Mechanism of the intrinsic arginine finger in heterotrimeric G proteins

Daniel Mann*, Christian Teuber*, Stefan A. Tennigkeit*, Grit Schröter*, Klaus Gerwert**,1, and Carsten Köttig*1

*Department of Biophysics, Ruhr University Bochum, 44780 Bochum, Germany; and **Partner Institute for Computational Biology, 200031 Shanghai, China

Heterotrimeric G proteins are crucial molecular switches that maintain a large number of physiological processes in cells. The signal is encoded into surface alterations of the Gα subunit that carries GTP in its active state and GDP in its inactive state. The ability of the Gα subunit to hydrolyze GTP is essential for signal termination. Regulator of G protein signaling (RGS) proteins accelerates this process. A key player in this catalyzed reaction is an arginine residue, Arg178 in Gαi1, which is already an intrinsic part of the catalytic center in Gα in contrast to small GTPases, at which the corresponding GTPase-activating protein (GAP) provides the arginine “finger.” We applied time-resolved FTIR spectroscopy in combination with isotopic labeling and site-directed mutagenesis to reveal the molecular mechanism, especially of the role of Arg178 in the intrinsic Gαi1 mechanism and the RGS4-catalyzed mechanism. Complementary biomolecular simulations (molecular mechanics with molecular dynamics and coupled quantum mechanics/molecular mechanics) were performed. Our findings show that Arg178 is bound to γ-GTP for the intrinsic Gαi1 mechanism and pushed toward a bidentate α-γ-GTP coordination for the Gαi2·γ-GTP4 mechanism. This movement induces a charge shift toward β-GTP, increases the planarity of γ-GTP, and thereby catalyzes the hydrolysis.

GTase | FTIR spectroscopy | QM/MM calculations | arginine finger | reaction mechanism

Heterotrimeric G proteins serve as a link between G protein-coupled receptors (GPCRs) and second messenger systems like adenyl cyclases in the cell (1). The inactive trimeric form consisting of the GTase Gα and the Gβγ complex gets activated by a GPCR that acts as a guanine nucleotide exchange factor (GEF). It promotes GDP release and enables GTP uptake at the active site of Gα, which results in structural changes in the switch I–III regions of the α-subunit (2), separation of the subunits, and signal transduction (3). Termination of the signal is initiated by GTP hydrolysis at the active center of Gα to GDP and P. This crucial mechanism is highly conserved among GTases and requires numerous mechanistic features the protein has to provide [e.g., Mg2+ incorporation (4), substrate coordination (5), charge neutralization (6), positioning of the nucleophilic water (7)]. Some of these functions are maintained by two highly conserved residues: an arginine side chain (arginine “finger” in small GTases) and a carboxyamide near the γ-phosphate (8). In contrast to small GTases, where the arginine is provided by a GTase-activating protein (GAP), heterotrimeric G proteins are equipped with an intrinsic arginine finger (Arg178 in Gαi1), which is located in switch I [residues 178–188 in Gαi1 (9)] and enables fast hydrolysis compared with small GTases [factor of 50 (10, 11)]. A GAP protein [e.g., regulator of G protein signaling 4 (RGS4) in the case of Gαi2] can further accelerate GTP hydrolysis (12). The importance of the arginine finger manifests in various diseases; for example, single point mutations in Gαi lead to McCune–Albright syndrome (13, 14) and ADP ribosylation of the arginine finger in Gαi by Vibrio cholerae leads to cholera disease (15).

We have demonstrated recently how FTIR spectroscopy on Gαi2 can monitor the GTase reaction label-free with high spatiotemporal resolution (10). This approach was originally established to elucidate the proton-pump mechanism of bacteriorthodospin via protein-bound water molecules (16). In this study, we will focus on the intrinsic arginine finger and elucidate its position and mechanism in intrinsic and RGS4-catalyzed Gαi2. Current models of the arginine finger mechanism rely on crystal structures that provide atomistic snapshots of the active GTP state using nonhydrolyzable GTP analogs (17, 18), the GDP·AlF4− intermediate state (17), and the inactive GTP state (19). Upon Gαi1, the arginine finger is variable; for example, in Gαi2·Mg2+·GTP·S, it is hydrogen-bonded toward the β-γ-bridging oxygen (20), and in Gαi1·Mg2+·GppNHp and Gαi1·Mg2+·GTP·γS, it is partially disordered, forming an ion pair with Glu43 (18). In all Gα isoforms resolved to date with GDP·AlF4−, the arginine finger is bound to the fluoride group, also facing the bridging β-γ-atom and the α-γ-GTP group (2, 21–25). The arginine finger of the isoform Gαi1 seems to be flipped away from the nucleotide in both the GTP state and the GDP state, and it only participates in nucleotide binding during the intermediate AlF4− state (Fig. 1). However, active structures of Gαi1 were solved in presence of sulfur- or nitrogen-substituted GTP analogs only, which may influence the arginine finger position. Furthermore, the position of the arginine finger in the AlF4− intermediate state could also be influenced by the strong electronegativity of this intermediate state analog. To overcome this problem, we applied time-resolved FTIR spectroscopy with photocaged para-hydroxyphenacyl cgGTP (pHPaGTP), which cleaves rapidly [107 s−1 (26)] and results in the natural GTP nucleotide that triggers the GTase reaction label-and analog-free. The resulting photolysis and hydrolysis difference spectra reflect the reaction with subangstrom spatial and millisecond temporal

**Significance**

The α-subunit of heterotrimeric G proteins is a molecular switch that mediates a great number of physiological processes such as vision, smelling, and blood pressure regulation. A GTase-activating protein (GAP) [e.g., regulator of G protein signaling 4 (RGS4) in the case of Gαi2] regulates the off-switch by catalyzing GTP hydrolysis. Here, we present the molecular reactions of GAP catalysis at atomic resolution using a combination of FTIR spectroscopy and biomolecular simulations. In contrast to X-ray structures, not GTP analogs but GTP itself is used. This approach is crucial to reveal now a previously undescribed GAP mechanism for Gαi. A key player in the hydrolysis reaction, the arginine finger, is pushed from a monodentate γ-GTP coordination toward a bidentate α-γ-GTP coordination by RGS4, and thereby catalyzes GTP-hydrolysis.

Author contributions: K.G. and C.K. designed research; D.M., C.T., S.A.T., and G.S. performed research; D.M. and C.K. analyzed data; and D.M., K.G., and C.K. wrote the paper. The authors declare no conflict of interest.

This article is a PNAS Direct Submission. E.F.P. is a Guest Editor invited by the Editorial Board. Freely available online through the PNAS open access option.

1To whom correspondence may be addressed. Email: canten.koetting@rub.de or gerwert@bph.rub.de.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1612394113/-/DCSupplemental.
resolution. We assigned individual phosphate and protein bands using isotopic labeling and site-directed mutagenesis, and were able to observe the arginine finger position and its mechanism with native GTP. Mutations were selected because of their catalytic relevance (e.g., Arg178, Glu204), effects in previous studies (e.g., Lys180), and spatial proximity to the active site. Positions of all point mutations are indicated in SI Appendix, Fig. 1. Molecular mechanics with molecular dynamics (MM-MD) and coupled quantum mechanics/molecular mechanics (QM/MM) simulations complemented the experiments and provided additional evidence for the arginine finger mechanism in heterotrimeric G proteins.

**Results**

Time-resolved FTIR spectroscopy monitors reactions label-free at atomic resolution and provides both rate constants and structural information that are coded into IR spectra. WT or mutant Ga\textsubscript{i1} was loaded with pHP\textsubscript{c}GTP, a photolabile GTP derivate that binds to Ga\textsubscript{i1} but is not hydrolyzed. FTIR measurements were triggered by a laser flash that removes the pHP-caged group, resulting in the natural substrate GTP (result \(a_0\) of global fit as detailed in Materials and Methods, termed photolysis in the following) that is subsequently hydrolyzed by Ga\textsubscript{i1} (result \(a_1\) of global fit, termed hydrolysis in the following). Both the photolysis and the hydrolysis reaction were monitored by time-resolved FTIR spectroscopy. The obtained data were evaluated using a global fit (Eq. 1) with one exponential function for Ga\textsubscript{i1}-WT [rate constant \(k = 0.02\) s\(^{-1}\) at 15 °C (10)] or two exponential functions when intermediate formation occurred (Table 1). RGS4-catalyzed measurements were also evaluated using one exponential function. All resulting \(t_{1/2}\) values and their SEs are depicted in Table 1. Spectral information is coded into a photolysis spectrum (negative bands correspond to the caged GTP state, and positive bands correspond to the GDP + P\textsubscript{i} state) and a hydrolysis spectrum (negative bands correspond to the GTP state, and positive bands correspond to the GDP + P\textsubscript{i} state). Several Ga\textsubscript{i1} mutants showed an intermediate during the hydrolysis reaction, and were therefore evaluated with an additional reaction rate. The importance of working label- and analog-free can be demonstrated when performing FTIR measurements using N-methylanthraniloyl (MANT)-GTP instead of natural GTP. Using MANT-GTP slows down hydrolysis kinetics by one order of magnitude and significantly, due to distortion of the catalytic center, the GTP and protein bands (SI Appendix, Fig. 2).

**Arg178 Is Bound to \(\gamma\)-GTP in the Active State of Ga\textsubscript{i1}.** FTIR measurements of Ga\textsubscript{i1}-WT and Ga\textsubscript{i1}-R178S are depicted in Fig. 2. We recently assigned the bands for \(\alpha\)-GTP, \(\beta\)-GTP, and \(\gamma\)-GTP (1.243 cm\(^{-1}\), 1.224 cm\(^{-1}\), and 1.156 cm\(^{-1}\), respectively); \(\alpha\)-GDP and \(\beta\)-GDP (1.214 cm\(^{-1}\) and 1.134/1.103 cm\(^{-1}\), respectively); and free phosphate (1.078/991 cm\(^{-1}\)) for Ga\textsubscript{i1}-WT (10). Surprisingly, in this study, the \(\gamma\)-GTP vibration of the mutant Ga\textsubscript{i1}-R178S appeared blue-shifted from 1.156 cm\(^{-1}\) to 1.165 cm\(^{-1}\) (Fig. 2), indicating changes in the direct environment of the \(\gamma\)-phosphate. Because the exchange of Arg178 to Ser178 was the only difference described for the small GTPase Ran (4). The mutation Ran-Y32A caused a \(\gamma\)-GTP shift of 12 cm\(^{-1}\). For Ga\textsubscript{i1}-R178S, the band shift was observable in both the photolysis spectrum and the hydrolysis spectrum for measurements in H\(_2\)O and measurements in D\(_2\)O. The vibrations of \(\alpha\)-GTP and \(\beta\)-GTP remained unaltered. To verify that the Ga\textsubscript{i1}-R178S mutation had no effect on \(\alpha\)-GTP, we applied isotopic labeling using \(^{13}\)O\textsubscript{2}pHP\textsubscript{c}GTP in Ga\textsubscript{i1}-R178S and assigned the band at 1.243 cm\(^{-1}\) clearly to \(\alpha\)-GTP, similar to Ga\textsubscript{i1}-WT (SI Appendix, Fig. 3). The vibration of \(\alpha\)-GDP appeared slightly redshifted from 1.214 cm\(^{-1}\) (Ga\textsubscript{i1}-WT) to 1.208 cm\(^{-1}\) (Ga\textsubscript{i1}-R178S).

Mutation of the intrinsic arginine finger slowed down the reaction by almost two orders of magnitude as we previously reported (10), and is comparable to the Ga\textsubscript{i1}-Q204A mutation (Table 1). Kinetics of the cleaved phosphate product bands are depicted in Fig. 2B.

**Several Ga\textsubscript{i1} Mutants Exhibit Intermediate Formation During Hydrolysis.** Mutation of the intrinsic arginine finger caused a rate separation during hydrolysis, showing an intermediate that proceeded hydrolysis with spectral features at 1.280 cm\(^{-1}\) and 1.230 cm\(^{-1}\) (Fig. 2A, cyan).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Temperature</th>
<th>(t_{1/2}) values (global fit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ga\textsubscript{i1}-WT</td>
<td>15 °C</td>
<td>32.7 ± 2.5 s</td>
</tr>
<tr>
<td>Ga\textsubscript{i1}-WT</td>
<td>5 °C</td>
<td>68.2 ± 5.1 s</td>
</tr>
<tr>
<td>Ga\textsubscript{i1}-E43Q</td>
<td>15 °C</td>
<td>(t_{1/2} = 1.9 \pm 0.8 s)</td>
</tr>
<tr>
<td>Ga\textsubscript{i1}-E43Q</td>
<td>5 °C</td>
<td>(t_{1/2} = 55.7 \pm 6.2 s)</td>
</tr>
<tr>
<td>Ga\textsubscript{i1}-T48A</td>
<td>15 °C</td>
<td>8.6 ± 1.2 s</td>
</tr>
<tr>
<td>Ga\textsubscript{i1}-T48A</td>
<td>5 °C</td>
<td>75.5 ± 9.4 s</td>
</tr>
<tr>
<td>Ga\textsubscript{i1}-D150N</td>
<td>15 °C</td>
<td>(t_{1/2} = 23.4 \pm 44.0 s)</td>
</tr>
<tr>
<td>Ga\textsubscript{i1}-D150N</td>
<td>5 °C</td>
<td>9.8 ± 0.9 s</td>
</tr>
<tr>
<td>Ga\textsubscript{i1}-R178S</td>
<td>15 °C</td>
<td>(t_{1/2} = 3437.7 \pm 426.8 s)</td>
</tr>
<tr>
<td>Ga\textsubscript{i1}-K180P</td>
<td>15 °C</td>
<td>(t_{1/2} = 68.9 \pm 26.0 s)</td>
</tr>
<tr>
<td>Ga\textsubscript{i1}-K180P</td>
<td>5 °C</td>
<td>(t_{1/2} = 537.4 \pm 58.0 s)</td>
</tr>
<tr>
<td>Ga\textsubscript{i1}-Q204A</td>
<td>15 °C</td>
<td>(t_{1/2} = 7.1 \pm 5.4 s)</td>
</tr>
<tr>
<td>Ga\textsubscript{i1}-Q204A</td>
<td>5 °C</td>
<td>(t_{1/2} = 3406 \pm 939.3 s)</td>
</tr>
<tr>
<td>Ga\textsubscript{i1}-W211A</td>
<td>15 °C</td>
<td>(t_{1/2} = 4.4 \pm 0.4 s)</td>
</tr>
<tr>
<td>Ga\textsubscript{i1}-W211A</td>
<td>5 °C</td>
<td>(t_{1/2} = 125.2 \pm 16.3 s)</td>
</tr>
<tr>
<td>Ga\textsubscript{i1}-D229N</td>
<td>15 °C</td>
<td>(t_{1/2} = 14.7 \pm 1.0 s)</td>
</tr>
<tr>
<td>Ga\textsubscript{i1}-D229N</td>
<td>5 °C</td>
<td>(t_{1/2} = 53.2 \pm 3.8 s)</td>
</tr>
<tr>
<td>Ga\textsubscript{i1}-E236Q/D237Q</td>
<td>15 °C</td>
<td>(t_{1/2} = 6.2 \pm 0.9 s)</td>
</tr>
<tr>
<td>Ga\textsubscript{i1}-E236Q/D237Q</td>
<td>5 °C</td>
<td>(t_{1/2} = 60.0 \pm 4.1 s)</td>
</tr>
<tr>
<td>Ga\textsubscript{i1}-E245Q</td>
<td>15 °C</td>
<td>(t_{1/2} = 5.1 \pm 3.4 s)</td>
</tr>
<tr>
<td>Ga\textsubscript{i1}-E245Q</td>
<td>5 °C</td>
<td>(t_{1/2} = 53.9 \pm 6.1 s)</td>
</tr>
<tr>
<td>Ga\textsubscript{i1}-WT-RGS4</td>
<td>5 °C</td>
<td>(t_{1/2} = 1.4 \pm 0.3 s)</td>
</tr>
<tr>
<td>Ga\textsubscript{i1}-R1785-RGS4</td>
<td>5 °C</td>
<td>(t_{1/2} = 12.6 \pm 2.7 s)</td>
</tr>
<tr>
<td>Ga\textsubscript{i1}-T48A-RGS4</td>
<td>5 °C</td>
<td>(t_{1/2} = 9.6 \pm 1.6 s)</td>
</tr>
</tbody>
</table>
found the band at 1,230 cm⁻¹ that appeared in the photolysis and disappeared in the hydrolysis (Fig. 3, red) (Complete photolysis and hydrolysis spectra from 1,800 cm⁻¹ to 950 cm⁻¹ are depicted in SI Appendix, Fig. 5). We applied isotopic labeling using α-¹⁸O₂-pHPcgGTP (SI Appendix, Fig. 6) and β-¹⁸O₂-pHPcgGTP (SI Appendix, Fig. 7), and could thereby determine that the α-GTP vibration caused the band at 1,182/1,184 cm⁻¹ for RGS4-catalyzed Gα₁. Moreover, this effect was completely reversible when the intrinsic arginine finger was missing due to the R178S mutation (Fig. 3, cyan), so we can conclude that RGS4 induces a conformational change of Arg178 in such a way that it binds to α-GTP. In addition, Arg178 is simultaneously bound to γ-GTP, because for Gα₁-R178S-RGS4, it was seen that in both the photolysis and hydrolysis spectra, the γ-GTP vibration was shifted from 1,156 cm⁻¹ to 1,165 cm⁻¹ (Fig. 3, cyan), like in intrinsic Gα₁ (Fig. 2). The magnitude of the RGS4-induced α-GTP shift indicates that RGS4 binding changes more than the α-GTP environment (e.g., induces structural changes like torsions or bond lengths and a different charge distribution). The α-GDP vibration was also shifted from 1,214 cm⁻¹ to 1,219 cm⁻¹ in RGS4-catalyzed measurements, which was again reversible when Arg178 was missing. For Gα₁-R178S-RGS4, no intermediate formation was observed. To exclude a temperature artifact, we also repeated these measurements at −5 °C and observed no spectral changes. Our measurements demonstrated that the intrinsic arginine finger is bound to γ-GTP for intrinsic Gα₁ and bound bidentately to α-GTP and γ-GTP for Gα₁-RGS4.

**RGS4 Stabilizes Switch I in MD Simulations.** We performed MD simulations of intrinsic and RGS4-bound Gα₁ in the GDP- and GTP-bound states to gain a structural interpretation of the IR

![Fig. 2.](image.png)

Fig. 2. (A) Photolysis and hydrolysis FTIR difference spectra (global fit) of Gα₁-WT and the mutant Gα₁-R178S. (B) Kinetics of cleaved phosphate (1078 cm⁻¹) for Gα₁-WT, Gα₁-R178S, and Gα₁-R178S + RGS4 at 15 °C and 5 °C. In A, positive bands in the photolysis spectra and negative bands in the hydrolysis spectra correspond to the GTP state. Positive bands in the hydrolysis spectra correspond to the GDP state. Arrows indicate the γ-GTP shift caused by the mutation. In B, mutation of Arg178 caused a slowdown of the GTPase reaction by two orders of magnitude. RGS4 reversed this effect. Kinetic constants obtained from global fits are depicted in Table 1. mAU, milli absorbance units; norm., normalized.

This intermediate was observable for various point mutations in Gα₁ (Gα₁-E43Q, Gα₁-K180P, Gα₁-D150N, Gα₁-E226Q/D227Q, Gα₁-D229N, Gα₁-W211A, and Gα₁-E245Q) and is not an exclusive feature of the arginine mutation. The t₁/₂ values of both rates are depicted in Table 1. We applied isotopic labeling of the Gα₁-E43Q mutant using α-¹⁸O₂-pHPcgGTP and β-¹⁸O₂-pHPcgGTP and found the band at 1,230 cm⁻¹ to be caused by both α-GTP and β-GTP (SI Appendix, Fig. 4).

**RGS4 Addition Accelerates GTP Hydrolysis in Gα₁ by Two Orders of Magnitude.** In addition to intrinsic FTIR measurements of Gα₁, we performed measurements with its GAP RGS4 in a 1:1 complex. Like in intrinsic Gα₁-WT measurements, no intermediate formation was observed. RGS4 addition accelerated the t₁/₂ value of the GTPase reaction in Gα₁ by almost two orders of magnitude from 68.2 ± 5.1 s (5 °C) to 1.4 ± 0.3 s (5 °C). Kinetics of the cleaved phosphate product bands are depicted in Fig. 2B.

**RGS4 Pushes Arg178 Toward a Bidentate α-γ-GTP Coordination.** The addition of RGS4 not only accelerated hydrolysis kinetics by two orders of magnitude but also changed the GTP and GDP vibrations. The most prominent change was a band at 1,184 cm⁻¹ for Gα₁-WT and the mutant Gα₁-R178S. This intermediate was observable for various point mutations in Gα₁ (Gα₁-D229N, Gα₁-G178S, Gα₁-E245Q) and is not an exclusion. Our measurements demonstrated that RGS4-induced α-GTP shift indicates that RGS4 binding changes more than the α-GTP environment (e.g., induces structural changes like torsions or bond lengths and a different charge distribution). The α-GDP vibration was also shifted from 1,214 cm⁻¹ to 1,219 cm⁻¹ in RGS4-catalyzed measurements, which was again reversible when Arg178 was missing. For Gα₁-R178S-RGS4, no intermediate formation was observed. To exclude a temperature artifact, we also repeated these measurements at −5 °C and observed no spectral changes. Our measurements demonstrated that the intrinsic arginine finger is bound to γ-GTP for intrinsic Gα₁ and bound bidentately to α-GTP and γ-GTP for Gα₁-RGS4.

**RGS4 Stabilizes Switch I in MD Simulations.** We performed MD simulations of intrinsic and RGS4-bound Gα₁ in the GDP- and GTP-bound states to gain a structural interpretation of the IR

![Fig. 3.](image.png)

Fig. 3. Hydrolysis FTIR spectra of intrinsic and RGS4-catalyzed Gα₁-WT and its mutant R178S. RGS4 shifted the α-GTP vibration from 1,243 cm⁻¹ to 1,184 cm⁻¹. The effect was reversible when Arg178 was mutated. Full spectra of the photolysis and the hydrolysis reaction are depicted in SI Appendix, Fig. 5.
structures. Contact matrix analysis of the Arg178-bound (black) and RGS4-bound (red) MD simulations in comparison to the starting structure of GTP-bound Gαi1 shows large conformational dynamics of Arg178, with the monodentate coordination at α-phosphate groups. This interaction was never interrupted in all five production runs (Fig. 4D).

IR Band Assignment of Gαi1-Arg178. Band assignments were performed using 15N-labeled arginine. Protein expression was performed in M9 minimal medium (28) with unlabeled arginine replaced by 15N-labeled arginine. Purity and labeling efficiency of recombinant proteins were checked via SDS-PAGE and liquid chromatography (LC)-MS (minimum 92% labeling efficiency). Isotopic labeling results in a red shift of arginine side-chain vibrations due to the increased reduced mass. Because isotopic labeling of arginine amino acids affects all arginine side chains of Gαi1, we also performed measurements of the mutant Gαi1 to ensure a site-specific assignment. In H2O, no isotopic shift could be determined because the 1H(CN3H5+) vibration at 1,630–1,680 cm⁻¹ (29) is superimposed by absorptions of water. We exchanged the solvent to D2O and observed isotopic shifts exclusively in the area between 1,580 cm⁻¹ and 1,610 cm⁻¹ that is described in the literature for the symmetrical and asymmetrical arginine side-chain vibrations (29) in the hydrolysis spectrum (Fig. 5A). The band at 1,604 cm⁻¹ that belongs to the GDP state is shifted to 1,600 cm⁻¹, and the band at 1,590 cm⁻¹ that belongs to the GDP state is shifted to 1,585 cm⁻¹ (Fig. 5B). These band shifts could not be observed in the photolysis spectrum, probably indicating that Arg78 is in the GDP caged GTP state in a similar position as in the Gαi1 GTP state. To ensure a site-specific assignment, we also measured the mutant Gαi1-R178S (Fig. 5A, orange) and observed that the corresponding bands at 1,604 cm⁻¹ and 1,590 cm⁻¹ were missing. Additional changes at 1,553 cm⁻¹ (negative, Amide II) and around 1,450 cm⁻¹ (positive, Amide II') are caused by deuterium exchange that follows the hydrolysis (increased flexibility of Arg78).

MD Simulations Confirm Monodentate (Intrinsic) and Bidentate (RGS4-Catalyzed) Coordination of Arg178. In addition to rmsd calculations, we performed contact matrix analysis of the production runs with a focus on the interaction between Arg178 and the individual phosphate groups. For intrinsic Gαi1, the arginine finger was bound monodentately to γ-GTP in three of five simulation runs (Fig. 4C), but the coordination to α-GTP (Fig. 4C, run 2) and even a bidentate coordination to α-GTP and γ-GTP were also observed (Fig. 4C, run 5) in replica runs. Simulations showed large conformational dynamics of Arg178, with the monodentate coordination at γ-GTP being the preferred interaction. It is notable that in all starting structures, the arginine pointed in the opposite direction, forming a stacked π-interaction to Glu43. This coordination was also disrupted, and Arg178 flipped down to the substrate in all simulations. We also repeated the simulations starting from intrinsic Gαi1-AlF₄⁻·GDP [Protein Data Bank (PDB) ID code 1GFI, with GDP-AlF₄⁻ replaced by GDP or GTP], where the arginine is already oriented toward the substrate to exclude starting structure artifacts, and found similar behavior. With RGS4 bound to Gαi1 (PDB ID code 1AGR), the intrinsic arginine finger was always bound bidentately to α-GTP and γ-GTP. The terminal NH₂ groups of the side chain were tightly bound to the oxygen atoms of the α- and γ-phosphate groups. This interaction was never interrupted in all five production runs (Fig. 4D).
switch regions in the GDP state) and is more pronounced for G\textsubscript{\alpha\textsubscript{1}}-R178S because of the slowed-down hydrolysis (two orders of magnitude). Therefore, the IR bands for Arg178 were assigned to 1,604 cm\(^{-1}\) (G\textsubscript{\alpha\textsubscript{1}}-GTP) and 1,590 cm\(^{-1}\) (G\textsubscript{\alpha\textsubscript{1}}-GDP). Complete hydrolysis spectra of unlabeled and labeled G\textsubscript{\alpha\textsubscript{1}}-WT and the G\textsubscript{\alpha\textsubscript{1}}-R178S mutants are depicted in SI Appendix, Fig. 8.

**IR Band Assignment of Arg178 in G\textsubscript{\alpha\textsubscript{1}}-K180P.** After the successful band assignment of Arg178 in G\textsubscript{\alpha\textsubscript{1}}-WT, we were also interested in which step the arginine finger takes action in rate-separated mutants. For this purpose, we performed measurements with \(^{15}\text{N}\) labeled G\textsubscript{\alpha\textsubscript{1}}-K180P (Fig. 5C). The bands at 1,230 cm\(^{-1}\) and 1,280 cm\(^{-1}\) that appeared in the first rate in H\(_2\)O were shifted to 1,238 cm\(^{-1}\) and 1,275 cm\(^{-1}\) in D\(_2\)O. Isotopic labeling of the first rate showed no band shifts [Fig. 5C, double-difference (\(\Delta\Delta\)) rate 1], whereas the second rate showed that the same bands at 1,604 cm\(^{-1}\) and 1,590 cm\(^{-1}\) were shifted to 1,600 cm\(^{-1}\) and 1,587 cm\(^{-1}\) like in WT G\textsubscript{\alpha\textsubscript{1}} (Fig. 5C, \(\Delta\alpha\) rate 2). Hence the arginine finger remains bound to GTP in the first rate and changes its conformation exclusively in the second rate of rate-separated G\textsubscript{\alpha\textsubscript{1}} mutants. The \(t_{1/2}\) values of the rates were \(t_{1/2\text{1}} = 69 \pm 26\) s and \(t_{1/2\text{2}} = 537 \pm 58\) s at 15 °C. Complete hydrolysis spectra of unlabeled and labeled G\textsubscript{\alpha\textsubscript{1}}-K180P are depicted in SI Appendix, Fig. 9.

**IR Bands of G\textsubscript{\alpha\textsubscript{1}}-Arg178 Differ from the Bands of the Arginine Finger in Ras-RasGAP.** The arginine finger mechanism is conserved among many small GTPases. For the RasGAP neurofibromin 1 (NFI), the band assignment of Arg1276 was already performed (30). A band at 1,589 cm\(^{-1}\) was assigned to arginine in a deuterated water environment, and a band at 1,571 cm\(^{-1}\) was assigned to arginine within the binding pocket of Ras-GAP. It was not possible to observe the transient state, where Arg1276 is coupled to GTP, because bond breakage is faster than the movement of Arg1276 into the binding pocket. G\textsubscript{\alpha\textsubscript{1}} is equipped with an intrinsic arginine finger, and we could observe here the arginine finger coupled to GTP (SI Appendix, Fig. 10).

**IR Bands of the Arginine Finger in G\textsubscript{\alpha\textsubscript{1}}-RGS4 Differ from Intrinsic G\textsubscript{\alpha\textsubscript{1}}.** After the observation that Arg178 is coupled bidentately to \(\alpha\)-GTP and \(\gamma\)-GTP upon RGS binding, we examined whether the vibration of Arg178 in G\textsubscript{\alpha\textsubscript{1}}-RGS4 also differs from intrinsic G\textsubscript{\alpha\textsubscript{1}}. Therefore, we performed FTIR measurements with \(^{15}\text{N}\) amino acid-labeled G\textsubscript{\alpha\textsubscript{1}} in a 1:1 complex with RGS4. Indeed, the bands of Arg178 were no longer found at 1,604 cm\(^{-1}\) and 1,590 cm\(^{-1}\) as assigned for intrinsic G\textsubscript{\alpha\textsubscript{1}} (SI Appendix, Fig. 11). Isotopic labeling revealed that for both the GTP and GDP states, two bands could be assigned. In the GTP state, bands at 1,601 cm\(^{-1}\) and 1,583 cm\(^{-1}\) were shifted to 1,596 cm\(^{-1}\) and 1,587 cm\(^{-1}\), respectively. In the GDP state, bands at 1,613 cm\(^{-1}\) and 1,593 cm\(^{-1}\) were shifted to 1,610 cm\(^{-1}\) and 1,590 cm\(^{-1}\), respectively. The difference in arginine vibrations supports the monodentate vs. bidentate binding mode we proposed for intrinsic and RGS4-catalyzed G\textsubscript{\alpha\textsubscript{1}}. We also performed measurements of the complex G\textsubscript{\alpha\textsubscript{1}}-R178S-RGS4 in D\(_2\)O to assign the site-directed arginine bands; however, in this case, the mutant is too invasive. The resulting photolysis and hydrolysis difference spectra differed significantly from G\textsubscript{\alpha\textsubscript{1}}-RGS4, disallowing specific assignments. The hydrolysis spectrum of G\textsubscript{\alpha\textsubscript{1}}-R178S-RGS4 matched the hydrolysis spectrum of intrinsic G\textsubscript{\alpha\textsubscript{1}} with missing bands at 1,604 cm\(^{-1}\) and 1,590 cm\(^{-1}\).

**Spectra from QM/MM Simulations Reproduce FTIR Experiments.** To decode the spectral data further, we performed coupled QM/MM simulations and calculated theoretical IR spectra of the individual phosphate groups. We calculated an ensemble of 15 snapshots of a 100-ns MD trajectory (details are provided in Materials and Methods) with the functionals B3LYP, M06, and PBE with the basis set 6-31G*. The geometry of the binding pocket, including the intrinsic arginine finger, is depicted in Fig. 6A. As shown via MD simulations and experiments, Arg178 is coupled to \(\gamma\)-GTP. Mean values and SDs of each of 15 QM/MM simulations for the functional M06 are depicted in Fig. 6E and compared with peak maxima and full width at half-maximum values of the experiment (FTIR). The functional M06/6-31G* exactly reproduces experimental peaks and band widths for \(\nu_{\text{AS}}\) Py-O\(_2\), \(\nu_{\text{AS}}\) P=O\(_2\), and \(\nu_{\text{AS}}\) Py-O\(_2\). Calculated IR vibrations of B3LYP/6-31G* and PBE/6-31G* showed slightly decreased absolute wavenumbers, but with comparable spacing and SDs, indicating the calculated geometry matches the experimental situation (SI Appendix, Fig. 12).

**Bidentate Coupling of Arg178 Causes Eclipsed (\(\alpha\)-\(\beta\)-\(\gamma\))-GTP Conformation.** Because the experiments predicted only a bidentate coupling of Arg178 to \(\alpha\)-GTP and \(\gamma\)-GTP, we calculated different starting positions of Arg178, with \(\gamma\) oriented away from \(\alpha\)-GTP (PDB ID code 1AGR) (Fig. 6B) and \(\alpha\) oriented toward \(\alpha\)-GTP (Fig. 6C), as suggested by \(^{13}\text{C}\) NMR of Rho-GAP (31). Both conformations caused significant shifts of the individual GTP vibrations (Fig. 6E), but only the latter conformation formed \(\alpha\)-\(\beta\)-\(\gamma\) eclipsed GTP (Fig. 6D). The eclipsed \(\alpha\)-\(\beta\)-oxygen in this geometry were proposed to stretch the bridging P-O-P bonds, and thereby catalyze GTP hydrolysis (11). Experimental band assignments for the G\textsubscript{\alpha\textsubscript{1}}-RGS4 complex were already performed, showing a slight RGS4-induced blue shift (\(+6\text{ cm}\(^{-1}\)) for \(\beta\)-GTP (SI Appendix, Fig. 7) and a large red shift of \(\alpha\)-GTP of \(-59\text{ cm}\(^{-1}\), resulting in a shift of the \(\alpha\)-GTP vibration between the \(\beta\)-GTP and \(\gamma\)-GTP vibrations (Fig. 3 and SI Appendix, Fig. 6). This behavior was not reproduced in calculations with the Ne group of Arg178 turned away from \(\alpha\)-GTP (Fig. 6E). In this case, the \(\beta\)-GTP band was shifted to the opposite direction than in the experiments and the \(\alpha\)-GTP band showed higher wavenumbers than the \(\beta\)-GTP band (Fig. 6E). Only with the Ne group of Arg178 pointing toward \(\alpha\)-GTP (Fig. 6C) could the experimental behavior be reproduced with a small blue shift of \(\beta\)-GTP and a large red shift of \(\alpha\)-GTP below the \(\beta\)-GTP frequency (Fig. 6E). However, the experimental \(\alpha\)-GTP shift of \(-59\text{ cm}\(^{-1}\) could not be reproduced quantitatively. The calculated average
\( \alpha \)-GTP shift was only \( -20 \text{ cm}^{-1} \), which still reproduced the experimental behavior qualitatively.

**Origin of the \(-59 \text{ cm}^{-1} \alpha \)-GTP Shift.** The QM/MM calculations suggested a tight coordination of \( \alpha \)-GTP between Arg178 on one side and Thr48 on the other side. Therefore, we performed FTIR measurements of intrinsic and RGS4-catalyzed \( \text{G}_{\text{a1}}-\text{T48A} \) and assigned the \( \alpha \)-GTP band via isotopic labeling (SI Appendix, Fig. 14), resulting in a \( \alpha \)-GTP band of 1,270 cm\(^{-1}\) for intrinsic \( \text{G}_{\text{a1}}-\text{T48A} \) and a red shift to 1,222 cm\(^{-1}\) for \( \text{G}_{\text{a1}}-\text{T48A}-\text{RGS4} \). This point mutation decreased the RGS4-induced \( \alpha \)-GTP shift from \(-59 \text{ cm}^{-1}\) (\( \text{G}_{\text{a1}}-\text{WT} \)) to \(-45 \text{ cm}^{-1}\) (\( \text{G}_{\text{a1}}-\text{T48A} \)), which is representative of a strong hydrogen bond. Hydrolysis kinetics of the \( \text{G}_{\text{a1}}-\text{T48A} \) mutant with and without RGS4 appeared slightly slowed down to \( t_{1/2} = 9.6 \pm 1.6 \text{ s} \) (5 \(^\circ\)C), which is one order of magnitude slower than \( \text{G}_{\text{a1}} \). RGS4\((t_{1/2} = 1.4 \pm 0.3 \text{ s})\) and in the same range as \( \text{G}_{\text{a1}} \)-R178S-RGS4\((t_{1/2} = 12.6 \pm 2.7 \text{ s})\) (Table 1), demonstrating that Thr48 is relevant for RGS4-catalyzed hydrolysis of \( \text{G}_{\text{a1}} \).

**QM/MM Simulations Show Charge Shifts and Structural Rearrangements That Assist GTP Hydrolysis.** We evaluated the QM/MM \( \text{G}_{\text{a1}} \)-GTP geometries and calculated Merz–Kollman partial charges [electrostatic potential fitting (ESP)] for each GTP atom. Charge distribution showed that upon RGS4 binding and rearrangement of Arg178, charges are transferred from \( \gamma \)-GTP to the bridging \( \beta\gamma\)-oxygen (Fig. 7 and SI Appendix, Fig. 13). The bridging oxygen becomes more negative from \(-0.5 \text{ e}^{-}\) (intrinsic) to \(-0.6 \text{ e}^{-}\) (RGS4-catalyzed). Upon product formation, one net charge is transferred to the \( \beta \)-phosphate \((q_{\beta\text{GTP}} = -1 \text{ e}^{-}; q_{\beta\text{GDP}} = -2 \text{ e}^{-})\). RGS4 binding already transfers 10% of this charge in QM/MM calculations of the educt state (Fig. 7). In addition, the terminal P\(_{\gamma}\)-oxygen atom that binds the nucleophilic attacking water becomes more positive, which might decrease the negative \( \gamma \)-GTP barrier the attacking water has to cross upon hydrolysis. Analysis of the \( \gamma \)-GTP angles showed that upon RGS4 binding and Arg178 movement, the \( \gamma \)-GTP group becomes more planar (Fig. 7). The corresponding angle is shifted below 100\(^\circ\), facilitating Walden inversion of the \( \gamma \)-GTP group that is needed for a second-order kinetics nucleophilic substitution (S\(_{\text{N2}}\)) hydrolysis mechanism.

**Fig. 7.** RGS4-catalyzed GTP hydrolysis model in \( \text{G}_{\text{a1}} \). (A) Binding pocket, electrostatic potential fitting (ESP) partial charges, and bond angle of GTP in intrinsic \( \text{G}_{\text{a1}} \) obtained from QM/MM calculations on the level M066-31G*\(^\dagger\). Shown are mean values of each of 15 snapshots. (B) Binding pocket, ESP partial charges, and bond angle of GTP in \( \text{G}_{\text{a1}} \)-RGS4. Shown are mean values of each of 15 snapshots. RGS4 pushes Arg178 from a monodentate coordination of GTP (A) to a bidentate coordination of GTP (B). Thereby, charges are shifted to the bridging P\(_{\gamma}\)-oxygen (\( \beta \), blue arrow), which stabilizes the product state, and the P\(_{\gamma}\)-group becomes more planar (\( \beta \), black arrow), which stabilizes the intermediate state and thereby catalyzes GTP hydrolysis. Detailed charge distributions on the levels B3LYP/6-31G*\(^\dagger\) and PBE/6-31G*\(^\dagger\) show the same trend and are depicted in SI Appendix, Fig. 13.

**Fig. 8.** Schematic representation of the mechanisms in \( \text{G}_{\text{a1}} \)-RGS catalysis. (A) RGS4 stabilizes the planar intermediate that corresponds to the GDP-AlF\(_4\)^− crystal structure. (B) RGS4 transfers charge toward \( \beta\gamma \)-GTP. Upon hydrolysis, the \( \beta \)-phosphate gets more negative from \(-1 \text{ to } -2 \text{ e}^{-}\). RGS4 binding already transfers 0.1 e\(^{-}\) in the prehydrolysis state. (C) RGS4 puts GTP into an \( \alpha\beta\gamma \)-glide conformation. This eclipsed conformation creates a strain in the substrate, and thereby facilitates hydrolysis.

**RGS4 Pushes \( \text{G}_{\text{a1}} \)-Arg178 from a Monodentate to a Bidentate GTP Coordination.** Our findings are summarized in Figs. 7 and 8. In contrast to crystal structures, Arg178 is bound to \( \gamma \)-GTP in the active state of \( \text{G}_{\text{a1}} \) in solution. RGS4 catalyzes GTP hydrolysis in \( \text{G}_{\text{a1}} \) by pushing Arg178 from a monodentate \( \alpha\gamma \)-GTP coordination to a bidentate \( \alpha\gamma \)-GTP coordination. This change is accompanied by increased planarity of the \( \gamma \)-GTP group (Fig. 8A), charge transfer toward \( \beta\gamma \)-GTP (Fig. 8B), and formation of \( \alpha\beta\gamma \)-glide GTP (Fig. 8C).

**Discussion.** Here, we have elucidated the intrinsic and GAP-catalyzed molecular reaction of the intrinsic arginine finger of \( \text{G}_{\text{a1}} \) label-free with high spatiotemporal resolution by a combination of FTIR spectroscopy and molecular simulations. GTP itself, and not GTP analogs as in X-ray structure analysis, was used. The arginine finger of heterotrimeric G proteins is hydrogen-bonded to \( \gamma \)-GTP in the active state of intrinsic \( \text{G}_{\text{a1}} \). RGS4 binding causes stabilization of the switch areas, and thereby pushes the arginine finger to a bidentate \( \alpha\gamma \)-GTP coordination. GTP hydrolysis in GTPases (e.g., EF-G, EF-Tu, Ras) is proposed to take place in an \( \text{S}_{\text{N2}} \) mechanism via in-line attack of a water molecule at \( \gamma \)-GTP, with inversion of coordination around the phosphate (32–37). Many of the proposed aspects were directly observed by combination of FTIR spectroscopy and QM/MM calculations in the present study. First, the inversion of \( \gamma \)-GTP (38) is prepared by the \( \text{G}_{\text{a1}} \)-RGS complex (Fig. 8A). The P\(_{\gamma}\)-O angle is twisted from a tetrahedral geometry toward a planar geometry that is proposed to be the transition state before inversion. A corresponding pentavalent planar transition state analog is resolved in a number of crystal structures with aluminum fluoride and GDP (2, 6, 39, 40). Displacement of Arg178 in \( \text{G}_{\text{a1}} \)-RGS4 toward \( \alpha \)-GTP pulls the hydrogen-bonded \( \gamma \)-oxygen atom toward a more planar geometry (Fig. 8A). Second, displacement of the arginine finger resulted in reorganization of the charge distribution: \( \beta \)-GTP becomes more negative (Fig. 8B). While in the GTP state, the \( \beta \)-phosphate moiety has a formal charge of \(-1 \), but its charge is increased to \(-2 \) in the
Biomolecular simulations revealed that the extent of the and found almost no spectral changes (SI Appendix, Figs. 3 and 4) and β-GTP (SI Appendix, Fig. 4). One might explain now why mutation of the arginine finger in G\textsubscript{\alpha} from regaining activity due to GAP binding: it might also (pH 8), 150 mM NaCl, 0.5 mM EDTA, and 5 mMD-norleucine. Cells were flashed per isotopic labeling, unlabeled arginine was exchanged for \( ^{15}N \)-labeled arginine in the \( \alpha \)-subunit. The reaction was performed in identical conditions using only kanamycin for plasmid selection. For G\textsubscript{\alpha}, genes were cloned into the vector pET27bmod with N-terminal 10×His-tag and tobacco etch virus site, and transformed into DH5\textsubscript{α} for amplification. Point mutants of G\textsubscript{\alpha} were created using overlap extension PCR. Each construct was verified by sequencing.

### Materials and Methods

**Chemicals.** The pHPP-glut and the isotopologues \( ^{18}O \)-Glut and \( ^{15}N \)-Glut were synthesized as described previously (17, 26, 54, 55). The \( \eta \)-N\(_{2} \)-labeled arginine was purchased from Cambridge Isotope Laboratories. Deuterium oxide was purchased from Deutero GmbH.

**Cloning.** Human G\textsubscript{\alpha} (UniProtKB P63096-1) and human G\textsubscript{\alpha} (UniProtKB P49798) were amplified as described previously (10). Briefly, genes were cloned into the vector pET27bmod with N-terminal 10×His-tag and tobacco etch virus site, and transformed into Escherichia coli DH5\textsubscript{α} for amplification. Point mutants of G\textsubscript{\alpha} were created using overlap extension PCR. Each construct was verified by sequencing.

**Protein Expression.** The plasmid encoding G\textsubscript{\alpha} was transformed into E. coli Rosetta2(DE3) (Novagen, Merck) and incubated at 37 °C overnight on LB agar plates supplemented with 0.2% (v/v) glucose, 50 μg/mL kanamycin, and 20 μg/mL chloramphenicol. Precultures were incubated overnight at 37 °C and shaking at 160 rpm in LB supplemented with the same components. The plasmid encoding G\textsubscript{\alpha} was transformed into E. coli BL21(DE3) (Novagen, Merck) under identical conditions using only kanamycin for plasmid selection. For G\textsubscript{\alpha}, main cultures, 1.5 L of M9 medium (50 mM glucose, 2 mM MgSO\(_4\), 0.2 mM CaCl\(_2\), 49 mM Na\(_2\)HPO\(_4\), 22 mM KH\(_2\)PO\(_4\), 11.5 mM NaCl, 23 mM NH\(_4\)Cl, 0.2 mM thiamine-HCl, 0.5 mM thymine, and 0.62 mM each standard amino acid) supplemented with 50 μg/mL kanamycin was inoculated with the pre-culture and grown at 37 °C and shaking at 100 rpm to an \( A_{595} \) of 0.5 absorbance units (AU). For isotopic labeling, unlabeled arginine was exchanged for \( ^{1}H\)-arginine in the main culture. Protein expression was induced at 18 °C by the addition of isopropl-1-thio-\( ^{15}O \)-galactopyranoside (IPGT) overnight. For G\textsubscript{\alpha}, main cultures contained 18 L of LB supplemented with 50 μg/mL kanamycin and 0.2% glucose. Cultures were incubated at 37 °C, shaking at 100 rpm, and 20 L/min airflow in a Biostat C20-3 Fermenter (Sartorius), induced with IPTG, and grown to an \( A_{595} \) of 1.5 absorbance units. Cells were harvested by centrifugation at 5,000 × g and 4 °C. G\textsubscript{\alpha} was suspended in buffer A containing 20 mM Tris (pH 8), 300 mM NaCl, 1 mM MgCl\(_2\), 0.5 mM EDTA, and 5 mM \( \eta \)-oxoroeleinuclease. G\textsubscript{\alpha} was suspended in buffer B containing 50 mM Tris (pH 8), 150 mM NaCl, 0.5 mM EDTA, and 5 mM \( \eta \)-oxoroeleinuclease. Cells were flash-frozen and stored at −80 °C until protein purification.
Protein Purification. Purification was performed as described (10). Briefly, cells were thawed, disrupted with a microfluidizer M-110L (Microfluidics Corp.), and centrifuged for 45 min at 45,000 × g and 4 °C to remove cell fragments. In contrast, RG54-containing cells were centrifuged with an additional low-speed step for 15 min at 18,000 × g and 4 °C, followed by a high-speed step for 45 min at 75,000 × g and 4 °C. Supernatants were applied to a 25 mL nickel-nitrilotriacetic acid superflow column (Qiagen) and eluted with buffers containing 200 mM imidazole. Fractions containing Gαi1 or RG54 were screened via SDS/PAGE, pooled, concentrated to 5 mL using a 10,000 molecular weight cutoff (MWCO) concentrator (Amicon Ultra-0.5; Merck Millipore). Protein concentrations were determined using Bradford reagent in a concentration of FTIR samples. Nucleotide exchange rates to GTP. Samples were flash-frozen in liquid nitrogen, and aliquoted, flash-frozen in liquid nitrogen, and stored at -80 °C until utilization.

Control of Labeling Efficiency with LC-MS. Efficiency of arginine N-terminals was checked via LC-MS in cooperation with Claudia Lindemann (Medical Center, Ruhr University, Bochum, Germany). Labeling efficiency was determined for four digested peptide fragments ([AVVNTYSOISISIR (92.6% labeled), EYQLDNASAYNYLNDLTAE-LAGVKIR (92.6% labeled), and IAPQNYPTQDLVR (92.3% labeled)] and resulted in an overall label efficiency of 92.8% ± 0.3%. Gαi1-K180P was arginine 15N2-labeled with an efficiency of 96.7 ± 0.5%. Both Gαi1:WT and Gαi1-K180P showed no scattering of heavy isotopes to amino acids other than arginine.

Nucleotide Exchange to pHPgGTP. Nucleotide exchange reactions of WT and mutant Gαi1 were performed in the presence of alkaline phosphatase coupled to agarose beads (Sigma-Aldrich Chemie GmbH). Phosphatase beads were washed five times with 50 mM Tris (pH 7.5) and 0.1 M ZnSO4, with each washing step followed by centrifugation at 10,000 × g until the supernatant was free of protein. Five fractions of WT or Gαi1-GFP were supplemented with 50 mM Tris (pH 7.5), 10 μM ZnSO4, and a twofold molar excess of unlabeled or oxygen-labeled pHPgGTP. Samples were incubated for 3 h and analyzed via reverse-phase HPLC (LC-2010; Shimadzu) [mobile phase: 50 mM P, (pH 6.5), 5 mM tetrabutylammonium bromide, 7.5% (vol/vol) acetonitrile; stationary phase: ODS-Hypersil C18 column] for remaining GDP. After >95% of GDP was hydrolyzed to GTP, samples were centrifuged at 10,000 × g for 2 min and the supernatant was rebuffered using a Nap5 column (GE Healthcare Life Sciences). Fractions containing the highest protein concentrations were pooled and concentrated using a 10,000 MWCO concentrator (Amicon Ultra-0.5; Merck Millipore). Protein concentrations were determined using Bradford reagent in a concentration of 100 mM Hepes (pH 7.5), 100 mM Tris (pH 7.5), 150 mM NaCl, 5 mM MgCl2, and 20 mM DTT, and applied to an Illustra HiLoad 26/60 Superdex 200-26 pg column (GE Healthcare Life Sciences). Peak fractions were collected and concentrated to ca. 20 mg/mL for Gαi1 or ca. 10 mg/mL for RG54. Protein concentrations were determined using Bradford reagent in triplicate. Proteins were aliquoted, flash-frozen in liquid nitrogen, and stored at -80 °C until utilization.

MD Simulations and Evaluation. Structures of active Gαi1 (PDB ID code 1GIA), inactive Gαi1 (PDB ID code 1GIA, chain A), GTP-αS-, and bound Gαi1 (PDB ID code 1GII), and Gαi1-RGS4 (PDB ID code 1AGR) were prepared in the Moppy program suite (57). Structure preparation included dihedral, angle, and bond corrections according to the UA amber84 force field (58), protonation according to pH calculations using the PAKA Max, UHJA83 algorithm in Moppy, and replacement of nucleotide analogs with natural GTP or GDP. Systems were subsequently solvated according to the protocols of the GROMACS 4.0.7 (60–63). Systems were energy-minimized using steepest descent and heated to 310 K using the Berendsen thermostat and barostat with a time step of 1 fs for 25 ps with restrained protein backbone positions in the optimized potentials for liquid simulations all atom (OPLS/AA) force field (64). Electrostatics were calculated using PME (0.9 nm) and a van der Waals cutoff of 1.4 nm. Projection runs were carried out without restraints for 100 ns in five replicas per system (total simulation time of 2 μs). Evaluation was performed using the GROMACS package (g_rms) and the contact matrix algorithm implemented in Moppy. Pictures were created using PyMOL (Schrodinger LLC) and Gnuplot 4.4 (65).

QM/MM Calculations. Snapshots from equilibrated MM simulations (25–100 ns in 5 ns steps for each system) were truncated substructure-based around 1.5 nm of the QM core (GTP + Mg2+ + Mg2+-coordinating water molecules). Nearest Na+ ions were taken into account to ensure the total charge of the system was zero. The QM core region (51 atoms) was embedded in a mobile MM region (0.5 nm), which was embedded in a fixed MM region containing protein and solvent centers (1.5 nm) that was again embedded by a fixed MM region containing protein centers beyond 1.5 nm of the QM core (MM layer ca. 6,000 atoms). Initially, a single point calculation was performed using our own N-layered integrated molecular orbital and molecular mechanics (ONIOM) QM/MM embedded method implemented in TILGS (68). Calculated Merz–Kollman (ESP) charges were transferred to an external quasi-newton Broyden–Fletcher–Goldfarb-Shanno minimizer (70, 71) implemented in the Moppy program suite. The mobile MM layer was optimized, and the updated coordinates were retransferred to the Gaussian program system, where a full optimization of the QM part with QM/MM embedding according to the ONIOM scheme was performed. This procedure was repeated two times, followed by spectra calculation in the Gaussian program using normal mode analysis. No imaginary frequencies were observed for each calculation, indicating a minimum structure was always reached. Even a normal mode analysis of the MM part showed no imaginary frequencies, indicating that a minimum structure for both parts was reached successfully. QM calculations were performed with the density functionals B3LYP, M06, and PBE and the basis set 6-31G*. Calculations using the B3LYP functional are well-characterized in the literature. Additional functionals were chosen because of their strengths in dispersion (M06) and small IR scaling factors (PBE). IR frequencies were scaled according to the Computational Chemistry Comparison and Benchmark Database of the National Institute of Standards and Technology. IR frequencies for each vibration were averaged over 15 snapshots for each simulation system, and the SE was calculated for comparison with the experimental bandwidth widths. We depicted only the asymmetrical vibrations of the individual phosphate groups, because their high transition dipole moment causes IR intensities that dominate the experimental spectrum. QM/MM calculations showed the distinct ν(PO2) band for γ-GTP, indicating that the calculations showed only ν(PO2) vibration, which was previously described for small GTPases in the literature (2, 72). Therefore, mean values for ν(PO2) were used to derive comparison with the experiment. The whole QM/MM calculation was performed in MATLAB R2012a (The MathWorks, Inc.) and OPUS (Bruker Corp.).
11. Rudack T, Xia F, Schlitter J, Kotting C, Gerwert K (2012) Ras and GTPase-activating protein for one snapshot was performed within a single day on eight parallel central processing unit cores (1.9 GHz; AMD Opteron).
12. Lindemann for LC-MS measurements at the Medical Proteome Center (Ruhr University). We thank PD Dr. Udo Höweler [chemistry-oriented program system (CHEOPS)] for helpful discussions. We further thank Dr. Jonas Schlitter and Dr. Yan Gu for synthesis of the caged compounds, Iris Boudos for excellent technical support, and the Deutsche Forschungsgemeinschaft Grant SF 642, TP A1 for financial support.
Supplemental Dataset:

Mechanism of the Intrinsic Arginine Finger in Heterotrimeric G-Proteins

Daniel Mann, Christian Teuber, Stefan Tennigkeit, Grit Schröter, Klaus Gerwert, and Carsten Kötting

Supplemental Figure 1: Locations of the investigated point mutants in \( \alpha_i \) (PDB-ID 1GIA)
Supplemental Figure 2: Exchange of GTP to MANT-GTP significantly alters kinetics and infrared spectra of $\alpha_{i1}$-WT

Positive bands in the photolysis correspond to the GTP state, negative bands correspond to the caged-GTP state. Positive bands in the hydrolysis reaction represent the GDP state of $\alpha_{i1}$ whereas negative bands represent the GTP state.
Supplemental Figure 3: Assignment of IR bands of α-GTP and α-GDP in Gα11-R178S in H2O

Positive bands in the photolysis correspond to the GTP state, negative bands correspond to the caged-GTP state. Positive bands in rate 1 and 2 of the hydrolysis reaction represent the GDP state of Gα11-R178S whereas negative bands represent the GTP state. Arrows indicate band shifts caused by the heavy isotopes.
Supplemental Figure 4: $\alpha^{18}\text{O}_2$ pHPcgGTP and $\beta^{18}\text{O}_3$ pHPcgGTP labeling of the rate that preceded hydrolysis in $G_{\alpha_{i1}}$-E43Q

The mutant $G_{\alpha_{i1}}$-E43Q shows an additional rate that precedes the hydrolysis reaction. Isotopic labeling using $\alpha^{18}\text{O}_2$ pHPcgGTP and $\beta^{18}\text{O}_3$ pHPcgGTP showed that the band at 1230 cm$^{-1}$ is caused by $\alpha$- and $\beta$-GTP. Double differences ($\Delta\Delta$) are the result of labeled minus unlabeled spectra. Arrows indicate band shifts caused by the heavy $^{18}\text{O}$ isotopes.
Supplemental Figure 5: Photolysis (A) and Hydrolysis (B) spectra of intrinsic and RGS4 catalyzed $\Gamma_{\alpha_1}$-WT and the mutant $\Gamma_{\alpha_1}$-R178S. RGS4 addition shifts the $\alpha$-GTP vibration to lower wavenumbers. This effect is reversible when Arg178 is mutated (C).
Supplemental Figure 6: $\alpha$-GTP / $\alpha$-GDP band assignments of $G_{\alpha_{11}}$·RGS4 in H$_2$O

Positive bands in the photolysis correspond to the GTP state, negative bands correspond to the caged-GTP state. Positive bands in the hydrolysis reaction represent the GDP state of $G_{\alpha_{11}}$·RGS4 whereas negative bands represent the GTP state. Arrows indicate band shifts caused by the heavy isotopes.
Supplemental Figure 7: β-GTP / β-GDP band assignments of $\alpha_{i1}$·RGS4 in H$_2$O

Positive bands in the photolysis correspond to the GTP state, negative bands correspond to the caged-GTP state. Positive bands in the hydrolysis reaction represent the GDP state of $\alpha_{i1}$·RGS4 whereas negative bands represent the GTP state. Arrows indicate band shifts caused by the heavy isotopes.
Supplemental Figure 8: Infrared band assignment of Gα1-Arg178

Deuterated hydrolysis spectra of unlabeled (black) and η¹⁵N₂-Arg labeled Gα1 (A). Detailed view (B) and double difference spectrum (ΔΔ) of the assignment. The bands at 1604 cm⁻¹ (GTP state) and 1590 cm⁻¹ (GDP state) are assigned to Arg178. This assignment is site specific since the bands were also missing in the mutant Gα1-R178S (C). Positive bands correspond to the GDP state, negative bands correspond to the GTP state. Arrows indicate band shifts caused by the heavy isotopes. Grayed out areas are superimposed by DOD bending of deuterated water.
Supplemental Figure 9: Infrared band assignment of Gαi1-Arg178 in the rate separated mutant Gαi1-K180P

A: rate 1 and 2 of unlabeled (black) and η15N2-Arg labeled Gαi1-K180P (red). B: double differences (ΔΔ) of rate 1 show a zero-line, exclusively rate 2 shows isotopic shifts for the bands 1604 cm⁻¹ and 1590 cm⁻¹ like in wildtype Gαi1. Positive bands correspond to the GDP state, negative bands correspond to the GTP state.
Supplemental Figure 10: Infrared bands of the arginine finger in Gαi1 and Ras•GAP and their corresponding $\eta^{15}N_2$ shifts (gray) in comparison.

In Gαi1 1604 cm$^{-1}$ represents the GTP-bound state of the arginine finger whereas 1590 cm$^{-1}$ represents the GDP state. In Ras•GAP 1589 cm$^{-1}$ represents the arginine in a water environment whereas 1571 cm$^{-1}$ represents the arginine finger within the binding pocket but after bond breakage (17). The GTP bound state is not resolved in Ras•GAP and could now be resolved for the first time in Gαi1.
Supplemental Figure 11: Isotopic labeling of the arginine finger in RGS4 catalyzed FTIR measurements of Go11.

The intrinsic vibrations of Arg178 at 1604 and 1590 cm\(^{-1}\) were no longer observable when RGS4 was added, probably due to altered GTP coordination. Positive bands represent the GDP state whereas negative bands represent the GTP state.
Supplemental Figure 12: QM/MM calculations of $G\alpha_{i1}$ and $G\alpha_{i1}$-RGS4.

Intrinsic (A) and RGS4 catalyzed $G\alpha_{i1}$ (C,E) and the corresponding calculated IR spectra from QM/MM simulations (B,D,G). QM/MM spectra calculation (B) of intrinsic $G\alpha_{i1}$-GTP (A) on the levels B3LYP/6-31G*, M06/6-31G* and PBE/6-31G* in comparison to the experiment (FTIR). Shown are mean values and standard deviations for 15 snapshots of a 100 ns MD simulation. (D,G) Shown are RGS4 induced $\alpha$-, $\beta$- and $\gamma$-GTP shifts for configurations (C) and (E), respectively. Calculation of configuration (E) resulted in eclipsed ($\alpha$-$\beta$-$\gamma$)-GTP (F). While calculation of geometry (C) with RGS4 resulted in higher $\alpha$-GTP vibrations in comparison to the $\beta$-GTP vibrations (solid lines) this trend is reversed (G) for the eclipsed geometry (E) (solid lines) which is in line with the experiment. All spectra were scaled according to CCCBDB (B3LYP/6-31G*: 0.96; M06/6-31G*: 0.95; PBE/6-31G*: 0.99).
Supplemental Figure 13: Detailed ESP Charge distribution of intrinsic and RGS4 bound Gαi1

(A) ESP partial charges of intrinsic and RGS4 bound Gαi1 on the levels B3LYP/6-31G*, M06/6-31G* and PBE/6-31G*. Charge is transferred towards the bridging β-γ-GTP oxygen (O6). Atom names match those in panel (C). (B) planarity of γ-GTP is increased when RGS4 is bound.
Supplemental Figure 14: Band assignment of $\alpha$-GTP for the mutant $G_{\alpha_{i1}}$-T48A+RGS4.

In $G_{\alpha_{i1}}$-T48A+RGS4 the $\alpha$-GTP band is shifted from 1184 cm$^{-1}$ to 1220 cm$^{-1}$, indicating the RGS4 induced 59 cm$^{-1}$ $\alpha$-GTP band shift is caused by strong binding of $\alpha$-GTP between Arg178 and Thr48.
Supplemental Figure 15: FTIR measurements of $G_{\alpha_{i1}}$-WT and $G_{\alpha_{i1}}$-Q204A

Photolysis and hydrolysis spectra almost match completely. The $\gamma$-GTP band at 1155 cm$^{-1}$ is slightly red-shifted to 1152 cm$^{-1}$ and $G_{\alpha_{i1}}$-Q204A shows an intermediate rate with a spectral feature at 1150 cm$^{-1}$. Half-live values of global fits are depicted in Table 1.
Supplemental Figure 16: FTIR measurements of $\alpha_1$-R178K

The $\gamma$-GTP vibration of $\alpha_1$-R178K resembles more the $\alpha$-WT $\gamma$-GTP vibration at 1155 cm$^{-1}$ than the $\gamma$-GTP vibration of $\alpha_1$-R178S, probably indicating that Lys178 is also bound to $\gamma$-GTP. However, hydrolysis kinetics are comparably slowed down, indicating not only the charge of the arginine finger is important but also its geometry.

![Image showing FTIR measurements and hydrolysis kinetics]

Supplemental Table 1: Charge sums (ESP) in QM/MM calculations (M06/6-31G*).

<table>
<thead>
<tr>
<th></th>
<th>$\Sigma P\gamma$-O$_3$</th>
<th>$\langle P\gamma \rangle$ O(-P$\beta$)</th>
<th>$\Sigma P\beta$-O$_2$</th>
<th>$\langle P\beta \rangle$ O(-P$\alpha$)</th>
<th>$\Sigma P\alpha$-O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_1$</td>
<td>-1.53</td>
<td>-0.52</td>
<td>-0.45</td>
<td>-0.50</td>
<td>-0.48</td>
</tr>
<tr>
<td>$\alpha_1$+RGS4</td>
<td>-1.45</td>
<td>-0.60</td>
<td>-0.50</td>
<td>-0.46</td>
<td>-0.50</td>
</tr>
</tbody>
</table>