Mechanism of Activation and Inactivation of Opsin: Role of Glu^{113} and Lys^{296} †

George B. Cohen, Daniel D. Oprian,* and Phyllis R. Robinson‡

Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254

Received August 10, 1992; Revised Manuscript Received September 30, 1992

ABSTRACT: In previous studies, mutation of either Lys^{296} or Glu^{113} in bovine rhodopsin has been shown to result in constitutive activation of the apoprotein form, opsin [Robinson et al. (1992) *Neuron 9*, 719–725]. In this report, pH–rate profiles for the rhodopsin-catalyzed exchange of GTPgS for GDP on transducin are established for the constitutively active opsin mutants. All of the mutants, including the double-mutant E113Q,K296G, show a bell-shaped pH–rate profile. Therefore, it is evident that at least two ionizable groups in addition to Lys^{296} and Glu^{113} control the formation of the active opsin state. The sole effect of mutation at position 113 or 296 is to alter the ionization constant of the group with the higher pK_a, called pK_{a_2}. pK_{a_2} decreases in the following order: rhodopsin/light (9.0) > K296E = K296G = E113Q,K296G (8.0) > E113Q (6.8) > K296H (6.6) ≫ wild-type opsin (<5.0). These results are consistent with a model where activation of opsin involves (i) breaking of the salt bridge between Lys^{296} and Glu^{113}, (ii) deprotonation of Lys^{296}, and (iii) the *net* uptake of a proton from the solvent. Furthermore, exogenous addition of the chromophore *all-trans*-retinal shifts the wild-type and E113Q opsin equilibrium to favor the active state. In all these respects, the light-independent activation of the opsin mutants appears to proceed by a mechanism similar to that of light-activated rhodopsin.

The visual pigment rhodopsin is a member of the large family of G protein-linked sensory, neurotransmitter, and hormone receptors that include the adrenergic, cholinergic, and olfactory receptors (Dohlman et al., 1987, 1991; Gilman, 1987; Birnbaumer et al., 1990; Strosberg, 1991; Buck & Axel, 1991; Khorana, 1992; Oprian, 1992; Hargrave & McDowell, 1992; Nathans, 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; Findlay & Pappin, 1986). The pK_a of the Schiff base nitrogen in rhodopsin is abnormally high, such that the protein denatures at pH 11–12 before the proton is lost from the chromophore (Radding & Wald, 1956). Stabilization of the protonated Schiff base is brought about by interaction with the negatively charged side chain of Glu^{113}, the Schiff base counterion (Zhukovsky & Oprian, 1989; Sakmar et al., 1989; Nathans, 1990). Mutation of Glu^{113} to Gln dramatically lowers the pK_a of the Schiff base nitrogen to 6 (Zhukovsky & Oprian, 1989; Sakmar et al., 1989), which is about the pK_a expected for a Schiff base of retinal free in solution (Baasov & Sheves, 1986).

A central question in rhodopsin chemistry is the mechanism by which light isomerization of the bound chromophore, to the *all-trans* form, activates rhodopsin. This isomerization initiates a sequence of changes in the protein that results in a conformation, that of the spectrally defined intermediate metarhodopsin II (MII)[1] (Matthews et al., 1963; Wald, 1968), which is capable of activating the G protein transducin (Emeis et al., 1982). In the active MII species the Schiff base nitrogen is not protonated (Matthews et al., 1963; Doukas et al., 1978). Rando and co-workers (Longstaff et al., 1986) have shown that deprotonation of the nitrogen is required for rhodopsin to achieve the active MII conformation. Presumably, the light-induced isomerization of the chromophore moves the protonated Schiff base nitrogen away from the Glu^{113} counterion, breaking the salt bridge between the two residues. Loss of the counterion leads to deprotonation of the nitrogen and allows the protein to shift to the active state.

Although the transition to the active MII intermediate involves the loss of a proton from the Schiff base nitrogen, MII formation is favored at low pH (Matthews et al., 1963; Bennett, 1978, 1980; Parkes & Liebman, 1984; Hofmann, 1986). Matthews et al. (1963) concluded from this apparent inconsistency that two other groups on the protein must undergo protonation upon formation of MII. Therefore, MII formation involves the *net* uptake of a proton from the solvent. MII forms transiently, and its decay is followed by hydrolysis of the Schiff base linkage and dissociation of *all-trans*-retinal from the protein. The resulting apoprotein, opsin, is inactive until once again it binds a molecule of 11-*cis*-retinal and is activated by light.

Recent mutagenesis studies from this laboratory have shown that mutation of either Lys^{296} or Glu^{113} in the apoprotein opsin results in constitutive activation of the protein (Robinson et al., 1992). That is, these mutants are fully active in the absence of added chromophore. These data indicate that the salt bridge between Glu^{113} and Lys^{296} is a dominant feature of the inactive state, and activation of either rhodopsin or opsin requires breaking of this salt bridge. We show here that pH–rate profile analysis of the constitutively active opsin mutants is consistent with the notion that activation of opsin involves (1) breaking the salt bridge between Lys^{296} and Glu^{113}, (2) moving the Lys^{296} residue to a hydrophobic pocket, where

† Supported by NIH Grant EY07965. G.B.C. was a Gillette fellow and was supported by Training Grant 5T32 GM0796. P.R.R. was a Bunting Science Scholar and was supported by O.N.R. Grant 01-7305/2 awarded to the Mary Ingraham Bunting Institute of Radcliffe College.

* To whom correspondence should be addressed.

‡ The order of authorship is alphabetical; all authors have contributed equally.

[1] Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TAPS, 3-((tris(hydroxymethyl)methyl)amino)propanesulfonic acid; GTPgS, guanosine 5′-*O*-(3-thiotriphosphate); PBS, phosphate-buffered saline; MI, metarhodopsin I; MII, metarhodopsin II; the Schiff base complex of 11-*cis*-retinal and *n*-propylamine is designated nPrSB; 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.

0006-2960/92/0431-12592$03.00/0

it deprotonates, and (3) protonation of at least two other residues on opsin. We further demonstrate that activation of wild-type opsin is promoted by the addition of *all-trans*-retinal in the dark. In all these respects, the mechanism of activation of the opsin mutants is highly reminiscent of the mechanism of activation of rhodopsin by light.

EXPERIMENTAL PROCEDURES

Materials. 11-*cis*-Retinal was the generous gift of Peter Sorter and Hoffman-LaRoche (Nutley, NJ). Dodecyl β-D-maltoside was from Calbiochem (La Jolla, CA). DE-52, BSA (98–99%, essentially fatty acid free), and the buffers MES, HEPES, TAPS, and Tris were from Sigma. Bovine retina were obtained from J. A. Lawson Co. (Lincoln, NE). The monoclonal antibody rho 1D4, which is specific for the carboxyl terminus of rhodopsin, has been described previously (Molday & MacKenzie, 1983). [^{35}S]GTPgS (1156 Ci/mmol) was from NEN, and nonradiolabeled GTPgS (tetralithium salt) was from Boehringer Mannheim.

Mutagenesis of Rhodopsin Gene and Expression of Mutants. All procedures for DNA manipulation, mutation of the rhodopsin gene, DNA sequence analysis, and transfection and expression of the rhodopsin gene in the COS cell system were performed as previously described (Ferretti et al., 1986; Oprian et al., 1987, 1991; Zhukovsky & Oprian, 1989; Zhukovsky et al., 1991; Robinson et al., 1992).

Preparation of COS Cell Membranes. Membranes from COS cells were harvested from 10 100-mm culture plates 72 h after transfection with DNA as previously described (Robinson et al., 1992).

Preparation of nPrSB. nPrSB, the Schiff base complex of 11-*cis*-retinal with *n*-propylamine, was prepared in ethanol solution as has been described (Zhukovsky et al., 1991). The Schiff base complex of *all-trans*-retinal and *n*-propylamine was prepared in an identical manner to that of nPrSB.

Purification of H211F. The mutant H211F was purified from dodecyl maltoside-solubilized COS cells by an immunoaffinity procedure using the monoclonal antibody rho 1D4 (Molday & MacKenzie, 1983) as has been described (Oprian et al., 1987), except that the protein was eluted from the immunoaffinity matrix in a solution containing 0.1% (w/v) dodecyl maltoside instead of CHAPS.

