Purification of Functional Heterotrimeric G-Protein α-Subunits Using the Profinity eXact™ Fusion-Tag System on the Profinia™ Protein Purification System

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Introduction

Due to low natural abundance, many eukaryotic proteins require heterologous expression in order to obtain the milligram amounts of material often required for high-resolution structural studies. Prokaryotic expression systems have obvious advantages over eukaryotic cell expression systems, including better controlled growth in a variety of liquid media that can be supplemented with various labeled compounds to facilitate structure determination. A key limiting factor for prokaryotic expression systems is whether the eukaryotic protein of interest can be isolated in a pure and functional form. The use of affinity tags for isolation of heterologously expressed eukaryotic proteins has now become commonplace. Because the affinity tags can often interfere with the structure and/or function of the expressed protein, particularly in those cases where the amino- and/or carboxyl-terminus may contain a functionally important site(s), a cleavage site for a sequence specific protease (e.g. TEV protease, thrombin, etc.) is typically introduced between the protein of interest and the affinity tag. However, subsequent removal of the tag from milligram quantities of protein after affinity purification can be relatively costly, necessitating additional purification steps to remove the protease, and in many cases, may result in additional amino acids remaining on the protein of interest.

Here we describe the application of the Profinity eXact fusion-tag system (Oganesyan and Strong 2008; He et al. 2008), which is based on the interaction between the prodomain region of subtilisin BPN' and an active, mutant form of subtilisin BPN' (Ruan et al. 2004), integrated with the automated Profinia protein purification system to isolate milligram amounts of wild-type and mutant heterotrimeric G-protein α-subunits (G_α). The wild-type form of G_α, as well as a mutant (W254F), were found to be as active as G_α isolated from bovine retina.

Methods

Materials

Restriction endonucleases were purchased from New England Biolabs and Complete EDTA-free protease inhibitor cocktail tablets were obtained from Roche Applied Science. Phenylmethylsulfonylfluoride (PMSF), isopropyl-β-D-thiogalactopyranoside (IPTG), and GDP were from Sigma-Aldrich, and bovine retinae were from W. Lawson Co. The Profinia purification system was from Bio-Rad Laboratories, Inc. A plasmid containing the gene for Chi6 (Skiba et al. 1996), an amino-terminal His6-tagged chimeric G_α protein that contains amino acids 1-215 and 295-350 from $G_{t\alpha}$ and an intervening sequence (amino acids 216-294) from $G_{i1\alpha}$ was used as a source for the G_α protein gene used to make the expression construct. The pPAL5 expression vector, a fusion vector encoding the Profinity eXact tag (the modified 76 amino acid prodomain region of subtilisin BPN'), the 1 ml Bio-Scale™ Mini Profinity eXact cartridges containing immobilized S189 subtilisin BPN', and the 10 ml Bio-Scale Mini Bio-Gel® P-6 desalting columns were from Bio-Rad.

Insertion of the Chimeric G_α Gene Into pPAL5 and Mutant Construction

The construction of the Chi6 gene lacking the amino-terminal His6-tag and linker region has been described (Abdulaev et al. 2005a). The resulting gene was excised from the plasmid using HindIII and EcoRI and introduced into the pPAL5 vector using these same restriction sites. pPAL5 vector is essentially similar to the pPAL7 expression vector, a component of the Profinity eXact fusion-tag system, except for a few modifications in the multiple cloning sites downstream of the Profinity eXact fusion tag. Similarly, genes encoding the W207F and W254F mutants (Abdulaev et al. 2005a) were introduced into the pPAL5 vector using the HindIII and EcoRI restriction sites.

Expression of G_α Using the Profinity eXact Fusion-Tag System

E. coli BL21 (DE3) cells harboring a pPAL5/G_α vector were grown in 200-250 ml of LB medium in the presence of 100 µg/ml ampicillin at 26ºC to A_{550} ~0.3, and then induced with 30 µM IPTG for 12 hr at 26ºC. The cell pellet was resuspended in 50 mM Tris-HCl, pH 8.0, containing 50 mM NaCl, 5 mM $MgCl_2$, 150 µM GDP, 5 mM β-mercaptoethanol, 0.1 mM PMSF, and a protease inhibitor tablet and then disrupted by sonication.

