Integration of Fourier Transform Infrared Spectroscopy, Fluorescence Spectroscopy, Steady-state Kinetics and Molecular Dynamics Simulations of $\alpha_{i1}$ Distinguishes between the GTP Hydrolysis and GDP Release Mechanism*

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Background: Multiple turnover GTPase assays of $\alpha$ are dominated by nucleotide exchange.

Results: FTIR elucidates single turnover rates and individual phosphate vibrations.

Conclusion: G$\alpha_{i1}$-R178S is slowed down in single turnover hydrolysis by 2 orders of magnitude, G$\alpha_{i1}$-Asp$^{229}$ and -Asp$^{231}$ are key players in Ras-like/all-$\alpha$ domain coordination.

Significance: With FTIR on G$\alpha$ established, detailed information on the reaction mechanism can be obtained.

$\alpha$ subunits are central molecular switches in cells. They are activated by G protein–coupled receptors that exchange GDP for GTP, similar to small GTPase activation mechanisms. G$\alpha$ subunits are turned off by GTP hydrolysis. For the first time we employed time-resolved FTIR difference spectroscopy to investigate the molecular reaction mechanisms of G$\alpha_{i1}$. FTIR spectroscopy is a powerful tool that monitors reactions label free with high spatio-temporal resolution. In contrast to common multiple turnover assays, FTIR spectroscopy depicts the single turnover GTPase reaction without nucleotide exchange/Mg$^{2+}$ binding bias. Global fit analysis resulted in one apparent rate constant of 0.02 s$^{-1}$ at 15 °C. Isotopic labeling was applied to assign the individual phosphate vibrations for $\alpha$-, $\beta$-, and $\gamma$-GTP (1243, 1224, and 1156 cm$^{-1}$, respectively), $\alpha$- and $\beta$-GDP (1214 and 1134/1103 cm$^{-1}$, respectively), and free phosphate (1078/991 cm$^{-1}$). In contrast to Ras-GAP catalysis, the bond breakage of the $\beta$-$\gamma$-phosphate but not the P$_{i}$ release is rate-limiting in the GTPase reaction. Complementary common GTPase assays were used. Reversed phase HPLC provided multiple turnover rates and tryptophan fluorescence provided nucleotide exchange rates. Experiments were complemented by molecular dynamics simulations. This broad approach provided detailed insights at atomic resolution and allows now to identify key residues of G$\alpha_{i1}$ in GTP hydrolysis and nucleotide exchange. Mutants of the intrinsic arginine finger (G$\alpha_{i1}$-R178S) affected exclusively the hydrolysis reaction. The effect of nucleotide binding (G$\alpha_{i1}$-D272N) and Ras-like/all-$\alpha$ interface coordination (G$\alpha_{i1}$-D229N/G$\alpha_{i1}$-D231N) on the nucleotide exchange reaction was furthermore elucidated.

Heterotrimeric G proteins are interaction partners of G protein–coupled receptors (GPCRs) and deliver external signals into the cell (1). They are switched on by exchange of GDP for GTP induced by the GPCR as exchange factor and switched off by GTP hydrolysis. The nucleotide is bound between two domains of the G$\alpha$-subunit, namely the Ras-like domain, which is similar to the G-domain of small GTPases, and the all-$\alpha$ domain. In its inactive state G$\alpha_\beta_\gamma$ exists GDP bound in its heterotrimeric form. Activation by guanosine nucleotide exchange factors, like GPCRs or non-receptor guanosine nucleotide exchange factors (2–4), leads to nucleotide exchange in the $\alpha$-subunit. Incorporation of GTP alters the protein conformation in the switch I-III regions (5), which causes separation of the G$\alpha_1$ and $\beta_\gamma$ subunits and signal transduction, e.g. by binding of G$\alpha_1$ to adenylate cyclase isoforms that in turn inhibit the production of cAMP from ATP (6). GTPase activity of G$\alpha_1$ leads to hydrolysis of GTP to GDP and P$_{i}$, inactivation, and reassociation with its $\beta_\gamma$ subunits. Heterotrimeric G proteins are equipped with an intrinsic arginine finger (Arg$^{178}$ in G$\alpha_{i1}$) usually provided in case of small GTPases by the GAP protein, which is known to function as a key residue for catalyzing the hydrolysis reaction. Therefore intrinsic GTPase rates of G$\alpha_{i1}$ are rather comparable with Ras-GAP than to Ras. The hydrolysis mechanism taking place in G$\alpha_{i1}$ thereby determines the duration of its active state, which can be pathogenic when hindered, e.g. by ADP-ribosylation catalyzed by pertussis toxin (7). As for Ras, the intrinsic hydrolysis activity of G$\alpha_{i1}$ can be further accelerated by GTPase activating proteins (GAPs), which are called regulators of G protein signaling (RGS), e.g. RGS4 in case of G$\alpha_{i1}$ (8). It is generally known that GDP/GTP exchange is the rate-limiting step in multiple turnover measurements of G$\alpha$ isoforms (9–12). Therefore, beside steady-state assays using $\gamma$-$^{32}$P labeling (13) or malachite green (14), pre-steady-state assays are used to characterize the hydrolysis reaction of G$\alpha$

