Allosteric Behavior in Transducin Activation Mediated by Rhodopsin

INITIAL RATE ANALYSIS OF GUANINE NUCLEOTIDE EXCHANGE*

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Photolyzed rhodopsin acts in a catalytic manner to mediate the exchange of GTP for GDP bound to transducin. We have analyzed the steady-state kinetics of this activation process in order to determine the molecular mechanism of interactions between rhodopsin, transducin, and guanine nucleotides. Initial velocities $V_o$ of the exchange reaction catalyzed by rhodopsin were measured for various transducin concentrations at several fixed levels of the GTP analog, $[^35S]$guanosine 5′-(3-O-thio)triphosphate (GTPyS). The initial rate data analysis rigorously demonstrates that rhodopsin mediates the activation of transducin by a double-displacement catalytic mechanism. The Michaelis-Menten curves determined as a function of $[^35S]$GTPyS reveal remarkable allosteric behavior; analysis of this data yields a Hill coefficient of 2. Lineweaver-Burk plots of $V_o$ versus $[^35S]$GTPyS display curvature indicative of positive cooperativity and a series of parallel lines are generated by plotting $V_o$ as a function of $[^35S]$GTPyS. The plots of $V_o$ versus $[^35S]$GTPyS show no evidence of allosterism and are parallel. Furthermore, the allosteric behavior observed in the activation of transducin is also witnessed in the rhodopsin-catalyzed guanine nucleotide exchange of the G protein's purified α subunit in the absence of the βγ subunit complex. The latter observation implies that the molecular basis for allosterism in the activation process resides in the interactions between the photoreceptor and transducin's α subunit.

A family of GTP-binding proteins, called G proteins, have been shown to provide a signal transduction mechanism for many cell surface receptors (for review, see Ref. 1). These receptors act catalytically to mediate the guanine nucleotide exchange of G proteins; this process is referred to as activation and results in the displacement of bound GDP for GTP. The concomitant dissociation of the G protein's α subunit, with GTP bound, from the βγ subunit complex initiates the signal to elicit the appropriate cellular response. One example of such a signal transduction system is the photon receptor, rhodopsin, and its G protein, transducin. Limited characterization of the activation of transducin mediated by rhodopsin has been accomplished by several investigators (2-5); however, molecular interactions between the receptor and G protein remain undefined. We have chosen to study this G protein-coupled receptor system in order to investigate molecular events involved in the activation process. Our approach is to model the catalytic activity of the receptor using established techniques of initial rate analysis employed in the study of enzyme catalysis. This kinetic analysis characterizes a double-displacement catalytic mechanism in the molecular interactions between rhodopsin, transducin, and guanine nucleotides. Furthermore, our findings demonstrate allosteric behavior during the activation process. The use of this method provides a conceptual basis for understanding the molecular interactions and regulation of the signal transduction mechanism utilized by the family of G protein-coupled receptors.

MATERIALS AND METHODS

Preparation of Transducin and Rhodopsin—Transducin and stripped membranes containing rhodopsin were prepared from rod outer segments (ROS) isolated from frozen, dark-adapted bovine retinas following protocols described by Peng et al. (2, 3). Briefly, thawed retinas were placed in ice-cold 20 mM Tris, pH 7.4, 1 mM CaCl₂, 45% (w/w) sucrose and passed several times through a syringe; ROS disrupted in this manner were collected and subjected to a series of extensive washes. The first series of four isotonic washes was carried out in a buffer of 10 mM Tris, pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA; the ROS were then washed four times in a hypotonic buffer of 10 mM Tris, pH 7.4, 1 mM DTT, 0.1 mM EDTA. These centrifugation steps were performed at 100,000 × g for 30 min at 4°C, and were subjected to a series of extensive washes. The first series of four isotonic washes was carried out in a buffer of 10 mM Tris, pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA; the ROS were then washed four times in a hypotonic buffer of 10 mM Tris, pH 7.4, 1 mM DTT, 0.1 mM EDTA. These centrifugation steps were performed at 100,000 × g for 15 min at 4°C.

