Kinetic and Hydrodynamic Properties of Transducin: Comparison of Physical and Structural Parameters for GTP-Binding Regulatory Proteins†

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Received August 20, 1986; Revised Manuscript Received March 6, 1987

ABSTRACT: Transducin is a member of the family of GTP-binding regulatory proteins that interact with cell surface receptors and that include G_s, G_i, and G_o. Kinetic and physical properties of purified bovine transducin were characterized by the following results: (1) Initial rate analysis demonstrates a dissociative-type mechanism for the guanine nucleotide exchange process of transducin in the absence of rhodopsin. A second-order rate constant of $k_f = (1.7–2.7) \times 10^{-7}$ M^{-1} s^{-1} was determined for this reaction. (2) Equilibrium binding measurements indicated a K_d of 0.05–0.10 μM for guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) binding to transducin. (3) Neither the rate nor the extent of GTPγS binding was affected in the presence of up to 50 mM Mg^{2+}, as compared to values obtained in the presence of excess ethylenediaminetetraacetic acid. (4) Sucrose density gradient ultracentrifugation gave $s_{20,w}$ values for transducin, its α subunit, and its $\beta\gamma$ subunit complex of 4.23 ± 0.25, 3.42 ± 0.37, and 4.04 ± 0.2, respectively. (5) Incubation of transducin in the presence of up to 20 mM Mg^{2+} did not alter its sedimentation behavior; however, the presence of guanine nucleotides did produce a shift in transducin's migration in the sucrose gradient. (6) Gel filtration over Sephacryl S-300 indicated that transducin elutes at a Stokes radius of 37.5 Å and that transducin's α subunit displays a Stokes radius of 24 Å. (7) A molecular mass of 68 kDa for transducin is derived from the determined hydrodynamic parameters. These results are compared with properties known for other G proteins, and functional differences between transducin and G_s, G_i, and G_o are proposed in relation to the proteins' primary sequences.

The relationships between members of the family of regulatory GTP-binding proteins (G proteins)[1] are well documented [for review, see Gilman (1984)]. These proteins interact with cell surface receptors such that, in response to stimuli, the receptor mediates the exchange of GTP for GDP bound to the G protein. Thus, G_s and G_i serve as stimulatory and inhibitory elements in the hormonal regulation of adenylate cyclase (Gilman, 1984). The retinal rod outer segment G protein, transducin, acts in the visual transduction system to stimulate cGMP phosphodiesterase (Stryer et al., 1981). Additional G proteins, including G_o and G_p, have been identified (Sternweiss & Robishaw, 1984; Neer et al., 1984; Evans et al., 1986), although their functional roles are not completely understood. The G proteins are heterotrimers of α, β, and γ subunits. The β and γ subunits appear to be common between G proteins (Katada et al., 1984; Manning & Gilman, 1983); however, there is evidence indicating heterogeneity in the γ-subunit species isolated from different G proteins (Roof et al., 1985; Hildebrandt et al., 1985). The α subunits, which contain the GTP binding site as well as sites for ADP-ribosylation by cholera and pertussis toxins, appear to be unique.

Several groups have reported the cDNA sequences coding for the α subunit of transducin (Lochrie et al., 1985; Tanabe et al., 1985; Medynski et al., 1985; Yatsunami & Khorana, 1985); nucleotide sequences have also been determined for the β and γ subunits (Sugimoto et al., 1985; Fong et al., 1986; Hurley et al., 1984; Yatsunami et al., 1985). More recently, cDNA clones coding for the α subunits of G_s, G_i, and G_o have been isolated and sequenced (Robishaw et al., 1986; Nukada et al., 1986; Itoh et al., 1986). With this knowledge, it has become increasingly important to study physical similarities and differences between members of this family in order to gain an understanding of structural relationships. To this end, investigations from several laboratories have focused on the physical characterization of G_s and G_i (Northup et al., 1982, 1983; Sternweis et al., 1981; Hanski et al., 1981; Bokoch et al., 1984; Katada et al., 1984; Codina et al., 1984a,b; Sunyer et al., 1984) and, recently, G_o (Huff et al., 1985; Huff & Neer, 1986; Sternweis & Robishaw, 1984). Although much work has been performed in order to characterize transducin functionally and structurally (Fung & Stryer, 1980; Fung et al., 1981; Fung, 1983; Baehr et al., 1982), a thorough investigation of its hydrodynamic and kinetic characteristics has not been completed. In this paper we report the results of such studies, with the purpose to compare transducin's physical properties to what has been reported for other G proteins. On the basis of this comparison, we propose functional differences between homologous domains of transducin, G_s, G_i, and G_o.

Materials and Methods

Preparative Methods. Transducin was isolated from dark-adapted bovine retinas following procedures previously described (Wessling-Resnick & Johnson, 1987). Retinas were illuminated and disrupted in ice-cold 20 mM Tris, pH 7.4, 1 mM $CaCl_2$, and 45% (w/w) sucrose by several passages through a syringe. Rod outer segments (ROS) were collected by flotation, washed in buffer without sucrose, and layered

† Supported by NIH Grants GM30324, AM37871, and CA39240.
‡ G.L.J. is an Established Investigator of the American Heart Association.

