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Crystallization and preliminary X-ray crystallographic analysis of the receptor-uncoupled mutant of $G\alpha_{i1}$

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In order to understand the molecular mechanisms by which G-protein-coupled receptors (GPCRs) activate G proteins, the K349P mutant of $G\alpha_{i1}$ (K349P), which is unable to couple to the muscarinic acetylcholine receptor, was prepared and its crystals were grown along with those of wild-type $G\alpha_{i1}$ protein (WT). The two proteins were crystallized under almost identical conditions, thus enabling a detailed structural comparison. The crystallization conditions performed well irrespective of the identity of the bound nucleotide (GDP or GTPγS) and the crystals diffracted to resolutions of 2.2 Å (WT·GDP), 2.8 Å (WT·GTPγS), 2.6 Å (K349P·GDP) and 3.2 Å (K349P·GTPγS).

1. Introduction

Heterotrimeric GTP-binding proteins (G proteins) transduce signals from membrane G-protein-coupled receptors (GPCRs) to downstream intracellular effectors. Upon ligand binding, a GPCR promotes the release of GDP from the inactive trimeric $G\alpha$(GDP)·$\beta\gamma$ complex, which allows the binding of cytosolic GTP to the empty $G\alpha$ subunit, thereby resulting in the dissociation of the trimeric $G\alpha$(GTP)·$\beta\gamma$ complex into $G\alpha$(GTP) and the $G\beta\gamma$ subunit complex. $G\alpha$(GTP) and $G\beta\gamma$ activate and inhibit various effector proteins, leading to appropriate cellular responses (Gilman, 1987; Cabrera-Vera *et al.*, 2003). Although the structures of active (GTP-bound) and inactive (GDP-bound) $G\alpha$ have been determined, the mechanism by which liganded GPCRs enhance GDP release from $G\alpha$ subunits is still under debate (Rondard *et al.*, 2001; Cherfils & Chabre, 2003; Nanoff *et al.*, 2006). Cocrystallization of GPCR and G protein, which would shed light on these issues, is presently quite difficult, primarily owing to difficulties in the preparation and crystallization of GPCR (Palczewski *et al.*, 2000).

Another approach to obtain insights into the interaction between GPCR and G protein would be a structural analysis of a mutant G protein that is unable to couple to GPCR. Such mutants are known for $G\alpha_s$ (the *unc* mutant; Haga *et al.*, 1977; Sullivan *et al.*, 1987) and for $G\alpha_{i1}$ (Tanaka *et al.*, 1998). These proteins have a proline residue in place of the sixth (basic) residue from the C-terminus (Arg342 for $G\alpha_s$ and Lys349 for $G\alpha_{i1}$). We attempted to crystallize the K349P mutant (K349P) and wild-type (WT) $G\alpha_{i1}$ proteins in order to compare their structures. However, for unknown reasons the crystallization conditions reported for $G\alpha_{i1}$ in the GDP form (Coleman, Lee *et al.*, 1994) failed to produce crystals for both our protein samples. We thus searched for appropriate crystallization conditions from scratch and found ones that yielded highly diffracting crystals of both proteins. In addition, our conditions can be employed for the active GTPγS-bound form with just a minor modification of the magnesium concentration, even though fairly different conditions had previously been employed for the GDP-bound and GTPγS-bound forms (Coleman, Lee *et al.*, 1994). Since there is still continued interest in the structures of G protein α subunits in the monomeric state (Kreutz *et al.*, 2006) and in complexes with other proteins (Chen *et al.*, 2005; Tesmer *et al.*, 2005), the crystallization conditions we describe in this study will also be useful as starting conditions for other $G\alpha$ proteins.

