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Purification, crystallization and preliminary X-ray diffraction studies of a complex between G protein-coupled receptor kinase 2 and $G\beta_1\gamma_2$

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G protein-coupled receptor kinase 2 (GRK2) phosphorylates activated G protein-coupled receptors (GPCRs), which ultimately leads to their desensitization and/or downregulation. The enzyme is recruited to the plasma membrane *via* the interaction of its carboxyl-terminal pleckstrin-homology (PH) domain with the β and γ subunits of heterotrimeric G proteins ($G\beta\gamma$). An improved purification scheme for GRK2 has been developed, conditions under which GRK2 forms a complex with $G\beta_1\gamma_2$ have been determined and the complex has been crystallized in CHAPS detergent micelles. Crystals of the GRK2–$G\beta\gamma$ complex belong to space group $C2$ and have unit-cell parameters $a = 187.0$, $b = 72.1$, $c = 122.0$ Å, $\beta = 115.2^\circ$. A complete data set has been collected to 3.2 Å resolution with Cu $K\alpha$ radiation.

1. Introduction

G-protein coupled receptors (GPCRs) are responsible for many signaling processes, including the regulation of heart rate and blood pressure and the sensations of sight, smell and taste (Pitcher *et al.*, 1998). G protein-coupled receptor kinases (GRKs) play a key role in the desensitization of GPCR signaling by phosphorylating the cytoplasmic loops or tails of activated receptors. Subsequently, arrestins bind to these phosphorylated loops and uncouple the receptors from heterotrimeric G proteins (Sterne-Marr & Benovic, 1995). GRK2 (previously referred to as β-adrenergic receptor kinase 1) is a ubiquitous enzyme that can phosphorylate many GPCRs, including cardiac adrenergic receptors (Pitcher *et al.*, 1999). Its physiological roles in the heart include the regulation of heart rate and the proper development of cardiac tissue (Jaber *et al.*, 1996). However, abnormally high GRK2 activity has been implicated in a variety of disorders including chronic heart failure and myocardial ischemia (Ungerer *et al.*, 1996). Inhibitors of GRK2 therefore hold great promise for the treatment of cardiovascular disease (Iaccarino *et al.*, 1999).

GRK2 is an 80 kDa protein that consists of three domains: an amino-terminal regulator of G-protein signaling (RGS) homology (RH) domain, a central kinase domain and a carboxyl-terminal pleckstrin-homology (PH) domain (Benovic *et al.*, 1986). GRK2 activity is stimulated by the interaction of its PH domain with $G\beta\gamma$ subunits (Pitcher *et al.*, 1992) and anionic phospholipids (Pitcher *et al.*, 1996; DebBurman *et al.*, 1996). These interactions help to recruit GRK2 to the plasma membrane near activated GPCRs. Many other important effector enzymes have PH domains that interact with $G\beta\gamma$ subunits, including Bruton's tyrosine kinase (Tsukada *et al.*, 1994) and phospholipase Cβ (Wang *et al.*, 1999). The molecular basis for the interaction between PH domains and $G\beta\gamma$ is not known.

The crystal structure of the GRK2–$G\beta\gamma$ complex will reveal not only the common molecular architecture of all GRKs, but also how $G\beta\gamma$ interacts with the PH domain of a *bona fide* effector target. The structure will also provide a model for how three modular signaling domains can successfully integrate their individual activities within a single polypeptide chain. Towards this end, we have developed an improved and efficient purification scheme for GRK2 and determined conditions under which GRK2 forms a 1:1 complex with prenylated $G\beta\gamma$ in detergent micelles. We have crystallized this complex and collected a preliminary data set to 3.2 Å resolution on a home X-ray source.

