Rapid Kinetics of Regulator of G-protein Signaling (RGS)-mediated Ga and Go Deactivation

Ga SPECIFICITY OF RGS4 AND RGS7*

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Regulator of G-protein signaling (RGS) proteins accelerate GTP hydrolysis by Ga subunits speeding deactivation. Ga deactivation kinetics mediated by RGS are too fast to be directly studied using conventional biochemical methods. We describe a stopped-flow spectroscopic approach to visualize these rapid kinetics by measuring the intrinsic tryptophan fluorescence decrease of Ga accompanying GTP hydrolysis and Ga deactivation on the millisecond time scale. Basal kcat values for Ga, Ga1, and Go at 20 °C were similar (0.025–0.033 s−1). Glutathione S-transferase fusion proteins containing RGS4 and an RGS7 box domain (amino acids 305–453) enhanced the rate of Ga deactivation in a manner linear with RGS concentration. RGS4-stimulated rates could be measured up to 5 s−1 at 3 μM, giving a catalytic efficiency of 1.7–2.8 × 106 M−1 s−1 for all three Ga subunits. In contrast, RGS7 showed catalytic efficiencies of 0.44, 0.10, and 0.02 × 106 M−1 s−1 toward Ga, Ga1, and Go, respectively. Thus RGS7 is a weaker GTPase-activating protein than RGS4 toward all three Ga subunits tested, but it is specific for Ga over Ga1 or Ga2. Furthermore, the specificity of RGS7 for Go does not depend on N- or C-terminal extensions or a Gβγ subunit but resides in the RGS domain itself.

G-protein1-coupled receptor-mediated signal transduction governs many important physiological functions. Upon binding of a ligand, such as light, neurotransmitter, hormone, chemokine, etc., to heptahelical receptors, the heterotrimeric G-proteins composed of α, β, and γ subunits are stimulated to release GDP and bind GTP. In the GTP-bound form, Ga dissociates from Gβγ and both interact with downstream effectors. This pathway is terminated when Ga hydrolyzes the bound GTP, thereby promoting reassociation of Ga and Gβγ and returning the system to inactive state (reviewed in Refs. 1–3).

Members of the recently described family of proteins, Regulators of G-protein signaling (RGS), act as negative regulators of G-protein function by enhancing GTP hydrolysis by Ga (4–6) or by functioning as effector antagonists (7). Although the first RGS protein, Sst2p, was identified in yeast (8, 9), more than 20 variants have been found in mammalian species, all being characterized by a conserved RGS domain of approximately 120 amino acids (reviewed in Refs. 10–12). The GTPase-activating property (GAP) of RGS proteins has been demonstrated by direct in vitro biochemical studies (13–15). Further studies using GDP-AlF4 suggest that RGS proteins enhance GTP hydrolysis by stabilizing the transition state conformation of Ga (16), which leads to the crystal structure of the RGS4-Gα1 complex (17). Mutagenesis analyses have also illustrated the importance of specific residues on the interface between Ga and RGS proteins (18–21). Besides being GAPs, many RGS proteins have also been found to serve as links to other cellular signaling pathways through non-RGS domains such as GGL, DEP, DH/PJ, and PDZ domains (reviewed in Refs. 11 and 12). The wide expression of RGS in various tissue and cell types (see Ref. 12 for review) thus indicate that RGS proteins may play important physiological roles directly or indirectly.

The specificity of RGS proteins for interactions with Ga subtypes (Ga, Ga/Gα, and Ga) is now being vigorously explored. The majority of the RGS proteins are GAPs for Ga and Ga but not for Ga subunit (reviewed in Refs. 10, 22–23). Numerous studies on the specificity between RGS proteins and Ga subunits have been reported using biochemical, immunological, and functional methods. RGS4 is one of the most extensively studied RGS protein and is a highly effective GAP for all Ga/Gα family members (reviewed in Refs. 10, 11, 24, 26). In contrast, limited and apparently inconsistent data on RGS7 have been reported. The RGS domain of RGS7 has been shown to have effective GAP activity toward Ga1 and Ga (21, 27), whereas it preferentially bound Ga and Ga, but not Ga1 or Ga2 (28). However, accurate quantitation of GAP activity is difficult, because rates of GTP hydrolysis are too fast to measure with standard assays (see below). Furthermore, full-length RGS7 was recently found to form a complex with Gβ (29, 30), and this complex was shown to have moderate but significant GAP activity toward Ga but not Ga1 or Ga2 (31), despite the fact that it did not seem to favor the physical binding of RGS7 to Ga (32). Therefore, it is not clear whether the specificity of full-length RGS7 for Ga over Ga1 and Ga2 is encoded in the RGS domain or it depends on Gβγ.