[1] Abbreviations: G protein, regulatory GTP-binding protein; G_s and G_i, stimulatory and inhibitory proteins of the adenylate cyclase system; G_o and G_p, GTP binding proteins isolated from brain and placenta, respectively; T_α and $T_{\beta\gamma}$, α and $\beta\gamma$ subunits of transducin; $\beta\gamma$, G protein subunit complex; $G_{s\alpha}$, $G_{i\alpha}$, and $G_{o\alpha}$, α subunits of the respective G proteins; ROS, retinal rod outer segments; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); Gpp(NH)p, guanosine 5′-(β,α-imidotriphosphate); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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onto a step gradient of 25% and 35% (w/w) sucrose in 20 mM Tris, pH 7.4, and 1 mM $CaCl_2$. ROS were obtained at the 25%/35% interface after centrifugation at 100000*g* for 50 min at 4 °C and washed 4 times in isotonic buffer of 10 mM Tris, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and 0.1 mM EDTA followed by a second series of four washes in hypotonic buffer of 10 mM Tris, pH 7.4, 1 mM DTT, and 0.1 mM EDTA. ROS were pelleted in these latter steps by centrifugation at 100000*g* for 15 min at 4 °C. Transducin was extracted from the washed ROS during a 15-min incubation with 40 μM GTP in 10 mM Tris, pH 7.4, 1 mM DTT, and 0.1 mM EDTA followed by centrifugation at 100000*g* for 15 min at 4 °C. The supernatant, containing transducin, was dialyzed against 10 mM Tris, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and 0.1 mM EDTA and concentrated 25-fold by vacuum dialysis. If necessary, the preparation was made 50% in glycerol and stored at −20 °C; otherwise, transducin was immediately employed for the kinetic experiments. Preparations of transducin obtained by this procedure were at least 90% pure, as judged by Coomassie staining on SDS–polyacrylamide gels. Western blotting of transducin preparations with antibodies prepared against rhodopsin provided evidence that contaminating rhodopsin was not present (see Figure 1). Transducin was column purified by chromatography over hexylagarose as described by Fung et al. (1981). The α and $\beta\gamma$ subunits of transducin were further purified and separated by chromatography over Blue Sepharose as previously described (Wessling-Resnick & Johnson, 1987), following protocols adapted from Watkins et al. (1985) and Shinozawa et al. (1980). Briefly, transducin was applied to a Blue Sepharose column (1 mg of protein/5-mL bed volume) in 10 mM Tris, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and 0.1 mM EDTA. The column was washed with the same buffer, and the $\beta\gamma$ subunits were collected in the flow-through and wash fractions. The α subunit was eluted in a 100–750 mM NaCl gradient, and the separated subunits were manipulated as described for holotransducin preparations. Measurements of the GTPase activity of transducin preparations in the presence of saturating rhodopsin were reproducibly in the range of 0.9–1.3 mol of PO_4 (mol of transducin)$^{-1}$ min^{-1} (Kelleher et al., 1986).

Stripped ROS membranes containing rhodopsin were prepared following the methods outlined above, with the exception that all steps were performed under dim red light. Following the hypotonic washes, ROS were extracted with urea, as described by Yamasaki et al. (1983). The urea-stripped ROS membranes containing rhodopsin were stored at −70 °C in 20 mM Tris, pH 7.4, 1 mM EDTA, and 0.5 mM DTT.

Kinetic Measurements. In order to investigate the properties of transducin's GTP-binding domain and exchange process, the nonhydrolyzable analogue GTPγS was employed. Use of this guanine nucleotide avoids possible interference from the GTPase activity native to transducin's α subunit. The time course of binding of [^{35}S]GTPγS to transducin was monitored by rapid filtration. A 300-μL assay mixture contained appropriate concentrations of transducin and [^{35}S]GTPγS, (1.5–2.0) × 10^5 cpm/pmol, in 10 mM Tris, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and 0.1 mM EDTA. Following the rapid addition of transducin, 50-μL aliquots were withdrawn at timed intervals, filtered through nitrocellulose filters (Schleicher & Schuell, BA85), and immediately washed with two 3.5-mL aliquots of ice-cold buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA). The amount of [^{35}S]GTPγS associated with the filters was measured in a Beckman LSC. It was found that 80% of the transducin present bound to the nitrocellulose, and this correction factor was incorporated into all calculations. Less than 0.01% of the bound cpm could be attributed to background [^{35}S]GTPγS associated with the filter. Initial rates of [^{35}S]GTPγS binding were determined from the slopes of [^{35}S]GTPγS bound vs. time, representing that portion of the progress curve that was linear, obtained within a time period in which no more than 10% of the maximum extent of reaction occurred.

Calculations. Initial rates were determined as described above for the reaction

$$\text{transducin} + \text{GTP}\gamma\text{S} \xrightarrow{k_f} \text{transducin–GTP}\gamma\text{S}$$

The rate equation for this reaction may be written in terms of initial velocity v_0:

$$v_0 = \frac{\text{d[transducin–GTP}\gamma\text{S]}}{\text{d}t} = k_f[\text{transducin}]^q[\text{GTP}\gamma\text{S}]^r$$

where $q + r = n$, n representing reaction order, and k_f indicates the rate constant for the reaction. In linear form

$$\ln v_0 = \ln k_f + q \ln [\text{transducin}] + r \ln [\text{GTP}\gamma\text{S}]$$

Accordingly, the parameters k_f, q, and r were calculated from the slopes and intercepts determined for plots of ln v_0 vs. ln [transducin] or ln [GTPγS], with respect to constant concentration of the appropriate reaction component (see, for example, Figures 3 and 4).

Other Methods and Reagents. Sucrose density gradient centrifugation experiments were accomplished with a 4.5-mL linear 5–20% gradient in 10 mM Tris, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and 0.1 mM EDTA. Samples containing protein of interest along with marker proteins (catalase, $s_{20,w}$ = 11.3 S; BSA, $s_{20,w}$ = 4.31 S; cytochrome *c*, $s_{20,w}$ = 1.71 S) were overlayed and subject to centrifugation at 40000 rpm for 13 h in a Beckman Ti 50.1 rotor. Following fractionation into 250-μL aliquots, samples were analyzed by SDS–PAGE and by densitometry of the resultant Coomassie-staining pattern. Gel filtration over Sephacryl S-300 (Pharmacia) was accomplished with a 1.5 × 80 cm column at a flow rate of 1 mL/min. The buffer employed in these studies contained 10 mM Tris, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and 0.1 mM EDTA. Void volume was measured by monitoring the migration of Blue Dextran; total volume was obtained with ^3H_2O. The column was calibrated with the following standards with their respective Stokes radii: chymotrypsinogen, 20.9 Å; BSA, 35.5 Å; catalase, 52.2 Å; β-galactosidase, 69.1 Å. One-milliliter fractions were collected, and protein content was assayed by the method of Bradford (1976) as well as by SDS–PAGE. GTPγS binding in the presence of rhodopsin was measured by the filtration assay described above in order to monitor the elution of transducin and its α subunit. Immunoblots were prepared with reagents and protocols described by Weiss et al. (1987).