Absorption Spectra. UV/visible absorption spectra were recorded using a Hitachi Model U-3210 spectrophotometer that was modified by the manufacturer for use in a dark room. Data were acquired with the aid of an Everex System 1700 microcomputer using Spectra Calc software from Galactic Industries Corp. (Salem, NH). Spectra were recorded on samples of 1.0-cm path length in thermostated cell holders with the temperature maintained at 4 °C by a Lauda RM6 constant temperature water circulator.

Purification of Transducin. Transducin was purified from retina according to the procedure of Wessling-Resnick and Johnson (1987) and then subjected to ion-exchange chromatography on DE-52 as described by Baehr et al. (1982). The protein preparations were routinely monitored for purity by gel electrophoresis and for contamination with rhodopsin by Western blot analysis with the rho 1D4 monoclonal antibody (Robinson et al., 1992).

Assay for Activation of Transducin. The rhodopsin mutants were assayed for their ability to catalytically activate transducin by following the binding of [^{35}S]GTPgS as has been described previously (Zhukovsky et al., 1991). Unless noted otherwise, the assays were conducted with rhodopsin (or opsin mutants) in COS cell membranes instead of in detergent-solubilized solution. There are two reasons for this. First, opsin, the apoprotein form of rhodopsin, is relatively unstable and does not survive purification in detergent. Second, all of the proteins are more resistant to the extremes of pH used in this study when embedded in membranes. For light-dependent reactions, membranes were preincubated in a solution of 10 mM Tris, pH 7.5, containing 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM EDTA, and chromophore at the concentrations indicated. The suspension was incubated in the dark for 1 h at 4 °C and then diluted 10-fold into the final reaction mixture.

Unless noted otherwise, the reaction mixtures contained 5 nM rhodopsin in 20 mM indicated buffer (see below), 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM EDTA, 1 mM DTT, 2.5 μM transducin, and 3 μM [^{35}S]GTPgS (5 Ci/mmol). NaCl was used to keep the ionic strength constant and varied in concentration from 165 to 185 mM. The reactions were initiated by addition of GTPgS. The temperature was 24–26 °C. Under these conditions, the reaction rate was directly proportional to rhodopsin concentration.

Buffers Used for the Transducin Assays. Several different buffers were used to cover the entire pH range from 4.9 to 9.5. MES (pK_a 6.1) was used for pH 4.9 to 6.7, HEPES (pK_a 7.5) was used for pH 6.7 to 8.1, and TAPS (pK_a 8.4) was used for 8.1 to 9.5. In initial experiments, the pH range of the buffers was overlapped to ensure that the activity did not vary with a change in the buffer component.

Simulation of the pH–Rate Profile Data. We simulated the pH–rate profile data in Figures 2–6 and 12B with curves derived as follows: We assumed that the uptake of a proton (with ionization constant K_{a_2}) was necessary to activate the opsin mutants as well as light-activated rhodopsin. However, further lowering of the pH led to the uptake of another proton (with ionization constant K_{a_1}) which inactivated opsin or light-activated rhodopsin. Thus, of the four possible species: R, R·H1, R·H2, and R·H1·H2 (where R is rhodopsin and R·H1 indicates that site 1 on rhodopsin is protonated, etc.), only R·H2 is active. We further assumed that the ionization of the groups responsible for K_{a_1} and K_{a_2} are independent. Therefore, at saturating levels of transducin, the observed velocity equals V_{max} times the fraction of the protein that is both deprotonated at site 1 and protonated at site 2. This is expressed mathematically as

$$\text{rate} = V_{max}[H/(H + K_{a_2})][K_{a_1}/(H + K_{a_1})] + T \quad (1)$$

where H is the proton concentration and the expression $H/(H + K_{a_2})$ is the fraction of the protein that is protonated at site 2 at proton concentration H. Similarly, the expression $K_{a_1}/(H + K_{a_1})$ is the fraction of the protein deprotonated at site 1. The term T has been added to account for the pH-dependent activation of the transducin that occurs in the absence of opsin (see Figure 2). The values that T assumes as the pH is varied are shown in the relevant curve in Figure 2. All of the simulated curves shown in Figures 2–6 and 12B were derived from eq 1 with the value of pK_{a_1} fixed at 5.9. The only variables that did vary among the mutants were K_{a_2} and V_{max}. The amount we had to vary V_{max} to fit the simulated data to the experimental data was small (it varied by ±20% among the different mutants). As $V_{max} = (k_{cat})(R_T)$, where R_T is the total concentration of opsin or light-activated rhodopsin, this variation is within the experimental error of our method of quantifying the concentration of protein (Western blot analysis). Therefore, *the primary effect of the constitutively active mutants, is on the ionization constants* K_{a_2}.

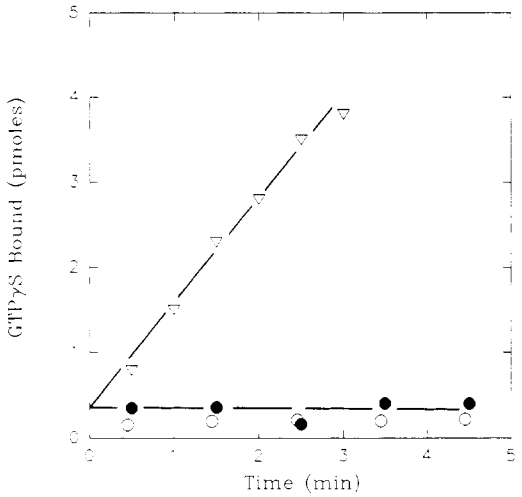


FIGURE 1: Transducin activation by K296H in the absence of chromophore. Transducin activity was assayed as described in the Experimental Procedures section. Open triangles: time course for the reaction catalyzed by K296H in the absence of added chromophore (pH 6.4). Open circles: time course for the reaction catalyzed by wild-type opsin. Closed circles: time course for the reaction catalyzed by membranes from cells that had been transfected with the expression vector not containing the rhodopsin gene.

RESULTS

We have recently shown that mutation of either Lys^{296} or Glu^{113} results in the constitutive activation of opsin as assayed by its ability to activate the rod G protein transducin. The mutants E113Q, K296G, K296A, and K296E were shown to be active in the absence of added chromophore and in the absence of light (Robinson et al., 1992). As shown in Figure 1, the mutant K296H is also constitutively active. Control membranes containing wild-type opsin or membranes prepared from COS cells transfected with the expression vector alone display no activity under similar conditions.

Dependence of Activity on pH. To further characterize these mutants, the dependence of activity on pH was examined. The activity of light-activated wild-type rhodopsin shows a bell-shaped dependence on pH (Figure 2). The two apparent pK_as will be referred to as pK_{a_1} and pK_{a_2}. pK_{a_1} and pK_{a_2} were determined by simulating the experimental data with the equation derived in the Experimental Procedures section (eq 1). For light-activated rhodopsin $pK_{a_1} = 5.9$ and $pK_{a_2} = 9.0$ (Figure 2).

In control experiments, pH–rate profiles for wild-type opsin (no chromophore added), for rhodopsin in the dark, for COS cell membranes containing no opsin, and for transducin alone (no membranes) were identical. These profiles showed some activity below pH 6, but none at higher pH values, as is illustrated for the reaction with transducin alone in Figure 2. Therefore, the activity at low pH does not appear to be from rhodopsin, but represents rather the activation of transducin by acidic pH. Almost no activity was detected at any pH in the absence of transducin whether or not membranes were present, or whether or not the membranes contained rhodopsin (not shown). In summary, wild-type opsin does not appear to show any ability to activate transducin in the pH range 5–10.

The pH–rate profile for the activation of transducin in the dark by wild-type opsin in the presence of *all-trans*-retinal is also shown in figure 2. Clearly, *all-trans*-retinal is able to activate wild-type opsin, although not at the same level as light-activated rhodopsin under these conditions.

