Protein Purification and Crystallization of Diabetes Related Peroxisome Proliferator-Activated Receptor-γ (PPARγ)

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Tech Note

Protein Purification

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Abstract

PPARγ plays a role in glucose metabolism. Activated PPARγ increases insulin sensitivity, making PPARγ an important receptor for treatment of type 2 diabetes. Obtaining a crystal structure of PPARγ aids in development of interacting ligands and beneficial anti-diabetic drugs. Here we describe the purification of PPARγ using Bio-Rad's NGC™ chromatography system and a three-step column purification approach. Well-ordered crystals of the purified PPARγ protein were obtained. X-ray diffraction data showed consistent results compared to established protocols.

Introduction

PPARγ is a member of the nuclear hormone receptor superfamily of ligand-dependent transcription factors, together with PPARα and β/δ. The active form of PPARγ has been correlated with an increase in insulin sensitivity. Thiazolidinediones (TZDs), a class of synthetic agonists of PPARγ, increase insulin sensitivity by activating PPARγ (Day 1999 and Pearson et al 1996). This has made the TZD class of anti-diabetic drugs, such as rosiglitazone and pioglitazone, ideal as a pharmaceutical treatment of type 2 diabetes. Despite the clinical benefits of these drugs, use of TZDs causes a substantial number of patients to experience negative pleiotropic effects, including fluid retention, weight gain, congestive heart failure, and loss of bone mineral density (Kahn et al. 2008 and Nesto et al. 2004). Although all TZDs lead to an increase in insulin sensitivity, they do not cause the same pleiotropic effects. This suggests that subtle changes in ligand-receptor interaction can produce substantial biological effects.

X-ray crystallography is a powerful method for studying the structures of proteins bound to their ligands. The information obtained from crystal structures can allow for the initial identification or continued design of a ligand with high affinity and selectivity toward a drug target. This can lead to development of a drug that effectively interacts with the protein target and elicits the desirable effect while diminishing or limiting the adverse side effects.

In order to acquire a high-resolution crystal structure, a pure, homogeneous PPARγ sample must first be obtained. Here we report a three-step column purification workflow utilizing Bio-Rad's NGC Quest™ chromatography system equipped with a sample pump. The three-step column purification approach allows for a pure and consistent end product suitable for crystallization trials and ultimately high resolution data collection for protein structure determination.

Materials and Methods

Expression

A glycerol stock of BL21 (DE3) *E. coli* harboring a pET12plasmid encoding the PPARγ ligand-binding domain (LBD) with an N-terminal 6x histidine tag was used to inoculate 50 ml LB-ampicillin (50 µg/ml) solution and was grown overnight at 37°C with aeration. The next day 20 ml of overnight culture was subcultured into 2 L of Terrific Broth-ampicillin (50 µg/ml) solution and incubated at 37°C with aeration in two Ultra Yield flasks (Thomson Instrument Co.). When the culture reached OD_{600} = 0.6, 0.5 mM IPTG was added to the culture and incubation/shaking continued overnight at 16°C. The overnight culture reached OD_{600} = 10. The cells were then harvested by centrifugation at 4,200 x g for 15 min at 4°C, resuspended in 66 ml Ni^{2+} IMAC buffer A (20 mM Tris pH 8.0, 500 mM NaCl, 10 mM imidazole, 10% glycerol, 5 mM 2-mercaptoethanol) and stored at –80°C for 2 weeks.

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Purification

Cells were thawed and lysed by passing through a cell disruptor at 30 kpsi. The resulting lysate was clarified at 45,000 x g for 45 min at 4°C. Using the NGC Quest chromatography system with sample pump (Bio-Rad Laboratories), 240 ml of the clarified lysate was loaded directly onto a 5 ml Nuvia™ IMAC Ni-charged column (Bio-Rad Laboratories) pre-equilibrated with Ni^{2+} IMAC buffer A (20 mM Tris pH 8.0, 500 mM NaCl, 10 mM imidazole, 10% glycerol, 5 mM 2-mercaptoethanol) at 4°C. The column was washed with 15 column volumes (CV) of buffer A + 3% buffer B (Buffer A + 0.5 M imidazole) before the protein was eluted using an isocratic step gradient of 50% buffer B (buffer A + 0.5 M imidazole). The resulting 3 ml fractions were analyzed by SDS-PAGE. Appropriate fractions were pooled together and dialyzed to anion exchange buffer C (20 mM Tris pH 8.0, 20 mM NaCl, 10% glycerol, 1 mM dithiothreitol) overnight at 4°C. Protein concentration was then assayed using a Shimadzu spectrophotometer with extinction coefficient of 0.385 and determined to be 0.6 mg/ml.