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The abbreviations used are: GPCR, G protein–coupled receptor; GAP, GTPase activating protein; pH, para-hydroxyphenacyl; NPE, 1-ortho-nitrophenyl ethyl; GTP$\gamma$S, guanosine 5’-3-O-(thio)triphosphate; RGS, regulators of G protein signaling; MD, molecular dynamics; PDB, Protein Data Bank.
isomers (15–18). We present here for the first time single turn-
over measurements of Go11 using time-resolved FTIR spectro-
copy, an ultrasensitive method that can be applied in solution and
has been successfully used for photoactivatable proteins like bac-
teriorhodopsin (19, 20), channelrhodopsin (21), and other rhodopsins (22). Adenylyltransferases (23), ATPases (24–27),
and GTPases (28–31) can also be investigated by usage of caged
nucleotides (28). The resulting photolysis and hydrolysis differ-
sements to decode the molecular reactions at the atomic level.

Materials and Methods

Gαi1-WT and mutant proteins were expressed, isolated, and
characterized by various biophysical and biochemical methods
(Fig. 1).

Michaelis-Menten multiple turnover kinetics were moni-
tored via reversed phase HPLC. The kinetics include both the
catalytic reaction and the dissociation/association kinetics
depicted as time per turnover. The isolated single turnover hy-
drolysis reaction was obtained via FTIR spectroscopy as half-
life values of the global fit. The isolated nucleotide exchange
kinetics were investigated via tryptophan fluorescence spec-
troscopy and depicted as half-life values of the intensity change.
Experiments were accompanied by molecular dynamics simu-
lations to decode the molecular reactions at the atomic level.

Chemicals—Lyophilized GDP, GTP, and GTPγS were pur-
based from Jena Bioscience (Jena, Germany). The photola-
beled nucleotide para-hydroxyphenacyl (pHP) cGTP and the
isotopologues α-18O2- and β-18O2-pHPcGTP were synthe-
sized as described previously (29, 35–37). 1-Ordo-nitrophenyl
ethyl (NPE) cGTP and γ-18O4-NPEcGTP were synthesized as
described previously (38). Alkaline phosphatase coupled to
agarose beads was purchased from Sigma (Munich, Germany).

Cloning—The gene for the human GNAI1 (UniProtKB acce-
sion number P63096–1; kind gift from C. Wetzel, University of
Regensburg, Germany (39)) was amplified by polymerase chain reaction using the oligonucleotide primers GCCG-
CCATGGGCTGTCAACGCGGC and GCCCGGATCATTA-
AAAGAGACCACAATCTTTTAG (restriction sites for NcoI and
BanHI are underlined). Resulting fragments were cut with
NcoI and BanHI and ligated into the vector pET27bmod (kind
gift from M. Engelhard, MPI Dortmund, Germany (40)) with a
N-terminal ×10 histidine tag and tobacco etch virus (TEV) site.
The plasmid was transformed into Escherichia coli DH5α for
amplification. Gαi1 mutants R178S, D229N, D231N, and
D272N were created by overlap PCR using appropriate primers.
Integrity of each construct was confirmed by sequencing.

Protein Expression—The plasmid encoding Gαi1 was trans-
formed into E. coli Rosetta 2 (DE3) (Novagen®, Merck, Darm-
stadt, Germany) and incubated overnight at 37 °C on LB agar
plates containing 0.2% (w/v) glucose as well as 50 μg/ml of
kanamycin and 20 μg/ml of chloramphenicol for plasmid and
strain selection. A preculture (LB medium, 50 μg/ml of N-
norleucine for Gαi1, ammokinase was inoculated and incubated overnight at 37 °C and 150 rpm.

The plasmid encoding RGS4 was transformed into E. coli
BL21(DE3) under identical conditions using only kanamycin
for plasmid selection. For the main culture, 18 liters of LB
middle supplemented with 50 μg/ml kanamycin and 0.2% gl-
ucose were inoculated with the preculture and grown at 37 °C,
100 rpm, and 20 liters/min airflow in a Biostat® C20-3 Fer-
menter (Sartorius, Göttingen, Germany). At an A600 of 0.5–0.6
the culture was cooled to 18 °C and protein expression was
induced by addition of 0.25 mM isopropyl 1-thio-β-D-galacto-
pyranoside. After 15–18 h the cells were harvested by centri-
figation at 5000 × g and 4 °C, suspended in buffer A (20 mM Tris,
ph 8, 300 mM NaCl, 1 mM MgCl2, 0.5 mM EDTA, 5 mM D-nor-
leucine for Gαi1 or 50 mM Tris, pH 8, 150 mM NaCl, 0.5 mM
EDTA, 5 mM D-norleucine for RGS4), flash frozen, and stored at
−80 °C.