The constitutively active mutant K296G, assayed in the absence of chromophore, shows a bell-shaped pH–rate profile

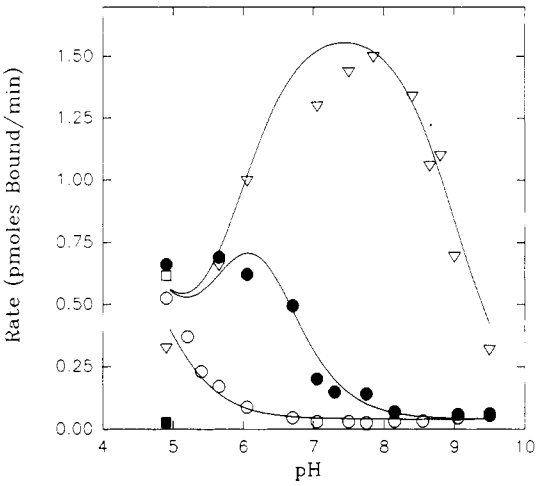


FIGURE 2: pH dependence of transducin activation by wild-type rhodopsin. Open triangles: pH–rate profile for reaction with light-activated wild-type rhodopsin. Closed circles: pH–rate profile for wild-type opsin preincubated with 100 μM *all-trans*-retinal and assayed in the dark. Open circles: pH–rate profile for transducin alone (no membranes added). Square symbols: control experiment showing that in the absence of membranes the pH-dependent activation of transducin is reversible. Closed square: transducin preincubated for 3 min at pH 4.9 and then brought to pH 7.6 for assay. Open square: transducin preincubated at pH 4.9 and assayed at pH 4.9. Rates were determined from the initial linear portion of each reaction time course. The solid lines were simulated as described in the Experimental Procedures section. pK_{a_2} for light-activated rhodopsin and opsin/*all-trans* retinal is 9.0 and 6.4, respectively.

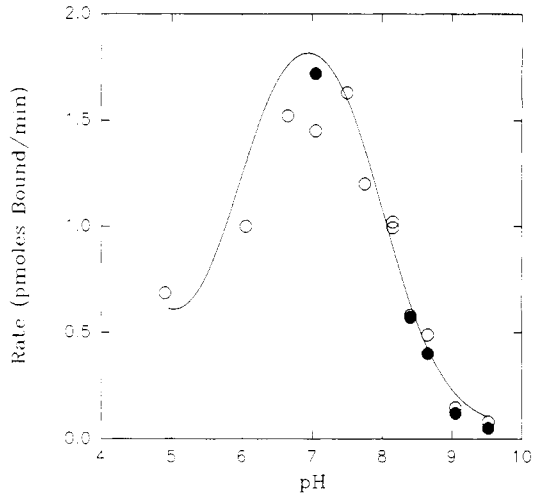


FIGURE 3: pH–rate profile for transducin activation by K296G. Open circles: pH–rate profile for K296G in the absence of added chromophore. Closed circles: pH–rate profile for K296G preincubated with 100 μM nPrSB and assayed after exposure to light. The solid line was simulated as described in the Experimental Procedures section. pK_{a_2} is 8.0.

(Figure 3) similar to that of light-activated wild-type rhodopsin except that pK_{a_2} is slightly shifted ($pK_{a_2} = 8.0$). When K296G is regenerated with the chromophore nPrSB (Zhukovsky et al., 1991) and then activated by light, the pH–rate profile does not appear to differ from that of K296G in the absence of chromophore (Figure 3). A possible explanation for the fact that these two reactions have similar pH–rate profiles is that at higher pH the noncovalently bound nPrSB chromophore spontaneously dissociates from the protein prior to illumination. However, in control experiments, K296G showed no activity at pH 7.1 or 8.7 when incubated with nPrSB and kept in the dark (not shown). Theses controls suggest that the noncovalently held nPrSB chromophore remains bound to the protein in the dark as the pH is varied, but upon light

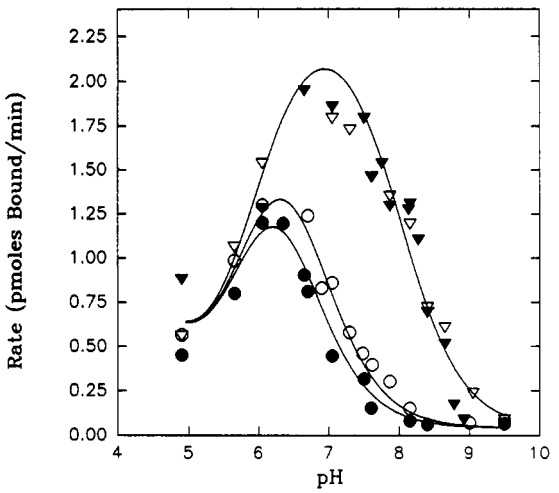


FIGURE 4: pH–rate profiles for opsin mutants in the absence of added chromophore. Symbols: closed circles, K296H; open circles, E113Q; closed triangles, K296G; open triangles, K296E. The pH–rate profile for the double mutant E113Q,K296G was superimposable on the profile for the single mutant K296G (not shown). The solid lines were simulated as described in the Experimental Procedures section. pK_{a_2} for K296H, E113Q, K296G, and K296E is 6.6, 6.8, 8.0, and 8.0, respectively.

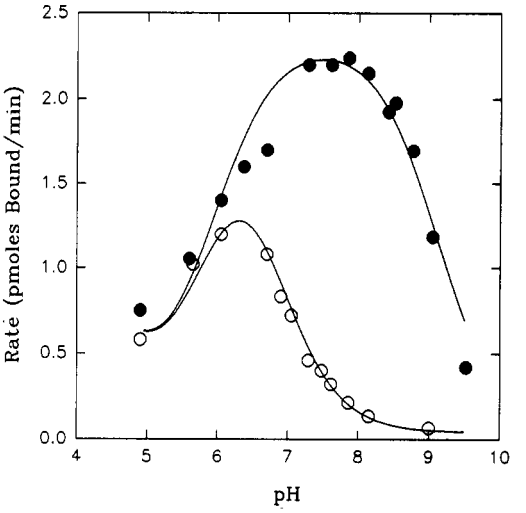


FIGURE 5: Comparison of the pH–rate profiles for E113Q without chromophore and with *all-trans*-retinal in the dark. Symbols: open circles, E113Q; closed circles, E113Q preincubated with 100 μM *all-trans*-retinal and assayed in the dark. The solid lines were simulated as described in the Experimental Procedures section. pK_{a_2} for E113Q with and without *all-trans*-retinal is 6.8 and 9.1, respectively.

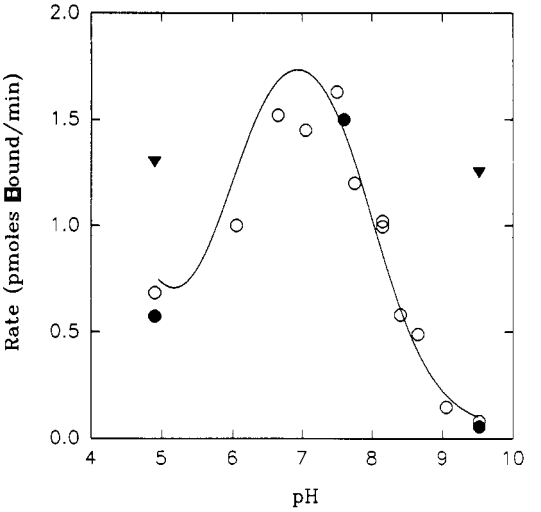


FIGURE 6: pH–rate profile of K296G showing that the change in activity with pH is reversible. Symbols: open circles, pH–rate profile for K296G in the absence of added chromophore; closed symbols, transducin and K296G were preincubated together at the pH indicated on the graph (4.9, 7.6, or 9.5) and then assayed at the same pH (closed circles) or after changing the pH to 7.6 (closed triangles). The solid lines were simulated as described in the Experimental Procedures section. pK_{a_2} is 8.0.

activation, the *all-trans* form of the chromophore quickly dissociates from the K296G mutant. The presence of *all-trans*-retinal in the active state of light-activated rhodopsin (MII) may account for the fact that pK_{a_2} in the wild-type protein is higher than in K296G.

