



Detergent Screening for NMR-Based Structural Study of the Integral Membrane Protein, Emopamil Binding Protein (Human Sterol $\Delta 8-\Delta 7$ Isomerase)

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Abstract Human sterol $\Delta 8-\Delta 7$ isomerase, commonly known as emopamil binding protein (EBP), is an essential protein in the cholesterol-synthetic pathway, and mutations of this protein are critically associated with human diseases such as Conradi-Hunermann–Happle or male EBP disorder with neurological defects syndrome. Due to such a clinical importance, EBP has been intensively investigated and some important features have been reported. EBP is a tetra-spanning membrane protein, of which 2nd, 3rd, and 4th membrane-spanning α helices play an important role in its enzymatic function. However, detailed structural feature at atomic resolution has not yet been elucidated, due to characteristic difficulties in dealing with membrane protein. Here, we over-expressed EBP using *Escherichia coli* and performed detergent screening to find suitable membrane mimetics for structural studies of the protein by NMR. As results, DPC and LMPG could be evaluated as the most favorable detergents to acquire promising NMR spectra for structural study of EBP.

Keywords emopamil binding protein, cholesterol, sterol $\Delta 8-\Delta 7$ isomerase, detergent screening, NMR

Introduction

Cholesterol, as a major sterol in mammalian cells, is a critical component to maintain and build membranes, and controls membrane fluidity over the range of physiological temperatures.¹⁻³ Biosynthesis of cholesterol is a complex pathway associated with a variety of intermediates and enzymes.⁴ After formation of lanosterol via the mevalonate pathway, cholesterol-synthetic pathway is divided into two branches, the Bloch pathway producing desmosterol and the Kandutsch-Russell pathway producing 7-dehydrocholesterol.^{5,6} Both pathways possess the same enzymatic system but differ in the stage at which the C24 double bond is reduced. For example, the sterol $\Delta 8-\Delta 7$ isomerase converts zymosterol to cholestadienol in Bloch pathway, whereas in Kandutsch-Russell pathway the enzyme is involved in the lathosterol formation from zymosterol. Enzymes working in the cholesterol synthesis pathway are conspicuously associated with diseases. Representatively, a variety of different mutations of $\Delta 7$ -sterol reductase cause a frequent malformation syndrome (Smith-Lemli-Opitz syndrome), and dysfunction of $\Delta 8-\Delta 7$ isomerase causes a Conradi-Hunermann–Happle (CHH) syndrome or male EBP disorder with neurological defects (MEND) syndrome.⁷⁻⁹ The human $\Delta 8-\Delta 7$ sterol isomerase, which can bind to the antiischemic drug emopamil, was named emopamil binding protein (EBP).¹⁰⁻¹² In addition, as a variety of structurally

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distinct pharmacological compounds including receptor σ -ligand SR31747A, trifluoperazine, and tamoxifen showed an ability to inhibit the $\Delta 8$ - $\Delta 7$ isomerase activity, it is needed to get structural information underlying the advanced mechanism of those inhibitors.¹³⁻¹⁵

Due to the biological importance, molecular feature of EBP has been intensively studied and some useful information has been reported. For example, Glossmann and colleagues revealed that H77, E81, E123, T126, N194 and W197 in human EBP are essential for its isomerase activity. Karst and colleagues also identified essential residues of EBP from *Zea mays*.^{16,17} In addition, EBP has been known as an integral membrane protein with four putative membrane spanning helices.¹² However, structural characterization of EBP at atomic resolution has not been achieved as its high proportion of hydrophobic region hindered atomic-resolution NMR and/or crystallographic studies.

Recently, several integral membrane proteins were successfully cloned and expressed in *Escherichia coli* system.¹⁸⁻²⁰ After acquiring an enough amount of membrane protein as a prerequisite for structural study by solution NMR, it is a critical starting point screening experimental conditions to get a well separated ¹H/¹⁵N chemical shift correlation spectra. High-quality NMR spectra can be obtained by a complicated optimization of conditions including temperature, protein preparation method, buffer components, pH, and suitable membrane mimetics.²¹ Particularly for membrane proteins, it exerts a great influence on the quality of NMR spectrum which kind of membrane mimetic environment is employed.²² In the present study we expressed the membrane protein EBP in *E. coli* and conducted detergent screening to get a good NMR spectrum. We tested various detergents and their mixed detergents, which are varied in length of hydrophobic tails and head group properties.^{23,24} We expect that this approach could critically contribute to progressing steps to determine tertiary structure of EBP.