Protein Isolation—Frozen cells were thawed, supplemented
with 0.3 mM PMSF, 5 mM β-mercaptoethanol, DNase (Gαi1
containing cells were additionally supplemented with 0.1 mM
GDP), and disrupted using a microfluidizer M-110L (Microfu-
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...the samples contained 10 mM Gαi1 in 20 mM Tris, pH 8, 150 mM NaCl, 0.5 mM MgCl2, and 0.1 mM DTT. After tempering for 5 min at 30 °C, 0.1 mM GTP (Gαi1-WT, -R178S, -D229N, -D231N) or 2.5 mM GTP (Gαi1 or RGS4) were added, and immediately the first aliquot of the sample was analyzed by reversed phase HPLC at 254 nm (Beckman Coulter System Gold, Pasadena CA) 

...used a AKTA purifier 100 system (GE Healthcare Life Sciences, Freiburg, Germany) at 6 °C with a flow rate of 1–2 ml/min. After a washing step with buffer C (buffer B + 4 mM MgCl2, 0.1 mM ATP) for 8–10 column volumes and a subsequent step with buffer B + 50 mM imidazole for another 8–10 column volumes, the proteins were eluted with buffer B + 200 mM imidazole. The fractions containing Gαi1 or RGS4 were selected after SDS-PAGE, pooled, supplemented with 5 mM DTT, and concentrated to 5 ml using a 10,000 MWCO concentrator. Protein concentrations and not disrupted cells, the suspension was centrifuged with an additional low-speed step for 15 min at 18,000 × g and 4 °C. The supernatant was applied to a 25-ml nickel-nitrilotriacetic acid superflow (Qiagen, Hilden, Germany) column, equilibrated with buffer B (buffer A + 0.3 mM PMSF + 5 mM β-mercaptoethanol + 20 mM imidazole), using a AKTApurifier 100 system (GE Healthcare Life Sciences, Freiburg, Germany) at 6 °C with a flow rate of 1–2 ml/min. After a washing step with buffer C (buffer B + 4 mM MgCl2, 0.1 mM ATP) for 8–10 column volumes and a subsequent step with buffer B + 50 mM imidazole for another 8–10 column volumes, the proteins were eluted with buffer B + 200 mM imidazole. The fractions containing Gαi1 or RGS4 were selected after SDS-PAGE, pooled, supplemented with 5 mM DTT, and concentrated to 5 ml using a 10,000 MWCO concentrator (Amicon Ultra-15, Merck Millipore, Darmstadt, Germany). For gel filtration chromatography, the pool was applied to an illustra HiLoad 26/600 Superdex 200 pg column (GE Healthcare Life Sciences, Freiburg, Germany) equilibrated with buffer D (20 mM Tris, pH 8, 300 mM NaCl, 1 mM MgCl2, 2 mM DTT, 0.1 mM GDP for Gαi1 or 50 mM Heps, pH 8, 100 mM KCl, 2 mM DTT for RGS4). Peak fractions were analyzed by SDS-PAGE. Purest fractions containing Gαi1 or RGS4 were mixed 1:2 with buffer E (20 mM Tris, pH 8, 1 mM MgCl2, 0.1 mM GDP for Gαi1 or 50 mM Heps, pH 8, 100 mM KCl for RGS4), pooled, and concentrated to ~20 mg/ml for Gαi1 or ~10 mg/ml for RGS4 using a 10,000 MWCO concentrator. Protein concentration was determined using Bradford reagent as triplicate. The concentrated pool was aliquoted, flash frozen, and stored at −80 °C until utilization. Coomassie-stained gels after SDS-PAGE of purified proteins are depicted in Fig. 2. ...
FTIR Spectroscopy and Detailed Kinetics of Go11