Purification of G_α From the Profinity eXact Fusion-Tag Protein on an Automated Profinia System

The supernatant obtained by centrifugation of the cell lysate at 100,000 x g for 60 min was filtered through a 0.2 µm filter, and then loaded onto a 1 ml cartridge packed with Profinity eXact purification resin integrated with a 10 ml Bio-Scale Mini Bio-Gel P-6 desalting cartridge on the Profinia protein purification system. All washing, elution, and column cleaning/regeneration steps were carried out using pre-programmed Bio-Rad methods and recommended buffers except that the binding and wash buffers also contained 5 mM $MgCl_2$, 150 µM GDP, and 5 mM β-mercaptoethanol. The pre-elution incubation step with 100 mM NaF was for 2 hr. For the W207F and W254F mutant G_α constructs, purification using the Profinity eXact purification method was performed in tandem on the Profinia system, under the same conditions as described above for wild-type G_α.

Other Methods

The retinal heterotrimeric G-protein transducin (G_t) was isolated from bovine retina using a standard protocol (Fung et al. 1981), and $G_{t\alpha}$ separated from the $G_{t\beta\gamma}$ subunits as described (Abdulaev et al. 2005b). The intrinsic tryptophan fluorescence of the various G_α subunits in the absence and presence of aluminum fluoride (AlF_4^-) was measured essentially as described (Abdulaev et al. 2005b). Protein samples were analyzed by SDS-PAGE using Criterion™ XT Bis-Tris gels 4-12% (Bio-Rad) and XT MES running buffer (Bio-Rad) and visualized by Coomassie Blue staining. Protein determinations were done using the method of Peterson (Peterson 1977) with BSA as the standard.

Results and Discussion

Expression and Purification of Profinity eXact Fusion-Tagged G_α Subunits

Wild-type and two mutant forms (W207F and W254F) of a chimeric G_α subunit were expressed in *E. coli* as a Profinity eXact fusion-tag and purified on Profinity eXact purification resin integrated with a Bio-Scale Mini Bio-Gel P-6 desalting cartridge on the Profinia protein purification system. Figures 1A and 1B show a representative chromatogram and results from SDS-PAGE analysis, respectively, for wild-type G_α. The chromatogram shows that G_α can be effectively eluted from the Profinity eXact resin and desalted on the Bio-Scale Mini Bio-Gel P-6 cartridges with a good yield. Consistent with earlier G_α chimera expression studies (Abdulaev et al. 2005a), results using the Profinity eXact fusion-tag encoded by the

