constants of αα, αβ, and ββ.

Tropomyosin from *R. esculenta* leg and back muscle, extracted and purified at low temperature where chain exchange is minimized or eliminated, consists of >90% αβ. The presence of ≈10% αα appears to be the consequence of a slightly greater relative amount of α over β in leg muscle. Thus, αβ is the preferred native composition, and excess αα appears to form after αβ. This explanation was used to explain the prevalence of αβ in several rabbit skeletal muscles in which the amounts of α and β were approximately equal (*3*).

Studies with *R. temporaria* αβ tropomyosin showed two thermal unfolding transitions (*20*). The first transition of this frog species also appears to be due to chain exchange (*18*). The heterodimer of smooth tropomyosin from fowl gizzard is the native species (*9*), but a 1:1 mixture of homodimers is produced after refolding guanidinium hydrochloride–unfolded chains (*11*). Incubation of the mixture of homodimers at physiological temperature, however, produces the αβ native composition (*12*). For rabbit skeletal tropomyosin, refolding from a 1:1 mixture of α and β chains on cooling from a high temperature gave a random mixture of dimer molecules (*6*). A tendency toward αβ formation was observed when refolding took place by dilution from denaturant into buffer at physiological temperature (7). It is possible that a greater fraction of native αβ would be produced if incubation times at the physiological temperature were long enough to allow chain exchange to reach equilibrium.

Although the assembly preference of the tropomyosin heterodimer can be understood thermodynamically, the significance of its predominance in several muscles (*3, 9*) and of the changing α/β tropomyosin ratio (which will change the dimer composition) in skeletal muscle development (*21, 22*) and in differentiating muscle cells in vitro (*23*) is not known. Frog muscle functions over a wide range of body temperatures, and it is possible that the composition and properties of the tropomyosin dimer may depend on the temperature at the time of biosynthesis, thus compensating for temperature effects on regulation. However, in preliminary studies, no significant differences were observed between the effects of *R. esculenta* αα and αβ tropomyosin on the Mg^{2+}-dependent adenosine triphosphatase activity of actomyosin subfragment 1 either at 15° or 25°C (*18*).

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Effect of Carboxylic Acid Side Chains on the Absorption Maximum of Visual Pigments

EUGENE A. ZHUKOVSKY AND DANIEL D. OPRIAN

The proposal that the absorption maximum of the visual pigments is governed by interaction of the 11-*cis*-retinal chromophore with charged carboxylic acid side chains in the membrane-embedded regions of the proteins has been tested by mutating five Asp and Glu residues thought to be buried in rhodopsin. Changing Glu^{113} to Gln causes a dramatic shift in the absorption maximum from 500 nanometers to 380 nanometers, a decrease in the pK_a (acidity constant) of the protonated Schiff base of the chromophore to about 6, and a greatly increased reactivity with hydroxylamine. Thus Glu^{113} appears to be the counterion to the protonated Schiff base. Wavelength modulation in visual pigments apparently is not governed by electrostatic interaction with carboxylate residues, other than the counterion.

RHODOPSIN CONTAINS AN 11-*CIS*-retinal chromophore covalently attached to the apoprotein opsin through a protonated Schiff-base linkage to the ε-amino group of Lys^{296} (*1, 2*). The absorption maximum for rhodopsin is at 500 nm, whereas that for a protonated Schiff base of 11-*cis*-retinal free in methanol solution is at 440 nm. This 60-nm (2700 cm^{-1}) bathochromic shift or "opsin shift" (*3*) is caused by interaction of the protonated chromophore with the protein. The difference in absorption maximum then between the unprotonated chromophore free in solution and the protonated form bound to the protein (8100 cm^{-1}) is thought to result from protonation of the Schiff base nitrogen (5400 cm^{-1}) and from the opsin shift.

Graduate Department of Biochemistry, Brandeis University, Waltham, MA 02254.

Several theories have been proposed for the mechanism of the opsin shift in rhodopsin (*4*). The same mechanism probably also accounts for wavelength modulation in the color pigments. Kropf and Hubbard (*5*) originally proposed that the photoexcited state of rhodopsin could be stabilized by an electrostatic interaction of a negative charge from an amino acid side chain with the delocalized positive charge from the protonated Schiff base in the polyene chain of the chromophore. Honig *et al.* (*6*) in their "external point-charge model" (Fig. 1A) proposed on the basis of semiempirical calculations and experiments with dihydro-retinal analogs that 11-*cis*-retinal interacts with two carboxylate residues: (i) a counterion for the protonated Schiff base nitrogen atom and (ii) a point charge located near carbon atoms 12 and 14 of the chromophore.

