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The Effect of GTP and Mg^{2+} on the GTPase Activity and the Fluorescent Properties of G_o*

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The structures of the guanosine 5′-*O*-(3-thio)triphosphate (GTPγS)-containing guanine nucleotide-binding regulatory proteins (G proteins) are distinct from those of the GDP-containing forms. One indication of the conformational change caused by GTPγS is a Mg^{2+}-sensitive increase in the intensity of the proteins' tryptophan fluorescence (Higashijima, T., Ferguson, K. M., Sternweis, P. C., Ross, E. M., Smigel, M. D., Gilman, A. G. (1987), J. Biol. Chem., 262, 762–766). GTP causes a similar change in the fluorescence of G_o, a G protein from bovine brain. When Mg^{2+} is also present, the increase in fluorescence is transient, and the rate of decline in the intensity of the fluorescence is the same as the rate of GTP hydrolysis by the protein. The steady-state rate of hydrolysis of GTP by G_o (0.3–0.4/min) is slower than the catalytic rate of the protein (2/min), because the rate-limiting step in the reaction is the release of GDP.

The G proteins[1] are a family of homologous, membrane-associated guanine nucleotide-binding regulatory proteins that act as transducers of receptor-mediated signals (1–3). Their properties are described briefly in the first of this series of papers. All of the G proteins hydrolyze GTP, albeit slowly, to GDP and P_i. Although the GTPase activity of the purified G proteins depends on the assay conditions and the particular protein, basal rates of hydrolysis are typically 0.01–1.5 min^{-1} (4, 5). Receptor-mediated stimulation of GTPase activity has been demonstrated by reconstitution of purified G proteins with receptor-containing membranes (6–11). Such reconstituted preparations yield hormone-stimulated activities that are similar to those noted in plasma membranes (12, 13).

The GTPase activity of the G proteins is a crucial aspect of their regulatory mechanism. With regard to stimulation of adenylate cyclase, for example, it is believed that agonist-bound receptors catalyze the exchange of tightly bound GDP on G_s for GTP (14). GTP-bound G_s stimulates adenylyl cyclase activity until hydrolysis of GTP terminates this action and restores the system to its inactive state. Several types of experiments support this general model (12, 15–18), although it is questioned whether the rate of release of GDP from G_s is always rate-limiting (7, 19–21).

Our previous experiments demonstrated that binding of GTPγS to either G_i or G_o induced a change in the fluorescence of these proteins. In the experiments reported here, we show that GTP causes a similar change in fluorescence, and that this change can be used to measure the intrinsic rate of GTP hydrolysis by G_o.

MATERIALS AND METHODS

Protein Preparation—G_o was purified as described (22). GDP-free $G_{o\alpha}$ was prepared by chromatography on Sephadex G-25 in an $(NH_4)_2SO_4$-containing buffer (23). The $(NH_4)_2SO_4$ was removed by chromatography on Sephadex G-25 in buffer A (50 mM NaHepes, pH 8.0, 1 mM NaEDTA, 1 mM dithiothreitol, 0.1% Lubrol) containing 20% glycerol.

GTP Hydrolysis—GTPase activity was measured as described (6) with some modifications. The G protein was incubated at 20 °C in buffer A containing the indicated concentrations of [γ-^{32}P]GTP and $MgSO_4$. For measurement of the time-dependent release of [^{32}P]P_i the reaction was initiated by the addition of protein that had been warmed to the temperature of the reaction. Aliquots (50 μl) were removed at the indicated intervals, added to 750 μl of 5% (w/v) Norit in 50 mM NaH_2PO_4 (0 °C), and vortexed. The charcoal was removed by centrifugation (2000 rpm for 10 min in a Beckman JA 4.2 rotor) and the amount of radioactivity in a 400-μl aliquot of the supernatant was determined by liquid scintillation counting. The rate of protein-independent formation of [^{32}P]P_i was subtracted to determine the GTPase activity. [γ-^{32}P]GTP was prepared as described (24). All other procedures have been described elsewhere (25).