Transducin and rhodopsin were obtained from frozen, dark-adapted bovine retinas purchased from J. Lawson and Co., Lincoln, NE. [^{35}S]GTPγS was from New England Nuclear, and GTPγS was obtained from Boehringer-Mannheim. All other materials were of reagent grade and purchased from Sigma.

Results

Several investigators have studied the guanine nucleotide binding of transducin mediated by rhodopsin (Godchaux & Zimmerman, 1979; Fung & Stryer, 1980; Fung et al., 1981; Baehr et al., 1982; Fung, 1983). Fung and Stryer (1980) observed that GDP, bound to transducin, is released in ex-

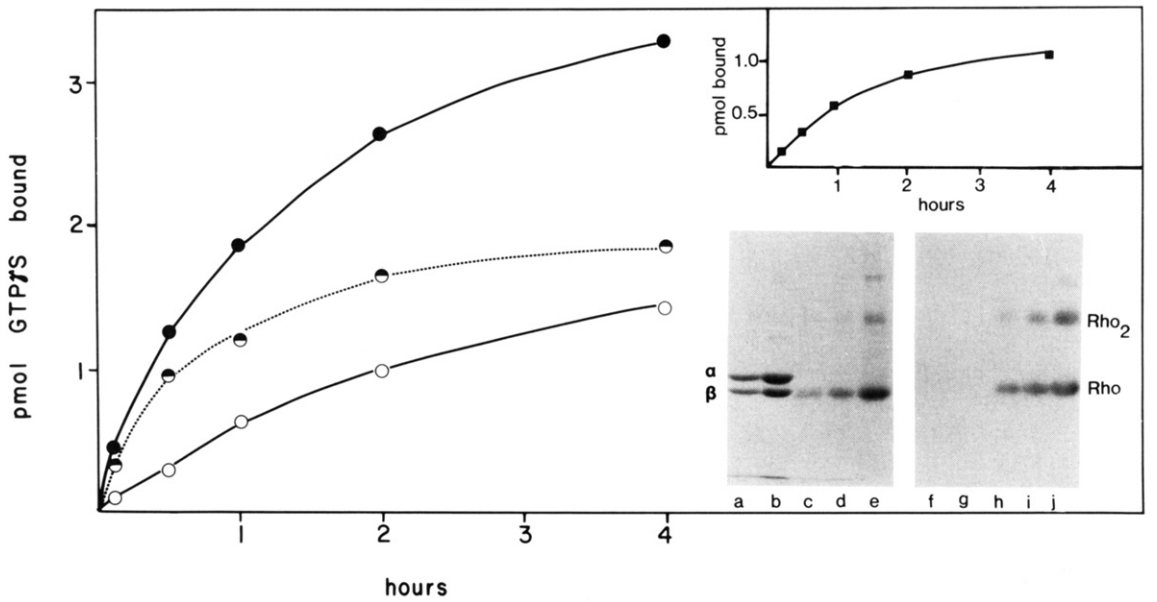

FIGURE 1: Time course of GTPγS binding. A 1-mL assay mixture was prepared containing 0.5 μM GTPγS and 0.05 μM transducin in the absence (○) or presence (●) of 5.4 nM rhodopsin. The binding reaction was initiated by the addition of GTPγS, and at the times indicated, 100-μL aliquots were withdrawn, filtered, and washed as described under Materials and Methods for kinetic measurements. The amount of GTPγS bound in the absence of rhodopsin was deducted from that determined in its presence to yield the amount bound to transducin due to interaction with the photorectpor (◓). Data represent the average of duplicate determinations. Also shown is the time course of GTPγS binding of hexylagarose-purified transducin (■), measured in the absence of rhodopsin under similar experimental conditions. Inset shows SDS–PAGE and immunoblot of preparations used in this study. Lanes a–e demonstrate the Coomassie staining pattern of transducin, 5 μg (a) and 25 μg (b), and UROS (urea-stripped rod outer segment membranes), 2 μg (c), 10 μg (d), and 20 μg (e). Protein was electrophoresed on a 10% polyacrylamide gel; indicated in the figure are rhodopsin, rhodopsin dimer, and the α and β subunits of transducin (the γ subunit does not resolve on this gel system). After electrophoresis, equivalent lanes were transferred to nitrocellulose by electroblotting at 380 mA for 20 min. The blot was incubated with a 1:100 dilution of antibodies (F_6) prepared against rhodopsin in Tween–PBS, it was washed, and ^{125}I-protein A was added. Immunoreactivity was detected by autoradiography as shown by lanes f–j, which correspond directly to lanes a–e. Presented is a 4.5-h autoradiograph; a series of exposures for longer times showed no evidence of the presence of rhodopsin in transducin preparations.

change for GTP in the presence of the photoreceptor, rhodopsin. Our own studies demonstrated that rhodopsin catalyzes this guanine nucleotide exchange reaction by a double-displacement mechanism (Wessling-Resnick & Johnson, 1987). However, the binding and exchange of guanine nucleotides by purified transducin independent of rhodopsin have not been characterized in detail. Figure 1 shows a comparison of the time course of GTPγS binding by transducin in the absence and presence of rhodopsin. As shown in this figure, transducin binds GTPγS in a time-dependent manner in the absence of the rhodopsin, albeit at a much slower rate. Also shown in Figure 1 is the difference between these two curves, which indicates rhodopsin-stimulated binding. For this experiment, we chose a minimal concentration of rhodopsin (5.4 nM) required to accelerate binding. This represents $^1/_{10}$ of the concentration of transducin employed in the assay. In order to verify that the GTPγS exchange observed in the absence of rhodopsin is due to the native capacity of the G protein to undergo this reaction and is not due to the presence of contaminating rhodopsin, immunoblots were performed on the transducin preparation with anti-rhodopsin antibodies (see inset). Overexposure of the autoradiograph for longer times (48 h) did not show any indication of the presence of rhodopsin in transducin preparations. Immunoblots performed with antibodies raised against synthetic peptides corresponding to specific rhodopsin sequences (Weiss et al., 1987) also failed to detect contaminating rhodopsin (not shown). The limits of detection by immunoblotting indicate that contaminating rhodopsin would be present at less than 0.1% of the transducin concentration. Furthermore, the inset of Figure 1 demonstrates the noncatalyzed GTPγS binding activity of hexylagarose-purified transducin, representing the native capacity of transducin to exchange guanine nucleotides. Finally, analysis of the kinetics of this reaction is consistent with the fact that the binding of GTPγS to transducin represents a noncatalyzed exchange reaction (see Figures 3 and 4 below).