The pH–rate profiles for the mutants K296E, K296G, E113Q, and K296H are shown in Figure 4. Although the bell-shaped profile is maintained in each mutant, it is clear that the amino acid substitutions have a significant effect on the position of maximum activity. This effect appears to result primarily from a change in pK_{a_2}, with little or no change evident in pK_{a_1}. pK_{a_1} and pK_{a_2} were determined by simulating the experimental data with the equation derived in the Experimental Procedures section (eq 1). Both pK_{a_1} and pK_{a_2} represent the uptake of a single proton from the solvent. The same equation (eq 1) was used to fit all the pH–rate profiles in this paper. The sole effect of the opsin mutations discussed in this paper appears to be on the value of pK_{a_2}. The order of values for pK_{a_2} in the wild-type and mutant proteins is rhodopsin/light (9.0) > K296E = K296G (8.0) > E113Q (6.8) > K296H (6.6) > opsin/*all-trans*-retinal/dark (6.4) ≫ opsin (pK_{a_2} for wild-type opsin is estimated to be less than 5 as it does not show any ability to activate transducin). The double mutant E113Q,K296G was also examined and found to have a pH–rate profile which was essentially indistinguishable from that of K296G (not shown).

The pH–rate profile for activation of the mutant E113Q by exogenously added *all-trans*-retinal in the dark is shown in Figure 5. The ability of *all-trans*-retinal to activate the mutant E113Q in the dark was first reported by Sakmar et al. (1989), where the reaction was performed in detergent solution at pH 7.2. As shown in Figure 5, the reaction shows the characteristic bell-shaped pH–rate profile with an apparent pK_{a_2} of 9.1. The maximal activity of E113Q in the presence of *all-trans*-retinal is almost twice that of E113Q in the absence of chromophore (see Discussion).

Characterization of pH-Dependent Change in Activity. A series of experiments were performed to determined if the pH-dependent changes were (1) reversible, (2) affecting V_{max} or the K_m for transducin, or (3) linked to the guanine nucleotide binding and dissociation steps. That the pH-dependent changes are reversible is demonstrated in Figure 6. When K296G and transducin were preincubated at pH 4.9 or 9.5 and then returned to pH 7.6 and assayed, most of the activity was recovered. There is a small amount of denaturation of the protein at the extremes of pH, but this cannot account for the observed pH–rate profiles.

As is shown in Figure 7, transducin activation with K296G displays a sigmoidal dependence on transducin concentration which is typical of the wild-type protein (Wessling-Resnick & Johnson, 1987; Nakayama & Khorana, 1990). To consider the possibility that the decrease in activity with pH on the basic side of the maximum (pK_{a_2}) might result from a change in the K_m for transducin with pH, we determined the dependence of activity on transducin concentration for K296H at the maximum, pH 6.4, and at pH 6.9 and 7.3. As is shown in Figure 8, the K_m for this reaction does not appear to depend

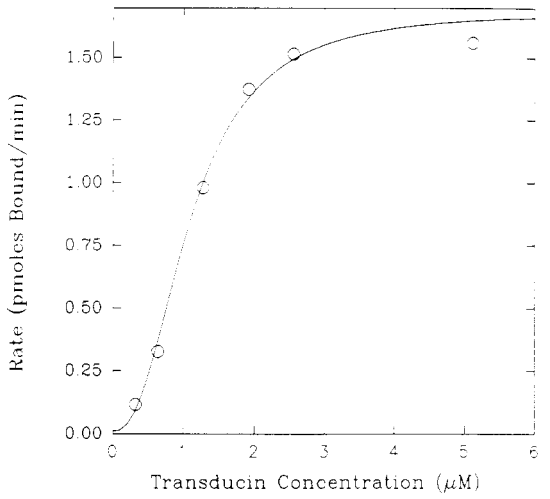


FIGURE 7: Dependence of reaction rate on transducin concentration for K296G without chromophore. Reaction was performed at different transducin concentrations as indicated. Rates were obtained from the initial linear portion of reaction time course. The solid line was simulated using eq 2 (see Results). V_{max}(Obs) is 1.7 pmol of GTPgS bound/min and K_m is 1.1 μM.

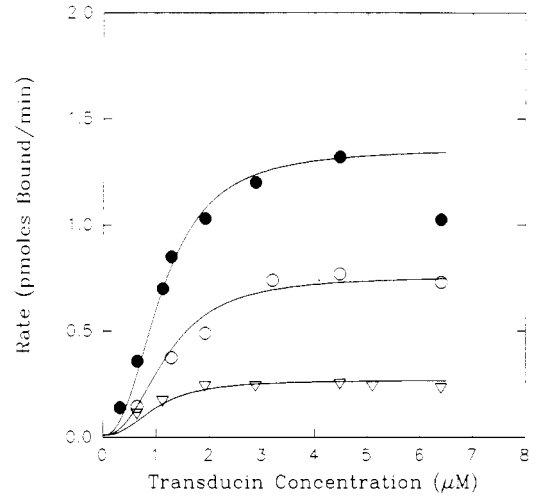


FIGURE 8: Dependence of V_{max}(Obs) and K_m on pH for reaction of K296H without chromophore. Activation of transducin by K296H was measured as a function of transducin concentration (0–6.5 μM) at three different pH values: solid circles, pH 6.4; open circles, pH 6.9; open triangles, pH 7.3. The solid lines were simulated using eq 2 (see Results). V_{max}(Obs) is 1.35, 0.75, and 0.25 pmol of GTPgS bound/min at pH values 6.4, 6.9, and 7.3, respectively. K_m is 1.1 ± 0.1 μM and did not vary with pH.

on pH, although the observed maximal velocity [V_{max}(Obs)] does.

The simulated curves in Figures 7 and 8 are based upon the following equation:

$$\text{rate} = [V_{max}(\text{Obs})](G_T)^n/[(K_m)^n + (G_T)^n] + c \quad (2)$$

where G_T is the concentration of transducin, K_m is the Michaelis constant for transducin, V_{max}(Obs) is the maximal velocity observed at saturating transducin concentrations (note that V_{max}(Obs) is pH-dependent and equals rate in eq 1), n is the Hill coefficient for transducin binding, and c is a correction term for the small amount of nonspecific binding of GTPgS to the membranes observed in the absence of transducin (ordinate intercept in Figures 7 and 8). We found that Hill coefficients between 2 and 3 gave reasonable fits to the data. The curves drawn in Figures 7 and 8 are based upon a Hill coefficient of 2.4. These results are in good agreement with those of Wessling-Resnick and Johnson (1987), who found

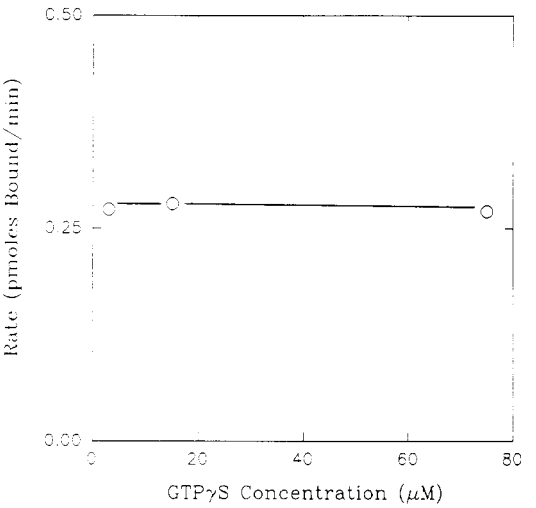


FIGURE 9: Dependence of reaction rate on GTPgS concentration. K296H was assayed at pH 7.3. The GTPgS concentration was varied from 3 to 75 μM. Increasing the concentration of GTPgS within this range did not alter the pH. The specific activity of GTPgS was kept constant throughout the experiment.

