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Biarsenical ligands bind to endogenous G-protein α -subunits and enable allosteric sensing of nucleotide binding

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Abstract

Background: Heterotrimeric G-proteins relay extracellular signals to intracellular effector proteins. Multiple methods have been developed to monitor their activity; including labeled nucleotides and biosensors based on genetically engineered G-proteins. Here we describe a method for monitoring unlabeled nucleotide binding to endogenous G-protein α -subunits in a homogeneous assay based on the interaction of 4',5'-bis(1,2,3-dithioarsolan-2-yl)-2',7'-difluorofluorescein (F₂FIAsH) with G-protein α -subunits.

Results: The biarsenic fluorescent ligand F₂FIAsH binds to various wild-type G-protein α -subunits (α i1, α i2, α i3, α s_{long}, α s_{short}, α olf, α q, α 13) via high affinity As-cysteine interactions. This allosteric label enables real time monitoring of the nucleotide bound states of α -subunits via changes in fluorescence anisotropy and intensity of their F₂FIAsH-complexes. We have found that different α -subunits displayed different signal amplitudes when interacting with F₂FIAsH, being more sensitive to nucleotide binding to α i, α s, α olf and α q than to α 13. Addition of nucleotides to F₂FIAsH-labeled α -subunits caused concentration-dependent effects on their fluorescence anisotropy. pEC₅₀ values of studied nucleotides depended on the subtype of the α -subunit and were from 5.7 to 8.2 for GTP γ S, from 5.4 to 8.1 for GppNHp and from 4.8 to 8.2 for GDP and lastly up to 5.9 for GMP. While GDP and GMP increased the fluorescence anisotropy of F₂FIAsH complexes with α i-subunits, they had the opposite effect on the other α β γ M complexes studied.

Conclusions: Biarsenical ligands interact allosterically with endogenous G-protein α -subunits in a nucleotide-sensitive manner, so the presence or absence of guanine nucleotides has an effect on the fluorescence anisotropy, intensity and lifetime of F₂FIAsH-G-protein complexes.

Keywords: G-proteins, Tetracysteine, F₂FIAsH, Fluorescence anisotropy, Nucleotide binding

Background

Heterotrimeric guanine nucleotide binding proteins (G-proteins) play an integral role in signal transduction and, when activated by G-protein coupled receptors (GPCRs), relay signals crossing the plasma membrane to intracellular effector proteins. They are composed of α - and β γ -subunits and bind guanosine diphosphate (GDP) in their inactive and guanosine triphosphate (GTP) in their activated state. In the latter case the heterotrimer may dissociate fully or partially and both subunits may subsequently

interact with downstream effectors [1]. Intrinsic GTPase activity of the α -subunit leads to eventual inactivation, completing the cycle as the subunits reassociate. Various means have been employed to measure the activity of G-proteins, with measurements of GTPase activity, intrinsic fluorescence and binding of radioactively or fluorescently labeled nucleotides being among the most common methods [2]. Genetic engineering has made possible the development of G-proteins that are either fused to fluorescent proteins or contain a motif that allows for their labeling with various small fluorescent molecules [3]. One such motif is the tetracysteine tag, which binds 4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein (FIAsH) analogues with high affinity and selectivity [4]. It has been used to label G-proteins to give resonance energy transfer pairs with either another also fluorescently labeled

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G-protein subunit [5,6], or other proteins that interact with G-proteins such as GPCRs or regulators of G-protein signaling [2,7]. One disadvantage of this method is that the interacting proteins have to be labeled with donor and acceptor fluorophores, which limits the range of interactions that can be measured and can lead to alterations in their function, while simultaneously conferring selectivity due to the requirement for close proximity between the donor and acceptor for efficient energy transfer.

Fluoresceine-based biarsenical fluorophores such as FLAsH or F₂FLAsH retain the parent compounds hydrophobicity and may bind nonspecifically to intracellular proteins and membranes [4], which can generate high background signals. This kind of interaction can become advantageous when FLAsH-analogues are used as sensitive reporters of their molecular environment: for example as conformation sensitive probes of protein structure [8]. FLAsH is also known to bind to cysteine-rich proteins *in vivo* [9]. Taking advantage of these properties, we have developed a method for *in vitro* monitoring of nucleotide binding to heterotrimeric G-proteins based on F₂FLAsH interactions with cysteine residues of endogenous G-protein α -subunits. We have used this method to characterize nucleotide binding to 8 different G-proteins and show that F₂FLAsH interactions with G-proteins are subtype specific.

Methods

Cell lines and reagents

Spodoptera frugiperda 9 (Sf9) cells were from Invitrogen Life Technologies (Carlsbad, CA, USA). HEPES, NaCl, EDTA, MgCl₂ were from Applichem GmbH (Darmstadt, Germany). GDP, guanosine monophosphate (GMP), guanosine 5'-O-[gamma-thio]triphosphate (GTP γ S), guanosine 5'-[β,γ -imido]triphosphate (GppNHp), dodecylsucrose, sodium cholate, polyoxyethylene (10) lauryl ether (C12E10), tris(2-carboxyethyl)phosphine (TCEP), ethanedithiol, desthiobiotin were from Sigma-Aldrich GmbH (Munich, Germany). AsCl₃ was from Reachim (Russia). β -mercaptoethanol was from Merck KGaA (Darmstadt, Germany). F₂FLAsH was synthesized according to published procedures [10]. FLAsH was from Toronto Research Chemicals (Toronto, Canada). G-protein α -subunits (α_q , $\alpha_{s_{long}}$, $\alpha_{s_{short}}$, α_{olf} and α_{13}) were from Kerastat Inc, (Boston, MA, USA). Tetracysteine-labeled peptide (FLNCCPGCCMEP) was from Bachem AG (Bubendorf, Switzerland). Pyruvate kinase was from Roche diagnostics GmbH (Mannheim, Germany), BSA was from PAA Laboratories GmbH (Pasching, Austria). Fluorescein was from Lambert Instruments (Roden, the Netherlands).

Protein expression and purification

G-protein α_1 , α_2 , α_3 and dual-tagged $\beta_1\gamma_2$ -subunits ($\beta\gamma M$) were expressed and purified as previously described [11] using tandem affinity chromatography [12]. Briefly,

Sf9 cells were grown in serum free medium in shaker flasks and infected with baculoviral stocks to simultaneously express either only $\beta\gamma M$ -subunits or $\beta\gamma M$ and α -subunits. Infected cells were harvested after 48 h. Cell pellets were homogenized in ice cold homogenization buffer (HB: 20 mM HEPES, pH = 8, 10 mM NaCl, 2 mM MgCl₂, 1 mM EDTA, 5 μ M GDP, 5 mM β -mercaptoethanol and protease inhibitors, diluted according to manufacturer's recommendations: Roche Complete EDTA-free, Roche diagnostics GmbH (Mannheim, Germany)). Cells were homogenized by sonication for 5 cycles of 10 sec (Bandelin SonoPuls, Bandelin electronic GmbH, Berlin, Germany). Homogenates were then centrifuged for 30 min at 40 000 \times g (Sigma 3 K30, SIGMA Laborzentrifugen GmbH, Osterode am Harz, Germany) and the resulting membrane pellets resuspended in solubilization buffer (HB with 1% Na-cholate, 0,1% C12E10 and 0,5% dodecylsucrose) and shaken for 1 h at 4°C at 250 rpm (ELMI DOS-20S, ELMI Ltd, Riga, Latvia). The solubilized proteins were separated by centrifugation for 30 minutes at 40 000 \times g and purified with affinity chromatography using Strep-Tactin Superflow high capacity resin (IBA GmbH, Göttingen, Germany) in Poly-Prep columns (Bio-Rad, Hercules, CA, USA). The columns were washed with washing buffer (WB: 20 mM HEPES, pH = 8, 10 mM NaCl, 1 mM EDTA, 0,5% C12E10, 5 mM β -mercaptoethanol) and the G-proteins eluted with elution buffer (WB +2 mM desthiobiotin). Eluates were aliquoted, frozen and kept until use at -80°C. Protein concentrations were determined by UV-absorbance at 280 nm using a Nanodrop 1000 spectrophotometer (NanoDrop products, Wilmington, DE, USA) and purities estimated using Ag-stained SDS-PAGE gels [11].