The observation that transducin undergoes receptor-independent exchange is analogous to what has been reported for the GTP-binding proteins of the hormone-sensitive adenylate cyclase system. It has been observed that the receptor-independent exchange of guanine nucleotides by these other G proteins exhibits a marked dependency on Mg^{2+}; that is, the rate of guanine nucleotide binding by G_s and G_i is stimulated by the presence of Mg^{2+}. Since dissociation of the α subunits from the βγ subunits of the G protein occurs concomitant with binding, Gilman and collaborators have postulated that the action of Mg^{2+} is to promote the release of the α subunit, which subsequently has an enhanced capacity to bind GTPγS (Northup et al., 1983; Katada et al., 1984; Gilman, 1984). Taking this idea into consideration, the properties of guanine nucleotide exchange by transducin were assessed by measuring initial rates of GTPγS binding as a function of [Mg^{2+}]. Figure 2 shows that, in contrast to what has been characterized for other G proteins, we observed that the rate of guanine nucleotide binding displayed little or no dependency on Mg^{2+} either in the presence or in the absence of rhodopsin (Figure 2, inset). Measurements obtained in the presence of excess EDTA indicate that Mg^{2+} is not required for binding. Furthermore, the time course of GTPγS binding in the presence of rhodopsin appears unaltered at concentrations between 1 and 50 mM Mg^{2+} as demonstrated by the results shown in Figure 2. No effect was observed on the extent of GTPγS binding at these concentrations. It is interesting to note that agonist-promoted guanine nucleotide exchange of G_s, when-reconstituted with the β-adrenergic receptor in a lipid bilayer, is also independent of [Mg^{2+}] (Asano et al., 1984).

Yamanaka et al. (1985) have found that Mg^{2+} is required for the GTPase activity of transducin; presumably, this is due

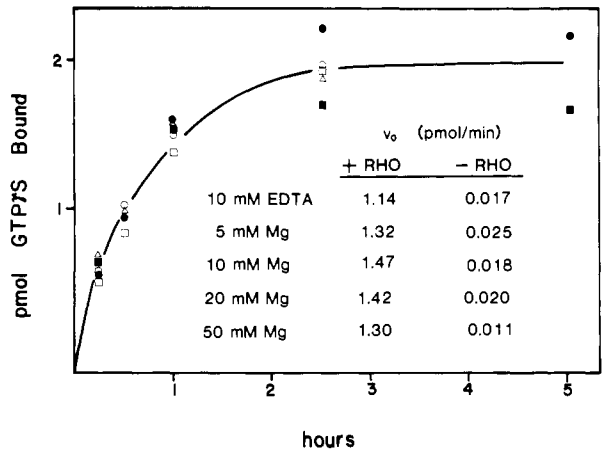

FIGURE 2: Effect of Mg^{2+} on the time course of GTPγS binding. The time course of rhodopsin-catalyzed binding to transducin was determined essentially as described for Figure 1, with the exception that measurements were accomplished with the following concentrations of Mg^{2+}: 1 (●), 5 (○), 10 (□), 20 (■) and 50 mM (△). Inset presents data from a separate experiment in which initial rates (v_o) of GTPγS exchange were measured in the presence or absence of 25 nM rhodopsin as indicated, under the experimental conditions shown.

Table I: Summary of Sedimentation Velocity Studies[a]

addition	$s_{20,w}$ (S)
5 mM $MgCl_2$	
$\alpha\beta$	4.23 ± 0.25
α	3.42 ± 0.37[b]
$\beta\gamma$	4.04 ± 0.20[b]
20 mM $MgCl_2$	
$\alpha\beta\gamma$	4.23 ± 0.32
20 mM $MgCl_2$ + 100 μM GTPγS	
α	3.45 ± 0.07
$\beta\gamma$	3.90 ± 0.14
10 mM EDTA + 100 μM GTPγS	
α	3.23 ± 0.04
$\beta\gamma$	4.15 ± 0.07

[a] Measurements were performed with holotransducin except where indicated. Measurements of $s_{20,w}$ were obtained from 5–20% (w/w) linear sucrose gradients in a buffer of 10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM DTT, 0.1 mM EDTA, and additions as indicated. Transducin was equilibrated in the appropriate buffers for 24 h at 4 °C. [b] Values determined for individual subunits, isolated and separated by chromatography over Blue Sepharose (see Materials and Methods).

to the participation of the divalent cation in the mechanism of GTP hydrolysis. In contrast to our results, these authors find that excess EDTA blocked rhodopsin-catalyzed binding of GTP to transducin and promoted the release of bound GDP. It is possible that the difference in these results reflect an alteration in the requirements for GTPγS binding. However, Yamanaka et al. (1985) do not directly address the question of whether the stimulatory effect of Mg^{2+} (2–20 mM) witnessed with other G proteins is also observed with GTP. Our results show that this effect is absent for transducin with respect to the binding of GTPγS.