Analysis of the Time-dependence of GTPase Activity—Estimates for the dissociation rate of GDP (k_{-2}) from G_o and the protein's rate of catalysis (k_{cat}) were obtained by fitting the data to one of two models:

For GDP-free G protein:

$$[P_i](t) = \frac{G_t k_{cat} k_{-2}}{k_{cat} + k_{-2}} \left(t - \frac{1}{k_{cat} + k_{-2}} + \frac{e^{-(k_{cat}+k_{-2})t}}{k_{cat} + k_{-2}} \right)$$

For GDP-containing G protein:

$$[P_i](t) = G_t k_{cat} \left[\frac{t k_{-2}}{(k_{cat} + k_{-2})} + \frac{k_{cat}}{(k_{cat} + k_{-2})^2} (1 - e^{-(k_{cat}+k_{-2})t}) \right]$$

In either case, the total amount of G_O in the reaction was determined by GTPγS binding as described (23). Thus, only k_{-2} and k_{cat} were permitted to vary during the nonlinear least squares parameter estimation procedure. Details of the derivation of these equations are provided in the Appendix.

RESULTS

The Influence of GTP on the Fluorescence of $G_{o\alpha}$—Binding of GTPγS to $G_{o\alpha}$ and $G_{i\alpha}$ changes the conformation of the proteins and causes an increase in the intensity of their tryptophan fluorescence. A similar effect is caused by GTP

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[1] The abbreviations used are: G proteins, guanine nucleotide-binding regulatory proteins; G_s, the G protein that stimulates adenylyl cyclase; G_i, the G protein that inhibits adenylyl cyclase; G_o, a G protein of unknown function purified from bovine brain; GTPγS, guanosine-5′-[γ-thio]triphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

When 10 μM GTP is added to a reaction containing 290 nM $G_{o\alpha}$, there is a slow, exponential increase in the fluorescence intensity; steady state is achieved in 5 min (Fig. 1). (The abrupt decrease in the intensity of the fluorescence emission immediately upon addition of GTP is due to the absorbance of the nucleotide.) The subsequent addition of 10 mM Mg^{2+} causes a further, rapid increase in the fluorescence intensity (>35% enhancement), which then declines exponentially (rate = 2.2 ± 0.1 min^{-1}) to a value that exceeds the baseline by 10%. The change in the fluorescence intensity caused by GTP in the absence of added Mg^{2+} is similar to that observed with GTPγS in both time course and intensity (25), suggesting that the alteration is caused by the exchange of bound GDP for GTP. (Recall that $G_{o\alpha}$ as purified contains 1 mol GDP/mol protein (Ref. 23).) Once steady state is achieved, addition of Mg^{2+} causes a rapid increase in the fluorescence intensity, also analogous to that observed when GTPγS was used. Unlike the results obtained with GTPγS, however, the Mg^{2+}-induced change in the presence of GTP is transient. These data suggest the following model:

$$\begin{array}{ccc} Mg^{2+} + G_{o\alpha} + GDP + GTP & \underset{k_o}{\overset{k_{-o}}{\rightleftharpoons}} & G_{o\alpha}\cdot GTP + Mg^{2+} \\ k_{-2}\downarrow\uparrow k_2 & & k_{-1}\downarrow\uparrow k_1 \\ Mg^{2+} + GTP + G_{o\alpha}\cdot GDP & \xleftarrow[k_{cat}]{P_i} & G_{o\alpha}\cdot GTP\cdot Mg^{2+} \end{array}$$

$G_{o\alpha}$·GTP is the form of the protein that has a 10% enhancement of the fluorescence intensity; $G_{o\alpha}$·GTP·Mg^{2+} has a higher fluorescence intensity (35% enhancement), but, because of the hydrolysis of GTP, it has a short lifetime determined by k_{cat}. Upon hydrolysis, the system returns to the GDP-bound form, which is the reference state for these experiments.

