Constitutive Activation of Opsin: Influence of Charge at Position 134 and Size at Position 296†

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ABSTRACT: In previous studies, mutation of Lys^{296} or Glu^{113} in opsin has been shown to result in constitutive activation of the protein—that is, these mutants can activate the G protein transducin in the absence of chromophore and in the absence of light. These and other data have led to the suggestion that a salt bridge between Lys^{296} and Glu^{113} helps to constrain opsin to an inactive conformation. It is shown here that of 12 different amino acids substituted at position 296, all, except Arg and the wild-type Lys, are constitutively active at neutral pH, lending further support to this suggestion. However, activation of opsin appears also to be influenced significantly by the size of amino acid side chain at position 296. Thus, there are multiple effects of the mutations. Wild-type opsin is also shown to be weakly active at pH 6.1. Five other charged amino acids in the membrane-embedded region of the protein (Asp^{83}, Glu^{122}, Glu^{134}, Arg^{135}, and Glu^{201}) were mutated to see if they affect constitutive activity. Of these amino acids, only mutation of Glu^{134} results in an increase in the activity of opsin. Changing Glu^{134} to Gln increases the activity of opsin, while changing Glu^{134} to Asp inhibits activity. These results suggest that a negative charge on Glu^{134} is important in stabilizing the inactive state of opsin. Glu^{134} is highly conserved in all visual pigments and most of the other G protein-linked receptors.

The visual pigment rhodopsin is a member of the large family of G protein-linked receptors (Birnbaumer *et al.*, 1990; Dohlman *et al.*, 1991; Strosberg, 1991; Hargrave & McDowell, 1992; Khorana, 1992; Nathans, 1992; Oprian, 1992). 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} (Bownds, 1967; Ovchinnikov *et al.*, 1982; Dratz & Hargrave, 1983). The protonated Schiff base is stabilized through an electrostatic interaction with the negatively charged side chain of Glu^{113}, the Schiff base counterion (Zhukovsky & Oprian, 1989; Sakmar *et al.*, 1989; Nathans, 1990).

Recent mutagenesis studies have shown that mutation of either Glu^{113} or Lys^{296} in the apoprotein, opsin, results in constitutive activation of the protein (Robinson *et al.*, 1992; Cohen *et al.*, 1992). That is, these mutants are fully able to activate the G protein transducin in the absence of added chromophore.

We show here that of 12 different amino acids at position 296, all, except Lys^{296} (wild-type) and Arg^{296}, cause constitutive activation of opsin at neutral pH. These results support a model in which a salt bridge between Glu^{113} and Lys^{296} helps to stabilize the inactive state of opsin. Interestingly, the steric bulk of the amino acid side chain at position 296 is found to influence the transition from inactive to active states.

We also show that mutation of the highly conserved carboxylate residue Glu^{134} to Gln favors the active state of opsin and results in an increase in constitutive activity. A Glu or Asp residue is found at position 134 in all visual pigments and most of the other G protein-linked receptors.

EXPERIMENTAL PROCEDURES

Expression of Opsin Mutants. The wild-type and mutant rhodopsins used in this study were obtained from transfected COS cells. All procedures for DNA manipulation, mutation of the rhodopsin gene, DNA sequence analysis, transfection of COS cells, reconstitution of the pigment with 11-*cis*-retinal chromophore, and purification of the protein were performed as previously described (Robinson *et al.*, 1992; Cohen *et al.*, 1992).

Activity Assays. Mutant and wild-type opsins (and rhodopsins) were assayed for ability to catalyze exchange of guanine nucleotides on transducin by following the binding of [^{35}S]GTPγS[1] as has been described (Zhukovsky *et al.*, 1991; Robinson *et al.*, 1992; Cohen *et al.*, 1992). Unless noted otherwise, COS cell membranes were used in these assays and the opsin concentration varied from 1 to 5 nM as estimated by Western blot analysis. For certain mutants, the assay was performed also in detergent solution with immunopurified rhodopsin. In this case, the concentration of protein was determined from the absorption spectrum of the pigment.

Absorption Spectroscopy. UV–visible absorption spectra were recorded as previously described (Cohen *et al.*, 1992) except that the temperature was 24 °C.