As discussed earlier, it had been postulated that Mg^{2+} promotes the dissociation of the subunits of other G proteins, with a concomitant increase in the binding of guanine nucleotides to the released α subunit. Since the kinetics of GTPγS binding by transducin did not display any sensitivity to the presence of Mg^{2+}, we questioned whether or not the dissociation of α and βγ subunits occurred. Table I summarizes these results. Sucrose density gradient experiments performed in the presence of 20 mM Mg^{2+} showed no alteration in the sedimentation behavior of transducin as compared to that observed in the presence of 5 mM Mg^{2+}. We have found that transducin displays a shift in sedimentation only

Table II: Comparison of Guanine Nucleotide Binding Characteristics between Members of the G Protein Family

(A) Determined Values of K_d, the Dissociation Constant, for GTPγS Binding

	K_d (μM)	references
transducin, G_T	0.05–0.10	this paper
	0.05	Kelleher et al., 1986
G_s	0.7	Northup et al., 1982
	0.005–0.010	Asano et al., 1984
	0.10	Brandt & Ross, 1985
G_i	0.012	Bokoch et al., 1984
	0.032	Huff & Neer, 1986
G_o	0.027	Huff et al., 1985
	0.030	Huff & Neer, 1986

(B) Relative Affinities for Guanine Nucleotides Reported for G Proteins

	relative rank of affinity	references
transducin, G_T	GTPγS > GTP ≫ GppNHp > GDP > GMP	Kelleher et al., 1986; Yamanaka et al., 1986
G_s	GTPγS > GTP > GDP G Gpp (NH)p	Northup et al., 1982
G_i	GTPγS ≥ GTP ≥ Gpp(NH)p = GDP > GMP	Bokoch et al., 1984; Sunyer et al., 1984
G_o	GTPγS > GTP > GDP ≫ GMP	Sternweis & Robishaw, 1984

when GTPγS is present. These results indicate that Mg^{2+} alone does not affect the state of transducin subunit association. Indirect evidence presented by Kuhn (1981) and Deterre et al. (1984) in relation to Mg^{2+} effects on the properties of transducin are also consistent with this conclusion. Furthermore, kinetic measurements performed with purified T_α indicate a decreased ability to bind guanine nucleotides in the absence of $T_{\beta\gamma}$ (Fung, 1983; Wessling-Resnick & Johnson, 1987). Thus, it appears that although transducin can undergo guanine nucleotide exchange independent of receptor, structural characteristics allowing for the stimulatory effect of Mg^{2+} observed for other G proteins are absent. Finally, binding of GTPγS and dissociation of transducin's subunits appear to be independent of the presence of divalent cation.

Equilibrium binding measurements were obtained in the presence and absence of rhodopsin. Similar curves for the binding of GTPγS to transducin were obtained for both conditions, demonstrating that rhodopsin does not influence the affinity of transducin for guanine nucleotides. These results emphasized the catalytic role of rhodopsin during the guanine nucleotide exchange reaction. Values for the dissociation constant, K_d, of 0.05–0.10 μM were obtained in three independent experiments. Table II compiles similar data reported for other members of the G protein family as shown in part A. It is obvious from this comparison that the regulatory GTP-binding proteins all demonstrate similar affinities for GTPγS. The relative rank of affinities for guanine nucleotides that have been determined for G proteins are listed in part B of Table II. The similarities demonstrated by this comparison underscore the relationship between transducin and other members of this protein family.

Initial rates of GTPγS binding were measured at several fixed concentrations of transducin in order to investigate the kinetics of the guanine nucleotide exchange reaction. Figure 3 shows the linear plots of ln v_0 vs. ln [transducin] obtained at different levels of GTPγS, as indicated in the figure panels. These data serve to characterize the noncatalyzed exchange reaction. The lower right hand panel demonstrates the linear relationship between the intercepts of these plots and ln [GTPγS]. Conversely, Figure 4 presents the same results, with

