Catecholamine-Stimulated Guanosine 5'-O-(3-Thiophosphosphate) Binding to the Stimulatory GTP-Binding Protein of Adenylate Cyclase: Kinetic Analysis in Reconstituted Phospholipid Vesicles

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ABSTRACT: The stimulatory GTP-binding protein of adenylate cyclase, Gs, and β-adrenergic receptors were reconstituted into unilamellar phospholipid vesicles. The kinetics of the quasi-irreversible binding of guanosine 5'-O-(3-thiophosphate) (GTPyS) to Gs, equivalent to Gs activation by nucleotide, was studied with respect to the stimulation of this process by β-adrenergic agonists and Mg2+ . The rate of GTPyS binding displayed apparent first-order kinetics over a wide range of nucleotide, agonist, and Mg2+ concentrations. In the absence of agonist, the apparent first-order rate constant, $k_{app}$, was 0.17–0.34 min⁻¹ and did not vary significantly with the concentration of nucleotide. At 50 mM MgCl₂, $k_{app}$ increased somewhat, to 0.26–0.41 min⁻¹, and remained invariant with the nucleotide concentration. In the presence of agonist, $k_{app}$ was dependent on nucleotide concentration. At 10⁻⁹ M GTPyS, the addition of (-)-isoproterenol caused at most a 2-fold stimulation of $k_{app}$. However, $k_{app}$ measured in the presence of isoproterenol increased as an apparently saturable function of the GTPyS concentration, such that isoproterenol caused a 17-fold increase in $k_{app}$ at 1 µM GTPyS. The effect of isoproterenol on $k_{app}$ also appeared to saturate at high isoproterenol concentration, yielding a $k_{app} \approx 6$ min⁻¹ at high concentrations of both nucleotide and agonist. These data suggest that the receptor–agonist complex acts by increasing the rate of conversion of a lower affinity Gs–GTPyS complex to the stable activated state.

The activity of hormone-sensitive adenylate cyclase primarily reflects the extent of activation of the stimulatory GTP-binding protein, Gs. Activation of Gs occurs upon the high-affinity binding of GTP or a GTP analogue such as GTPyS, and is manifest as the ability of nucleotide-ligated Gs to bind to and stimulate the catalytic unit of adenylate cyclase. Activation is terminated either by the slow dissociation of nucleotide or, in the physiological case, by the hydrolysis of GTP to GDP. GDP does not activate Gs. Receptors for stimulatory hormones function by increasing the rate of activation of Gs by guanine nucleotides. This increase in the rate of activation increases the steady-state concentration of Gs–GTP or, in the case of nonhydrolyzed analogues, increases the rate at which the active Gs–nucleotide complex is formed [reviewed by Ross & Gilman (1980) and Smigel et al. (1984)].

The mechanism of activation of Gs by nucleotides has been addressed most thoroughly by Gilman's group using purified, detergent-solubilized Gs (Northup et al., 1982, 1983; Smigel et al., 1982). Gs is a dimer of a 45,000-Da (or 52,000-Da) GTP-binding α subunit and a 35,000-Da β subunit. These authors showed that the free α subunit–nucleotide complex is the active form of Gs. GTPyS binds to Gs and activates it by a slow reaction that is tightly coupled to the dissociation of the 35,000-dalton β subunit. The binding reaction is first order in Gs, and the observed first-order rate constant for binding, $k_{app}$, was found to vary less than 2-fold between 10⁻¹⁸ and 10⁻¹⁴ M GTPyS (Northup et al., 1982). These findings and other data led to the following proposed mechanism for

1 Abbreviations: Gs, stimulatory GTP-binding protein of hormone-sensitive adenylate cyclase; GTPyS, guanosine 5'-O-(3-thiophosphate); $k_{app}$, apparent first-order rate constant for GTPyS binding to Gs; Da, dalton; Heps 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