Experimental Methods

Cloning and Plasmid Construction The cDNA of human EBP was amplified by polymerase chain reaction (PCR), followed by ligation into a pET16b vector using two restriction endonuclease sites, NcoI and BamHI. The final protein construct contained an N-terminal poly-histidine tag to facilitate purification.

Recombinant Expression of EBP in *E. coli* *E. coli* Rosetta2(DE3) cells transformed with the constructed plasmids were plated on LB-agar containing ampicillin and chloramphenicol and incubated overnight at 37 °C. A single colony was inoculated into an LB broth medium containing 100 µg/mL ampicillin and 50 µg/ml chloramphenicol. This starter culture was grown for 10 h at 37 °C. 1.5 mL of the culture was then transferred into 1 L of M9 minimal medium, which contained ampicillin, chloramphenicol, glucose, MEM vitamins, 0.1 mM CaCl₂, 1 mM MgSO₄, and ¹⁵NH₄Cl for isotopic labeling. This large-scale culture was performed at room temperature until the OD₆₀₀ reached 0.6-0.7, followed by induction of protein expression with 1 mM IPTG added to the cultures. Following 24-hours induction, the cells were harvested by centrifugation. Expression of recombinant EBP was confirmed by Western blot using a monoclonal anti-5X His mouse antibody (Cell Signaling Technology, Danvers, MA).

Purification of Human EBP- Harvested cells were resuspended in 20 ml lysis buffer (75 mM Tris, pH 7.8, 300 mM NaCl) per gram of wet cells. The lysis buffer also contained 2 mM ethylenediaminetetraacetic acid (EDTA), 5 mM magnesium acetate, 2 mg/mL lysozyme, 0.2 mg/mL of both DNase and RNase, and either 50 µL of protease cocktail inhibitor (Sigma-Aldrich, St. Louis, MO) or 0.2ml of phenylmethylsulfonyl fluoride (PMSF) per gram of cells. The suspension was tumbled for 90 min at room temperature followed by sonication on ice for 5 min with a 50% duty cycle at approximately 57 W using a Misonix sonicator

(Farmingdale, NY). Inclusion bodies were pelleted by centrifugation at 20,000 rpm for 30 min using a Beckman-Coulter JA 25.5 rotor (approximately 48,000 g) (Indianapolis, IN). The inclusion bodies were then solubilized in 20 mL of buffer A (40 mM HEPES pH 7.8 and 300 mM NaCl) containing 3% (v/v) Empigen detergent (Sigma-Aldrich, St. Louis, MO) per gram of cells. The solution was then tumbled at 4°C until the mixture was clarified (approximately 2 h) and then centrifuged to remove any remaining insoluble particulates. Ni-NTA resin (1.2 mL/g of cells) equilibrated with buffer A was added to the supernatant. The mixture was then tumbled for 1 h at room temperature. The resin was loaded into a chromatography column and sequentially washed with the buffer A containing 3% (v/v) Empigen and with the buffer A containing 75 mM imidazole and 1.5% (v/v) Empigen, which eluted non-His6-tagged proteins from the resin. Empigen was subsequently exchanged with other detergents by re-equilibrating the column with 12 column volumes of 20 mM sodium phosphate (pH 7.2) containing either 0.5% β -n-decylmaltoside (DM), 0.5% dodecylphosphocholine (DPC), 0.2% n-tridecylphosphocholine (Fos-13), 0.2% n-tetradecylphosphocholine (TDPC), 0.2% lyso-myristoylphosphatidylcholine (LMPC), 0.1% lyso-palmitoylphosphatidylcholine (LPPC), 0.1% lyso-myristoylphosphatidylglycerol (LMPG), 0.1% lyso-palmitoylphosphatidylglycerol (LPPG), 0.5% sodium dodecyl sulfate (SDS), 0.5% DPC/LPPG (7:3), 0.5% DPC/TDPC (5:5), 0.5% DPC/LMPG (8:2), 0.5% DMPC/LMPG (2:8), 0.5% DMPC/DPC (2:8), 0.5% DMPC/LMPG (2:8), or 4% bicelle DMPC/DHPC (q: 0.3). EBP was finally eluted from the column by washing with the buffer A containing 250 mM imidazole (pH 7.8), 2 mM DTT, and designated detergents. After adjusting pH by adding acetic acid, the eluted protein was concentrated at 20 °C using a centrifugal concentrator (10 kDa Amicon Ultra 4ml, EMD Millipore).