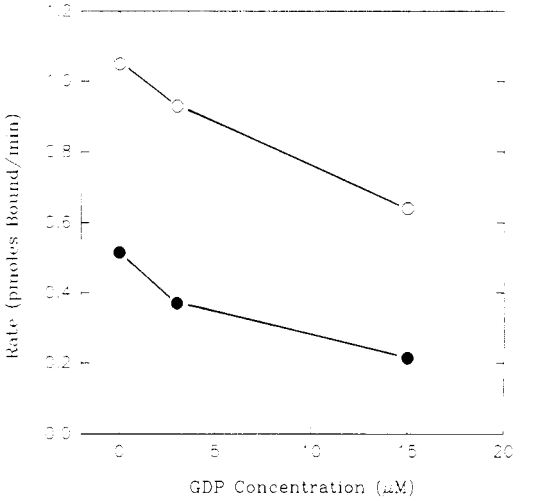


FIGURE 10: Inhibition of reaction rate by GDP. K296H was assayed at pH 6.4 (open circles) and pH 6.9 (closed circles) in the presence of 0, 3, and 15 μM added GDP.

evidence for cooperativity and a Hill coefficient of 2.2 for light-activated bovine rhodopsin. We also find that a K_m value of 1.1 ± 0.1 μM fits the experimental data. K_m does not vary significantly with pH (Figure 8), nor does it vary between mutants K296H and K296G (compare Figures 7 and 8).

The dependence of activity on pH also does not result from a change in the K_m for GTPgS as is shown for the reaction with K296H at pH 7.3 in Figure 9. The reaction velocity is independent of GTPgS concentration in the range of 3–75 μM.

Finally, we considered if the change in activity with pH might be a consequence of a differential change in affinity of transducin for GTPgS and GDP with pH. There are two sources of GDP in our assay mixture. The first is a small contaminant (<10%) in the commercial GTPgS preparations (maximum of 0.3 μM GDP in our reaction mixtures). The second is the transducin itself which releases a molecule of GDP for each molecule of GTPgS bound. The concentration of transducin is 2.5 μM in the assays, but under our experimental conditions the reaction is routinely quenched before the reaction is 15% complete. Therefore, transducin can contribute up to 0.4 μM GDP to the reaction mixture. To determine if the GDP could account for the change in activity

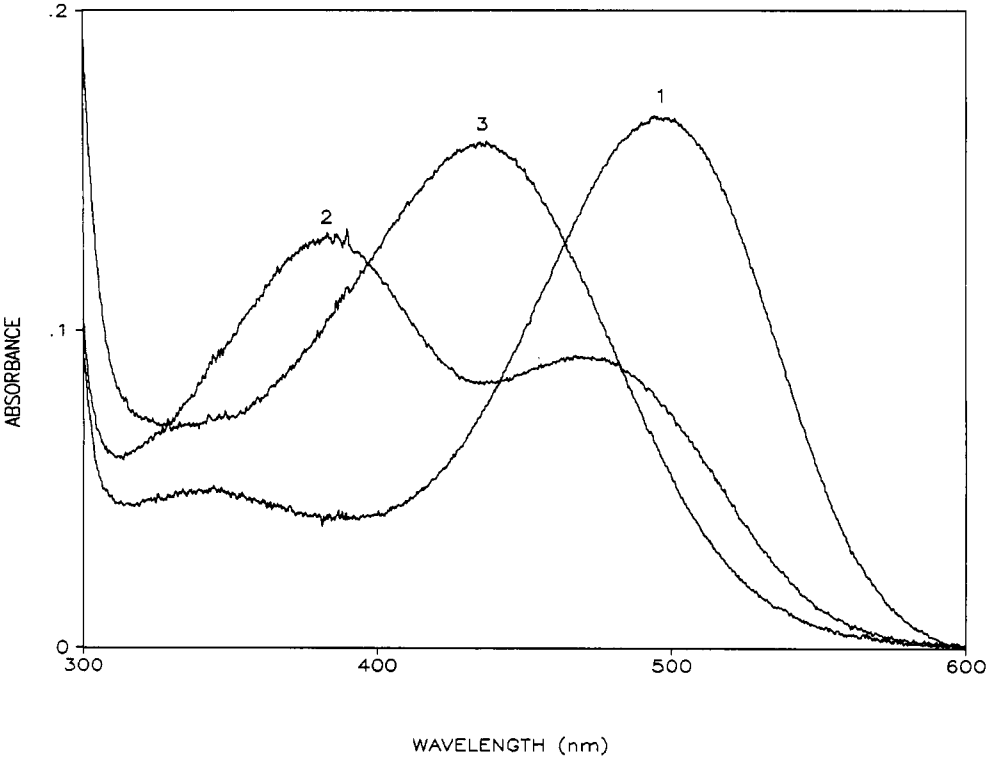


FIGURE 11: Absorption spectra of H211F. (1) Absorption spectrum for a sample of H211F purified from 30 plates of transfected COS cells. (2) Absorption spectrum for the photostationary state of H211F. The sample in spectrum 1 was exposed to light filtered through a Schott 515-nm cut-on filter for 30 s from a 300-W bulb. Subsequent exposure to light did not alter the spectrum. (3) Absorption spectrum 2 after addition of 1% (v/v) 12 N HCl to trap intermediates containing covalently bound chromophore.

with pH, the reaction with K296H was undertaken at pH 6.4 and 6.9 with concentrations of GDP up to 15 μM. As seen in Figure 10, the reaction at both pH values is inhibited by GDP. However, GDP inhibition does not seem to vary significantly with pH and cannot account for the difference in rates observed in the pH–rate profile of Figure 4. Therefore, a pH-dependent change in transducin's affinity for GDP and GTPgS cannot account for the observed pH-dependent change in activity.

His^{211} Is Not Essential for Activity. The spectrally defined intermediate, MII, is the active species for light-activated rhodopsin. In 1963, Wald and co-workers showed that MII is in a pH-dependent equilibrium with MI. The apparent pK_a for this equilibrium was determined to be 6.3. Because this pK_a is close to the pK_a of an imidazolium ion, they suggested that the titratable group was a His side chain to the protein. Recently, Weitz and Nathans (1992) concluded that His^{211} was the residue chiefly responsible for the pH-dependent equilibrium. This conclusion was reached on the basis of the observation that the mutant H211F did not form MII in response to lower pH. Additionally, the MI/MII equilibrium was unaffected by a synthetic peptide from transducin which is known to bind to MII (in wild-type rhodopsin) and shift the equilibrium away from MI (Hamm et al., 1989).

To determine if His^{211} is responsible for pK_{a_2}, we prepared two mutants: the single mutant H211F, and the double mutant H211F,K296G. As is shown in Figure 11, the purified 11-*cis*-retinal-reconstituted mutant H211F had an absorption spectrum similar to that of wild type, although the maximum was slightly shifted (495 nm). Upon exposure to light, a photostationary state was established consisting primarily of MII (380 nm) with a smaller amount of isorhodopsin and, presumably, MI (478 nm). Under identical conditions, wild-type rhodopsin was quantitatively converted to MII (not shown). That the 380-nm absorption was due to MII and not free *all-trans*-retinal was demonstrated by acid trapping of the protonated Schiff base (Figure 11). As shown in Figure 12A, purified H211F was fully able to activate transducin in a light-dependent manner. The activity was indistinguishable from that of wild-type rhodopsin. Further, the double mutant H211F,K296G was constitutively active, as would be expected of the K296G mutant if residue 211 was not essential for activity (Figure 12B). pK_{a_2} for light-activated H211F is shifted relative to the pK_{a_2} for light-activated wild-type rhodopsin (7.3 and 9.0, respectively). However, the characteristic bell-shaped curve is still evident (Figure 12B), indicating that the ionizable group titrating with apparent pK_{a_2} is still very much present. Furthermore, the experimental data can still be simulated with the same simple model (eq 1) that we used to fit all the previous pH–rate profiles. Therefore, His^{211} is not the group titrating with apparent pK_{a_2}, nor is His^{211} essential to the activity of rhodopsin.

DISCUSSION

Activation/Inactivation of Opsin. In a previous report, we have shown that mutations at position 296 and 113 result in the ability of opsin to constitutively activate transducin. That is, these mutants catalytically activate transducin in the absence of chromophore and in the absence of light (Robinson et al., 1992).

