Maximal Rate and Nucleotide Dependence of Rhodopsin-catalyzed Transducin Activation

INITIAL RATE ANALYSIS BASED ON A DOUBLE DISPLACEMENT MECHANISM* 

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Despite the growing structural information on receptors and G proteins, the information on affinities and kinetics of protein-protein and protein-nucleotide interactions is still not complete. In this study on photoactivated rhodopsin (R*) and the rod G protein, Gt, we have used kinetic light scattering, backed by direct biochemical assays, to follow G protein activation. Our protocol includes the following: (i) to measure initial rates on the background of rapid depletion of the GtGDP substrate; (ii) to titrate GtGDP, GTP, and GDP; and (iii) to apply a double displacement reaction scheme to describe the results. All data are simultaneously fitted by one and the same set of parameters. We obtain values of $K_m = 2200 \text{ Gt/} \mu \text{m}^2$ for GtGDP and $K_m = 230 \mu \text{m}$ for GTP; dissociation constants are $K_p = 530 \text{ Gt/} \mu \text{m}^2$ for R*GtGDP dissociation and $K_d = 270 \mu \text{m}$ for GDP release from R*GtGDP, once formed. Maximal catalytic rates per photoexcited rhodopsin are 600 Gt/s at 22 °C and 1300 Gt/s at 34 °C. The analysis provides a tool to allocate and quantify better the effects of chemical or mutational protein modifications to individual steps in signal transduction.

In retinal rod cells, absorption of a photon by the visual pigment rhodopsin initiates a cascade of biochemical reactions that eventually generates an electrical signal (1). Much of the tremendous overall gain (10$^5$–10$^9$) of the visual cascade depends on two enzymatic amplification stages, namely the receptor-catalyzed nucleotide exchange in the rod G protein, transducin, and the hydrolysis of the second messenger cGMP by the effector, cGMP phosphodiesterase.

Rhodopsin (R) and transducin (Gt) display fundamental similarities to other receptors and G proteins. However, the function of the rod as a single photon sensory cell requires both low basal activities of R and Gt and high speed of catalytic nucleotide exchange in the G protein. Any catalytic activity of rhodopsin is effectively blocked in the dark by the covalently bound inverse agonist 11-cis-retinal. Although a mammalian rod cell contains $<10^7$ rhodopsin molecules, thus providing the necessary target for efficient light absorption, spontaneous single photon-like activity originates only every 100 s from one of the many dormant receptors (2). On absorption of a photon, light-induced isomerization converts the chromophore to the agonist all-trans-retinal, thereby triggering conformational changes in the receptor protein that result within milliseconds in the formation of the enzymatically active intermediate metarhodopsin II (for review, see Refs. 3 and 4). Once activated, rhodopsin finds by diffusion its substrate Gt. In its inactive, GDP-bound state (GtGDP), the heterotrimeric Gt holoprotein is peripherally bound to the disc membrane by weak hydrophobic and ionic interactions (5–7). The activation of the Gt protein proceeds through a sequence of two mutual displacements (R* for GDP and GTP for R*; so-called double displacement mechanism). Collisional interaction between light-activated rhodopsin (R*) and GtGDP (Fig. 1A, step 1) triggers a conformational change that opens the GtGDP nucleotide-binding site. Upon GDP release, a stable R*Gt complex with an empty nucleotide-binding site on the Gt-GDP subunit is formed (step 2). Binding of GTP to the GtGDP subunit within the R*Gt complex enables a second conformational change (step 3) that eventually induces the dissociation of active GtGTP (Gt*) from the receptor (step 4) and the (simultaneous or unmeasurably delayed) separation of the α- and βγ-subunits (GtαGTP and Gtβγ). At least in vitro, activation is further accompanied by immediate (delay <1 ms (8)) dissociation of both GtαGTP and Gtβγ from the disc membrane. The high rate of R*-catalyzed nucleotide exchange leads to the rapid (transient) accumulation of Gt*. Active GtαGTP in turn binds to the cGMP phosphodiesterase (PDE) within less than 5 ms (8). The noncatalytic, stoichiometric interaction keeps the PDE active, and hydrolysis of cGMP leads to the closure of cGMP-dependent ion channels in the plasma membrane of the rod outer segment (for review, see Refs. 9 and 10).

Despite the detailed knowledge of the reaction mechanisms and despite the growing information about the underlying structures (11, 12), sufficiently accurate estimates for the affinities and rates of the protein-protein and protein-nucleotide interactions are still not available. In this study we focus on the crucial activation reaction of the G protein, its rate of catalytic activation, and its dependence on GTP and GDP. Together with the kinetic parameters of PDE activation and cGMP turnover, this is the key parameter for any quantification of the gain of phototransduction in the rod cell (see Refs. 13 and 14). Efforts...
nucleotide exchange on Gt includes (at least) four microsteps as follows. Following the classical approach of Kuhn and co-workers (5, 14), however, data obtained with low time resolution underestimates the actual rate, when not accounting for rapid depletion of 5% was used. Both methods yield osmotically intact disc vesicles with reproducible quantification, arrestin was added to the sample buffer as a reference signal (N$_D$ signal) measured on a sample without added G$_t$ as described (19). All measurements were performed in 10-mm path cuvettes with 300-μl volumes in isotonic buffer (20 mM BTP, pH 7.4, 130 mM NaCl and 5 mM MgCl$_2$) at 22 °C unless specified otherwise. Reactions are triggered by flash photolysis of rhodopsin with a green (500 μM) flash, attenuated by appropriate neutral density filters. The flash intensity is quantified photometrically by the amount of rhodopsin bleached and expressed either in terms of the mole fraction of photoexcited rhodopsin (R*/R) or in the surface density of R* (R*/μm$^2$). Dissociation signals were recorded with a 0.5–5-ms dwell time of the A/D converter (Nicolet 400, Madison, WI). To suppress base-line activation, NH$_2$OH was added to the membrane stock at a concentration of 4.5 mM. The final concentration of NH$_2$OH in the samples never exceeded 300 μM NH$_2$OH to keep the decay of the flash-induced R* small. For calibration of the LS monitor, dissociation signals were induced by saturating flashes (R*/R = 0.5%). For the kinetic steady state analysis dissociation signals were routinely measured at R*/R = 2.3×10$^{-4}$ (5.7 R*/μm$^2$), i.e. in the linear range of the light titration curve (Fig. 3). Binding signals (R*/R = 32%) were corrected by a reference signal (N signal) measured on a sample without added G$_t$, as described (19). All data were taken at pH 7.4, i.e. in the maximum of the bell-shaped pH/rate profile (31); at this pH, membrane binding of G$_t$GDP (in the dark) is also near its maximum. 2