All of the preparative methods were performed with illumination in order to obtain transducin, which was extracted from the washed ROS by incubation for 15 min in 10 mM Tris, pH 7.4, 1 mM DTT, 0.1 mM EDTA, in the presence of 40 μM GTP, followed by centrifugation at 100,000 × g for 15 min at 4°C. The supernatant, containing transducin, was collected and subjected to dialysis against 10 mM Tris, pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, and concentrated 25-fold by vacuum dialysis. This procedure yielded preparations of transducin which were at least 90% pure, as judged by Coomassie staining on sodium dodecyl sulfate-polyacrylamide gels routinely run to assess the quality of the product. Equimolar amounts of transducin's subunits were present, as verified by their co-migration in sucrose gradient centrifugation analysis. Typically, transducin was immediately employed in the kinetic assays if stored, the preparation was made 50% in glycerol and kept at −20°C. Transducin's α subunit was further purified and separated from the βγ subunit complex by chromatography over Blue Sepharose (5). Purified α subunit was concentrated, dialyzed, and manipulated as described for holotransducin.

Striped membranes containing rhodopsin were prepared under dim red light following the steps outlined above. After the isotonic and hypotonic washes, the ROS were further extracted with area,
according to methods described by Yamazaki et al. (6). ROS were suspended in 20 mM Tris, pH 7.4, 1 mM EDTA, 2 mM MgCl₂, 0.5 mM DTT, and a solution of 5 mM urea, 50 mM HEPES, pH 8.0, 5 mM EDTA, initial velocity, was determined by the linear correlation between the extent of reaction, product formation is a function of enzyme concentration and time; therefore, for any given extent of reaction, the value of enzyme concentration multiplied by time must be a constant. As Fig. 2 shows, this relationship was verified by the linear correlation between the extent of reaction, picomole of [³⁵S]GTPyS bound, and [rhodopsin] × time, at concentrations between 5.4 and 10.7 nM rhodopsin during time periods used in the study. If time-dependent inactivation of rhodopsin were to conflict with this measurement, distinct progress curves would be distinguishable for different concentrations of rhodopsin. The results of this experiment eliminate the possibility that photoactivation interferes with the kinetic measurements. Finally, the isomerization of rhodopsin’s

\[ V_0 = \frac{V_{\text{max}}}{K_{\text{m}} + [S]} + [S] \]  

\[ V_0 = \frac{V_{\text{max}}}{K_{\text{m}} + [S] + [S]^2} \]  

The values of \( K_{\text{m}} \) and \( V_{\text{max}} \) were provided with standard errors of their estimates.

**RESULTS**

Initial rate analysis provides a powerful tool in order to investigate the mechanisms of catalyzed reactions. We have utilized this method to study catalytic interactions between photoactivated rhodopsin and its substrates, transducin and guanine nucleotides. The validity of this kinetic approach relies on the capacity to measure initial rates of the reaction, thereby allowing an interpretation of the data based on steady-state assumptions. A functional means to ensure initial velocity measurements is to employ experimental conditions such that measured rates are proportional to enzyme concentration. Fig. 1 presents the linear relationship between initial velocity and rhodopsin concentration, determined in the catalyzed reaction between transducin, GT, and [³⁵S]GTPyS:

\[ G_T(GDP) + GTPyS \rightarrow \text{rhodopsin} \rightarrow G_T(GTPyS) + GDP \]

The nonhydrolyzable GTP analog, GTPyS, was employed to study the guanine nucleotide-exchange reaction in order to avoid possible interference arising from the slow GTPase activity intrinsic to T. The rate of production of G₂₅ (GTPyS) was monitored by rapid filtration through nitrocellulose filters which bind transducin. Experimental details of the exchange reaction assay are described under “Materials and Methods” and discussed below. Typically, a rhodopsin concentration of 5.4 nM was employed in the initial rate assays, within the linear range indicated by the result presented in Fig. 1 which confirms that initial velocities were measured under steady-state conditions. A second concern was the possibility of inactivation of rhodopsin complicating the measurement of reaction rates. A simple test of this situation is provided by Selwyn’s plot, shown in Fig. 2. This method is based on the reasoning that for any catalyzed reaction, product formation is a function of enzyme concentration and time; therefore, for any given extent of reaction, the value of enzyme concentration multiplied by time must be a constant. As Fig. 2 shows, this relationship was verified by the linear correlation between the extent of reaction, picomole of [³⁵S]GTPyS bound, and [rhodopsin] × time, at concentrations between 5.4 and 10.7 nM rhodopsin during time periods used in the study. If time-dependent inactivation of rhodopsin were to conflict with this measurement, distinct progress curves would be distinguishable for different concentrations of rhodopsin. The results of this experiment eliminate the possibility that photoactivation interferes with the kinetic measurements. Finally, the isomerization of rhodopsin’s
chrome. 11-cis-retinal to all-trans initiates the bleaching of the photopigment producing a series of spectral changes, eventually transforming rhodopsin to the metarhodopsin II form. To ensure a homogeneous population of rhodopsin molecules, the reaction components were photoolyzed a full minute prior to initiation of guanine nucleotide exchange. Preincubation in the light for up to 30 min did not qualitatively alter results (data not shown). Thus, possible aberrations which might occur at early time points due to the phototransition seem unlikely.

