METHODS AND APPLICATIONS

Solution NMR resonance assignment strategies for β -barrel membrane proteins

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Abstract: Membrane proteins in detergent micelles are large and dynamic complexes that present challenges for solution NMR investigations such as spectral overlap and line broadening. In this study, multiple methods are introduced to facilitate resonance assignment of β -barrel membrane proteins using Opa₆₀ from *Neisseria gonorrhoeae* as a model system. Opa₆₀ is an eight-stranded β -barrel with long extracellular loops (~63% of the protein) that engage host receptors and induce engulfment of the bacterium. The NMR spectra of Opa₆₀ in detergent micelles exhibits significant spectral overlap and resonances corresponding to the loop regions had variable line widths, which interfered with a complete assignment of the protein. To assign the β -barrel residues, trypsin cleavage was used to remove much of the extracellular loops while preserving the detergent solubilized β -barrel. The removal of the loop resonances significantly improved the assignment of the Opa₆₀ β -barrel region (97% of the resonances corresponding to the β -barrel and periplasmic turns were assigned). For the loop resonance assignments, two strategies were implemented; modulating temperature and synthetic peptides. Lowering the temperature broadened many peaks beyond detection and simplified the spectra to only the most dynamic regions of the loops facilitating 27 loop resonances to be assigned. To further assign functionally important and unstructured regions of the extracellular loops, a synthetic 20 amino acid peptide was synthesized and had nearly complete spectral overlap with the full-length protein allowing 17 loop resonances to be assigned. Collectively, these strategies are effective tools that may accelerate solution NMR structure determination of β -barrel membrane proteins.

Keywords: outer membrane protein; NMR; assignments; β -barrel membrane protein

Introduction

Solution NMR structure determination of membrane proteins allows the investigation of membrane

protein dynamics and their role in protein function.^{1–6} However, the number of high-resolution polypeptidic membrane protein structures is limited. To date, there are six NMR solution structures of β -barrel membrane proteins^{1–6} (compared to the 89 unique β -barrel structures deposited in the Protein Data Bank), all except for OprH have corresponding X-ray crystal structures.^{7–12} The first β -barrel membrane protein structure determined with solution NMR was in 2001⁴ and the latest structure was determined in 2011³ yielding an average of 0.6 structures a year. The dearth of solution NMR membrane

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protein structures is likely due to the difficulty of obtaining quality NMR spectra, which interferes with assigning the resonances and obtaining quality structural restraints.

There have been many NMR developments to overcome some of the challenges in studying large complexes including membrane proteins. Deuteration¹³ and transverse relaxation optimized spectroscopy (TROSY) pulse sequences¹⁴ circumvent relaxation effects that hinder NMR spectral quality of larger biomolecules (>25 kDa). In addition, assignment of resonances in regions with significant spectral overlap can be addressed with specific amino acid labeling^{15–17} as well as compensating for the loss of proton–proton distance restraints due to deuteration.^{18,19} Other methods, such as residual dipolar couplings²⁰ and paramagnetic relaxation enhancement,²¹ have provided additional structural restraints crucial for large α -helical proteins (e.g., α -helical membrane proteins in detergent micelles). With these advances, six solution NMR β -barrel membrane protein structures (varying in the number of β -strands (8–19) and molecular weight (16–31 kDa) have been determined.^{1–6}

In this study, the difficulties of assigning Opa₆₀, a β -barrel membrane protein from *Neisseria gonorrhoeae*,²² are outlined and strategies for circumventing these challenges are demonstrated. Opa₆₀ is a putative eight-stranded β -barrel with four extracellular loops, which comprise approximately 63% of the protein.²³ The two main difficulties in assigning Opa₆₀ were: (1) the abundant loop resonances occluded some of the β -barrel resonances, which are much less intense than the loop resonances and (2) the loops have variable dynamics with some resonances sharp and intense, while others are significantly line broadened. In addition to previously established methods such as specific amino acid labeling, several new strategies were used to assign the different regions of Opa₆₀. The β -barrel resonances (97%) were assigned by cleaving the extracellular loops with trypsin, which improved spectral quality and allowed resonances occluded by the loops to be observed. Assignment of loop resonances (24%) required both the manipulation of temperature and a synthetic peptide. Overall, 49% of Opa₆₀ was assigned and sufficient for structure calculations of the full-length Opa₆₀.

