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Conformational dynamics of human FXR-LBD ligand interactions studied by hydrogen/deuterium exchange mass spectrometry: Insights into the antagonism of the hypolipidemic agent *Z*-guggulsterone

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ABSTRACT

Farnesoid X receptor (FXR) is a member of the nuclear receptor superfamily of transcription factors that plays a key role in the regulation of bile acids, lipid and glucose metabolisms. The regulative function of FXR is governed by conformational changes of the ligand binding domain (LBD) upon ligand binding. Although FXR is a highly researched potential therapeutic target, only a limited number of FXR-agonist complexes have been successfully crystallized and subsequently yielded high resolution structures. There is currently no structural information of any FXR-antagonist complexes publically available. We therefore explored the use of amide hydrogen/deuterium exchange (HDX) coupled with mass spectrometry for characterizing conformational changes in the FXR-LBD upon ligand binding. Ligand-specific deuterium incorporation profiles were obtained for three FXR ligand chemotypes: GW4064, a synthetic non-steroidal high affinity agonist; the bile acid chenodeoxycholic acid (CDCA), the endogenous low affinity agonist of FXR; and *Z*-guggulsterone (GG), an in vitro antagonist of the steroid chemotype. A comparison of the HDX profiles of their ligand-bound FXR-LBD complexes revealed a unique mode of interaction for GG. The conformational features of the FXR-LBD–antagonist interaction are discussed.

1. Introduction

The farnesoid X receptor (FXR) is a therapeutic target with the potential to modulate metabolic pathways associated with diverse liver and metabolic disorders. FXR's prominence stems from its role as a key regulator of bile acid homeostasis, and glucose and lipoprotein metabolisms [1,2]. FXR has been identified as a bile acid sensor and is expressed mainly in the liver, intestine, and kidney. Many reviews have discussed the physiologically relevant role of FXR regulation and the potential of FXR as a target for synthetic ligands to prevent, manage or treat metabolic diseases, such as hyperlipidemia and type 2 diabetes [3–5].

FXR is a member of the nuclear receptor (NR) superfamily and a ligand-activated transcription factor. Since chenodeoxycholic acid (CDCA) was identified as the endogenous agonist of FXR that modulates the expression of target genes related to bile acid metabolism, FXR activation rapidly became the objective of intense research efforts. Synthetic FXR agonists have been developed with the potential to treat cholestatic liver diseases, including primary biliary cirrhosis, and metabolic disorders [6]. For instance, FXR agonists have been identified that improve myocardial fatty acid metabolism in obese (fa/fa) Zucker rats, and to counteract pro-atherogenic lipoprotein profiles and thereby confer protection against aortic plaque formation in ($ApoE^{-/-}$) mice, a model of accelerated atherosclerosis [7–10]. Ursodeoxycholic acid (UDCA), an isomer of CDCA, was the first FXR-targeting drug approved by the FDA to treat primary biliary cirrhosis [11].

The X-ray structures of agonist-bound FXR complexes show the classical NR fold, consisting of 12 helices that form a three-layer sandwich harboring the LBC. Ligand binding is primarily facilitated by interactions with residues located in helices 3, 5, 6, 7, and 11. Ligand binding changes the position of the AF-12 helix and facilitates recruitment of co-activator proteins. Despite FXR's importance as a potentially therapeutically relevant receptor, only a limited number of X-ray structures of the FXR-LBD bound to high affinity synthetic ligands are available that may guide structure–activity relationship (SAR) studies. The published X-ray crystal structures of FXR-ligand complexes illustrate the binding modes of five different ligand chemotypes: three steroid-like agonists [12,13], ten stilbene-based agonists (GW4064 and derivatives) [14–18], seven

Abbreviations: HDX, hydrogen/deuterium exchange; LC–ESI-MS, liquid chromatography electrospray ionization mass spectrometry; FXR-LBD, farnesoid X receptor ligand binding domain; LBC, ligand binding cavity; CDCA, chenodeoxycholic acid; GG, *Z*-guggulsterone; 6ECDCA, 6-ethylchenodeoxycholic acid; UDCA, ursodeoxycholic acid; DMSO, dimethylsulfoxide; TCEP, Tris (2-carboxyethyl) phosphine hydrochloride; TOF, time of flight; PPARγ, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; THR, thyroid hormone receptor; ER, estrogen receptor; VDR, vitamin D receptor; GR, glucocorticoid receptor