In order to fully investigate the catalytic mechanism of transducin activation mediated by rhodopsin, it is necessary to explore the relationships between initial velocities measured at different substrate concentrations, [GTPyS] and [GTPγS]. Fig. 3 shows a series of time course measurements of guanine nucleotide exchange in reaction mixtures which contained varying transducin concentrations and 0.167 μM [35S]GTPγS, in the presence or absence of 5.4 nM rhodopsin. As depicted, linear portions of reaction progress curves were measured under the experimental conditions used throughout this investigation. Initial rates were obtained as the slopes of the generated lines; the rate of exchange measured in the absence of rhodopsin was deducted from that determined in its presence in order to obtain a value for the catalyzed reaction. Serial determinations in this manner allow analysis of the relationship between velocity and substrate concentration. The lower right panel of Fig. 3 presents the Michaelis-Menten curve of the initial velocities, Vₐ, determined in this experiment, as a function of transducin concentration. The substrate-velocity relationship revealed an unexpected finding. The sigmoid nature of this curve is indicative of positive cooperative allosteric behavior. Analysis of the rate of the guanine nucleotide-exchange reaction for transducin in the absence of rhodopsin showed no evidence of sigmoidal behavior. This latter result implies that the cooperative effect resides in the interactions between rhodopsin and transducin rather than between GTPγS and transducin.

Since transducin is a heterotrimer of αβγ subunits, it is possible that the observed allosterism could involve molecular events between Tₐ and rhodopsin, Tβ and rhodopsin, or Tγ and rhodopsin. In order to discriminate between these possibilities, purified Tβ was separated from Tγ, by column chromatography and employed in similar kinetic assays, the results of which are presented in Fig. 4. It should be noted that the initial rates measured in experiments with free Tα were markedly decreased when compared to those obtained at similar concentrations of holotransducin. This can be expected since Tα has been shown to enhance the interaction of Tγ with rhodopsin (9). The Eadie-Hofstee plot shows the downward curvature expected of a system displaying positive cooperative behavior. The fact that cooperativity is observed in the absence of Tγ indicates that the basis for this phenomenon must arise from interactions between transducin's α subunit and the photoreceptor. The dashed line in Fig. 4 represents the curvature expected from a positive cooperative interaction with nH = 2, whereas the dotted line indicates the straight line observed in the absence of cooperative behavior. This allosteric response is corroborated by the Hill plot of data from a separate experiment with holotransducin, shown in Fig. 5. The Hill coefficient determined for a series of similar experiments was found to be 1.97 ± 0.22 (n = 16). Analysis of kinetic data obtained using purified Tβ, yielded a value of nH = 1.95 ± 0.44 (n = 4).

To complete the kinetic investigation, initial velocities as a function of transducin concentration were measured at several fixed levels of [35S]GTPγS concentration. Fig. 6 shows the results from one such experiment presented in double-reciprocal form. The Lineweaver-Burk plots with respect to reciprocal transducin concentration (panel B) show the curvature