Direct measurement of the kinetics of GTP hydrolysis and deactivation of Ga mediated by RGS would be the most straightforward approach to determining specificity. The current challenge in this regard is that the conventional biochem-
ical assays can only delineate kinetics as fast as $t_{1/2} \sim 10 \text{ s}$, whereas the RGS-mediated Ga deactivation, as well as the turn-off of many physiological processes, takes place on the subsecond scale. One recent study in a m1AChR-Gq vesicle system using a quench-flow method has reported more than a 1000-fold enhancement of GTP hydrolysis rate mediated by RGS4 with $t_{1/2} \sim 50 \text{ ms}$, indicating that data acquisition on the millisecond time scale is required to study the effect of RGS on Ga deactivation kinetics under physiological conditions (33).

The present study aimed to develop a stopped-flow spectroscopic approach to visualize these rapid kinetics and to quantitatively assess RGS-Ga specificity. The rapid-mix stopped-flow fluorescence spectroscopy utilizes the intrinsic tryptophan fluorescence of Ga. Intrinsic tryptophan fluorescence is high in GTP-bound, active Ga subunits and low for GDP-bound Ga (34–37). The intrinsic fluorescence change is due to alterations in the environment of the α2 helix tryptophan residue, $T_{\text{m}}^{212}$ in Ga (38), and $T_{\text{m}}^{207}$ in transducin (39). With purified Gaα, Gaβ1, and Gaγ2 proteins in solution, we successfully employed this stopped-flow method to quantitate catalytic efficiencies of RGS4 and RGS7 and obtained new evidence on the specificity of RGS4 and RGS7 (box domain) toward Ga subunits.

**EXPERIMENTAL PROCEDURES**

**Materials**—[γ-32P]GTP was obtained from PerkinElmer Life Sciences (Boston, MA). Dithiothreitol (DTT) was purchased from Calibochem (San Diego, CA). Glutathione was from Sigma. Rut RGS4 (full-length) and RGS8 (nucleotides 315–857) in expression vectors pGEX-2T and pGEX-4T, respectively, were provided by Dr. Robert Mackenzie (Parke-Davis Research). The human RGS7 box cDNA fragment (nucleotides 915–1359, or amino acids 305–453) in the expression vector pGEX were generously provided by Dr. Maurine E. Linder (Washington University). The myristoylated Gaα protein was provided by Dr. Ron Taussig.

**Purification of His-tagged Gaα and Gaγ2, and Myristoylated Gaα and Gaβ1 Proteins**—Hisα, and Hisα, were expressed from the vector, pQE60, in Escherichia coli strain BL21/DE3 and purified as described previously (21). Hisα, and Hisα, eluted from nickel resin columns (Qiagen, Santa Clar, CA) were approximately 80% pure by Coomassie Blue staining of SDS gels. Myristoylated Gaα and Gaγ2 were expressed from the pQE6 expression vector cotransfected in the E. coli strain BL21/DE3 with the N-myristoyl transferase vector based on the protocol of Mummy and Linder (40). The specific activities of the Ga proteins determined by [γ-32P]GTP-SepS binding assay (41) ranged from 6 to 18 pmol/μg.

**Purification of GST-RGS Fusion Proteins**—The GST-RGS fusion proteins were expressed and purified using PGEX expression vectors in E. coli strain BL21/DE3 as described previously (21). The bacterial supernatant was incubated with glutathione-agarose beads (Amersham Pharmacia Biotech) at 4°C overnight. After washing with ice-cold PBS buffer (pH 7.3), the GST-RGS fusion proteins were eluted with 15 mgl glutathione in PBS and dialyzed against PBS buffer. Where indicated, the fusion proteins were cleaved by incubation overnight at 4°C with 10 units of thrombin/ng of fusion protein followed by incubation with glutathione-agarose to remove GST and any uncleaved GST-RGS proteins. Cleavage of the GST to yield RGS4 alone did not alter the rate constants for Ga deactivation (data not shown). RGS proteins were approximately 60–90% pure by Coomassie Blue staining of SDS gels.