caged compounds. Phosphatase beads were washed 5 times in buffer 1 (50 mM Tris, pH 7.5, 100 μM ZnSO4) to remove free phosphatase. Each washing step was followed by centrifugation at 10,000 × g and the supernatant was checked for free phosphatase using a colorimetric assay with para-nitrophenylphosphate (43). 5 mg of wild type or mutant Go11 were supplemented with 50 mM Tris, pH 7.5, 10 μM ZnSO4 and a 2X molar excess of the caged nucleotide. Hydrolysis of free and protein-bound GDP to guanosine was monitored via HPLC. After 3 h at room temperature >95% of GDP was hydrolyzed. Samples were centrifuged at 10,000 × g for 2 min and the supernatant was re-buffered through a Nap5 column (GE Healthcare Life Sciences) that was equilibrated with 10 mM Hepes, pH 7.5, 5 mM NaCl, 0.25 mM MgCl2, 1 mM DTT at 7 °C. Protein fractions were pooled and concentrated in a 10,000 MWCO concentrator (Amicon Ultra-0.5, Merck Millipore, Darmstadt, Germany). Concentrations were determined using Bradford reagent and determination via HPLC (>95%). Samples were aliquoted into 107.5 μg portions (5 mM final concentration in FTIR measurements), flash frozen in liquid nitrogen, and stored at −80 °C. Subsequently samples were lyophilized for 3 h at −55 °C/0.05 mbar in a Christ Alpha-1−2 LDPlus Lyophilizer (Martin Christ GmbH, Osterode am Harz, Germany) and stored light protected in paraffin and aluminum foil at −20 °C.

FTIR Measurements on Go11—FTIR measurements were performed using 5 mM Go11-25GTP in 200 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM MgCl2, 20 mM DTT, 0.1% (v/v) ethylene glycol at 15 °C. RGS4 catalyzed measurements were performed by the addition of 5 mM RGS4 to establish a 1:1 complex with Go11. Sample preparation was done under red light to protect the photolabile caged group. Composition of the required residual buffer depends on the protein concentration of the samples after nucleotide exchange to achieve the above named ion concentrations. FTIR samples were prepared between two CaF2 windows (Ø 2 cm, 2 mm thickness, one of them with a 10-μm deepened area 1 cm in diameter). One lyophilisate of Go11-25GTP was dissolved in 0.5 μl of the appropriate residual buffer at the center of the deepened window and subsequently covered with the second window, whose rim had been lubricated with a thin ~1 mm wide silicon grease film. The windows were fixed in a metal cuvette and mounted in the spectrometer (Bruker IFS 66v/S or Vertex 80 v (Bruker, Ettlingen, Germany)). After sample equilibration, background spectra were taken (400 scans) and photolysis of the caged compounds was carried out with an LPX 240 XeCl excimer laser (Lambda Physics, Göttingen, Germany) by 12 flashes within 24 ms (20 ps per flash) or 40 flashes within 80 ms (NPEcgGTP) at 308 nm (100−200 mJ/flash, 20 ns pulse duration) (28). Measurements were performed in the rapid-scan mode of the spectrometer for 30 min (Go11-WT, -D229N, -D231N, -D272N) or 3 h (Go11-R178S) using a liquid nitrogen-cooled mercury cadmium telluride detector. Data between 1800 and 950 cm−1 was collected with a spectral resolution of 4 cm−1 using an aperture of 5 mm in the double-sided forward−backward data acquisition mode with a scanner speed of 120 kHz. Data were analyzed via global fit (44). The absorbance change (ΔA(ν,t)) was fitted with a sum of exponential functions n describing the apparent rate constants ki and amplitudes ai of the hydrolysis reaction and the amplitudes ai of the photolysis reaction for every wavenumber ν.

\[ \Delta A(\nu,t) = a_0(\nu) + \sum_{i=1}^{n} a_i(\nu)(1 - e^{-k_i t}) \]  

(Eq 3)

In the figures disappearing bands face downward and appearing bands face upward. Data were averaged over at least 3 measurements. Half-lives were calculated as arithmetic means, variation was calculated as standard deviations.

Molecular Dynamics Simulation and Evaluation—Molecular dynamics (MD) simulations were performed starting with the Go11-Mg2+-GTPγS structure of Protein Data Bank (PDB) code 1GIA (5) that depicts the truncated (Δ1−32 Δ345−354) active state of Go11. Structure preparation was performed in Moby (45) and included correction of dihedrals, angles, and bonds according to the UA amber84 forcefield (46), protonation of ionizable side chains using the PKA,MAX,UX,JAB3 algorithm as well as replacement of the GTPγS for a GTP molecule (total charge: −4) and initial solvation by the Vedani algorithm (47). Point mutations were realized in Moby and were followed by a short headgroup optimization. Simulation systems were set up in GROMACS 4.0.7 (48−52). The prepared structures were thoroughly solvated in a cubic simulation cell filled with 154 mM NaCl in explicit TIP4P water. Simulations were carried out in the all atom OPLS forcefield (53) with GTP parameters from T. Rudack (54) at 310 K using the berendsen thermo− barostat and a time step of 1 fs. Long range electrostatics were calculated using PME (cutoff 0.9 nm), short range electrostatics were calculated using a VDW cutoff of 1.4 nm. Bonds were constrained using LINCS. Systems were energy minimized and equilibrated for 25 ps with restrained protein positions followed by three free MD runs, each to a simulation time of 100 ns (total simulation time 1.5 μs).