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\[2 \text{M. Wessling-Resnick and G. L. Johnson, manuscript in preparation.}\]
**Fig. 3.** Time course of guanine nucleotide exchange of transducin. A 300-μl assay mixture was prepared by incubating 5.4 nM rhodopsin and 0.167 μM [35S]GTP·S in the light for 1 min and initiating the exchange reaction by the addition of the appropriate amount of transducin (as indicated in the figure panels). At times shown, a 50-μl sample was withdrawn, rapidly filtered through nitrocellulose filters, and immediately washed with ice-cold buffer. The amount of radioactivity counted is proportional to the amount of [35S]GTP·S exchanged on transducin and is plotted as a function of time. The reaction time course was monitored in the presence (●) and absence (○) of rhodopsin and initial rates were determined from the slopes of these lines. The exchange reaction rate in the absence of rhodopsin was deducted from that measured in its presence in order to obtain the initial velocity for the catalyzed exchange. As shown, the linear portion of the reaction time course was studied. Lower right panel, Michaelis-Menten curve for the initial velocities of the rhodopsin-mediated guanine nucleotide exchange determined as a function of transducin concentration. This plot shows the relationship between V, picomole of [35S]GTP·S bound per min, and transducin, μM, determined for the data shown.

**Fig. 4.** Kinetics of T, subunit activation. Purified α subunit from transducin was employed in kinetic assays as described in the legend to Fig. 3. Initial rates were determined at concentrations of T, from 0.048 to 0.592 μM and [GTP·S] of 0.15 μM. Shown is the Eadie-Hofstee plot of V/[T,] versus V (filled circles). The dashed line represents the normalized curve expected from a system having a Hill coefficient of 2. The dotted line depicts the normalized straight line which is obtained in the absence of allosteric behavior. The approximated initial velocity equation predicts that plots of reciprocal velocity as a function of the reciprocal of the square of transducin concentration would generate a series of parallel lines. Fig. 7 shows that this latter prediction is met; the linear transformations are presented using data from panel B in Fig. 6. From the intercepts and slopes of the secondary plots, determinations of the kinetic parameters may be made. Values of Kα = 0.1–1.5 × 10⁻⁶ M², K_{GTP·S} = 0.1–1.7 × 10⁻⁶ M, and Vmax = 0.9–6.0 × 10⁻⁵ M/min were obtained from data analysis of three individual experiments performed in this fashion. All of the experimental data were found to agree quite well with the approximated initial rate equation. However, in order to obtain highly accurate values of the kinetic parameters given above, it is necessary to include experimental data from rate assays containing concentrations of substrates an order of magnitude higher than Kα. This is the single limitation of the kinetic studies, imposed by the constraints of obtaining a large enough quantity of transducin within realistic means. It should be emphasized, however, that this limitation by no means invalidates the information obtained in support of the rate equation, it only indicates that always found to be of a normal hyperbolic form (data not shown). Data from these experiments were analyzed as detailed under “Materials and Methods.” Secondary plots of the slopes of the double-reciprocal lines (1/V blank/V max) were found to be independent of the square of transducin concentration, [G-¹]², and GTP·S concentration. Secondary plots of the intercepts (1/V blank) were found to be linear with respect to [G-¹]² or [GTP·S] accordingly (data not shown). The data were found to be in agreement with the following initial rate equation, derived from principles of steady-state kinetics and discussed under “Appendix.”
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FIG. 5. Allosterism observed in substrate-velocity plot. Initial velocities, $V_0$, were determined for the rhodopsin-catalyzed reaction between 0.5 μM [35S]GTPγS and transducin at concentrations as indicated in the figure. Measurements were performed as described in the legend to Fig. 3, and similarly, the Michaelis-Menten curve generated by the plot of $V_0$ as a function of transducin concentration is sigmoidal in character. A value of $V_{max} = 18.34 \pm 0.86$ pmol of GTP+ bound per min was obtained for the experimental data using nonlinear regression analysis as detailed under "Materials and Methods." Inset shows Hill plot of initial velocity data. Log $[V_0/\text{V}_{\text{max}} - V_0]$ is plotted as a function of log[transducin] in order to analyze data according to the Hill equation (8). As shown, a positive slope corresponding to $n_h = 2.2$ was determined. (See text for further discussion of the latter parameter.)

