Transducin Activation by Rhodopsin Without a Covalent Bond to the 11-*Cis*-Retinal Chromophore

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Rhodopsin and the visual pigments are a distinct group within the family of G-protein–linked receptors in that they have a covalently bound ligand, the 11-*cis*-retinal chromophore, whereas all of the other receptors bind their agonists through noncovalent interactions. The retinal chromophore in rhodopsin is bound by means of a protonated Schiff base linkage to the ε-amino group of Lys-296. Two rhodopsin mutants have been constructed, K296G and K296A, in which the covalent linkage to the chromophore is removed. Both mutants form a pigment with an absorption spectrum close to that of the wild type when reconstituted with the Schiff base of an *n*-alkylamine and 11-*cis*-retinal. In addition, the pigment formed from K296G and the *n*-propylamine Schiff base of 11-*cis*-retinal was found to activate transducin in a light-dependent manner, with 30 to 40% of the specific activity measured for the wild-type protein. It appears that the covalent bond is not essential for binding of the chromophore or for catalytic activation of transducin.

THE VISUAL PIGMENT RHODOPSIN IS a member of a large family of sensory, neurotransmitter, and hormone receptors that includes the β-adrenergic and muscarinic acetylcholine receptors (*1–3*). This family is defined by three salient features: (i) all are membrane receptors that enable a cell to communicate with its external environment; (ii) all share significant homology in amino acid sequence and inferred similarities in secondary and tertiary structure, most notably the presence of seven transmembrane segments; and (iii) all exhibit a common mechanism of action in that they bring about their intracellular biochemical effects through the activation of a guanosine triphosphate (GTP)–binding regulatory protein, or G-protein (*3, 4*).

Despite the structural and functional similarities that link these various proteins together as a family, rhodopsin and the other visual pigments are distinguished as a group from the hormone and neurotransmitter receptors by the fact that their ligand, the 11-*cis*-retinal chromophore, is bound to the protein by a covalent bond whereas the agonists for all of the other receptors are bound through noncovalent interactions. The 11-*cis*-retinal chromophore of rhodopsin is bound by means of a protonated Schiff base linkage with the ε-amino group of Lys-296, present in the seventh transmembrane segment of the protein (*1*). This difference is difficult to reconcile with the concept that the binding pocket for 11-*cis*-retinal in rhodopsin is an evolutionary variant of the binding pocket for agonists found in the other receptors (*5*). In this report we show by using site-directed mutagenesis that the covalent bond to the chromophore in rhodopsin is not essential for the formation of a pigment or the ability to activate transducin.

We prepared two mutants of rhodopsin in which Lys-296 was changed to either Ala or Gly. Neither mutant binds 11-*cis*-retinal as judged by the fact that the purified proteins showed no visible absorption spectra (*6*). However, both mutants readily bind chromophore when it is provided in the form of a Schiff base with an *n*-alkylamine (refer to Fig. 1 for a schematic representation of the chromophore binding pocket). As is shown in Fig. 2, the mutant K296A (7) binds EtSB to form a pigment with an absorption maximum at 493 nm (absorption maximum of wild type is 500 nm). K296A did not bind, however, the larger nPrSB chromophore. This suggests that the alkylamine chain of the chromophore binds to rhodopsin with little room to spare in the binding pocket. Four carbon atoms (the four methylene carbons of the Lys side chain) separate the α-carbon at Lys-296 from the Schiff base nitrogen in the native protein. Four carbons would also separate these two atoms if K296A bound nPrSB. Presumably the greater van der Waal's radii of two methyl groups relative to two methylenes preclude accommodation of the chromophore in the binding pocket of the mutant. Removal of one carbon atom from the alkylamine chain of the chromophore gives a slightly smaller ligand that binds effectively to the protein. Conversely, the chromophore binding pocket can be made larger by removing one carbon atom from the side chain of Ala-296. As is shown in Fig. 2, the mutant K296G binds the larger nPrSB chromophore as well as EtSB, but binds nBuSB, with a four-carbon chain, poorly. The spectral absorption maximum for K296G bound to either EtSB or nPrSB was 486 nm.

The absorption maxima for K296G and K296A are shifted toward the blue relative to the 500-nm maximum for the wild-type protein. This shift can be accounted for by proposing that the Schiff base nitrogen is less restricted in the noncovalently bound chromophore and can therefore approach more closely the Schiff base counterion Glu-113 (*8, 9*) than can the ε-amino nitrogen of Lys-296 in the native pigment (*10*).