Next, 69 ml of dialyzed protein sample was filtered through a 0.22 µm filter and loaded onto a pre-equilibrated 1 ml ENrich™ Q high capacity anion exchange chromatography column (Bio-Rad Laboratories) using the sample pump at 4°C. The protein was then eluted using a 15 column gradient from 0 to 70% buffer D (buffer C + 1 M NaCl) followed by three column volumes of 100% buffer D with 1 ml fractions collected. The fractions were analyzed using SDS-PAGE and acceptable fractions containing PPARγ-LBD were pooled for the final purification step.

The 2 ml sample containing approximately 4.4 mg of PPARγ-LBD obtained after anion exchange was mixed with sphingosine-1-phosphate (S1P) at a 5:1 molar ratio of ligand:protein in a final volume of 12 ml buffer C. This protein-ligand mixture was incubated on ice for 20 min before being concentrated to 1.1 ml using a 10,000 NMWL Amicon Ultra centrifugal filter (Millipore).

Finally, 1 ml of PPARγ-LBD + S1P protein was filtered through a 0.22 µm filter and loaded using a 1 ml static loop onto a 24 ml ENrich 650 size exclusion column (Bio-Rad Laboratories) pre-equilibrated with buffer E (20 mM Tris pH 8.0, 50 mM NaCl, 1 mM dithiothreitol) at 4°C. The protein was eluted isocratically with buffer E and 1 ml fractions were collected. The fractions were analyzed using SDS-PAGE and acceptable fractions were pooled. The final pool of PPARγ-LBD + S1P protein was concentrated using a 10,000 NMWL Amicon Ultra centrifugal filter to a final volume of approximately 450 µl to give a concentration of 9 mg/ml.

Crystallization

PPARγ-LBD + S1P crystals were grown using the hanging drop vapor diffusion method at 16°C. Approximately 1 µl of PPARγ was mixed with 0.5, 1, and 1.5 µl reservoir solution (0.8 M sodium citrate tribasic dihydrate, 20 mM Tris pH 8.0) on a silanized glass coverslip and inverted over 0.5 ml reservoir solution. Vacuum grease was used to seal the coverslip to the reservoir well. Crystals were observed after 3 days and left to continue growing until 14 days.

Data Collection

Crystals were harvested using loops appropriate to crystal size and transferred to a cryo solution containing reservoir buffer + 25% ethylene glycol for 10 sec. The crystals were plunged into liquid nitrogen and kept at liquid nitrogen temperatures (100°K) until data collection. Crystals were mounted at the macromolecular crystallography beamline (MX2) of the Australian Synchrotron and 360 1-sec diffraction images were collected. HKL-2000 software (HKL Research, Inc.) was used to calculate diffraction resolution and dataset ranges.

Results

A traditional capture, intermediate, and polish purification workflow was followed. To capture the ligand-binding domain (LBD) of PPARγ for structural studies, PPARγ-LBD was linked to a 6x histidine-tagged sequence and overexpressed in *E. coli*. The initial capture of the PPARγ histidine-tagged protein was accomplished using a Nuvia IMAC column charged with nickel to capture histidine-tagged proteins. Use of the Nuvia IMAC column resulted in high selectivity for the target protein and low nonspecific binding (Figures 1 and 4).

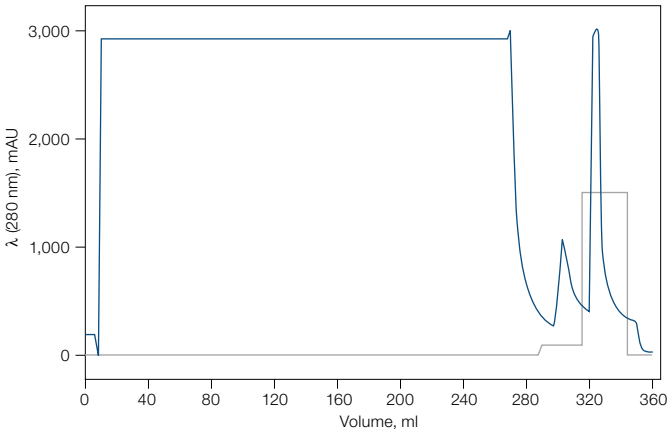

Fig. 1. PPARγ-LBD purification using 5 ml Nuvia IMAC on Bio-Rad NGC Quest system plus sample pump. Using the sample pump, 240 ml of clarified lysate was loaded onto a 5 ml Nuvia IMAC column. The protein was eluted with a 50% buffer B after a 3% buffer B wash to remove weakly binding contaminants. λ (280 nm) (—); %B (—).

