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Refolding of G protein α subunits from inclusion bodies expressed in *Escherichia coli*

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Abstract

Heterotrimeric G proteins relay signals from G protein-coupled receptors (GPCRs) to the interior of the cell. The signaling cascades induced by G protein activation control a wide range of cellular processes. The α subunit is believed to determine which G protein couples to each GPCR, and is the primary determinant of the type of signal transmitted. Several members of the G_α family have been expressed in active form in *E. coli*. However, production levels of these proteins are limited: in most cases only ~10 % of total G_α protein expressed is active; the rest accumulates in inclusion bodies. Although $G_{i\alpha}$ has been readily expressed in soluble form (to 10 mg/L), other α subunits are minimally soluble, and many are exclusively expressed to inclusion bodies. Previous efforts to solubilize and refold G_α from inclusion bodies have not been successful. Here we did a thorough study of the characteristics of G_α subunits (human $G_{i\alpha(1)}$, human $G_{s\alpha}$ (short), human $G_{11\alpha}$ and human $G_{t\alpha}$ (cone)), solubilized and purified from inclusion bodies. We find that we can obtain soluble protein both by on-column and rapid-dilution techniques. Comparison to native, soluble $G_{i\alpha}$ expressed from *E. coli* showed that although the refolded G_α subunits were soluble and retained partial α -helicity characteristic of the native, folded G_α subunit, they did not bind GDP or GTP as effectively as native protein. We conclude that the refolded $G_{i\alpha}$ protein has a native-like secondary structure, but is predominately in a molten globular state.

Guanine nucleotide-binding proteins (G proteins) are an essential component in G-protein coupled receptor (GPCR) mediated signaling. They are stimulated by a ligand-induced conformational change in the receptor, which catalyzes the exchange of GDP, bound to the 'resting' state of the G_α subunit, with GTP. It is thought that the GTP-bound G protein dissociates into two signaling entities: the G_α subunit bound to GTP and the $G_{\beta\gamma}$ subunit, both of which activate downstream effectors to initiate a physiological response. That response is then terminated when the G_α subunit hydrolyzes GTP to GDP and re-associates with the $G_{\beta\gamma}$ subunit, and the process can begin anew [reviewed by ¹].

The α subunit is the primary determinant for which receptor the G protein couples and the defining feature of the G protein. Therefore, G proteins are categorized by the G_α subunit. Together they make up four subfamilies: the G_s subfamily stimulates adenylyl cyclase [²]; the G_i subfamily inhibits adenylyl cyclase and regulates ion channels [³, ⁴]; the $G_q/11$ subfamily activates phospholipase C beta and results in an influx of Ca^{2+} [⁵]; and the $G_{12/13}$ subfamily regulates small GTP binding proteins, such as rho, which modulates the cytoskeleton [⁶].

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Approximately twenty different subtypes of the α subunit exist [7]. Five subtypes of the β subunit [8] and 11 subtypes of the γ subunit [9, 10] have been identified as well. Combinations of the different α , β and γ subtypes provide a great diversity of signaling pathways that can be regulated by GPCRs, other activators of G protein signaling (AGS) or regulators of G protein signaling (RGS). It is interesting to note that very little is known about how GPCRs selectively couple to a single subtype of the α subunit. This phenomenon is especially striking considering there are a thousand GPCRs of diverse primary structure [11], yet there are only four subfamilies of G proteins. High-resolution structural insight into the receptor- G protein complex is difficult to obtain due to the dynamic nature of the interaction. Currently, static images of the various states of the G_{α} subunit [12–16], the inactive structure of rhodopsin [17] and recently the structure of $G_{i\alpha}$ in the presence of ICL3 receptor mimic and $\beta\gamma$ -interacting peptides [18] have been described. These images, along with biophysical studies [19–21], have supplied knowledge about the receptor- G protein interaction, but a complete depiction of the ternary complex is still elusive [22]. The G protein interacts with many non-GPCR related proteins, or activators of G protein signaling (AGS), that share no sequence homology and activate G proteins by different signaling mechanisms [23]. In order to understand the differential activation and the fluctuation of G protein states, a comprehensive collection of G protein structures needs to be obtained [1]. Heterologous expression systems have been successful at generating the copious amount of isotope-labeled soluble protein needed for high-resolution NMR studies in other protein systems. As a step towards these structural studies of G protein states, we need to utilize and improve the use of *Escherichia coli* (*E. coli*), which can provide a scalable production source of recombinant proteins. Advantages include speed, inducibility for control of optimal expression, isotope labeling for high-resolution NMR studies, cost and ease of purification.

Of the twenty different subtypes of the G_{α} subunit identified, five have been successfully expressed in *E. coli*: wild-type and mutant forms of $G_{s\alpha}$, $G_{i1\alpha}$, $G_{i2\alpha}$, $G_{i3\alpha}$ and $G_{o\alpha}$, all of which only differ from their mammalian sources by post-translational covalent modification. However, the expression of these proteins is not without its challenges. The current protocol requires long growth times (24–36 h) at moderate temperature (30 °C) and often multi-step purification to obtain active protein [24]. Expression levels of active protein vary depending on the α subunit expressed; $G_{s\alpha}$ expression typically yields 0.1–1 mg/L, while $G_{i\alpha}$ expression yields 40 mg/L [24, 25]. Many of the G_{α} subunits ($G_{q\alpha}$ family, $G_{12\alpha}$ family and $G_{t\alpha}$ (transducin) from the $G_{i\alpha}$ family) have not been successfully obtained from bacterial systems. Manipulation at the DNA level to produce chimeras of different α subunits has been successful at producing proteins that resemble these difficult-to-express proteins: a transducin/ $G_{i\alpha}$ chimera was made to achieve soluble active transducin-like protein, expressed in *E. coli*, at levels of 3–6 mg/L for biophysical studies [26, 27]. Chimeras of $G_{12\alpha}$ and $G_{13\alpha}$, also with $G_{i\alpha}$, were used to produce these hard-to-express G_{α} subunits in baculovirus systems [28]. Interestingly these chimeras only differ from their parent protein by 11 amino acids. The 11 amino acid exchange allowed for difficult-to-express G_{α} production in the recombinant systems while maintaining the parent G_{α} selectivity. Table 1 summarizes the literature available on the current expression systems used to acquire the representative members of the G_{α} subunits for structural characterization.

While Sf9 cells have been successful at producing some of the more challenging G_{α} subunits they have several drawbacks. Recombinant protein production from insect cells is time-consuming and laborious and often gives rise to heterogeneous populations of proteins, due to variable posttranslational modifications. Sf9 cells also natively express certain G alpha subunits – $G_{s\alpha}$, $G_{o\alpha}$ and $G_{q\alpha}$ – which account for 10–40% of total active G_{α} subunit. These contaminating endogenous G_{α} subunit require additional purification procedures, with final yields in the μ g/L range [29].