Fluorescence lifetime measurements

We determined fluorescence lifetimes in the frequency domain using an imaging attachment (LIFA-X, Lambert Instruments, Roden, The Netherlands) consisting of a signal generator, Multi-LED excitation source with a 3 W light emitting diode (477 nm LED), and an intensified CCD Li²CAM-X with GEN-III GaAs photocathode. The CCD was mounted on the side port of an iMIC inverted digital fluorescence microscope (Till Photonics GmbH, Gräfelfing, Germany) through a TuCam adapter with 2 \times magnification (Andor Technology, Belfast, UK). Multi-LED was fiber coupled to the epicondenser of iMIC. The filter cube comprised of a BrightLine HC 475/35 nm (Semrock, New York, USA) exciter, a zt 491 rdcxt dichroic (Chroma, Bellows Falls, USA) and a BrightLine HC 525/45 nm (Semrock, New York, USA) emitter. For all samples and references a series of images with an exposure time of 150 ms was taken at 11 modulating frequencies (from 1–120 MHz, with increasing LED AC from 0.1 until 2.5 V) and 12 phase-shifts between LED and image intensifier per every modulating frequency. MCP gain used

was 750. Photons were collected with 4 × UPLSAPO objective (Olympus, Japan). To increase efficiency of primary photon collection and to decrease the effect of photobleaching 4 images/phase were averaged. Camera binning of 4 by 4 was used. For lifetime calibration, a solution of fluorescein (0.1 μM, at pH > 10) was used as reference with τ = 4.02 ns. The background correction was performed automatically by subtracting an image obtained with blocked excitation using Li-FLIM v1.2.22 software (Lambert Instruments, Roden, The Netherlands).

Samples were measured in 4 well CMS Chamlide chambers (Live Cell Instrument, Seoul, Korea) with 1.5H cover glasses (Paul Marienfeld GmbH & Co, Lauda-Königshofen, Germany).

By using nonlinear optimization routines (Levenberg-Marquart) the distance between measured modulation depths $m_{\omega m}$ and phase shifts $\vartheta_{\omega m}$ and the calculated $m_{\omega c}$ and $\vartheta_{\omega c}$ was minimized by finding optimal values for lifetimes τ_i and fractions α_i [13].

$$\vartheta_{\omega c} = \arctan \frac{\sum_i \frac{a_i \omega \tau_i}{1 + (\omega \tau_i)^2}}{\sum_i \frac{a_i}{1 + (\omega \tau_i)^2}} \quad (1)$$

$$m_{\omega c} = \sqrt{\left(\sum_i \frac{a_i}{1 + (\omega \tau_i)^2} \right)^2 + \left(\sum_i \frac{a_i \omega \tau_i}{1 + (\omega \tau_i)^2} \right)^2} \quad (2)$$

Error function was given by:

$$x^2 = \sum_{\omega} (\vartheta_{\omega m} - \vartheta_{\omega c})^2 + \sum_{\omega} (m_{\omega m} - m_{\omega c})^2 \quad (3)$$

Measurements were made in two independent experiments using 50 nM F₂FLAsH in the presence or absence of either 60 nM βγM or αolf and α13 (with or without 10 μM GTPγS) or 600 nM of tetracysteine-labeled peptide (FLNCCPGCCMEP). F₂FLAsH and its complexes were preincubated at 28°C for 6 h before lifetime measurements at room temperature.

Spectrophotometric measurements of F₂FLAsH-complexes

Fluorescence emission spectra of free F₂FLAsH (5 nM) and its complexes with G-proteins (12.5 nM αolf, with or without 10 μM GTPγS, or 15 nM βγM subunits) were determined using a Perkin-Elmer LS 55 luminescence spectrometer (PerkinElmer Inc, Waltham, MA, USA) with excitation at 480 nm and a 10 nm emission slit width at 100 nm/min scan speed.

Fluorescence anisotropy measurements

All fluorescence anisotropy measurements were carried out at 28°C in 96-well half area microtiter plates (Corning

Product No.3993, Corning Life Sciences, Lowell, MA, USA) in a Pherastar platereader (BMG LABTECH GmbH, Ortenberg, Germany) [14]. Fluorescence anisotropies were measured using (polarized) excitation at 485 nm (20 nm bandwidth) and simultaneous dual (polarized) emission at 520 nm (20 nm bandwidth), which enabled recording of fluorescence emission intensities that are parallel and perpendicular to the plane of excitation light. Erythrosin B was used for fluorescence polarization calibration [15].

Measurements were conducted in duplicate or quadruplicate in two or three independent experiments. The apparent affinities of GDP, GMP, GTPγS and GppNHp were determined by their abilities to modulate F₂FLAsH fluorescence anisotropy in the presence of αβγM heterotrimers. For experiments with nucleotide-depleted αi-heterotrimers, 5 nM F₂FLAsH was used to label approximately 40 nM G-proteins and nucleotide affinities were determined at 2 h from the start of the experiment. For experiments with αq, αs_{long}, αs_{short}, αolf and α13, 12.5 nM α-subunits were preincubated on ice with 15 nM βγM for 60 min, to allow the subunits to associate, before addition of 15 nM F₂FLAsH and nucleotides. Nucleotide affinities for αs_{long} + βγM, αs_{short} + βγM, αq + βγM and αolf + βγM were determined at 6 h from the start of the experiment and for α13 + βγM at 14 h. All measurements (unless specified otherwise) were made in 20 mM HEPES buffer, pH = 7.8, with 1 mM EDTA, 2 mM MgCl₂, 10 mM NaCl, 2 mM β-mercaptoethanol, 1 mM TCEP and 0.1% C12E10. All reagents, proteins and microtiter plates were kept on ice prior to the initiation of the measurements. In experiments where F₂FLAsH and G-proteins were subjected to heat denaturation, microtiter plates that had been measured in the platereader for 6 h were sealed with AbsorbMax adhesive film (Excel Scientific Inc, Victorville, CA, USA) and heated at 70°C for 1 h in an Eppendorf Thermomixer Comfort (Eppendorf AG, Hamburg, Germany) with shaking at 400 rpm. The plates were then cooled to room temperature, unsealed and remeasured in the platereader. Data was then collected at 30 min from the start of the measurement.