**Slow Time-based Fluorescence Measurements**—Slow time-based fluorescence measurements were determined using a PTI Alphascan fluorometer (Photon Technology, Monmouth Junction, NJ) with a water-cooled 150-watt xenon arc lamp as described (42). Hisα, proteins were diluted into buffer containing 50 mM Hepes, 1 mM EDTA, 1 mM DTT, 10 mM MgSO4, 20 mM of deionized Lubrol, pH 8.0 (HEDML buffer), placed in customized 5-mm round quartz cuvettes in temperature-controlled (20°C) sample holders, and continuously stirred. Time course measurements of intrinsic fluorescence ($λ_{ex}$ 280, $λ_{em}$ 340 nm, 2-nm slits) were performed with 500 nM Hisα, proteins and 2 μM GTP in the presence of various concentrations of RGS4 (0–30 nM) in a final volume of 0.2 ml.

**Stopped-flow Ga Deactivation Kinetics**—The rapid kinetics of Ga deactivation in the presence or absence of RGS proteins was measured in an Applied Photophysics IX-17MV stopped-flow fluorometer. The intrinsic fluorescence change of Ga proteins was measured at an excitation wavelength of 290 nm with 2.3-nm slits. Emission light was detected with a photomultiplier tube behind a WG320 band pass filter (Corion, Holliston, MA). Gaα (400 nM), Gaβ1 (600 nM), or Gaγ2 (600 nM) proteins were preloaded with 2 μM GTP in the presence of magnesium in HED buffer (50 mM Hepes, 5 mM EDTA, 2 mM DTT, pH 8.0). Incubations were for 20 min at 20°C for Gaα and for 15 min at 30°C for Gaβ1 and Gaγ2. Samples were then stored on ice until use (<2 h). GTP-loaded G-protein was equilibrated in the instrument at 20°C for at least 3 min, then samples were mixed 1:1 with magnesium-containing HEDM buffer (50 mM Hepes, 5 mM EDTA, 30 mM MgSO4, pH 8.0) in the presence of RGS proteins as indicated. Tryptophan fluorescence was recorded for at least 100 s in the absence of RGS and for 20 or 50 s in the presence of RGS, depending on the rates achieved. Data from four to six shots were averaged and fit to a one-phase exponential decay equation using Prism v.3.0 for Windows (GraphPad Software, San Diego, CA).

**Determination of Single Turnover $k_{\text{cat}}$ for Hydrolysis of [γ-32P]GTP**—Single turnover [γ-32P]GTPase assays at low temperature (4°C) were performed as described previously (21). Single turnover [γ-32P]GTP hydrolysis was also determined at room temperature (24°C) as below. Hisα, or Hisα, (10 nM) was preloaded in magnesium-free HED buffer (50 mM Hepes, 1 mM EDTA, 1 mM DTT, 20 ppm deionized Lubrol, pH 8.0) with 1 μM [γ-32P]GTP (7500 cpm/mmol). The preloading incubation was for 20 min at room temperature for Gaα and for 15 min at 30°C for Gaβ1 and Gaγ2. After equilibration at room temperature, the single turnover hydrolysis reaction was then started by addition of MgSO4 and GTP/S to final concentrations of 20 mM and 200 μM, respectively. MgSO4 activates Hisα, and triggers catalysis, while GTP/S prevents [γ-32P]GTP from rebinding to the G-protein. Aliquots (50 μl) were taken and diluted in 1 ml of 15% (w/v) charcoal solution (50 mM NaH2PO4, pH 2.3, 0°C) at the indicated time points. Background hydrolysis was determined in the absence of protein and represents less than 10% of total [γ-32P]IP, release. The amount of [γ-32P]IP, released at each time point was fit to an exponential function, $cpm(t) = cpm_0 + Δcpm \times (1 - e^{-kt})$.