Methods for expressing the protein in inclusion bodies and then efficiently refolding the G_α subunits would be of great value for characterization of the structure and properties of the G_α subunit. In addition, G_α is quite prone to loss of activity upon storage at 4 °C, regardless of the original source of expression, requiring storage in small samples at -80 °C in order to prevent denaturation. A refolding method would allow for *E. coli* production of G_α subunits that can be stored in denaturant at room temperature or as inclusion bodies and quickly refolded when needed. Successfully refolding G_α would enable a rescue of the protein lost during expression, a simplified method of production for those G_α that have proven recalcitrant to other production methods, and sufficient quantities of isotope-labeled G_α subunits for high-resolution NMR studies.

Materials and Methods

Materials

Reagents were obtained from the following sources: GTP γ S, GTP, GDP and CHAPS were purchased from Sigma. [35 S]GTP γ S was purchased from PerkinElmer. BODIPY GTP γ S FL was purchased from Invitrogen. Glycerol and imidazole were purchased from Fisher Scientific. The cDNA of the α subunits (human $G_{i\alpha(1)}$, human $G_{s\alpha(\text{short})}$, human $G_{11\alpha}$ and human $G_{t\alpha(\text{cone})}$) was obtained from a non-profit cDNA resource center at University of Missouri-Rolla (cDNA.org) and the vector used for expression (pET15b) was purchased from Novagen, EMD Biosciences. The expression plasmid for the $G_{i\alpha}$ positive control, pProEXHT-H $_6$ - $G_{i\alpha(1)}$ was a generous gift from Dr. Jason Snyder (University of North Carolina, School of Medicine). The $G_{s\alpha(\text{short})}$ positive control, human $G_{s\alpha(\text{short})}$ recombinantly expressed in Sf9 insect cells, was purchased from Jena Bioscience.

G_α constructs

The pET15b vector was altered slightly to include new restriction endonuclease (RE) sites for insertion of the α subunits. Oligonucleotide cassettes, containing our novel RE sites, were synthesized to correspond to novel sites in the multiple cloning region of pET15b. To allow for cloning of the $G_{s\alpha}$ and $G_{11\alpha}$ subunits, a cassette containing an NheI site was cloned into pET15b. To allow for cloning $G_{i\alpha}$ and $G_{t\alpha}$, a cassette containing a KpnI site was cloned into pET15b. The pET15b vector also contains a thrombin-cleavable N-terminal His $_6$ tag. The N-terminal His $_6$ tag is desirable for the α subunit because its receptor-coupling region is known to exist primarily on the C-terminal end of the protein [30]. The calculated molecular weights of the G_α subunit alone as well as the fusion histidine purification tag followed by a thrombin cleavage site are as follows: Human $G_{i\alpha(1)}$ 40,375Da (His $_6$ -thrombin 2,303 Da), human $G_{s\alpha(\text{short})}$ 44,266 Da (His $_6$ -thrombin 3,358 Da), human $G_{11\alpha}$ 42,074 Da (His $_6$ -thrombin 3,174 Da) and human $G_{t\alpha(\text{cone})}$ 40,175 Da (His $_6$ -thrombin 3,174 Da).

Expression and Purification of G_α subunits

E. coli BL21 (DE3) cells harboring the pET15b-H $_6$ - G_α constructs were grown in 1.5 L enriched media (2% tryptone, 1% yeast extract, 0.5% NaCl, 0.2% glycerol, and 50 mM KH $_2$ PO $_4$, pH 7.2) in the presence of 50 μ g/ml ampicillin at 37 °C to an OD $_{600}$ of 0.6 in baffled shaker flasks and then induced with 1 mM IPTG for 4 hours (multiple conditions were tested and under all conditions the G_α subunits formed inclusion bodies). Cell cultures were harvested by centrifugation at 8,000 rpm for 15 min at 4 °C. At the time of harvest, OD $_{600}$ was ~2.0 for the G_α subunits expressing cells. Cell pellets were rapidly frozen in liquid nitrogen and stored at -80 °C. Cell pellets were resuspended to 1/10 culture volume (150 ml) in buffer containing 50 mM Tris-HCl (pH 8.0), 20 mM β -mercaptoethanol (β Me), and 8 M urea. To facilitate lysis, the cell lysate was sonicated on ice for 15 min at 30 % duty cycle with 30 % output using a Branson 450 sonicator. The lysate was incubated with pre-equilibrated Ni-NTA (Qiagen) resin. The column was washed with 20 mM Tris-HCl (pH 8.0), 20 mM β Me, 500 mM NaCl, 8 M

urea, and 40 mM imidazole. The G_{α} subunits were eluted with 20 mM Tris-HCl (pH 8.0), 20 mM β Me, 300 mM NaCl, 8 M urea, 10 % glycerol, with a series of increasing step gradients of imidazole in the range of 60 – 250 mM. The eluted protein fractions were stored at room temperature.

E. coli cells harboring the pProEXHT-H₆-G_{1 α} construct were grown as instructed by Dr. Jason Snyder (University of North Carolina, School of Medicine) [31]. One liter LB culture was grown in the presence of 50 μ g/ml ampicillin at 37 °C until the cell density reached an OD₆₀₀ of 0.8. Then the temperature was reduced to 25 °C and protein expression was induced by addition of 0.2 mM IPTG for 8–10 hours. Cell cultures (OD₆₀₀ ~ 2.0) were harvested by centrifugation at 8,000 rpm for 15 min at 4 °C. Cell pellets were rapidly frozen in liquid nitrogen and stored at –80 °C. Cell pellets were resuspended in 50 ml cold N1 buffer (20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 10 % glycerol, 5 mM MgCl₂). To facilitate lysis, the cell lysate was sonicated on ice for 15 min at 30 % duty cycle with 30 % output using a Branson 450 sonicator. The crude lysate was cleared by centrifugation at 30,000 rpm for 30 min. The supernatant was collected and incubated with pre-equilibrated Ni-NTA (Qiagen) resin. The resin was washed with N1 buffer and G_{1 α} was eluted in N1 buffer with a gradient of imidazole. Superdex-200 gel filtration column (GE Healthcare) pre-equilibrated with 20 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 5 mM MgCl₂ was used as a final purification step to isolate the monomeric G_{1 α} subunit. Amicon ultrafiltration devices (Millipore, 30,000 molecular weight cut-off) were used to concentrate purified protein in HED buffer (50 mM HEPES, pH 8.0, 0.1 mM EDTA, 1 mM DTT) for storage at –20 °C.

Refolding purified denatured G_{1 α} subunits

A rapid dilution technique was employed to efficiently refold the G_{1 α} subunits. The G_{1 α} subunits were diluted 20-fold (<10 μ g/ml) into refolding buffer (20 mM Tris-HCl, pH 8.0, 20 % glycerol, 0.1 mM EDTA, 1 mM DTT, 0.1 % CHAPS, 100 μ M GDP) at room temperature. The G_{1 α} subunits were added dropwise (~1 drop per 20 seconds) into refolding buffer under rapid stirring at room temperature, then incubated 16–72 hr at 4 °C with gentle stirring. Amicon Ultra (Millipore 30,000 molecular weight cut-off) device was used to concentrate the sample. Samples were dialyzed against 1 L refolding buffer at 4 °C, without GDP, to remove excess unbound GDP. G_{1 α} subunits were passed through a Millex syringe driven 0.22 μ m filter unit (Millipore) to eliminate any large aggregates that may have formed in the refolding process.