2. Methods

2.1. Purification of GRK2

Bovine GRK2 and bovine $G\beta_1\gamma_2$ were co-expressed in Sf9 insect cells using the baculovirus expression system. Co-expression was used to enhance the expression levels of both $G\beta\gamma$ and GRK2, but was not exploited to isolate the complex. A volume of 8 l (16 × 500 ml) of Sf9 cells in log phase was co-infected with baculoviruses containing genes for GRK2(S670A), wild-type $G\beta_1$ and a mutant

$G\gamma_2$ engineered to have an amino-terminal hexahistidine (H_6) tag. The S670A mutation of GRK2 removes a MAP kinase phosphorylation site that results in heterogeneously phosphorylated preparations of wild-type GRK2 (Pitcher *et al.*, 1999). The multiplicity of infection (MOI) for all viruses was between 5 and 10. The infected cells were allowed to grow for 48 h, then pelleted (2500*g* for 15 min) and resuspended in ~80 ml of ice-cold 10 m*M* HEPES pH 7.2, 100 m*M* NaCl, 5 m*M* EDTA containing protease inhibitors (1 m*M* PMSF, 1 m*M* benzamidine, 1 µ*M* leupeptin and 1 µ*M* pepstatin A). This solution was flash-frozen in liquid nitrogen and stored at 193 K. The cells were thawed in a 310 K water bath and resuspended in a total volume of 200 ml buffer *A* (20 m*M* HEPES pH 8.0, 50 m*M* NaCl, 2.5 m*M* EDTA, 2 m*M* DTT) containing protease inhibitors (1 µ*M* leupeptin, 1 m*M* lima bean trypsin inhibitor, 1 m*M* PMSF and 1 m*M* TPCK). All subsequent steps of the purification were performed at 277 K. The cells were lysed with a dounce homogenizer and then subjected to ultracentrifugation at 186 000*g* for 40 min (40 000 rev min^{-1} in a Beckman Ti-45 rotor). The supernatant and cell pellets were reserved. Cell pellets were resuspended with a Vertishear tissue homogenizer (VirTis) in 100 ml of buffer *A*, dounced a second time and subjected to a second round of ultracentrifugation. The supernatants from the two ultracentrifugation runs were pooled for the purification of GRK2 and the cell pellets were saved for the subsequent purification of G$\beta\gamma$.

GRK2 was subsequently purified using three successive cation-exchange steps. The pooled ultracentrifugation supernatant was applied at 3 ml min^{-1} to a 25 ml Macroprep High S support column (Bio-Rad) using a 25 ml Macroprep DEAE column (Bio-Rad) as a pre-filter. The DEAE column was then removed and GRK2 was eluted from the High S column with a 50–500 m*M* NaCl gradient in buffer *B* (20 m*M* HEPES pH 8.0 and 1 m*M* dithiothreitol). Fractions containing GRK2 were identified using SDS–PAGE, pooled and diluted to a final NaCl concentration of 50 m*M*. The resulting mixture was loaded onto a 35 ml FF heparin Sepharose column (Amersham Pharmacia Biotech) and eluted with a 50–500 m*M* NaCl gradient in buffer *B*. GRK2 fractions were pooled, diluted to 50 m*M* NaCl, loaded onto an 8 ml Source 15 S column (Amersham Pharmacia Biotech) and eluted with a 50–300 m*M* NaCl gradient in buffer *B*. Pooled fractions of GRK2 were concentrated to ~5 ml and purified on a 16/60 Superdex S200 preparative gel-filtration column (Amersham Pharmacia Biotech) pre-equilibrated in a buffer containing 20 m*M* HEPES pH 8.0, 200 m*M* NaCl and 2 m*M* DTT. Fractions containing GRK2 were then concentrated using a 50 kDa Centriprep (Millipore) to at least 10 mg ml^{-1} and frozen as 50 µl pellets in liquid nitrogen. The typical yield from 8 l of Sf9 cell culture varied from 40 to 200 mg of near-homogenous GRK2.