The noncovalent mutant K296G with nPrSB chromophore is the most stable of the different species and was characterized further. The absorption coefficient of nPrSB-bound K296G was determined to be 32,300 M^{-1} cm^{-1} (*11*), which is substantially smaller than 42,700 M^{-1} cm^{-1} observed for the wild-type pigment (*12*). The mutant was found to be stable to hydroxylamine (10 mM) in the dark, but was bleached rapidly upon exposure to light, as is the case for native rhodopsin (*13*). Perhaps most significant is the fact that nPrSB-bound K296G catalyzes light-dependent GTPγS binding by transducin, as shown in Fig. 3. The mutant shows about 40% as much activity as native rhodopsin. The fact that the mutant was functional demonstrates that the covalent linkage is not essential for rhodopsin to catalyze light-dependent activation of transducin. Essentially identical results were obtained when activation of transducin was measured by following light-

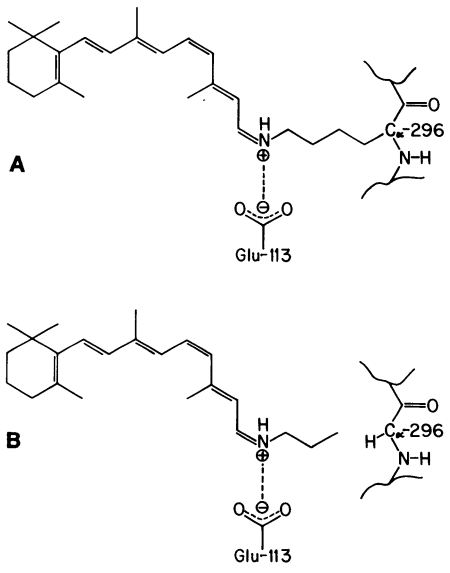

Fig. 1. Schematic diagram of the chromophore binding pocket in (**A**) rhodopsin and (**B**) the mutant K296G with nPrSB. The figure shows two amino acid residues: Lys-296 (Gly in the mutant), which is located in the seventh transmembrane segment and donates the Schiff base nitrogen atom in the wild-type protein, and Glu-113, which is located in the third transmembrane segment and has been shown to be the counterion to the protonated Schiff base (*8, 9*). The figure is drawn to emphasize the similarity between the wild-type and mutant binding pockets, particularly with regard to the aliphatic amine chain.

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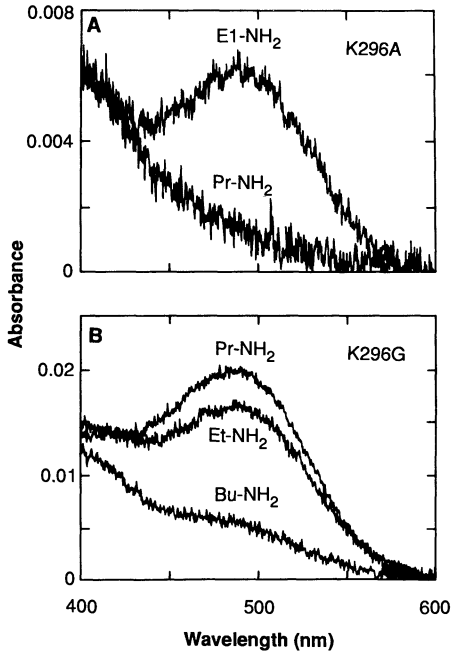

Fig. 2. Absorption spectra for (**A**) rhodopsin mutants K296A and (**B**) K296G that have been reconstituted with various Schiff bases of 11-*cis*-retinal. The experimental system used to produce and purify mutants of rhodopsin has been described (*8, 20–22*).

dependent GTP hydrolysis where the mutant was 30% as active as wild-type rhodopsin (8 and 28 pmol of GTP hydrolyzed per minute per picomole of rhodopsin, respectively).

The fact that no pigment was formed from either K296G or K296A when reconstituted with 11-*cis*-retinal, whereas both mutants readily formed long-wavelength absorbing pigments when reconstituted with the alkylamine Schiff base analogs of 11-*cis*-retinal suggests that the electrostatic interaction between the charged Schiff base nitrogen and the carboxylate group of Glu-113 contributes significantly to the stability of the noncovalent complex (Fig. 1). To test this, we prepared the double mutant E113Q,K296G in which the Schiff base counterion was removed, by changing Glu to Gln, and covalent bonding to the chromophore is not possible [the single mutant E113Q causes deprotonation of the Schiff base nitrogen, but does not prevent binding of the chromophore to the protein (*8, 9*)]. E113Q,K296G did not bind the nPrSB chromophore which is consistent with the hypothesis that electrostatic forces make a significant contribution to the overall binding energy (*15*). It is interesting that the E113Q,K296G mutant, which can be thought of as a counterion mutant of K296G, behaves similarly to the D113N counterion mutant in β_2-adrenergic receptor where the affinity for the antagonist propranolol is greatly reduced (*16*).