Alternatively, the G_{1 α} subunits were refolded during purification utilizing an on-resin batch refolding method. The cleared lysate was loaded on Ni-NTA, washed and diluted 20-fold in refolding buffer at 4 °C with gentle stirring in batch for 16–48 hr. The G_{1 α} subunit was eluted from the resin by loading the resin into a column and using a gradient of 60 – 250 mM imidazole in refolding buffer. The purified refolded G_{1 α} subunits were dialyzed and filtered as described above.

Circular Dichroism

CD spectra were recorded on G_{1 α} subunits at 25°C using an Aviv model 202 spectrophotometer. A 0.1 cm path-length quartz cuvette was used for all far-UV measurements. Spectra were collected from 180 nm to 260 nm using a 1 nm step resolution with 3 sec integration time. Sample spectra were subtracted from reference spectra containing buffer at the same composition; spectra reported represents an average of three runs.

GTP γ S Binding Assay

Method used was essentially as described previously [27]. Briefly, the binding reaction was started by adding 5 μ l G_{1 α} subunits (final concentration of 1 μ M) in refolding buffer to 15 μ l of radioactive assay buffer (100 mM HEPES, pH 8.0, 1 mM EDTA, 10 mM MgSO₄, 10 mM

dithiothreitol) with 5 μ M [35S]GTP γ S (5000 cpm/pmol) and carried out at room temperature. Samples were withdrawn at indicated times and filtered through the Millipore Multiscreen-HA 96-well filtration plate and washed 10 times with ice cold filtration buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl and 25 mM MgCl₂). Scintillation fluid was added and radioactivity was measured directly using a Perkin-Elmer 1450 Microbeta scintillation counter with Microbeta workstation software version 3.01.005.

BODIPY GTP γ S Activity Assay

Commercially available BODIPY GTP γ S FL analog (Invitrogen, Molecular Probes) was used to determine G α subunit activity [32, 33]. Fluorescence was monitored for samples of 50 nM BODIPY GTP γ S and 200 nM α subunit (G_{ia}, G_{sa}, G_{11a} and G_{ta}) in HEM buffer (10 mM HEPES, pH 8.0, 0.1 mM EDTA, 1 mM MgCl₂). Fluorescence emission spectra were collected using an ISS PC-1 spectrofluorimeter. Excitation wavelength was 485 nm and emission spectra were collected between 500–600 nm. Intensity data was converted to spectral center-of-mass ($\langle \nu \rangle$) as follows [34]:

$$\langle \nu \rangle = \int \nu I(\nu) d\nu / \int I(\nu) d\nu$$

Alternately, emission was monitored at 510 nm over time for kinetic scans. The emission and excitation polarizer were oriented at 0° and a 10 mm $\times$ 10 mm quartz cuvette was used for all measurements. BODIPY GTP γ S FL alone was subtracted from all other spectra time points for kinetic plots.

Trypsin Protection Assay

Assay performed as described previously [35]. Trypsin G_{ta} fragments were probed using TF15 monoclonal primary antibody (EMD Bioscience) and peroxidase-labeled goat anti-mouse secondary antibodies (Kirkegaard & Perry Laboratories). Supersignal West Pico chemiluminescent substrate (Pierce) was used for detection.

GDP determination

Spectroscopic assay performed as described previously [36]. Samples were boiled for 5 minutes then applied to a 10-kDa molecular weight cut-off Microcon device (Millipore). The collected flow-through allowed for determination of GDP content due to the linear relationship between GDP concentration and maxima absorbance at 253 nm. GDP concentration in the flow-through was compared to the protein concentration of the boiled protein sample and moles of GDP bound/moles of G α subunit were calculated.

Protein Concentrations

Purified G α subunit concentration was determined by BCA protein assay (Pierce) using a BSA standard curve and by UV-visible spectroscopy at 280 nm using an extinction coefficient calculated from the primary sequence of each α -subunit; The extinction coefficients were calculated to be 33,771 M⁻¹cm⁻¹ for G_{ia}, 40,680 M⁻¹cm⁻¹ for G_{sa}, 38,830 M⁻¹cm⁻¹ for G_{11a} and 28,880 M⁻¹cm⁻¹ for G_{ta} (obtained from primary sequence analysis at <http://us.expasy.org/tools/protparam.html>). Purity was determined by SDS-PAGE in conjunction with Western blot. For immunoblotting, samples were transferred using nitrocellulose filter paper and blotted using mouse anti-histidine primary and peroxidase labeled goat anti-mouse secondary antibodies (Kirkegaard & Perry Laboratories). Supersignal West Pico chemiluminescent substrate (Pierce) was used for detection. The presence of G α was verified using MALDI mass spectroscopy (Ann Snellinger, Johnston Laboratory, University of Delaware) and N-terminal sequencing (Yu Cue Huang, Colman Laboratory, University of Delaware).

Characterization of the G α subunits

Near-UV CD spectra were collected at 5 μ M G α concentration using an Aviv model 202 spectrophotometer with Peltier temperature control. A 1 cm path-length quartz cuvette was used for all near-UV measurements. Spectra were collected from 180 nm to 400 nm using a 1 nm step resolution, 0.1 nm bandwidth, and 3 s integration time. Sample spectra were corrected by subtraction of the reference spectra, containing buffer at the same composition. CD spectra are expressed in mean residue molar ellipticity (deg cm² dmol⁻¹).

Fluorescence emission spectra were collected using an ISS PC-1 spectrofluorimeter. Solutions of 5 μ M G α and 5 μ M G α in 8 M urea were mixed to obtain intermediate concentrations of urea. All samples were allowed to equilibrate for 3 hrs prior to analysis. Excitation wavelength was 280 nm and emission spectra were collected from 300 to 400 nm. Sample spectra were corrected by subtraction of reference spectra containing buffer at the same composition. Intensity data was converted to spectral center-of-mass ($\langle \nu \rangle$) as described above [34].

ANS Fluorescent dye binding

ANS was incubated with 200 nM G α subunit and fluorescence spectra were collected using an ISS PC-1 spectrofluorimeter. Excitation wavelength was 390 nm and emission spectra were collected between 420–600 nm. A 10 mm $\times$ 10 mm quartz cuvette was used for all measurements.