Structure analysis was performed using the GROMACS evaluation tools and the contact matrix algorithm implemented in Moby. Pictures were created using PyMOL 1.7.1.1 (Schrödinger LLC, Portland, OR) and Gnuplot 4.4 (55).

Results

FTIR Measurements of Go11—Time-resolved FTIR spectroscopy enables label-free detection of the GTP/GDP vibrations as well as determination of the apparent kinetics of the hydrolysis reaction. The protein was loaded with caged GTP and the sample was excited at 308 nm with a laser flash to remove the caged group (28) that cleaves rapidly (107 s−1) for pHpcGTP (36)). The resulting difference spectrum is referred to as photolysis spectrum. Subsequently the intrinsic hydrolysis reaction in Go11 takes place (Fig. 3).

The reaction (Scheme 1) is observed in FTIR. Global fit analysis of the absorbance changes revealed a monoexponential function that describes the hydrolysis (Fig. 4). No intermediate enrichment was observed in the measurements of Go11-WT. Global fit analysis of five independent Go11-WT measurements at 15 °C resulted in a half-life of 32.7 ± 2.5 s (kH2O = 0.02 ± 0.001 s−1).
Data analysis according to Equation 3 resulted in photolysis and hydrolysis spectra that represent the transition from the pHPCgGTP to the GTP bound active state of Gα11 and the transition from the active GTP bound state to the inactive GDP bound state, respectively. Bands facing downward represent the educt state, bands facing upward represent the product state. Both spectra show numerous highly reproducible bands in the protein (1680–1350 cm\(^{-1}\)) and the phosphate (1350–950 cm\(^{-1}\)) region (Fig. 5). Surprisingly a band at 1784 cm\(^{-1}\) appeared in the photolysis and disappeared in the hydrolysis reaction, indicating a protonation of a carboxyl group from an Asp or Glu (56) in the GTP state (Fig. 5). To our knowledge this is the first time a protonation change has been observed in GTPases. For a clear cut assignment further studies with site-directed mutations have to be performed.

Phosphate vibrations were assigned using isotopically labeled nucleotides, namely \(\alpha\-^{18}\text{O}_2\)-pHPcgGTP, \(\beta\-^{18}\text{O}_2\)-pHPcgGTP, and \(\gamma\-^{18}\text{O}_2\)-pHPcgGTP. Double difference spectra of FTIR measurements using unlabeled and labeled nucleotides showed exclusively band shifts caused by the isotopes and allow band assignments of the phosphate region. In the photolysis spectrum the bands at 1240, 1224, and 1155 cm\(^{-1}\) were assigned to the asymmetric stretching vibrations of \(\alpha\-, \beta\-,\) and \(\gamma\-\text{GTP (Fig. 6, A and B). The vibrations for \(\beta\-\text{and} \gamma\-\text{GTP appear as clear bands, the \(\alpha\-\text{band appears as a shoulder only in the photolysis spectrum but is more distinct in the hydrolysis spectrum. Band assignments of the hydrolysis reaction confirmed \(\alpha\-, \beta\-,\) and \(\gamma\-\text{GTP vibrations at 1243, 1224, and 1156 cm}\(^{-1}\). The vibrations of the product state were assigned to 1214 cm\(^{-1}\) for \(\alpha\-\text{GDP, 1134 and 1103 cm}\(^{-1}\) for \(\beta\-\text{-GDP, and 1078 and 991 cm}\(^{-1}\) for the cleaved free phosphate (Fig. 6, C and D). The cleaved phosphate is not protein bound, as the vibrations at 1078 and 991 cm\(^{-1}\) are typical for free phosphate. Protein-bound phosphate intermediates are blue-shifted, e.g. in case of RasGAP an intermediate band appears at 1192 cm\(^{-1}\) (57). Because a protein-bound phosphate intermediate was not observed as in case of the RasGAP catalyzed reaction, bond breakage is the rate-limiting step in the hydrolysis reaction of Gα11 (Fig. 4). Summarizing, the hydrolysis reaction of Gα11-bound GTP to GDP and Pi was monitored label free at atomic resolution and in the millisecond time scale. Individual asymmetric stretching modes of GTP and GDP bound to Gα11, and Pi were assigned clear cut.

In addition, we performed the same experiments with the Gα11-RGS4 1:1 complex at 5°C. Addition of RGS4 further catalyzed the hydrolysis reaction by almost 2 additional orders of magnitude (Fig. 7). As for the intrinsic measurements, global fit analysis resulted in one exponential rate, which demonstrates that again bond breakage is rate-limiting. No protein-bound
FTIR Spectroscopy and Detailed Kinetics of Gαi1

![FTIR Spectroscopy and Detailed Kinetics of Gαi1](image)

FIGURE 6. Band assignment via isotopically labeled α-18O2-pHPCGTP, β-18O2-pHPCGTP, and γ-18O2-NPecGTP of the photolysis (A) and hydrolysis (C) reaction of Gαi1, at 15 °C. Measurements with NPecGTP were scaled by the factor of 5 (photolysis) or 10 (hydrolysis). Arrows indicate band shifts caused by the 18O isotopes. Double differences (Δ and D) represent measurements with labeled minus unlabeled nucleotides and reveal the band shift only. The positions of 18O labeling are depicted in panel E. The 18O-labeled phosphoester in γ-18O2-NPecGTP leads to β-18O2-GDP, corresponding band shifts are marked in cyan.