According to this model, the rate of approach to steady state is $k_{-2} + k_{cat}$ when both Mg^{2+} and GTP are added to the G protein. In the absence of Mg^{2+}, the rate is k_{-2}. A consequence of the hydrolytic reaction is that the enhancement of steady-state fluorescence intensity in the presence of Mg^{2+} is $f_2k_{-2}/(k_{-2} + k_{cat})$, where f_2 is the increase in fluorescence characteristic of $G_{o\alpha}$·GTP·Mg^{2+}. When 1 μM GTP is added to a reaction containing both Mg^{2+} and $G_{o\alpha}$, there is a rapid increase in the fluorescence intensity to a steady-state enhancement of 5% (Fig. 2). The rate of this increase (2–3 min^{-1}) is much faster than that observed when Mg^{2+} is omitted (Fig. 1). Given the magnitude of the increase in fluorescence (5%) and using estimates of f_2 =35% and k_{cat} = 2 min^{-1}, one would predict k_{-2} to approximate 0.3 min^{-1} (see below). If this increase in fluorescence is caused by the steady-state concentration of $G_{o\alpha}$·GTP·Mg^{2+}, the addition of an excess of GDP should block the formation of $G_{o\alpha}$·GTP·Mg^{2+}, and the hydrolysis of GTP should cause a decrease in fluorescence. When 10 μM GDP is added to a reaction at steady state that contains $G_{o\alpha}$, 1 μM GTP, and 10 mM $MgSO_4$, there is a rapid decline in the intensity of the fluorescence (Fig. 2). This relaxation process is complete in 1 min and is absent when 10 μM GTP is substituted for GDP.

The Rate of Hydrolysis of GTP—To test the hypothesis that hydrolysis causes the decline in fluorescence following the rapid increase upon addition of Mg^{2+} to GTP-containing $G_{o\alpha}$ (Fig. 1), $G_{o\alpha}$ was incubated with 1 μM [γ-^{32}P]GTP for 16 min to allow sufficient time for GTP to replace the GDP on $G_{o\alpha}$. During this incubation there was little release of [^{32}P]P_i (Fig. 3). When 10 mM Mg^{2+} was added, there was a relatively rapid release of phosphate, followed by a slower rate of GTP hydrolysis. These data were analyzed as described under "Materials and Methods" to obtain estimates for k_{cat} (1.8 ± 0.3 min^{-1}) and k_{-2} (0.4 ± 0.05 min^{-1}). The decay in fluorescence intensity that followed addition of Mg^{2+} in Fig. 1 should occur with rate = $k_{cat} + k_{-2}$. This value (2.2 ± 0.1 min^{-1}) is thus in excellent agreement with the values of k_{cat} and k_{-2} determined by analysis of the data of Fig. 3. These data indicate that the release of GDP and not the hydrolysis of GTP is the rate-limiting step in the GTPase reaction at steady state.

The Effect of Bound GDP on the Hydrolysis of GTP—If the dissociation of GDP is rate-limiting, incubation of GDP-free G_o with [γ-^{32}P]GTP should cause a rapid release of phosphate. This hypothesis was tested by incubation of GDP-containing G_o and GDP-free G_o with 1 μM [γ-^{32}P]GTP (100 nM [γ-^{32}P]GTP for GDP-free G_o) and 10 mM $MgSO_4$ at 20 °C (Fig. 4). The release of [^{32}P]P_i catalyzed by GDP-containing G_o lagged slightly before a steady-state rate of hydrolysis of 0.3 min^{-1} was achieved. The production of [^{32}P]P_i in the reaction that contained GDP-free G_o was initially faster and then slowed to the same steady-state rate of hydrolysis as found for GDP-containing G_o. Analysis of these data according to the equations described under "Materials and Methods" indicates that both forms of the protein catalyze hydrolysis at similar rates (2.1 ± 0.9 min^{-1} for GDP-free G_o; 2.4 ± 0.4 min^{-1} for GDP-