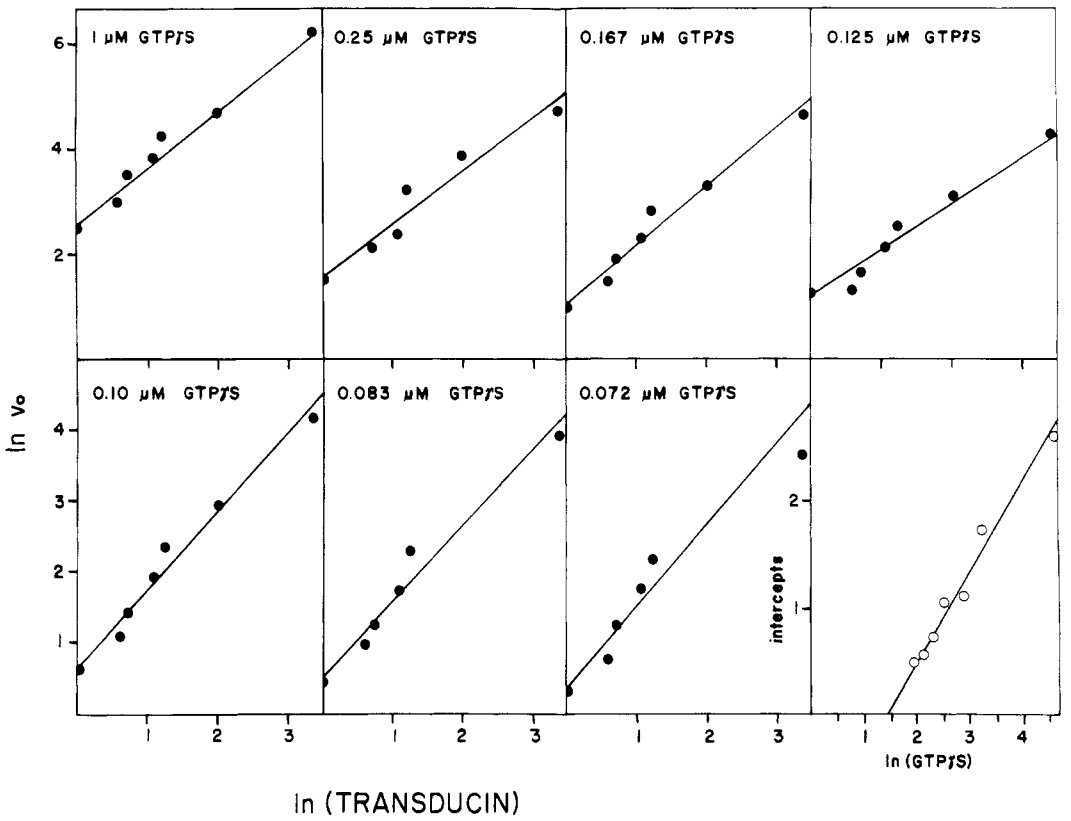

FIGURE 3: Plots of ln v_0 vs. ln [transducin] as a function of [GTPγS]. Initial rates (v_0) of GTPγS binding to transducin in the absence of rhodopsin were measured with the filtration assay detailed under Materials and Methods. A 300-μL reaction mixture contained the indicated concentrations of transducin, in the presence of different [GTPγS]. At 10-, 20-, 30-, and 60-s time points, 50-μL aliquots were withdrawn, filtered, and washed with ice-cold buffer, and the amount of GTPγS bound was determined. Initial rates were obtained from the slopes of time course plots from these data. Shown are the plots of ln v_0 (10^{-2} pmol/min) vs. ln [transducin] (10^{-2} μM) determined with levels of GTPγS as displayed; the lower right hand panel presents the plot of the intercepts of these lines as a function of ln [GTPγS] (10^{-2} μM). The linear relationship shown correlates well with the mathematical description of the guanine nucleotide exchange reaction as described under Calculations.

ln v_0 shown as a function of ln [$GTP_\gamma S$] at several concentrations of transducin. The inset of Figure 4 is the plot of the intercepts of these lines vs. ln [transducin]. From the latter relationship, and that shown in the lower right hand panel of Figure 3, and evaluation of k_f, the forward rate constant for binding, and n, the reaction order, may be made (for details please refer to Materials and Methods). Values of k_f between 1.7×10^7 and 2.7×10^7 M^{-1} s^{-1} were determined in three separate experiments, and the results of these experiments indicate a reaction order of 2; that is, a 1:1 stoichiometry is observed for the binding of GTPγS to transducin.

Several relevant conclusions may be drawn from characteristics of the relationship presented between v_0 and transducin. Our investigations of the enzymatic behavior in the rhodopsin-catalyzed guanine nucleotide exchange reaction revealed allosteric behavior with respect to transducin (Wessling-Resnick & Johnson, 1987). The linear relationship determined for ln v_0 as a function of ln [transducin] does not display any indication of similar behavior for the noncatalyzed reaction. This observation implies that the basis for the allosteric phenomenon resides in interaction between transducin and rhodopsin. The linear correlation demonstrated in Figures 3 and 4 is also indicative of the presence of a single population of binding sites for GTPγS. Under identical experimental conditions, purified T_α exhibits much slower exchange rates.[2] Since the presence of free T_α subunit would be detected due to its altered kinetics, this result implies that over the experimental range of concentration transducin remains as a stable heterotrimer (in the absence of GTPγS) with minimal dissociation of T_α from $T_{\beta\gamma}$. Finally, the linear correlation between ln v_0 and ln [GTPγS] as well as ln [transducin] is compatible with a dissociative-type mechanism; this is consistent with the double-displacement mechanism demonstrated for the rhodopsin-catalyzed guanine nucleotide exchange mechanism (Wessling-Resnick & Johnson, 1987).

Although rigorous kinetic analysis has not been reported on the nucleotide exchange mechanism for all of the G proteins, the demonstration of a dissociative-type mechanism for transducin is in good agreement with what is known. The second-order rate constants for G_i (10^6 M^{-1} s^{-1}) and the α subunit of G_o (10^7 M^{-1} s^{-1}) have been reported by Ferguson et al. (1986). These values are also comparable to what we observe for transducin (10^7 M^{-1} s^{-1}). The difference in the intrinsic rate constant between G_i and transducin may reflect contributions due to a Mg^{2+} effect, as discussed earlier. It is also possible, however, that this difference reflects alterations in the G proteins tertiary configuration, which contribute to the exchange process.

It is with respect to the physical nature of transducin that we sought to characterize its hydrodynamic properties. Sucrose density gradient experiments were performed with transducin as well as isolated T_α and $T_{\beta\gamma}$. Results presented in Table I show that holotransducin migrates at a greater rate relative to the individual subunits, T_α and the $T_{\beta\gamma}$ complex. The $s_{20,w}$ values determined from three separate experiments were as follows: transducin, 4.23 ± 0.25; T_α, 3.42 ± 0.37; $T_{\beta\gamma}$, 4.04 ± 0.20. To complement this study, gel filtration experiments were performed as described under Materials and Methods. Transducin was found to have a Stokes radius of

[2] M. Wessling-Resnick and G. L. Johnson, unpublished observations.