Although the results of the present study do not prove that the chromophore binding site in rhodopsin is a variant of the agonist binding site in the β-adrenergic receptor, they do undermine arguments for any necessary differences. Clearly a pigment with a long-wavelength absorption maximum can be formed in the absence of the covalent bond. This pigment is also capable of activating transducin in a light-dependent manner. Therefore, the covalent bond is not essential to the mechanism of action, and this fact should be reflected in models for activation of the protein. For example, rhodopsin has been shown to store light energy in a step that is essential to activation (*17*). This is thought to occur by a charge-separation mechanism involving the protonated Schiff base nitrogen and the glutamate counterion (*18*). If this is the case, the two charges must separate without the assistance of a covalent bond linking the nitrogen to the seventh transmembrane segment.

What then is the role of the covalent bond? Presumably one function is to ensure maximal occupancy of the chromophore binding site so that the protein is continuously poised for absorption of light. Once a photon is absorbed, activation of rhodopsin proceeds by a thermally irreversible process (*19*). In contrast, the neurotransmitter receptors must respond rapidly to changes in the concentration of the ligand itself, a process that would be hindered by a covalent bond.

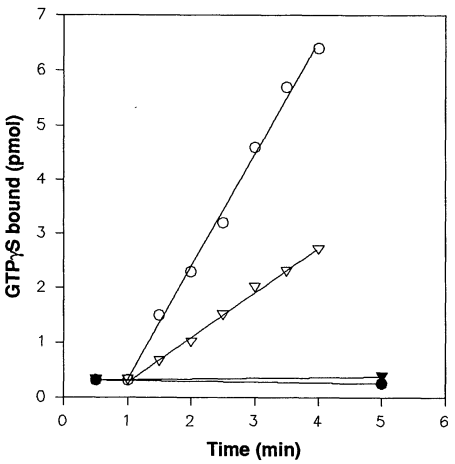

Fig. 3. Light-dependent activation of transducin by wild-type rhodopsin and the mutant K296G containing the nPrSB chromophore. Triangles, time course for the reaction catalyzed by nPrSB-bound K296G (4.8 nM). Circles, time course for the reaction catalyzed by wild-type rhodopsin (5 nM) purified from transfected COS cells. Transducin activity was assayed by measuring the binding of ^{35}S-labeled GTPγS (*14*). Closed symbols are data for the dark reaction, open symbols are data for the light reaction. The light reactions were initiated after 1-min incubation in the dark. The specific activities determined for wild-type rhodopsin and the K296G mutant were 41 and 18 pmol of GTPγS bound per minute per picomole of rhodopsin, respectively.