Amplitudes of the signals are expressed as relative scattering intensity changes (ΔI/I, where I represents the intensity measured before the flash and M the amplitude was routinely performed prior to each set of experiments by measuring dissociation and binding signals induced by saturating flashes on aliquots of disc membranes (3 μM R) supplemented with 0.5 μM G$_t$ (see Fig. 2B). As described below (see under “Results”), the dissociation signal exclusively monitors activation to measure the actual rate of the catalytic power of rhodopsin date back to the late 70s. To account for the rapid cGMP hydrolysis, Liebman and co-workers (15) concluded that light activation of a single molecule of rhodopsin results in the activation of several hundred molecules of PDE. After identification of transducin (16–18), direct GDP release and GTP binding studies yielded lower G$_t$ activation rates (see Refs. 13 and 14). However, data obtained with low time resolution underestimates the actual rate, when not accounting for rapid depletion of the substrate, and are contaminated by both the onset of deactivation reactions and by slow activation of soluble G$_t$.

Complementary to the biochemical assays are the light-scattering (LS) techniques, which follow flash-induced changes in the scattering of near infrared light as an endogenous probe of specific molecular changes (see Ref. 19). The LS monitor allows one to measure G$_t$ activation continuously and in real time, so that the decisive 500 ms after flash excitation are obtained.

The experimental data can then be used to extract initial rates of G$_t$ activation, thereby separating them from slower, associated reactions, such as membrane interaction, rhodopsin kinase interference (21), and the decay of the active receptor. To account for the influence of nucleotide concentration, we titrate G$_t$GDP, GDP, and GTP. The double displacement reaction scheme that takes into account of all these components (22) is then applied to the experimental data.

**EXPERIMENTAL PROCEDURES**

Membrane and Transducin Preparations—Rod outer segments were prepared from frozen bovine retinas using a sucrose gradient procedure as described (23). Hypotonically stripped disc membranes were prepared from rod outer segments either by two consecutive extractions with low salt buffer as described (24) or by the Ficoll floating procedure similar to the procedure described (25) except that 2% w/v Ficoll instead of 5% was used. Both methods yield osmotically intact disc vesicles with a vesicle size ~400 nm. No significant difference was found between the two types of preparations. Contamination by vesicle aggregates was removed by a 2-μm filter (Nucleopore). Membranes were kept on ice and used within 4 days without any loss of activity. Rhodopsin concentration was determined from its absorption spectrum using ε$_{280}$ = 40,000 M$^{-1}$ cm$^{-1}$.

Transducin was purified as described (8). Subunits were further purified on Blue-Sepharose (1 ml of HiTrap Blue, Amersham Pharmacia Biotech) at a flow rate of 1.2 ml/h. Proteins eluted with starting buffer (20 mM BTP, pH 7.5, 1 mM MgCl$_2$, 2 mM dithiothreitol) contain inactive G$_t$βγ (26). Active G$_t$βγ was eluted with a linear gradient of 0–0.3 M NaCl (15 ml). G$_t$α was eluted with 1 M NaCl. The subunits were dialyzed against measuring buffer (20 mM BTP, pH 7.7, 5 mM MgCl$_2$, 2 mM dithiothreitol, concentrated (Amicon, YM-10), and stored at ~4 °C. G$_t$βγ concentration was determined by the method of Bradford (27) using bovine serum albumin as the standard. The amount of intact, activatable G$_t$α was determined precisely by fluorometric titration with GTPyS (28).

**Nucleotides—**GTP (>98%, Fluka) was used without further purification. GDP (80%, Fluka) was further purified by two successive ion exchange chromatographies on QAE-Sepharose (Amersham Pharmacia Biotech) using a linear gradient of 0.2–1 M triethylammonium hydrogen carbonate buffer, pH 7.7. The procedure yields GDP virtually free of contaminating GTP. Concentration of the nucleotides was determined spectrophotometrically using ε$_{252}$ = 13,700 M$^{-1}$ cm$^{-1}$.

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of the membrane-bound pool of Gt, (Gtmb), whereas the binding signal exclusively measures transition of the soluble fraction of Gt,GDP (Gt,sol) to the membranes. Thus, the (absolute values of the) maximum scattering change of the signals are proportional to the concentration of Gtmb and Gt,sol, respectively. Together with the known amount of total Gt added to the samples, the amplitude of the signals can be converted to concentration units by the scaling factor (F) as shown in Equations 1–3.

\[ \text{[Gt,sol]} + [\text{Gtmb}] = [\text{Gt}]_{\text{added}} \]  

\[ (\Delta I/I)_{\text{max}} \cdot F = [\text{Gtmb}] \]  

\[ (\Delta I/I)_{\text{max}} \cdot F = [\text{Gt,sol}] \]  

where

\[ [\text{Gt,sol}] + [\text{Gtmb}] = [\text{Gt}]_{\text{added}} \]  

Once determined for a given preparation, the scaling factor was used to transform the maximum amplitude of individual dissociation signals (Equation 1) to yield the respective initial (i.e. at the time of the flash) volume concentration of Gtmb ([Gtmb]). Due to the membrane localization of the proteins, both the R*-GtGDP dissociation constant and Michaelis constant for Gtmb are only properly specified in two-dimensional (2D) terms (see under “Appendix”). Conversion of [Gtmb] to surface concentrations is obtained with the known surface density of rhodopsin (25,000 R/μm², see Ref. 32), and the volume concentration of rhodopsin, [R], in the sample (see Equation 4).

\[ \frac{[\text{Gtmb}]}{[\text{R}]} = \frac{25,000 \mu m^2}{[\text{R}]} \]  

where [Gtmb] is given in units of molecules per μm² (see Equation 7 under “Appendix”). Analogously, the maximum slope of the dissociation signal (ΔI/I s⁻¹) was transformed to the surface concentration of Gtmb activated per s (Gtmb μm⁻² s⁻¹).