![Kinetics of the GTPase reactions of intrinsic Gαi1, and Gαi1, RGS4 measured via FTIR spectroscopy (β-GDP band at 5 °C)](image)

FIGURE 7. Kinetics of the GTPase reactions of intrinsic Gαi1, and Gαi1, RGS4 measured via FTIR spectroscopy (β-GDP band at 5 °C)

exchange (koff,GDP and koff,GTP) and the hydrolysis rate khyd was investigated by reversed phase HPLC at 30 °C according to Equation 1. Results can be grouped into four classes. One turnover is a cycle consisting of GDP release, GTP binding, and GTP hydrolysis that took 12.7 ± 0.2 min for Gαi1-WT. The arginine finger mutant Gαi1-R178S was slowed down to 29.6 ± 1.6 min per turnover. Gαi1-D229N and -D231N shared an accelerated turnover time of 3.9 ± 0.03 min and Gαi1-D272N was accelerated even more to 0.5 ± 0.02 min per turnover (Fig. 9A). It is generally accepted that the GDP release step is rate-limiting in multiple turnover measurements of Gα-proteins (59). To further investigate the underlying rate constants we additionally performed nucleotide exchange and single turnover hydrolysis experiments.

Nucleotide Exchange Experiments—In contrast to multiple turnover experiments, tryptophan fluorescence spectroscopy can monitor solely the nucleotide exchange reaction from GDP to GTPγS of Gαi1 as Trp211 is sensitive for binding of the third phosphate group (Fig. 10). Hydrolysis cannot proceed as GTPγS is a non-hydrolyzable GTP analogue. The results of nucleotide exchange can be grouped into three classes. In contrast to multiple turnover measurements, the half-life value for nucleotide exchange to GTPγS of Gαi1-R178S was similar to Gαi1-WT (12.8 ± 0.9 and 10.7 ± 0.2 min, respectively). Nucleotide exchange was accelerated by a factor of about 3 in Gαi1-D229N and -D231N to 3.3 ± 0.2 and 3.7 ± 0.1 min and even more accelerated in Gαi1-D272N (0.8 ± 0.03 min) (Fig. 9B). Hence it can be concluded that the acceleration in multiple turnover measurements of Gαi1-D229N, -D231N, and -D272N can be explained by accelerated dissociation times for GDP and/or association times for GTP. On the other hand the decelerated multiple turnover time for Gαi1-R178S is not caused by nucleotide exchange, indicating that nucleotide exchange is not the rate-limiting step for this mutant.

Single Turnover Hydrolysis Measurements Using FTIR—In contrast to multiple turnover measurements, time-resolved FTIR spectroscopy can determine the hydrolysis reaction label free under actual single turnover conditions with high spatio-temporal resolution. The half-life value was obtained from the

phosphate intermediate was observed and the absorptions of protein-bound GTP disappeared with the same rate as the absorptions of free P

in nucleotide binding (G

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results of the global fit procedure (44). The measured kinetics can again be grouped into three classes. Go11-WT and Go11-D231N had similar half-life values of 32.7 ± 2.5 and 27.8 ± 2.6 s, respectively. The single turnover hydrolysis reaction of Go11-D229N and -D272N was slightly slowed down to 50.2 ± 4.5 or 49.8 ± 4.1 s. The hydrolysis reaction of Go11-R178S was noticeably slowed down by 2 orders of magnitude to 3400 ± 400 s (Fig. 9C). Thereby the deceleration of Go11-R178S in multiple turnover measurements can be explained solely by the slowed down single turnover hydrolysis reaction. Due to the change of the rate-limiting step, the slowdown in the multiple turnover assay appears to be only about 2-fold, whereas the single turnover FTIR assay yield the true slowdown by 2 orders of magnitude.

Molecular Dynamics Simulations—To further examine the molecular interactions taking place in the interface between the Ras-like and all-α domain of Go11, molecular dynamics simulations were performed to elucidate the role of Asp229 and Asp231 at atomic detail. Simulations of wild type Go11 and mutants Go11-D229N and -D231N were performed for 100 ns each. Subsequently contact matrix analysis was carried out for every simulation. Contacts were sampled in time windows of 1 ns and the interaction partners of Asp/Asn229 and Asp/Asn231 were depicted in Fig. 11. Polar contacts of the side chain groups are indicated by black bars. Asp229 formed a stable interdomain contact to the all-α domain through Arg242 that bound Gln147 in wild type Go11 (Fig. 11A). When mutated to Asn229, this interdomain contact triad was interrupted after the first 30 ns. Thereby the contact loss between Asn229 and Arg242 happened simultaneously to the contact loss of Arg242 to Glu147 and Gln147. Hence Asp229 seems to position Arg242 allosterically, so that Arg242 forms an interdomain contact that tightly binds and stabilizes the Ras-like and the all-α domain.