2 A 8000-Da polypeptide also frequently copurifies with Gs (Hildebrandt et al., 1984) and probably is a γ subunit, analogous to the γ subunit of transducin (Fung, 1983). When Gs dissociates, the β and γ subunits apparently remain associated.


the activation of Gs (Smigel et al., 1982, 1984) (N is nucleotide):

\[ aB \xrightarrow{k_1} \gamma \]

\[ a - N \xrightarrow{k_4} a \]

Either reaction 2 or reaction 4 is the first-order, rate-limiting step. Because \( k_{\text{app}} \) does not vary with the concentration of GTP\( \gamma \)S, either the forward rates of reaction 2 and 4 are equal or \( k_1 \) is not in the range of \( 10^6-10^8 \) M\(^{-1}\). Subunit dissociation, and therefore activation, is promoted by free Mg\(^{2+}\) also leads to the loss of total Gs activity. Thus, the measured extent of Gs activation and GTP\( \gamma \)S binding is highly dependent on the nucleotide concentration because the extent of binding reflects the competing reactions of nucleotide binding to the \( \alpha \) subunit and that subunit’s denaturation (Smigel et al., 1982). This behavior was also observed for Gs that had been reconstituted into phospholipid vesicles (Asano et al., 1984).

The hormonal regulation of Gs activation has been described in much less detail. Some of the best information on the stimulation of Gs activation comes from studies of the turkey erythrocyte \( \beta \)-adrenergic adenylate cyclase system by Levitizki’s group (see Tolkovsky (1983) for review). They stressed that agonist-stimulated activation by nonhydrolyzable GTP analogues is also an apparent first-order process (Tolkovsky et al., 1982) whose rate constant is proportional to the number of agonist-ligated receptors in the membrane (Tolkovsky & Levitizki, 1978). They inferred from these and other kinetic data that the agonist–receptor complex acts formally as a catalyst to promote the activation of multiple molecules of adenylate cyclase at an elevated rate that remains first order.

The adenylate cyclase system is quite complex, and its detailed analysis will be vastly simplified by focusing on the interaction of its purified components. Since receptors and Gs do not interact functionally in detergent solution, we have reconstituted purified Gs and partially purified (1,000–4,000-fold) \( \beta \)-adrenergic receptors with phospholipids to yield a liposomal system that expresses rapid, agonist-stimulated adenylate cyclase at an elevated rate that remains first order.

The data were fit by an unweighted linear least-squares formula. GTP\( \gamma \)S concentrations and the values of \( B_t \) were \( 10^8 \) (A) (24 fmol), \( 10^7 \) (B) (55 fmol), \( 10^6 \) (C) (82 fmol), and \( 10^5 \) M (D) (82 fmol).

dithiothreitol, 0.1 mM ascorbic acid, and varying amounts of [\( ^{35} \)S]GTP\( \gamma \)S, isoproterenol, and Mg\(^{2+}\) as shown in the text. Binding was assayed at 30 °C, and the binding reaction was initiated by the addition of warmed receptor–Gs vesicles to warmed assay medium. The reactions were quenched as described by Asano et al. (1984). The binding of GTP\( \gamma \)S to the vesicles was stable in the quenched reaction volumes for several hours at 0 °C. "Basal" binding is defined as that which occurs in the presence of 1 mM free Mg\(^{2+}\). Stimulation of binding by isoproterenol was also measured at this concentration of Mg\(^{2+}\). Stimulation of binding by Mg\(^{2+}\) was achieved with 50 mM MgCl\(_2\).

Binding rates were generally analyzed according to apparent first-order kinetics. Apparent first-order rate constants, \( k_{\text{app}} \), were obtained as the negative slopes of plots of ln \([ (B_t - B)/B_t ] \) vs. time, where \( B_t \) is the total amount of GTP\( \gamma \)S bound at long times (after the plateau was reached) and \( B \) is the amount of GTP\( \gamma \)S bound at individual shorter times. Values of \( B_t \) were the average of three samples in each experiment, but individual values of \( B \) were usually single determinations. Values of \( k_{\text{app}} \) were determined from unweighted least-squares fits of these data in the range of \([ (B_t - B)/B_t ] = 0.1-0.9. Reported values of \( k_{\text{app}} \) are the means from experiments performed by using at least two separate preparations of vesicles. Error bars in the figures indicate the standard deviations.

**Results**

The rate of slowly reversible GTP\( \gamma \)S binding to Gs in reconstituted receptor–Gs vesicles was investigated in the presence of various concentrations of GTP\( \gamma \)S, the \( \beta \)-adrenergic agonist isoproterenol, and Mg\(^{2+}\). As an example, Figure 1 demonstrates that, for the agonist-stimulated binding reaction, increasing concentrations of GTP\( \gamma \)S increased both the initial rate of binding and the final plateau level of bound ligand. This effect on the plateau level of binding appeared to saturate as would a freely reversible binding equilibrium (Asano et al., 1984). However, the apparent saturation is probably the result of the competition between the binding of ligand to Gs and the irreversible denaturation of Gs (Smigel et al., 1982; Asano et al., 1984). Because the total concentration of GTP\( \gamma \)S was generally much greater than that of Gs, binding was analyzed as a possibly pseudo-first-order reaction. First-order replot
The apparent first-order rate constant for GTPγS binding to reconstituted G, is either much below 1 mM or much above 50 mM [see Asano et al. (1984)]. In contrast to the basal and Mg2+-stimulated binding reactions, the rate of agonist-stimulated GTPγS binding was strongly influenced by the concentration of GTPγS (Figures 1 and 2). Figure 2 shows the effect of the GTPγS concentration in the presence or absence of 1 mM isoproterenol. At 1 mM GTPγS, the agonist-stimulated value of \( k_{app} \) was 0.41 ± 0.07 min⁻¹, rather low and only 2-fold greater than the basal \( k_{app} \), 0.17 ± 0.05 min⁻¹. Increasing concentrations of GTPγS increased the isoproterenol-stimulated value of \( k_{app} \) to an observed maximum of 4.5 ± 0.5 min⁻¹ at 1 mM GTPγS. Thus, while the agonist-stimulated binding reaction is clearly dependent on the GTPγS concentration, the nonlinear relationship between \( k_{app} \) and the concentration of GTPγS indicates that it is not a simple, second-order bimolecular reaction either (Figure 2). For a bimolecular binding reaction where one reactant (GTPγS) is in excess, the rate should be pseudo first order in the other reactant (G,). The pseudo-first-order rate constant should be proportional to the concentration of the reactant that is in excess. This is clearly not the case, and \( k_{app} \) appears to saturate at high nucleotide concentrations. The agonist-stimulated binding reaction has not yet been studied in detail at higher concentrations of GTPγS because nonspecific binding becomes large and because the high rates are not measurable by manual procedures. However, the data shown here suggest that agonist-stimulated GTPγS binding behaves formally as a reaction that is first order in G, but whose rate constant, \( k_{app} \), increases as a saturable function of the GTPγS concentration.

If the estimated initial rate of binding, rather than \( k_{app} \), was considered as a function of GTPγS, the basal rate increased in parallel with the plateau amount of GTPγS bound. The data were consistent with the relationship initial rate = \( k_{app}B_t \). This relationship held for isoproterenol-stimulated binding as well. Because agonist increases \( B_t \), (Asano et al., 1984) as well as \( k_{app} \), the relative stimulation of initial rate caused by agonist was greater than the relative effect on \( k_{app} \) shown in Figure 2.

The effect of the isoproterenol concentration on the GTPγS-binding reaction was also studied by using similar protocols. Isoproterenol increased the initial rate of GTPγS binding and also increased the maximal amount of ligand that was specifically bound to the vesicles (Asano et al., 1984). The initial rate of GTPγS binding to the receptor–G, vesicles appeared to be first order at all concentrations of agonist tested. At low nucleotide concentrations, there was little effect of agonist on the first-order rate constant for GTPγS binding.

### Table 1: Effect of the Concentration of GTPγS on MgCl₂-Stimulated GTPγS Binding

<table>
<thead>
<tr>
<th>[GTPγS] (M)</th>
<th>( k_{app} ) (min⁻¹)</th>
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<tbody>
<tr>
<td>10⁻⁹</td>
<td>0.26 ± 0.06 (n = 5)</td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>0.36 ± 0.05 (n = 4)</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>0.41 ± 0.13 (n = 4)</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>0.33 ± 0.05 (n = 4)</td>
</tr>
<tr>
<td>3 × 10⁻⁶</td>
<td>0.34 (mean of 0.23 and 0.45)</td>
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*The apparent first-order rate constant for GTPγS binding to receptor-G, vesicles was determined in the presence of 50 mM MgCl₂ as described in the legend to Figure 2. Data were obtained by using two or three different preparations of vesicles. The error statistic is the standard deviation. The values of \( k_{app} \) observed at 3 × 10⁻⁶ M GTPγS, while similar to those seen at other concentrations, are not highly reliable due to high nonspecific binding.
stimulated rate constants that have been observed for the activity of adenylate cyclase in membranes from S49 lymphoma cells (Ross et al., 1977), turkey erythrocytes (Tolkovsky & Levitzki, 1978), or rat liver (Birnbaumer et al., 1980a,b) or in a reconstituted system composed of crude G, and receptors from turkey erythrocytes (Gal et al., 1983). The high basal rate and the large hormonal activation that are observed in the reconstituted system can be taken to indicate either highly efficient reconstitution or the nonphysiological nature of the reconstituted vesicles. Clearly, a system that combines proteins from different tissues and different vertebrate classes in synthetic membranes is not, strictly speaking, physiological. However, the highest rates observed here for the activation of G, by GTPyS are no higher than those observed when native membranes are activated by an agonist plus GTP, a rapid process that is usually complete in less than 10 s.

The first-order kinetics of G, activation indicates most simply that the rate-limiting step in the process is not the bimolecular association of G, with GTPyS. The activation process that is observed in the vesicles can be explained generally in terms of the model described under the introduction that ascribes the first-order, rate-limiting step to a process closely linked to subunit dissociation (eq 1) (Smigel et al., 1982). We have not directly demonstrated such dissociation during activation of vesicle-bound G, but the data of Katada et al. (1984) suggest strongly that the subunits of G, do dissociate upon activation in native membranes.

The most intriguing aspect of our data is the interaction between agonist and nucleotide in regulating the rate of GTPyS binding. In the absence of agonist, the rate constant for activation was essentially invariant with nucleotide concentration (Table I and Figure 2), as is the case for soluble G, (Northup et al., 1982). In terms of eq 1, this might indicate that rapid GTPyS binding (reaction 3) is obligately preceded by the rate-limiting dissociation step (reaction 2). Alternatively, the αβ–N complex may form at higher concentrations of nucleotide, such that the $K_d \rightarrow K_a$ pathway becomes significant. The αβ–N complex would presumably be of low affinity and not detectable by the filtration assay used to measure bound [35S]GTPyS or by the reconstitution assay for functional G, activation. If the $K_a \rightarrow K_d$ pathway is significant, then the basal and Mg$^{2+}$-stimulated forward rate constants for reactions 4 and 2 must be virtually identical if $k_{app}$ is to be invariant with the GTPyS concentration. The finding that the rates of activation of G, by GTPyS and either Gpp(NH)p or Gpp(CH)$_2$p are different (Birnbaumer et al., 1980a) suggests that the $K_a \rightarrow K_d$ pathway.

In contrast to basal or Mg$^{2+}$-stimulated binding, the agonist-stimulated value of $k_{app}$ increased more than 10-fold with increasing concentrations of GTPyS and appeared to saturate. There was almost no effect of hormone at low concentrations of GTPyS ($10^{-9}$ M). It thus appears that increasing concentrations of GTPyS shift $k_{app}$ from a low, hormone-insensitive level to a higher, hormone-stimulated level. This suggests that receptor-mediated stimulation of the binding reaction takes place via the $K_d \rightarrow K_a$ pathway in which the equilibrium of reaction 1 is shifted from $\alpha \beta \rightarrow \alpha \beta - N$. The rate-limiting first-order step becomes reaction 4 rather than reaction 2. Reaction 4 appears to be the size of hormonal stimulation. We propose that the agonist–receptor complex acts by accelerating reaction 4 via a transient N–αβ–R–H complex (R–H; receptor–hormone). Since R–H can promote the activation of multiple molecules of G, (Asano et al., 1984; Pedersen & Ross, 1982), it evidently behaves formally as a catalyst of this reaction, and R–H need not be assumed to shift
the dissociation equilibrium of the G subunits. This conclusion agrees with the tendency of R-H to stabilize reconstituted G and to increase its apparent affinity for nucleotide (Asano et al., 1984).

While the mechanism we propose for the hormonal stimulation of G activation seems to be consistent with our kinetic and quasi-equilibrium data, it is still clearly speculative. Direct physical measurements of the receptor-G interaction and the dissociation of the subunits of G must be made in the vesicles, and the reconstitution of receptor with the resolved subunits of G will simplify this analysis.

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References


Variation of Transition-State Structure as a Function of the Nucleotide in Reactions Catalyzed by Dehydrogenases. 1. Liver Alcohol Dehydrogenase with Benzyl Alcohol and Yeast Aldehyde Dehydrogenase with Benzaldehyde†

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ABSTRACT: Primary intrinsic deuterium and 13C isotope effects have been determined for liver (LADH) and yeast (YADH) alcohol dehydrogenases with benzyl alcohol as substrate and for yeast aldehyde dehydrogenase (ALDH) with benzaldehyde as substrate. These values have also been determined for LADH as a function of changing nucleotide substrate. As the redox potential of the nucleotide changes from −0.320 V with NAD to −0.258 V with acetylpyridine-NAD, the product of primary and secondary deuterium isotope effects rises from 4 toward 6.5, while the primary 13C isotope effect drops from 1.025 to 1.012, suggesting a trend from a late transition state with NAD to one that is more symmetrical. The values of 13C (again the product of primary and secondary isotope effects) and 13C for YADH with NAD are 7 and 1.023, suggesting for this very slow reaction a more stretched, and thus symmetrical, transition state. With ALDH and NAD, the primary 13C isotope effect on the hydride transfer step lies in the range 1.3-1.6%, and the secondary deuterium isotope effect on the same step is at least 1.22, but 13C isotope effects on formation of the thiohemiacetal intermediate and on the addition of water to the thio ester intermediate are less than 1%. On the basis of the relatively large 13C isotope effects, we conclude that carbon motion is involved in the hydride transfer steps of dehydrogenase reactions.

Very little is known about the structure of the transition states for hydride transfer in enzymatic reactions catalyzed by dehydrogenases, since until recently it was not even possible to determine the intrinsic isotope effects on the hydride transfer step except in rare cases where this step was totally rate limiting (as for formate dehydrogenase; Blanchard & Cleland, 1980). Hermes et al. (1982), however, have developed methods that allow the determination of the intrinsic isotope effects within narrow limits by measurement of 13C isotope effects on C-H cleavage with an unlabeled substrate and one that is deuterated in the primary position. Further, if the 13C isotope effect can be measured with a nucleotide deuterated at the 4-position (this causes an α-secondary deuterium isotope effect), one can obtain an exact solution for all of the intrinsic isotope effects and commitments in the system (Hermes et al., 1982). These techniques allow one to vary the nucleotide substrate and determine the effects on the intrinsic primary