Results

Our goal was to obtain high-level recombinant G α expression in *E. coli* for four G α subunits (human G $\alpha_{i(1)}$, human G α_{sa} (short), human G $\alpha_{11\alpha}$ and human G α_{ta} (cone)). Previously, the G $\alpha_{i(1)}$ had been reported to have a high level of soluble expression [31], and the soluble protein would serve as a positive control for our studies; clearly, we did not expect that our inclusion body refolding method would outperform the high level of expressed protein for G $\alpha_{i(1)}$ (40 mg/L) – this comparison would serve merely as a benchmark for our studies. The G α_{sa} had been reported to express between 0.3 mg/L [25] and 3.5 mg/L [24]. The expression of this subunit is challenging and variable, often resulting in no, or low levels (< 1 mg/L), of soluble protein (Dr. Jason Snyder, person. communication). For the G α_{sa} , our goal was to refold protein at mg/L levels. Since neither G $\alpha_{11\alpha}$ nor G α_{ta} had been expressed successfully in *E. coli*, recovery of any active protein (in the absence of co-expressed G β or G γ) would be an improvement to current *E. coli* methods.

Expression and Purification

All expressed pET-H₆-G α constructs produced G α subunits that were exclusively located in the insoluble fraction of the *E. coli* lysate. Expression parameters were altered (cell line, IPTG concentration and temperature) without any detectable amount of soluble G α expressed. Optimal inclusion body yields were obtained after a 4 hr induction at 37 °C with 1 mM IPTG. Detergents and reducing agents failed to gently solubilize G α subunits from inclusion bodies. A chaotropic reagent – urea or guanidinium chloride – was necessary to liberate G α from the insoluble fraction and all purification steps were performed in 8 M urea. Figure 1A-C depicts samples from the expression, solubilized inclusion bodies, and purified fractions of G α_{sa} . MALDI mass spectroscopy verified the predicted molecular weight of the G α subunit while SDS-PAGE was used to determine purity (shown for G α_{sa} , Figure 1D).

An alternative approach to recover active protein was on-column refolding. The G α subunits were bound to Ni-NTA resin in the presence of 8 M urea and refolded and purified in the absence of 8 M urea, as described in material and methods (Figure 1E). G α_{ta} required an additional purification step, Superdex200 size exclusion chromatography, to rid purified G α_{ta}

of N-terminal fragments, verified via N-terminal sequencing (not shown). Other G_{α} subunits were purified simply using the nickel affinity resin and an imidazole eluent. The N-terminal hexa-histidine tag of the G_{α} subunit fractions was identified using anti-His western blot and N-terminal sequencing (not shown). The G_{α} subunit expression varied, with $G_{i\alpha}$ yields the highest, even under insoluble conditions, and $G_{11\alpha}$, the lowest; Table 2 details total protein yields from solubilized inclusion bodies.

Refolding

Several approaches were investigated to determine the optimal refolding conditions. Rapid dilution is a useful technique for proteins that have a natural tendency to aggregate and form precipitants [37, 38]. The technique allows the protein to fold into its correct conformation rapidly by giving the protein ample solvent, preventing close interaction due to a concentration effect or from problematic intermediates, as present in step-wise dialysis methods. Using rapid dilution in the presence of CHAPS, a zwitterionic detergent; glycerol; DTT; and GDP, a natural ligand for the G_{α} subunits, helped to minimize the tendency of G_{α} subunits to precipitate out of solution after concentration to 1 mg/ml protein. Glycerol was critical to the optimal recovery of refolded G_{α} subunit. Increasing the glycerol content from 10% to 20% stabilized G_{α} from precipitation and ultimately increased the activity of the α subunits to a detectable level. It has been noted previously that there is a good correlation between the refolding yields and the suppression of protein aggregation by glycerol [39]. However, increasing the glycerol content above 20% did not increase the level of active G_{α} subunits. All other additives tested (see Table 3), including small molecules (e.g. arginine), various protecting osmolytes (e.g. sucrose), and multiple detergents, resulted in precipitation of G_{α} subunits and/or large aggregates as indicated by apparent absorbance at 280 nm due to light scattering.

One criterion structural biologists have used to determine if G_{α} is in its active conformation is that it is monomeric in the absence of denaturant. Often, GTP-exchange activity of the G_{α} has not been reported in high-resolution structural studies [for example, 15, 16, 26, 40, 41]; however, these scientists typically rely on a method of expression where GTP-exchange activity was measured directly or indirectly previously [24, 26, 42]. In other cases where characterization of different G_{α} proteins is desirable, activity is reported for variants in terms of the relative wild-type levels [for example, 25, 27]. Thus, the standard of comparison is the soluble native-like G_{α} protein, where the assumption is that solubility and activity are equivalent.

G_{α} is quite prone to precipitation, so its solubility in the absence of denaturants may be suggestive of a correctly folded conformation, although soluble aggregates are possible. Therefore, UV spectroscopy was used as a first screen of solubility to detect the presence of G_{α} precipitation, since ultraviolet scattering above 280 nm would indicate the presence of large aggregated protein. Refolded G_{α} subunits showed little scattering (Figure 2), suggesting that the proteins were soluble upon refolding. The solubility of the G_{α} subunits varied; $G_{i\alpha}$ was soluble above 1 mg/ml while the others were less soluble ($G_{s\alpha}$ was more soluble than $G_{t\alpha}$ and $G_{t\alpha}$ was more soluble than $G_{11\alpha}$). $G_{11\alpha}$ was soluble up to 1 mg/ml but would precipitate out of solution upon multiple concentration steps using an Amicon Ultra filtration device. Baseline subtraction was used to accurately determine protein concentrations from estimated extinction coefficients and the absorbance at 280 nm.

Circular Dichroism

Figure 3A, shows that the refolded $G_{i\alpha}$ subunit has partial α -helical structure and closely resembles the secondary structure characteristic of the native, solubly expressed $G_{i\alpha}$ subunit (pPro $G_{i\alpha}$ purified from *E. coli* in its active, primarily soluble form [31], as verified by GDP binding and GTP exchange). The other refolded G_{α} subunits also maintain partial α -helicity

(Figure 3B). It has been shown previously [43] that G_{α} subunits can misfold irreversibly in the absence of GDP or even during the exchange of GDP for GTP *in vitro* [36, 44, 45]. The misfolded G_{α} subunits have an α -helical character [43]; therefore the presence of secondary structure does not necessarily mean the protein is active as defined by nucleotide binding.

Determination of GDP content of refolded G_{α} subunits

To further understand the nature of the refolded G_{α} subunits we sought to determine the GDP content of the refolded protein. In order to exchange GTP, the proteins must first bind GDP effectively. The spectroscopic determination of GDP is possible due to the linear relationship between absorbance at 253nm and concentration. To determine GDP-associated protein content, the refolded samples were subjected to repeated dilution-concentration steps to ensure complete removal of excess GDP and interfering reducing agents present during refolding. The refolded $G_{i\alpha}$ and $G_{s\alpha}$ subunits were found to have GDP bound, while $G_{t\alpha}$ did not (Table 2). $G_{11\alpha}$ was also found to be GDP-free but it is possible that this assay was unable to detect the level of $G_{11\alpha}$ -GDP present. The main weakness of this assay is that while straightforward to carry out once the sample is in a buffer free of UV interfering reagents, it requires relatively high concentrations of GDP (>500 nM); therefore the assay lacks the sensitivity to detect low levels of GDP. The GDP spectroscopic assay was very reproducible for $G_{i\alpha}$ with samples from separate refolding trials repeatedly demonstrating a 0.5 mol bound GDP/mole $G_{i\alpha}$. In contrast, the $G_{s\alpha}$ GDP content ranged from 0.18 – 0.50 mol bound GDP/mole $G_{s\alpha}$ depending on the refolding trial.

