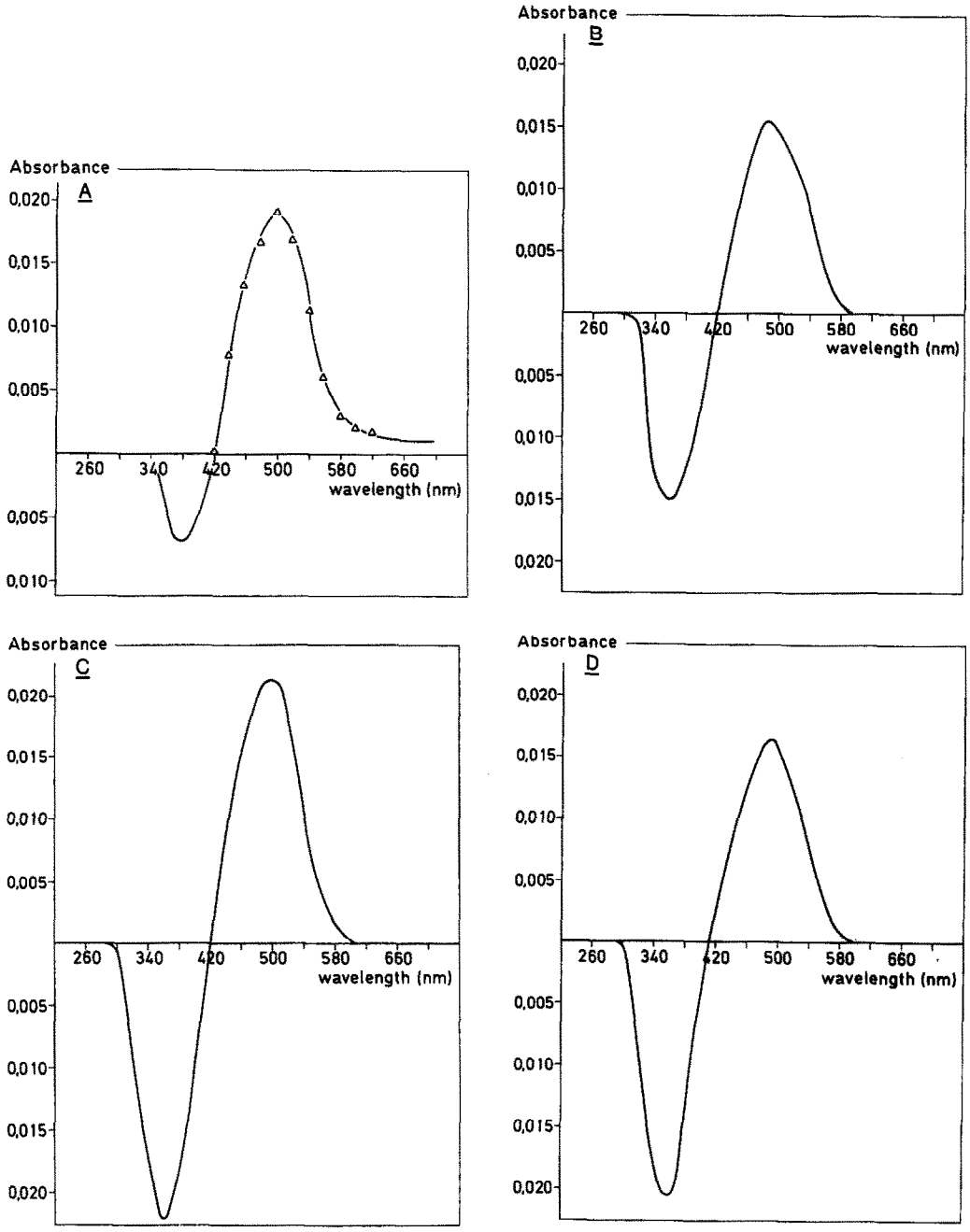

Fig.2. Difference spectra of 'wild-type' and mutant bovine rhodopsins produced by recombinantt baculovirus. Panel A represents wild-type and panel B–D present bovine rhodopsin carrying the following AA substitutions; $Asp_{83} \rightarrow Asn$ (panel B), $Glu_{134} \rightarrow Asp$ (panel C) and $Glu_{113} \rightarrow Gln$ (panel D). Triangles in panel A show the difference spectrum of native bovine rhodopsin normalized to the same maximal adsorbance at 498 nm. The relative low 365/500 ratio in A is due to the lower v-rho content and a decrease in turbidity < 400 nm upon bleaching of this sample. In darkness all pigments are stable in the presence of 50 mM hydroxylamine.