REFERENCES AND NOTES

1. E. A. Dratz and P. A. Hargrave, *Trends Biochem. Sci.* **8**, 128 (1983); M. L. Applebury and P. A. Hargrave, *Vision Res.* **26**, 1881 (1986); J. B. C. Findlay and D. J. C. Pappin, *Biochem. J.* **238**, 625 (1986).
2. H. G. Dohlman, M. G. Caron, R. J. Lefkowitz, *Biochemistry* **26**, 2657 (1987).
3. G. L. Johnson and N. Dhanasekaran, *Endocrine Rev.* **10**, 317 (1989).
4. A. G. Gilman, *Annu. Rev. Biochem.* **56**, 615 (1987).
5. It may be more appropriate to compare the binding site for 11-*cis*-retinal with that for an antagonist rather than an agonist since rhodopsin is not active in the 11-*cis*-bound form. However, the binding sites for agonists and antagonists overlap and no confusion should be introduced by ignoring this point in the present context.
6. Due to the fact that the proteins are purified before their absorption spectrum is recorded, a mutant with greatly reduced affinity for chromophore would be grouped with those that do not bind at all.
7. 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. The following abbreviations are used to designate the Schiff bases of ethylamine, *n*-propylamine, and *n*-butylamine, respectively: EtSB, nPrSB, and nBuSB.
8. E. A. Zhukovsky and D. D. Oprian, *Science* **246**, 928 (1989).
9. T. P. Sakmar, R. R. Franke, H. G. Khorana, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 8309 (1989).
10. P. E. Blatz, J. H. Mohler, H. V. Navangul, *Biochemistry* **11**, 848 (1972); B. Honig, A. D. Greenberg, U. Dinur, T. G. Ebrey, *ibid.* **15**, 4593 (1976). The closer that the counterion and protonated Schiff base come to one another, the more difficult is delocalization of the positive charge over the polyene chain to achieve the photoexcited state.
11. The absorption coefficient was determined by acid denaturation of the pigment as described by R. A. Morton and G. A. J. Pitt [*Biochem. J.* **59**, 128 (1955)] and Sakmar *et al.* (*9*).
12. K. Hong and W. L. Hubbell, *Biochemistry* **12**, 4517 (1973).
13. Rhodopsin mutants in which the counterion Glu-113 is changed to a neutral amino acid react with hydroxylamine in the dark (*8, 9*).
14. The activity of rhodopsin was determined by following the light-dependent binding of [^{35}S]GTPγS essentially as described by M. Wessling-Resnick and G. Johnson, *J. Biol. Chem* **262**, 3697 (1987). Transducin concentration was 1.0 μM, [^{35}S]GTPγS (2.1×10^{12} cpm/mmol) was 3 μM, and the detergent dodecylmaltoside was present at 0.01%. The data were collected from 10-μl aliquots of the reaction mixture. Activity was also followed by measuring the light-dependent activation of GTP hydrolysis (*21*) using [γ-^{32}P]GTP under the same assay conditions as described above for the binding reaction.
15. These data suggest that electrostatic forces are involved in binding of the chromophore to the protein. They do not bear on whether or not Glu-113 is directly involved in any of these interactions.
16. C. D. Strader *et al.*, *J. Biol. Chem.* **263**, 10267 (1988). We note that bacteriorhodopsin has been shown by T. Schreckenbach *et al.* [*Eur. J. Biochem.* **76**, 499 (1977)] to bind noncovalently an all-*trans*-retinol chromophore to give a three-peaked absorption spectrum centered at about 360 nm, although this species was not shown to be active.
17. A. Cooper, *Nature* **282**, 531 (1979).
18. B. Honig, T. Ebrey, R. H. Callender, U. Dinur, M. Ottolenghi, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2503 (1979).
19. G. Wald, *Science* **162**, 230 (1968).
20. L. Ferretti *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 599 (1986).

21. D. D. Oprian, R. S. Molday, R. J. Kaufman, H. G. Khorana, *ibid.* **84**, 8874.
22. Reconstitution of the mutant rhodopsins with the Schiff bases of 11-*cis*-retinal was performed essentially as has been described for the normal chromophore 11-*cis*-retinal (*21*). To prepare nPrSB and nBuSB, 13 mM 11-*cis*-retinal was reacted with a tenfold excess of amine in ethanol at 4°C overnight in the dark. EtSB was prepared by reacting 4 mM 11-*cis*-retinal with a tenfold excess of ethylammonium hydrochloride in the presence of 4 mM triethylamine also at 4°C overnight in the dark. The reactions were followed spectrally by the shift in absorption maximum from 380 nm for the aldehyde to 355 nm for the unprotonated Schiff base.
23. We thank W. P. Jencks, C. Miller, and I. Levitan for reading of the manuscript. We thank G. Johnson for advice regarding preparation of ROS membranes and transducin and W. P. Jencks for indispensable discussions throughout the course of these studies. We greatly appreciate the gift of 11-*cis*-retinal from P. Sorter and Hoffmann–La Roche. Supported by NIH grants EY07965 and BRSG S07 RR07044; E.A.Z. was supported by NIH training grant 5T32 GM07596-11 awarded to the Graduate Department of Biochemistry at Brandeis University.

19 September 1990; accepted 6 December 1990

Inhalational Anesthetics Stereochemistry: Optical Resolution of Halothane, Enflurane, and Isoflurane

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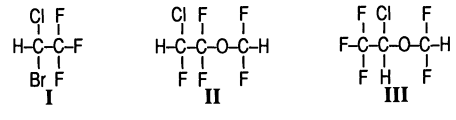
Halothane (I), enflurane (II), and isoflurane (III), which are among the most important inhalation anesthetics, are currently administered as racemic mixtures. The pure enantiomers have not been described, and no analytical method for resolving the commercially available racemic mixtures has been reported. Complete optical resolution of (±)-I and (±)-III on per-*n*-pentylated α-cyclodextrin (Lipodex A) and of (±)-II and (±)-III on octakis(6-O-methyl-2,3-di-O-pentyl)-γ-cyclodextrin capillary columns has been achieved, making rapid and convenient determination of enantiomeric ratios in samples of all three of these anesthetics possible.