Determination of pK_{a2}. pH–rate profiles for all constitutively active opsin mutants, as well as for light-activated rhodopsin, are bell-shaped with the lower apparent pK_a referred to as pK_{a1} and the higher apparent pK_a referred to as pK_{a2}

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[1] Abbreviations: GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); MII, metarhodopsin II; mutant forms of rhodopsin are designated by the one-letter code for the wild-type amino acid, followed by the position of mutation, followed by the one-letter code for the new amino acid.

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[see Figure 2 of this paper for a representative example and Cohen *et al.* (1992) for a more complete characterization of these reactions]. To determine the values of pK_{a1} and pK_{a2}, the data were simulated with the equation [see Cohen *et al.* (1992)]

$$\text{obsd rate} = V_{max}[H^n/(H^n + K_{a2}^n)][K_{a1}^k/(H^k + K_{a1}^k)] + T \quad (1)$$

where H is the proton concentration, k is the number of protons going onto the protein during the transition described by K_{a1}, and n is the number of protons going onto the protein during the transition described by K_{a2}. Therefore, the expression $[H^n/(H^n + K_{a2}^n)][K_{a1}^k/(H^k + K_{a1}^k)]$ is the fraction of the protein that is both protonated at site(s) 2 and unprotonated at site(s) 1 at proton concentration H. It is important to emphasize here that the number of groups ionizing in either of these transitions (pK_{a1} or pK_{a2}) is unknown. Only the *net* number of protons involved in the transition may be determined from the data. The term T (eq 1) has been added to account for the pH-dependent activation of transducin that occurs in the absence of opsin (Cohen *et al.*, 1992).

All of the data reported in this paper were fit well using a value of pK_{a1} that ranged from 5.8 to 6.0 (except for the E113Q,K296E mutation, which was better fit using a value of 5.4) and with values of n and k which ranged from 0.9 to 1.1. The only variables that did vary significantly among the mutants were K_{a2} and V_{max}. V_{max} varied among the mutants by a factor of 7. Much of this variation is accounted for by differences in the expression level for the different opsin mutants. However, the expression levels were determined by Western blot analysis, which is an imprecise method for the determination of active protein. Therefore, it is possible that there are real variations in the activity for these mutants on the order of 2–3-fold. In contrast, the value of K_{a2} was found to vary by over 4 orders of magnitude for these same mutations. By analyzing and comparing pK_{a2} for the different mutants, which is an intrinsic property of the protein, we have tried to avoid comparisons that may be influenced by expression level of the various mutants.

RESULTS

Effect of Size and Charge at Position 296 on Opsin Activity. pH–rate profiles for the constitutively active opsin mutants E113Q, K296E, K296G, and K296H are bell-shaped, with the characteristic that the value for the more alkaline of the two apparent pK_as (pK_{a2}) is highly influenced by the particular amino acid substitution (Cohen *et al.*, 1992). To further explore this effect, we examined a total of 12 different amino acid residues at position 296 in the apoprotein. All were constitutively active at pH 6.7 with the exception of Arg^{296} and the wild-type Lys^{296}. All of the constitutively active mutants had bell-shaped pH–rate profiles, and in agreement with our previous results using a more limited number of mutations, pK_{a2} is highly influenced by the identity of amino acid side chain at position 296 (Table I). With the exception of the charged residues Lys, Arg, and Glu, the value of pK_{a2} correlates well with the volume (Figure 1A) and surface area (Figure 1B) of amino acid side chain but poorly with hydrophobicity (Figure 1C).

The fact that the Arg^{296} mutant opsin is inactive at pH 6.7 could result from (i) the ability of the positively charged Arg residue to substitute for the wild-type Lys at position 296 or (ii) a global disruption of protein structure in this mutant. It is unlikely that Arg^{296} disrupts the structure of opsin since the single mutant, K296R, was expressed at normal levels and, more importantly, the double mutant, E113Q,K296R, displays robust constitutive activity (Figure 2). We note that the ability of a positive charge at position 296 to inactivate opsin is lost upon mutation of Glu^{113}, as is shown by the series of double mutants in Table II. The effect of negative charge at 296 (E113Q,K296E) and size appears to be preserved in these mutants.