2. Experimental

2.1. Protein expression and purification

The cDNA encoding the wild-type $G\alpha_{i1}$, corresponding to full-length *Rattus norvegicus* $G\alpha_{i1}$ (NCBI database accession No. NP_037277; gene ID 25686), was amplified using the pET24a(+)/His_{10}-$G_i\alpha$ vector (Tanaka *et al.*, 1998) as the template, with forward primer 5′-ATGGGCTGCACACTGAGCGCTGAGGAC-3′ and reverse primer 5′-CCGGTCGACTTAGAAGAGACCACAGTCTT-TTAG-3′. The amplified DNA fragment was blunted and digested with *Sal*I. The blunted/*Sal*I-digested DNA fragment was cloned into the *Sma*I/*Sal*I sites of a modified pET24a(+) expression vector (Novagen), thus producing pET24a(+)/GST-WT-$G\alpha_{i1}$, to express $G\alpha_{i1}$ as a glutathione *S*-transferase (GST) fusion protein at the N-terminus. Between GST and $G\alpha_{i1}$, a PreScission Protease (GE Healthcare Biosciences) cleavage site (LEVLFQGP) was introduced. The expression plasmid for the K349P mutant of $G\alpha_{i1}$ was prepared by ligating the *Dra*I/*Sal*I fragment of the pET24a(+)/K349P-$G_i\alpha$ vector (Tanaka *et al.*, 1998), which has Lys349 replaced by Pro in His_{10}-$G_i\alpha$, into the *Dra*I/*Sal*I sites of pET24a(+)/GST-WT-$G\alpha_{i1}$, thus producing pET24a(+)/GST-K349P-$G\alpha_{i1}$. After confirmation of the sequences, the resultant expression plasmids were transformed into *Escherichia coli* BL21(DE3).

The transformed *E. coli* BL21(DE3) cells were grown in LB medium at 310 K to an OD_{600} of 0.5. Expression of the GST-fusion proteins was induced with 1 m*M* IPTG for 12 h at 303 K. After induction, the cells were harvested by centrifugation and stored at 193 K. The cell pellet was resuspended in ice-cold lysis buffer (50 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 200 m*M* NaSCN, 1% Triton X-100 and 1 m*M* DTT). The cells were disrupted by sonication and the lysate was centrifuged at 30 000*g* for 30 min at 277 K. The supernatant was loaded onto a glutathione Sepharose column (GE Healthcare Biosciences). After washing, the GST-affinity tag was cleaved on the column with PreScission Protease (0.25 units per milligram of fusion protein) overnight at 277 K. The resultant protein contains two additional residues (GP) at the N-terminal flanking region derived from the PreScission Protease recognition sequence. The cleaved product was passed through a fresh glutathione Sepharose column to remove the GST protein and PreScission Protease, which is also a GST-fusion protein. The eluate was further purified by anion-exchange (Resource Q, GE Healthcare Biosciences) and gel-filtration (Superdex 200HR, GE Healthcare Biosciences) chromatography steps. The purified protein was concentrated to 30 mg ml^{-1} in 20 m*M* HEPES–NaOH pH 8.0 and 10 m*M* DTT and was stored at 193 K. About 10 mg (WT) and 3 mg (K349P) purified protein was obtained from 1 l cultures using the procedure described above. The purity was at least 99% as estimated by SDS–PAGE.

Table 1
Supplements to the protein solution and dimensions of crystals obtained.

Crystal	Supplements to protein solution	Dimensions (mm)
WT·GDP	2.5 m*M* $MgSO_4$	1.0 × 0.1 × 0.1
WT·GTPγS	2.5 m*M* $MgSO_4$, 1 m*M* GTPγS	0.4 × 0.04 × 0.04
K349P·GDP	6.25 m*M* $MgSO_4$	0.2 × 0.1 × 0.03
K349P·GTPγS	6.25 m*M* $MgSO_4$, 1 m*M* GTPγS	0.4 × 0.03 × 0.03

2.2. Crystallization

Preliminary crystallization trials were carried out using the sitting-drop vapour-diffusion method according to a previously published protocol (Coleman, Lee *et al.*, 1994). The crystallization drops, which contained 1 µl protein solution [8–20 mg ml^{-1} $G\alpha_{i1}$ protein (WT or K349P), 50 m*M* EPPS–NaOH pH 8.0, 5 m*M* GDP, 5 m*M* EDTA pH 8.0, 5 m*M* DTT and 0–200 m*M* $MgSO_4$] and 1 µl reservoir solution