Similar to Asp229, Asp231 formed an interdomain contact to Arg144 within a 100-ns MD simulation (Fig. 11B). It is notable, that the contact Asp231-Arg144 does neither exist in the starting crystal structure generated from PDB code 1GIA, nor in the original structure, but formed de novo in the simulation. The initial contact to Lys277 was weakened, but the contact to Arg144 was completely lost. Summarizing the results from multiple and single turnover measurements, nucleotide exchange experiments and simulation data, we understand the effects of the point mutations in Go11. Go11-R178S was slowed down in multiple turnover measurements, but even slower in single turnover measurements that depict only the hydrolysis reaction itself. Its nucleotide exchange ability appeared unaltered. Slowdown of multiple turnover is solely from hydrolysis in this case. Thus Arg178 only participates in the hydrolysis reaction as described elsewhere (5, 58).

The interface mutations D229N and D231N were both accelerated in multiple turnover measurements. Investigations of the nucleotide exchange reaction showed that the exchange time for both mutants was accelerated. Simulation data suggest an allosteric (Asp229) and direct (Asp231) interdomain binding mode of both amino acids. Mutations affecting the guanosine binding moiety Asp272 resulted in accelerated half-life values in multiple turnover measurements that can be originated to an accelerated nucleotide exchange behavior as shown via fluorescence spectroscopy.
Discussion

Molecular mechanisms that take place in Gi have been investigated by numerous studies including structural (5, 34, 60), computational (61), and biochemical (13, 14, 33) assays. In particular, multiple turnover GTPase assays like malachite green or radiometric phosphate tests using [γ-32P]GTP are widely used even though it is commonly known that GDP/GTP exchange is the rate-limiting step in intrinsic multiple turnover measurements (9–12) and thereby determines k
\text{obs}. Pre-steady-state measurements using GTP or [γ-32P]GTP pre-loaded Gi subunits that are triggered via Mg
2+ addition are also able to depict single turnover conditions, but the percentage of nucleotide loading (Gi/GDP versus Gi/GTP) and the altered GTP binding affinity due to Mg
2+ -binding (62) may cause systematic errors such as side reactions like nucleotide exchange. However, in FTIR measurements the percentage of loaded cgGTP versus GDP does not influence the kinetics due to the method of phototriggered difference spectroscopy. Additionally we checked the loading rate via HPLC (always >95% cgGTP) and

![Figure 9](image)

**FIGURE 9.** Summary of the results of multiple turnover measurements via HPLC at 30 °C (A), nucleotide exchange experiments via fluorescence spectroscopy at 30 °C (B) and single turnover hydrolysis measurements via FTIR spectroscopy at 15 °C (C) of wild type and mutant Gi1.

![Figure 10](image)

**FIGURE 10.** Negative control of the mutant Gi1-W211A in fluorescence spectroscopy. After baseline monitoring for 5 min, 2.5 μM GTPγS (×5 molar excess) was added to trigger nucleotide exchange.

![Figure 11](image)

**FIGURE 11.** Contact matrix analysis of the molecular interactions taking place in the surrounding of Asp229 (D229) (WT) and the mutant D229N (A) or Asp231 (D231) (WT) and its mutant D231N (B) during a MD simulation. Black bars indicate H-bonds, white spaces indicate no H-bond formation.
removed non protein-bound nucleotides. The determined single turnover rate for wild type \( \alpha_{i1} \) measured via FTIR spectroscopy (0.02 s\(^{-1}\) at 15°C) is in good agreement to the literature (0.03 s\(^{-1}\) at 30°C (58) and 0.03 s\(^{-1}\) to 0.04 s\(^{-1}\) at 20°C (13, 64)). In addition the ensemble of methods enables a classification of effects caused by point mutations in high detail. Effects caused by the intrinsic arginine finger mutant \( \alpha_{i1}-R178S \) were quantified correctly in single turnover measurements (2 orders of magnitude) but not in multiple turnover measurements (factor of 2). The unaltered nucleotide exchange rate of \( \alpha_{i1}-R178X \) mutants has already been described elsewhere (5).