Activity of Wild-Type Opsin. At pH 6.7 we cannot detect in our assay activity for wild-type opsin (data not shown). However, at pH 6.1, wild-type opsin displays a small, but significant, ability to activate transducin. This activity can

Table I: Effect of Volume, Surface Area, and Hydrophobicity of the Position 296 Side Chain on pK_{a2}

amino acid	pK_{a2}	volume[a] (Å^3)	surface area[a] (Å^2)	hydrophobicity[a]
Glu (E)[b]	8.3	138	190	−0.74
Gly (G)[b]	8.0	60	75	+0.48
Ala (A)	7.9	89	115	+0.62
Thr (T)	7.4	116	140	−0.05
Cys (C)	7.3	109	135	+0.29
Gln (Q)	7.0	144	180	−0.85
Val (V)	6.7	140	155	+1.08
His (H)[b]	6.5	153	195	−0.40
Leu (L)	6.2	167	170	+1.06
Phe (F)	6.1	190	210	+1.19
Arg (R)	<5.0	173	225	−2.53
Lys (K)	<5.0	169	200	−1.50

[a] Volume and surface area of a side chain are from Creighton (1984); values for hydrophobicity are from Eisenberg *et al.* (1989). [b] The values of pK_{a2} for these mutants have been reported previously (Cohen *et al.*, 1992). The values of pK_{a2} reported in this table for Glu^{296} and His^{296} differ, slightly, from the values previously reported (8.0 and 6.6, respectively).

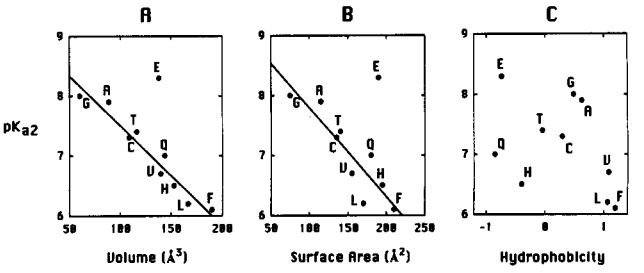

FIGURE 1: Dependence of pK_{a2} on size of side chain at position 296. (A) Plot of pK_{a2} against the volume of the side chain substituted at position 296. (B) Plot of pK_{a2} against the surface area of the side chain substituted at position 296. (C) Plot of pK_{a2} against the hydrophobicity of the side chain substituted at position 296. All of the values used in this figure (pK_{a2}, volume, surface area, and hydrophobicity) are from Table I. The lines drawn in parts A and B are linear regressions of all data points excluding Glu^{296}.

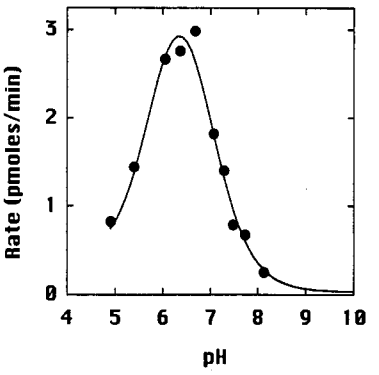

FIGURE 2: pH–rate profile for transducin activation by E113Q,K296R opsin in the absence of chromophore. The solid line was generated from eq 1 under Experimental Procedures. pK_{a2} is as in Table II.

Table II: pK_{a2} Values for Constitutively Active Double Mutants of E113Q

mutant	pK_{a2}	mutant	pK_{a2}
E113Q[a]	6.8	E113Q,K296H	7.3
E113Q,K296E	8.7	E113Q,K296R	6.9
E113Q,K296G[a]	8.0	E113Q,K296F	6.6

[a] The values of pK_{a2} for these mutants have been reported previously (Cohen *et al.*, 1992).