**RESULTS**

**Slow Time-based Fluorescence Measurements**—To determine the feasibility of using fluorescence spectroscopy to study RGS function, we first examined the intrinsic fluorescence changes of Gaα upon addition of GTP. The time course of intrinsic fluorescence changes of Gaα (500 nM) was monitored using a slow time-based fluorometer before and after addition of 2 μM GTP in the presence of 10 mM Mg2+ (Fig. 1A). Similar to the findings reported previously (34, 36), GTP and Mg2+ caused a rapid increase in the fluorescence intensity followed by a slow decrease in fluorescence. Fluorescence approached the basal level at approximately 30 min (1800 s). The fluorescence increase represents GTP binding to Gaα, to induce an active conformation. As the bound GTP is hydrolyzed and GDP produced, an increasing percentage of Gaα becomes GDP-bound, which displays a lower intrinsic fluorescence. When a small amount of RGS4 (3 nM) was added with GTP and Mg2+, the peak of intrinsic fluorescence decreased and the time for the fluorescence to return to baseline was shortened. A more dramatic effect was observed when a higher concentration of RGS4 (30 nM) was added.

Because our stopped-flow approach required preloading of GTP with Ga, we also tested the effect of adding RGS4 at the peak of the intrinsic fluorescence increase (Fig. 1B). RGS4 markedly accelerated the rate of fluorescence decrease.

**Stopped-flow Measurements of Ga Deactivation Kinetics**—Most previous studies of G protein-stimulated GTP hydrolysis by G-proteins have been done at 0–4°C to slow the single turnover kinetics (15, 14, 43). Although low temperature halts the measurement of the $k_{\text{cat}}$ of basal GTPase, which had rates of 0.002 and 0.004 s–1 for Gaα and Gaβ1, respectively, the RGS4-enhanced $k_{\text{cat}}$ was still too fast to be measured (21). The intrinsic GTPase activity of Hisα, (Fig. 2A) and Hisα, (not shown), as determined by [γ-32P]GTP single turnover assays at room temperature, were 0.019 ± 0.002 s–1 (n = 5) and 0.028 ±
Rapid Gα Deactivation Mediated by RGS4 and RGS7

Fig. 1. Slow time-based measurement of Gα, fluorescence changes induced by GTP and RGS4. A, His6Gα (500 nM) was equilibrated 3 min at room temperature in HEDM (50 mM Hepes, pH 8.0, 1 mM EDTA, DTT 2 mM, and 2 mM MgSO4). At 1 min, GTP and RGS4 were added to give the final concentrations of 1 μM and 0–30 nM, respectively. The time course of Gα, intrinsic fluorescence was monitored (290-nm excitation, 340-nm emission); B, GTP (1 μM) was added to 500 nM His6Gα, as in A, however, RGS4 was added at the peak of fluorescence (t = 150 s). For all traces, the fluorescence contributed by RGS4 was subtracted (290-nm excitation, 340-nm emission); C, intrinsic fluorescence changes induced by GTP and RGS4.

The rate constants were 0.022 s−1 and 0.002 s−1 for His6Gα and myrGα, respectively. The time course of Gα fluorescence decrease depended on the GTP hydrolysis and Gα state (GDP-AlF4, or Gpp(NH)p, a non-hydrolyzable GTP analog, in the presence of 100 nM RGS4. None of these reactions led to detectable fluorescence changes (Fig. 3A). Thus it was not simply binding of RGS4 to either the active form (Gpp(NH)p) or the transition state (GDP-AlF4) of Gα that produced the fluorescence signal.