Crosslinking experiment- The eluted sample from Ni-NTA resin was enforced to pass through an

Econo-Pac 10 DG buffer exchange column (10 ml of bed volume) pre-equilibrated with 25 mM Phosphate buffer (pH 6.5) containing 20 mM NaCl, 1 mM DTT, 1 mM EDTA, and 0.0 5% LMPG, to remove imidazole in the protein solution which can randomly reacts with glutaraldehyde. For crosslinking, 20 μ g of protein was mixed with glutaraldehyde at the concentrations ranging from 0 to 16 mM, followed by a 3-hour incubation at room temperature or at 42°C.

NMR Spectroscopy- 2D [$^1\text{H}/^{15}\text{N}$]TROSY NMR spectra²⁵ were acquired using Bruker 600, 800, and 900 MHz spectrometers equipped with TXI cryoprobes. NMR data were processed with the NMRPIPE²⁶ and analyzed using the SPARKY program.²⁷

Results and Discussion

Gene cloning and protein purification- Gene of human membrane protein EBP was successfully amplified by PCR and subcloned into pET16b. The final construct contains poly histidine at the N-terminus which facilitates a simple purification of the protein. EBP were successfully overexpressed in M9 media using 1 mM IPTG at room temperature. We first examined the location of expression in *E. coli* using ultracentrifugation and western blotting. As a result (Figure 1), the expressed EBP wholly existed in the inclusion body, which implies *E. coli* bacterial expression system is not compatible with correct folding of this protein. Alternatively, therefore, EBP was prepared by a refolding method using harsh detergents as described previously.²² Refolding from inclusion body usually provides an additional benefit of a very efficient purification. We could also obtain a clearly purified EBP through the single step of his-tag affinity chromatography (Figure 1).

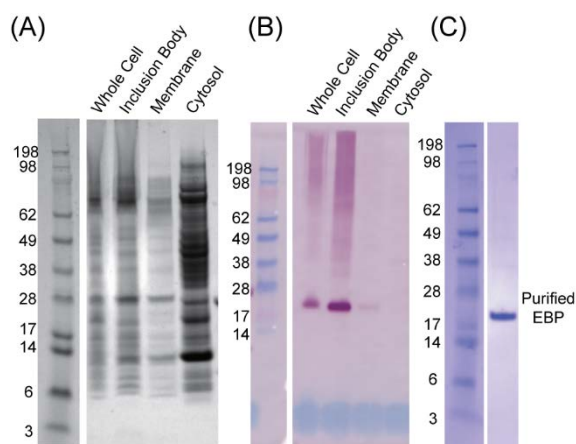


Figure 1. Detection of EBP in cells (A and B) and in purified solution (C), by SDS-PAGE (A and C) and Western blot (B). Majority of expressed EBP was located in inclusion body (A and B), which was successfully purified (C).

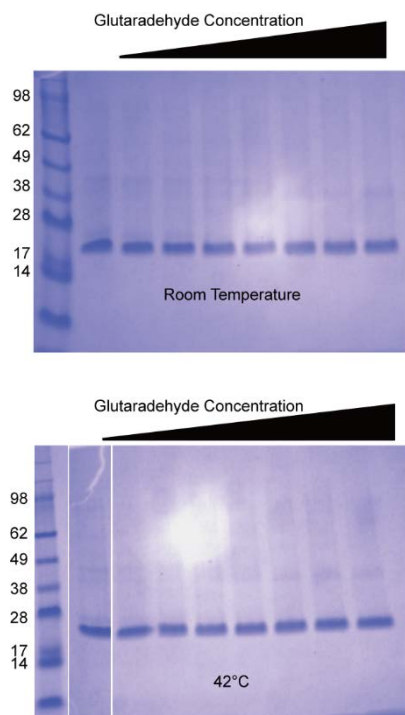


Figure 2. Cross-linking experiment of EBP using glutaraldehyde. Experiments were performed at room temperature and 42 °C.