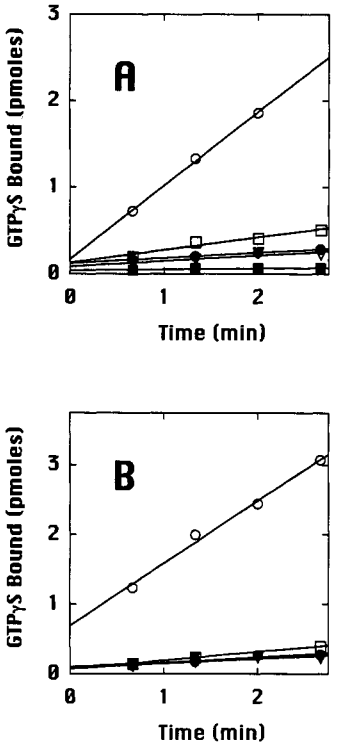

FIGURE 3: Transducin activation by wild-type opsin and by R135Q rhodopsin. (A) Weak constitutive activity of wild-type opsin at pH 6.1. (Open circles) Wild-type opsin with 11-*cis*-retinal in the light; (solid circles) wild-type opsin with 11-*cis*-retinal in the dark; (open squares) wild-type opsin without 11-*cis*-retinal; (solid squares) wild-type opsin in the absence of transducin; (open triangles) membranes from COS cells transfected with the expression vector not containing the opsin gene. Two other controls were performed but were left out of the figure for clarity: (i) membranes from COS cells transfected with the expression vector not containing the opsin gene, with 11-*cis*-retinal in the light; and (ii) transducin in the absence of opsin and membranes. These controls were essentially indistinguishable from wild-type opsin with chromophore in the dark (solid circles). All reactions were at pH 6.1. (B) Activation of transducin by R135Q. (Open circles) R135Q opsin with 11-*cis*-retinal in the light; (solid circles) R135Q opsin with 11-*cis*-retinal in the dark; (open squares) R135Q opsin without 11-*cis*-retinal; (open triangles) membranes from COS cells transfected with the expression vector not containing the opsin gene; (solid triangles) the same membranes with 11-*cis*-retinal in the light. All reactions were at pH 6.1.

be suppressed by the addition of 11-*cis*-retinal in the dark (Figure 3A). This activity is above that seen for transducin either in the absence of membranes or in the presence of membranes that lack opsin (Figure 3A). The activity of wild-type opsin at pH 6.1 is only about 10% of that observed for light-activated wild-type rhodopsin under the same conditions.

Glu^{134}. It is evident from the bell-shaped pH–rate profiles observed for all constitutively active mutants that there are ionizable groups in addition to Glu^{113} and Lys^{296} which control the active state of opsin (Cohen *et al.*, 1992). To identify other charged residues involved in the activation of opsin, we screened mutants of Asp^{83}, Glu^{122}, Glu^{134}, Arg^{135}, and Glu^{201} for ability to constitutively activate transducin.

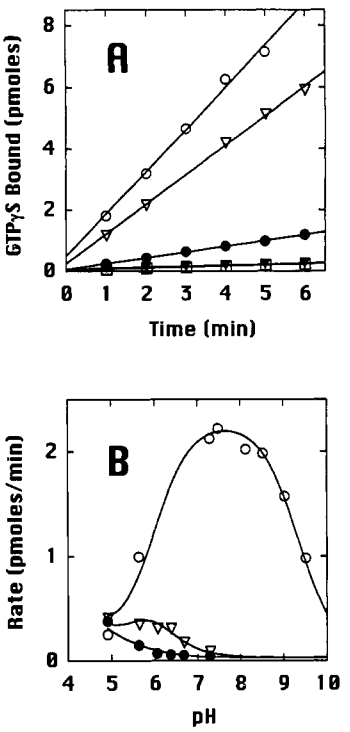

FIGURE 4: Activation of opsin by mutation of Glu^{134}. (A) Constitutive activity of E134Q at pH 6.7. (Open circles) Reaction catalyzed by E134Q opsin with 11-*cis*-retinal in the light; (solid circles) reaction catalyzed by E134Q opsin without 11-*cis*-retinal; (open triangles) reaction catalyzed by D83N opsin with 11-*cis*-retinal in the light; (solid triangles) reaction catalyzed by D83N opsin without 11-*cis*-retinal; (open squares) reaction catalyzed by membranes transfected with the expression vector not containing the opsin gene. The reaction for wild-type opsin was essentially indistinguishable from that of D83N opsin (solid triangles). All reactions were at pH 6.7. (B) pH–rate profiles for E134Q. (Open circles) pH–rate profile for E134Q opsin with 11-*cis*-retinal in the light; (solid circles) pH–rate profile for E134Q opsin with 11-*cis*-retinal in the dark; (open triangles) pH–rate profile for E134Q opsin without 11-*cis*-retinal. The solid lines were generated from eq 1 under Experimental Procedures. pK_{a2} values are 5.6 and 9.4 for constitutive and light-dependent activity, respectively.