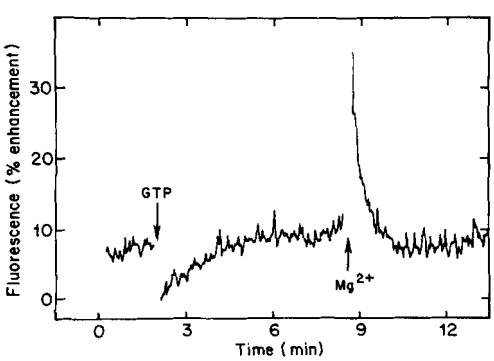

FIG. 1. **The GTP-induced change in the fluorescence of $G_{o\alpha}$.** The fluorescence emission of $G_{o\alpha}$ (290 nM, 392 μl) was measured in buffer A at 20 °C in a 5 × 5-mm fluorescence cuvette (equipped with a magnetic stir bar to provide continuous mixing). After 2.5 min, a 4-μl aliquot of 1 mM GTP was added. At 9 min, a 4-μl aliquot of 1 M $MgSO_4$ was added. The abrupt decrease in fluorescence intensity following addition of GTP is due to a decrease in the intensity of the exciting light caused by the absorbance of the guanine nucleotide.

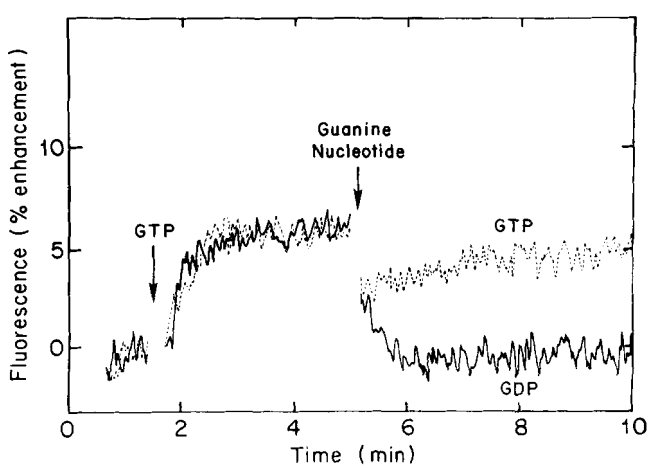

FIG. 2. **The effect of GDP on the GTP-induced change in the tryptophan fluorescence of $G_{o\alpha}$.** $G_{o\alpha}$ (290 nM, 392 μl) in buffer A except containing 10 mM $MgSO_4$ was incubated as described in Fig. 1. After 1.5 min, a 4-μl aliquot of 100 μM GTP was added to the cuvette, followed by the addition, after 5 min, of 4 μl of 1 mM GTP. The experiment was repeated except GDP was added at 5 min.

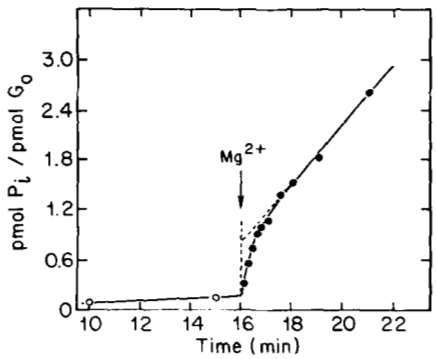

FIG. 3. **The hydrolysis of GTP by $G_{o\alpha}$.** $G_{o\alpha}$ (100 nM, 20 °C) was incubated in buffer A containing 1 μM [γ-^{32}P] GTP (8500 cpm/pmol). Aliquots (50 μl) were taken during the first 15 min (○) of the incubation (data prior to 10 min not shown) and release of [^{32}P]P_i was measured as described under "Materials and Methods." $MgSO_4$ (10 mM final concentration) was added when indicated (●), aliquots were removed at the indicated times, and the release of [^{32}P]P_i was quantitated.