Activity of the Refolded G_{α} subunits

The activity of the G_{α} subunits is tightly linked to their ability to bind GTP. The G_{α} subunits are endogenously bound to GDP and are often purified in buffer containing GDP to prevent protein denaturation during multi-step purification. In this study the proteins were refolded in the presence of GDP to facilitate the formation of this native, GDP-bound resting structure. Upon binding at the cell surface to a GPCR, agonists promote the release of GDP from the G_{α} subunit, resulting in GTP binding. Some G_{α} subunits will exchange GDP for GTP *in vitro* over time. The solubilized and refolded G_{α} subunits were tested for their ability to exchange GDP for GTP in the absence of their signaling partners. In order to determine functionality, two activity assays were used; one utilized the radioactive GTP γ S and another, a fluorescent GTP γ S analog. If active, the α subunits exchanges GDP for [³⁵S] GTP γ S or BODIPY GTP γ S, which, for the purposes of this assay, binds irreversibly because the α subunit cannot hydrolyze the nucleotide analog.

The radioactive assay is problematic because the noise from the GTP γ S³⁵ radioactive counts (or non-specific binding) disguised low levels of active protein. Our positive control demonstrated ~30% maximal binding in the absence of receptor and the $\beta\gamma$ -subunit, as shown previously for recombinant, non-myristoylated $G_{i\alpha}$ [27, 46]. Refolded $G_{i\alpha}$ demonstrated ~10% activity (~3% maximal binding) compared with this control $G_{i\alpha}$ (Figure 4). We purchased a positive control for $G_{s\alpha}$, expressed in insect cells, but unfortunately under our assay conditions separate stocks repeatedly yielded low and highly variable binding activity, illustrating the common difficulty in storing and maintaining activity in these G_{α} subunits.

We sought to utilize a more convenient way to monitor small amounts of active G_{α} subunit. The BODIPY fluorescent GTP γ S analog has been used to efficiently test for activity of G_{α} subunits [33]. This analog is convenient because no filtering or washing away of excess ligand is required. The interaction between the fluorophore and the purine base of GTP interact to quench the fluorescence of BODIPY in solution, and BODIPY fluorescence increases upon binding to GTP-binding proteins. Unfortunately, the positive $G_{s\alpha}$ control expressed in insect cells is palmitoylated and anchored to Sf9 membranes. The more constrained, membrane-

anchored G_{α} subunits have been shown to have low affinity for the BODIPY analog possibly due to the large BODIPY group [47]. Therefore the $G_{i\alpha}$ control was used to ensure that the analog was working properly.

The fluorescence of BODIPY GTP γ S is more sensitive to small amounts of active protein and was useful in detecting activity of refolded $G_{i\alpha}$, $G_{s\alpha}$, and $G_{11\alpha}$ (Figure 5A depicts a wavelength scan and center of mass measurement for $G_{11\alpha}$). Addition of $G_{i\alpha}$, $G_{s\alpha}$ and $G_{11\alpha}$ to BODIPY GTP γ S resulted in a time-dependent increase in BODIPY fluorescence monitored at 510 nm (Figure 5B). This assay did not detect an increase in fluorescence signal upon the addition of $G_{t\alpha}$ to BODIPY GTP γ S, as expected since $G_{t\alpha}$ requires signaling partners to demonstrate nucleotide exchange. It is reasonable to believe that the subunits demonstrating signal are $\leq 10\%$ active from refolding, although it should be noted that the subunits vary in ability to exchange nucleotide in nature. Therefore the “native-like” recombinantly expressed $G_{i\alpha}$ is not useful as a reference to quantify active amounts of $G_{11\alpha}$ and $G_{s\alpha}$.

$G_{t\alpha}$ requires rhodopsin and the $\beta\gamma$ subunits to demonstrate any activity measured by nucleotide exchange, therefore, an AlF_4^- trypsin protection assay was used to test $G_{i\alpha}$ activity. AlF_4^- mimics the γ -phosphate upon addition to G_{α} -GDP, simulating the active form of G_{α} bound to GTP without the requirement for the receptor induced exchange of GDP for GTP [40, 48]. Upon trypsin digest, pPro $G_{i\alpha}$ demonstrated the trypsin cleavage fragment indicative of active G_{α} subunit, but we were unable to detect any active $G_{t\alpha}$ protein by trypsin digestion (data not shown).

Tertiary Structure Determination of Refolded versus Native $G_{i\alpha}$

In order to understand why the $G_{i\alpha}$ and $G_{s\alpha}$ subunits were binding GDP, but were not as active as solubly expressed $G_{i\alpha}$, we sought to compare the tertiary structure of the refolded $G_{i\alpha}$ to that of the native, soluble $G_{i\alpha}$. Near ultraviolet circular dichroism spectroscopy was used to determine if the refolded G_{α} subunit had defined tertiary structure. The near UV circular dichroism (250–350 nm) is dominated by aromatic side chain positioning. If the aromatic residues are held rigid, as would be expected in a native structure, then the side chain has an asymmetric environment and exhibits circular dichroism. If, however, the protein has a less defined tertiary structure the aromatics would be mobile and the near-UV CD spectra would be very weak [49]. Refolded $G_{i\alpha}$ has no near-UV CD signal as compared to that of the native $G_{i\alpha}$ near-UV CD spectra (Figure 6A), indicating that multiple aromatics ($G_{i\alpha}$ has 3 tryptophans, 13 tyrosines and 19 phenylalanines) are not ordered.

To further probe the useful properties of the aromatic residues, we monitored a wavelength shift in intrinsic fluorescence upon denaturation. Aromatic residues are expected to move from their typical well-shielded hydrophobic environment to an exposed or unburied environment upon protein denaturation. We found that both demonstrated a blue shift with increasing urea but that the environment of the aromatics in the refolded $G_{i\alpha}$ structure was not identical to that of native $G_{i\alpha}$ (not shown). Also, the protein did not exhibit a clear two-state unfolding transition (unlike the native $G_{i\alpha}$, data not shown). This further indicates a lack of ordered tertiary structure in the refolded $G_{i\alpha}$.