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benzimidazole-based agonists [19,20], two azepino [4,5-b] indole agonists (XL335 and FXR 450) [21,22], and one fexaramide y-shaped ligand [23]. To date, there are no published crystal structures available for antagonist-bound FXR-LBD complexes or the apo-protein. The conformational plasticity of FXR's LBD has been discussed as one of the possible reasons for the limited success of X-ray structural analyses of FXR-LBD ligand complexes [12].

HDX-MS has been previously used successfully for studying the conformational properties of diverse nuclear receptors and their conformational changes upon binding to diverse ligands [24–27]. In the current study, we used HDX-MS for studying conformational changes of the FXR-LBD upon binding to different classes of ligands: (1) CDCA, a primary bile acid ligand and a low affinity agonist; (2) GW4064, a synthetic non-steroidal stilbene-based high affinity agonist; and (3) *Z*-guggulsterone (GG), a natural steroidal FXR ligand and one of the few ligands described as an in vitro FXR antagonist ($K_d > 5$ μM) [28]. A comparison of the deuterium incorporation profiles obtained in the absence and presence of the chemically diverse ligands enabled the identification of distinct regions of the FXR-LBD that exhibit ligand-specific exchange behaviors. Insights into the ligand-dependent modulation of the conformational properties within the LBD may aid the development of selective bile acid receptor modulators (SBARMs) which show promise to manipulate FXR's pleiotropic regulation of metabolic networks.

2. Materials and methods

2.1. Materials

Deuterium oxide (D_2O, 99.9% deuterium) was from Sigma-Aldrich Chemical Co. (St. Louis, MO). GG was purchased from ChromaDex™ Corporate (Irvine, CA). GW4064 and CDCA were obtained from Tocris (Ellisville, MO). All ligands were prepared as 10 mM stock solution in dimethylsulfoxide (DMSO). All other materials were obtained from standard commercial sources.

2.2. Protein expression and purification

The pET 15B vector containing hFXR-LBD, residues 193–472, was transformed into *Escherichia coli* BL21 (DE3) pLysS and grown on LB agar plates. A single colony was used to inoculate 100 mL of 2XYT medium with antibiotics (Carbenicillin 100 μg/mL and Chloramphenicol 35 μg/mL) and grown overnight at 37 °C. The overnight culture was centrifuged for 10 min at room temperature and then the pellet was resuspended in 6 mL of fresh 2XYT medium. Each liter of fresh 2XYT medium with antibiotics (ampicillin 150 μg/mL and Chloramphenicol 35 μg/mL) was inoculated with 1 mL of the resuspended cells (a total of 6 L) and grown at 37 °C to $A_{600} = 0.6$. Protein expression was induced with 0.8 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and the cells were allowed to continue growing for 4 h at 20 °C. The cell pellets were harvested by centrifugation (4000 rpm, 25 min, 4 °C), resuspended in cell wash solution (150 mM NaCl, 50 mM Tris and 10% w/v sucrose), centrifuged again and frozen at −80 °C.

The frozen cell pellets were resuspended in buffer solution (50 mM sodium phosphate, 0.5 M NaCl, 0.5 mM CHAPS, 15 mM imidazole, 0.5 M sucrose, pH 7.3) and centrifuged. The His_6-tagged FXR-LBD was mixed with Clontech Talon Co^{2+} polyhistidine affinity resin (equilibrated with the above buffer) at 4 °C for 45 min. The proteins were eluted into a solution containing 50 mM sodium phosphate, 0.5 M NaCl, 0.5 mM CHAPS, 200 mM imidazole, and 0.5 M sucrose, pH 7.3. The His tag was removed by thrombin digestion at 4 °C (48 h) followed by purification on a column packed with Co^{2+} resin to yield purified human FXR-LBD monomer which was used for all experiments. The protein concentration was determined spectrophotometrically at 280 nm, and the purity (over 95%) was judged by sodium dodecyl sulfate-acrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry (Fig. S1).