These data suggest that the presence of Lys^{296} is required to maintain the inactive state of native opsin. Since the most prominent feature of Lys^{296} is the positive charge associated with the ϵ-amino group, we presumed that loss of the charge results in a shift to the active conformation. The charge on Lys^{296} can be lost by mutation of either Lys^{296} or the Schiff base counterion Glu^{113}. Mutation of Glu^{113} substantially lowers the pK_a of the Schiff base nitrogen in rhodopsin, from an estimated pK_a of 11 or 12 in the wild-type (Radding &

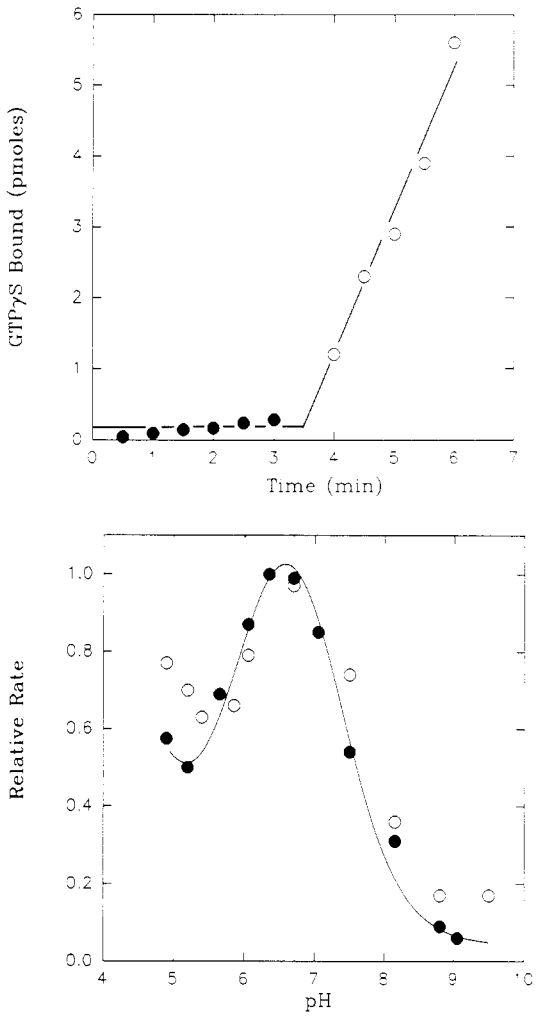


FIGURE 12: Activation of transducin by His^{211} mutants. (A, top) Light-dependent activation of transducin by purified H211F rhodopsin. The H211F sample used was the same as that for spectrum 1 in Figure 11: closed circles, time course for the reaction in the dark; open circles, time course for the reaction after exposure to light (at 3.5 min). The H211F concentration was 5 nM and the reaction was performed as described previously (Zhukovsky et al., 1991) except that the pH was 6.7. The turnover number for H211F was 36 pmol of GTPgS bound/(min·pmol of H211F). (B, bottom) pH–rate profile for H211F mutants. Membranes were used in these experiments. Closed circles: pH–rate profile for light-dependent activation of transducin by H211F. Membranes of H211F were preincubated with 40 μM 11-*cis*-retinal and assayed after exposure to light. Open circles: pH–rate profile for the double mutant H211F,K296G without chromophore. The pH–rate profiles have been normalized to facilitate the comparison. The solid lines were simulated as described in the Experimental Procedures section. pK_{a_2} for both H211F with 11-*cis*-retinal and H211F,K296G without chromophore is 7.3.

Wald, 1956) to 6 in the mutant (Sakmar et al., 1989; Zhukovsky & Oprian, 1989). Presumably, Glu^{113} has a similar effect on the ϵ-amino group of Lys^{296} in opsin. Mutation of Glu^{113} to Gln would lower the pK_a of the Lys side chain, and as a consequence, activate the protein.

The fact that mutation of either Lys^{296} or Glu^{113} both lead to constitutive activation of opsin suggests that the protein is held in an inactive conformation by a salt bridge formed between these two residues. However, it is evident from the bell-shaped pH–rate profiles for light-activated rhodopsin, and all of the constitutively active mutants including the double mutant E113Q,K296G, that there are at least two other ionizable groups (in addition to Lys^{296} and Glu^{113}) that exert control over the inactive and active states of the protein. The two apparent pK_as for these groups are referred to as pK_{a_1} and pK_{a_2}. pK_{a_1} appears to be relatively unaffected by the mutations, whereas pK_{a_2} is highly influenced by the nature of amino acid at position 113 or 296 (Figure 4). Therefore, we must account for two observations: first, mutation of either Glu^{113} or Lys^{296} leads to constitutive activation of opsin; second, pK_{a_2} for the various mutants follows the order K296E = K296G > E113Q > K296H $\gg$ opsin.

To account for these observations, we propose the following model. Opsin exists in two functionally distinct states, active and inactive. In the inactive state, Lys^{296} and Glu^{113} are located close to each other and form a salt bridge that stabilizes the charge on both residues [see Honig et al. (1979) and references therein for a discussion of the involvement of a salt bridge in the photochemical activation of rhodopsin]. Opsin is constrained to the inactive state by this salt bridge. To switch to the active state three changes must take place: (1) the salt bridge is broken, (2) the Lys moves to a more hydrophobic pocket, which is incapable of supporting the positive charge, and the proton is lost to some other group on the protein, and (3) another ionizable amino acid is exposed to solvent and becomes protonated. As the pH is lowered, protonation of this last residue drives the equilibrium to favor the active conformation. A key element of this model is that there are ionizable groups, in addition to Glu^{113} and Lys^{296}, that control activation of opsin. Protonation of these groups is thermodynamically linked to breaking the salt bridge and deprotonation of the Lys. Therefore, the apparent pK_a for this equilibrium (pK_{a_2}) will depend on (1) the ease of breaking the salt bridge; (2), the pK_a for Lys^{296}, and (3) the intrinsic pK_as of the other ionizable groups. For wild-type opsin, the energetic cost of breaking the salt bridge and deprotonating Lys^{296} is great, and wild-type opsin is not active at any pH tested ($pK_{a_2} < 5$).

The influence of the mutations is easily understood within the context of this model. Mutation of Glu^{113} or Lys^{296} will break the salt bridge and promote the transition to the active conformation. In the mutant K296G, the salt bridge is broken and the equilibrium favors the active state. It is only at high pH values that deprotonation of the other ionizable groups results in inactivation of opsin. Therefore, in K296G the apparent pK_{a_2} is high. Similarly, in K296E the salt bridge has been broken. The carboxylic acid side chain would be next to Glu^{113} in the inactive state and in a hydrophobic pocket in the active state. This residue may be protonated in both states and it would not contribute much to the energy difference between the active and inactive states outside of breaking the salt bridge. This would explain the fact that the apparent pK_{a_2} of K296E is the same as that of K296G.

In the mutant E113Q, the salt bridge is also broken. This will promote formation of the active state. However, Lys^{296} can still be protonated by recruiting an anion from the solvent (Zhukovsky & Oprian, 1989; Sakmar et al., 1989, 1991; Nathans, 1990). Therefore, the problem of deprotonating the positively charged Lys side-chain remains. The transition to the active state will be more difficult for this mutant than for K296G. Accordingly, pK_{a_2} for E113Q is lower than that for K296G, since it is only at low pH values that the driving force to the active state will be strong enough to overcome the energy needed to deprotonate the Lys (recall that the active state of opsin involves the net uptake of one proton from the solvent and is therefore favored at low pH).

Finally, in the mutant K296H, the His side chain can form a salt bridge with Glu^{113}; however, the His side chain has a much lower intrinsic pK_a than does Lys. It is therefore easier to deprotonate His than it is to deprotonate Lys. This accounts

for the apparent pK_{a_2} for K296H being higher than the pK_{a_2} for wild-type opsin but lower than the pK_{a_2} for K296G.