Finally, to understand the tertiary structure differences between the refolded and native state of the protein, we utilized a hydrophobic dye-binding assay to determine the exposed hydrophobic surface profile of the refolded $G_{i\alpha}$. ANS, a fluorescent dye whose intensity increases upon binding hydrophobic regions of a protein, was used to detect the presence of hydrophobic surfaces. The emission profile of the bound dye is similar for both the refolded and native $G_{i\alpha}$ (not shown). A titration experiment was used to ensure that the amount of dye was in excess over binding sites (Figure 6B). Fluorescence emission of ANS increased upon addition of protein (final protein concentration of 200 nM). At saturation, the fluorescence

emission intensity of the ANS upon binding refolded $G_{i\alpha}$ is four-fold higher than native $G_{i\alpha}$. There is a strong correlation between ANS binding affinity and the exposure of available hydrophobic surface. Solubly-expressed $G_{i\alpha}$ protein is less accessible to binding by ANS than the refolded $G_{i\alpha}$; therefore, a greater ANS emission intensity is observed because the dye is more strongly associated with the refolded $G_{i\alpha}$ protein. This is consistent with a more disordered structure in the refolded $G_{i\alpha}$.

Discussion

Our goal was to express a representative member of G_{α} subunit from three of the four major subfamilies of G proteins in *E. coli* for study with corresponding GPCRs. As reported previously, $G_{i\alpha}$ and $G_{s\alpha}$ have been expressed in *E. coli* under conditions that allow the protein to fold properly (i.e., low temperature, low IPTG concentration and long post-induction incubation) [24]. We were able to duplicate protocols for active $G_{i\alpha}$ expression only under promoter control that was known to produce soluble protein [50]. We were not able to duplicate $G_{s\alpha}$ expression at the reported levels in vectors known to produce soluble protein previously [24]. The active yield of G_{α} obtained in *E. coli* varies from subtype to subtype; $G_{i\alpha}$ was readily expressed in its active form, utilizing a specific expression vector, but $G_{s\alpha}$ expression was difficult at best and many other α -subunits appear recalcitrant under any conditions in *E. coli*. Our experience suggests that G_{α} subunits are easily expressed in *E. coli* but are found completely or predominately as inclusion bodies. Inclusion bodies have the advantage of being protected from proteases, are relatively easy to purify and therefore, an efficient refolding technique for large-scale production of these proteins is desirable.

Previously, the criteria for successful purification from bacteria depended on the presence of G_{α} in the soluble fraction of the cell lysate because efforts to solubilize and refold the G_{α} subunit failed [27, 51]. We have shown that a small percentage of G_{α} subunits can be rescued from the inactive, insoluble state by a rapid dilution technique either through use of solid support or by dilution followed by a concentration step. The concentration step can be problematic due to aggregate losses from the device itself; therefore an on-column method allowed for a greater yield of refolded G_{α} subunits.

It has been suggested that inclusion bodies form from partially folded protein intermediates and that mild solubilization techniques assist in improving native-like recovery of the insoluble protein [52]. However, the G_{α} subunit was resistant to solubilization via reducing agents or detergents and complete solubilization required harsh denaturants. G_{α} subunits contain many cysteines (~8 cysteines); therefore, it was necessary to have reducing agents present. After solubilizing the G_{α} subunits in 8 M urea, histidine tag assisted nickel purification was straightforward. If further purification was deemed necessary, gel filtration was sufficient to rid the sample of proteolytic fragments.

Table 3 lists refolding reagents commonly used for recovery of proteins solubilized from inclusion bodies (<http://refold.med.monash.edu.au/>) [53, 54]. We tested many of the common reagents listed in Table 3 and found that rapid dilution in the presence of 0.1% CHAPS, 100 μ M GDP, 1 mM DTT, and 20% glycerol in a 20 mM Tris-Cl, pH 8.0 buffer supported the greatest recovery of soluble G_{α} subunit. We believe the presence of CHAPS enhanced folding of hydrophobic regions, the presence of solvophobic-glycerol and DTT prevented aggregate formation, and that partial activity was regained when G_{α} subunits ultimately formed the correct conformation in the presence of the ligand, GDP. As a preliminary test we checked to see if the solubilized subunit demonstrated α -helical character. Circular dichroism spectroscopy indicated partial α -helical structure; therefore, either the refolded G_{α} subunit has partial helicity or the solubilized G_{α} subunit has a population that is properly folded and maintains the characteristic G_{α} subunit 2° structure [55].

The ability to bind GTP is a definitive feature for active G_{α} subunits. Refolded $G_{i\alpha}$ binds GTP in a characteristic fashion similar to that of the positive control, pPro- $G_{i\alpha}$. The rate-limiting step in the exchange of GDP for GTP is the release of GDP. The $t_{1/2}$ is identical to binding data obtained on G_{α} subunits that have GDP bound, indicating that the active fraction of the refolded G_{α} subunits is GDP bound. We were able to determine that ~10% of the refolded $G_{i\alpha}$ demonstrated activity. Typically, moles of GDP bound G_{α} correlate with moles of GTP binding sites. A maximum of 1 mole of GDP per mole of GTP binding sites exists, suggesting that the stability of the protein is influenced by the GDP content [36]. Spectroscopic determination of GDP content demonstrated that the refolded G_{α} subunits have varying amounts of GDP present. Interestingly, $G_{i\alpha}$ contained more moles of GDP bound than its demonstrated ability to bind GTP. In the case of $G_{i\alpha}$, it may be that G_{α} is prone to aggregation even in a GDP-bound state, thereby hindering GTP exchange.

Experimental procedures for the remaining G_{α} subunits presented difficulties. Our positive control for $G_{s\alpha}$ demonstrated low active levels by our radioactive GTP γ S binding assay, and subsequently binding data on the refolded $G_{s\alpha}$ was unreliable. The other subunits had other inherent difficulties; positive controls for either $G_{t\alpha}$ or $G_{11\alpha}$ were not available and detection of activity using this radioactive assay is problematic and unpredictable without positive controls. Purified $G_{11\alpha}$ from insect cells requires 10 μ M GTP γ S, 100-fold more than is needed in a membrane suspension, to demonstrate GTP γ S binding [45]. In turn this makes characterization of the soluble, refolded $G_{11\alpha}$ subunit difficult. Addition of both $G_{s\alpha}$ and $G_{11\alpha}$ to BODIPY GTP γ S caused an increase in BODIPY fluorescence indicating that they had partial activity upon refolding. The BODIPY GTP exchange assay results combined with the alpha helicity lead us to believe we are obtaining some native-like $G_{11\alpha}$ protein. However, these results have some inconsistency with the GDP spectroscopy assay, which showed no observable bound GDP. Further studies with other partners will likely be needed to test the activity of this subunit.