2.3. Fluorescence titration experiment

Steady state fluorescence measurements were performed with a Perkin Elmer LS 50 luminescence spectrophotometer. The protein sample was excited at 295 nm, and the sample temperature was maintained at 25 °C. The ligand solutions (1×10^{-3} M) were titrated into a fixed volume (400 μL) of FXR-LBD (1×10^{-5} M) until the ligand/protein ratio reached 5:1. The fluorescence intensity was measured over a wavelength range of 305–480 nm. The fluorescence spectra were corrected (F_{cor}) for background fluorescence, instrument-dependent monochromator and photomultiplier response emission and dilution factor effects: $F_{cor} = (F_o - F_B) \times 100 \times A/F_S$, where F_o is the measured fluorescence intensity, F_B is the background fluorescence of the specific ligand, A is the dilution factor of the protein sample, and F_S is the emission correction factor. Based on the FXR-LBD-GG binding data, the dissociation constant (K_d) of GG was derived by fitting a curve to the Hill equation [29] using the GraphPad Prism software program.

2.4. HDX-MS analysis

The purified FXR-LBD protein (15 μL, 98 μM, in 50 mM sodium phosphate, 0.5 M NaCl, 0.5 mM CHAPS, 1 mM TCEP, 0.5 M sucrose and 10% glycerol, pH = 7.4) was equilibrated for 30 min in the presence of the respective DMSO solution (0.5 μL, ±10 mM ligand). The ligand/monomeric protein molar ratio was 3.2:1. The small amount of DMSO (3.3% v/v) did not affect protein binding as indicated by fluorescence titration data as well as by intact protein HDX data. The D_2O buffer (50 mM sodium phosphate, 0.5 M NaCl, 0.5 mM CHAPS, 1 mM TCEP, 0.5 M sucrose and 10% glycerol, pH 7.3) was equilibrated for 30 min in the presence of the same percentage of DMSO. The exchange reaction was initiated by adding 10-times D_2O buffer (150 μL). At different reaction time points, 0.5, 1, 2, 5, 10, 30 and 60 min, aliquots (15 μL) were added to pre-chilled vials containing the same volume of quenching solution (15 μL, 0.42% phosphoric acid, pH 2.5). The quenched sample was immediately frozen in liquid nitrogen for subsequent LC–ESI-MS analysis. Experiments were performed in triplicate.

The experimental conditions for the ligand binding studies in combination with the HDX approach were chosen to ascertain that both the FXR-LBD protein and the respective low affinity ligand retained solubility throughout the period of labeling. Even in the presence of structure promoting and stabilizing additives (CHAPS, sucrose, glycerol, DMSO) the FXR-LBD protein showed a high tendency to precipitate in the presence of low affinity ligands. This limited our flexibility in using large excess of low affinity ligands. We believe that the absence of X-ray structures of FXR-LBD in complex with low affinity ligands is at least in part caused by this shortcoming. The reported dissociation constant/binding affinity values for GW4064, CDCA, and GG are 0.06 μM [30], 10 μM [31], and GG > 5 μM [28], respectively. The bound fraction of FXR-LBD in each ligand binding study before initiation of the HDX reaction is ~100% as estimated by applying (Eq. (1)) [32]:

$$\text{fraction of protein bound} = \frac{(L_T + P_T + K_d) - \sqrt{(L_T + P_T + K_d)^2 - 4L_T P_T}}{2P_T} \quad (1)$$

where L_T and P_T are the total ligand concentration and protein concentration used, respectively, and K_d is the dissociation constant. In the present experimental protocol we kept the mole ratio between protein and ligand constant (being 3.2:1) during the time course of labeling. The EX2-type mass isotope distributions of the peptides evaluated indicated that the fraction of 'protein bound' was the predominant population present in solution (Fig. S5).

For intact protein HDX-MS, the protein was eluted through a Micro Trap™ C4 column (1 × 8 mm) with a steep of 10–90% (v/v) B gradient within 10 min (mobile phase A: 0.1% HCOOH in H_2O, mobile phase B: