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Catecholamine-Stimulated Guanosine 5′-*O*-(3-Thiotriphosphate) 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, G_s, and β-adrenergic receptors were reconstituted into unilamellar phospholipid vesicles. The kinetics of the quasi-irreversible binding of guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) to G_s, equivalent to G_s activation by nucleotide, was studied with respect to the stimulation of this process by β-adrenergic agonists and Mg^{2+}. The rate of GTPγS binding displayed apparent first-order kinetics over a wide range of nucleotide, agonist, and Mg^{2+} 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 $MgCl_2$, 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 GTPγS, 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 GTPγS concentration, such that isoproterenol caused a 17-fold increase in k_{app} at 1 μM GTPγS. The effect of isoproterenol on k_{app} also appeared to saturate at high isoproterenol concentration, yielding a $k_{app} \sim 6$ 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 G_s–GTPγS 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, G_s.[1] Activation of G_s occurs upon the high-affinity binding of GTP, or a GTP analogue such as GTPγS, and is manifest as the ability of nucleotide-liganded G_s 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 G_s. Receptors for stimulatory hormones function by increasing the rate of activation of G_s by guanine nucleotides. This increase in the rate of activation increases the steady-state concentration of G_s–GTP or, in the case of nonhydrolyzed analogues, increases the rate at which the active G_s–nucleotide complex is formed [reviewed by Ross & Gilman (1980) and Smigel et al. (1984)].

The mechanism of activation of G_s by nucleotides has been addressed most thoroughly by Gilman's group using purified, detergent-solubilized G_s (Northup et al., 1982, 1983; Smigel et al., 1982). G_s is a dimer of a 45 000-Da (or 52 000-Da) GTP-binding α subunit and a 35 000-Da β subunit.[2] These authors showed that the free α subunit–nucleotide complex is the active form of G_s. GTPγS binds to G_s and activates it by a slow reaction that is tighly coupled to the dissociation of the 35 000-dalton β subunit. The binding reaction is first order in G_s, and the observed first-order rate constant for binding, k_{app}, was found to vary less than 2-fold between 10^{-8} and 10^{-4} M GTPγS (Northup et al., 1982). These findings and other data led to the following proposed mechanism for

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[1] Abbreviations: G_s, stimulatory GTP-binding protein of hormone-sensitive adenylate cyclase; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); k_{app}, apparent first-order rate constant for GTPγS binding to G_s; Da, dalton; Hepes 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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

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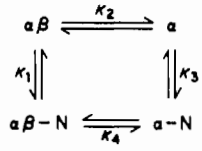
the activation of G_s (Smigel et al., 1982, 1984) (N is nucleotide):



(1)

Either reaction 2 or reaction 4 is the first-order, rate-limiting step. Because k_{app} does not vary with the concentration of GTPγS, either the forward rates of reaction 2 and 4 are equal or K_1 is not in the range of 10^4–10^8 M^{-1}. Subunit dissociation, and therefore activation, is promoted by free Mg^{2+} in the 10–200 mM range. Because the free α subunit is labile to denaturation, the stimulation of dissociation by Mg^{2+} also leads to the loss of total G_s activity. Thus, the measured extent of G_s activation and GTPγS binding is highly dependent on the nucleotide concentration because the extent of binding reflects the competing reactions of nucleotide binding to the α subunit and that subunit's denaturation (Smigel et al., 1982). This behavior was also observed for G_s that had been reconstituted into phospholipid vesicles (Asano et al., 1984).

The hormonal regulation of G_s activation has been described in much less detail. Some of the best information on the stimulation of G_s activation comes from studies of the turkey erythrocyte β-adrenergic adenylate cyclase system by Levitzki'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-liganded receptors in the membrane (Tolkovsky & Levitzki, 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 G_s do not interact functionally in detergent solution, we have reconstituted purified G_s and partially purified (1000–4000-fold) β-adrenergic receptors with phospholipids to yield a liposomal system that expresses rapid, agonist-stimulated GTPase activity, [^{35}S]GTPγS binding, and G_s activation by GTPγS (Brandt et al., 1983; Asano et al., 1984). In this report, we describe the kinetics of the hormone-stimulated activation of G_s in these vesicles with respect to the concentrations of agonist and nucleotide. The kinetic pattern is distinctly different from that observed in the Mg^{2+}-promoted activation of soluble G_s and suggests a possible sequence of molecular interactions that lead to activation.

Experimental Procedures

Most of the procedures and the sources of the materials used in this study were reported in the preceding paper (Asano et al., 1984). Receptor–G_s vesicles were prepared from β-adrenergic receptors that were partially purified (0.5–2 nmol/mg) from turkey erythrocytes (Brandt et al., 1983), purified rabbit hepatic G_s (Sternweis et al., 1981), and a mixture of dimyristoylphosphatidylcholine and turkey erythrocyte polar lipids (Brandt et al., 1983). The molar ratio of receptor to G_s in the vesicles used in experiments reported here varied from 3 to 10.

GTPγS binding to reconstituted G_s was performed as described by Asano et al. (1984). The binding reaction contained 20 mM NaHepes (pH 8.0), 1 mM EDTA, 0.1 M NaCl, 1 mM dithiothreitol, 0.1 mM ascorbic acid, and varying amounts of [^{35}S]GTPγS, isoproterenol, and $MgCl_2$ as shown in the text. Binding was assayed at 30 °C, and the binding reaction was initiated by the addition of warmed receptor–G_s vesicles to warmed assay medium. The reactions were quenched as described by Asano et al. (1984). The binding of GTPγS 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 $MgCl_2$.

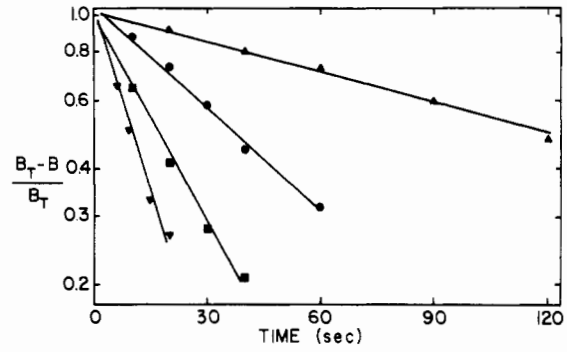


FIGURE 1: Time courses of isoproterenol-stimulated binding of [^{35}S]GTPγS to receptor–G_s vesicles at various concentrations of nucleotide. Vesicles were incubated at 30 °C for the indicated times in a binding assay mixture containing 1 mM EDTA, 2 mM $MgCl_2$, and various concentrations of [^{35}S]GTPγS, with 1 μM (−)-isoproterenol or 0.1 μM (−)-propranolol. The incremental amounts of binding due to isoproterenol are shown as first-order replots. Plateau levels of bound [^{35}S]GTPγS were averaged for each concentration of nucleotide to yield B_t and used to normalize binding at shorter times. The data were fit by an unweighted linear least-squares formula. GTPγS concentrations and the values of B_t were 10^{-9} (▲) (24 fmol), 10^{-8} (●) (53 fmol), 10^{-7} (■) (82 fmol), and 10^{-6} M(▼) (82 fmol).

Binding rates were generally analyzed according to apparent first-order kinetics. Apparent first-order rate constants, k_{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 GTPγS bound at long times (after the plateau was reached) and B is the amount of GTPγS 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_{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_{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 GTPγS binding to G_s in reconstituted receptor–G_s vesicles was investigated in the presence of various concentrations of GTPγS, the β-adrenergic agonist isoproterenol, and Mg^{2+}. As an example, Figure 1 demonstrates that, for the agonist-stimulated binding reaction, increasing concentrations of GTPγS 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 G_s and the irreversible denaturation of G_s (Smigel et al., 1982; Asano et al., 1984). Because the total concentration of GTPγS was generally much greater than that of G_s, binding was analyzed as a possibly pseudo-first-order reaction. First-order replots

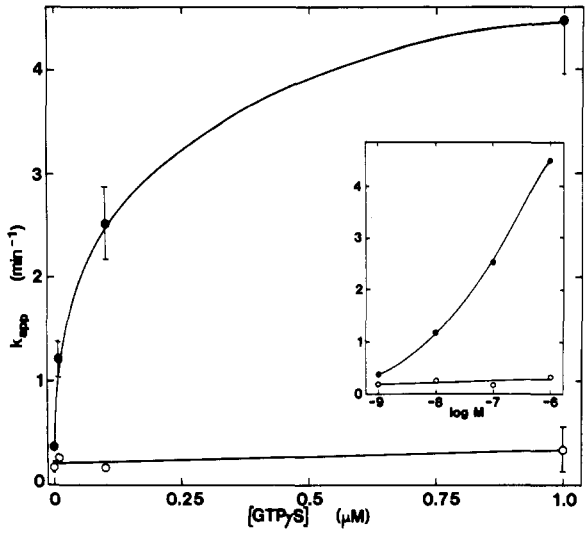


FIGURE 2: Influence of GTPγS concentrations on the apparent first-order rate constant for the binding of GTPγS to receptor–G_s vesicles in the presence or absence of hormone. Apparent first-order rate constants (k_{app}) for the binding of GTPγS stimulated by 1 μM (−)-isoproterenol (●) were determined from data of the sort shown in Figure 1. Values for k_{app} in the absence of agonist (○) were determined in the presence of 0.1 μM (−)-propranolol. Data are averages from two, three, four, (one point each), or more experiments. The standard deviation of the mean is indicated by brackets where significant. The inset shows k_{app} plotted vs. the logarithm of the GTPγS concentration.

of the binding reactions were linear under all conditions studied. When the G_s concentrations were varied by altering the concentration of vesicles in the binding reaction, the rate constants obtained from the slopes of the replots did not change over a 4-fold range of G_s concentrations. This was also consistent with a pseudo-first-order reaction. Even at 10^{-9} M GTPγS, only 10% of total ligand was bound when the plateau was reached. Thus, the change in rate due to the depletion of free GTPγS would not have been detectable during the course of the reaction, and the binding reaction might have been pseudo first order.

The data in Figure 2 describe the dependence of the apparent first-order rate constant for GTPγS binding, k_{app}, on the concentration of GTPγS. In the absence of agonist (basal rate), k_{app} was relatively independent of the concentration of GTPγS, suggesting that the binding reaction was simply first order in G_s. This behavior of the GTPγS binding reaction was similar to that described for detergent-solubilized G_s (Northup et al., 1982), although the rate of binding observed here for reconstituted G_s is about 5–10-fold higher than that observed with the soluble protein. This high basal rate was due at least in part to the effect of placing G_s in a phospholipid bilayer, since elevated rates were also observed when G_s was simply diluted into sonicated dispersions of several different phospholipids. It is also likely that an interaction of G_s with the unliganded β-adrenergic receptor contributes to this high basal rate (S. E. Pedersen and E. M. Ross, unpublished results).

In the absence of agonist, the extent of GTPγS binding to reconstituted G_s was markedly increased by the addition of $MgCl_2$ if the concentration of GTPγS was relatively high (Asano, et al., 1984). This also was the case with soluble G_s (Northup et al., 1982). Consequently, the absolute initial rate of binding was also increased by Mg^{2+}. However, the first-order rate constant for binding was only increased about 2-fold by 50 mM $MgCl_2$, and the rate constant measured in the presence of high Mg^{2+} remained essentially independent of the GTPγS concentration (Table I). The low relative stimulation of the binding rate by 50 mM Mg^{2+} may simply reflect

Table I: Effect of the Concentration of GTPγS on $MgCl_2$-Stimulated GTPγS Binding[a]

[GTPγS] (M)	k_{app} (min^{-1})
10^{-9}	0.26 ± 0.06 (n = 5)
10^{-8}	0.36 ± 0.05 (n = 4)
10^{-7}	0.41 ± 0.13 (n = 4)
10^{-6}	0.33 ± 0.05 (n = 4)
3×10^{-6}	0.34 (mean of 0.23 and 0.45)

[a] The apparent first-order rate constant for GTPγS binding to receptor–G_s vesicles was determined in the presence of 50 mM $MgCl_2$ 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.

the fact that the basal rate was already rather high. It is also possible that the concentration of free Mg^{2+} needed to stimulate the rate of binding to reconstituted G_s is either much below 1 mM or much above 50 mM [see Asano et al. (1984)].

In contrast to the basal and Mg^{2+}-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 μM isoproterenol. At 1 nM 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 μM 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_s), but 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_s 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 $\approx k_{app}B_t$. 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_s 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,

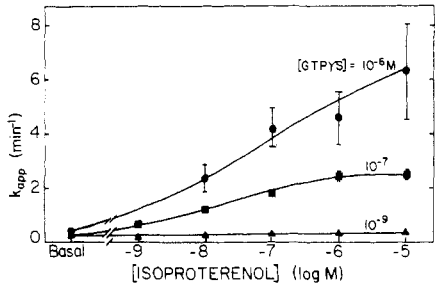


FIGURE 3: Influence of the isoproterenol concentration on the first-order rate constant for GTPγS binding to receptor–G_s vesicles at various GTPγS concentrations. Binding was measured as described in the legend to Figure 1 at either 10^{-9} (▲), 10^{-7} (■), or 10^{-6} M(●) [^{35}S]GTPγS. The apparent first-order association constant was determined as described in Figure 1. The basal values were determined in the presence of 0.1 μM (−)-propranolol. Data are averages from two experiments (three points), three experiments (five points), four experiments (three points), or more (six points).