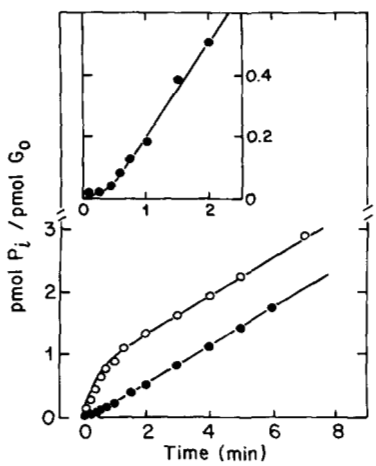

FIG. 4. **Time-dependent hydrolysis of GTP by GDP-free and GDP-containing G_o.** GDP-free G_o (○) (10 nM, prepared by mixing 1 mol of $G_{o\alpha}$ with 2 mol of $G_{\beta\gamma}$) was incubated at 20 °C in buffer A containing 10 mM $MgSO_4$ and 100 nM [γ-^{32}P]GTP (42,000 cpm/pmol). Aliquots (50 μl) were withdrawn at the indicated times to determine the amount of [^{32}P]P_i produced. The hydrolysis of GTP by GDP-containing G_o (●) (75 nM, prepared as for GDP-free G_o) in buffer A containing 10 mM $MgSO_4$ and 1 μM [γ-^{32}P]GTP (7500 cpm/pmol) was measured as described for GDP-free G_o. The *inset* presents an expansion of the early time points for GDP-containing G_o. The data shown are normalized to the amount of G protein assayed (pmol P_i/pmol G_o).

containing G_o) and release GDP at the same rate (0.4 ± 0.06 min^{-1} for GDP-free G_o, 0.4 ± 0.01 min^{-1} for GDP-containing G_o).

The rate of dissociation of GDP from $G_{o\alpha}$ was measured directly using [α-^{32}P]GDP (see Table I, Ref. 26). This value averaged 0.3/min at 20 °C and was independent of the concentration of Mg^{2+}. It is in excellent agreement with those estimated above by consideration of the level of fluorescence enhancement achieved in the presence of Mg^{2+} and GTP (Fig. 2) and by examination of the rate of GTP hydrolysis (Figs. 3 and 4).

DISCUSSION

Activation of G proteins by guanine nucleotides changes the proteins' conformation (8, 27–30). Concomitant with this structural change is an increase in the intensity of fluorescence of tryptophan residues (25). Both GTPγS and GTP can cause this change, suggesting that a similar alteration in protein structure is caused by the different nucleotides. When GTP is added to GDP-containing $G_{o\alpha}$, there is a slow increase in the intensity of tryptophan fluorescence as GDP dissociates and GTP then binds. After GDP has been replaced with GTP, the addition of Mg^{2+} causes a rapid increase in fluorescence intensity, reflecting the formation of $G_{o\alpha}\cdot GTP\cdot Mg^{2+}$. Unlike the complex formed in the presence of GTPγS, the GTP-containing form of the protein is transient, because GTP is hydrolyzed to GDP and P_i in the presence of Mg^{2+}. The hydrolysis is accompanied by a decrease in the fluorescence intensity, providing a direct measure of the catalytic reaction. The rate of the catalytic reaction (as distinguished from the rate of steady-state hydrolysis) found for G_o and G_i[2] is similar to that noted for G_s (4 min^{-1}) and transducin (1 min^{-1}) (5, 7, 18). The rate of GTP hydrolysis by $G_{o\alpha}$ and $G_{i\alpha}$ measured at steady state is the same as the rate of dissociation of GDP. These rates are influenced by several factors, including Mg^{2+}, $G_{\beta\gamma}$, and anions (see accompanying articles).