It has been speculated that loss of association between activated $G_{q\alpha}$ and $G_{\beta\gamma}$ disrupts $G_{q\alpha}$ mediated signaling [56]. These findings, together with the inability to express active protein, suggest that the $G_{q\alpha}$ family may operate differently than some of the other G_{α} subunits. $G_{t\alpha}$ presents additional difficulties for determining activity. $G_{t\alpha}$ relies on the presence of receptor (rhodopsin) and its $\beta\gamma$ partner subunits to demonstrate full activity [27, 57]. Therefore the traditional G_{α} GTP binding assays are not useful for detection of $G_{t\alpha}$ activity in the absence of rhodopsin and $G_{\beta\gamma}$. A classic G_{α} assay, involving AlF_4^- , circumvents the need for receptor or $\beta\gamma$ subunits. It does, however, require that $G_{t\alpha}$ be bound to GDP. Addition of AlF_4^- to $G_{t\alpha}$ -GDP forms a $G_{t\alpha}$ -GDP- AlF_4^- complex that mimics the active conformation of $G_{t\alpha}$ -GTP [40, 48]. The active conformation of $G_{t\alpha}$ has a trypsin resistant fragment (~32 kDa) that is specific to the presence of the active state and therefore, can be detected by a Western blot by probing with an antibody specific for this trypsin-resistant cleavage fragment. Unfortunately, we were unable to detect active $G_{t\alpha}$ subunits using this method. Our results were also consistent with the GDP assay, although the AlF_4^- assay is much more sensitive than the spectroscopic technique. Thus, we conclude that refolded $G_{t\alpha}$ is inactive and GDP-free.

In an attempt to understand the nature of these soluble but largely inactive G_{α} subunits, we sought to determine if the refolded $G_{i\alpha}$ protein maintained any defined tertiary structure. In comparing refolded and native $G_{i\alpha}$, we found that the refolded $G_{i\alpha}$ was largely disordered, as determined by intrinsic fluorescence and ANS-dye binding assays, and maintained little to no tertiary structure, as determined by circular dichroism. The near-UV spectrum signal indicated that the aromatic residues are constrained and not freely moving in the native $G_{i\alpha}$, whereas the refolded $G_{i\alpha}$ had no near-UV signal indicating freely moving aromatic residues. These findings were supported by the lack of a folding transition in the urea-induced intrinsic fluorescence unfolding experiment and the high-magnitude fluorescence emission upon ANS dye binding.

It is likely that the majority of the refolded G_{α} subunit is misassociated, resulting in a structure that is lacking the native hydrophobic surfaces and exposing aggregation prone, high-affinity ANS regions that are buried in the native G_{α} subunit. This supports the conclusion that there is a combination of protein states in solution, a small percentage that is correctly folded or can adopt an active conformation and a problematic population that maintains a compact secondary structure but has an exposed hydrophobic region causing the protein to be aggregation prone. These techniques demonstrate that the refolded protein is not simply slightly misfolded, indistinguishable from native G_{α} , as described by Zelent et al., 2001 [43], but rather lacks crucial tertiary structure that enable a stable and active G_{α} subunit.

Alternate common refolding procedures (dialysis and step-wise dilution), and not so common procedures (hydrostatic pressure), were tried with no improvement. Hydrostatic pressure was somewhat effective at obtaining soluble protein but no population of the soluble protein was found to be active (data not shown). Dialysis and step-wise dilution techniques resulted in precipitation of the protein and addition of common refolding additives (Table 3) in the rapid dilution buffer were not helpful; arginine, detergents, protecting osmolytes, polymers and small concentrations of denaturant were all tested without improvement in refolding yield as indicated by activity (data not shown). Refolding additives herein provided the best case refolding conditions in terms of overall soluble yield and attainable active protein.

Although higher or equivalent levels of G_{α} subunit were obtained compared to soluble expression, there was no clear advantage in terms of the active yield as judged by the BODIPY assay, except perhaps for $G_{11\alpha}$. This is the first time, of which we are aware, where *E. coli* production of $G_{11\alpha}$ has been described. Other methods for obtaining sufficient quantities of G_{α} subunit from multiple expression systems are available [58].

Taken together, these results suggest that high-level production of many G_{α} subunits in *E. coli* may require identification of necessary cellular components that assist in proper G_{α} subunit folding. It may be that some of these proteins are so dynamic at the cellular level that the correct active conformation is not achieved until they associate with the $\beta\gamma$ subunit or other cellular signaling component found in eukaryotic hosts, thereby leaving them extremely aggregation prone in bacterial systems. Interestingly, the $\beta\gamma$ subunit is difficult to express in *E. coli* as well and it may be that they require each other for optimal expression in any system. Co-expression of the α -subunit with the $\beta\gamma$ subunit is necessary to purify properly folded α subunits of the G_q family from Sf9 cells [29, 42]. It is also likely that post-translational modifications may be required for certain G_{α} to find a natural, low-energy conformation. To improve refolding yields, addition of chaperone proteins or other G protein signaling components may be worthy of investigation, but conventional refolding approaches are unlikely to result in better refolding yields than described here.

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Abbreviations

β Me	β -mercaptoethanol
DTT	dithiothreitol

GDP	Guanosine diphosphate
Ni-NTA	nickel-nitrotriacetic acid
GPCR	G protein-coupled receptor
<i>E. coli</i>	Escherichia coli
CHAPS	(3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate)
IM	imidazole
ANS	8-anilino-1-naphthalene sulfonic acid

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FIGURE 1. Expression, Purification, and Refolding of G_{α} subunit

Expression and purification scheme for $G_{s\alpha}$ subunit (48 kDa) A) Western blot detection of the His-tag on the N-terminal region of $G_{s\alpha}$ subunit. Western blot indicates that 8 M urea is necessary to liberate protein from insoluble fraction of cell lysate. Histidine molecular weight ladder (Invitrogen): 10, 15, 20, 30, 40, 50, 60, 80, 100–220 kDa. Magic Mark Western blot ladder (Invitrogen): 20, 30, 40, 50, 60, 80, 100, 120, 200 kDa. B) Coomassie stained SDS-PAGE of G_{α} fractionation on a Ni-chelate column. Purification of G_{α} subunit using imidazole (IM). **Lanes:** 1 marker, 2 blank, 3 crude lysate, 4 soluble, 5 FT (flow through), 6 FT2, 7 wash, 8 80 mM imidazole (IM), 9 100 mM IM, 10 120 mM IM, 11 150 mM IM, 12 250 mM IM. Molecular weight ladder assignment is 10, 15, 20, 25, 30, 40, 50, 60, 80–220 kDa. C) The presence of large molecular weight species was suspected to be aggregates and indeed after the addition of DTT those bands collapsed to the monomeric band of the same molecular weight as G_{α} . D) N-terminal sequencing of the first 16 amino acids, which were found to match the His tag and $G_{s\alpha}$ subunit (GSSHHHHHSSGLVPR). MALDI mass spectroscopy was used to verify the identity of the G_{α} subunits. Mass spectra of eluates: 2 ionization states that match G_{α} molecular mass prediction. E) SDS PAGE illustrating on-column refolding purification results. Lanes from left to right (1–7): 1 flow through from binding urea-denatured G_{α} subunits, 2 8 M urea wash, 3 flow through after O/N (overnight) incubation with refolding buffer, 4 60 mM IM, 5 100 mM IM, 6 200 mM IM and 7 500 mM IM eluates. Star indicates 50 kDa on

molecular weight ladder. Note: N-terminal sequencing verified that the lower molecular weight species are N-terminal fragments of the full-length G_{α} subunits. Arrow indicates MW of full-length G_{α} subunits. Molecular weight ladder assignment for $G_{s\alpha}$, $G_{11\alpha}$ and $G_{i\alpha}$ are 10, 15, 25, 30, 40, 50, 60, 70, 80, 90–220 kDa. Molecular weight ladder assignment for $G_{t\alpha}$ is 15, 19, 26, 37, 49, 64, 82, 115, 180 kDa. The calculated molecular weights of the full His₆-thrombin- G_{α} subunit are as follows: Human $G_{i\alpha(1)}$ 42,679 Da, human $G_{s\alpha}$ (short) 47,625 Da, human $G_{11\alpha}$ 45,249 Da, and human $G_{t\alpha}$ (cone) 43,350 Da.