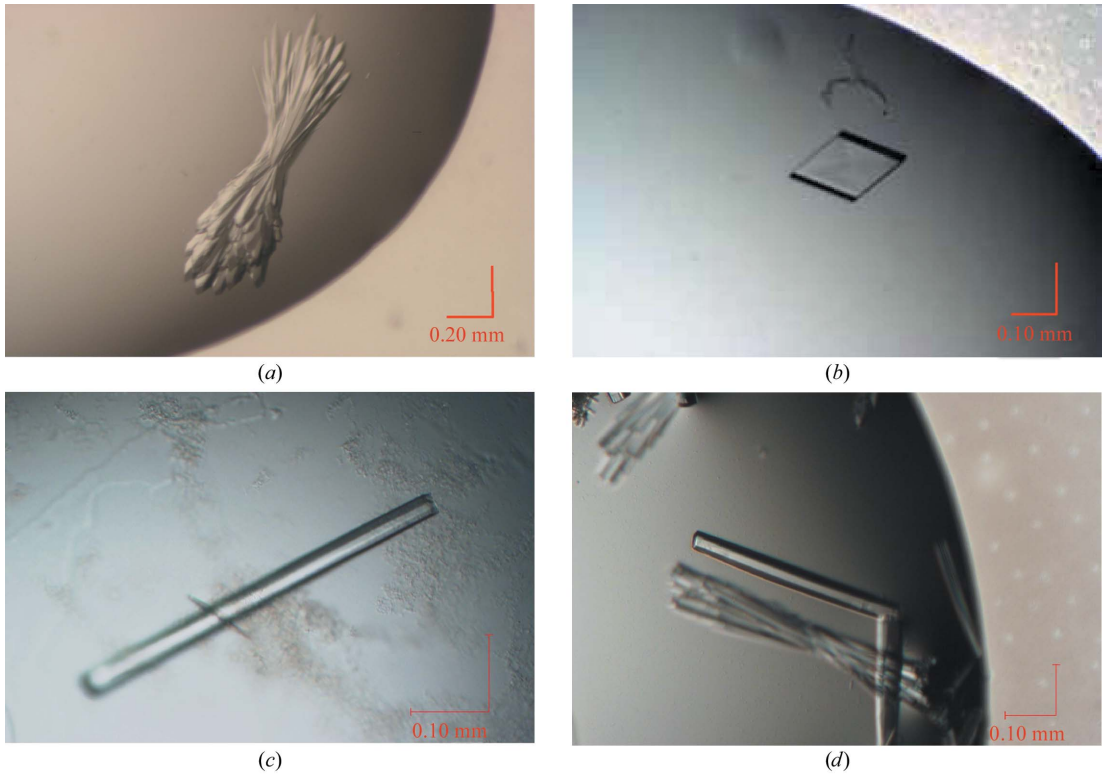

Figure 1
Crystals of $G\alpha_{i1}$ proteins. (*a*) WT·GDP, (*b*) K349P·GDP, (*c*) WT·GTPγS, (*d*) K349P·GTPγS.

Table 2
Data-collection and processing statistics.

Values in parentheses refer to the highest resolution shell.

	K349P·GDP	K349P·GTPγS
X-ray source	SPring-8, BL24XU	Rigaku RA-Micro7
Wavelength (Å)	0.82656	1.5418
Temperature (K)	100	100
Space group	$P2_12_12$	$P3_221$
Unit-cell parameters		
a (Å)	101.38	78.72
b (Å)	164.07	78.72
c (Å)	53.41	105.59
Resolution range (Å)	20–2.6 (2.69–2.6)	50–3.2 (3.31–3.2)
Measured reflections	133853	35722
Unique reflections	27996	11997
Completeness (%)	99.8 (99.7)	98.4 (98.9)
Redundancy	4.8 (4.8)	3.0 (2.8)
$\langle I/\sigma(I)\rangle$	21.5 (2.6)	32.8 (5.5)
R_{sym}† (%)	6.3 (31.9)	9.4 (30.4)

† $R_{sym} = \sum_h \sum_i |I(h,i) - \langle I(h)\rangle| / \sum_h \sum_i I(h,i)$, where $I(h,i)$ is the intensity of the ith measurement of reflection h and $\langle I(h)\rangle$ is the mean value of $I(h,i)$ for all i measurements.

