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Efficient stable isotope labeling and purification of vitamin D receptor from inclusion bodies

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ABSTRACT

Vitamin D receptor (VDR) plays a crucial role in many cellular processes including calcium and phosphate homeostasis. Previous purification methods from prokaryotic and eukaryotic expression systems were challenged by low protein solubility accompanied by multi purification steps resulting in poor protein recovery. The full-length VDR and its ligand binding domain (LBD) were mostly (>90%) insoluble even when expressed at low temperatures in the bacterial system. We describe a one-step procedure that results in the purification of rat VDR and LBD proteins in high-yield from *Escherichia coli* inclusion bodies. The heterologously expressed protein constructs retained full function as demonstrated by ligand binding and DNA binding assays. Furthermore, we describe an efficient strategy for labeling these proteins with ^{2}H, ^{13}C, and ^{15}N for structural and functional studies by nuclear magnetic resonance (NMR) spectroscopy. This efficient production system will facilitate future studies on the mechanism of vitamin D action including characterization of the large number of synthetic vitamin D analogs that have been developed.

Introduction

1α,25-Dihydroxyvitamin D_3 [$1,25(OH)_2D_3$][2], the hormonally active form of vitamin D_3, plays an essential role in calcium and phosphate homeostasis and an important role in specific cell differentiation in the immune system and elsewhere [1–3]. Consequently, vitamin D deficiency has been associated with osteoporosis, cancers, autoimmune disorders, cardiovascular diseases, and infections [1–3]. The pleiotropic actions of vitamin D have highlighted $1,25(OH)_2D_3$ and its analogs as potential therapeutic agents in a variety of diseases beyond those relating to calcium, phosphate, and bone [4,5].

$1,25(OH)_2D_3$ and its analogs exert their function by binding to the vitamin D receptor (VDR), which in turn forms a heterodimer with retinoid X receptor. The ternary complex binds to specific DNA sites on target genes (vitamin D response elements or VDREs) and recruits an array of various co-activators and co-repressors that regulate gene expression [6]. Defects in the VDR gene causes hereditary vitamin D resistant rickets type II (HVDRR) [7]. Genetic ablation of VDR in mice leads to phenotypic traits characteristic of HVDRR as a result of loss of hormonal control [8–11].

VDR is highly conserved among vertebrates ranging from fish to human and displays broad tissue distribution with the highest expression in the intestine. Nevertheless, coinciding with the subnanomolar affinity for its natural ligand, VDR is present in low abundance, making direct purification difficult [12–14]. The VDR genes from multiple species have been cloned [15–22], and the heterologous expression and purification of recombinant VDR has facilitated its biochemical characterization and extended our understanding of the mechanism of vitamin D action [23–30]. Crystallographic studies of VDR have provided insight into its structure–activity relationships [31–34]. However, it remains unclear why VDR in complex with $1,25(OH)_2D_3$ and its analogs having distinct structures and unique biological effects, exhibit essentially the same protein structure in X-ray crystal studies [32,35–40]. NMR studies on VDR have been limited [29], largely owing to the unavailability of active and stable proteins in sufficient quantity. Previous purification of soluble forms of VDR or its ligand binding domain (LBD) from prokaryotic and eukaryotic systems usually involved time consuming multistep purification protocols to achieve adequate purity. Subsequently, after final removal of the purification tag the overall yield was rather low [23–30].

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[2] *Abbreviations used:* 1,25(OH)2D3, 1,25-dihydroxyvitamin D3; VDR, vitamin D receptor; VDRE, vitamin D response element; HVDRR, hereditary vitamin D resistant rickets; NMR, nuclear magnetic resonance; LBD, ligand binding domain; IPTG, isopropyl-β-Dthiogalactopyranoside; DTT, dithiothreitol; LB, Luria broth; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; *E. coli, Escherichia coli*.

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In this study, we describe an efficient overexpression and one-step purification protocol with high yields (25–70 mg/L culture) for full-length VDR and LBD proteins that allows economically feasible isotope labeling strategies required for structure–function characterization of these large molecular weight proteins by NMR spectroscopy.

Materials and methods

Reagents

All isotopic labeled ISOGRO® Complex Growth Media were purchased from ISOTEC™ (Sigma–Aldrich). Ammonium chloride (^{15}N, 99%), D-glucose (1,2,3,4,5,6,6-D7, 98%), and D-glucose (U-^{13}C6, 99%; 1,2,3,4,5,6,6-D7, 97–98%) were purchased from Cambridge Isotope Laboratories. Deuterium oxide (99% D), isopropyl-β-d-thiogalactopyranoside (IPTG), and dithiothreitol (DTT) were from Sigma–Aldrich. 1,25$(OH)_2D_3$ was purchased from SAFC-Pharma (Madison, WI). 26,27-[^{3}H]-1,25$(OH)_2D_3$ was provided by Perkin-Elmer (Boston, MA). All other chemicals were from Fisher or Sigma.

Plasmid constructs and Escherichia coli expression strains

Plasmid p29LBD codes for the production of rat VDR LBD residues 116–423 (Fig. 1) with a C-terminal six-histidine tag flanked by a linker sequence containing a thrombin cleavage site [29]. Plasmid p29LBDm is derived from p29LBD as previously described [32]. This new construct encodes LBD in which the flexible 47 amino acid (165–211) loop has been truncated. Plasmid p29LBDm-NT is derived from p29LBDm without the C-terminal His-tag. Constructs p29VDR, p29VDRm, and p29VDRm-NT are the full-length counterparts of p29LBD, p29LBDm, and p29LBDm-NT; we refer to these constructs as VDR, VDRm, VDRm-NT and LBD, LBDm, LBDm-NT, respectively. *E. coli* strains Rosetta 2(DE3) (Novagen, Madison, WI, USA) and BL21-CodonPlus(DE3)-RIPL (Stratagene, Agilent Technologies, Inc., Santa Clara, CA, USA) are designated as Rosetta and BL21/RIPL, respectively.

