Stereochemistry of the Guanyl Nucleotide Binding Site of Transducin Probed by Phosphorothioate Analogues of GTP and GDP†

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ABSTRACT: The stereochemistry of the guanyl nucleotide binding site of transducin from bovine retinal rod outer segments was probed with phosphorothioate analogues of GTP and GDP. Transducin has markedly different affinities for the five thio analogues of GTP, as measured by their effectiveness in inhibiting GTPase activity, competing with GTP for entry into transducin, and displacing GDP bound to transducin. The order of binding affinities is GTPγS = (S_P)-GTPαS > (R_P)-GTPαS > (S_P)-GTPβS ≫ (R_P)-GTPβS. The affinity of transducin for GTPγS is $>10^4$ higher than that for (R_P)-GTPβS. These five analogues have the same relative potencies in eliciting the release of transducin from the membrane and in activating the phosphodiesterase. Transducin hydrolyzes (S_P)-GTPαS with a $1/e$ time of 55 s, compared with 28 s for GTP. In contrast, (R_P)-GTPαS, like GTPγS, is not hydrolyzed on the time scale of several hours. The order of effectiveness of thio analogues of GDP in displacing bound GDP is (S_P)-GDPαS > GDP > (R_P)-GDPαS > GDPβS. The affinity of transducin for (S_P)-GDPαS is about 10-fold higher than that for GDPβS. Mg^{2+} is required for the binding of GTP and GDP to transducin. Cd^{2+} does not lead to a reversal of stereospecificity at either the α- or β-phosphorus atom of GTP. These results lead to the following conclusions: (1) The *pro-R* oxygen atom at the α-phosphorus of GTP does not bind Mg^{2+} but instead interacts with the protein. (2) The *pro-S* oxygen at the α-phosphorus does not appear to be involved in a critical interaction with transducin. (3) In contrast, both the *pro-R* and *pro-S* oxygen atoms at the β-phosphorus of GTP are involved in key interactions with the protein or Mg^{2+}. (4) The α-phosphoryl groups of GDP and GTP bind similarly to transducin. Guanyl nucleotide binding proteins exhibit many patterns of stereoselectivity in their uptake and hydrolysis of thio analogues of GTP. Transducin resembles elongation factor Tu [Leupold, C. M., Goody, R. S., & Wittinghofer, A. (1983) *Eur. J. Biochem. 135*, 237–241], another protein that undergoes translocations coupled to interconversions between GTP and GDP forms, in its stereospecificity at the α-phosphorus of GTP but may differ at the β-phosphorus atom. An attractive prospect is that thio analogues can be used to probe the evolutionary kinship of guanyl nucleotide binding proteins.

Transducin is the signal-amplifying protein in retinal rod outer segments that carries the excitation signal from photoexcited rhodopsin to the cyclic-GMP phosphodiesterase (Fung et al., 1981; Stryer, 1983). This peripheral membrane protein consists of a 39-kDa α-subunit containing the guanyl nucleotide binding site, a 36-kDa β-subunit, and an 8-kDa γ-subunit (Kühn, 1980). Transducin cycles between an inactive T–GDP[1] form in the dark and an active T_α–GTP form following illumination. The exchange of GTP for GDP bound to transducin is catalyzed by R*. GTP binding leads to the dissociation of transducin into T_α–GTP and $T_{\beta\gamma}$. T_α–GTP then activates the phosphodiesterase by relieving an inhibitory constraint. The GTPase activity of the α-subunit takes transducin back to the inactive T–GDP state. The significance of transducin extends beyond vision. Transducin is structurally and mechanistically similar to the G proteins of the adenylate cyclase cascade of hormone-sensitive cells (Bitensky et al., 1981; Stryer et al., 1981; Gilman, 1984). Moreover, recent studies suggest that the p21 *ras* protein, which is encoded by the *ras* oncogene of RNA tumor viruses and its cellular counterpart (Gibbs et al., 1985), is a member of this family of signal-coupling proteins (Hurley et al., 1984). Interest in evolutionary relationships has been heightened by the finding of amino acid sequence similarities between procaryotic elongation factor Tu and the G protein family (Halliday, 1983; Leberman & Egner, 1984; Lochrie et al., 1985; Medynski et al., 1985; Tanabe et al., 1985). Indeed, the light-activated amplification cycle of rod outer segments is reminiscent of the elongation factor Tu-Ts cycle in bacterial protein synthesis (Kaziro, 1978). The controlled uptake and release of macromolecules coupled to GTP–GDP exchange and hydrolysis were perfected early in evolution and retained over several billion years.

The guanyl nucleotide binding site of transducin is central to its function. The conformations of the GTP and GDP states of transducin are markedly different, as expressed by their contrasting interactions with R* and the phosphodiesterase (Kühn, 1984; Stryer, 1983). The GTP and GDP forms also differ markedly in their accessibility to labeling reagents and

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[1] Abbreviations: ROS, rod outer segment; R, rhodopsin; R*, photoexcited rhodopsin or photoexcited rhodopsin in urea-stripped disc membranes; T, transducin; PDE, cyclic-GMP phosphodiesterase; EF-Tu, procaryotic elongation factor Tu; GDPαS, guanosine 5′-*O*-(1-thiodiphosphate); GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); GTPαS, guanosine 5′-*O*-(1-thiotriphosphate); GTPβS, guanosine 5′-*O*-(2-thiotriphosphate); GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); TEAB, triethylammonium bicarbonate; P_α, α-phosphorus atom of GTP or GDP; P_β, β-phosphorus atom of GTP or GDP; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate.

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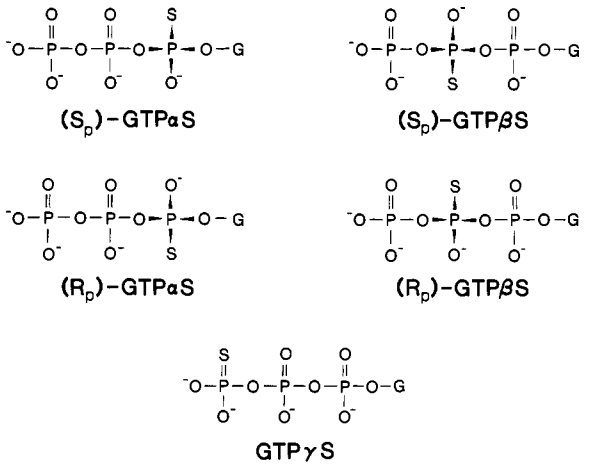

FIGURE 1: Absolute configurations of thio analogues of GTP.