[100 m*M* sodium acetate pH 6.0 and 1.9–2.0 *M* $(NH_4)_2SO_3$ pH 8.0], were equilibrated against 100 µl reservoir solution in 96-well plates consisting of Crystal Clear strips (Hampton Research) at 278 and 293 K. However, these trials failed to produce crystals for both of our protein samples. This may be because our proteins have two additional residues (GP) at the N-termini derived from the PreScission Protease recognition sequence. Therefore, we searched for new crystallization conditions for the Gα_{i1} proteins. Initial crystallization attempts were carried out as described above at 293 K with the sitting-drop vapour-diffusion method using Crystal Screens 1 and 2 (Hampton Research) and Wizard Screens I and II (Emerald Biostructures). Several microcrystals were obtained within 3–5 d from condition No. 39 of Crystal Screen 1 [100 m*M* HEPES–NaOH pH 7.5, 2%(*v*/*v*) PEG 400 and 2.0 *M* $(NH_4)_2SO_4$] and this condition was further optimized by varying the pH, protein concentration, precipitants and additives. Finally, diffraction-quality crystals were obtained at 293 K using the hanging-drop vapour-diffusion method. The total drop size was 6 µl, made up of 4.8 µl protein solution [10 mg ml^{-1} Gα_{i1} protein (WT or K349P), 80 m*M* HEPES–NaOH pH 8.0, 120 m*M* succinic acid and 8 m*M* DTT, with the supplements listed in Table 1] and 1.2 µl reservoir solution [2.0–2.2 *M* $(NH_4)_2SO_3$ pH 8.0]. The drops were equilibrated against 1000 µl reservoir solution in 24-well VDX plates (Hampton Research). Rod-shaped crystals of WT·GDP (Fig. 1*a*), WT·GTPγS (Fig. 1*c*) and K349P·GTPγS (Fig. 1*d*) and diamond-shaped crystals of K349P·GDP (Fig. 1*b*) typically grew within two weeks.

2.3. Data collection

The crystals were soaked in reservoir solution containing 17.5%(*v*/*v*) glycerol as a cryoprotectant and were flash-cooled in a nitrogen-gas stream at 100 K. X-ray diffraction images were collected from the crystals at 100 K using a Rigaku R-AXIS V with 0.8266 Å synchrotron radiation at BL24XU of SPring-8 or a Rigaku R-AXIS VII with 1.5418 Å in-house Cu *K*α radiation provided by a Rigaku RA-Micro7 generator. The diffraction data sets were processed and scaled using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

Complete data sets were collected to 2.2 Å (WT·GDP), 2.8 Å (WT·GTPγS), 2.6 Å (K349P·GDP) and 3.2 Å resolution (K349P·GTPγS). From the diffraction data collection, the space group of the K349P·GDP crystal was determined to be orthorhombic $P2_12_12$, with unit-cell parameters a = 53.41, b = 101.38, c = 164.07 Å, $\alpha = \beta = \gamma = 90^\circ$. With two monomers in the asymmetric unit, the Matthews volume (V_M; Matthews, 1968) was calculated to be 2.75 Å^3 Da^{-1}; the estimated solvent content is thus 55.23%, which is in the range normally observed for protein crystals. The K349P·GDP orthorhombic crystals represent a new crystal form for Gα_{i1} proteins. The space group of the K349P·GTPγS bound state was determined to be trigonal $P3_221$, with unit-cell parameters $a = b$ = 78.72 Å, c = 105.59, $\alpha = \beta = 90$, $\gamma = 120^\circ$. With a monomer in the asymmetric unit, the V_M was calculated to be 2.34 Å^3 Da^{-1}; the estimated solvent content is thus 47.36%, which is also in the range normally observed for protein crystals. The K349P·GTPγS trigonal crystals represent the same crystal form as the WT·GTPγS crystals described by Coleman and coworkers. The space groups of the WT·GDP and WT·GTPγS crystals were essentially the same as those reported previously (data not shown; Coleman, Lee *et al.*, 1994). The statistics of data collection are summarized in Table 2.

The preliminary crystal structures of K349P·GDP and K349P·GTPγS were solved using the molecular-replacement method implemented in the program *CNS*, with the structures of the wild-type Gα_{i1} GDP-bound state (PDB code 1gdd, residues 30–342; Mixon *et al.*, 1995) and GTPγS-bound state (PDB code 1gia, residues 34–343; Coleman, Berghuis *et al.*, 1994) as search models, respectively. Model fitting and further refinement are in progress.

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