The mutants D83N, E122Q, E134Q, R135Q, and E201Q can all be reconstituted with 11-*cis*-retinal (Zhukovsky & Oprian, 1989; Sakmar *et al.*, 1989) and, when assayed in membranes at pH 6.7, all activated transducin in a light-dependent manner with activity comparable to that of wild type (data not shown). When assayed in the absence of the chromophore, only the mutant E134Q was constitutively active, and this activity was suppressed by 11-*cis*-retinal in the dark (Figure 4). pH–rate profiles for the constitutively active E134Q apoprotein and the light-activated holoprotein are shown in Figure 4B. The V_{max} (eq 1) values for these two reactions are approximately the same (2.25 and 2.0 pmol/min for the light-dependent and constitutive reactions, respectively), and the chief difference between them appears to be in the value of pK_{a2}, which differs by about 4 units (9.4 and 5.6 for the light-dependent and constitutive reactions, respectively).

In contrast to these results, the mutant opsin E134D, in which an Asp residue is substituted for the Glu, did not appear to be constitutively active, even at pH 6.1. The rhodopsin form of E134D displayed light-dependent activity comparable in magnitude to that of wild type.

The effect of mutation at position 134 is dramatically illustrated in the pH–rate profiles and associated pK_{a2} values for a series of constitutively active double mutants of K296H (Table III and Figure 5). Of the amino acids mutated, only

Table III: pK_{a2} Values for Constitutively Active Double Mutants of K296H

mutant	pK_{a2}	mutant	pK_{a2}
K296H[a]	6.5	D83N,K296H	6.3
E134Q,K296H	8.8	R135Q,K296H	6.0
E201Q,K296H	6.5	E134D,K296H	5.8
E122Q,K296H	6.4		

[a] The value of pK_{a2} for this mutant has been reported previously (Cohen *et al.*, 1992).

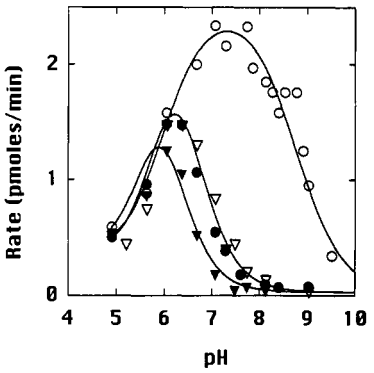

FIGURE 5: Effect of Glu^{134} on the constitutive activity of K296H as shown by pH–rate profiles for E134Q,K296H opsin (open circles), K296H opsin (solid circles), E201Q,K296H opsin (open triangles), and E134D,K296H opsin (solid triangles). All reactions were in the absence of 11-*cis*-retinal. To facilitate comparison of their respective pK_{a2} values, the activity of K296H has been normalized (multiplied by a factor of 1.25) to that of E201Q,K296H.

substitution of Glu^{134} significantly perturbs pK_{a2} in this series; pK_{a2} in the mutant E134Q,K296H (8.8) is greatly increased over that of the single mutant K296H (6.5), and pK_{a2} is slightly decreased in the mutant E134D,K296H (5.8).

The R135Q mutant deserves further comment. As stated above, light-activated R135Q rhodopsin activates transducin with a specific activity comparable to that observed for wild type when assayed in membranes (Figure 3). This mutant has been shown previously to be about 12 times less active than wild-type rhodopsin when purified and assayed in detergent solution (Sakmar *et al.*, 1989; Franke *et al.*, 1990, 1992). We also found detergent-solubilized and purified R135Q rhodopsin to be 10–20-fold less active than wild-type rhodopsin. The reduced ability of detergent-solubilized R135Q to activate transducin is not due to an inability to form metarhodopsin II (MII) since we observe that MII (as defined spectrally; Matthews *et al.*, 1963) formed quantitatively under the assay conditions and it was stable, as shown by acid-trapping (Morton & Pitt, 1955), over the entire time of the assay (data not shown). We do note, however, that the double mutant R135Q,K296H opsin had a slightly lower pK_{a2} value than the single mutant K296H (Table III), suggesting that even in membranes the mutation of $Arg^{135} \rightarrow$ Gln may inhibit transducin activation.