An Enrich Q ion exchange column was chosen as the intermediate step of the purification workflow due to the pI of PPARγ and the buffer pH. In order to lower the ionic strength, the sample was dialyzed in 20 mM NaCl to allow efficient binding of PPARγ-LBD to the ENrich Q ion exchange column. The large-volume dialyzed sample was loaded onto the NGC Quest chromatography system using the sample pump, which is a simple and fast means of protein purification. The use of the ENrich Q ion exchange column provided high resolution, as well as concentrated PPARγ-LBD protein (Figures 2 and 4).

Even slight changes in buffer composition from one batch run to another can generate changes in crystal structure formation, creating inconsistencies in the diffraction data. An ENrich SEC 650 size exclusion chromatography column was used for the final polishing step in order to maintain batch-to-batch consistency of buffer composition and to remove any aggregate species. Prior to the final column purification step, sphingosine-1-phosphate (S1P) was mixed with the protein sample to allow for the formation of a protein-ligand complex. S1P was added to stabilize PPARγ-LBD conformation, which aids in crystal formation. The final purification step using the ENrich 650 size exclusion column resulted in 97% purity for PPARγ-LBD + S1P protein (Figures 3 and 4). The purity for each column purification step was monitored using SDS-PAGE (Figure 4).

The resulting purified PPARγ-LBD + S1P protein was concentrated for crystallization and crystals were grown using the hanging drop vapor diffusion method. Well ordered, single protein crystals were produced (Figure 5) and their diffraction limit tested at the Australian Synchrotron. Diffraction data were collected (Figure 6) and showed unit cell dimensions consistent with previous studies (Bruning 2007). The space group was assigned as C2 with a = 92.9, b = 61.2, c = 118.3, α = 90.0, β = 102.8, and γ = 90.0 cell constants. The dataset was processed to 2.1 Å showing a completeness of 96% and R_{merge} = 9.9%.

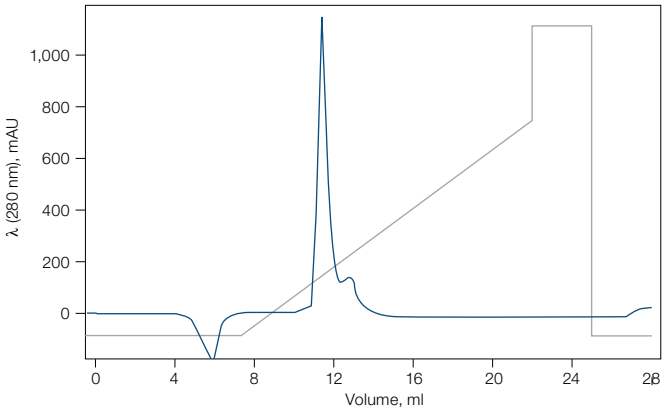

Fig. 2. PPARγ-LBD purification using 1 ml Enrich Q anion exchange column on NGC Quest system plus sample pump. The protein was eluted with a 15 CV linear gradient from 0–70% buffer B. λ (280 nm) (—); %B (—). **Note:** Buffer B is the same as Buffer D described in Materials and Methods.

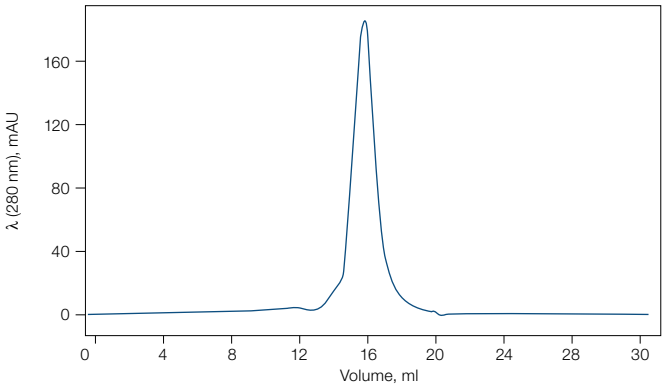

Fig. 3. PPARγ-LBD purification using 24 ml Enrich SEC 650 size exclusion column on NGC Quest system. The protein was eluted with a 1 CV isocratic flow of buffer A. λ (280 nm) (—). **Note:** Buffer A is the same as Buffer E described in Materials and Methods.