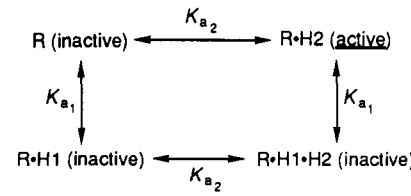
H211F. In this discussion, the groups ionizing with apparent pK_{a_2} are taking up protons at the same time that the positive charge on the 296 side chain is lost. This is reminiscent of the MI/MII equilibrium described by Wald and co-workers (Matthews et al., 1963) where the transition from MI to MII is accompanied by the net uptake of at least one proton from the solvent although the proton on the Schiff base is lost at the same time [see also Bennett (1978, 1980), Parkes and Liebman (1984), and Hofmann (1986)]. Since the MI/MII equilibrium determines the amount of rhodopsin in the active state (Emis et al., 1982), it is tempting to speculate that the same groups are responsible for both pK_{a_2} and the MI/MII equilibrium. Weitz and Nathans (1992) have recently proposed that the group chiefly responsible for the MI/MII equilibrium is His^{211}. We have shown here that His^{211} is not the residue titrating with apparent pK_{a_2}. Mutation of His^{211} clearly has an effect on pK_{a_2}, resulting in a more difficult transition to the active state for the protein. However, it is not the residue undergoing ionization. We found that at pH 6.7 the mutant H211F is as active as light-activated wild-type rhodopsin.

As obvious goal of future studies will be to identify the amino acids associated with pK_{a_2}. Although it seems likely that these are groups on opsin, it is possible that pK_{a_2} involves ionizable groups on transducin. This possibility deserves further consideration since transducin was shown here to be active (as defined by its ability to bind GTPgS) in the absence of rhodopsin at low pH values (Figure 2).

Activation of Opsin by all-trans-Retinal. We have also shown that opsin can be activated by incubation with *all-trans*-retinal in the dark. This has been reported previously for frog rhodopsin (Fukada et al., 1982) but has not been observed for bovine (Calhoon & Rando, 1985). We attribute the lack of activity with bovine opsin to the conditions under which the assays were conducted. As is shown in Figure 2, the pH optimum for this reaction is less than 6. Very little activity would be expected at pH 8, which was the pH used in the earlier studies (Calhoon & Rando, 1985). Recently, Hofmann et al. (1992) have shown that *all-trans*-retinal reacts with opsin to form a MII-like intermediate which interacts tightly with arrestin and rhodopsin kinase.

all-trans-Retinal also activates the mutant E113Q (Sakmar et al., 1989). pK_{a_2} for E113Q in the presence of *all-trans*-retinal is 9.1, while in the absence of chromophore it is 6.8 (Figure 5). These results are consistent with the model described above for the constitutively active mutants. *all-trans*-Retinal can form a Schiff base with Lys^{296}. In addition to any specific interactions between the all-trans isomer and the retinal binding pocket of the activated protein which may favor the active state, the Schiff base has a lower pK_a than the primary amine (by about 3–4 pH units) and, therefore, requires less energy to deprotonate. As a result, pK_{a_2} for E113Q, and also wild-type opsin, is shifted to higher pH upon addition of *all-trans*-retinal.

Simulation of the pH–Rate Profiles. We attempted to simulate the pH–rate profile data on the basis of a model where the ionization state of two independent groups controls the activity of opsin or light-activated rhodopsin. Protonation of group 2 activates the protein, while protonation of group 1 inactivates the protein. This is described by the following scheme:



where R is rhodopsin and R•H1 indicates that site 1 on rhodopsin is protonated, and so on. Only R•H2 was assumed to be active. A simple equation (eq 1) was then derived to simulate the pH–rate profile data (see Experimental Procedures).

The equilibrium described by K_{a_1} and K_{a_2} are likely to be linked to changes in the protein's conformation. Large conformational changes, like those associated with the MI/MII transition for light-activated rhodopsin, could conceivably alter the ionization state of many groups on the protein. In this case, the net number of protons taken up may be either greater or less than one. However, we found that the simple assumption that only one proton is taken up in the K_{a_1} and K_{a_2} transitions gave a substantially better fit to the data in Figures 2–6 and 12B than did an assumption of 2 or more protons. Furthermore, all of the simulated curves drawn in these figures are derived from eq 1 with the value of pK_{a_1} fixed at 5.9. The only variable in eq 1 that was found to vary significantly among the mutants was K_{a_2}.

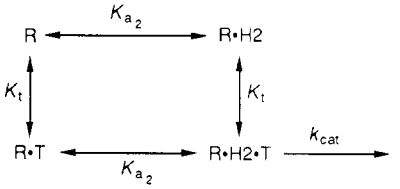
The simulated data explain why the maximal activity of E113Q opsin is only half that of E113Q in the presence of *all-trans*-retinal (Figure 5). Both of these assays were done using the same membrane preparation. The only difference between them is the addition of the chromophore. For E113Q opsin, pK_{a_1} is 5.9 and pK_{a_2} is 6.8. Since pK_{a_1} and pK_{a_2} are close in value, at pH values where site 2 is almost fully protonated, site 1 becomes partially protonated. Much of the protein will then be in the inactive R•H1•H2 state. Therefore, there is no pH where all of the E113Q opsin is in the active R•H2 state. Evaluation of eq 1, using these pK_a values, leads to a curve with maximal activity at pH 6.35. At this pH, only 55% of the protein will be in the active protonation state R•H2. Adding *all-trans*-retinal to E113Q shifts pK_{a_2} to 9.1. In this case, on the basis of eq 1, the maximal activity is at pH 7.5, where 95% of the protein is in the active R•H2 state. If k_{cat} for E113Q opsin is the same as that for E113Q with *all-trans*-retinal, then the maximal velocity for E113Q with *all-trans*-retinal is expected to be almost 75% greater (95%/55%) than that of E113Q opsin. This is what was actually found (Figure 5).

The above analysis can also be used to compare wild-type opsin, preincubated with *all-trans*-retinal, with light-activated wild-type rhodopsin (pK_{a_2} equals 6.4 and 9.0, respectively). Here, eq 1 predicts the light-activated rhodopsin will be more than two times as active at the optimum as opsin preincubated with *all-trans*-retinal. This agrees well with the experimental results (Figure 2).

The mutant K296G deserves further comment. If activation of opsin involves deprotonation of Lys^{296} and protonation of two other groups on the protein, then mutation of Lys^{296} to Gly might change the net number of protons picked up during the pK_{a_2} transition, since position 296 would no longer be losing a proton. Our curve fitting did not find this to be the case. pH–rate profiles for K296G in Figures 3, 4, and 6 were simulated best under the assumption that only one proton is taken up during the transition to the active state (as was the case for all the other mutants). It may be that Lys^{296} is in close proximity to one of the two groups that must be

protonated during the transition to the active state. Loss of the positive charge at position 296 may allow the group close to Lys[296] to become protonated in the inactive state. Activation of the K296G mutant would then depend solely on the titration of the second ionizable group.

Kinetic Model. The relation between MI/MII and the constitutively active mutants will be explored in future studies. Despite some obvious similarities, there are also important differences as shown in the following kinetic model which accounts well for our data with the constitutively active mutants:



where R is the inactive state, R·H2 is the active state, T is transducin, H is a proton, K_t is the dissociation constant for transducin, and K_{a_2} is the equilibrium constant for the active and inactive states. The salient features of this scheme are (1) the mutants affect the position of equilibrium between active and inactive states (K_{a_2}), (2) k_{cat} is the same for the different mutants, (3) K_t is the same for different mutants, and (4) K_t is independent of pH, that is, the inactive and active forms of the mutants bind transducin with about the same affinity. The fact that transducin does not affect the position of equilibrium between active and inactive states suggests that K_{a_2} may not be strictly equated with the MI/MII equilibrium, which is known to be affected by the transducin concentration (Emeis et al., 1982; Hofmann, 1986).