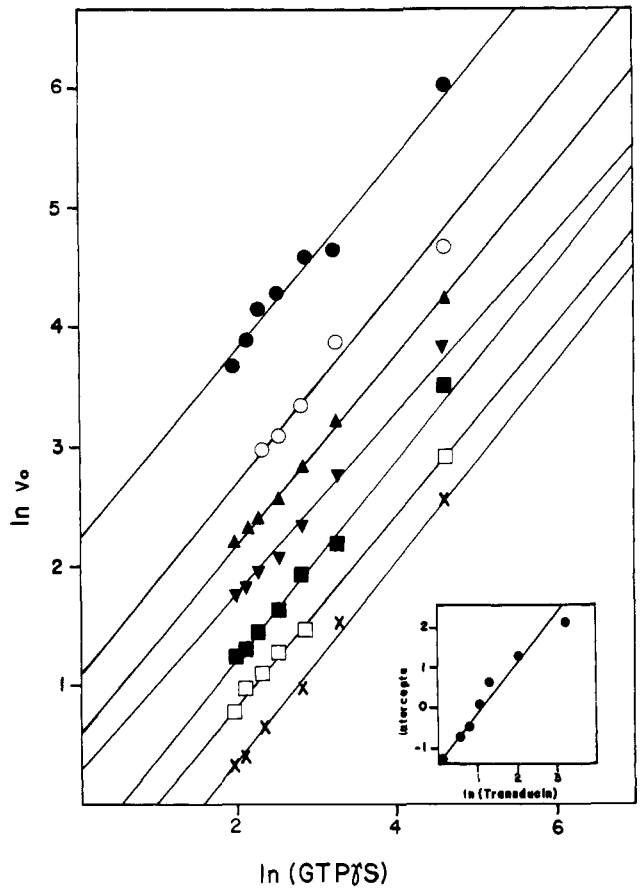

FIGURE 4: Plots of ln v_0 vs. ln [GTPγS] as a function of [transducin]. Data from Figure 5 are presented in the converse plotting form of ln v_0 (10^{-2} pmol/min) vs. ln [GTPγS] (10^{-2} μM) as measured with the following concentrations of transducin: 3 (●), 0.75 (○), 0.34 (▲), 0.30 (▼), 0.21 (■), 0.19 (□) and 0.10 μM (×). Inset shows the intercepts of these lines as a function of ln [transducin] (10^{-2} μM). The linear relationships demonstrated above, along with the data presented in Figure 5, indicate a dissociative-type mechanism for transducin's guanine nucleotide exchange process.

37.5 Å, and T_α migrated with a Stokes radius of 24 Å. $T_{\beta\gamma}$ eluted in a position similar to that observed for T_α.

Table III summarizes the hydrodynamic data in comparison with results reported for other G proteins. The experimental data we obtain for T_α and $T_{\beta\gamma}$ are in close agreement with the results of gel filtration on Bio-Gel P-100 reported by Fung (1983), which yielded molecular mass values of 41 and 43 kDa, respectively. The hydrodynamic parameters we measure for holotransducin yield a molecular mass value of 68 kDa, well below the expected value of ~80 kDa. However, this value is in quite close agreement with the molecular mass estimation of 70 kDa from small-angle neutron scattering measurements reported by Deterre et al. (1984). Taken together, these results indicate that the heterotrimeric form of transducin has hydrodynamic characteristics, suggestive of a compact shape relative to other globular proteins. By comparison, the information obtained for other G proteins is necessarily from detergent solutions, with a correction made for detergent binding. It is interesting to note that molecular mass values for G proteins that were determined in cholate solutions (see Table III) may actually be overestimated since no correction for detergent binding may be made. As transducin is the only soluble protein in the G protein family, the data we report here might imply that complications arising from detergent may obscure similar anomolous behavior for G_i, G_s, and G_o.

Baehr et al. (1982) have reported the results of nondenaturing gel electrophoresis and analytical ultracentrifugation

Table III: Comparison of Hydrodynamic Parameters Reported for Purified G Proteins

	$s_{20,w}$ (S)[a]	Stokes radius (Å)	mass[b]	references
transducin, G_T, bovine retina	4.23	37.5	68 000	present study
G_s				
rabbit liver[c]	3.9	40	68 000	Sternweis et al., 1981
turkey erythrocyte[c]	4.55	41.6	81 000	Hanski et al., 1981
human erythrocyte	4.11	59	96 000	Codina et al., 1984a,b
G_i				
rabbit liver[c]	4.31	44.3	82 000	Bokoch et al., 1983
human erythrocyte	4.09	56	96 000	Codina et al., 1984a,b
G_o				
bovine brain	4.2	47	80 000	Huff et al., 1985

[a] Except for transducin, $s_{20,w}$ values for other G proteins were obtained in Lubrol solutions unless otherwise indicated. Molecular mass values for these other proteins are listed with the appropriate correction made for other detergent binding (refer to references cited). [b] Molecular mass was calcd according to mass = $(6\pi N\eta_{20,w}/[1 - (\nu\rho_{20,w})])as_{20,w}$, where N is Avogadro's number, $\eta_{20,w}$ is the viscosity of water at 20 °C, $\rho_{20,w}$ is the density of water of 20 °C, and a is the Stokes radius. A value for partial specific volume, ν = 0.735, was employed in this calculation for transducin. [c] Measurements of $s_{20,w}$ performed in cholate solutions; since cholate has a density similar to that of protein, no estimate of detergent binding may be made (refer to references cited).

experiments. These investigators concluded that, in solution, transducin consists of a population of T_α dimers and $T_{\beta\gamma}$ dimers and tetramers. However, on the basis of the results presented here, we conclude that T_α and $T_{\beta\gamma}$ can be isolated in monomeric form and that the heterotrimeric species exhibits unique hydrodynamic characteristics that are perhaps indicative of an unusual configuration other than that displayed by simple globular proteins. The conditions under which possible oligomeric assembly may be functionally relevant remain to be determined, but it should be emphasized that transducin's physiological activity occurs in a membrane environment. Although hydrodynamic studies of transducin in solution are vital to understanding the biophysical nature of the protein in relation to other G proteins, it will also be necessary to explore these relationships as they pertain to the cell surface.

Discussion

The results presented here characterize the kinetic, physical, and hydrodynamic properties of transducin. Previously, these parameters have not been described, and it is of interest, therefore, to compare this information with properties that have been reported for other members of the family of G proteins. The dissociative-type guanine nucleotide exchange mechanism demonstrated for transducin is analogous to the behavior of other G proteins. Transducin's kinetic parameters and equilibrium binding properties are also comparable to values reported for G_s, G_i, and G_o. It is not surprising that transducin displays kinetic behavior and guanine nucleotide binding characteristics similar to those of the other members of this class of proteins because of the remarkable sequence homology between their α subunits (Itoh et al., 1986). Alterations in the properties of the G proteins must therefore reflect variations in the amino acid sequence between these proteins that affect their tertiary structure and characteristics of their regulation.