proteolytic enzymes (Fung & Nash, 1983). Knowledge of the detailed mode of binding of GTP and GDP to transducin is essential for understanding how the state of the bound guanyl nucleotide governs the translocations of this signal-coupling protein. Phosphorothioate analogues of adenyl and guanyl nucleotides have been advantageously used to delineate the stereochemistry of nucleotide binding sites in a variety of proteins (Eckstein, 1983a,b, 1985). We were especially stimulated by studies of the stereochemistry of the GTP and GDP complexes of elongation factor Tu that made use of phosphorothioates (Goody & Leberman, 1979; Leupold et al., 1983; Wittinghofer et al., 1982). These analogues contain sulfur in place of a nonbridging oxygen atom in one of the phosphoryl units of GTP or GDP (Figure 1). Sulfur coordinates Mg^{2+} far less strongly than does oxygen. The van der Waals radius, charge distribution, acidity, and hydrogen-bonding properties of the phosphoryl group are significantly altered when oxygen is replaced by sulfur (Frey & Sammons, 1985). Substitution of sulfur for oxygen produces a chiral center at the α- and β-phosphorus atoms of GTP and at the α-phosphorus atom of GDP. The diastereomers are designated as having the S_P or R_P absolute configuration. Thus, there are five thio-GTP analogues [(S_P)-GTPαS, (R_P)-GTPαS, (S_P)-GTPβS, (R_P)-GTPβS, and GTPγS] and three GDP analogues [(S_P)-GDPαS, (R_P)-GDPαS, and GDPβS] (Burgers & Eckstein, 1978; Jaffe & Cohn, 1978; Bryant & Benkovic, 1979). We report here studies of the binding of these analogues to transducin and of their effectiveness in inducing subunit dissociation and activation of transducin in the presence of Mg^{2+} or Cd^{2+}. Our findings provide information concerning the stereochemistry of the guanyl nucleotide binding site of transducin and its degree of similarity to that of elongation factor Tu.

Materials and Methods

Materials. [α-^{32}P]GTP and [γ-^{32}P]GTP were from Amersham. [^{35}S]GTPγS was from New England Nuclear and $H_3{}^{32}PO_4$ was from ICN. GTPγS, GDPβS, and phosphoglycerate kinase/glyceraldehyde-3-phosphate dehydrogenase were from Boehringer Mannheim. (S_P)-GTPαS, (R_P)-GTPαS, (S_P)-GTPβS, (R_P)-GTPβS, (S_P)-GDPαS, and (R_P)-GDPαS were synthesized as described (Connolly et al., 1982). The GDP thio analogues were purified by DEAE-Sephadex A-25 chromatography. The (S_P)-GTPαS sample contained 9% R_P isomer, and the (R_P)-GTPαS sample contained <1% S_P isomer.

Protein Isolation. ROS were isolated from frozen bovine retinas (J. A. Lawson, Lincoln, NE) as described earlier (Fung & Stryer, 1980). Urea-stripped rhodopsin membranes devoid of transducin were prepared by sequentially washing ROS in the following solutions: (1) 5 mM Tris-HCl, pH 7.4, 1 M NH_4Cl, and 1 mM DTT; (2) 10 mM Tris-HCl, pH 7.4, 3 mM EDTA, and 1 mM DTT; (3) 20 mM Tris-HCl, pH 7.4, 4 M urea, and 1 mM DTT; (4) 20 mM Tris-HCl, pH 7.4, 120 mM NaCl, 30 mM KCl, 2 mM $MgCl_2$, and 1 mM DTT (buffer A). Membranes were suspended to a rhodopsin concentration of 5 μM for each wash and centrifuged at 17000g for 20 min (solutions 1 and 2) or 100000g for 30 min (solutions 3 and 4). The final membrane pellet was resuspended in buffer A to 50 μM rhodopsin and stored in aliquots at −70 °C. Fresh aliquots were used for each experiment and bleached just prior to use. Rhodopsin concentration in membranes was determined from the absorbance at 500 nm of a solution in 1.5% lauryl dimethylamineoxide, with a molar absorbance coefficient of 4×10^4 cm^{-1} M^{-1}. R* refers to photoexcited rhodopsin in these bleached membranes. Transducin was extracted from bleached ROS (Kühn, 1980), purified as previously described (Fung et al., 1981; Fung, 1983), and stored in 50% glycerol at −20 °C. Typical preparations were >95% pure as judged by SDS–polyacrylamide gel electrophoresis and Coomassie blue staining.

Synthesis of (S_P)-[γ-^{32}P]GTPαS. This radioactive analogue was synthesized by enzyme-catalyzed exchange of the γ-phosphoryl group of (S_P)-GTPαS with [^{32}P]P_i (Connolly et al., 1982; Romaniuk & Eckstein, 1981). The reaction mixture (100 μL) contained 50 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$, 100 μM (S_P)-GTPαS, 1 mM 3-phosphoglycerate, 0.1 mM NAD, 65 μg/mL phosphoglycerate kinase, 300 μg/mL glyceraldehyde-3-phosphate dehydrogenase, and 1.1 mCi of [^{32}P]P_i (carrier free). After 4 h at 24 °C, the mixture was loaded onto a column of DEAE-Sephadex A-25 (0.5 × 10 cm), eluted with a linear gradient of 15 mL each of 50 and 800 mM triethylammonium bicarbonate, pH 8.0 (TEAB), and washed with 800 mM TEAB. The (S_P)-[γ-^{32}P]GTPαS fractions eluted at 800 mM TEAB and were pooled and dried under a stream of dry N_2. The yield of radioactivity was 16%, and the specific activity was 19 Ci/mmol.

Assays. All assays involving the binding of radioactive nucleotides to transducin were performed in buffer A at 23 °C, unless otherwise noted. The uptake of [α^{32}P]GTP or [^{35}S]GTPγS by transducin was assayed by measuring the conversion of added nucleotide to a form that could be retained on a nitrocellulose filter (Millipore HA or Gelman GN-6). Transducin and membranes containing R* were combined in buffer A to the final concentrations described in each figure legend. Reactions were initiated by the addition of radioactive nucleotide. Aliquots (100 μL) were removed at the times indicated and quenched by dilution into 3 mL of ice-cold buffer A (minus DTT), followed by filtration through a 0.45 μm pore nitrocellulose filter and two 3-mL washes with ice-cold buffer. Filters were counted in 6 mL of Aquasol (New England Nuclear). Release of radioactive nucleotide was assayed in a similar manner. Transducin and R* were first mixed with [α-^{32}P]GTP to allow the nucleotide to be taken up. Nonradioactive nucleotides were then added, and the amount of the ^{32}P radiolabel remaining bound was measured.