DISCUSSION

Effect of Size at Position 296. The size of amino acid side chain at position 296 is a reasonably good inverse correlate of constitutive activity in opsin. Large residues favor the inactive state, and small residues favor the active state. The degree to which the active state is favored may be expressed quantitatively in the form of the apparent ionization constant for this transition, K_{a2}, or, more conveniently, pK_{a2}. The difference in pK_{a2} from the smallest (Gly^{296}) to the largest (Phe^{296}) residue examined was 1.9 units, corresponding to a difference in free energy of 2.6 kcal/mol.

The mechanism by which the size of amino acid exerts this effect is not clear. It is unlikely to be due to the hydrophobic effect since there is a poor correlation with hydrophobicity for the various amino acid side chains. It is noteworthy that the regression line in Figure 1B has a slope of 0.0149 unit/Å^2 or about 20 cal/Å^2 of surface area. This is roughly the same magnitude determined by Matthews and co-workers for the effect of cavity-creating mutations on the thermal stability of T4 lysozyme: about 20 cal/Å^2 of surface area for the cavity created by mutation (Eriksson *et al.*, 1992). Therefore, one simplistic interpretation of these data is that van der Waals contacts between Lys^{296} and opsin are lost upon going to the active state. There are, however, other equally likely interpretations that account for these results.

Contribution of the Salt Bridge. What then is the energetic contribution of the salt bridge to the inactive state? It is difficult to determine this unambiguously from the available data, but we can make a crude estimate. First, we note that there are three amino acids which do not fit the correlation in Figure 1A. These are the charged residues Lys^{296}, Arg^{296}, and Glu^{296}, and it is a fair guess that they fit the line poorly because they are charged.[2] The positively charged Lys and Arg lie below the line (see Table I), and the negatively charged Glu lies above the line. From the regression in Figure 1A, we would expect an opsin with a neutral side chain of equivalent size to Lys at position 296 to have a pK_{a2} of 6.4. The difference between this value and the actual value of pK_{a2} would give an estimate of the stabilization from the salt bridge. However, we do not know the exact value of pK_{a2} for wild-type opsin. Based on the observation that wild-type opsin displays a small, but significant, amount of activity at pH 6.1 (Figure 3A), we estimate that the pK_{a2} of wild-type opsin is between 4 and 5. Therefore, the contribution of the salt bridge is 1.4–2.4 pH units, or 2–3 kcal/mol. This is roughly the same amount of energy found by Dahlquist and co-workers for the contribution of a salt bridge between His^{31} and Asp^{70} to the thermal stability of T4 lysozyme (Anderson *et al.*, 1990).

This relatively weak salt bridge is to be contrasted with the situation found in rhodopsin, where the loss of the counterion shifts the pK_a of the Schiff base nitrogen by over 5 pH units, or about 7 kcal/mol (Zhukovsky & Oprian, 1989; Sakmar *et al.*, 1989). That the salt bridge between Lys^{296} and Glu^{113} in opsin is weak is not surprising, since the Lys must be able to deprotonate to attack the incoming 11-*cis*-retinal aldehyde during the formation of the pigment. A salt bridge of high stability would hinder this reaction.