Data analysis

All data were analyzed using nonlinear regression with GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Fluorescence anisotropy data was baseline (F₂FLAsH-G-protein complex with no added nucleotides) corrected for each experiment before data was pooled and fitted for determination of nucleotide affinities. Kinetic curves (Figure 1) were also baseline corrected to show the appearance of nucleotide-sensitivity. Apparent affinity values (logEC₅₀) were calculated using three parameter competitive binding equations ($Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{(1 + 10^{(X - \text{LogEC}_{50})})}$), where X corresponds to logarithm of molar concentration of nucleotide.

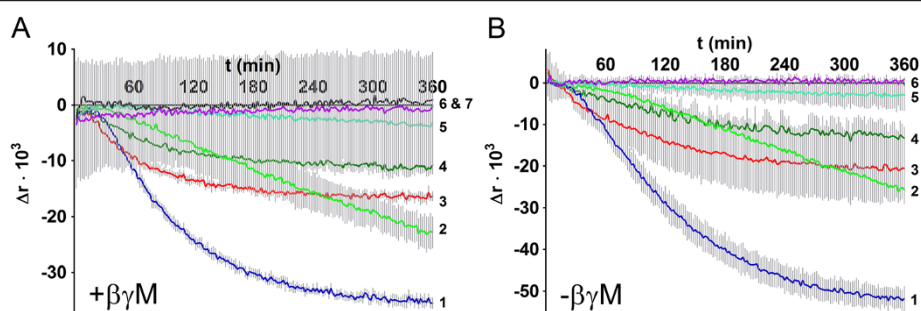


Figure 1 The effect of 10 μM GTP γ S on the formation of complexes between F₂FlAsH and G-proteins in the presence (A) or absence of $\beta\gamma$ M subunits (B). Data are presented as the time-dependent attenuation of fluorescence anisotropy in comparison to GTP γ S-untreated F₂FlAsH-G-protein complexes. The preparations used were αolf (1), αq (2), $\alpha\text{S}_{\text{short}}$ (3), $\alpha\text{S}_{\text{long}}$ (4), α13 (5), none (6) and $\beta\gamma\text{M}$ (7). Data is from two or three independent experiments carried out in duplicate and presented as mean \pm SD as error bars.

Results and discussion

Fluorescence intensities and lifetimes of F₂FlAsH-complexes

The fluorescence lifetime and quantum yield of fluorophores can be altered by interactions with their molecular environment. As the fluorescence intensity of F₂FlAsH was increased up to twofold by the addition of G-protein α -subunits – with no significant shift in emission or absorbance maxima in comparison to free F₂FlAsH (Additional file 1: Figure S1) – we decided to investigate whether this increase in fluorescence intensity would also be reflected in alterations of the fluorescence decay rates of F₂FlAsH complexes, as higher fluorescence intensities suggest higher quantum yields, with proportionally longer lifetimes of fluorescence decay.

We measured the effects of a tetracysteine-labeled peptide, $\beta\gamma\text{M}$, α13 and αolf (with or without 10 μM GTP γ S) on the fluorescence lifetimes of F₂FlAsH (Table 1, Additional file 2: Figure S2). Free F₂FlAsH exhibited a

two component exponential decay rate with a shorter component of 1.0 ± 0.2 ns and a longer component of 4.3 ± 0.1 ns, with the shorter lifetime component comprising $34 \pm 3\%$ of the signal. Addition of GTP γ S to free F₂FlAsH did not significantly alter fluorescence decay rates or their relative proportions. Addition of a tetracysteine labeled peptide however decreased the proportion of the faster decaying component down to $11 \pm 2\%$, as did the addition of $\beta\gamma\text{M}$, which decreased the 1.0 ns component down to $25 \pm 2\%$ of the total. When αolf subunits were added to F₂FlAsH the proportion of the faster decaying component was decreased to $12 \pm 5\%$ in the absence of GTP γ S, but when the nucleotide was present we observed a fluorescence decay rate that was best described by a 3-component fit with a previously undetected very rapidly decaying component ($\tau < 0.1$ ns, 17% of the signal), while the proportion of the 1.0 ns component was increased to 31%. Addition of α13 , which had very low sensitivity to nucleotide-dependent changes in fluorescence anisotropy, also had limited effects of the fluorescence decay rates of F₂FlAsH: the 1.0 ns component was not greatly altered at $37 \pm 4\%$ (in comparison to free F₂FlAsH, where this component was at $34 \pm 3\%$) in the absence of GTP γ S and was at $38 \pm 3\%$ in the presence of the nucleotide. No rapid 0.1 ns component was detected in the presence of GTP γ S for α13 .

These results indicate that the nucleotide-dependent changes in fluorescence anisotropy, which will be described in the following paragraphs, could be the result of quenching of F₂FlAsH by guanine nucleotides that bind to G-protein α -subunits in close proximity to the fluorophore. This quenching would (by decreasing the time available for rotation) increase fluorescence anisotropy. However, if the reaction is accompanied by a change in the rotational correlation time (as a result of altered binding or conformational changes) it would have the opposite effect on steady state fluorescence anisotropy measurements.

Table 1 Fractions of F₂FlAsH and its complexes' fluorescence with a lifetime of 1 ns

	Fraction $\tau_{(1.0 \text{ ns})}^a$ %	χ^2
F ₂ FlAsH	34 ± 3	1.9
F ₂ FlAsH + GTP γ S	29 ± 1	3.7
CCPGCC-peptide	11 ± 2	3.8
$\beta\gamma\text{M}$	25 ± 2	4.6
αolf	12 ± 5	1.67
αolf + GTP γ S	56 ± 4^b	12^b
α13	37 ± 4	6.7
α13 + GTP γ S	38 ± 3	2.5

^aFluorescence lifetimes were fitted to equations (1–3) with two components (1.0 ± 0.2 ns and 4.3 ± 0.1 ns) and fractions corresponding to 1.0 ns are reported as mean \pm SD.

^bA better fit was obtained by including a third very fast component corresponding to 17% of fluorescence, but with a lifetime that was below the measurement range of the instrument ($\tau < 0.1$ ns).

Thus, the final results would depend on the extent of changes in fluorescence lifetime in comparison to changes in the rotational correlation time.

The effects of G-proteins and nucleotides on the fluorescence anisotropy of F₂FLAsH

We also investigated the kinetics and nucleotide-dependence of F₂FLAsH binding to various G-protein subunits by using fluorescence anisotropy. When purified tetracysteine-tagged βγM-subunits were added to F₂FLAsH, a time-dependent increase in fluorescence anisotropy of F₂FLAsH was seen. The fluorescence anisotropy of free F₂FLAsH or F₂FLAsH-βγM complexes was not significantly affected by the presence or absence of GTPγS (Figure 1 lines 6 and 7). Addition of purified α-subunits to F₂FLAsH (in the presence of βγM-subunits) caused an additional increase in fluorescence anisotropy in comparison to F₂FLAsH-labeled βγM. The magnitude of the increase depended on the α-subunit subtype and this increase in fluorescence anisotropy could be attenuated by the addition of 10 μM GTPγS for all 8 α-subunits studied (Figure 1, Figure 2 solid lines).

When F₂FLAsH was added to purified wild type α-subunits in the absence of tetracysteine-tagged βγM subunits, the large GTPγS-sensitive signal was still evident (Figure 1B) and this time-dependent increase in nucleotide-sensitive fluorescence anisotropy was practically unaltered by the addition of βγM-subunits to the mixture (Figure 1A), except for α_{olf}, which had the largest nucleotide-sensitive signal amplitude and this was reduced in the presence of βγM (Figure 1 line 1). These results indicate that F₂FLAsH interacts directly with G-protein α-subunits and nucleotide-dependent changes in anisotropy are not the results of α-subunit binding to F₂FLAsH-labeled βγM subunits in a way that alters the latter's fluorescent properties.

In the case of βγM complexes with α_{s_{short}}, α_{s_{long}} and α_{olf}, addition of GTPγS depressed the fluorescence anisotropy signal close to F₂FLAsH-labeled βγM-levels (Figure 3A), which we interpret (based of fluorescence lifetime measurements) as possible quenching of the fluorophore by the guanine nucleotide in close-proximity to the binding site of F₂FLAsH, or possibly also by some conformational rearrangements of the G-protein heterotrimers (or just α-subunits) that made the F₂FLAsH-binding sites on α-subunits less favorable for F₂FLAsH interactions upon nucleotide binding. These conformational rearrangements may have even led to complete loss of binding for some α-subunits, although the appearance of a sub 0.1 ns fluorescence lifetime component (Table 1) in the presence of GTPγS would not be explained by this. Additionally: as G-protein α-subunits may aggregate under certain conditions [16], it is possible that nucleotide-dependent changes may have been caused by dissociation of α-subunit oligomers (GTPγS is thought to disaggregate α-subunits). These oligomers could have presented multiple cysteine residues to F₂FLAsH in close proximity (in the absence of added nucleotides), so F₂FLAsH could have cross-linked these α-subunits (leading to large increases in fluorescence anisotropy).

Large nucleotide-sensitive changes in fluorescence anisotropy for the aforementioned α-subunits were also observed in the absence of βγM-subunits, where addition of nucleotides depressed the signal close to free F₂FLAsH levels (Figure 3C), indicating that if there are any energy transfer processes between the F₂FLAsH-labeled α- and βγM-subunits, they do not contribute significantly to the observed signals.

In contrast to α_{s_{short}}, α_{s_{long}} and α_{olf} the fluorescence anisotropy remained higher for α_q and α₁₃ in the presence of GTPγS (Figure 3A, 3C), when compared to F₂FLAsH-βγM or F₂FLAsH. This was the case in experiments done both in the presence or absence of βγM. This seems to indicate that F₂FLAsH was still able to associate with α_q and α₁₃ in their nucleotide-bound states - but as this interaction with F₂FLAsH had limited nucleotide sensitivity the F₂FLAsH-binding site on these subunits is probably not close enough or oriented properly for quenching (or other mechanisms that lower fluorescence anisotropy such as disaggregation or conformational rearrangements) by guanine nucleotides. Additionally: low nucleotide sensitivity could also be caused by slower nucleotide binding to α_q and α₁₃ (Figure 1 lines 2 and 5).

GTPγS also caused a time-dependent decrease of fluorescence anisotropy with F₂FLAsH-α_i-subunit complexes (Figure 2 solid lines). Conversely to the other 5 α-subunits studied, when GDP was added to these nucleotide depleted F₂FLAsH-α_iβγM complexes, an increase in fluorescence anisotropy was seen (Figure 2, dotted lines). For the other 5 α-subunit subtypes studied GDP decreased the

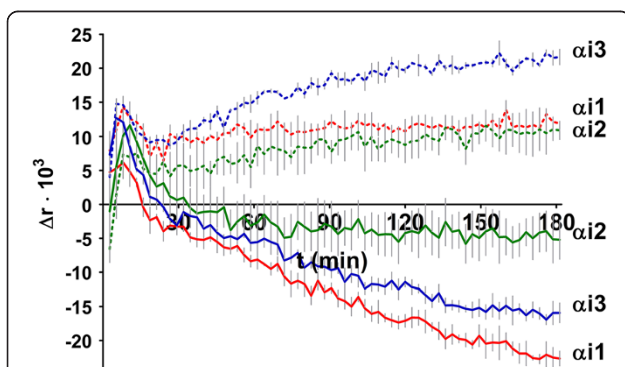


Figure 2 The effect of 10 μM GTPγS (solid lines) and 10 μM GDP (dotted lines) on the formation of complexes between F₂FLAsH and G_i-protein heterotrimers. Data are presented as the time-dependent change in fluorescence anisotropy in comparison to nucleotide-untreated F₂FLAsH-G_i-protein complexes. Data is from two or three independent experiments carried out in duplicate and presented as mean ± SD as error bars.

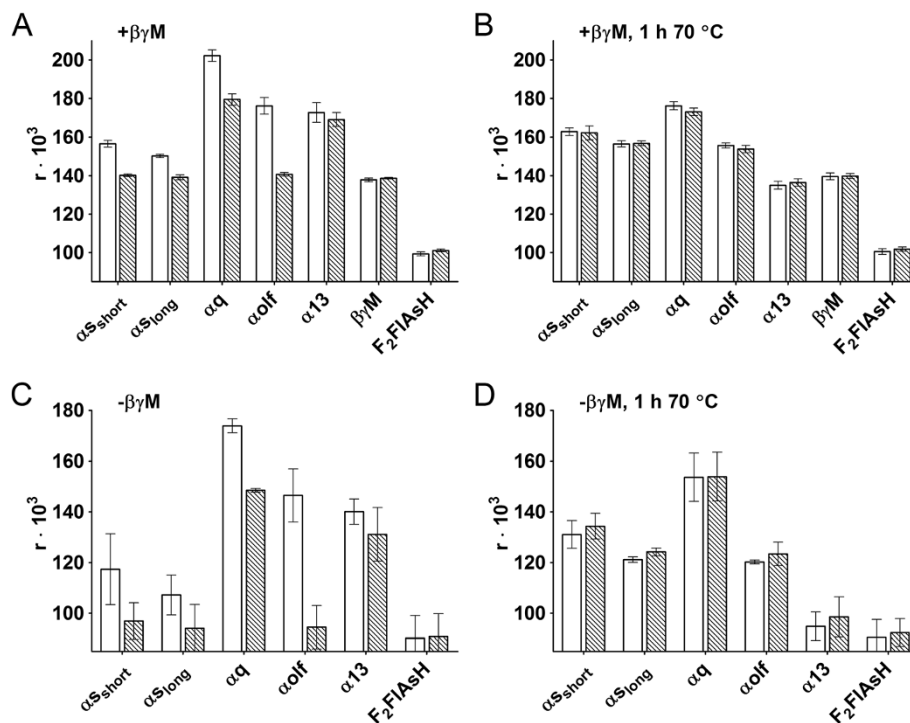


Figure 3 Effect of GTP γ S on the fluorescence anisotropy of $F_2\text{FlAsH}$ -G-protein preparations. The fluorescence anisotropy of α -subunits with (A, B) or without (C, D) $\beta\gamma M$ subunits, in the absence (solid bars) or presence of 10 μM GTP γ S (striped bars), was measured after 6 h of incubation at 28°C (A and C) and again after subsequent heat treatment for 1 h at 70°C (B and D). Data is from two independent experiments carried out in duplicate, presented as mean \pm SD as error bars.

fluorescence anisotropy of their $F_2\text{FlAsH}$ complexes, similarly to GTP γ S, although to a lesser extent (Figure 4A-E).

There were also some differences between the three α -subtypes themselves: $F_2\text{FlAsH}$ complexes with α_2 had smaller nucleotide-sensitive signal amplitude than with α_1 or α_3 . This could indicate that there are specific differences between the interaction of $F_2\text{FlAsH}$ and the α_2 subunit, when compared to α_1 and α_3 . Alternatively this effect could be caused by differences in protein composition, which was evaluated by using Ag-stained SDS-PAGE gels (as described in [11]). This could have resulted in under- or overestimation of G-protein α -subunit concentrations in the purified protein preparations of different α -subtypes, even though the samples were resolved and stained in parallel and had very similar compositions. Due to this uncertainty, no direct comparison between $F_2\text{FlAsH}$ - $\beta\gamma M$ and GTP γ S-treated and untreated $F_2\text{FlAsH}$ - $\alpha\beta\gamma M$ complexes was undertaken, as had been for the other 5 α -subunit preparations (Figure 1A, Figure 3A).

The fluorescence anisotropy of $\alpha_{S\text{short}}$, $\alpha_{S\text{long}}$, α_Q and α_{olf} , α_1 , α_2 and α_3 complexes with $F_2\text{FlAsH}$ all yielded robust changes caused by the addition of guanine nucleotides (Figure 1, Figure 2). In comparison the nucleotide-dependent fluorescence anisotropy signal of $F_2\text{FlAsH}$ - α_{13} complexes (in the presence or absence of $\beta\gamma M$) was very

small and it appeared very slowly (Figure 1 line 5). So even after 14 h of incubation at 28°C only a small effect of nucleotides on the fluorescence anisotropy could be detected, but longer incubation times could not be applied due to evaporation of the solvent. Similarly slow emergence of nucleotide-dependent effects was observed for $F_2\text{FlAsH}$ - α_Q complexes, but this signal had higher amplitude (Figure 1, line 2). This is consistent with previously published results as both α_{13} - and α_Q -subunits have been found to have slow GDP release rates and to require high concentrations of GTP γ S for activation [17]. The nucleotide-sensitive signal of the α_{olf} - $F_2\text{FlAsH}$ complex, which had the highest amplitude, stabilized within 6 h - while the nucleotide-sensitive changes in $F_2\text{FlAsH}$ - $\alpha_{S\text{short}}$ or $F_2\text{FlAsH}$ - $\alpha_{S\text{long}}$ complexes reached their maximum values in approximately 3 h.

Nucleotide-sensitive changes in $F_2\text{FlAsH}$ fluorescence anisotropy appeared rapidly for α -subunit preparations as well (Figure 2), although they were assayed at higher concentrations (~40 nM $\alpha\beta\gamma M$ heterotrimer) and in an approximately 8-fold stoichiometric excess when compared to $F_2\text{FlAsH}$ (5 nM in the α -subunit assays). This seemed to indicate that nucleotide-dependent $F_2\text{FlAsH}$ binding to G-protein α -subunits could not be easily displaced by an apparent excess of $\beta\gamma M$ -subunits, even

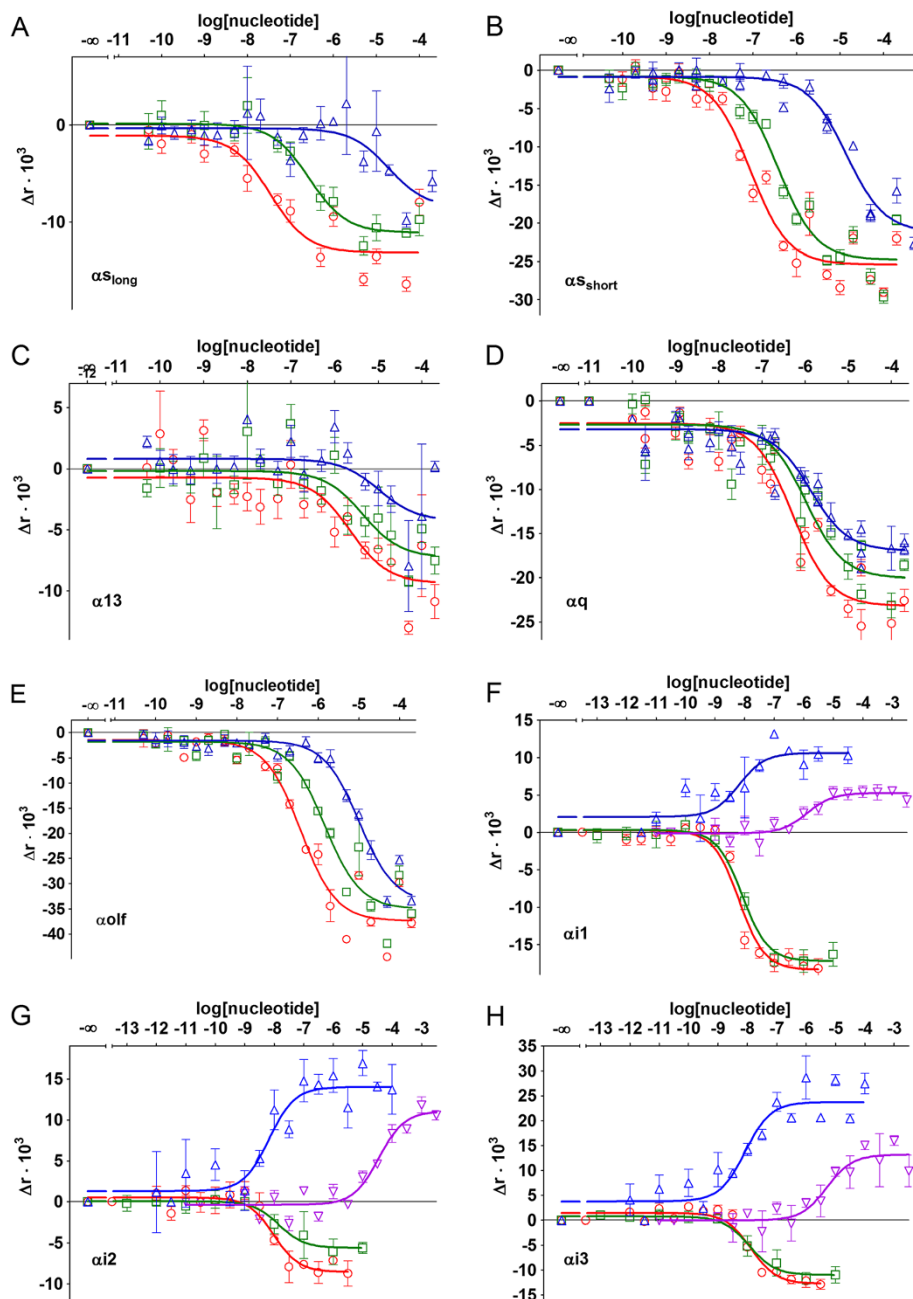


Figure 4 Concentration-dependent effects of nucleotides on the fluorescence anisotropy of $F_2FIAsh-\alpha\beta\gamma M$ complexes. The data was collected after incubation of the complexes with different concentrations of GTP γ S (red circles), GppNHp (green squares), GDP (blue pyramids) or GMP (magenta triangles). Measurements were taken at different timepoints for the various α -subunits: **A** - αS_{long} at 6 h; **B** - αS_{short} at 6 h; **C** - $\alpha 13$ at 14 h; **D** - αq at 6 h; **E** - αolf at 6 h; **F** - $\alpha i1$ at 2 h; **G** - $\alpha i2$ at 2 h; and **H** - $\alpha i3$ at 2 h. Data is from two or three independent experiments in duplicate, presented as mean \pm SD as error bars.

though the tetracysteine-tag on $\beta\gamma M$ has a very high affinity for FIAsh-analogues and would be expected to bind nearly all of the F_2FIAsh present. This should make the fluorophore unavailable for presumable less favorable interactions with α -subunits, as the latter do not contain engineered tetracysteine motifs.