Cross-linking experiment- Verifying oligomerization state of integral membrane protein in detergent solution is notoriously challenging because the amount of detergents associated with the protein is usually ambiguous. Sedimentation equilibrium centrifugation and static light scattering, which represent two classical methods determining the membrane protein molecular weight, requires expensive and hard-to-use equipment, and should be very carefully performed to get an accurate result. Thus, alternatively, we attempted to check the oligomerization states of EBP by cross-linking experiment as an easy-to-use method. We reasoned that monomeric characteristic would lead to a single protein band corresponding to monomeric size in SDS-PAGE even after cross-linking reaction. The cross-linking experiment on EBP with glutaraldehyde showed a single band at the size corresponding to a monomer, which provided sketchy information that EBP behaved as a monomer under the solution condition employed (Figure 2).

Detergent screening of EBP- Particularly for membrane proteins, NMR spectral quality including the criteria of number of observed peaks, degree of broadening, and degree of separation, is very sensitive to the feature of detergents used, depending on their head groups, kind of charges, and tail length.²¹ In this work, we tested various detergents including single-detergent micelles, mixed micelles and bicelles, for acquisition of promising 2D [¹H/¹⁵N] TROSY spectrum, which is generally used as a basic fingerprinting spectrum of proteins for starting NMR study. Unfortunately, the purified EBP coordinated with DM or bicelles couldn't be eluted at the purification step using his-tag affinity resin (data not shown), whereas other detergent systems efficiently worked. As shown in Figure 3, the [¹H/¹⁵N] TROSY spectra of EBP in the presence of nine different detergents varied in quality. The largest number of