GTPase assays were performed by measuring the release of [^{32}P]P_i from [γ-^{32}P]GTP (Abrams et al., 1974; Yamazaki et al., 1983). Reactions contained 0.1 μM transducin and 2 μM R* and were initiated by the addition of 1 μM [γ-^{32}P]GTP (5 Ci/mmol), either alone or in the presence of thio analogue. Reactions were quenched by removing 100-μL aliquots into 300 μL of a quench solution (5% activated charcoal, 10%

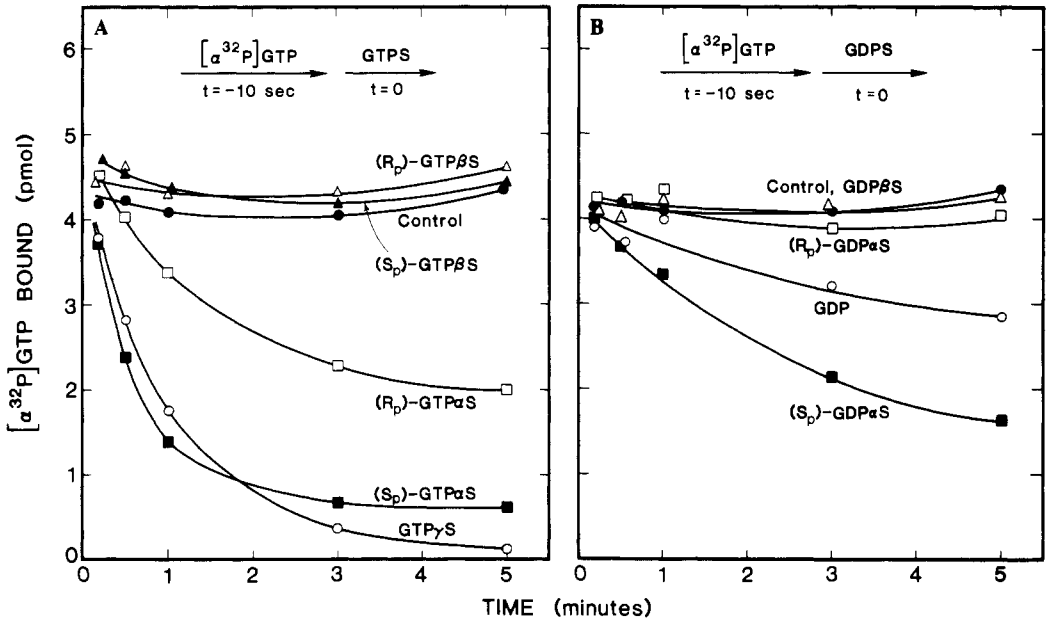

FIGURE 2: Effectiveness of thio analogues of GTP and GDP thio analogues in competing with GTP for binding to transducin. Transducin and R* were added to a reaction mixture to final concentrations of 0.15 and 2.0 μM, respectively. At $t = -10$ s, 1 μM [α-^{32}P]GTP was added, followed by the addition of 1 μM guanyl nucleotide thio analogue at $t = 0$. Aliquots (100 μL) were removed at the times indicated, and the amount of [α-^{32}P]GTP remaining bound was measured. (A) GTP thio analogues: control, no competing nucleotide (●), (S_P)-GTPαS (■), (R_P)-GTPαS (□), (S_P)-GTPβS (▲), (R_P)-GTPβS (△), and GTPγS (○). (B) GDP thio analogues: control, no competing nucleotide (●), (S_P)-GDPαS (■), (R_P)-GDPαS (□), and GDPβS (△).

trichloroacetic acid, 5 mM $NaPO_4$). The charcoal was removed by centrifugation, and 200 μL of the supernatant was counted in 3 mL of Aquasol (New England Nuclear). GTP hydrolysis was linear with time for the first 5 min. Single-turnover GTP hydrolysis rates were assayed by measuring the release of ^{32}P from GTP–transducin produced by binding a substoichiometric amount of [γ-^{32}P]GTP or [γ-^{32}P]-(S_P)-GTPαS to transducin in the presence of saturating R*. Reactions were initiated by the addition of nucleotides and quenched by removing 100-μL aliquots into 3 mL of ice-cold buffer, followed by filtration through nitrocellulose membranes.

Transducin-release experiments were performed by eluting 0.5 μM transducin from 10 μM R* membranes with varying concentrations of guanyl nucleotides. Solubilized transducin was separated from transducin bound to R* membranes by centrifugation in a Beckman airfuge (2 min at 30 psi at room temperature) and analyzed by sodium dodecyl sulfate gel electrophoresis.

Phosphodiesterase activity was assayed by using bleached ROS suspensions (containing 5 μM rhodopsin) with 2 mM cGMP as substrate. The buffer contained 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 8.0, 150 mM KCl, and 2 mM $MgCl_2$. Reactions were initiated by the addition of varying concentrations of guanyl nucleotides. The hydrolysis of cGMP was followed by monitoring the production of protons with a pH electrode (Liebman & Evanczuk, 1982). Complete hydrolysis of 2 mM cGMP changed the pH from 8.00 to 7.73.

RESULTS

Transducin Requires a Divalent Metal Ion for Nucleotide Binding. Magnesium ion (or another divalent metal ion, such as manganese) is known to be required for the catalytic activity of a wide variety of kinases, phosphatases, and phosphodiesterases (Mildvan, 1970). The effect of EDTA on the GTPase of transducin was measured to determine whether Mg^{2+} is also needed for the action of transducin. The GTPase activity of transducin was inhibited 50% by 0.25 mM EDTA when the total Mg^{2+} concentration was 0.2 mM. Under these conditions, the free Mg^{2+} concentration was <10 nM. EDTA at 1 mM was also found to block the R*-catalyzed binding of [α-^{32}P]GTP by transducin. Moreover, [α-^{32}P]GDP bound to transducin was released by the addition of 1 mM EDTA. These findings indicate that a divalent metal ion is required for the binding of GDP or GTP to transducin.

The effects of several divalent metal ions on transducin were measured. Both Mn^{2+} and Co^{2+} can substitute for Mg^{2+} in enabling GTP to be hydrolyzed. In contrast, Zn^{2+} and Cd^{2+} were inhibitory at 0.2 mM. Cd^{2+} was especially potent, inhibiting GTP hydrolysis by 50% when present at 15 μM.

Effect of Thio Analogues of GTP on the GTPase and GTP Binding Activities of Transducin. The interaction of thio analogues of GTP with transducin was surveyed by measuring their inhibitory effect on GTPase activity. The addition of 1 μM (S_P)-GTPβS or (R_P)-GTPβS had virtually no effect on the initial rate of hydrolysis of 1 μM GTP. In contrast, GTP hydrolysis was inhibited 63% by (S_P)-GTPαS, 17% by (R_P)-GTPαS, and 95% by GTPγS when 1 μM GTP was added together with 1 μM thio analogue.

The effectiveness of these thio analogues in competing with GTP for binding to transducin was then measured with an [α-^{32}P]GTP binding assay (Figure 2A). [α-^{32}P]GTP (1 μM) was added to transducin (0.15 μM) and R* (2 μM), followed 10 s later by thio analogue (1 μM). A high concentration of R* was used to ensure that the formation of R*–T would not be rate limiting. At the time of addition of thio analogue ($t = 0$), nearly all of the binding sites were occupied by [α-^{32}P]GTP. Hydrolysis of bound GTP on the time scale of about 30 s led to the formation of R*–T, which reacted rapidly with either the added thio analogue or [α-^{32}P]GTP. In this assay, the kinetics and extent of displacement of [α-^{32}P]GTP by thio analogue depend on their relative rates of entry and also on their relative rates of exchange and hydrolysis when bound. As expected from the GTPase assay, neither GTPβS isomer at 1 μM competed significantly with GTP in binding to transducin (Figure 2A). In contrast, (S_P)-GTPαS, like

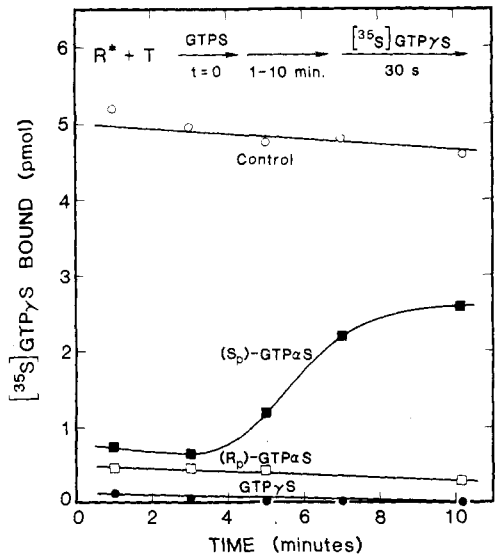

FIGURE 3: (S_p)-GTPαS is hydrolyzed by transducin. Transducin (0.13 μM) and R* (0.05 μM) were mixed with GTP thio analogues (0.25 μM) at $t = 0$ and incubated for times ranging from 1 to 10 min. Aliquots (80 μL) were removed, added to tubes containing 20 μL of [^{35}S]GTPγS (5.0 μM, 50 Ci/mmol), and thoroughly mixed. Reactions were quenched after 30 s, and the amount of [^{35}S]GTPγS bound was measured. Control, no nucleotide added prior to [^{35}S]GTPγS (○), (S_p)-GTPαS (■), (R_p)-GTPαS (□), and GTPγS (●).

GTPγS, was highly effective in competing with GTP. The extent of displacement by (R_p)-GTPαS was less, and its rate of entry into transducin was 3.5-fold slower than that with (S_p)-GTPαS or GTPγS. The order of effectiveness of these analogues in competing with GTP was

$$\text{GTP}\gamma\text{S} = (S_p)\text{-GTP}\alpha\text{S} > (R_p)\text{-GTP}\alpha\text{S} \gg (S_p)\text{-GTP}\beta\text{S} = (R_p)\text{-GTP}\beta\text{S}$$

A similar experiment was carried out for thio analogues of GDP (Figure 2B). The order of effectiveness in displacing bound [α-^{32}P]GDP was

$$(S_p)\text{-GDP}\alpha\text{S} > \text{GDP} > (R_p)\text{-GDP}\alpha\text{S} > \text{GDP}\beta\text{S}$$

The relative effectiveness of these GDP analogues parallels that of the GTP analogues.

(S_p)-GTPαS Is Rapidly Hydrolyzed by Transducin. The GTPase and competition binding assays described above showed that both isomers of GTPαS bind effectively to transducin. Are they hydrolyzed by tranducin? This question was answered by taking advantage of the fact that bound GTP (or the triphosphate form of an analogue) does not rapidly undergo nucleotide exchange, whereas bound GDP (or the diphosphate form of an analogue) does (G. Yamanaka and L. Stryer, unpublished results). A 2-fold excess of (S_p)-GTPαS or (R_p)-GTPαS was added to R*–T, followed 1–10 min later by the addition of a 5-fold excess of [^{35}S]GTPγS. After 30 s, the amount of [^{35}S]GTPγS bound to transducin was measured. (S_p)-GTPαS bound to T_α is not displaced in 30 s by GTPγS, whereas (S_p)-GDPαS is displaced during this interval. Hence, this assay reveals whether (S_p)-GTPαS was hydrolyzed during the preceding 1–10-min incubation period. As shown in Figure 3, (R_p)-GTPαS, like GTPγS, blocks the entry of subsequently added [^{35}S]GTPγS. This means that (R_p)-GTPαS, like GTPγS, is not hydrolyzed on the time scale of 10 min. In fact, this blocking effect of (R_p)-GTPαS persisted for at least 3 h (data not shown). In contrast, the inhibitory effect of (S_p)-GTPαS on the uptake of subsequently added [^{35}S]GTPγS decreased within a few minutes. This experiment showed that (S_p)-GTPαS is hydrolyzed in times of minutes, whereas (R_p)-GTPαS is not.

The rate of hydrolysis of (S_p)-GTPαS was then directly measured with (S_p)-[γ-^{32}P]GTPαS as substrate. This radioactive analogue was synthesized from nonradioactive (S_p)-GTPαS and [^{32}P]P_i by enzyme-catalyzed exchange reactions. Transducin and (S_p)-[γ-^{32}P]GTPαS were mixed in the presence of R* to form T–(S_p)-[γ-^{32}P]GTPαS. Aliquots were removed at intervals, and the amount of γ-^{32}P remaining bound to transducin was measured. This single-turnover assay gave a $1/e$ time of 55 s for the hydrolysis of (S_p)-GTPαS, about 2-fold slower than the value of 28 s for GTP (G. Yamanaka and L. Stryer, unpublished results).

Affinities of Thio Analogues of GTP and GDP for Transducin. The affinities of thio analogues of GTP and GDP for transducin were determined in equilibrium binding experiments that monitored the displacement of [α-^{32}P]GDP bound to transducin as a function of the concentration of thio analogue. T–[α-^{32}P]GDP was formed first and then mixed with varying concentrations of thio analogues in the presence of a catalytic amount of R*. A low concentration of R* was used to minimize the amount of R*–T and maximize the amount of T–[α-^{32}P]GDP. After 5 h, the amount of [α-^{32}P]GDP remaining bound to transducin was measured by a filter binding assay (Figure 4A). The equilibrium measured by this experiment is

$$\text{T–[}\alpha\text{-}^{32}\text{P]GDP} + \text{GTPS} \overset{\text{R*}}{\rightleftharpoons} T_\alpha\text{–GTPS} + T_{\beta\gamma} + \text{[}\alpha\text{-}^{32}\text{P]GDP}$$

Of the five thio analogues of GTP, GTPγS was most effective, displacing GDP half-maximally at a concentration of less than 0.1 μM, which corresponds to nearly stoichiometric binding. (R_p)-GTPαS was slighly less effective, followed by (S_p)-GTPβS, which binds less tightly by 1 order of magnitude. The weakest binding was exhibited by (R_p)-GTPβS. The affinity of (S_p)-GTPαS for transducin could not be measured in this experiment because it is rapidly hydrolyzed. The concentrations of thio analogue that gave half-maximal binding were <0.1 μM for GTPγS, 0.1 μM for (R_p)-GTPαS, 1.2 μM for (S_p)-GTPβS, and >100 μM for (R_p)-GTPβS.

The affinities of thio analogues of GDP for transducin were measured similarly (Figure 4B). The equilibrium measured in this experiment is

$$\text{T–[}\alpha\text{-}^{32}\text{P]GDP} + \text{GDPS} \overset{\text{R*}}{\rightleftharpoons} \text{T–GDPS} + \text{[}\alpha\text{-}^{32}\text{P]GDP}$$

(S_p)-GDPαS has a higher affinity for transducin than does GDP, whereas (R_p)-GDPαS and GDPβS have slightly lower affinities. The concentrations of thio analogue that gave half-maximal binding were 0.3 μM for (S_p)-GDPαS, 2 μM for (R_p)-GDPαS, and 3 μM for GDPβS, compared with 1.2 μM for GDP.

Elution of Transducin from R by Thio Analogues of GTP.* We next examine the effectiveness of the thio analogues of GTP in releasing transducin from illuminated disc membranes, where it is complexed to R*. Varying concentrations of thio analogue were added to membranes containing R*–T. The membranes were pelleted after 5 min, and the amount of tranducin in the supernatant was measured. The equilibrium measured by this experiment is

$$\text{R*–T} + \text{GTPS} \rightleftarrows \text{R*} + T_\alpha\text{–GTPS} + T_{\beta\gamma}$$

As shown in Figure 5, the amount of thio analogue required to elute half of the transducin was 0.4 μM for GTPγS, 0.8 μM for (R_p)-GTPαS, 1 μM for (S_p)-GTPαS, 60 μM for (S_p)-GTPβS, and >100 μM for (R_p)-GTPβS. A significant amount of (S_p)-GTPαS was hydrolyzed during the 5-min incubation of this experiment. We estimate that the concentration of (S_p)-GTPαS at the time of centrifugation was

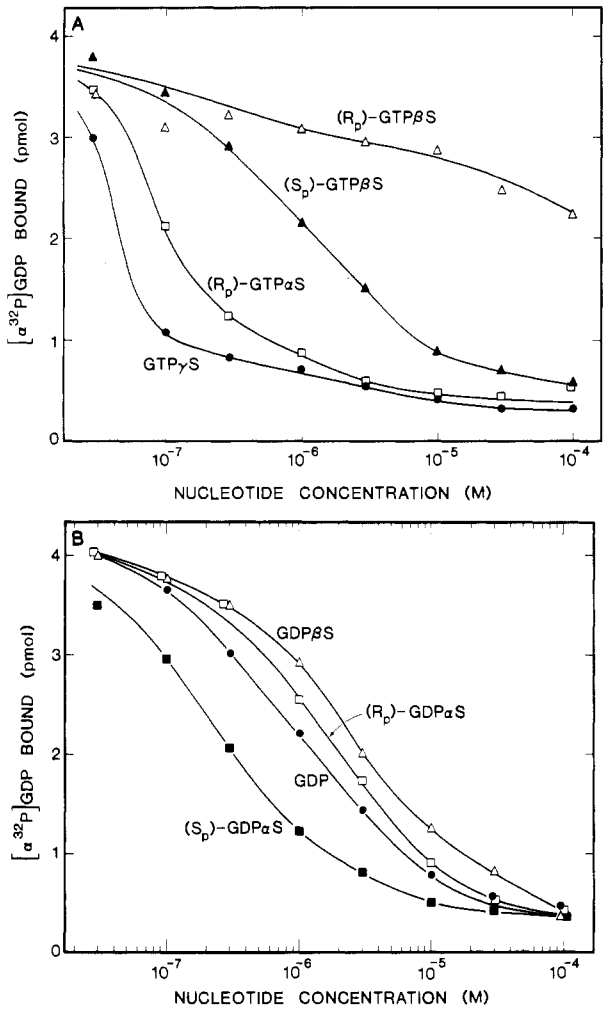

FIGURE 4: Relative affinities of GTP and GDP thio analogues for transducin. T-[^{32}P]GDP was first formed by mixing 0.4 μM transducin with 1.0 μM [α-^{32}P]GTP (20 Ci/mmol) in the presence of 0.2 μM R*. After a 2-h incubation to allow the transducin to take up and hydrolyze all of the [α-^{32}P]GTP, aliquots were removed and diluted 2-fold into solutions of guanyl nucleotide thio analogues. After a 5-h incubation period, sufficient for the system to come to equilibrium, the amount of [α-^{32}P]GDP remaining bound to transducin was measured. Each assay was performed in a total reaction volume of 100 μL. (A) GTP thio analogues: GTPγS (●), (R_P)-GTPαS (□), (S_P)-GTPβS (▲), and (R_P)-GTPβS (△). (B) GDP thio analogues: GDP (●), (S_P)-GDPαS (■), (R_P)-GDPαS (□), and GDPβS (△).

close to 0.5 μM. Hence, the order of effectiveness of these analogues in eluting transducin from R* is

GTPγS > (S_P)-GTPαS > (R_P)-GTPαS > (S_P)-GTPβS > (R_P)-GTPβS

Effect of Cd^{2+} and Other Divalent Cations on Transducin. The preceding studies showed that substitution of sulfur for oxygen at either the *pro-S* or *pro-R* position at the β-phosphorus atom markedly lowers the affinity of the nucleotide for transducin. A plausible reason is that one of these oxygen atoms might normally coordinate Mg^{2+}. Many kinases and nucleoside triphosphatases bind their substrates as β,γ-bidentate or α,β,γ-tridentate chelates with Mg^{2+} (Eckstein 1983a,b, 1985; Leyh et al., 1985). The outcome of substituting Cd^{2+} for Mg^{2+} on the effectiveness of a thio analogue can be used as a criterion of whether a particular oxygen atom in GTP coordinates Mg^{2+} (Jaffe & Cohn, 1978, 1979). The basis of this test is that Mg^{2+} chelates oxgyen 31 000 times more strongly than it does sulfur, whereas Cd^{2+} chelates sulfur 55 times more strongly than it does oxygen (Pecoraro et al., 1984). A reversal in the order of effectiveness of a pair of S_P and R_P isomers of a thio analogue in going from Mg^{2+} to Cd^{2+} points to a particular oxygen atom as a likely chelator of Mg^{2+}.

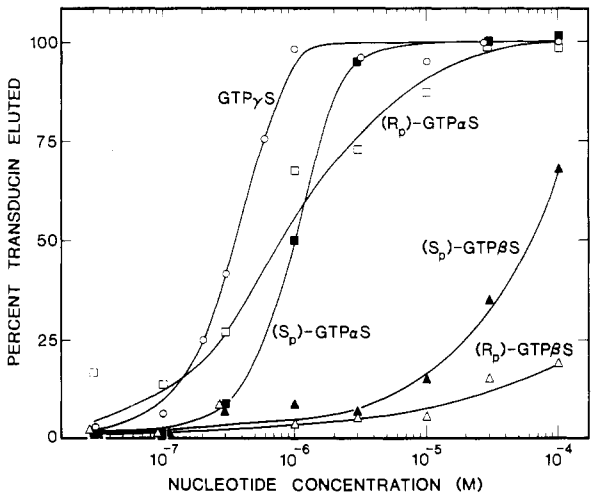

FIGURE 5: Relative effectiveness of GTP thio analogues in eluting transducin from R* membranes. R*-T was formed by mixing transducin (1.0 μM) with R* (20 μM) in the presence of 50 μg/mL carbonic anhydrase (added as an internal standard). Aliquots (50 μL) were removed and diluted 2-fold with GTP thio analogues. After 5 min, reactions were quenched by centrifugation in a Beckman airfuge (2 min at 30 psi), followed by immediate removal of 80 μL of the supernatant. Supernatants were examined on SDS gels with Coomassie blue staining and normalized against carbonic anhydrase (which did not interact with R* membranes). GTPγS (○), (S_P)-GTPαS (■), (R_P)-GTPαS (□), (S_P)-GTPβS (▲), and (R_P)-GTPβS (△).

We applied this Cd^{2+} test by using the transducin elution assay, which was chosen because Cd^{2+} does not interfere with the binding of transducin to R*. An experiment like the one shown in Figure 5 was carried out except that 0.2 mM Cd^{2+} was substituted for 2 mM Mg^{2+}. The resulting plots were similar to those depicted in Figure 5, except that the one for (R_P)-GTPαS was shifted to the right by a factor of 10. In other words, the Cd^{2+} chelate of (R_P)-GTPαS was 10-fold less effective in eluting transducin than was the Mg^{2+} chelate of this analogue. The substitution of Cd^{2+} for Mg^{2+} did not enhance the binding of either GTPβS isomer to transducin. Thus, a Cd^{2+}-induced reversal of stereospecificity at the α- or β-phosphorus atom was not observed with this elution assay. Likewise, Cd^{2+} did not lead to a reversal in the effectiveness of (S_P)-GTPβS and (R_P)-GTPβS in inhibiting the GTPase activity of transducin. In the presence of 600 μM thio analogue, the ratio of GTPase activity in the presence of S_P to that in the presence of R_P isomer was 0.4 when Mg^{2+} was present and 0.5 when Cd^{2+} was present. Cd^{2+} blocked the GTPase activity 90% in this assay but did not change the relative effectiveness of the S_P and R_P isomers of GTPαS in inhibiting GTPase activity.

Effectiveness of Thio Analogues of GTP in Activating the Phosphodiesterase. $T_α$-GTPγS, like $T_α$-GTP, is known to be a highly effective activator of the cGMP phosphodiesterase in rod outer segment membranes (Liebman & Pugh, 1982; Bennett, 1982). Our finding that (S_P)-GTPαS and (R_P)-GTPαS bind tightly to transducin and induce its elution from the membrane (Figures 4A and 5) suggested that these thio analogues can also promote the activation of the phosphodiesterase. This was tested by determining whether cyclic GMP hydrolysis, as evidenced by proton release, is initiated by the addition of thio analogue to illuminated membranes. As shown in Figure 6, the phosphodiesterase is rapidly activated on addition of (S_P)-GTPαS and remains active for about 1 min. The 1/*e* time for deactivation of the phosphodiesterase, due to hydrolysis of bound (S_P)-GTPαS, is 48 s. For GTP, the

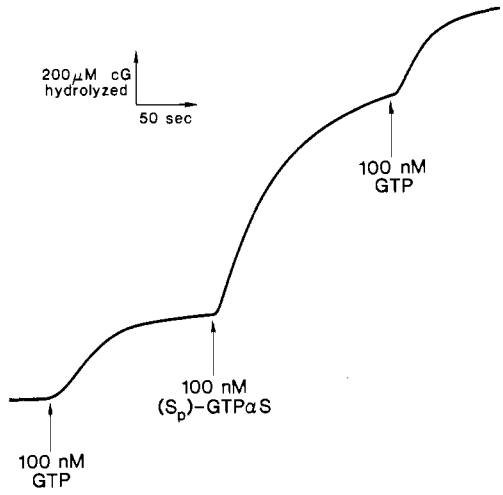

FIGURE 6: Activation of the cGMP phosphodiesterase upon addition of (S_P)-GTPαS (100 nM) to illuminate disc membranes (5 μM R*) containing transducin. The hydrolysis of cGMP (initial concentration of 2 mM) was followed by proton release. The activating effect of GTP (100 nM) is shown for comparison.

corresponding value is 22 s. These deactivation times are similar to hydrolysis times determined from measurements of the release of [^{32}P]P_i from [γ-^{32}P]GTP. The concentration of nucleotide required for 50% activation of the phosphodiesterase is about 0.1 μM for (S_P)-GTPαS, (R_P)-GTPαS, and GTPγS and 0.15 μM for GTP. As expected from the binding and elution experiments, (S_P)-GTPβS and (R_P)-GTPβS are much less effective in stimulating the phosphodiesterase. At 100 μM, 30% activation was obtained with (S_P)-GTPβS and only 10% with (R_P)-GTPβS. Thus, the effectiveness of the thio analogues in activating the phosphodiesterase parallels their affinity for transducin and their capacity to elute transducin from the membrane.