Does Opsin Activate Transducin under Physiological Conditions? Recently, the results of several laboratories have suggested that opsin is capable of activating transducin (Okada et al., 1989; Corson et al., 1990; Jin et al., 1992; Cornwall & Fain, 1992; Fain & Cornwall, 1993). Although we could not detect transducin activation by wild-type opsin under our assay conditions, such activity might be detectable under physiological conditions by electrophysiological techniques. The results presented here suggest a mechanism by which opsin becomes active. We estimate that pK_{a_2} for opsin will be between 3 and 4. This estimate is based upon the assumption that *all-trans*-retinal will alter the pK_{a_2} of wild-type opsin to the same degree as it does that of the mutant E113Q. For the mutant E113Q, the addition of *all-trans*-retinal shifts pK_{a_2} by 2.3 units (Figure 5). Since pK_{a_2} for wild-type opsin in the presence of *all-trans*-retinal is 6.4 (Figure 2), the pK_{a_2} for wild-type opsin is calculated to be 4.1.[2] As a consequence, under physiological conditions a small amount of wild-type opsin will be deprotonated at Lys[296] and, therefore, in the active state. At pH 7.5, one out of every 3000–30 000 opsin molecules would be in the active state. This level of activity would account for the persistent desensitization observed during bleaching adaptation in isolated rod cells (Corson et al., 1990).

It is also interesting to speculate that in dark-adapted rods the source of discrete-event noise is the activation of transducin by opsin rather than thermal isomerization of the chromophore (Baylor et al., 1980).

[2] An alternate estimate of pK_{a_2} for opsin may be obtained as follows. A lysine residue is 3–4 orders of magnitude more difficult to deprotonate than a His residue. Since pK_{a_2} for K296H is 6.6, the pK_{a_2} for opsin, which has lysine at position 296, will be around 3.

Therefore, it appears that the 11-*cis*-retinal chromophore in rhodopsin has two distinct functions. First, it binds to opsin and allows the molecule to absorb light. Second, the chromophore keeps the protein in an inactive conformation. The inactivation of opsin by 11-*cis*-retinal allows the photoreceptor cell to pack an extraordinary number of photopigments into the cell, creating a highly sensitively photodetector with little dark noise.

Conclusion. We have shown in this paper that kinetic and pH–rate profile analysis of the constitutively active opsin mutants is consistent with the idea that activation of the opsin mutants and activation of rhodopsin by light proceed by similar mechanisms. Both processes involve (1) breaking of the salt bridge between Lys[296] and Glu[113], (2) deprotonation of the residue at position 296, and (3) the net uptake of a proton from the solvent. Furthermore, the mutants and light-activated rhodopsin have similar k_{cat} values (30 turnovers/min) and K_m for transducin (1–2 μM). Finally, addition of the chromophore *all-trans*-retinal shifts the equilibrium for both wild-type opsin and E113Q to favor the active state. In all these respects, activation of the opsin mutants appears to proceed by a mechanism similar to that of light-activated rhodopsin.

ACKNOWLEDGMENT

We thank C. Miller and W. Jencks for reading the manuscript. We thank R. Franke for advice regarding preparation of COS cell membranes and J. Lisman, K. Palczweski, and G. Fain for helpful discussions.

REFERENCES

Baasov, T., & Sheves, M. (1986) *Biochemistry 25*, 5249–5258.
Baylor, D. A., Matthews, G., & Yau, K.-W. (1980) *J. Physiol. 309*, 591–621.
Baehr, W., Morita, E. A., Swanson, R. J., & Applebury, M. L. (1982) *J. Biol. Chem. 257*, 6452–6460.
Bennett, N. (1978) *Biochem. Biophys. Res. Commun. 83*, 457–465.
Bennett, N. (1980) *Eur. J. Biochem. 111*, 99–103.
Birnbaumer, L., Abramowitz, J., & Brown, A. M. (1990) *Biochim. Biophys. Acta 1031*, 163–224.
Bownds, D. (1967) *Nature 216*, 1178–1181.
Buck, L., & Axel, R. (1991) *Cell 65*, 175–187.
Calhoon, R. D., & Rando, R. R. (1985) *Biochemistry 24*, 6446–6452.
Cornwall, C. M., & Fain, G. L. (1992) *Invest. Ophthalmol. Vis. Sci. 33* (4), 2055.
Corson, D. W., Cornwall, M. C., MacNichol, E. F., Jin, J., Johnson, R., Derguini, F., Crouch, R. K., & Nakanishi, K. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 6823–6827.
Dohlman, H. G., Caron, R. J., & Lefkowitz, R. J. (1987) *Biochemistry 26*, 2657–2664.
Dohlman, H. G., Thorner, J., Caron, M. G., & Lefkowitz, R. J. (1991) *Annu. Rev. Biochem. 60*, 653–688.
Doukas, A. G., Aton, B., Callender, R. H., & Ebrey, T. G. (1978) *Biochemistry 17*, 2430–2435.
Dratz, E. A., & Hargrave, P. A. (1983) *Trends Biochem. Sci. 8*, 128–131.
Emeis, D., Kuhn, H., Reichert, J., & Hofmann, K. P. (1982) *FEBS Lett. 143*, 29–34.
Fain, G. L., & Cornwall, M. C. (1993) in *The Fifth Annual Retina Research Foundation Symposium, Contrast Sensitivity: from Receptors to Clinic* (Shapley, R., & Lam, D.-K., Eds.) MIT Press Cambridge, MA (in press).
Ferretti, L., Karnik, S. S., Khorana, H. G., Nassal, M., & Oprian, D. D. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 599–603.
Findlay, J. B. C., & Pappin, D. J. C. (1986) *Biochem. J. 238*, 625–642.

Fukada, Y., Yoshizawa, T., Ito, M., & Tsukida, K. (1982) *Biochim. Biophys. Acta 708*, 112–117.
Gilman, A. G. (1987) *Annu. Rev. Biochem. 56*, 615–649.
Hamm, H. E., Deretic, D., Arendt, A., Hargrave, P. A., Koenig, B., & Hofmann, K. P. (1989) *Science 241*, 832–835.
Hargrave, P. A., & McDowell, J. H. (1992) *FASEB J. 6*, 2323–2331.
Hofmann, K. P. (1986) *Photobiol. Photophys. 13*, 309–327.
Hofmann, K. P., Pulvermuller, A., Buczylko, J., Van Hooser, P., & Palczewski, K. (1992) *J. Biol. Chem. 267*, 15701–15706.
Honig, B., Ebrey, T., Callender, R. H., Dinur, U., & Ottolenghi, M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2503–2507.
Jin, J., Cornwall, M. C., Corson, D. W., Katz, B. M., & Crouch, R. K. (1992) *Invest. Ophthalmol. Vis. Sci. 33* (4), 2046.
Khorana, H. G. (1992) *J. Biol. Chem. 267*, 1–4.
Longstaff, C., Calhoon, R. D., & Rando, R. R. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4209–4213.
Matthews, R. G., Hubbard, R., Brown, P. K., & Wald, G. (1963) *J. Gen. Physiol. 47*, 215–240.
Molday, R. S., & MacKenzie, D. (1983) *Biochemistry 22*, 653–660.
Nakayama, T. A., & Khorana, H. G. (1990) *J. Biol. Chem. 265*, 15762–15769.
Nathans, J. (1990) *Biochemistry 29*, 9746–9752.
Nathans, J. (1992) *Biochemistry 31*, 4923–4931.
Okada, D., Nakai, T., & Ikai, A. (1989) *Photochem. Photobiol. 49*, 197–203.
Oprian, D. D. (1992) *J. Bioenerg. Biomembr. 24*, 211–217.
Oprian, D. D., Molday, R. S., Kaufman, R. J., & Khorana, H. G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8874–8878.
Oprian, D. D., Asenjo, A. B., Lee, N., & Pelletier, S. L. (1991) *Biochemistry 30*, 11367–11372.
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.
Parkes, J. H., & Liebman, P. A. (1984) *Biochemistry 23*, 5054–5061.
Radding, C. M., & Wald, G. (1956) *J. Gen. Physiol. 39*, 909–922.
Robinson, P. R., Cohen, G. B., & 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.
Sakmar, T. P., Franke, R. R., & Khorana, H. G. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3079–3083.
Strosberg, A. D. (1991) *Eur. J. Biochem. 196*, 1–10.
Wald, G. (1968) *Science 162*, 230–239.
Weitz, C. J., & Nathans, J. (1992) *Neuron 8*, 465–472.
Wessling-Resnick, M., & Johnson, G. L. (1987) *J. Biol. Chem. 262*, 3697–3705.
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.

Registry No. Glu, 56-86-0; Lys, 56-87-1; *all-trans*-retinal, 116-31-4.