FIG. 6. Lineweaver-Burk plots for the rhodopsin-catalyzed guanine nucleotide-exchange reaction. Initial velocities were determined at varying concentrations of transducin at several fixed levels of [35S]GTPγS. Shown is representative data from one of three experiments in which initial rates were obtained. Panel A, double-reciprocal plots of $V_0^{-1}$ as a function of [GTPγS]$^{-1}$, with transducin concentrations of 3 μM (○), 0.75 μM (■), 0.5 μM (▲), 0.375 μM (▼), and 0.3 μM (●). A series of parallel lines are observed, providing evidence of a double-displacement catalytic mechanism for rhodopsin. Panel B, double-reciprocal plots of $V_0^{-1}$ as a function of [transducin]$^{-1}$, at GTPγS concentrations of 1 μM (○), 0.25 μM (□), 0.167 μM (▲), 0.125 μM (▼), and 0.1 μM (●). The curvilinearity depicted in these plots is indicative of positive cooperative behavior. Units are: $V_0^{-1}$ in terms of (picomole of GTP;S bound per min)$^{-1}$ and substrate concentrations as (μM)$^{-1}$. 
sites for transducin on rhodopsin, equivalent schemes could be formulated based on dimerization of transducin and/or rhodopsin. Here we consider that transducin interacts with rhodopsin in a manner which is sensitive to the presence of a second molecule of transducin (as witnessed in the curvilinearity of the double-reciprocal plots of panel B in Fig. 6 and supported by the linear transformations as the square of transducin concentration plotted in Fig. 7). As indicated by Plowman (10), models of this type can be obtained if one of the sites is essentially a regulatory rather than catalytic site. The fact that the allosterism is observed with the α subunit of transducin (Fig. 4) suggests that the cooperativity is localized to interactions between rhodopsin and Tα. This interaction promotes the displacement of the bound guanine nucleotide (GDP) (2, 3), generating a “substituted enzyme complex” between rhodopsin and transducin, now devoid of guanine nucleotide. The formation of the latter complex is predicted by the nature of the parallel double-reciprocal lines in Figs. 6 and 7. Convergent lines would otherwise indicate the presence of a “ternary complex” which might be thought of in this case to represent a complex between rhodopsin, transducin, and both guanine nucleotides. The GTP analog, GTPγS, stimulates with the rhodopsin-transducin “substituted enzyme” complex and causes activated transducin, with GTPγS bound, to exit the reaction complex. The character of the rate equation and double-reciprocal plots requires that the first guanine nucleotide (GDP) exit from the reaction complex prior to the addition of GTPγS, defining a double-displacement mechanism for the catalytic interactions between rhodopsin, transducin, and guanine nucleotides. Although a double-displacement type mechanism has been generally assumed, this is the first direct demonstration of this reaction mechanism.

An alternative model may be developed to explain the observed allosterism, depicted in Fig. 8B. As shown, the exchange reaction proceeds through a double-displacement mechanism, which is required by the character of the double-reciprocal plots. However, the possibility that rhodopsin is a hysteretic enzyme must be considered (see “Appendix” for further details). In this model, transducin binds to an inactive form of rhodopsin, Rho', promoting the conversion to the active form, Rho, which participates in the exchange reaction. Our results suggest that Tα would be intimately involved in this molecular event. The conversion of Rho' → Rho is otherwise a slow process representing hysteretic activity. Since this isomerization is dependent on transducin concentration, it is possible that sigmoidal behavior could be described by incorporating hysteresis into models describing the kinetics of the exchange process. Neet (11) and Ainsworth (12) have discussed this aspect of allosteric behavior to some length, which presents a distinct alternative to models which involve cooperative interactions between multiple binding sites.

**DISCUSSION**

In this article we report the results of a kinetic investigation of the catalytic mechanism through which rhodopsin mediates the guanine nucleotide exchange of transducin. These methods have not been previously applied and evidence is presented confirming the validity of the steady-state approach. The initial rate studies demonstrate that rhodopsin’s catalytic activity proceeds via a double-displacement mechanism. During the course of this investigation, we found remarkable allosteric behavior in the rhodopsin signal transduction system. The results presented show positive cooperative behavior in the substrate-velocity curves of transducin with respect to $V_o$, and a Hill coefficient of $n_H = 2$ was determined. Michaelis-
Menten plots of GTPγS concentration versus initial velocity did not display this sigmoidal character. However, the cooperative effect was also observed in kinetic experiments performed with purified Tc, implying that the molecular basis for the allosteric behavior is due to interactions between rhodopsin and transducin’s α subunit.