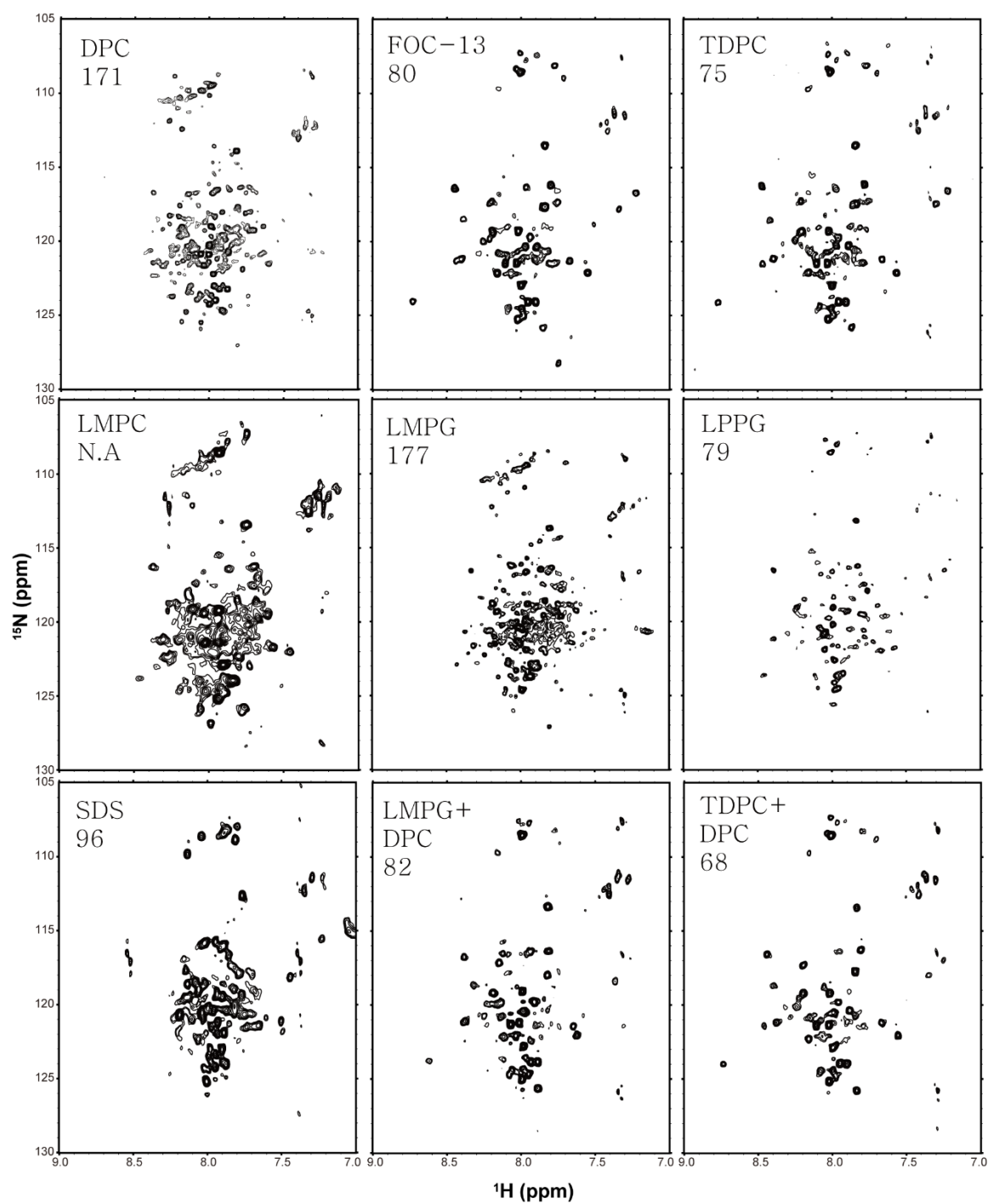


Figure 3. Comparison of the 2D $^1\text{H}/^{15}\text{N}$ TROSY spectra of EBP in various detergent micelles. The detergents used and the number of resolvable backbone amide peaks are indicated in each panel (N.A., not available)

amide resonances were observed in LMPG micelles (177 of the 228 expected peaks), which is slightly

larger than in the next favorable detergent DPC micelles (171 peaks). This result confirms that the NMR spectral quality of EBP can be also modulated by detergent properties particularly regarding the acyl chain length and the kind of head group. As in cases of DPC, FOC-13 and TDPC, which commonly possess phosphocholine-type head groups, detergents with the shorter tail were the more suitable for obtaining higher-quality spectrum of EBP. In comparison between phosphatidylglycerol-type head groups,

LMPG that has shorter tail than LPPG was also better for NMR study. Collectively, it depended on the length of acyl chain which one is more suitable for NMR studies of EBP between phosphocholine and phosphatidylglycerol detergents. As a result, DPC and LMPG could be selected in our screening as the best detergents for NMR-based structural studies of EBP. Therefore, we tried to test the detergent mixture of DPC and LMPG, but the result was not positive (Figure 3). Meanwhile, interestingly, the resonances from indole NHs in the eleven tryptophan residues of EBP couldn't be fully resolved in both LMPG (5 or 6 of the 11 expected peaks) and DPC (3 peaks). In contrast, 10 peaks of indole NH were nicely observed in LPPG detergent (Figure 4), in spite of less resolved backbone amide resonances (Figure 3). The reason for the apparent discrepancy in spectral quality between backbone and indole side chain remains to be investigated. However, as the indole

NH side chain peaks usually serves as a criterion assessing structural order of a membrane-spanning protein, we can infer that the structure of EBP is likely well-ordered in LPPG micelles, although some backbone amide signals didn't show up in the [$^1\text{H}/^{15}\text{N}$] TROSY spectrum. In conclusion, the system using LPPG as well as DPC and LMPG is worthy of further optimization in order to establish the most suitable membrane-mimetic system for EBP in terms of NMR study. Although the molecular behaviors of membrane proteins in membrane-mimetic systems are not easily predictable, we expect that the present results could contribute to the progressing structural study of EBP, by providing a fundamental information for suitable experimental conditions for NMR.

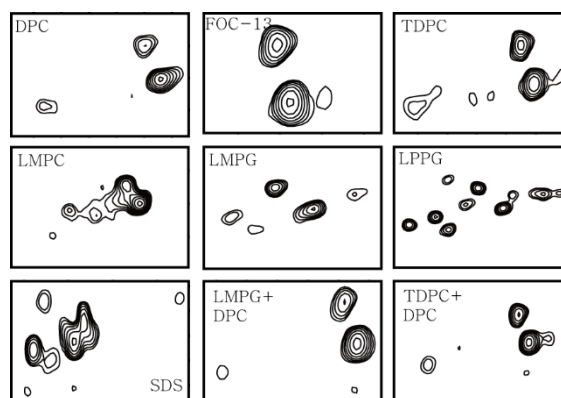


Figure 4. Comparison of the tryptophan indole peaks of EBP in various detergent micelles.

Acknowledgements

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