One of the most notable differences between transducin and

the other G proteins (G_o, G_i, and G_s) is the lack of any Mg^{2+} effect on the guanine nucleotide exchange reaction. Our results indicate that Mg^{2+} is not required for GTPγS binding and subunit dissociation. The acidic side chains of aspartic or glutamic acid have been demonstrated to be the major sites for Mg^{2+} interaction with proteins. For example, Asp-80 of EF-Tu has recently been shown to form a salt bridge with Mg^{2+} liganded to GDP situated in the guanine nucleotide binding site of the protein (Jurnak, 1985). A second regulatory Mg^{2+} binding site has been previously proposed for G proteins (Sternweis et al., 1981; Iyengar & Birnbaumer, 1982; Brandt & Ross, 1986). The data presented here indicate that this site is absent in transducin.

Examination of the α subunit primary sequences offers one rather striking domain in which a substitution of a neutral for an acidic amino acid occurs in transducin relative to the other G proteins. An alteration such as this could account for the specific loss of a Mg^{2+} binding site. In T_α, residue 162 is a glycine, whereas the corresponding residue is aspartic acid in $G_{s\alpha}$ (189), $G_{i\alpha}$ (167), and $G_{o\alpha}$ (123, partial sequence) (Itoh et al., 1986). This residue is located within one of the most highly conserved domains recognized between the α subunits of transducin (Pro^{161}-Ser^{186}), G_s (Ala^{188}-Gln^{213}), G_i (Ser^{166}-Thr^{191}), and G_o (Ala^{122}-Thr^{147}). Although other sites in the α subunit sequences must also be considered, this region is attractive since a structural basis for the different Mg^{2+} effects between the G proteins may be accommodated by the substitution of glycine for aspartic acid at this site in particular. This substitution occurs between consensus domains involved in the formation of the GTP-binding site; for transducin, these include Lys^{31}-Met^{49}, Phe^{195}-Arg^{201}, Ile^{217}-Ala^{225}, and Ser^{259}-Val^{269} (Medynski et al., 1985). Therefore, binding of Mg^{2+} to the aspartic acid in G_s, G_i, and G_o can easily be envisioned to induce conformational changes that would directly alter the properties of guanine nucleotide exchange. This effect would be lost in transducin because Gly^{162} would be unable to form an ionic bond with Mg^{2+}.

Evidence for conformational alterations induced by Mg^{2+} has been provided by sucrose density gradient ultracentirfugation experiments performed by Codina et al. (1984b). These investigators have shown that Mg^{2+} promotes the formation of a species of G_s and G_i that displays unusual sedimentation behavior, referred to as a "preactivated" form. The preactivated forms of G_s and G_i, in the presence of Mg^{2+}, subsequently display subunit dissociation when incubated with guanine nucleotides, as shown by sedimentation characteristics. In contrast, we do not observe any evidence for a preactivated form associated with transducin, as suggested by the lack of an effect by Mg^{2+} on the kinetics of GTPγS binding. However, we also observe that Mg^{2+} does not promote subunit dissociation; sucrose density gradient experiments with transducin show a shift in sedimentation only in the presence of GTPγS. Attention should be given to the physical properties of the preactivated forms of G_s and G_i, which migrate at a rate slower than expected for the heterotrimers, in comparison with our own hydrodynamic analysis, which yields a molecular mass value below the expected 80 kDa for transducin. Thus, it appears that Mg^{2+} promotes a physical association between $G_{s\alpha}$ and $G_{i\alpha}$ and their βγ subunits, which may be structurally analogous to heterotrimeric transducin; these forms are subsequently capable of exchanging guanine nucleotides.

Taken together, these results suggest that subtle structural differences between G proteins may produce profound alterations in the association of α and βγ subunits, affecting regulation, physical characteristics, and kinetic activities in response to Mg^{2+}. Our comparison of the similarities and differences between members of this class of proteins provides a rationale for future research in site-directed mutagenesis and synthetic peptide studies, as well as providing further understanding of the physical properties of transducin in relation to it function.

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Denaturation and Renaturation Studies of Benzo[*a*]pyrene Metabolite Modified DNAs†

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ABSTRACT: Evidence from absorbance, fluorescence, and circular dichroism (CD) measurements strongly suggests that adduct conformations at the binding sites are grossly different before and after thermal denaturation of (+)-*trans*-7,8-dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene [(+)-*anti*-BPDE] modified DNAs. This conclusion is reached through the following observations: (1) upon melting and cooling, the (+)-*anti*-BPDE-modified DNA exhibits pronounced hypochromic effects with concomitant spectral red shifts for the pyrenyl absorbance; (2) the pyrenyl CD spectrum reverses sign upon thermal denaturation–renaturation; (3) the fluorescence emission spectra resulting from excitations at 353 nm (10 nm to the red of hydrolyzed and unbound *anti*-BPDE) exhibit enhanced intensities and spectral red shifts for the thermally denatured and cooled adducts; and (4) in contrast to the absence of a shoulder prior to melting, the postmelt adducts exhibit a prominent 355-nm maximum (evidence of stacking interactions) in the excitation spectrum when 384–387-nm emission is monitored. Studies with synthetic polynucleotides further reveal that (+)-*anti*-BPDE-modified poly(dG)·poly(dC) exhibits the greatest nonreversible renaturation at the binding sites, possibly as a consequence of pyrenyl self-stacking. This, coupled with the previous findings that this polymer suffers the most extensive (+)-*anti*-BPDE modification, appears to suggest that $(dG)_n$·$(dC)_n$ regions may be responsible for such observed effects in native DNA.

Covalent modification of DNA by reactive metabolites is generally believed to be the initial critical event in the carcinogenesis of some polycyclic aromatic hydrocarbons (PAHs) (Harvey, 1981). There is strong evidence to suggest that the ultimate carcinogenic metabolite of benzo[*a*]pyrene (BP), the most widely studied PAH, is (+)-*trans*-7,8-dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene [(+)-*anti*-BPDE] (Brookes & Osborne, 1983; Conney, 1982; Newbold et al., 1979). This metabolite is strongly guanine specific as

† Research supported by USPHS Grant CA-42682 and by a subproject of MBRS Grant S06RR0892.

0006-2960/87/0426-4323$01.50/0