The model presented under "Results" provides a quantitative explanation for the hysteresis found in the time-dependence of GTP hydrolysis by the GDP-containing G protein. When Mg^{2+} is present, the rate of approach to the steady-state concentration of $G\cdot GTP\cdot Mg^{2+}$ is $k_{cat} + k_{-2}$. The lag in the release of phosphate (defined as the extrapolation of the steady-state release of P_i to the abscissa) is $(k_{cat} + k_{-2})^{-1}$ or about 30 s. Thus, even though the rate of binding of GTP is limited by the rate of dissociation of GDP, the rate of approach to steady state is not.

Another consequence of the relatively rapid k_{cat} is that the fraction of G protein with bound GTP and Mg^{2+} (presumably the active form of the protein) cannot exceed $k_{-2}/(k_{cat} + k_{-2})$, even when very high concentrations of GTP are present. Thus, the hydrolytic reaction holds the protein in the GDP-containing, presumably inactive form. Agonist-bound receptors increase the rate of dissociation of guanine nucleotide (7, 15, 17), apparently by a catalytic mechanism that is analogous to that of the interaction of elongation factors Tu and Ts (31). As the rate of dissociation of GDP increases, the velocity of the GTPase reaction will increase to a limit of k_{cat} and the fraction of the activated form of the G protein will approach 100%. Thus, the receptor alters the state of activation of the G protein merely by changing the rate of dissociation of GDP.

Even though k_{cat} is 5- to 20-fold greater (depending on the particular G protein)[2] than the observed steady-state rate of GTP hydrolysis, it is still an exceedingly slow turnover rate. This too is an important part of the function of the G protein. The turnover rate for adenylyl cyclase, estimated from the activity of the purified protein (32), is 1200 min^{-1}. If $G_{s\alpha}\cdot GTP\cdot Mg^{2+}$ activates adenylyl cyclase and hydrolysis of GTP terminates this activation, the slow rate of destruction of GTP permits the production of 500–1000 molecules of cyclic AMP. Since a single receptor can presumably activate many molecules of G_s, the amplification achieved is significantly greater than this. An identical model explains the gain in the light-activated cyclic GMP-sensitive phosphodiesterase of the retinal rod outer segment (3).

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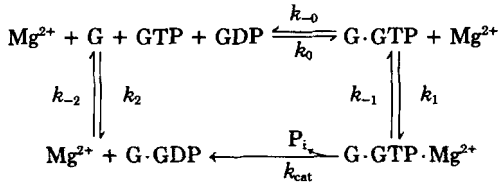
[2] Preliminary data using partially GDP-free G_i show k_{cat} = 1.9 min^{-1}. The rate of GDP dissociation is about 0.04 min^{-1} (Ref. 23).

APPENDIX

A Model for the Hydrolysis of GTP by G Proteins—The hydrolysis of GTP is assumed to occur according to the model:



The time-dependence of the formation of the various states in this model is determined by the differential equations below:

$$[G\cdot GTP]' = k_0[GTP][G] + k_{-1}[G\cdot GTP\cdot Mg^{2+}] - k_1[Mg^{2+}][G\cdot GTP] - k_{-0}[G\cdot GTP] \quad (1)$$

$$[G\cdot GTP\cdot Mg^{2+}]' = k_1[Mg^{2+}][G\cdot GTP] - (k_{-1} + k_{cat})[G\cdot GTP\cdot Mg^{2+}] \quad (2)$$

$$[G\cdot GDP]' = k_2[GDP][G] + k_{cat}[G\cdot GTP\cdot Mg^{2+}] - k_{-2}[G\cdot GDP] \quad (3)$$