in agreement with the data of Figure 2. As the nucleotide concentration was increased, agonist had a more marked effect on the rate of binding, increasing k_{app} to 2.7 ± 0.8 min^{-1} at 100 nM GTPγS (Figure 3). Under these submaximal conditions, the stimulatory effect of isoproterenol appeared to saturate. The agonist produced a half-maximal effect at about 20 nM, well below its equilibrium dissociation constant of 870 nM (Asano et al., 1984). At higher concentrations of GTPγS, isoproterenol caused a 17-fold increase in k_{app} to 5.8 ± 2.1. Technical limitations on the speed of the binding assay has limited our ability to measure the maximum possible rate with great accuracy. Because a saturating effect of isoproterenol on k_{app} was not clearly demonstrated, it is not certain from Figure 3 that k_{app} behaved as a single saturable function of isoproterenol concentration. However, the data of Figure 3 suggest that, in the presence of 1 μM GTPγS, the half-maximally effective concentration of isoproterenol is at least 100 nM, about 5-fold higher than that observed at the intermediate concentration of nucletide and somewhat closer to the equilibrium K_d for the agonist.

Discussion

The rate at which GTP binds to and activates G_s is a major factor in determining the activity of adenylate cyclase. It is the principal site of hormonal stimulation. The experiments reported here represent our initial kinetic description of the activation process in reconstituted receptor–G_s vesicles. In these vesicles, β-adrenergic receptors and G_s have been substantially purified, the catalytic unit and other receptors have been removed, and the two proteins are present in known and manipulatable concentrations in a predetermined lipid environment. By using GTPγS as the activating ligand, we could study only the activation step itself, since hydrolysis of GTPγS is negligible and reversal of binding is extremely slow.

The high-affinity binding of GTPγS to reconstituted G_s is a first-order reaction, as is the activation of adenylate cyclase by nonhydrolyzable analogues of GTP in native membranes (Tolkovsky, 1983). The rate, the reaction order, and the regulation of the rate all deserve discussion. The activation of reconstituted G_s by GTPγS is considerably faster than that of detergent-solubilized G_s under comparable conditions. The basal rate constant reported here, 0.17–0.34 min^{-1}, is at least 5 times greater than that observed for solubilized hepatic G_s when stimulated by 10 mM $MgCl_2$ (Northup et al., 1982). Likewise, the agonist-stimulated k_{app} displayed by reconstituted G_s is at least 10-fold greater than the maximum hormone-stimulated rate constants that have been observed for the activation of adenylate cyclase by guanyl-5′-yl imidodiphosphate 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_s 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_s by GTPγS 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_s activation indicates most simply that the rate-limiting step in the process is not the bimolecular association of G_s with GTPγS. 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_s, but the data of Katada et al. (1984) suggest strongly that the subunits of G_s 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 GTPγS 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_s (Northup et al., 1982). In terms of eq 1, this might indicate that rapid GTPγS 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 $K_1 \rightarrow K_4$ pathway becomes significant. The αβ–N complex would presumably be of low affinity and not detectable by the filtration assay used to measure bound [^{35}S]GTPγS or by the reconstitution assay for functional G_s activation. If the $K_1 \rightarrow K_4$ 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 GTPγS concentration. The finding that the rates of activation of G_s by GTPγS and either Gpp(NH)p or Gpp(CH_2)p are different (Birnbaumer et al., 1980a) supports the $K_1 \rightarrow K_4$ path.

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 GTPγS and appeared to saturate. There was almost no effect of hormone at low concentrations of GTPγS (10^{-9} M). It thus appears that increasing concentrations of GTPγS 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 $K_1 \rightarrow K_4$ pathway in which the equilibrium of reaction 1 is shifted from αβ to αβ–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_s (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_s subunits. This conclusion agrees with the tendency of R–H to stabilize reconstituted G_s and to increase its apparent affinity for nucleotide (Asano et al., 1984).

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

Acknowledgments

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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 ^{13}C 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 ^{13}C 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 ^{D}k (again the product of primary and secondary isotope effects) and ^{13}k 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 ^{13}C isotope effect on the hydride transfer step lies in the range 1.3–1.6%, and the α-secondary deuterium isotope effect on the same step is at least 1.22, but ^{13}C 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 ^{13}C 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 ^{13}C isotope effects on C–H cleavage with an unlabeled substrate and one that is deuterated in the primary position. Further, if the ^{13}C 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

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