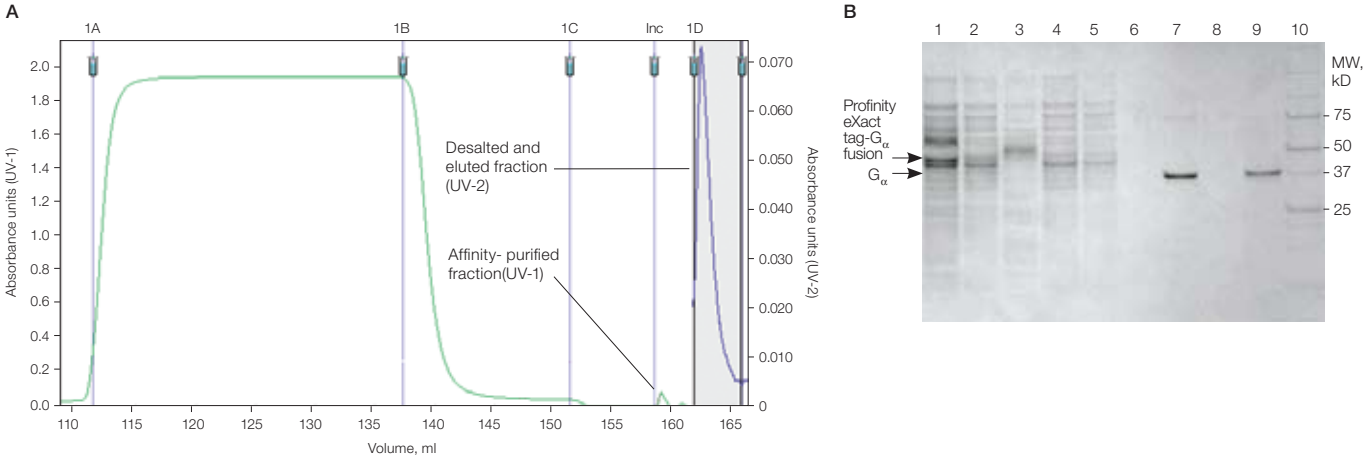

Fig. 1. G_α purification using Profinity eXact purification plus integrated desalting method on the Profinia system. A, chromatogram of Profinity eXact-tag-G_α fusion purification using Profinity eXact purification resin and a Bio-Scale Mini Bio-Gel P-6 desalting cartridge. The soluble fraction of the cell lysate was loaded onto the 1 ml Bio-Scale Mini Profinity eXact cartridge (1A, flowthrough) and the column was washed with 14 column volumes (CVs) of binding/wash 1 buffer, 100 mM sodium phosphate, pH 7.2 (1B, wash 1), followed by 6 CVs of wash 2 buffer, 100 mM sodium phosphate, 300 mM sodium acetate, pH 7.2 (1C, wash 2). Both buffers were supplemented with 5 mM $MgCl_2$, 150 µM GDP, and 5 mM β-mercaptoethanol. The Bio-Scale Mini Profinity eXact cartridge was primed with 1 CV of elution buffer, 100 mM sodium phosphate, 100 mM sodium fluoride, pH 7.2, supplemented with 150 µM GDP and 5 mM β-mercaptoethanol, and allowed to incubate for 2 hours (Inc, enzyme cleavage). G_α, obtained by fluoride-triggered cleavage from the bound Profinity eXact tag, was eluted in 3.5 CVs of the same buffer and desalted using a 10 ml Bio-Scale Mini Bio-Gel P-6 cartridge (1D, elution). (—) peaks detected by UV1 monitor. (—) detected by UV2 monitor; **B**, SDS-PAGE analysis of fractions obtained at various steps of the purification process. Lane 1, total cell extract from IPTG-induced cells; 2, 3, soluble and insoluble extracts respectively, from IPTG-induced cells; 4, column flowthrough (1A); 5, column wash 1 (1B); 6, column wash 2 (1C); 7, elution fraction following desalting (1D); 8, blank; 9, $G_{t\alpha}$ from bovine retina; and 10, Precision Plus Protein™ standards. Protein bands were visualized using Coomassie Blue stain and the positions of some of the molecular mass standards are shown on the right. The arrows on the left indicate the position of the Profinity eXact tag-G_α fusion and tag-free G_α.

pPAL5 vector clearly show that a majority of the fusion protein is present in the supernatant of the cell lysate (Figure 1B, compare lanes 1-3). Further, the soluble fraction containing the fusion protein binds to the Profinity eXact purification resin (Figure 1B, lane 4) and after two rounds of washing to remove contaminating proteins (Figure 1B, lanes 5 and 6) is subsequently cleaved from the Profinity eXact fusion-tag by the immobilized protease in the presence of 100 mM NaF and desalted on the integrated Bio-Scale Mini Bio-Gel P-6 desalting cartridge on the Profinia system. The resulting protein, which we refer to as ChiT (Abdulaev et al. 2005a), is present in a highly purified form (Figure 1B, lane 7) and co-migrates with $G_{t\alpha}$ isolated from bovine retina (Figure 1B, lane 9). Similar results were obtained for the W207F and W254F mutant forms of G_{α} when expressed and purified using the Profinity eXact fusion-tag system on the Profinia system (data not shown).

AlF_4^--Dependent Tryptophan Fluorescence Changes of G_{α}

Adduct formation with AlF_4^- and the GDP/Mg^{2+} bound form of G_{α} results in an increase in the intrinsic tryptophan fluorescence of the protein. In $G_{t\alpha}$, this increase in fluorescence has been largely attributed to a change in the environment of Trp-207 (Faurobert et al. 1993). As shown in Figure 2A, $G_{t\alpha}$ shows a ~30% increase in intrinsic fluorescence upon AlF_4^- treatment. Similarly, ChiT also shows ~30% increase in fluorescence emission upon AlF_4^- adduct formation (Figure 2B), suggesting that G_{α} prepared using the Profinity eXact fusion-tag system on the Profinia system is as functionally active as native $G_{t\alpha}$ isolated from bovine retina. Further, we have previously shown that G_{α} subunits expressed and purified using similar approaches are able to form stable heterotrimers with $G_{t\beta\gamma}$ subunits and undergo receptor-catalyzed guanine nucleotide exchange (Abdulaev et al. 2005b; Ridge et al. 2006a).