The sensitivity of F_2FIAsh -G-protein complexes to their molecular environment: indications of As-cysteine interactions

F_2FIAsh could interact with G-protein α -subunits through either nonspecific hydrophobic interactions or through more specific binding modes such as arsenic-thiol

interactions. Our results support the latter and indicate that G-protein α -subunits compete with high affinity for F₂FLAsH binding with other cysteine-rich proteins or dithiol motifs present in the reaction medium. For example, the presence of 2 mM β -mercaptoethanol in the assay buffer (in comparison to experiments done in the presence of 5 μ M β -mercaptoethanol) had only a small effect on nucleotide-sensitive changes in fluorescence anisotropy. This indicates that F₂FLAsH interactions with G-protein α -subunits are not easily displaced by monothiols. We also tested whether the interaction of F₂FLAsH with G-protein α -subunits could be blocked with dithiols. In this case no increase (compared to free F₂FLAsH) in fluorescence anisotropy or any GTP γ S-sensitivity changes could be detected when F₂FLAsH was added to G-proteins in the presence of 200 μ M ethanedithiol, which indicates that the F₂FLAsH- α -subunit interactions were completely blocked by this reagent. This result seems to indicate that F₂FLAsH binds multiple cysteine residues on G-protein α -subunits. As no such residues are present in the highly conserved guanine nucleotide binding site of G-proteins [18], it appears that F₂FLAsH acts as an allosteric sensor of nucleotide binding. We also tested whether the F₂FLAsH- α -subunit interaction could be blocked by the addition of arsenous acid (100 μ M hydrolyzed AsCl₃) and found that in this case the nucleotide-dependent fluorescence anisotropy signal was attenuated, but not completely lost. This indicates that biarsenical ligands have a higher affinity for cysteine residues on G-protein α -subunits than arsenic itself. Unfortunately we were not able to directly determine the affinity of F₂FLAsH for G-protein α -subunits as a non-fluorescent biarsenical ligand was not available to us for measuring nonspecific F₂FLAsH binding.

We also tested whether nonfluorinated FLAsH (Lumio Green) would associate in a nucleotide-sensitive manner with G-protein α -subunits (α_{short} , α_{long} , α_q , α_{olf} and α_{13}). The results were comparable to experiments done using F₂FLAsH, indicating that the formation of nucleotide-sensitive complexes with G-protein α -subunits is not a unique property of F₂FLAsH but a more general interaction with biarsenical ligands. We also tested whether fluorescein could associate with G-proteins in a nucleotide-dependent manner (presumably through hydrophobic interactions): no changes in fluorescence anisotropy caused by the addition of G-proteins could be detected; neither could we detect any nucleotide effects (data not shown). This further supports the hypothesis that FLAsH analogues bind to cysteine residues on α -subunits via high affinity arsenic-thiol interactions.

Of course it is probable that nucleotide-sensitive FLAsH and F₂FLAsH binding is not an effect that is unique to these eight G-protein α -subunits tested. Instead similar effects might be found for other proteins that have multiple cysteine residues near structural motifs that undergo

conformational rearrangements that alter their interaction with biarsenical ligands or bring the fluorophores in close proximity with quenching moieties. If this is so then FLAsH-analogues could be applied as an allosteric probe to study the functioning of such proteins *in vitro*.

Heterotrimeric G-proteins are known to be thermolabile [19,20] so we could test whether disruption of their active conformational state would have an effect on their nucleotide-dependent interaction with F₂FLAsH. When F₂FLAsH-G-protein complexes were heated at 70°C for 1 h after 6 h of measurements at 28°C, differences between the fluorescence anisotropy signals of nucleotide-treated and untreated F₂FLAsH-G-protein complexes were lost (Figure 3B, 3D). However, the fluorescence anisotropy of most F₂FLAsH-G-protein complexes, with the exception of α_{13} , was not decreased down to the levels of free F₂FLAsH (Figure 3D) or F₂FLAsH- $\beta\gamma$ M (Figure 3B) by the heat-treatment, indicating incomplete dissociation of the F₂FLAsH- α complexes. This suggests that some interactions of the thermally denatured α -subunits with F₂FLAsH remained for most of them even after nucleotide binding capability was lost, which could be explained by F₂FLAsH binding to α -subunit cysteine residues, or possibly by some nonspecific interactions of F₂FLAsH with the denatured proteins. For some α -subunits (α_{short} , α_{long}) the absolute fluorescence anisotropy values even increased after heat treatment, which could indicate increased exposure (or more favorable binding geometry) of cysteine residues that were able to associate with F₂FLAsH on these α -subunits after denaturation. Nucleotide-dependent changes in fluorescence anisotropy could also be abolished for α -heterotrimers by heat-treatment, but no direct comparison to F₂FLAsH- $\beta\gamma$ M was undertaken as the composition of these samples was not determined quantitatively and thus we could not be certain that identical amounts of the relevant G-protein subunits were compared.

The influence of other, non-specific proteins on the nucleotide sensitivity of F₂FLAsH- $\alpha\beta\gamma$ M complexes (tested with $\alpha_{13}\beta\gamma$ M) was measured in the presence of BSA or pyruvate kinase. BSA caused a 50% increase in F₂FLAsH-fluorescence intensity and (in both the absence and presence of $\alpha_{13}\beta\gamma$ M), but no such increase was detected when pyruvate kinase was used as a non-specific protein even at maximal concentrations (100 μ g/ml) tested. Both absolute levels of fluorescence anisotropy and nucleotide-dependent effects were unaltered by pyruvate kinase. BSA increased absolute anisotropy by about 30% and this was accompanied by complete disappearance of nucleotide-dependent effects with an EC₅₀ of about 0.5 μ g/ml, which for this assay was in the same range as the concentration of the G-protein α -subunit itself.

There are multiple reports in the literature that BSA binds fluorescein analogues with high affinity [21], while for pyruvate kinase no such reports were found. This

attenuation of nucleotide-sensitivity by BSA indicates that the F₂FLAsH fluorophore is either relatively exposed to the solvent in the F₂FLAsH- α i3 β γ M complex and non-specific interactions with BSA (which contains multiple cysteine residues) can completely block the sensitivity of F₂FLAsH fluorescence anisotropy to G-protein α -subunits in their various nucleotide bound states. Alternatively the interaction between F₂FLAsH and BSA is of a comparable or higher affinity than the interaction between F₂FLAsH and G-protein α -subunits, thus limiting the availability of F₂FLAsH for forming a fluorescent complex with the α -subunits.

Characterization of the nucleotide sensitivity of F₂FLAsH-G-protein complexes

As guanine nucleotides had robust effects on the fluorescence anisotropy of F₂FLAsH- α β γ M complexes we utilized this system for the characterization of nucleotide binding to G-proteins. We note that due to slow nucleotide-exchange kinetics and low nucleotide-affinities of some α -subunits, traditional orthosteric ligands (labeled nucleotides) would have been limited in their applicability, whereas F₂FLAsH as an allosteric probe could be used to monitor their nucleotide-bound states without being as limited by a nucleotide exchange requirement.

Eight different α -subunits in combination with F₂FLAsH and β γ M were studied to reveal the effects of GDP, GMP, GTP γ S and GppNHp on these complexes. Depending on the α -subunit, large differences in signal amplitudes, nucleotide affinities and even in the type of the effect (increase or decrease of fluorescence anisotropy) upon addition of guanine nucleotides, was seen (Figure 4).

The four nucleotides tested had a similar order in their apparent binding affinities for all of the G-proteins studied, with GTP γ S having the highest and GMP the lowest affinity (Table 2). GDP was equipotent with GTP γ S and GppNHp for α i-complexes, but less potent for all other G-protein preparations tested. Similar trends were seen for the amplitude of the nucleotide-dependent change in fluorescence anisotropy, with GTP γ S causing the biggest change, while for GDP and GMP the changes were smaller for most α -subunits (with the exception of α i2 and α i3). This could indicate that GTP γ S binding to α -subunits can cause a conformational rearrangement that brings the quenching guanine moiety closer to the F₂FLAsH binding site than either GDP or GMP - or alternatively: GTP γ S leads to disaggregation of α -subunit oligomers while GDP and GMP cause smaller conformational rearrangements.

Gi-proteins differed from the other five G-protein subtypes tested in several ways and there were also some specific differences between the α i-subunits themselves: these three subunits (purified in nucleotide-depleting conditions) exhibited striking differences between the direction of the effects of activating guanine nucleotides

Table 2 Apparent affinities of nucleotides in F₂FLAsH- α β γ M complexes

	pEC ₅₀ ^a			
	GTP γ S	GppNHp	GDP	GMP
α S _{long}	7.47 ± 0.09	6.56 ± 0.07	4.72 ± 0.18	ND
α S _{short}	7.06 ± 0.04	6.42 ± 0.04	4.85 ± 0.05	< 3
α i3	5.65 ± 0.13	5.36 ± 0.14	4.98 ± 0.23	ND
α q	6.27 ± 0.05	5.96 ± 0.07	5.87 ± 0.06	< 3
α olf	6.43 ± 0.04	5.84 ± 0.05	5.00 ± 0.03	< 3
α i1	8.19 ± 0.05	8.08 ± 0.04	8.19 ± 0.16	5.89 ± 0.12
α i2	8.02 ± 0.09	7.84 ± 0.14	8.17 ± 0.13	4.45 ± 0.07
α i3	7.87 ± 0.06	7.93 ± 0.07	8.08 ± 0.10	5.29 ± 0.13

^anegative logarithm of concentration of the nucleotide causing 50% of fluorescence anisotropy changes of F₂FLAsH- α β γ M complexes (mean ± SD), determined after 6 h incubation for α S_{long}, α S_{short}, α q and α olf, after 14 h for α i3, after 2 h for α i1, α i2 and α i3.

Data is from two to three independent experiments carried out in duplicate. Data were pooled and then fitted to three parameter competitive binding equations.

(nonhydrolyzable GTP-analogues) and GDP or GMP (thought to preferentially bind to and stabilize inactive G-protein conformations). In comparison to experiments done without added nucleotides, the addition of GDP and GMP caused an increase in fluorescence anisotropy, whereas the addition of GTP γ S and GppNHp resulted in a decrease (Figure 4F-4H). This could indicate that the nucleotide-depleted α i-subunit preparations partially denatured at 28°C and could no longer interact with F₂FLAsH (when no additional GDP or GMP was present), whereas the addition of GDP or GMP could stabilize the nucleotide-free pool of α -subunits and preserve their F₂FLAsH binding ability, thus increasing the signal. Rapid degradation of nucleotide-depleted α i-subunits at 28°C is also consistent with our previous measurements, where these nucleotide-depleted protein preparations lost their ability to bind Bodipy-FL-GTP γ S quickly ($\tau_{1/2}$ = 20 ± 5 min) at 28°C [11], but addition of saturating amounts of nucleotides could stabilize the complex. Alternatively the stabilizing effects of GDP and GMP could be connected with the stability of the G-protein heterotrimer: GDP-bound α i-subunits would be expected to be associated with β γ M-subunits, which would reduce their rotational mobility, while GTP γ S treatment would be expected to dissociate the heterotrimer and lead to a decrease in fluorescence anisotropy. Disaggregation of α i-subunits by GTP γ S is also a possible mechanism behind the different effects of activating and nonactivating guanine nucleotides as α i subunits are thought to be especially likely to aggregate [16]. Additionally, as we had determined affinity of these three Gi protein preparations for GDP and GTP γ S previously using a Bodipy-FL-GTP γ S-based nucleotide displacement assay [11] in a similar environment (except no TCEP was added to the buffer in the Bodipy-FL-GTP γ S assay), we

could compare the results from the two methods: the affinity of $\alpha\beta\gamma$ M complexes for GDP and GTP γ S determined using Bodipy-FL-GTP γ S was in close agreement with the values obtained by using the F₂FLAsH-based assay. The nucleotide-depleted $\alpha\beta\gamma$ M preparations also had the highest nucleotide affinities of all the G-protein preparations tested (Table 2).

α i-subunits also exhibited specific differences in the relative amplitudes of nucleotide effects: F₂FLAsH complexes of α i2 and α i3 exhibited a greater change in fluorescence anisotropy upon addition of GDP, instead of GTP γ S (Figure 4G, 4H). This could reflect the large stabilizing effect of GDP on the nucleotide-free α -subunit pool in those protein preparations as the relatively smaller effect GDP and GMP had on the α i1-subunit could be caused by its resistance to nucleotide depletion during protein purification, so the α i1-protein preparation had a greater GDP content. This would explain why there seemed to be less degradation of the nucleotide depleted F₂FLAsH- α i1-protein complex in the absence of added nucleotides.

In contrast to α i-subunits, all of the other α -subunits tested exhibited similar effects in complexes with F₂FLAsH for GTP γ S, GppNHp, GDP and GMP: a decrease in fluorescence anisotropy (Figure 4F-4H), indicating increased quenching of the F₂FLAsH-fluorophore or possibly a change in α -subunit conformation/aggregation that makes them less accessible to F₂FLAsH upon nucleotide binding. For α i-subunits the reverse was true and GDP and GMP seemed to stabilize the F₂FLAsH-G-protein interaction.

There may be several reasons why the effects GDP and GMP had on α i-subunits differed from other G-protein subtypes. One reason may be that α s_{short}, α s_{long}, α q, α olf and α 13-proteins were not purified in nucleotide-depleting conditions, so they could have been saturated with GDP. These 5 α -subunits had all been purified using GST-Ric8 association [17] instead of StrepII-labeled γ 2-subunits, as was the case for α i-heterotrimers. So these two purification approaches may have yielded protein preparations that had significantly different compositions in terms of nucleotide and cofactor content and also in G-protein subunit stoichiometry: tandem affinity chromatography would be expected to yield α i-subunits at up to equimolar concentrations with $\beta\gamma$ M as each immobilized $\beta\gamma$ M-subunit can bind up to one α -subunit. Whereas when the GST-Ric8-purified α -subunits (12.5 nM, determined by the manufacturer) were reconstituted with purified $\beta\gamma$ M subunits, the possibility existed that they could have been present in a stoichiometric excess when compared to $\beta\gamma$ M, which we estimate should have been present at approximately 15 nM (based on UV-absorbance and analysis of Ag-stained SDS-PAGE gels). Control experiments with 15 nM F₂FLAsH, 12.5 nM α q and 30 nM $\beta\gamma$ M did not, however, yield significantly different nucleotide effects on F₂FLAsH- α q $\beta\gamma$ M fluorescence anisotropy.

α i $\beta\gamma$ M preparations were also assayed at higher protein and lower F₂FLAsH concentrations than α s_{long}, α s_{short}, α q, α olf and α 13. The influence of the stoichiometry of F₂FLAsH-labeling (ratio of F₂FLAsH to G-protein heterotrimers) on nucleotide-dependent changes in fluorescence anisotropy was investigated using α q $\beta\gamma$ M preparations (2 to 20 nM F₂FLAsH and 12.5 nM α q and 15 nM $\beta\gamma$ M) and found to mainly affect the signal to noise ratio of the assay, while having little effect on the amplitude of the nucleotide-dependent change in fluorescence anisotropy. This suggests that the nucleotide binding experiments done with α s_{short}, α s_{long}, α q, α olf and α 13 could be similarly representative of their nucleotide binding affinities (in comparison to experiments with labeled nucleotides) as those done using α i $\beta\gamma$ M preparations, even though the subunit stoichiometries and F₂FLAsH to G-protein ratios were not identical for all of the F₂FLAsH-G-protein complexes studied. Indeed, our estimation of the apparent affinity of GTP γ S for α s-subunits is close to previously published values determined using assays based on the displacement of fluorescent nucleotides [22] and high concentrations (10–30 μ M) of GTP γ S have previously been found necessary to activate α q and α 13 [17], which is reflected by our results: these two proteins had the lowest apparent affinity for GTP γ S out of all the G-protein subtypes studied.

When comparing the effects of nucleotides on F₂FLAsH-G-protein complexes, it was apparent that F₂FLAsH- α i $\beta\gamma$ M protein complexes had the highest fluorescence anisotropy in the presence of GDP or GMP, while the same was true for F₂FLAsH- α s_{short}, α s_{long}, α q, α olf and α 13 complexes in the absence of any added nucleotides. It has been suggested that α i-subunits in their GDP-bound state could have multiple binding sites for $\beta\gamma$ -subunits [23] (and are likely to aggregate themselves [16]), which could also (in addition to inhibition of denaturation of nucleotide-free α i-subunits) explain the increase in fluorescence anisotropy upon GDP and GMP binding to α i-subunits: $\beta\gamma$ M subunits were probably present at a slight excess in the α i $\beta\gamma$ M protein preparations, so they could form complexes with at least some α i $\beta\gamma$ M-heterotrimers. The added mass of a second $\beta\gamma$ M binding to the G-protein heterotrimer would be expected to further decrease the rotational mobility of the F₂FLAsH-G-protein complex and result in a slightly higher anisotropy. Thus absence of any GDP or GMP-induced increase in the fluorescence anisotropy of α s_{short}, α s_{long}, α q, α olf and α 13 complexes with F₂FLAsH and $\beta\gamma$ M could be explained by the lack of a second $\beta\gamma$ M-subunit binding site on these α -subunits. Alternatively the effects of GDP or GMP on fluorescence anisotropy of α i $\beta\gamma$ M complexes could be explained by interactions with some regulatory proteins that were copurified alongside the heterotrimers using $\beta\gamma$ M-tandem affinity chromatography, but not with GST-Ric8-purified α -subunits.

Conclusions

F₂FLAsH bound to G-protein α -subunits allosterically via As-thiol interactions. The presence or absence of guanine nucleotides had an effect on the fluorescence anisotropy, intensity and lifetime of F₂FLAsH-G-protein complexes. F₂FLAsH was sensitive to nucleotide binding to α 1, α 2, α 3, α _{short}, α _{long}, α q and α olf, but showed low sensitivity to nucleotide binding to α 13. Nucleotide affinities (as determined by changes in the fluorescence anisotropy of F₂FLAsH-G-protein complexes) for GTP γ S, GppNHp, GDP and GMP were comparable to values obtained in assays based on the displacement of fluorescently labeled nucleotides [11]. GDP and GMP seemed to stabilize nucleotide-depleted F₂FLAsH- α -subunit complexes, whereas for all other α -subunits tested the fluorescence anisotropy decreased in the presence of these nucleotides. GTP γ S and GppNHp decreased fluorescence anisotropy for F₂FLAsH- α -subunit complexes for all G-protein subtypes studied.

Additional files

Additional file 1: Figure S1. Fluorescence emission and excitation spectra of F₂FLAsH (solid line), F₂FLAsH+ α olf (dotted line) or F₂FLAsH+ α olf+GTP γ S (dashed line). Emission was recorded at 550 nm and excitation at 480 nm. Data are presented as mean \pm SD.

Additional file 2: Figure S2. Fluorescence lifetime decay curves in the frequency domain of fluorescein (A), F₂FLAsH (B), F₂FLAsH+GTP γ S (C), F₂FLAsH+CCPGCC-motif containing peptide (D), F₂FLAsH+ α 13 (E), F₂FLAsH+ α 13+GTP γ S (F), F₂FLAsH+ β γ M (G), F₂FLAsH+ α olf (H) and F₂FLAsH+ α olf+GTP γ S (I). Fluorescence lifetimes and corresponding fractions were calculated by global fitting of phase shifts (blue) and demodulations (red) that were beforehand computed from measured data as a function of 11 frequencies. Curves are presented as 1-component (dotted line), 2-component (solid line) or 3-component (dashed line, in case I) fits.

Abbreviations

G-protein: Guanine nucleotide binding protein; GPCR: G-protein coupled receptor; GDP: Guanosine diphosphate; GMP: Guanosine monophosphate; GTP γ S: Guanosine 5'-O-[gamma-thio]triphosphate; GppNHp: Guanosine 5'-[β , γ -imido]triphosphate; C12E10: Polyoxyethylene (10) lauryl ether; TCEP: tris (2-carboxyethyl)phosphine; F₂FLAsH: 4',5'-bis(1,2,3-dithioarsolan-2-yl)-2',7'-difluorofluorescein; FLAsH: 4',5'-bis(1,2,3-dithioarsolan-2-yl)fluorescein; β γ M: Dimer of β 1 and Strepl and CCKACC labeled γ 2-subunits.

Competing interests

The authors declare that there are no competing interests.

Authors' contributions

LT expressed and purified Gi proteins, carried out spectrophotometric and fluorescence anisotropy measurements and analyzed and interpreted most of the data and drafted the manuscript. SK carried out fluorescence lifetime measurements, interpreted the data and contributed to overall experimental design, conception and interpretation of the results. AR contributed to overall experimental design, conception and interpretation of results and gave final approval for publication of the results. All authors read and approved the final manuscript.

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