FIGURE 2. UV spectra of G_{α} subunits

UV spectra in refolding buffer (minus excess GDP) to illustrate absorbance above 280 nm, which indicates the presence of aggregate species to varying degrees for each G_{α} subunit. Aggregation tendency from lowest to highest is $G_{i\alpha}$ (control pPro; filled circles), $G_{i\alpha}$ (open circles), $G_{s\alpha}$ (open squares), $G_{t\alpha}$ (filled squares), $G_{11\alpha}$ (crosses).

FIGURE 3A

FIGURE 3B

FIGURE 3. Secondary structure characterization of the G_α subunits

Measurements of far-UV CD at 25 °C. Protein concentration was 5 μM diluted in phosphate buffer. A) CD spectra comparison between G_{iα} refolded (open circles) and control (pPro, filled circles). B) Far CD spectra of G_{sα} (open squares), G_{tα} (filled squares), G_{11α} (crosses).

FIGURE 4A

FIGURE 4B

FIGURE 4. Time course of GTP γ S binding to G α

G α control (A, filled circles) and G α refolded (B, open circles) (1 μ M) were incubated at room temperature in radioactive buffer containing 5 μ M [35 S]GTP γ S. Samples were withdrawn at the indicated times and held on ice. At 90 min, samples were filtered, washed and counted. Binding of GTP γ S is expressed as percent maximal, calculated based on protein concentration and assuming one GTP bound per G α subunit.

FIGURE 5 A)

B)

FIGURE 5. Binding of BODIPY GTPγS to $G_{i\alpha}$, $G_{s\alpha}$, $G_{11\alpha}$ and $G_{t\alpha}$

A) Wavelength scan of $G_{11\alpha}$ over time after addition of BODIPY GTPγS. Inset represents center of mass calculation at each time point. B) Binding of BODIPY GTPγS to $G_{i\alpha}$, $G_{s\alpha}$, $G_{11\alpha}$ and $G_{t\alpha}$. The fluorescence of 50 nM of BODIPY GTPγS was measured at 25 °C, following the addition of 200 nM G_{α} subunit. G_{α} subunit was kept in excess to keep the noise associated with excess BODIPY GTPγS low. Data is representative of 3 experiments showing similar results. BODIPY GTPγS alone was subtracted for each time point. $G_{i\alpha}$ (control pPro; filled circles), $G_{i\alpha}$ (open circles), $G_{s\alpha}$ (open squares), $G_{t\alpha}$ (filled squares), $G_{11\alpha}$ (crosses).

FIGURE 6A)

FIGURE 6B)

FIGURE 6. Tertiary structure determination comparison of refolded and control $G_{i\alpha}$

A) Near UV CD spectra of native $G_{i\alpha}$ (pPro; filled circles) and refolded $G_{i\alpha}$ (open circles). Final protein concentration was 5 μM . CD spectra represent an average of 3 runs. B) Titration of ANS binding. Binding of ANS to native (open circles) or refolded (filled circles) was measured. Fluorescence intensity at 485 nm was collected upon addition of increasing concentrations of ANS. Final protein concentration was 200 nM.

TABLE 1

Previously reported expression yields for representative G_α subunits.

G _α subunit	Expression Host	G _α subunit Modification	Expression(mg protein/L culture)	Activity (mol GTP-γS bound/mol G _α) in absence of receptor	NMR* or crystal structure†
G _{12a(2)}	<i>E. coli</i> (BL21/DE3)	NA	40(1), 4(2) [24] 3–5(1) [27]	~0.3 [27] ~0.8 [24]	[15] †
G _{sa}	<i>E. coli</i> (BL21/DE3)	NA	0.3–0.6 [25] 3 [24]	~0.8 [24]	[13] †, [59] *
G _{q(1)a}	Sf9	Requires co- expression of β ₂ & γ ₂	0.01(1)/0.0013(1) [29]	~0.6–1.0/NA [29]	NA
G _{12a}	<i>E. coli</i> (BL21/DE3)	Chimera G _{12a(1)} /G _{12a}	3–5 [26, 27]	NA Rhodopsin/β ₂ stimulated ~1 [27]	[26] *, [40, 60] †
G _{12a}	Sf9	NA	0.1 [61]	0.3–0.5 relative to bovine G _{12a} in activating cGMP phosphodiesterase [61]	

NA, Not applicable

TABLE 2Comparison of expression and active yields obtained for the refolded G_{α} subunits.

G_{α} subunit	Expression(mg/L) ⁺	Activity (mol GTP γ S bound/mol G_{α}) [*]	GDP bound yield(mg/L) ⁺⁺
$G_{i\alpha(1)}$	70	0.3	35 (0.5 mol/mol)
$G_{sa(short)}$	40	~0.03	≥ 7.2 (0.18–0.5 mol/mol)
$G_{11\alpha}$	5	>0.05	ND
$G_{i\alpha}$	15	ND	ND

⁺ Expression levels were calculated after purification in 8 M urea using UV280nm measurements and calculated extinction coefficients.

^{*} Active yield was measured for $G_{i\alpha}$ using GTP γ S binding curve and estimated for the other G_{α} subunits. ND, None detected.

⁺⁺ GDP bound yield was determined using spectroscopic assay (see experimental procedures). ND, None detected.

TABLE 3Common refolding additives tested for G_α solubilization

Refolding Additives:		Refolding Additives:	
	Enhances Solubility		Enhances Solubility
<i>Denaturants:</i>		<i>Small molecule:</i>	
0.4–1 M GdnHCl	no	0.5 M Arginine	no
0.4–2 M urea	no	100 μM GDP	yes
<i>Detergents:</i>		<i>Polymer:</i>	
0.1% CHAPS	yes	0.05% polyethylene glycol	no
0.5% Triton X-100	no	20% Glycerol	yes
1% Tween			
0.1–1% CTAB		<i>Protecting osmolyte:</i>	
0.5–1% SB3–14		0.5–1 M sorbitol	no
		1 M TMAO	
<i>Reducing Agents:</i>		0.4 M Sucrose	No
1 mM DTT	yes	proline	
2 mM TCEP	no	glycinebetaine	
20 mM β-Mercaptoethanol	yes		

CTAB, cetyl trimethylammonium bromide; SB3-14, *N*-tetradecyl-*N*, *N*-dimethyl-3-ammonio-1-propane-sulfonate; TMAO, trimethylamine N-oxide; TCEP, Tris (2-carboxyethyl) phosphine Hydrochloride