Single turnover FTIR spectroscopy unravels for the first time the rate-limiting step of the intrinsic GTP hydrolysis in \( \alpha_{i1} \). Analogue experiments with small GTPases revealed that in some cases the bond breakage and in others the P\(_i\) release is rate-limiting (57). For \( \alpha_{i1} \) no protein-bound cleaved phosphate intermediate could be observed, thus bond breakage is the rate-limiting step in this reaction. This is surprising due to the tight coordination of the nucleotide by \( \alpha_{i1} \). The narrow protein environment is still able to release free phosphate to the periphery, probably through a small channel located near the \( \gamma \)-phosphate. The measured IR bands for GTP and GDP are very sensitive to changes in the protein environment and depict for the first time the coordination of the natural nucleotides GDP and GTP in \( \alpha_{i1} \) in contrast to GTP analogues, which were described to have poor affinities for \( \alpha_{i1} \) (65). After we have successfully assigned the \( \alpha \), \( \beta \), \( \gamma \), and the free phosphate vibrations it will be possible to assess the effect of point mutations in the binding pocket of \( \alpha_{i1} \), in the future supported by theoretical IR spectra calculation from QM/MM simulations as performed for the small GTPase Ras (66) to further decode the experimental spectra. In addition to the phosphate bands, various bands caused by the protein itself were nicely resolved, which will enable investigations of the hydrolysis mechanism taking place in \( \alpha \) proteins with improved spatio-temporal resolution. The observed band at 1784 cm\(^{-1}\) is the first protonation change observed in GTPases to our knowledge. In fact, heterotrimeric G proteins have been speculated to function as pH sensors (67) and a protonation change close to the surface of \( \alpha_{i1} \) could function as a key player in this reaction.

In addition to the intrinsic GTPase reaction of \( \alpha_{i1} \) we were also able to measure the hydrolysis reaction catalyzed by RGS4 via FTIR spectroscopy. Hydrolysis was thereby accelerated by almost 2 orders of magnitude (Fig. 7). As for intrinsic \( \alpha_{i1} \), bond breakage is the rate-limiting step.

We were able to show with our orchestration of different methods that two point mutations in the \( \alpha_{i1} \) Ras-like/all-\( \alpha \) interface (\( \alpha_{i1}-D229N/\alpha_{i1}-D231N \)) are able to weaken the coordination in the protein domain interface. Our measurements together with MD simulations demonstrate the importance of the amino acid triad Asp\(^{231}\)-Arg\(^{242}\)-Glu\(^{147}\) for the interface coordination in \( \alpha_{i1} \). Asp\(^{229}\) holds Arg\(^{242}\) in a position to bridge the interface to Glu\(^{147}\). Investigations on the mutant \( \alpha_{i1}-R242A \) confirmed its role (nucleotide exchange: 3.09 ± 0.21 min/single turnover hydrolysis: 32.5 ± 3.5 s) and resulted in similar values as \( \alpha_{i1}-D229N \). In agreement, accelerated nucleotide exchange for \( \alpha_{i1}-R242A \) has recently been described for the analogue R243H in \( \alpha_{i1} \) (68). Our findings on the other amino acid in the domain interface, Asp\(^{231}\), suggest a direct binding mode of the side chain of Asp\(^{231}\) across the interface to Arg\(^{144}\). This contact is not observable in any of the deposited structures of \( \alpha_{i1} \) in the Protein Data Bank, except structure 4PAQ where the side chain of Arg\(^{144}\) is slightly tilted toward Asp\(^{231}\) with occupancy of 0.46 (69). In contrast to tightly packed crystal structures, the dynamics of \( \alpha_{i1} \) in our experiments and in our simulations are much more comparable with physiological conditions, so we hereby demonstrate the importance of the salt bridge Asp\(^{231}\)-Arg\(^{144}\), which is not observable in the crystal structures. Our findings are summarized in an advanced interface binding model of \( \alpha_{i1} \) as shown in Fig. 12.

In summary, we were able to measure the isolated rates of nucleotide exchange and GTP hydrolysis, which both contribute to the signaling state of \( \alpha_{i1} \). In addition we identified the individual phosphate vibrations of GTP, GDP, and P\(_i\) during the hydrolysis reaction of \( \alpha_{i1} \). We demonstrated the importance of the intrinsic arginine finger for the hydrolysis reaction and the relevance of Asp\(^{272}\) for nucleotide binding. Furthermore, we identified novel key players in the coordination of the Ras-like/all-\( \alpha \) interface. Asp\(^{229}\) stabilizes the interface allosterically via Arg\(^{242}\) and Asp\(^{231}\) forms a direct H-bond to Arg\(^{144}\). Orchestration of our methods will further elucidate the molecular mechanisms taking place in \( \alpha_{i1} \) in the future.

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Molecular Biophysics: Integration of Fourier Transform Infrared Spectroscopy, Fluorescence Spectroscopy, Steady-state Kinetics and Molecular Dynamics Simulations of Gαi1 Distinguishes between the GTP Hydrolysis and GDP Release Mechanism

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