Role of Glu^{134}. To test for the involvement of other charged residues in the activation of opsin, we examined mutants of Asp^{83}, Glu^{122}, Glu^{134}, Arg^{135}, and Glu^{201} for constitutive activity and for ability to increase the activity (i.e., pK_{a2}) of another constitutively active mutant, K296H. Of these amino acids, only Glu^{134} was found to contribute significantly to the active state of opsin. Changing Glu^{134} to Gln increased the constitutive activity of opsin and increased pK_{a2} of K296H, while changing Glu^{134} to Asp decreased the constitutive activity of opsin and decreased pK_{a2} of K296H. These data agree well with those of Sakmar *et al.* (1989), who found that detergent-solubilized and purified E134Q and E134D rhodopsins are, respectively, 50% more and 50% less active than wild type in the light-dependent reaction. The data also suggest

[2] Note that the interpretation in this paper is slightly modified from that of our previous work (Cohen *et al.*, 1992), which was based on a more limited set of mutants. Here we suggest that the Glu side chain in the K296E mutant is charged and that, from Figure 1A, His^{296} probably is not charged.

that the role of charge is important for the effect of the mutations.

Other G Protein-Linked Receptors. Finally, we note that Glu^{134} is a highly conserved amino acid. It is a Glu in all of the visual pigments and an Asp or Glu in the other G protein-linked receptors (Dohlman *et al.*, 1991). It is very likely that mutation of Glu^{134} similarly will result in activation of the other receptors.

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REFERENCES

Anderson, D. E., Becktel, W. J., & Dahlquist, F. W. (1990) *Biochemistry 29*, 2403–2408.

Birnbaumer, L., Abramowitz, J., & Brown, A. M. (1990) *Biochim. Biophys. Acta 1031*, 163–224.

Bownds, D. (1967) *Nature 216*, 1178–1181.

Cohen, G. B., Oprian, D. D., & Robinson, P. R. (1992) *Biochemistry 31*, 12592–12601.

Creighton, T. E. (1984) in *Proteins*, p 7, Freeman, New York.

Dohlman, H. G., Thorner, J., Caron, M. G., & Lefkowitz, R. J. (1991) *Annu. Rev. Biochem. 60*, 653–688.

Dratz, E. A., & Hargrave, P. A. (1983) *Trends Biochem. Sci. 8*, 128–131.

Eisenberg, D., Wesson, M., & Wilcox, W. (1989) in *Prediction of Protein Structure and the Principles of Protein Conformation* (Fasman, G. D., Ed.) pp 635–646, Plenum Press, New York.

Eriksson, A. E., Baase, W. A., Zhang, X.-J., Heinz, D. W., Blaber, M., Baldwin, E. P., & Matthews, B. W. (1992) *Science 255*, 178–183.

Franke, R. R., Konig, B., Sakmar, T. P., Khorana, H. G., & Hofmann, K. P. (1990) *Science 250*, 123–125.

Franke, R. R., Sakmar, T. P., Graham, R. M., & Khorana, H. G. (1992) *J. Biol. Chem. 267*, 14767–14774.

Hargrave, P. A., & McDowell, J. H. (1992) *FASEB J. 6*, 2323–2331.

Khorana, H. G. (1992) *J. Biol. Chem. 267*, 1–4.

Matthews, R. G., Hubbard, R., Brown, P. K., & Wald, G. (1963) *J. Gen. Physiol. 47*, 215–240.

Morton, R. A., & Pitt, G. A. J. (1955) *Biochem. J. 59*, 128–134.

Nathans, J. (1990) *Biochemistry 29*, 9746–9752.

Nathans, J. (1992) *Biochemistry 31*, 4923–4931.

Oprian, D. D. (1992) *J. Bioenerg. Biomembr. 24*, 211–217.

Ovchinnikov, Yu. A., Abdulaev, N. G., Feigina, M. Y., Artamonov, I. D., Zolotarev, A. S., Kostina, M. B., Bogachuk, A. S., Miroshnikov, A. I., Marinov, V. I., & Kudelin, A. B. (1982) *Bioorg. Khim. 8*, 1011–1014.

Robinson, P. R., Cohen, G. B., Zhukovsky, E. A., & Oprian, D. D. (1992) *Neuron 9*, 719–725.

Sakmar, T. P., Franke, R. R., & Khorana, H. G. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8309–8313.

Strosberg, A. D. (1991) *Eur. J. Biochem. 196*, 1–10.

Zhukovsky, E. A., & Oprian, D. D. (1989) *Science 246*, 928–930.

Zhukovsky, E. A., Robinson, P. R., & Oprian, D. D. (1991) *Science 251*, 558–560.