2.2. Purification of G$\beta_1\gamma_2$

G$\beta_1\gamma_2$ subunits were purified from the membrane-containing Sf9 cell pellets as previously described (Kozasa, 1999) with some modifications. Briefly, the cell pellets were resuspended in 1% sodium cholate (Sigma) to solubilize membrane proteins. The G$\beta\gamma$ subunits were then bound to a 4 ml Ni–NTA column (Qiagen) by virtue of the H_6 tag on $G\gamma_2$ and the column was treated with GDP and AlF_3 to activate and release endogenous Gα subunits. G$\beta\gamma$ was then eluted with 200 m*M* imidazole and further purified using a 1 ml Mono Q column (from Amersham Pharmacia Biotech). Finally, G$\beta\gamma$ was purified by gel-filtration chromatography on two Pharmacia 10/30 Superdex 200 columns connected in tandem that had been pre-equilibrated with a buffer containing 20 m*M* HEPES, 100 m*M* NaCl, 10 m*M* 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and 1 m*M* DTT. G$\beta\gamma$ was concentrated to at least 7 mg ml^{-1} and frozen as 50 µl pellets in liquid nitrogen. The typical yield of G$\beta\gamma$ from membranes isolated from 8 l of cell culture varied from 6 to 16 mg.

2.3. Reconstitution and purification of the GRK2–G$\beta\gamma$ complex

GRK2 (80 kDa) was mixed with excess G$\beta\gamma$ (45 kDa) in order to facilitate separation of their complex from uncomplexed proteins by gel-filtration chromatography. GRK2 and G$\beta\gamma$ concentrations were determined by the Bradford assay and the proteins were then mixed together in a mass ratio of 0.85 (a 2:3 molar ratio of GRK2:G$\beta\gamma$) in a buffer containing 20 m*M*

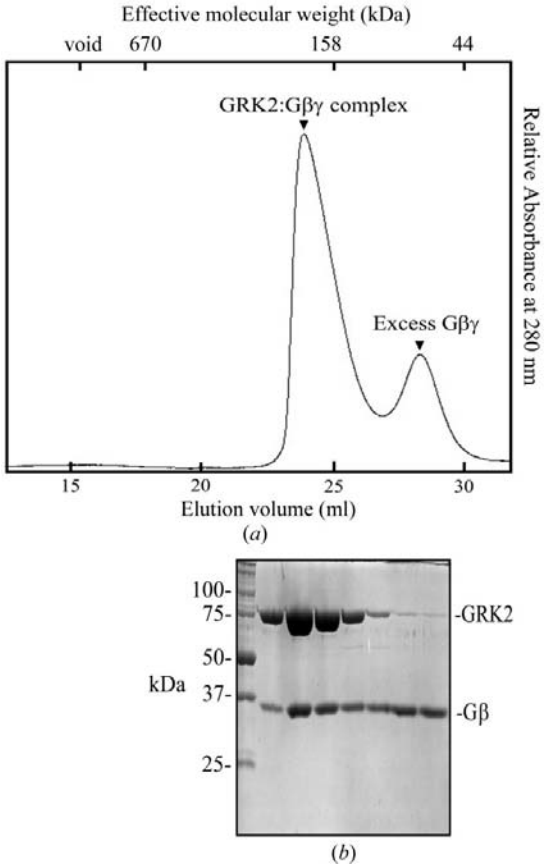

Figure 1
Purification of the GRK2–G$\beta\gamma$ complex. (*a*) Separation of the GRK2–G$\beta\gamma$ complex from excess G$\beta\gamma$ using two tandem Superdex 200 gel-filtration columns. The molecular weights listed at the top of the chromatogram correspond to the molecular weights of gel-filtration standards (Bio-Rad) eluted under the same conditions. (*b*) SDS–PAGE analysis of fractions eluted from the gel-filtration column. The leftmost lane contains Bio-Rad Precision protein standards.