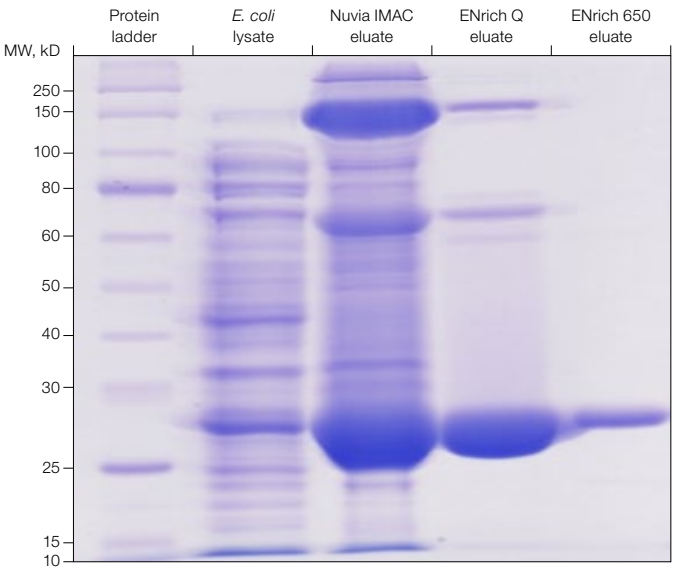

Fig. 4. SDS-PAGE of the PPARγ-LBD purification workflow. Approximately 10 µl of the pooled fraction after each step was run on an SDS-PAGE gel resulting in a protein with >97% purity for crystallization trials.

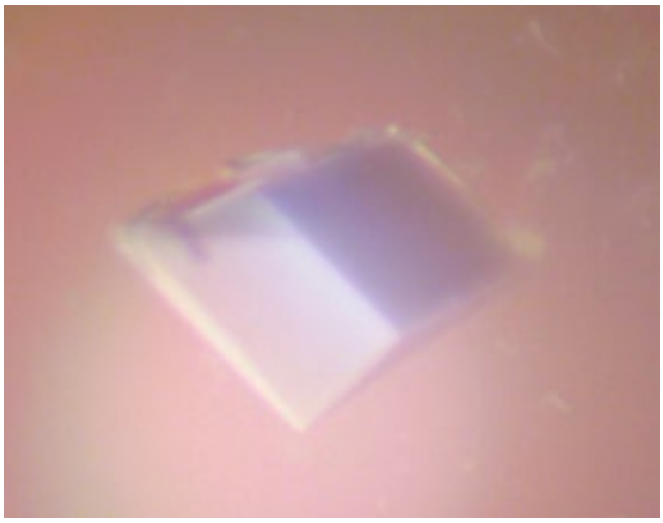

Fig. 5. Image of PPARγ-LBD + S1P crystal. Single crystals with a dimension of ~250 Å were successfully grown using the hanging drop vapor diffusion method.

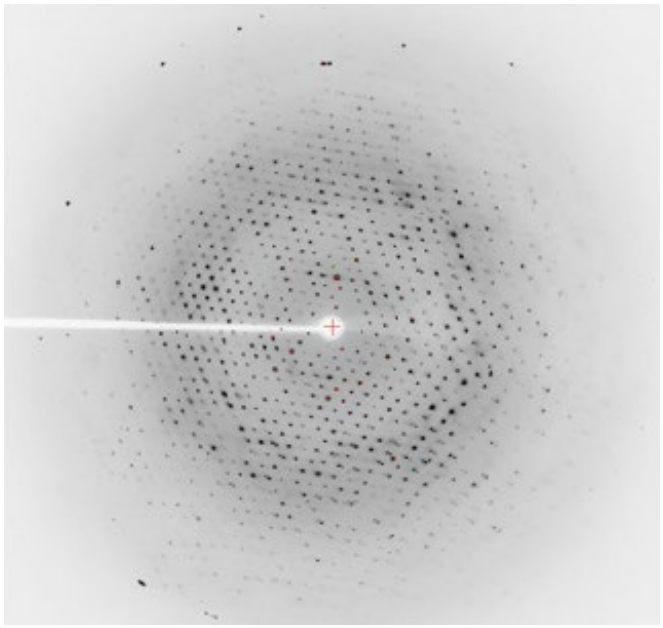

Fig. 6. Representative diffraction pattern of PPARγ-LBD + S1P. Crystals were mounted and data collected at beamline MX2 of the Australian Synchrotron. Diffraction images of 1 sec intervals were collected and processed to a final resolution of 2.1 Å.

Conclusions

Recent research has looked to identify ligands that phosphorylate the same sites on PPARγ that rosiglitazone and other anti-diabetic drugs do but without the serious adverse side effects (Choi et al. 2010). In order to create a suitable ligand for PPARγ that provides similar benefits as the current anti-diabetic drugs but minimizes the side effects, protein crystallization of PPARγ is of utmost importance. Protein crystallography requires the highest quality samples for consistent crystal generation. Using Bio-Rad's NGC Quest chromatography system equipped with a sample pump, and a three-step column purification approach, a very homogenous PPARγ-LBD protein was obtained. Well ordered crystals were generated from the purified PPARγ-LBD protein. The X-ray diffraction data generated from PPARγ-LBD protein purified using the NGC chromatography system is consistent with previously generated data that resulted in structure solution. Thus, the NGC chromatography system and Bio-Rad chromatography columns provide the vehicle to generate consistently pure samples for the formation of diffraction-quality protein crystals.

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