One approach in order to study structure-function relationships is to employ the kinetic methods which have provided a fundamental basis in mechanistic studies of soluble enzymes. Rhodopsin in this sense catalyzes the guanine nucleotide exchange of transducin in an analogous manner through which elongation factor Ts serves as an exchange catalyst for elongation factor Tu (13, 14). For example, both of these proteins mediate guanine nucleotide exchange by a double-displacement mechanism. Transducin, itself, has been characterized to be a member of the family of GTP-binding regulatory proteins (G proteins) which provide a signal transduction system for many cell surface receptors, mediating changes in cell function, metabolism, and growth (1). By analogy, such G protein-coupled receptors would also appear to fall into the class of exchange catalyst enzymes. Recent efforts of several investigators have focused on the molecular cloning of G protein subunits (15-22) and this knowledge will lay the foundation to explore structure-function relationships. Towards the latter goal, complete characterization of the molecular interactions between receptors and G proteins in the process of signal transduction is lacking, primarily due to the relatively low abundance of these regulatory elements (1). However, transducin is an exception since milligram quantities may be readily obtained from bovine retinal rod outer segments and biochemical characterization of this G protein has been the subject of intense study. Furthermore, structural information is also available for the photoreceptor, rhodopsin (23). Therefore, this system provides a paradigm within which a conceptual basis of the molecular events of these signal transduction mechanisms may be developed. The observations reported in this paper draw attention to the following possible explanations of allosteric behavior in the rhodopsin-transducin signal transduction mechanism, the merits of which are discussed below.

Positive Cooperative Interactions in Transducin Binding—The simplest interpretation of the initial rate data is that two transducins act in a positive cooperative manner in the interaction with rhodopsin as shown in Fig. 8A (see “Appendix” for kinetic development of model). It should be emphasized that such models do not discriminate between oligomeric forms of substrate and enzyme or the presence of multiple binding sites. It should also be noted that the analysis presented cannot differentiate between two binding sites with strong cooperativity, pairs of sites with equivalent cooperativity, or four sites with relatively poor cooperativity. Bennett and Dupont (5) have concluded that four transducins interact with a single rhodopsin based on light scattering measurements, although they indicate this result might vary by a factor of two. The findings reported in the present article are supported by the work of this group. Our experiments indicated that this binding interaction specifically involves the α subunit of transducin.

A point should be made that interactions between multiple transducins and monomeric rhodopsin must be understood in terms of structural relationships. Hargrave and co-workers (23) have discussed the structural organization of the 41 kDa rhodopsin molecule in the rod outer disc membrane and conclude that the native protein is monomeric. There has been some controversy, however, about the possible existence of rhodopsin dimers (24). With the determination of structural information concerning transducin, it is hopeful that structure-function relationships will become evident to increase the current understanding of this G protein-coupled receptor system and provide insight into its allosteric behavior. In this regard, the possible existence of a transducin dimer has been suggested by light scattering measurements (5). Nondenaturating gel electrophoresis and ultracentrifugation experiments performed by Baehr et al. (4) have shown the presence of dimeric forms of α subunits and the βγ complex. Indeed, oligomers of G proteins from other systems have been described by target size analysis (25). It is also interesting that elongation factor Tu, itself a GTP-binding protein, has been characterized as a dimer by neutron scattering (26). Another GTP-binding protein, the ADP-ribosylation factor ARF, interacts with the regulatory protein G, in such a way as to enhance ribosylation by cholera toxin (27). Taken altogether, these phenomena indicate that GTP-binding proteins may associate with one another under certain conditions. However, our own hydrodynamic analysis of transducin has failed to support the presence of oligomeric forms of the protein (4). Such physical studies of transducin in solution would not reveal structural alterations which may occur at the membrane surface, and therefore provide only speculative information. The kinetic analysis presented here does provide a sensitive measure of molecular interactions occurring on the surface of the membrane and affecting the activation process. Hence the possible involvement of oligomeric forms of transducin and/or the presence of multiple sites of binding on rhodopsin must be considered in future studies.