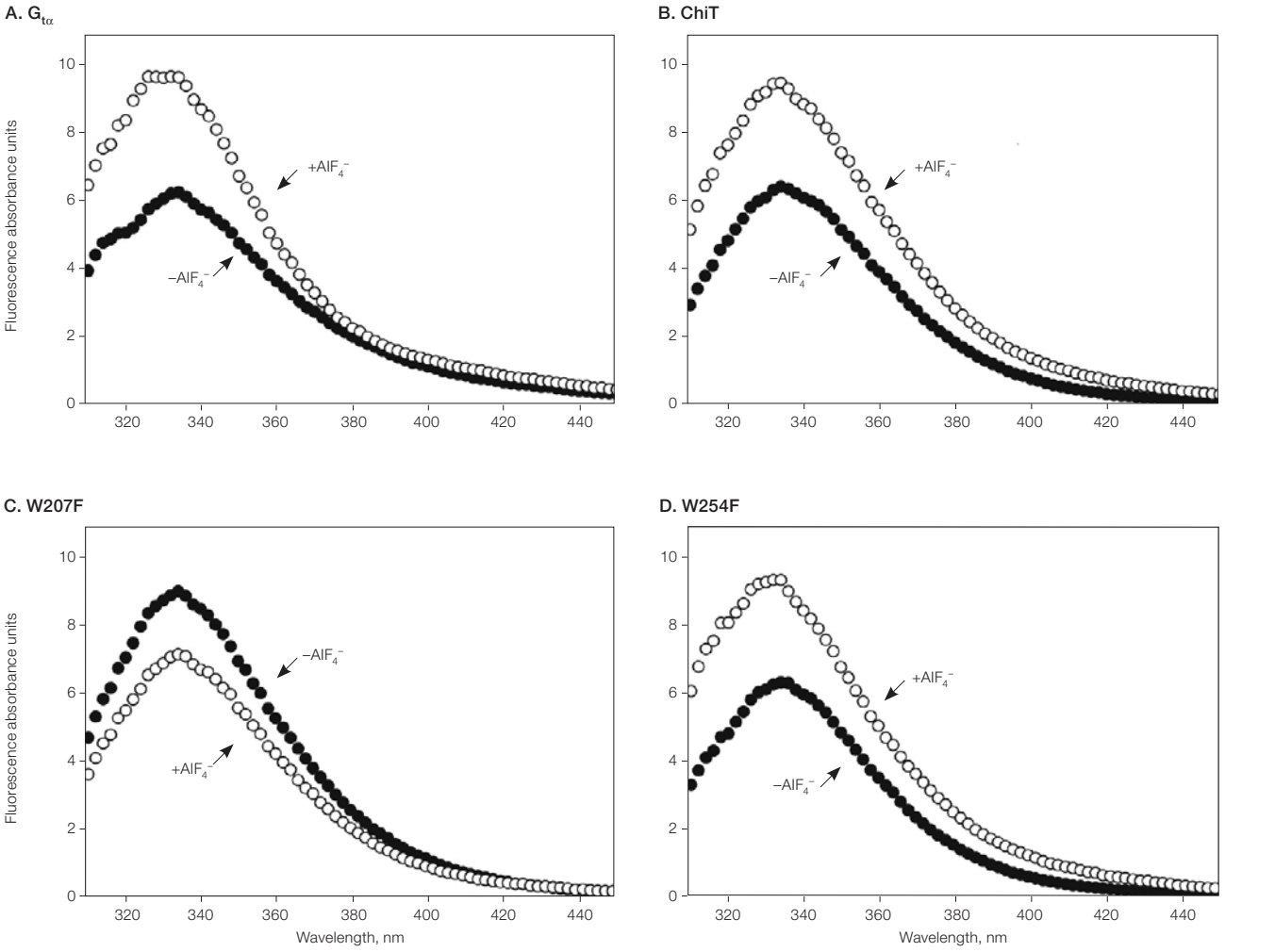

Fig. 2. Fluorescence changes accompanying AlF_4^- treatment of the GDP/Mg^{2+}-bound form of wild-type and mutant forms of G_{α} isolated using the Profinity eXact fusion-tag purification method on the Profinia system. A, B, C, and D, represent emission spectra for $G_{t\alpha}$, ChiT, W207F G_{α}, and W254F G_{α} respectively. (●), fluorescence before and (O), fluorescence after the addition of AlF_4^-.

As expected, the W207F mutant did not show the corresponding fluorescence increase upon addition of AlF_4^-, but in fact showed a ~10% decrease (Fig. 2C). This may be attributed to a change in the environment of W254F. In this respect, the W254F mutant showed a similar increase in fluorescence upon addition of AlF_4^- as $G_{t\alpha}$ and ChiT.

Summary

We have shown using the Profinity eXact fusion-tag system integrated with the Profinia protein purification system that milligram quantities of a highly purified wild-type G protein α-subunit chimera (ChiT) as well as mutant forms of the protein can be isolated in a relatively quick and reproducible fashion. Further, purified ChiT and the W254F mutant of G_α are as functionally active as G_α isolated from natural sources. This technical advance should further facilitate efforts using high-resolution structural methods to investigate the conformation and dynamics of G_α in various states (Abdulaev et al. 2005a; Abdulaev et al. 2005b; Ridge et al. 2006a: Ridge et al. 2006b; Abdulaev et al. 2006).

Acknowledgment

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