**Reaction Scheme and Mathematical Analysis**—The R*-catalyzed steady state Gtmb activation rate depends on the Gtmb surface concentration as well as on the volume concentrations of both GDP and GTP (Fig. 4C). The multifactorial dependence is well described by the double displacement scheme (formally equivalent to the ping-pong scheme; see Fig. 1B and Refs. 22 and 33).

By using the steady state approach, the following rate Equation 5 can be derived for the initial rate of Gtmb activation (v_Gt) in absence of the product GtGTP (see e.g. Ref. 34).

\[ v_{\text{Gt}} = \frac{K_{\text{mb}}}{K_{\text{mb}} + K_{\text{mbGDP}} + K_{\text{mbGTP}}} + \text{Gtmb} \frac{1 + K_{\text{mbGTP}}}{K_{\text{mbGTP}} + \text{Gtmb}} \]  

\[ + \frac{K_{\text{mbGDP}}}{K_{\text{mbGDP}} + \text{Gtmb}} \text{Gtmb} \]  

\[ + \frac{K_{\text{mbGTP}}}{K_{\text{mbGTP}} + \text{Gtmb}} \text{Gtmb} \]  

where Gtmb is the initial surface density of membrane-bound Gt,GDP, and GDP and GTP denote the volume concentrations of the respective nucleotide. The kinetic parameters are defined as Kmb, for the Michaelis constant for Gtmb, KmbGTP, for the dissociation constant for GTP, KmbGDP, for the dissociation constant of GDP release from R*,GtGDP, KmbGDP, for the dissociation constant of Gtmb dissociation from R*,GtGDP, and Vmax for the maximum value of v_Gt. The definition of the parameters in terms of microscopic rate constants is given in Table I. The factor F is the fraction of active receptor (without G protein), relative to the total amount of light-activated rhodopsin (see under “Appendix”).

Each set of experiments comprises the titration of the dissociation signal with exogenous Gt, in the presence of fixed concentrations of GTP and GDP. Both the initial activation rate of Gtmb (v_Gt) and the initial Gtmb surface concentration were determined from the maximum slope and the maximum amplitude of the dissociation signal, respectively (see above and “Results”). The Gtmb titrations were repeated (i) at different concentrations of GTP (10–3000 μM) and (ii) at 200 μM GTP with different concentrations of GDP (75–2000 μM). In this way the dependence of v_Gt on the three variables (Gtmb, GTP, and GDP) was obtained (Fig. 4).

The data points of 23 independent sets of titration experiments (232 data points overall) were numerically fitted with Equation 5, using a
multiple least squares fit, i.e. the simultaneous fit to all 23 titrations using one and the same set of the parameters $K_{d(G)}$, $K_{d(GT)}$, $K_{d(GDP)}$, $K_{d(GTP)}$, and $V_{max}$. In the fit procedure (Scientist Software, MicroMath), the concentrations of GTP and GDP were fixed for each individual titration (i.e. for each pair of [GTP] and [GDP]), and each of the five kinetic parameters was allowed to vary. The turnover number ($V_{max}/R^*$) was then calculated with the known surface density of $R^*$ (5.7 $R^*/\mu$m$^2$).

Note that Equation 5 is converted into a simple Michaelis-Menten type of hyperbolic function for any given (fixed) set of nucleotide concentrations to yield Equation 6,

$$v_{BO} = \frac{V_{max} G_{mb}}{K_m + G_{mb}}$$  \hspace{1cm} (Eq. 6)

where $V_{max}$ and $K_m$ denote the apparent maximum $G_t$ activation rate and the apparent Michaelis constant for $G_{mb}$, respectively. In Equation 6, both the apparent values depend on the nucleotide concentration.

The validity of the steady state approach is essentially based on the following: (i) the initial $G_{mb}$ concentration is high as compared with $R^*$ even at the lowest $G_{mb}$ concentrations investigated, and (ii) the time until the maximum slope was obtained, less than 5% of $G_{mb}$ is activated, thus rendering depletion of the substrate negligible. We have also found that neither lowering the membrane concentration (1.8 $\mu$m instead of 3 $\mu$m R) nor addition of excess $G_t\beta\gamma$ leads to a significant change of the observations (a putative persistent $G_t\beta\gamma$-receptor interaction may be integrated in the reaction scheme and the respective rate equations derived (22)). Note that both $K_{d(GT)}$ and $K_{d(GDP)}$ do not depend on the correct determination of $G_{mb}$ (in contrast to $V_{max}$, $K_{d(GT)}$, and $K_{d(GDP)}$ which linearly depend on any error in the $G_{mb}$ concentration).