Hysteretic in the Activity of Rhodopsin—The active state of rhodopsin has been characterized spectrophotometrically as the meta II form of the photopigment. There has been discussion about the possible existence of two conformic forms of rhodopsin in the meta II state as identified by spectrophotometric measurements (28, 29) and light scattering techniques (30); biochemically these states have yet to be defined. Such analysis might provide the necessary basis for a hysteretic model: an alternate enzyme state with different physical characteristics and catalytic properties. The availability of this additional state for rhodopsin would be affected by the presence of transducin as depicted in Fig. 8B (see “Appendix” for kinetic development of model). It is interesting, therefore, to compare rhodopsin to other G protein-coupled receptors which have been characterized. In the latter systems, two ligand binding states have been identified which are sensitive to the presence of guanine nucleotides (31, 32). Could conformational alterations of metarhodopsin II be analogous to the so-called coupled and uncoupled receptor? Indeed, if such forms do exist and display different kinetic behavior, allosterism would be observed.

A key point in order to distinguish this possibility is that hysteretic cooperativity can be witnessed only in kinetic studies; measurements from equilibrium binding techniques would not reveal this behavior. Instead, oligomeric associations arising from cooperative interactions would be observed (this is demonstrated by the discussion under “Appendix”). Bennett and Dupont (5) have used the light scattering method to obtain the latter measurements. While these investigators do not report allosteric behavior, they do present results indicating that multiple transducins interact with rhodopsin. Our results also support the latter idea, as discussed above. Current efforts in our laboratory are directed toward accomplishing equilibrium binding studies; these experiments will also provide insight into the molecular interactions between rhodopsin and transducin in the membrane environment.

Finally, in view of the broad analogies between G protein-
coupled receptor systems, it is tempting to speculate that the allostery behavior reported in this article may be a conserved phenomenon of this family. Considering the requirement for amplification in signal transduction systems, it is not surprising that transducin would display allostery regulation in its interaction with rhodopsin. We have presented initial observations describing this phenomenon; further work is necessary to explore the exact nature of this regulation, its implications, and its role in cellular processes. At present, similar experiments involving other members of the regulatory G protein family would appear to be difficult, being restricted by the low quantities of receptors and G proteins which may be obtained and constrained by the fact that these systems must be reconstituted into phospholipid vesicles (1, 33, 34). The double-displacement model for catalytic interactions between rhodopsin, transducin, and guanine nucleotides is consistent with what is known about the mechanism of molecular interaction for other members of this family (35). It remains to be seen whether or not the allostery behavior we report is another of the many analogies between G protein-coupled receptor systems.

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**APPENDIX**

The most direct and obvious interpretation of the characteristics of the double-reciprocal plots shown in Figs. 6 and 7, is that rhodopsin catalyzes guanine nucleotide exchange by a double-displacement mechanism and exhibits allostery with respect to the GTP-binding protein, transducin. The formulation of an initial rate equation for the exchange reaction is required to incorporate both of these experimental criteria.

One interpretation of the sigmoidal substrate-velocity curves is that two transducins (or two T subunits) interact in a cooperative manner during exchange for a single GTPγS. This can be thought of as arising from dimerization of the GTP-binding protein, dimerization of rhodopsin, or the presence of two equivalent binding sites for transducin on a single rhodopsin. The latter idea may be represented by the scheme depicted below, considering that the binding of the first molecule of transducin to rhodopsin alters the intrinsic dissociation constant, by a factor of a, towards a second molecule of transducin.

\[
\begin{align*}
Rho + T & \rightleftharpoons \frac{K_d}{\alpha K_d} Rho \cdot T + T \\
T + T & \rightleftharpoons \frac{K_d}{\alpha K_d} T \cdot Rho + T
\end{align*}
\]

If the allostery behavior is due to strong cooperativity between two sites, that is, if the interaction factor \(\alpha\) is small, all complexes with rhodopsin containing a single occupied site will be negligible at [transducin] > \(K_d\). In order to simplify our analysis, we will make this assumption, as suggested by Segel (8). The justification for this assumption is based on the goodness of fit to the Hill equation (demonstrated in the logarithmic plotting form shown in the inset of Fig. 5) as well as the apparent Hill coefficient, determined to be close to an integer value. The catalytic mechanism can then be expressed in the scheme presented below, which depicts a double-displacement mechanism for the exchange reaction.

\[
\begin{align*}
Rho + T & \rightleftharpoons \frac{K_d}{\alpha K_d} Rho \cdot T + T \\
T + T & \rightleftharpoons \frac{K_d}{\alpha K_d} T \cdot Rho + T
\end{align*}
\]