We have undertaken identification of the amino acid residues that function as coun-

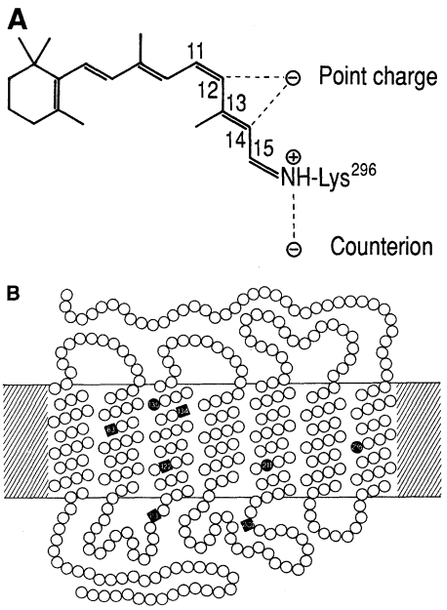

Fig. 1. (**A**) Diagram of 11-*cis*-retinal chromophore bound to opsin by a protonated Schiff base linkage to Lys^{296} of the polypeptide chain. (**B**) Schematic diagram for the structure of rhodopsin in a lipid bilayer, as proposed by Dratz and Hargrave (*1*). Charged amino acids relevant to this work (highlighted as solid diamonds for carboxylic acid residues or solid circles for positively charged side chains) are Asp^{83}, Glu^{113}, Glu^{122}, Glu^{134}, Arg^{135}, Glu^{201}, His^{211}, and Lys^{296}.

terion to the Schiff base and as negative point charge for wavelength modulation of the chromophore. The experimental system used for mutagenesis of rhodopsin has been described (*7–12*). We used restriction fragment replacement to facilitate mutagenesis of bovine rhodopsin and purified the altered proteins by an immunoaffinity procedure. Absorption spectra of the purified proteins allowed us to determine the ability of the pigment to be reconstituted with chromophore and the position of the absorption maximum.

Since the 11-*cis*-retinal chromophore is bound to the protein within the lipid bilayer (*1, 2*), we restricted our search to potential negative amino acid residues located within or near the seven transmembrane segments of the protein in the Dratz and Hargrave model (*1*) of rhodopsin (Fig. 1B). We focused exclusively on carboxylic acid residues because these were most likely to be ionized. Only three amino acids within the membrane were candidates according to these criteria, Asp^{83}, Glu^{122}, and Glu^{134}. We also studied two residues near the membrane surface, Glu^{113} and Glu^{201}.

Proteins resulting from mutation of Asp^{83} to Glu, Asn, and Ala bound the 11-*cis*-retinal chromophore and displayed a 500-nm absorption maximum similar to the wild type (Fig. 2A; D83E and D83A not shown). Rhodopsin is apparently insensitive to changes of polarity and volume at position 83. The reconstituted mutant of Glu^{134} to Gln also displayed the 500-nm absorption. We conclude that neither Asp^{83} nor Glu^{134} function as the counterion or as an external point charge.

Substitutions for Glu^{122} do result in modification of the absorption spectrum (Fig. 2B). However, the shifts are much smaller than expected for neutralization of the counterion or point charge. Substitution of a Gln for the Glu results in a shift of only 20 nm to 480 nm compared with 60 nm expected from the point-charge model. The interaction of Glu^{122} with the polyene chain is unlikely to be electrostatic since substitution by Asp results in a further blue shift to 475 nm. A simple model for interaction of a negatively charged amino acid side chain with the chromophore predicts that Asp^{122} would exhibit a longer wavelength peak than Gln^{122}. In that Ala^{122} also displays an absorption maximum at about 475 nm, the effect at position 122 may be steric.

The cone pigments have a different arrangement of carboxylic acids than in rhodopsin (*13*). To test if the variation in position and number of these carboxylic acid side chains may be directly responsible for variation of absorption maxima in the different pigments, we constructed two double mutants of rhodopsin (Fig. 2C). The D83N,E122Q mutant, which has the same arrangement of acidic amino acids in the membrane-embedded region as the blue pigment, has an absorption maximum at 480 nm, which is the value expected from the change at position 122 alone, and not at 420 nm, that of the native blue pigment. This result also makes unlikely a model for wavelength modulation based on total charge in the membrane-embedded region (*13*). The absorption maximum for M86E,E122Q, which corresponds to the arrangement of acidic residues found in the red and green pigments, is also at 480 nm, rather than at 565 or 535 nm for the native red or green pigments, respectively.

We studied mutations of Glu^{113} and Glu^{201}, which are near the membrane surface in the Dratz and Hargrave model. The absorption maximum of E201Q is at 500 nm, excluding this position from further consideration. However, mutation at position 113 had a dramatic effect on the visible absorption spectrum.

Absorption spectra for rhodopsin and the mutant E113Q are shown in Fig. 3A. The large blue shift of E113Q giving a maximum at 380 nm is apparent. Deprotonation of the Schiff base nitrogen in this mutant is indicated by the shift in the spectrum below 440

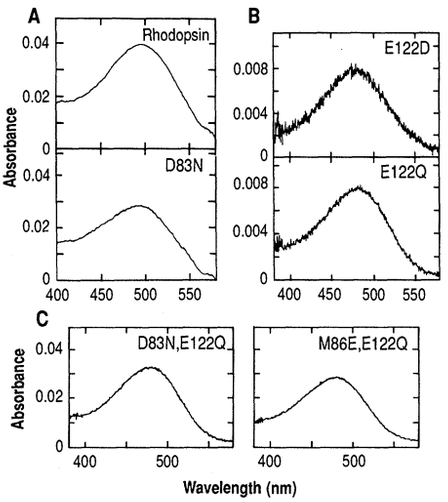

Fig. 2. Visible absorption spectra for (**A**) rhodopsin and the mutant form of rhodopsin, D83N; (**B**) mutant forms of rhodopsin E122D and E122Q; and (**C**) two double mutants of rhodopsin, D83N,E122Q and M86E,E122Q.

nm, the absorption maximum for the protonated Schiff base free in solution. Mutation of Glu^{113} to an acidic residue (E113D) restored the long wavelength absorption maximum (507 nm), and shows a modest red shift relative to the native spectrum (Fig. 3B).

The effect of *p*H on the absorption spectrum of E113Q (purified from COS cells in a buffer at *p*H 6.0) is shown in Fig. 4A. The spectrum shows two absorption maxima, one at 380 nm and a second long wavelength absorbance at about 490 nm. When the *p*H was raised to 7.2, the long wavelength maximum disappeared with a concomitant increase in absorbance at 380 nm. Reversing this experiment (purifying at *p*H 7.5 and then bringing the sample to *p*H 6.2) results in similar spectra. The interconversion of long and short wavelength absorbing species in both experiments indicates that the reaction is reversible. These data also indicate that the chromophore is bound to E113Q in the correct binding site.

We conclude that the long wavelength absorbing species of E113Q at low *p*H is a protonated Schiff base. The E113Q mutation causes a dramatic decrease in the pK_a for the Schiff base such that it is now around *p*H 6. The exact magnitude of this change is not known because the pK_a of this group in the native protein is so high that rhodopsin denatures before the proton can be removed. The protonated Schiff base of E113Q observed at low *p*H is probably associated with a counterion, most likely an anion recruited from the solvent. The absorbance maximum for the protonated form of E113Q (490 nm) is shifted relative to that of the wild type (500 nm), which may

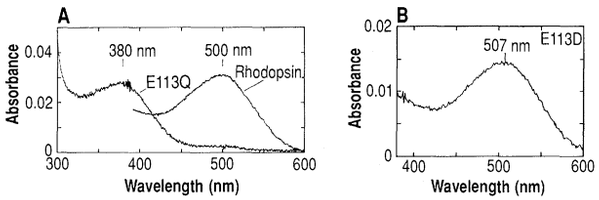

Fig. 3. (**A**) Absorption spectra for E113Q and rhodopsin. The pigment concentrations in these two samples were not identical, and therefore the relative extinctions cannot be compared. (**B**) Absorption spectrum of E113D.

indicate that the counterion is closer to the nitrogen atom than in the native protein. Similarly, the red shift observed with D113 (Fig. 3B) could result from a larger distance between the protonated nitrogen and the carboxylate group (*14*).

To determine the effect of mutation at position 122 on the absorption spectrum for the mutant E113Q, we constructed the double mutant E113Q,E122Q, which had an absorption maximum of 372 nm at *p*H 7.5. This blue shift of about 8 nm (570 cm^{-1}) relative to that for E113Q is similar to the effect of this Gln^{122} mutation on the wild type (830 cm^{-1}). We argue from this that the 11-*cis*-retinal chromophore of E113Q is in the same binding pocket that it occupies in the native protein. When the *p*H is changed from 7.5 to 6.2, the absorption spectrum of E113Q,E122Q also shows two maxima, one at 372 nm and another at longer wavelength (Fig. 4B).

Hydroxylamine reacted with the E113Q chromophore in the dark to form the oxime of 11-*cis*-retinal, with an absorption maximum at about 360 nm (Fig. 4C). This reactivity associated with the Schiff base nitrogen is in sharp contrast to that of native rhodopsin, which reacts with hydroxylamine only after exposure to light (*15*). Of the mutant proteins we report, only E113Q,E122Q also reacted with hydroxylamine in the dark.

The most straightforward interpretation of these results is that Glu^{113} is the counterion to the Schiff base, although these data are also consistent with a model in which Glu^{113} provides a crucial tertiary interaction in the protein that brings a different anion into contact with the Schiff base.

Because Glu^{113} is conserved in all of the cone pigments (*13*), the blue pigment may also have a protonated Schiff base, in agreement with the conclusions of Loppnow *et al.* (*16*). In *Drosophila* rhodopsin (*17*) the counterion is probably Tyr^{126}. This residue is in exactly the same position as is Glu^{113} in bovine rhodopsin relative to amino acids Cys^{110}, Glu^{134}, and Arg^{135}, which are strictly conserved in all of the visual pigments. Tyr^{126} is also conserved in the *Drosophila* R8 cell pigment (*18*), but in the ultraviolet pigments which would be expected not to have a counterion, it has been changed to a Phe (*19*). Significantly, octopus rhodopsin (*20*), which has been shown to contain a protonated Schiff base by resonance Raman spectroscopy (*21*) as has bovine rhodopsin (*22*), also has a Tyr at this position.

We note that Glu^{113} and Glu^{122} appear to account for about 7300 cm^{-1} of the observed 8100 cm^{-1} difference between the absorption maxima of the Schiff base free in solution and bound to the protein. Also, the shift observed for deprotonation of the Schiff base nitrogen in E113Q (6300 cm^{-1}) is larger than that expected based on the same reaction in methanol solution (5400 cm^{-1}). The fact that we find evidence for only a single charged carboxylate interacting with the chromophore is consistent with a model for rhodopsin put forward by Birge *et al.* (*23*) to account for two-photon absorption spectra of rhodopsin reconstituted with a locked 11-*cis*-retinal analog. Finally, the fact that Glu^{113} is located outside of the membrane-embedded region of the Dratz and Hargrave model suggests that the model be modified to bring this residue into the membrane, which will result in residues Glu^{134} and Arg^{135} being positioned at the membrane surface.

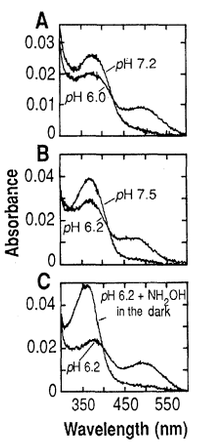

Fig. 4. The effect of *p*H and hydroxylamine on the spectra of E113Q and the double mutant E113Q,E122Q. (**A**) The spectrum of E113Q purified in 10 m*M* sodium phosphate buffer at *p*H 6.0, containing 150 m*M* sodium chloride and 1% dodecyl-maltoside. The buffer was then brought to 100 m*M* sodium phosphate, *p*H 7.2; this spectrum was expanded by 10% to correct for dilution accompanying addition of the buffer. (**B**) The spectrum of E113Q,E122Q purified in a 10 m*M* sodium phosphate buffer, *p*H 7.5. The buffer was then adjusted to 100 m*M* sodium phosphate, *p*H 6.2; this spectrum was also corrected by 10% for dilution. (**C**) Spectra of E113Q purified at *p*H 7.5, and then brought to *p*H 6.2 and reacted with hydroxylamine in the dark.

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