RESULTS

Quantification of Membrane-bound Transducin—Fig. 2A shows the typical, variable membrane binding of $G_t$ and its dependence on light and nucleotides as analyzed by the centrifugation assay (18, 19, 21) (see “Experimental Procedures”). Upon dilution of the membranes, $G_tGDP$ (unlike other $G$ proteins) is in equilibrium between a membrane-bound ($G_{mb}$) and a soluble form ($G_{sol}$) in the dark (Fig. 2A, lanes 3 and 4). The extent of this “dark binding” depends on several factors, including overall protein and membrane concentration, temperature, pH, ionic strength, and divalent cations (see Refs. 32 and 35). Photoactivation of excess rhodopsin in the absence of GTP or GDP locks $G_t$ in its nucleotide-free, receptor-bound conformation ($R^*G_t$), which is seen in a complete shift of $G_t$ to the membrane (Fig. 2A, lanes 5 and 6). Conversely, light activation of rhodopsin in the presence of GTP leads to a complete dissociation of $G_t$ from the membranes (Fig. 2A, lanes 7 and 8). Due
to the low membrane concentration used in the experiments (equivalent to 3 μM R), both Gα and Gβγ-subunits almost quantitatively dissociate from the membrane upon activation (Fig. 2A), even at high Gc concentration (data not shown).

The gain of mass of a membrane, when Gc is bound from the solution, and the loss of mass with its dissociation produces large and readily measurable changes in near-infrared light scattering (LS signals; see Ref. 19). Representative light-induced LS signals (measured on the samples used for the centrifugation assay shown in Fig. 2A) are shown in Fig. 2B. Transition of all Gsol to the membrane induced by bleaching excess rhodopsin in the absence of GTP gives rise to an increase of the scattered light (“binding signal”) (20, 36; Fig. 2B, trace a). Accordingly, dissociation of Gmb from the membrane upon activation is seen as decrease of LS (“dissociation signal”) (20, 36; Fig. 2B, trace b). Since the dissociation step itself is not rate-limiting (see below), the rising phase of the dissociation signal is a real time monitor of Gc activation. Notably, binding signals are generally slow as compared with dissociation signals. Previous work resolved this apparent conflict by the finding that, although binding of Gmb to R* is fast, interaction of Gsol with the membrane is slow (36). Consequently, the activation rate of Gsol is limited by its membrane binding, thereby leading to artificially slowed activation rates when total active Gc* formation is assayed under conditions where a significant fraction of Gc-GDP is solubilized.

As indicated in Fig. 2B, the dissociation signal exclusively monitors fast activation of Gmb. Its maximum amplitude is not contaminated by a contribution of Gsol, since the slow mass gain of the membrane upon its binding is just canceled by the fast subsequent dissociation of Gc*. Accordingly, the maximum amplitude of the dissociation signal is proportional to the Gmb pool. On the other hand, the maximum amplitude of binding signals induced by saturating flashes is proportional to Gsol, since binding of Gmb to R* does not lead to any LS changes of the membrane suspension (note that this is in contrast to LS measurement on rod outer segment preparations (37, 38)). Consequently, comparison of the amplitudes of the dissociation and binding signals allows determination of the sizes of the Gmb and Gsol pools (Fig. 2, B and C). The relative fraction of Gmb varied with different membrane and Gc preparations between 40 and 60% of the total Gc added. Densitometry on gels as shown in Fig. 2A was used to independently quantify dark binding of Gc under the conditions of the LS experiments. Comparison of the results obtained by the two methods (Fig. 2C) shows good agreement, which further confirms the interpretation of the LS signals.

The sum of the maximum amplitudes (absolute values) of the dissociation and binding signals is proportional to the known amount of Gc added (i.e. Gmb + Gsol), thereby allowing the calibration of the LS monitor. A calibration factor (F) was calculated for every set of experiments, which relates the relative scattering intensity change to Gc concentration units (see Equations 1–3). The calibration factor varied only little between different preparations (7.0 < F < 8.3 μM). The scaling factor was used to transform the maximum amplitude of individual dissociation signals to yield the respective initial (i.e. at time of the flash) volume concentration of Gmb. Based on the known surface density of rhodopsin in the disc membrane (25,000 R/μm²; see Ref. 32), the Gmb volume concentration is then easily transformed to surface concentration units (Equations 4 and 7; see Fig. 2D).

Titration of the amplitude of the dissociation signal with Gc yields the dependence of Gmb on added Gc (Fig. 2D). The slight but reproducible sigmoidal dependence of Gmb on added Gc may be explained by a relatively weak Gα-Gcβγ-subunit interaction in solution and a negligible interaction (relative to the Gα-holoprotein) of the isolated subunits with the membrane, which is in agreement with gel filtration and centrifugation experiments.2

Dependence of Gmb Activation Rate on the Concentration of R*—Gmb activation is accelerated with increasing concentration of R*. Accordingly, the rise time of the dissociation signal depends on the mole fraction of photoexcited rhodopsin (Fig. 3). The light titration curve saturates at high bleaching levels due to the local depletion of Gc on the disc membrane (and possibly rate limitation by dissociation of Gc* from the membranes). At low bleaching levels (R*/R < 10⁻³) the maximum slope of the dissociation signal depend linear on the R* concentration (dashed line in Fig. 3), which proves that the dissociation of Gc* from the membrane is not rate-limiting under these conditions and establishes the rising phase of the dissociation signal as a real time monitor of Gc* formation.

Furthermore, the linearity is an important criterion for the analysis of the activation rates since it allows us to normalize the activation rates to R*, i.e. to calculate the turnover number (Vmax/R*) of R*–catalyzed Gc activation. Consequently, we routinely measured the dissociation signal at R*/R = 2.3 × 10⁻⁴ (5.7 R*/μm²).

With decreasing R* the amplitude of the dissociation signal decreases (Fig. 3, inset), since it is proportional to the number of individual vesicles hit by at least one photon (the slow activation of Gmb initially bound to vesicles that do not contain an R* is not detected on the time scale of the experiments). It is seen that under the experimental standard conditions (R*/R = 2.3 × 10⁻⁴) more than 80% of the maximum amplitude is evoked. Fit of the data points to a Poisson sum (solid line in inset of Fig. 3) yield 15,000 R per domain (i.e. vesicle). With the known rhodopsin surface density of 25,000/μm², a vesicle size of 440 nm (diameter) is calculated (see Ref. 39 for details), which is in good agreement with the size as estimated from electron micrographs of our preparations (not shown). The slight systematic deviation of the data points with the best fitting Poisson sum is most likely due to slightly nonuniform vesicle size (and contamination by slow GTPase reaction, significant at very low R*).