Expression media

Luria broth (LB) medium was used as a control for recombinant protein expression, and M9 minimal medium was the basis for stable isotope labeling. For deuterium labeling, H_2O based M9 was replaced with 99% D_2O based M9 supplemented with 1 mL/L 100× BME vitamin solution (Sigma–Aldrich, St. Louis, MO, US), 200 mM $MgSO_4$, 100 μM $CaCl_2$, and 0.2% D-glucose (1,2,3,4,5,6,6-D7, 98%) as the sole carbon source. For additional isotope labeling, $^{15}NH_4Cl$ and/or D-glucose (U-^{13}C6, 99%; 1,2,3,4,5,6,6-D7, 97–98%) were used. To improve the expression of isotopically-labeled VDR and LBD, 0.5% ISOGRO (5 g/L) was added as a supplement to the M9 medium, and the pH was adjusted to 7.2. Kanamycin and chloramphenicol were added to final concentrations of 50 and 34 μg/L, respectively, for antibiotic selection.

Fig. 1. Domain organization of full length VDR. The scheme shows that VDR consists of several domains, including a DNA binding domain (DBD; C domain) and a ligand binding domain (LBD; E domain), linked by a hinge region (D domain) and preceded by a short A/B domain at the N-terminus. The area indicated by the cross in LBD denotes the flexible insertion region that was removed for the NMR studies. The numbers designate amino acid residues in rat VDR.

Expression of LBD and VDR

LBD and VDR were expressed as previously described [24–27] with some modifications. Chemically competent cells of BL21/RIPL and Rosetta were freshly transformed with the desired DNA construct following the general DNA transformation method. A starter culture in LB (20 mL) was inoculated with single colonies and grew at 30 °C overnight. The starting culture was added to 1 L of expression media (LB, M9 or 0.5% ISOGRO) with (50 μg/mL) kanamycin and (34 μg/mL) chloramphenicol and grown at 37 °C until A600 nm of 0.5–0.8 was reached. Cells were induced by addition of IPTG to a final concentration of 250 μM. Temperature after induction was varied to 18, 23, 30 or 37 °C, and the cultures were allowed to grow for up to 24 h. The cells were harvested by centrifugation at 2,500*g* at 4 °C for 20 min and rinsed with 0.9% (w/v) NaCl. The level of protein production was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE).

Purification of LBD and VDR from inclusion bodies

The refolding protocol used derives from a method developed for nuclease-brazzein fusion protein [41], but was tailored to the chemical properties of VDR. The refolding protocol was particularly modified for the first time to account for the p*I* and *redox state of cysteines* of VDR or LBD. These modifications included adjustment for buffer, pH, and addition of reducing agent to keep free cysteines in VDR in the reduced state. Briefly, the cell pellet prepared above was suspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 10 mM DTT, 0.3 mM phenylmethylsulfonyl fluoride, and 0.5 mg/mL lysozyme). Cells were sonicated with a sonic dismembrator (Fisher, Hanover Park, IL, USA) followed by centrifuging at 14,000*g* at 4 °C for 15 min. Cell pellets including inclusion bodies were washed once with 9 volumes of wash buffer (50 mM Tris–HCl, pH 8.0, and 100 mM NaCl), and once with the same wash buffer containing 0.5% (v/v) Triton X-100. After each addition, the slurry was stirred gently for 5 min and then centrifuged at 21,000*g* at 4 °C for 20 min. The final pellet was resuspended gently in 6 M guanidinium chloride dissolved in 40 mM Tris–acetate (pH 7.6) containing 100 mM DTT, and stirred for 2–3 h at room temperature. A clear solution was achieved by centrifugation at 21,000*g* at 4 °C for 20 min. The supernatant was dialyzed against 100 volumes of dialysis buffer containing 25 mM NaH_2PO_4/Na_2HPO_4 (pH 7.4), 50 mM KCl, and 2 mM DTT at 4 °C overnight followed by two more changes of the same buffer over 24 h to remove the denaturant and the reducing agent. The protein solution was concentrated to a final protein concentration of 0.5–1 mg/mL in an Amicon centrifugal filter (Millipore Ireland Ltd., Tullagreen, Carrigtwohill CO., CORK IRL).

The purity of the protein was analyzed by 12% SDS–PAGE. The yield of the protein was measured by the Bradford method (Bio-Rad, Hercules, CA). The protein was further characterized by electrospray ionization mass spectrometry (ESI–MS) at the University of Wisconsin Biotechnology Center.

Ligand binding assay

Ligand binding activity was determined using a competition assay as previously described [42]. Briefly, VDR or LBD (final 1 nM) was incubated with 0.16 μCi/mL 26,27-[^{3}H]-1,25$(OH)_2D_3$ (157.9 Ci/mmol) and increasing concentrations of 1,25$(OH)_2D_3$ on ice overnight in a buffer containing 50 mM Tris (pH 7.4), 1.5 mM EDTA, 150 mM KCl, 5 mM DTT and 0.2% CHAPS. Hydroxyapatite gel (Bio-Rad, Hercules, CA) as 50% slurry was added and mixed at 10 min intervals for 30 min to bind the protein. The resin was washed three times with a buffer containing 50 mM Tris (pH 7.4), 1.5 mM EDTA and 0.5% Triton X-100, and subjected to tritium