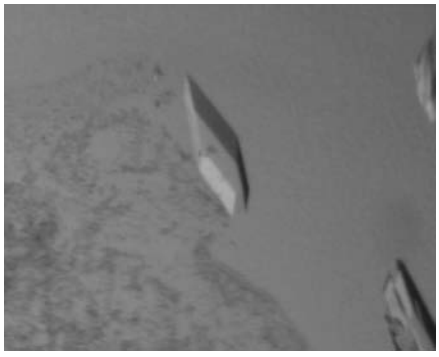

Figure 2
Crystal of the GRK2–$\beta\gamma$ complex. Crystals of the GRK2–G$\beta\gamma$ complex nucleate over the course of 10–20 d at 277 K and reach maximum dimensions (typically 350 × 150 × 50 µm) after one month. Crystals are shaped like thin diamonds, with the 350 µm and 150 µm lengths corresponding to the long and short axes of the diamond, respectively. The crystal shown has dimensions 300 × 100 × 50 µm and belongs to space group *C*2 (a = 187.0, b = 72.1, c = 122.0, β = 115.2°).

HEPES pH 8.0, 100 m*M* NaCl, 10 m*M* CHAPS, 5 m*M* ATP, 10 m*M* $MgCl_2$ and 1 m*M* DTT. After incubation on ice for 1 h, the GRK2–G$\beta\gamma$ complex was injected onto two 10/30 Superdex 200 columns in tandem that had been pre-equilibrated with 20 m*M* HEPES pH 8.0, 100 m*M* NaCl, 10 m*M* CHAPS, 1 m*M* ATP, 5 m*M* $MgCl_2$ and 2 m*M* DTT (Fig. 1). Compared with gel-filtration standards (Bio-Rad), the GRK2/G$\beta\gamma$/detergent micelle mixture eluted in two peaks with effective molecular weights of 180 and 70 kDa (Fig. 1*a*). The 180 kDa peak was consistent with a 1:1 complex between GRK2 and G$\beta\gamma$ bound to a CHAPS detergent micelle and the 70 kDa peak to a G$\beta\gamma$–CHAPS detergent micelle complex. GRK2 eluted from the same gel-filtration columns with an effective molecular weight of 90 kDa. SDS–PAGE analysis corroborated these assignments (Fig. 1*b*). Omission of ATP in the running buffer delayed the elution time of the GRK2–G$\beta\gamma$ complex, indicating that the complex loses affinity in the absence of ATP. Crystals grown from protein thus purified were also much smaller in size and ill-formed compared with those grown in the presence of ATP. Peak fractions of the complex were pooled, concentrated to $\sim$12 mg ml^{-1} and frozen as 50 µl pellets in liquid nitrogen. Excess G$\beta\gamma$ was saved from multiple runs, concentrated and recycled for use in future reconstitutions.

We have also formed complexes between G$\beta\gamma$ and the independently expressed GRK2 PH domain using the C68S mutant of $G\gamma_2$ (Koch *et al.*, 1993), which eliminates its isoprenylation site and renders G$\beta\gamma$ soluble. The PH–G$\beta\gamma$ complexes do not require detergent micelles to form and can be readily purified by gel-filtration chromatography, but to date have not yielded crystals. Surprisingly, full-length GRK2 fails to form a complex with $G\beta_1\gamma_2$C68S that can be isolated by gel-filtration chromatography.

2.4. Crystallization

Small plate-like crystals of GRK2–G$\beta\gamma$ complex were initially observed in a two-dimensional hanging-drop screen using PEG 3350 (5–20%) *versus* pH (5–7.5) as the variable parameters. All wells also contained 1 *M* NaCl. The best quality crystals grow from hanging drops containing 1 µl complex at $\sim$12 mg ml^{-1} and 1 µl of a well solution containing 100 m*M* MES pH 5.25, 200 m*M* NaCl, 1 m*M* inositol-3,4,5-triphosphate (IP_3), 5 m*M* $MgCl_2$ and 6.9–7.8% PEG 3350 suspended over 1 ml of the well solution. IP_3 is included as a soluble mimic of the head group of PIP_2, a regulator of GRK2 (Pitcher *et al.*, 1996; DebBurman *et al.*, 1996) (Fig. 2). SDS–PAGE analysis of protein recovered from crystals indicates that both GRK2 and G$\beta\gamma$ are present in the crystals (data not shown).