In terms of this model, the equation for initial velocity, \(V_o\), may be written as follows.

\[
V_o = k_o[Rho \cdot T_3(GDP)] + k_o[Rho \cdot T_3(GTP\gamma S)]
\]

The experimental conditions we imposed in this kinetic study are such that the concentrations of substrates, transducin and GTPγS, were much greater than rhodopsin concentration, and no products, GDP or T(GTPγS), were present at time 0. This allows straightforward analysis along the established description of steady-state enzyme kinetics (8). The following rate equations may be written in view of the steady-state.

\[
d[Rho]/dt = d[Rho \cdot T_3(GDP)]/dt = d[Rho \cdot T_3(GTP\gamma S)]/dt = 0
\]

\[
d[Rho \cdot T_3(GDP)]/dt = k_o[T(GDP)][Rho] - k_o[Rho \cdot T_3(GDP)] - k_o[Rho \cdot T_2(GDP)]
\]

\[
d[Rho \cdot T_3(GTP\gamma S)]/dt = k_o[T(GDP)][Rho] - k_o[Rho \cdot T_3(GTP\gamma S)] - k_o[Rho \cdot T_2(GTP\gamma S)]
\]

By rearrangement and substitution, the velocity equation may be rewritten.

\[
V_o = k_o k_o[T(GDP)]^2[Rho]
\]

Using the expression for the total concentration of rhodopsin, \([Rho]_e = [Rho] + [Rho \cdot T_3(GDP)] + [Rho \cdot T_2(GTP\gamma S)]\) the final form of the rate equation may be determined, which is expressed in double-reciprocal form below.

\[
1/V_o = 1/[Rho]_e \left[ \frac{(k_3 + k_4)}{k_1 k_2} \left[ \frac{1}{[T(GDP)]} + \frac{(k_3 + k_4)}{k_3 k_4} \right] \right]
\]

We prefer to express this equation as follows:

\[
1/V_o = \frac{K_m^T}{[T(GDP)]} + \frac{K_m^{GTP\gamma S}}{[GTP\gamma S]} + 1
\]

where

\[
V_{max} = \frac{(k_3 + k_4)[Rho]_e}{k_3 k_4} = \frac{K_m^T}{h_1 h_2 + h_3} + \frac{K_m^{GTP\gamma S}}{h_1 h_2 + h_3} = \frac{h_2 h_3}{h_1 h_2 + h_3}
\]

The rate equation developed is a 2/0 function, describing the sigmoidicity observed in the substrate-velocity plots. Alternative models which yield a similar function should also be considered. One consideration is that the allostery behavior may be the result of hysteresis in the activity of rhodopsin. A distinguishing feature of a hysteric enzyme is the manifestation of an enzyme state which has a different physical form and kinetic properties; the availability of this additional state is altered by the presence of substrate (11). This idea is
depicted in the following scheme:

$$
\text{Rho'} \xrightarrow{k_{\text{on}}} \text{T(GDP)} \xrightarrow{1'} \text{Rho} + \text{GDP} + T(GTP_\gamma S)$$

As this model suggests, two conformational isomers of rhodopsin exist, of which Rho' may be considered an inactive form. The interconversion process Rho' → Rho is slow compared to all other steps. T(GDP) binds Rho' to produce Rho'.T(GDP) and to promote the conversion to Rho'.T(GDP), which then participates in the exchange reaction, generating the products and the active form of Rho. Rabin (36) has discussed this kinetic model as an extension of Koshland's induced-fit hypothesis by placing the restriction that $k_{-a} \ll k_{-b}$, such that $k_{-a}/k_{+} \ll k_{-b}/k_{+}$. Thus, the $K_1$ of Rho'.T(GDP) is then smaller than that of Rho'.T(GDP) and the capacity of rhodopsin to bind transducin increases with the progress of the reaction; that is, the conversion of Rho'.T(GDP) → Rho'.T(GDP) causes an increase in the strength of interaction between rhodopsin and transducin. Since the conversion of Rho' into Rho is a function of transducin concentration, it is possible that the allosteric behavior witnessed experimentally could be described by models incorporating this idea.

REFERENCES