Steady State Analysis of Gc Activation Rate—Fig. 4A shows a typical set of dissociation signals, each evoked by a small flash producing 5.7 R*/μm² (i.e. in average about 3.4 R* per disc membrane vesicle) with increasing amounts of purified Gc added. As expected, both the maximum amplitude (Fig. 2D) and the maximum slope of the signal (Figs. 4 and 5) increase with Gc concentration. Expansion of the scales (Fig. 4B) shows that the maximum slope is not immediately reached after light activation of the receptor but is delayed by a short, R*–depending period (8). It is the time it takes all the individual reactions, R* formation, nucleotide exchange, and dissociation of Gc* from the membrane, to reach a steady state. The maximal rate of Gc* production is reached 40–100 ms after the flash under the condition of the experiments; despite of this delay, we will refer to it as “initial” rate of Gc activation. After the short linear period, the slope of the dissociation signal slows due to depletion of Gmb and increasing activation of Gsol. Because membrane binding of Gsol is slow, distortion of the maximum slope by this effect was neglected. Rapid substrate depletion is a consequence of the membrane-bound state of the proteins, which limits the “reservoir” of the substrate as compared with reactions in solution. As calculated from the dependence of Gmb on Gc added (Fig. 2D), Gmb membrane binding saturates at about 7500 Gc/μm² (0.3 Gmb per R), which is in good agreement with previous estimates (40, 41).

The maximum slope of the signals (dashed lines in Fig. 4B)
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**Fig. 5. Temperature dependence of R*-catalyzed G_{mb} activation.** A, steady state G_{i} activation rate as a function of initial G_{mb} concentration (both obtained from dissociation signals as described in Fig. 4) at 34 (a), 28 (b), 22 (c), 16 (d), 11 (e), and 6 °C (f). Solid lines are best fit to the data points of each titration to Equation 6. Experimental conditions: 3 mM GTP, no GDP added, pH 7.4, 6 R*/mM. B, Arrhenius plot of the apparent turnover number (V_{max}/R^*). was taken from the linear period, which lasts 100–300 ms, depending on G_{i} concentration (Fig. 4B). It was then converted to the steady state G_{i}^{a} formation rate by the calibration procedure described above (see Equations 1–4). Importantly, the initial G_{mb} concentration was calculated from the maximum amplitude of each signal (see above and under "Experimental Procedures"), thus avoiding errors inferred from variations of total G_{i} added (see Fig. 2D).

The titration of the dissociation signal with exogenous G_{i} (Fig. 4A) was repeated (i) with [GDP] = 0 and different concentrations of GTP (10–3000 μM; Fig. 4C, upper panel) and (ii) with 200 μM GTP and different concentrations of GDP (75–2000 μM, Fig. 4C, lower panel). The resulting dependence of the initial rate of G_{mb} activation (v_{RC}) on the three variables (G_{mb}, GTP, and GDP) was then fitted by a simultaneous fit to the data, using Equation 5 and 23 sets of dissociation signals; different colors in Fig. 4C identify different concentrations of nucleotide, and different symbols identify different preparations. The kinetic parameters thereby obtained are summarized in Table I.

**Dependence of G_{mb} Activation Rate on Temperature**—The temperature dependence of G_{mb} activation was studied by varying G_{mb} at each temperature in the presence of 3 mM GTP (no GDP added; Fig. 5A). Because the experiments were done at a single, fixed nucleotide concentration, the data were fit to a simple hyperbolic function (Equation 6). The apparent turnover number of R*-catalyzed G_{mb} activation (V_{max}/R^*) and the apparent Michaelis constant (K_{m}) for R*-G interaction as a function of temperature are summarized in Table II. Assuming K_{m}(GTP) as temperature-independent, the true values of V_{max}/R^* would be about 8% larger as compared with the apparent ones.

The temperature dependence of the apparent turnover number of R^* catalyzed G_{i} activation (V_{max}/R^*) is shown in Fig. 5B in Arrhenius coordinates. Below 22 °C the data points are well described by a linear relation, yielding an apparent activation energy of the G_{i} activation of 111 kJ/mol. At higher temperatures a pronounced nonlinearity of the Arrhenius plot is observed, indicating an activation energy <30 kJ/mol above 28 °C.

**DISCUSSION**

We have studied the receptor-catalyzed nucleotide exchange of a heterotrimeric G protein with the aim to quantify both the maximal catalytic activity of the activated receptor (turnover number) and the influence of GTP and GDP on the velocity of G protein activation. For such analysis, the visual system is well suited because (i) neither the receptor nor the G protein show any measurable basal activity; (ii) the G protein (G_{i}) is easily isolated and available in the quantities required for titration; and (iii) the light trigger allows us to load the system with defined doses of active receptor.

**Initial Rate Analysis**—Enzyme-catalyzed reactions are commonly assayed by steady state product formation with a high initial substrate concentration (S_{0} >> E_{i}). In the case of G protein-coupled systems, the membrane localization of the substrate (the G protein) limits its initial concentration. Bovine rod disc membranes operate at a G_{i}/R^* (S_{0}/E_{i}) ratio of 3000:1 (single photon detection). The low amount of membrane-bound G_{i}GDP (G_{mb}) leads to rapid depletion of the substrate and causes a very short steady state period. It is obvious that the rapid depletion of the G protein substrate cannot be overcome by overloading the membranes with G protein. Even if, as in the visual system, the G protein is in vitro in equilibrium between a membrane-bound and soluble form, the problem is not solved by a large excess of G protein in solution. The transition of soluble G_{i}GDP (G_{i}sol) to the membrane is far too slow to maintain a high concentration of membrane-bound G_{i}GDP. In other words, the rate of activation of soluble G_{i}GDP is limited by the slow membrane binding step, which is particularly evident when G_{i} activation is assayed at low membrane concentrations. As a consequence, true activation rates are only obtained when the initial rate of G_{i} activation is assayed in the presence of sufficiently high membrane concentrations. The initial rate approach kinetically separates the rapid activation of membrane-bound G_{i}GDP from the slower activation of the soluble G_{i}GDP pool (and from other slow reactions that influence the rate, such as R^* deactivation and GTPase reaction). The keys to reliable activation rates are thus a millisecond time resolution and an accurate quantification of the membrane-bound fraction of the G protein.

Unfortunately, biochemical assays so far applied to this system lack the necessary time resolution. Stopped-flow techniques, which were successfully applied to the study of nucleotide uptake or GTPase reaction of various systems, including small G proteins (see e.g. Refs. 42 and 43) and EF-Tu (see e.g. Ref. 44), are hard to apply to the visual system with its light sensitivity. We thus used the kinetic light-scattering technique to obtain initial rates of G_{mb} activation. Previous work established the dissociation signal as a real time monitor of the fast activation of the membrane-bound fraction of G_{mb}GDP (20, 36, 37). A precise calibration can be obtained from the dependence on [R^*] (37, 38) and/or from classical biochemical assays (this work). Both the initial surface concentration of G_{mb} and the
The parameters were derived by global fit to the data points shown in Fig. 4 according to Equation 5 (S.D. taken from global fit). \( f^A \) is the relative fraction of active receptor, taken as the relative fraction of MII (\( f^{MII} \)) to yield corrected values (see under “Appendix”).

| Parameter          | Kinetic definition                                                                 | Observed value | Corrected value |
|--------------------|-----------------------------------------------------------------------------------|----------------|-----------------|
| \( K_{mG/GTP} \)   | Observed Michaelis constant for \( Gmb \)                                        |                |                 |
| \( K_{mG/GDP} \)   | Michaelis constant for GTP                                                        |                |                 |
| \( K_{dG/GDP} \)   | Dissociation constant for GDP                                                      |                |                 |
| \( V_{max}/R^s \)  | Turnover number                                                                   |                |                 |

**Table I**

| Temperature (°C) | \( G^*/R^s \) | \( f^{MII} \) | \( K_m \)      |
|------------------|---------------|---------------|----------------|
| 6                | 45 ± 10       | 0.282         | 956 ± 434      |
| 11               | 102 ± 8       | 0.417         | 1085 ± 181     |
| 16               | 216 ± 14      | 0.560         | 1617 ± 221     |
| 22               | 583 ± 37      | 0.712         | 2706 ± 310     |
| 28               | 1044 ± 42     | 0.824         | 3073 ± 226     |
| 34               | 1290 ± 69     | 0.896         | 2977 ± 307     |

**Table II**

**Double Displacement Scheme**—The dependence of the \( G_t \) activation rate on nucleotide or initial \( G_t \)GDP concentration is commonly analyzed employing a Michaelis-Menten type of hyperbolic function (e.g. Equation 6). For example, each titration with \( G_t \) (Figs. 4 and 5) can be fitted with Equation 6. The resulting parameters (\( K_m \) and \( V_{max} \)), however, are only apparent values when multiple substrates are involved. The \( K_m \) for one particular substrate (e.g. \( G_t \)GDP) measured at one fixed set of cofactor (e.g. GTP) concentration changes as the cofactor concentration varies. Analogously, apparent values are obtained when the \( K_m \) for GTP is evaluated from the dependence of \( G_t \) activation rate on [GTP] at a fixed (sub-saturating) \( G_t \)GDP concentration (see below and Fig. 6A). Generally, the true \( K_m \) for a particular substrate is the one observed when all other substrates are saturating. Similarly, the true \( V_{max} \) is seen when all substrates are present at saturating concentrations and in the absence of any product (for a detailed description, see Ref. 34).

We have used the classical double displacement reaction scheme to describe adequately the receptor-catalyzed activation of a G protein (Fig. 1 (22)). By using the steady state approach, rate equations can be derived (e.g. Equation 5) that explicitly account for the concentrations of all components involved (\( R^s \), \( G_t \)GDP, GTP, GDP) and allow us to extract the true kinetic parameters for the individual steps.

We note that the inclusion of the transitory complex (\( R^sG_t\)GDP) in the reaction scheme is justified by the experimental data: as seen in Fig. 4C, \( V_{max} \) is not approached in the presence of GDP even at infinite \( G_t \)GDP concentrations. This shows that GDP is not a competitive inhibitor implying that binding of \( G_t \)GDP to \( R^s \) and the release of GDP are separated by a transitory \( R^sG_t\)GDP complex with finite lifetime.

**Affinity of \( G_t \)GDP for Photoactivated Rhodopsin**—The affinity of \( G_t \)GDP for \( R^s \) depends on the equilibrium of the active
receptor conformation with its tautomeric forms (see under “Appendix”). For the following discussion, we use the corrected parameters $K_{mb}(G)$ and $K_{mb}(G)$ to characterize the interaction of $G_{mb}$ with $R^*$. As compared with the native surface concentration of $G_i (3000 \mu M)$ the value of $K_{mb}(G)$ ($530 \pm 260 \mu M^{-2}$) reflects a surprisingly weak interaction of the proteins. However, this does not contradict the experimentally confirmed stability of the $R^*G$ complex (i.e. the complex without bound nucleotide (45, 46)), because formation of the latter is composed of two reactions, namely the initial interaction of $G_i$GDP with $R^*$ (step 1 in Fig. 1A) and the succeeding GDP release (step 2 in Fig. 1A). Consequently, the formation of the complex depends on both $K_{mb}(G)$ and $K_{mb}(G)$ (see under “Appendix”). Despite the low affinity of $G_i$GDP to $R^*$ (high value of $K_{mb}(G)$), the reaction is almost quantitatively shifted toward formation of the $R^*G$ complex under typical experimental conditions (i.e. low membrane concentration, no added GDP) because the concentration of the endogenous GDP released is too low to dissociate the complex ($K_{mb}(G) = 270 \mu M$; see Table I). At high membrane concentrations, however, the endogenous GDP can significantly affect $R^*G$ formation.

When volume concentrations are used and/or the influence of the endogenous GDP is omitted (see e.g. Refs. 47 and 48), the resulting $G_iR^*$ affinity depends on all the concentrations used in the experiment. Thus the proper definition and separation of the individual reaction steps is not an academic problem but has an immediate impact on the reaction mechanism.

**Dependence on Nucleotides**—An important result of this study is the high $K_{mb}$ (low affinity) of GDP to $R^*G$, once formed ($K_{mb}(G)$), and the high $K_m$ of GTP ($K_{mb}(GTP)$). This may seem surprising in view of the higher apparent affinities of the nucleotides obtained 1) by G activation assays performed at low concentration of $G_i$ and/or membrane, or 2) under equilibrium conditions. Reasons for these apparent discrepancies are as follows. 1) As described above, low $G_i$ (as compared with $K_{mb}(G)$) necessarily leads to lower apparent $K_{mb}$ for GTP, because $R^*-GDP$ association becomes rate-limiting (see Fig. 6A). When even slower reactions limit the reaction rate (e.g. membrane binding of soluble G$G_i$GDP or association of the detergent-solubilized proteins), the apparent $V_{max}$ can even saturate at micromolar GTP (Ref. 28 and for review see Ref. 49). 2) Equilibrium measurements (e.g. binding studies employing labeled nucleotides) are not suited to quantify the affinity of GTP or GDP to the $R^*G$ complex. The reason is that the dissociation of GTP$G_i$ (or G$G_i$GDP) from the active receptor, the $G_i$-membrane and $G_i$-subunit interactions, the metarhodopsin equilibrium, and quasi-irreversible reactions such as $R^*$ decay and the GT$P_i$ase reaction all affect the equilibrium. As a consequence equilibrium studies generally overestimate affinities (i.e. underestimate $K_{mb}$).

The low affinity of GDP to $R^*G$ obtained in this study ($K_{mb}(G) = 270 \mu M$) is necessary to explain the essentially quantitative formation of the $R^*G$ complex at low overall concentrations and in the absence of added GDP (see above). The low Michaelis constant for GTP ($K_{mb}(GTP) = 230 \mu M$) is in agreement with conclusions drawn from independent analyses of GTP-induced dissociation of the $R^*G$ complex (50).

**$G_i$ Activation Rate**—Published estimates of the $G_i$ activation rate vary from about 10 to $>3000$ $G_i/s$ per $R^*$ formed (for review see Ref. 13). The extreme variation is at least partially due to differences with respect to the method, preparation, and measuring conditions used. For example, when aliquots for a filter assay of GTP uptake are taken in seconds intervals (see e.g. Ref. 51), not only the depletion of membrane-bound $G_i$GDP but also slow reactions may severely affect the results. Activation of soluble $G_i$GDP will be the predominant artifact in broken rod outer segment preparations or reconstituted systems, whereas deactivation of $R^*$ may interfere in more intact systems. Evidently, measurements of $G_i$ activation on rod outer segments require a sufficiently fast assay that measures the rising phase of $G_i$ activation before these influences cut in (<100 ms for mammalian rods). With such approaches, initial rates in the order of 1000 $G_i$/s at $R^*$'s are obtained (38, 52, 53).

In the present study on isolated membranes, measuring initial rates of $G_{mb}$ activation with known $G_{mb}$ surface concentrations, the steady state approach yields a turnover number of 600 and 1300 $G_{mb}$/s at 22 and 34 °C, respectively. With the kinetic parameters obtained at 22 °C (Table I), the activation rate can be plotted as a function of GTP and GDP (Fig. 6).

The **maximal rate** is in good agreement with a previous study on whole rod outer segments (800 $G_i$/s at 21 °C (38)). However, there is a discrepancy because with the isolated membranes and the assumed native $G_i$ concentration, $V_{max}$ is not approached (see Fig. 6A). This is due to an increased $K_{mb}(G)$ and $K_{mb}(G)$ for $R^*-G$GDP interaction in the reconstituted preparation. The intact membranes may reserve more quantitative formation of active metarhodopsin II and/or more efficient collisional coupling. The latter is likely to depend on the reversible carboxymethylation of the $G_i$-subunit (26).

**What Is the Rate-limiting Step?**—Given the complexity of the reaction scheme (Fig. 1), the question arises whether the maximum rate of receptor-catalyzed G protein activation is limited by the rate of diffusion of the G protein or GTP or by protein conformation changes.

In the Arrhenius representation, the dependence of $V_{max}/R^*$ on temperature (Fig. 5B) yields a low temperature (<22 °C) branch with an activation energy of 111 kJ/mol. This value is significantly lower than the one determined for $G_i$ activation between ~2 and 12 °C and at very low [GTP] (175 kJ/mol (50)). Although the reasons for the differences remain unknown, the high activation energy indicates that a protein conformational change within the reaction sequence is likely to be rate-limiting in this range (54). At sufficiently high temperature, a process with smaller activation energy ($E_a < 30$ kJ/mol) takes over, possibly a diffusion limited process (54). However, it is difficult to extract an individual step when the kinetic parameters are composed of various individual rate constants (see Table I), each of which may have its own temperature dependence.

The lower limit of the $R^*-G_i$GDP encounter rate obtained from our analysis (see under “Appendix”) is 820 s$^{-1}$ at 22 °C, i.e. well below its theoretical limit of 7000 s$^{-1}$ (13). The lower limit for the bimolecular rate constant of formation of the encounter complex $R^*-G_i$GTP is 2.6-10$^6$ M$^{-1}$ s$^{-1}$ (see under “Appendix”), which is much smaller than the diffusional limit of about 10$^8$-10$^9$ M$^{-1}$ s$^{-1}$. Thus, at least in the reconstituted system, neither binding of the G protein nor GTP uptake is diffusion-controlled.

**Application to Other Receptors**—We have shown that the four-step analysis adequately describes the experimental data obtained with the visual system. Modifications of the analysis, to account for ligand binding (22), should make it applicable to other receptor systems, thus providing a basis for the assignment and quantification of chemical and mutational probing.

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**APPENDIX**

**Membrane Localization of the Proteins**—It is obvious that the density of rhodopsin in the disc membrane does not change upon dilution of the disc membrane suspension. As a consequence, neither the equilibrium of the $R^*-G_i$GDP interaction nor the rate of the $R^*-G_i$GDP complex formation depends on the
dilution of the membranes for a given amount of membrane-bound G,GDP (G,,m,b). Hence, both the concentrations and the corresponding kinetic parameters are only properly specified in two-dimensional terms. On the other hand, any surface density, [X] 2D (including e.g. the values of Km(G), Kd(G), and microscopic rate constants), is easily transformed to a volume concentration, [X] 3D (or vice versa), by the relation shown in Equation 7.

\[
\frac{[X]_{3D}}{[X]_{2D}} = \frac{[R]_{3D}}{[R]_{2D}}
\]  
(Eq. 7)

where [R] 3D is the actual volume concentration of rhodopsin, and [R] 2D is 25,000 μm⁻² (3D is three-dimensional and 2D is two-dimensional). However, it should be kept in mind that only the two-dimensional values are true constants, whereas the three-dimensional values depend on the experimentally chosen dilution of the membrane suspension. This becomes evident by the following example. The dissociation constant of R*,G,GDP (Kd(G)) is 530 μm⁻² (Table I) indicating a rather low affinity interaction of the proteins as compared with the native surface density of G, (3000 μm⁻²). The corresponding three-dimensional value (Kd(G) = 0.06 μm⁻1) obtained under the conditions of our experiments (3 μm R), however, could be mistaken as a very high affinity of the proteins under cellular conditions (300 μm G,).

The problem becomes even more evident when reactions are investigated that depend on both soluble components (such as GDP and GTP) and membrane-bound proteins. For instance, in the coupled equilibrium (see Equation 8),

\[
K_{G,GDP} \frac{R^* \cdot G,GDP}{R^* \cdot G,GDP} = \frac{K_{G,GDP}}{K_{G,GDP}} = \frac{G,GDP}{G,GDP} (Eq. 8)
\]

the formation of the R*,G complex depends on both the surface concentration of R* and G,GDP as well as on the volume concentration of GDP. The overall equilibrium is given by Equation 9,

\[
K_{G,GDP} = \frac{R^* \cdot G,GDP}{G,GDP} = \frac{K_{G,GDP}}{K_{G,GDP}} = \frac{G,GDP}{G,GDP} (Eq. 9)
\]

where Kd(G,GDP) is the dissociation constant of the R*,G complex in the presence of GDP. The value of Kd(G,GDP) (2 μm⁻²) is only properly specified in the respective two- and three-dimensional concentration units.

Influence of the Relative Fraction of Active Receptor on the Kinetic Parameters—A general characteristic of G protein-coupled receptors is the equilibrium of inactive and active receptor conformations. As a consequence, the actual amount of active receptor may be lower than the total amount of “activated” receptor (R*). The receptor equilibrium does not affect the interaction of the nucleotides with the R*,G complex once formed nor the turnover number Vmax/R* (due to the stabilizing effect of the G protein on the active receptor conformation). However, it strongly influences the interaction between R* and the G protein; the more the equilibrium is shifted to the inactive receptor conformation, the higher the observed values of Km(G) and Kd(G). The true values of Km(G) and Kd(G) are obtained by multiplying the observed values by the fraction (fA) of the active receptor (A) (measured in the absence of G protein).

The active rhodopsin conformation can be identified with metarhodopsin II (MII), which is in a pH and temperature dependent equilibrium with its precursor metarhodopsin I (MI; for review see Ref. 55) (Equation 10):

\[
MI \rightleftharpoons MII \quad with \quad K = \frac{MI}{MII} \quad (Eq. 10)
\]

The relative fraction of MII (fMII) is given by Equation 11,

\[
f_{MII} = \frac{MI}{MI + MII} = \frac{K}{1 + K} \quad (Eq. 11)
\]

The dependence of the equilibrium constant (K) on temperature and pH is well known for preparations of isolated disc membranes (48, 56). The resulting values of fMII under the conditions of the experiments (see Table II) were used to correct Km(G) and Kd(G).

Lower Limit of the Rate of G Protein and GTP Binding—It is important to note that the steady state treatment does not yield values of microscopic rate constants. However, lower limits of k1 and k3 (Fig. 1) can be calculated from koff(Km) (see Ref. 34). The minimum second-order rate constant of the formation of the encounter complex R*,G,GDP (k1) is thus given by Equation 12,

\[
V_{max}^f = \frac{k_1 \cdot k_2}{k_1 \cdot k_3 + k_4} \quad (Eq. 12)
\]

which yields k3 > 0.27 μm² s⁻¹ (using f = fMII = 0.714). With the native surface density of G, (3000 μm⁻²), the lower limit of the R*,G,GDP encounter rate is 820 s⁻¹ at 22 °C.

Analogously, a lower limit for the second-order rate constant of the formation of the encounter complex R*,G,GTP (k3) is given by

\[
V_{max}^r = \frac{k_3 \cdot k_4}{k_3 \cdot k_4 + k_4} \quad (Eq. 13)
\]

yielding k3 > 2.6·10⁶ m⁻¹ s⁻¹ at 22 °C.

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