Table I
Comparison of GTP hydrolysis/deactivation time courses as determined by single turnover [32P]GTPase and intrinsic fluorescence using stopped-flow fluorimetry. A, single round GTP hydrolysis by His6Gα was measured as described under “Experimental Procedures.” His6Gα protein (10 nM) was incubated at room temperature (24 °C) in magnesium-free buffer (HEDL) with 1 μM [γ-32P]GTP (7500 cpm/pmol) for 20 min. The hydrolysis reaction was started at time zero by addition of 20 mM MgSO4 and 200 μM GTP-γS. Nonenzymatic [γ-32P]GTP hydrolysis, determined in the absence of Gα protein, represented less than 10% of total [γ-32P]GMP release and was subtracted from the data. Data are averages (± S.E.) of five experiments, expressed as the fraction of the total GTP hydrolyzed and are fitted to single exponential association function (GraphPad Prism). B and C, intrinsic fluorescence changes of Gα were measured using stopped-flow as described in “Materials and Methods.” Reactions took place in HED buffer: 50 mM Hepes, 5 mM EDTA, 2 mM DTT. His6Gα (400 nM) and His6Gα1 (600 nM) were incubated with 2 μM GTP for 20 min at 20 °C or for 15 min at 30 °C, respectively. Samples were then loaded into the stopped-flow fluorimeter, GTP hydrolysis was triggered by mixing with an equal volume of 30 mM MgSO4 in HED buffer (15 mM final free Mg2+), then the intrinsic fluorescence changes were recorded with 290-nm excitation and a WG320 filter for emission. Intrinsic fluorescence at the first time point was set to zero. Graphs are averages of multiple experiments (His6Gα, five; His6Gα1, three), each averaged from four to six shots.

| Gα     | GTPase assay       | Stopped-flow assay |
|--------|--------------------|--------------------|
|        | kcat (s−1)         | t1/2 (s)           | kcat (s−1)         | t1/2 (s) |
| His6Gα | 0.019 ± 0.002 (5)  | 36                 | 0.022 ± 0.002 (8)  | 32       |
|        | myrGα              | 0.045 ± 0.002 (4)  | 15                 |
| His6Gα1| 0.028 ± 0.002 (5)  | 25                 | 0.027 ± 0.006 (3)  | 25       |
|        | myrGα1             | 0.024 ± 0.002 (6)  | 28                 |
|        | myrGα2             | 0.033 ± 0.001 (4)  | 21                 |

The rate of fluorescence decrease (kcat) by stopped-flow and the rate of GTP hydrolysis (kcat) by single-turnover [32P]GTPase assay were both determined at 20 °C as described under “Experimental Procedures.” Rates are expressed as mean ± S.E. from the number of experiments indicated in parentheses. Half-times (t1/2) were calculated from the rates.

100 nM RGS4. None of these reactions led to detectable fluorescence changes (Fig. 3A). Thus it was not simply binding of RGS4 to either the active form (Gpp(NH)p) or the transition state (GDP-AlF4) of Gα that produced the fluorescence signal.
Furthermore, the RGS-insensitive mutant G183S Ga11 (21) was utilized to test whether or not the effect of RGS4 depended on Ga/RGS4 interactions seen in biochemical studies. As expected, RGS4 did not increase the rate of Ga activation for G183S Ga11 (without RGS4, 0.027 ± 0.00, n = 2; with 100 nM RGS4, 0.013 ± 0.001, n = 2) compared with that for wild type His6Ga (Fig. 3B). This result is in agreement with our previous biochemical measurements at 4 °C (21). Therefore, the intrinsic fluorescence changes were dependent on and reflected the kinetics of GTP hydrolysis and Ga activation. To simplify discussion, we will use the designation $k_{\text{deact}}$ throughout the text to represent the rate of intrinsic fluorescence change that represents the rate of GTP hydrolysis and Ga activation kinetics.

To try to determine the maximal rate $V_{\text{max}}$ and the $K_m$ value of the RGS-facilitated Ga activation, we used increasing concentrations of RGS4 (Fig. 4A). The rate of Ga activation increased linearly with the concentration of RGS4 up to 3 μM, as shown in Fig. 4C. Due to the deteriorating signal-to-noise ratio contributed by intrinsic fluorescence of the high concentration of RGS4, 3 μM was the highest concentration of RGS4 for which acceptable data could be obtained. Similarly, the linear relationship between $k_{\text{deact}}$ and [RGS] was also found for RGS7 (Fig. 4B and D) and RGS8 (Fig. 4C) as well as for the three RGS proteins toward His6Ga11 (Fig. 4E and F).

**Specificity of RGS4 and RGS7 toward Ga11** —Given that we cannot separately define a $V_{\text{max}}$ and a $K_m$ for the RGS-mediated enhancement of GTP hydrolysis by Ga, we used the slope of the plot of $k_{\text{deact}}$ versus [RGS] as a measure of the “catalytic efficiency” of that RGS toward particular Ga subunits. This is equivalent to a $k_{\text{cat}}/K_m$ in classical enzyme mechanisms. The catalytic efficiencies of RGS4 and RGS8 toward His6Ga and His6Ga11 were similar, being $2 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ (Fig. 4 and Table II). In contrast, the catalytic efficiency of the RGS7 box construct toward His6Ga was approximately eight times lower than those of RGS4 and RGS8, being 0.27 ± 0.01 $\times 10^6 \text{ M}^{-1} \text{s}^{-1}$ (Fig. 4D, note scale change, and Table II). More surprisingly, very little effect was seen for RGS7 toward His6Ga11, yet we were still able to detect a linear relationship of rate versus RGS7 concentration (Fig. 4F). The catalytic efficiency of RGS7 toward His6Ga11 was 0.03 ± 0.01 $\times 10^6 \text{ M}^{-1} \text{s}^{-1}$, nine times lower than that toward His6Ga, and nearly 60 times lower than that of RGS4 toward His6Ga11 (Table II). This clarifies the confusion in the literature and demonstrates that the Ga specificity of RGS7 resides in the RGS box itself and doesn’t depend on complex formation with Gβγ. Furthermore, we examined whether myristoylation of the Ga subunits altered the kinetics or specificity of the RGS interactions. Comparable catalytic efficiencies were obtained for both RGS4 and RGS7 toward myrGa compared with the results for His6Ga (Fig. 5 and Table

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**Fig. 3.** GTP hydrolysis and Ga deactivation dependence of intrinsic fluorescence changes. A, stopped-flow experiments were conducted at 20 °C as described in the legend to Fig. 2 and under “Experimental Procedures.” Wild type His6Ga11 (600 nM) was preincubated with 2 μM GTP, GDP, Gpp(NH)p or 2 μM GDP plus 20 μM AlCl3 and 10 mM NaF (GDP-AlF4). Samples were then mixed with an equal volume of 30 mM MgSO4 in HED in the presence or absence of 100 nM RGS4 and intrinsic fluorescence of Ga was measured. B, similarly, deactivation of the RGS-resistant mutant G183S Ga11 prebound to GTP was measured in the presence or absence of 100 nM RGS4. Intrinsic fluorescence at the first time point was set to zero. Shown are averages of experiments repeated at least three times with multiple “shots” per experiment.

**Fig. 4.** Specificity of His6Ga and His6Ga11 deactivation kinetics accelerated by RGS4, RGS7, and RGS8. The rate of deactivation of GTP-preloaded His6Ga (200 nM final) or His6Ga11 (300 nM final) was determined by stopped-flow fluorescence at 20 °C as described under “Experimental Procedures” and in the legend to Fig. 2. Data were collected for at least 100 s in the absence of RGS and for 20–100 s in the presence of RGS. A and B, the effect of RGS4 and RGS7 on the intrinsic fluorescence changes (ΔF) of His6Ga. The rate of intrinsic fluorescence decrease (or Ga deactivation) was obtained by fitting the data to a single exponential decay curve. A and B are each from a single experiment representing a total of four and seven determinations, respectively. C–F, plots of rates as determined in A and B versus RGS concentrations. Linearity was shown for RGS4 up to 3 μM and similarly for RGS7 and RGS8 up to 1 μM for both His6Ga (C, D) and His6Ga11 (E, F). All lines shown are the linear fits of averaged data points at various RGS concentrations from at least three experiments (except for RGS8, n = 2). Error bars representing S.E. are too small to be visible. The slopes of all lines are summarized in Table II.
Table II

Catalytic efficiencies of RGS4 and RGS7 toward Ga subunits

| Ga       | By Stopped-flow |   | By GTPase assay |   |
|----------|----------------|---|----------------|---|
|          |                 |   | µm⁻¹s⁻¹ | Slope | µm⁻¹s⁻¹| Slope |
| RGS4     | RGS7            | Selectivity | RGS4/RGS7 | Selectivity |
| His₆Gaₒ  | 1.82 ± 0.06 (4) | 0.27 ± 0.01 (7) | 8 | 1.53 ± 0.21 (3) | 0.13 ± 0.03 (3) | 12 |
| myrGaₒ  | 2.81 ± 0.11 (3) | 0.61 ± 0.04 (4) | 5 |             |             |  |
| His₆Ga₁₁ | 1.72 ± 0.09 (3) | 0.03 ± 0.01 (3) | 75 |            | 1.28 ± 1.20 (3) | 0.03 ± 0.03 (3) | 43 |
| myrGa₁₁ | 2.07 ± 0.11 (6) | 0.02 ± 0.01 (5) | 104 |            |             |  |
| myrGa₂2 | 2.07 ± 0.20 (4) | 0.10 ± 0.02 (3) | 21 |             |             |  |

![Myristoylated Ga](image)

**Fig. 5.** Linear plot of deactivation rate of myristoylated Ga versus RGS concentration. Experiments were performed similarly to those shown in Fig. 4, except myrGaₒ, myrGa₁₁, and myrGa₂2 were used. All lines shown are the linear fits of averaged data points at various RGS concentrations from at least three experiments. The slopes of all lines are reported in Table II. A, RGS4; B, RGS7.

II). Thus the myristoylation of Ga did not affect the activity of RGS. A similar but less pronounced difference in catalytic activity was also seen between RGS4 and RGS7 toward myrGa₂2. To summarize these results, RGS4 was essentially equally active on all Ga subunits tested with a catalytic efficiency of \(2 \times 10^6 \text{ M}^{-1} \text{s}^{-1}\). RGS7 had lower activity overall, however, it was specific for Gaₒ, being \(10^{-10}-30\) times more active on Gaₒ than on Ga₁₁ and six times more active on Gaₒ than on Ga₉ (Fig. 5B and Table II).

To ensure that the specificity seen above by stopped-flow was not an artifact of the spectroscopic method, we used the standard single turnover [³²P]GTPase assay to check RGS4 and RGS7 at 27°C and 31°C. As shown in Fig. 6, His₆Gaₒ and His₆Ga₁₁ alone hydrolyzed GTP at similar rates at 4°C \((k_{cat} \approx 0.04 \pm 0.0002 \text{ s}^{-1} \text{ and } 0.007 \pm 0.0008 \text{ s}^{-1}, \text{ respectively, } n = 3)\), RGS4 at a concentration of 50 nM increased these rates about 10-fold \((k_{cat} \approx 0.08 \pm 0.01 \text{ s}^{-1} \text{ and } 0.07 \pm 0.01 \text{ s}^{-1}, \text{ respectively, } n = 3)\). RGS7, at a concentration of 100 nM, produced more modest increases \((k_{cat} \approx 0.017 \pm 0.003 \text{ s}^{-1} \text{ and } 0.010 \pm 0.003 \text{ s}^{-1}, \text{ respectively, } n = 3)\). We made qualitative estimates of catalytic efficiency from these single point data, and, as summarized in Table II, the results are consistent with the more extensive and quantitative data obtained by stopped-flow fluorimetry.

**DISCUSSION**

The recently identified RGS proteins are responsible for the fast physiological turnover of G-protein-mediated signaling by accelerating the GTPase activity of Ga (10, 23, 44, 45). Conventional biochemical assays to determine single turnover rates are not sufficiently fast to study the subsecond kinetics of GTP hydrolysis in the presence of RGS proteins. We describe here a novel approach, stopped-flow fluorimetry, that permits the quantitative study of the effects of RGS proteins on Ga on the millisecond time scale.