Results and Discussion

Challenges encountered in NMR investigations of Opa₆₀

The optimized solution condition yielded quality ¹⁵N, ¹H-HSQC (TROSY versions of pulse sequences were used throughout) spectra of Opa₆₀ with approximately 225 unique resonances observed, with many downfield resonances (>8.5 ppm) typically associated

with β -strands. Elevated temperature (40°C) was required to observe resonances corresponding to the β -barrel region. Despite the observation of a number of peaks that correspond to the β -barrel, the assignment was hindered due to spectral overlap with the more intense loop resonances. In addition, the high temperature necessary to observe the β -barrel resonances resulted in line broadening of a subset of the loop resonances, which interfered with assigning loop sequences. As a result, an assignment of Opa₆₀ proved to be unobtainable from a single condition.

Proteolysis facilitates assignment of β -barrel

In order to observe the less intense β -barrel peaks in the crowded region of the spectrum, a protease was used to cleave the extracellular loops from the protein–detergent complex. Based on the amino acid sequence, the predicted topology of the β -barrel, and the inaccessibility of the β -barrel to proteases,²⁴ trypsin is predicted to remove 94 residues [Fig. 1(A) and Supporting Information Fig. S1, red regions]. The remaining micelle-protected β -barrel would have an approximate molecular weight of 15 kDa (~30–35 kDa including the micelle). The Opa₆₀ β -barrel remains intact after proteolysis even upon boiling in sodium dodecyl sulfate (SDS) loading buffer (Fig. 2) despite being composed of five transmembrane fragments [topology shown in Fig. 1(A)]. The ¹⁵N, ¹H-HSQC of the trypsin-cleaved Opa₆₀ results in 147 unique resonances with approximately equal intensities that overlap with the full-length Opa₆₀ spectrum [Fig. 1(B)]. In addition to reduction of spectral overlap, significant improvement was observed in the 3D spectral quality (Supporting Information Fig. S2). Thus, using this methodology 97% of the transmembrane β -barrel and periplasmic turns of full-length Opa₆₀ was assigned (Supporting Information Fig. S3).

To determine whether this method is generally applicable to NMR investigations of β -barrel structures, two other proteins, Opa₅₀ and OprH, were investigated. Opa₅₀ and Opa₆₀ differ by one amino acid in the β -barrel region (Supporting Information Fig. S1); however, three of the extracellular loops differ significantly in sequence. The trypsin-treated Opa₅₀, as compared to the full-length protein, migrates on an SDS-PAGE (polyacrylamide gel electrophoresis) gel similarly to Opa₆₀ and indicates the β -barrel is maintained after cleavage even after boiling in SDS loading buffer (Fig. 2). The ¹⁵N, ¹H-HSQC spectrum of Opa₅₀ after trypsin cleavage overlaps with the corresponding peaks of the spectrum of the full-length protein [Fig. 1(C)]. The overlap with the full-length is essential for structure determination because the NOESY spectra of the full-length should be used for the final structure calculation. In addition, ¹⁵N, ¹H-HSQC of the trypsin treated Opa₅₀ and Opa₆₀ are nearly superimposable,

Figure 1. Trypsin cleavage of β -barrel membrane proteins. A: Topology diagram of Opa₆₀, Opa₅₀, and OprH with the extracellular residues predicted to be removed with trypsin colored red and the remaining residues colored in blue. The outlined red circles indicate the residues that were selected for the synthetic peptide. Extracellular loops 1–4 are labeled L1–L4. A sequence alignment of the three proteins is provided in Supporting Information Figure S1. B–D: Full-length (red) and trypsin-treated (blue) ^{15}N , ^1H -HSQC spectra of ^2H , ^{15}N -labeled Opa₆₀ (B), Opa₅₀ (C), and OprH (D).

indicating that the β -barrel structure of the different Opa proteins is likely identical (Supporting Information Fig. S4).