INHALATIONAL ANESTHETICS CAN BE used to induce unconsciousness, analgesia, amnesia, and muscle relaxation. In the decades following W. J. G. Morton's demonstration in 1846 that diethyl ether could be used to induce a state of reversible insensibility (*1*), most available volatile chemicals were tested for anesthetic potential. Among these were chloroform and nitrous oxide, the latter of which remains an important supplemental anesthetic (*1*). By 1930, it was understood that anesthetics with the desired characteristics of noncombustibility, volatility, potency, low toxicity, and stability would likely be organic fluorides (*2*). This realization led eventually to the synthesis of halothane (I) in 1951 (*3*), enflurane (II) in 1963 (*4*), and isoflurane (III) in 1965 (*4*). Together with the weaker agent nitrous oxide, these three compounds are the most important agents in use today (*5*); interestingly, all three have chiral structures.



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Although the physiological effects of these drugs have been studied in detail, the definition of a molecular mechanism for general anesthesia has remained an elusive goal (*6*). Since a wide variety of molecules, including some inert gases, possess anesthetic properties, it is suspected that all such agents depress the nervous system by nonspecific perturbation of the fluid characteristics of nerve membranes (*6*). A more stereoselective interaction with the protein components of such membranes has not, however, been excluded. That the enzyme activity of luciferase is inhibited by clinically effective concentrations of anesthetics (*7*), and that saturable rat brain binding sites for halothane have been demonstrated by ^{19}F nuclear magnetic resonance (NMR) (*8*), strongly suggest direct protein binding. Early experiments in which halothane of modest enantiomeric enrichment was used failed to demonstrate differences between the two optically active preparations in their ability to depress conduction in a rat cervical ganglion or to increase disorder in a lipid bilayer (*9*). It is likely, however, that putative stereoselective interactions are weak; differences between anesthetic enantiomers may only be revealed with optically pure compounds. Thus if stereochemically homogeneous anesthetics were available, they would at the least be valuable research tools. It also is apparent that a convenient analytical technique for determining enantiomeric enrichments would in itself be extremely useful. We report chromatographic methods for the rapid resolution of all three of these racemic anesthetics on a microscale.

Preliminary gas chromatographic experiments were discouraging, although not surprising in view of the expected weak interactions of a chiral stationary phase with such lipophilic substrates. Experiments with chiral NMR shift reagents were similarly unrewarding. Our first success was with a capillary column using per-*n*-pentylated α-cyclodextrin (Lipodex A) (*10*) as the liquid phase (*11*). (±)-Halothane was completely resolved on this phase. We found that this same column gave complete resolution of (±)-isoflurane as well. (±)-Enflurane, however, was not resolved at all (Fig. 1A). By switching to another cyclodextrin derivative, octakis(6-O-methyl-2,3-di-O-pentyl)-γ-cy-clodextrin (*12*), we were also able to resolve (±)-II as well as (±)-III (Fig. 1B). With this column, the previously resolved (±)-I showed no separation. Thus (±)-III is readily resolved on both of these columns, while I and II are resolved on one or the other of the chiral liquid phases. The physical basis for the selectivity of these resolutions is unclear.

These results promise to be useful in several contexts. They provide a method of determining the enantiomeric purity of synthetic samples of I, II, and III. This method is rapid, convenient, and sensitive. Stereochemical analyses of many other similarly constituted volatile, lipophilic racemic substances that have hitherto resisted resolution may be possible with the use of these columns. It should now be possible to determine whether there is stereochemical selectivity in the enzymatic or metabolic deg-

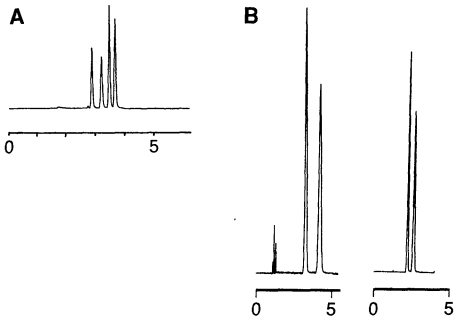

Fig. 1. Enantiomeric resolution of (**A**) halothane (I, longer retention times) and isoflurane (III, shorter retention times) on a 25-m fused-silica column with hexakis(2,3,6-tri-O-pentyl)-α-cyclodextrin and (**B**) of isoflurane (III, longer retention times) and enflurane (II, shorter retention times) on a 25-m Pyrex glass capillary column with octakis(6-O-methyl-2,3-di-O-pentyl)-γ-cyclodextrin. Retention times are in minutes. Capillary columns were coated according to the static procedure (*15*) as described earlier (*16*). A Carlo Erba Model 4160 gas chromatograph with split injection and flame-ionization detection was used. Conditions: headspace injection; column temperature, 30°C; carrier gas, 0.4 bar of hydrogen.