Note: $[x]' = d[x]/dt$, $[x]'' = d^2[x]/dt^2$
The rate of P_i production is

$$[P_i]' = k_{cat}[G\cdot GTP\cdot Mg^{2+}] \quad (4)$$

and requires the integration of (2) before the time dependence of the release of phosphate can be solved. To simplify the solution of Equation 2, Equation 1 is assumed to be at steady state (a valid assumption at relatively high concentrations of GTP). Then Equations 1–3 can be combined into the second order differential equation:

$$[G\cdot GTP\cdot Mg^{2+}]'' + A[G\cdot GTP\cdot Mg^{2+}]' + B[G\cdot GTP\cdot Mg^{2+}] = C \quad (5)$$

Where A, B, and C are combinations of the rate constants in Equations 1, 2, and 3. The solution of Equation 5 is:

$$[G\cdot GTP\cdot Mg^{2+}] = c_1e^{m_1t} + c_2e^{m_2t} + C/B \quad (6)$$

where m_1 and m_2 are the roots of the polynomial:

$$m^2 + Am + B = 0$$

The constants c_1 and c_2 are determined by the initial conditions. Evaluating Equation 6 when $t = 0$

$$c_1 + c_2 + C/B = 0 \quad (7)$$

The derivative of Equation 6 evaluated at $t = 0$ equated with Equation 2 at $t = 0$ is

$$c_1m_1 + c_2m_2 = G_t(1 - f)E \quad (8)$$

where G_t is the total concentration of G protein, f is the fraction of the protein with bound GDP at $t = 0$, and

$$E = \frac{k_1[Mg^{2+}]k_0[GTP]}{k_1[Mg^{2+}] + k_{-0}} \quad (9)$$

Integration of Equation 6 between the limits of 0 and t gives

$$[P_i](t) = k_{cat}(c_1/m_1(e^{m_1t} - 1) + c_2/m_2(e^{m_2t} - 1) + (C/B)t) \quad (10)$$

For the case of saturating GTP and Mg^{2+} and low GDP the rate constants m_1 and m_2 are

$$m_1 \cong -k_0[GTP] \qquad m_2 \cong -(k_{cat} + k_{-2}) \quad (11)$$

The magnitude of c_1 and c_2 are determined by the fraction of the G protein with bound GDP. When all the protein has bound GDP, $f = 1$, and

$$c_1 = -G_tk_{-2}/k_0[GTP]$$

$$c_2 = -G_tk_{-2}/(k_{cat} + k_{-2})$$

Then Equation 10 reduces to

$$[P_i](t) = \frac{G_tk_{cat}k_{-2}}{k_{cat} + k_{-2}}\left(t - \frac{1}{k_{cat} + k_{-2}} + \frac{e^{-(k_{cat}+k_{-2})t}}{k_{cat} + k_{-2}}\right) \quad (12)$$

The rate of hydrolysis at steady state is

$$V_{ss} = \frac{G_tk_{cat}k_{-2}}{k_{cat} + k_{-2}} \quad (13)$$

After steady state is attained, extrapolation to $[P_i](t) = 0$ gives

$$t = 1/(k_{cat} + k_{-2}) \quad (14)$$

When none of the G protein contains GDP at the start of the reaction ($f = 0$)

$$c_1 = -G_t \qquad c_2 \cong g_tk_{cat}/(k_{cat} + k_{-2})$$

In this case Equation 10 is

$$[P_i](t) = G_tk_{cat}\left[\frac{tk_{-2}}{(k_{cat}+k_{-2})} + \frac{k_{cat}}{(k_{cat}+k_{-2})^2}(1 - e^{-(k_{cat}+k_{-2})t})\right] \quad (15)$$

The rate of hydrolysis after steady state is attained is

$$V_{ss} = \frac{G_tk_{cat}k_{-2}}{k_{cat} + k_{-2}} \quad (16)$$

which is the same as for the previous case ($f = 1$, Equations 12 and 13). Extrapolation of the steady-state solution to $t = 0$ shows that there is a burst of P_i release:

$$[P_i](0) = G_t\left[\frac{k_{cat}}{k_{cat} + k_{-2}}\right]^2 \quad (17)$$

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