Crystals of the GRK2–G$\beta\gamma$ complex also grow in the presence of 10 m*M* EDTA, which should effectively inhibit the binding of ATP, in the absence of IP_3 and in the presence of phosphatidylserine. These crystals diffract similarly to those described above and have nearly identical unit-cell parameters. Either these compounds are not bound to the crystallized form of GRK2 or they do not provoke a large conformational change when they bind.

2.5. X-ray diffraction data collection and processing

Crystals were equilibrated in a cryoprotectant solution containing 25% ethylene glycol, 8% PEG 3350, 20 m*M* HEPES pH 8.0, 100 m*M* MES pH 5.25, 300 m*M* NaCl, 1 m*M* DTT, 2 m*M* $MgCl_2$, 1 m*M* IP_3, 1 m*M* ATP and 10 m*M* CHAPS by gradually adding cryoprotectant to the hanging drop in 0.5 µl aliquots. Crystals were then transferred to a 100% cryoprotectant solution using an appropriately sized cryoloop (Hampton Research) and allowed to equilibrate for 5–10 min. The crystals were then suspended in a cryoloop and frozen in liquid nitrogen or a nitrogen cold stream.

A complete data set (346 frames with 30 min 0.5° oscillations totalling 173°) was collected with a Rigaku RU-200H Cu $K\alpha$ X-ray source using Osmic confocal mirrors and a MAR345 imaging-plate system. A cold stream from an Oxford Instruments Cryojet maintained the temperature of the crystal at 90 K. The GRK2–G$\beta\gamma$ complex crystals belonged to space group *C*2, with unit-cell parameters a = 187.0, b = 72.1, c = 122.0 Å, β = 115.2° (Table 1). The crystals have a Matthews coefficient (V_M) of 2.5 Å^3 Da^{-1} (Matthews, 1968), suggesting that only one complex is found in each asymmetric unit. Longer 0.5° exposures for 1 h yield higher resolution diffraction, indicating that a synchrotron source would allow collection of superior data. The data were reduced and scaled together using the *HKL* package (Otwinowski & Minor, 1997). Analysis using *TRUNCATE* (Collaborative Computational Project, Number 4, 1994) indicated that the diffraction intensities are severely anisotropic, with the highest resolution data extending in a direction nearly bisecting the a^* and c^* axes and the weakest extending along the b^* axis. This anisotropy obscures the presence of well measured reflections in the highest resolution shells (Table 1). A preliminary molecular-replacement solution using the crystal structures of $G\beta_1\gamma_2$ (Wall *et al.*, 1995) and the homologous kinase domain of protein kinase A (Smith *et al.*, 1999) as search models has unambiguously located both G$\beta\gamma$ and the kinase domain of GRK2 within the unit cell.

Table 1
Summary of crystallographic data collection and processing.

Values in parentheses refer to the highest resolution shell (3.3–3.2 Å).

Wavelength (Å)	1.5418
Resolution limits (Å)	25–3.2
Unit-cell parameters (Å, °)	a = 187.0, b = 72.1, c = 122.0, β = 115.2
Unique (total) reflections	23600 (81000)
Mosaicity (°)	1.202
Completeness (%)	98.2 (97.3)
I_{AVG}/σ_{AVG}	12.5 (2.6)
R_{sym}† (%)	9.6 (50)

† $R_{sym} = \sum |I - I_{AVG}| / \sum I$, where the summation is over all symmetry-equivalent reflections, excluding reflections observed only once.

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