Discussion

Transducin has markedly different affinities for the five thio analogues of GTP. The order of binding affinities is

$$\text{GTP}\gamma\text{S} = (S_P)\text{-GTP}\alpha\text{S} > (R_P)\text{-GTP}\alpha\text{S} > (S_P)\text{-GTP}\beta\text{S} \gg (R_P)\text{-GTP}\beta\text{S}$$

The affinity of transducin for GTPγS is $>10^4$ times higher than that for (R_P)-GTPβS. These five analogues have the same relative potencies in inducing effector functions, namely, the release of the α-subunit of transducin from the membrane and the activation of the phosphodiesterase.

Magnesium ion (or another divalent metal ion, such as manganese) is required for the binding of GTP and GDP and for the hydrolysis of GTP by transducin, as shown by the inhibitory actions of EDTA. This fact is important in interpreting the interaction between transducin and the thio analogues. (S_P)-GTPαS most closely resembles GTP in its interactions with transducin. This analogue binds strongly to transducin and is hydrolyzed with a single-turnover time of 55 s, compared with 28 s for GTP. (R_P)-GTPαS binds less tightly than does (S_P)-GTPαS and is hydrolyzed very slowly if at all. This finding raised the question as to whether the *pro-R* oxygen at P_α coordinates Mg^{2+}. If so, the substitution of Cd^{2+} for Mg^{2+} would be expected to make the R_P isomer more effective than the S_P isomer of GTPαS. However, in the transducin elution assay, the replacement of Mg^{2+} by Cd^{2+} resulted in a weakening of the interaction of (R_P)-GTPαS with transducin. The stereoselectivity at P_α was not reversed, providing strong evidence that the *pro-R* oxygen at P_α does not coordinate Mg^{2+}. The lower affinity of (R_P)-GTPαS compared with (S_P)-GTPαS suggests that the *pro-R* oxygen at P_α interacts instead with groups on the protein. Furthermore, the striking resistance of (R_P)-GTPαS to hydrolysis indicates that substitution of sulfur for the *pro-R* oxygen at P_α produces conformational changes in the protein or triphosphate chain that are transmitted to the γ-phosphoryl group. In contrast, the *pro-S* oxygen at P_α does not appear to be involved in a critical interaction with transducin. This pattern of stereospecificity at P_α is like that of the proton-translocating ATPase from a thermophilic bacterium (Senter et al., 1983).

A different picture emerges for the β-phosphoryl group of bound GTP. Substitution of its *pro-S* oxygen lowers the binding affinity at least 30-fold. A larger effect, a greater than 1000-fold decrease in affinity, is produced by substitution of its *pro-R* oxygen atom. Hence, both the *pro-S* and *pro-R* oxygen atoms at P_β are involved in key interactions. Do they interact with Mg^{2+} or with groups on the protein? In two types of assays with transducin, no Cd^{2+}-induced reversal of stereospecifity at P_β was observed. Only two other nucleotide binding proteins, 3-phosphoglycerate kinase and carbamate kinase, are known to retain the same stereospecificity at P_β on replacement of Cd^{2+} for Mg^{2+} (Jaffe et al., 1982; Pillai et al., 1980). 3-Phosphoglycerate kinase is absolutely selective for the S_P isomer compared with the R_P isomer of GTPβS in the presence of Mg^{2+} or Zn^{2+} (Jaffe et al., 1982). In contrast to transducin, 3-phosphoglycerate kinase showed a reversal of stereospecificity at P_α upon substitution of Co^{2+} or Zn^{2+} for Mg^{2+}, indicating that the *pro-R* oxygen at P_α coordinates Mg^{2+} in this kinase. In transducin, it is evident that a P_α oxygen atom does not coordinate Mg^{2+}, but we are uncertain as to whether a P_β oxygen atom participates in chelation. The absence of a Cd^{2+}-induced reversal of stereospecificity indicates either that the phosphoryl group is not involved in coordinating a divalent metal ion or that protein interactions force the metal to bind to the same atom (oxygen or sulfur), irrespective of whether the metal is Mg^{2+} or Cd^{2+} (Jaffe et al., 1982).

The effectiveness of GTPγS in serving as a highly potent, hydrolysis-resistant analogue of GTP in interactions with the G protein (Cassel & Selinger, 1977) and transducin (Liebman & Pugh, 1982) is well-known. The high affinity of transducin for GTPγS shows that not all three oxygens at the γ-phosphorus participate in essential interactions. The freedom of rotation of the γ-phosphoryl group enables transducin (or Mg^{2+}) to select oxygen instead of sulfur in interacting with the γ-phosphoryl group of GTPγS. The resistance of GTPγS to hydrolysis is common to the G protein, transducin, and elongation factor Tu (Thompson & Karim, 1982).

For the thio analogues of GDP, the order of effectiveness of binding to transducin is

$$(S_P)\text{-GDP}\alpha\text{S} > \text{GDP} > (R_P)\text{-GDP}\alpha\text{S} > \text{GDP}\beta\text{S}$$

The affinity of transducin for (S_P)-GDPαS is about 10-fold higher than that for GDPβS. The tighter binding of (S_P)-GDPαS compared with (R_P)-GDPαS is like that observed for the triphosphate form of these thio analogues. This finding suggests that the α-phosphoryl groups of GDP and GTP bind similarly to transducin. GDPβS binds to transducin only 3-fold less tightly than does GDP. With the GTP analogues, substitution of sulfur for an oxygen atom at P_β, particularly the *pro-R* oxygen, has a much more deleterious effect. The sulfur atom of GDPβS is well tolerated, probably because it can rotate out of the way, as can the sulfur atom of GTPγS.

The interactions of thio analogues of GTP and GDP with bacterial elongation factor Tu have been studied in detail (Goody & Leberman, 1979; Wittinghofer et al., 1982; Leupold

et al., 1983). It is interesting to compare the stereospecificity of transducin with that of elongation factor Tu because of their functional resemblances. Both proteins undergo translocations that are coupled to interconversions between GTP and GDP forms. Furthermore, nucleotide exchange is catalyzed by another protein, R* in the case of transducin and EF-Ts for EF-Tu. The inhibition of GTPase activity of EF-Tu by thio analogues provides information concerning their relative binding affinities. In the presence of Mg^{2+}, the sequence of binding affinities to EF-Tu is (Leupold et al., 1983)

$$GTP\gamma S = (S_P)\text{-}GTP\alpha S > (R_P)\text{-}GTP\alpha S > (R_P)\text{-}GTP\beta S > (S_P)\text{-}GTP\beta S$$

Transducin resembles EF-Tu in having higher affinity for GTPγS and (S_P)GTPαS than for (R_P)-GTPαS. Both proteins have low affinity for (S_P)- and (R_P)-GTPβS. Transducin differs from EF-Tu in that it prefers (S_P)-GTPβS to (R_P)-GTPβS and does not exhibit a Cd^{2+}-induced reversal of stereospecificity at P_β. We conclude that the mode of binding of the β-phosphoryl group is significantly different in the two proteins. In contrast, the interactions at the α-phosphoryl group of transducin and EF-Tu appear to be similar. The *pro-S* oxygen atom at P_α is not involved in an important interaction with protein group or divalent metal ion, since both transducin and EF-Tu hydrolyze (S_P))-GTPαS at an appreciable rate. Finally, transducin and EF-Tu are alike in that GTPγS is bound about as tightly as is GTP but is hydrolyzed very much more slowly (Thompson & Karim, 1982; G. Yamanaka and L. Stryer, unpublished results).