We were able to quantify the rates of Ga₁₁ and Gaₒ deactivation up to 5 s⁻¹ (t₁/₂ 140 ms) in the presence of micromolar concentrations of RGS4. This is approximately five times faster than the deactivation of muscarinic receptor-activated potassium channels in the heart (46). To put these results in context, Popov et al. (43) also found a linear relationship between the \(k_{cat}\) of Ga₉ and the concentration of RGS using single turnover [³²P]GTPase assay at 0°C but only studied the kinetics up to 60 nM RGS4. Their calculated catalytic efficiency of \(0.9 \times 10^6\)
transition state conformation of Ga.

ature dependence of the second order rate constant for RGS

the specificity of RGS7 using accurate results, in comparison, provide quantitative measurements on paired with RGS4) toward Gα stimulated GTPase rates of Gα revealed in our results is quite interesting. Although the unstimulated GTPase rates of Gα subunits are reduced approximately 10-fold upon reducing the temperature from 20 °C to 4 °C (0.02–0.03 s⁻¹ to 0.002–0.004 s⁻¹), there is little temperature dependence of the second order rate constant for RGS catalytic efficiency. For RGS4 we observed only a 15–25% lower slope of k_{cat}/[RGS] at 20 °C versus 4 °C. Similarly, the value reported by Popov (0.9 × 10⁶ m⁻¹ s⁻¹) at 0 °C is within a factor of two of our results at 20 °C. It has been proposed that RGS proteins enhance GTP hydrolysis by Ga by stabilizing the transition state conformation of Ga (16). The lack of temperature dependence indicated in our results supports the suggestion that there may be no large conformational change of the G-protein upon Gα binding and that Gα simply binds to those G-proteins already in the transition state conformation and stabilizes them. Therefore, the enhanced rates are largely dependent on the collision between RGS and Ga in the transition state conformation, as suggested in the “asparagine knuckle” hypothesis (17). Thus diffusion might be the rate-limiting step, and a plateau of the Ga deactivation rate may not be observed until extremely high concentrations of RGS protein are reached. This stopped-flow method, however, opens up a window of RGS-mediated fast Ga deactivation kinetics beyond those available with the conventional approaches.

Structural Basis of Specificity of RGS4 and RGS7 toward Gaα.—The quantitative data obtained with this rapid stopped-flow method provides a more accurate picture of RGS-Ga specificity. We previously reported that 100 nM RGS4 had k_{cat} values of >5 min⁻¹ toward Gaα₁ (21), which is consistent with our present results, shown in Table II with a k_{cat} value of 14 min⁻¹. The catalytic efficiency of RGS4 for all three Gaα subunits is quite comparable.

We have also demonstrated the specificity of the RGS7 box domain (amino acids 305–453) for Gaα over Gaγ using both stopped-flow fluorimetry and single turnover [32P]GTPase methods (Table II). These data on the specificity of RGS7 are largely consistent with and extend other studies previously reported. These include the preferential binding of an RGS7 box domain (amino acids 303–470) to Gaα over Gaα₁ or Gaα₂ (28) and the moderate GAP activity of RGS7-Gaα complex (compared with RGS4) toward Gaα, but not Gaα₁, or Gaα₂ (31).

Our results, in comparison, provide quantitative measurements on the specificity of RGS7 using accurate k_{cat} values, especially for RGS at higher concentrations. In contrast, Shuey et al. (27), reported that the RGS7 box (amino acids 317–474) was active toward both Gaα₁ and Gaα₂. This conclusion, however, was only qualitative, because they did see slower k_{cat} for Gaα₂ than for Gaα₁. The high concentration of RGS7 they used (800 nM) makes the estimation of rates in the manual assay very difficult. The apparent difference between the results of Shuey et al. and those reported here does not seem to be due to the different RGS7 region they expressed, because a shorter version of our RGS7 construct (amino acids 327–452) still retained specificity for Gaα over Gaα₁ (data not shown). Thus the specificity of RGS7 GAP activity for Gaα over Gaα₁ and Gaα₂ resides in the RGS box domain itself and does not require the full-length RGS7 or a complex with the β₃ subunit.

In summary, we show results from a novel stopped-flow method to characterize the rapid Ga-RGS interactions. Our data are the first to report the effects of RGS proteins on Gaα or Gaα₁ on the millisecond time scale. The rates of Gaα deactivation observed here are clearly fast enough to account for the physiological turning off of ion channels. Furthermore, using this approach, we quantitatively evaluate the specificity of Ga-RGS interactions. It is also clear that the mechanism of RGS-mediated Ga GAP activity does not require substantial, slow conformation changes in the structure of the Go subunit.

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Rapid Ga Deactivation Mediated by RGS4 and RGS7

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