3.4. *Corollaries*

All visual pigments investigated (λ_{max} range: 340–500 nm) use a protonated Schiff base to bind their chromophore [24–26]. Protonation in itself is sufficient to red-shift the absorbance maximum of a retinal Schiff base from 360 to 440 nm. Several groups have proposed that additional shifts of the absorbance maximum originate from the presence of charged or strongly dipolar amino acid residues, most likely involving carboxyl group residues in the vicinity of the retinal polyene chain. Most of these 'external charge models' assume that at least one Asp or Glu residue protonates and is in close association with the Schiff base. For additional wavelength modulation a second charged residue is often placed near the polyene chain (C-12 to C-14) [17,27–29]. The magnitude of the observed shifts can be

Table 2

λ_{max} of native rhodopsin and recombinant species

		λ_{max}* (nm)
Native rhodopsin		498
'Wild-type'	v-rho	498
$D_{83}N$	v-rho	496
$E_{113}Q$	v-rho	495
$E_{134}D$	v-rho	498

*Average of at least 3 samples. SD lies within spectroscopic accuracy (±3 nm)

shown to depend on the proximity of the charged residues to the polyene π-electron system [27–29], hence the large λ_{max} variation in the visual pigment family is often explained to reflect small variations in the distance between some crucial residues and the polyene chain of the retinal. Several investigators have compared the amino acid sequence of a large number of visual pigments (vertebrate and invertebrate) and combined this with the potential constraints in 3D models of the rhodopsin structure [17,28–30]. Such arguments led to the conclusion that the carboxyl groups in the 2nd and 3rd transmembrane segment of opsin should be responsible for the Schiff base protonation and further wavelength modulation. Such a mechanism predicts that replacement of a Schiff base stabilizing Glu or Asp residue by Gln or Asn, which cannot donate a proton or act as counter-ion, will blue-shift the absorbance spectrum by at least 60–80 nm. The same substitution in a residue with a wavelength modulating function would result in shifts of several tens of nanometers. Likewise, replacement of Glu by the ca 0.15 nm shorter Asp will under both conditions shift the absorbance maximum by several tens of nanometers [17].

In complete contrast, the substitutions we have introduced have only marginal effects on the position of the absorbance band: Asp_{83}→Asn effectuates a blue-shift of 2 nm, Glu_{113}→Gln effectuates a blue-shift of 3 nm and Glu_{134}→Asp does not result in a detectable shift. Hence, we conclude that none of these residues acts as the counterion for the Schiff base and none of these residues is solely involved in wavelength modulation.

As yet, it cannot be excluded that other highly conserved membrane residues like Tyr, which has been implied in bacteriorhodopsin [31], or Cys in fact perform the role hitherto postulated for Asp or Glu. However, substitution of all membrane-located Cys residues for Ser does not influence the absorbance spectrum [32]. We have so far introduced substitutions in two other potential candidates (Tyr_{268} and Tyr_{301}) and again have only observed very small shifts in the absorbance maximum (<3 nm; Janssen et al., unpublished). Alternatively, it is conceivable that wavelength modulation and even Schiff base protonation are attended by a network of residues, e.g. stabilized by H-bond interactions [33], which would provide the correct charge distribution and electrochemical environment to produce the 'desired' absorbance maximum in the retinylidene moiety. Changing one residue in such a network might result in only small shifts in the net charge distribution and the resulting absorbance maximum. If we extrapolate our results to such a situation, this could imply that Asp_{83} and Glu_{113} contribute to such a network, while Glu_{134} probably serves a function during biosynthesis (Janssen et al., unpublished). In order to map such an alleged network, the production and analysis of hybrid proteins and mutants with a combination of selected substitutions is in progress.

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