It is evident that guanyl nucleotide binding proteins exhibit many different patterns of stereoselectivity in their uptake and hydrolysis of thio analogues of GTP (Jaffe et al., 1982; Eckstein, 1985). This diversity raises the possibility that thio analogues can be used to probe the kinship of proteins that bind GTP. A pertinent precedent here is that stereospecificities of pyridine nucleotide dehydrogenases with respect to the A and B sides of the nicotinamide ring are highly conserved in evolution (Popjak, 1970; Garavito et al., 1977; You, 1985). We think it likely that the steric relationship of protein groups that bind the triphosphate moiety of Mg–GTP and participate in its hydrolysis are also preserved over long evolutionary periods. The stereospecificities of the G proteins and the *ras* proteins will be informative in this regard. We expect that the G proteins will be most similar to transducin in its pattern of stereospecificity, the *ras* proteins less so, and procaryotic EF-Tu the least similar.

Registry No. (S_P)-GTPαS, 81570-51-6; (R_P)-GTPαS, 81570-50-5; (S_P)-GTPβS, 81570-53-8; (R_P)-GTPβS, 81570-52-7; GTPγS, 37589-80-3; GDPβS, 71376-97-1; GDP, 146-91-8; (R_P)-GDPαS, 71481-44-2; (S_P)-GDPαS, 71481-45-3; GTPase, 9059-32-9; GTP, 86-01-1; Mg, 7439-95-4; cGMP phosphodiesterase, 9068-52-4.

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Hydrodynamic Characterizations of Estrogen Receptors Complexed with [^{3}H]-4-Hydroxytamoxifen: Evidence in Support of Contrasting Receptor Transitions Mediated by Different Ligands†

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ABSTRACT: Size-exclusion high-performance liquid chromatography was used to characterize the hydrodynamic molecular properties of estrogen receptors complexed with estradiol and the antiestrogen 4-hydroxytamoxifen. Cytoplasmic estrogen receptors complexed with [^{3}H]-4-hydroxytamoxifen did not undergo reductions in hydrodynamic size after exposure to KCl or urea. Nuclear receptors complexed with 4-hydroxytamoxifen eluted as hydrodynamically larger molecules than nuclear receptors complexed with estradiol. Because identical hydrodynamic characterizations were obtained with the covalent ligand [^{3}H]tamoxifen aziridine, these differences in chromatographic behavior are due to differences in ligand-mediated receptor properties and are not the result of ligand dissociation. When estrogen receptors, complexed with either [^{3}H]estradiol or [^{3}H]-4-hydroxytamoxifen, were exposed to trypsin, the receptors complexed with 4-hydroxytamoxifen eluted as larger hydrodynamic forms than receptors complexed with estradiol. These observations are interpreted to indicate that estradiol and 4-hydroxytamoxifen mediate contrasting transitions in the molecular orientation of estrogen receptors. The consequences of the transitions mediated by 4-hydroxytamoxifen appear to be that intermolecular associations become difficult to disrupt with KCl or urea and that the accessibility of trypsin-sensitive proteolytic sites becomes altered. Chromatin fractionation using DNase I and hypotonic Mg^{2+} solubilization identified a chromatin region that was less readily penetrated by receptors complexed with 4-hydroxytamoxifen than receptors complexed with estradiol. This observation supports the hypothesis that one consequence of different ligand-mediated receptor transitions is that receptors become positioned distinctively in chromatin by agonistic and antagonistic ligands. We suggest that these transitions may be related to mechanisms that separate the actions of estrogen agonists and antagonists.

Estradiol enters the intact mouse uterus and associates with estrogen receptor proteins, which then interact with chromatin (Jensen et al., 1968; Shymala & Gorski, 1969). Some of these interactions appear to take place in a particular chromatin region, which previously has been identified as Mg^{2+}-soluble chromatin through DNase I mediated chromatin fractionation (Scott & Frankel, 1980; Pavlik & Katzenellenbogen, 1982). The action of estradiol can be antagonized by a series of typically nonsteroidal compounds that have a characteristic triphenylethylene structure and that are categorized as "anti-estrogens" (Clark & Peck, 1979; Katzenellenbogen et al., 1979). These antagonists compete with [^{3}H]estradiol for receptor binding sites. Some of these compounds are capable of activating receptors to varying degrees (deBoer et al., 1981; Katzenellenbogen et al., 1981) and of bringing about their retention in chromatin (Clark & Peck, 1979; Katzenellenbogen et al., 1979). Since antagonists ultimately cause receptor sites to be deposited in chromatin, it has so far been difficult to reconcile antagonism through the identification of peculiar or defective receptor–ligand interactions. However, it is well recognized that anti-estrogens promote a more lengthy retention of nuclear receptors than estrogens (Clark & Peck, 1979; Katzenellenbogen et al., 1979). The antagonist 4-hydroxytamoxifen has a high affinity for estrogen receptors (Borgna & Rochefort, 1981) and mimics estradiol with respect to many interactions within the mouse uterus. In this paper we have used SEHPLC[1] analyses to characterize the hydro-

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[1] Abbreviations: CD assay, dextran-coated charcoal assay; DES, diethylstilbestrol (α,α'-diethyl-4,4'-stilbenediol); DME, Dulbecco's modified Eagle's medium; DMF, dimethylformamide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; 17β-estradiol, estra-1,3,5(10)-triene-3,17β-diol; HAP, hydroxylapatite; P_{100} buffer, KH_2PO_4/K_2HPO_4 (100 mM), pH 7.0; SEHPLC, size-exclusion high-performance liquid chromatography; TAM, tamoxifen [1-[4-[2-(dimethylamino)ethoxy]-phenyl]-1,2-diphenylbut-1(*Z*)-ene]; TAMAZ, tamoxifen aziridine [(*Z*)-1-[4-[2-(*N*-aziridinyl)ethoxy]phenyl]-1,2-diphenylbuty-1(*Z*)-ene]; TOT, 4-hydroxytamoxifen [1-[4-[2-(dimethylamino)ethoxy]phenyl]-1-(4-hydroxyphenyl)-2-phenylbut-1(*Z*)-ene]; Tris, tris(hydroxymethyl)-aminomethane.

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