OprH has the longest extracellular loop (29 amino acids) of the six β -barrel membrane protein structures determined with NMR³ and, thus, was used to investigate the general applicability of the trypsin methodology for β -barrel membrane protein NMR resonance assignment. As both the ^{15}N , ^1H -HSQC spectra [Fig. 1(D)] and SDS-PAGE mobility (Fig. 2) indicate, the β -barrel remains unperturbed after trypsin cleavage. However, there were a few notable differences compared to the Opa proteins. Upon boiling in SDS loading buffer, the OprH β -barrel did not remain intact (Fig. 2) implying that the fold may be less stable than the Opa proteins. After cleavage, broadening of some extracellular loop resonances is observed [Fig. 1(D) inset] in OprH, which was not observed for Opa₅₀ or Opa₆₀ [Fig. 1(B, inset C)]. In contrast to the Opa proteins, cleavage of three of the OprH extracellular loops leaves most of the loop residues still attached to the β -barrel, which may alter the dynamics of the loops and result in the observed line broadening. Thus, the proteolytic cleavage may be generally applicable

to β -barrel membrane proteins with variable overall stabilities; however, significant cleavage (which can be predicted from amino acid sequence) should be achieved for optimal results.

Facilitating loop assignments: temperature

All solution NMR β -barrel membrane protein structures determined to date have contained relatively

Figure 2. SDS-PAGE of Opa₆₀ (Lanes 1–4), Opa₅₀ (Lanes 5–8), and OprH (Lanes 9–12) unfolded in 8M urea, folded via rapid dilution into detergent micelles, cleaved with trypsin, and boiled after trypsin cleavage.

Figure 3. ^{15}N , ^1H -HSQC spectra of ^1H , ^{15}N -labeled Opa₆₀ recorded at 40°C (red) and 10°C (blue). Backbone resonances assigned at low temperatures are labeled.

short extracellular loops [the longest loop that of OprH containing 29 amino acid; Fig. 1(A)]; thus, spectral overlap and line broadening from loop resonances did not hinder the assignment of the majority of the protein. Opa₆₀ has three long extracellular loops with two loops composed of over 40 amino acids. The loops are unstructured based on circular dichroism (data not shown), amide proton chemical shift, and the ^{15}N -edited NOESY spectrum (data not shown). NMR relaxation data suggests some loop regions are mobile on the nanosecond timescale ($\tau \approx 2\text{--}5$ ns compared to $\tau \approx 20$ ns for the β -barrel (and associated detergent, data not shown)). A subset of loop resonances exhibit significant line broadening at 40°C (Fig. 3; red spectrum) and could not be assigned. At lower temperatures, the intensity of the β -barrel peaks decrease beyond detection and the line widths of a population of loop resonances decrease. Of the 149 potentially observable resonances from the extracellular loops and N-terminus, 34 disperse well-defined resonances are observed (Fig. 3; blue spectrum). As the temperature decreases, the overall tumbling of the protein-detergent complex slows, causing the β -barrel peaks (and likely many of the loop residues near the β -barrel) to broaden beyond detection. However, the loop regions most extended from the micelle fluctuate more rapidly than the β -barrel-micelle complex and, therefore, may be observed at lower temperatures. Using a series of 3D NMR experiments, the amide

proton, nitrogen, and α -carbon were assigned for 27 of the 34 observed resonances (corresponding to residues 1–6 in the N-terminus; 109–110 in loop 2; 152–154, 160, 162–164, 166–175, and 177–178 in loop 3). A series of ^{15}N , ^1H -HSQC spectra were recorded at 10, 20, 30, and 40°C and the peaks were tracked through each spectrum in order to correlate the low temperature assignments to the assignment of the full length Opa₆₀ at 40°C.

Facilitating loop assignments: synthetic peptide

After observing that the most extended regions of the loops lacked secondary structure, unique individual peptides were considered.²⁵ Different peptide sequences were selected based on the biological relevance (two regions in loops 2 and 3 are identified to be important in engaging host receptors²⁶), solubility, and amino acid composition. There are several hydrophobic residues in the loop regions, which dramatically affected the GRAVY value²⁷ and likely the solubility of many peptides that were considered. The peptide selected corresponded to residues 159–178 in extracellular loop 3 [Fig. 1(A)] and had a GRAVY value²⁷ of -0.835 . Near complete spectral overlap was observed in the ^{15}N , ^1H -HSQC of the peptide and full-length Opa₆₀ [Figs. 4 and 5(A)].

Figure 4. An overlay of the ^{15}N , ^1H -HSQC spectrum of the synthetic peptide T*-V*-P-S*-N-A*-P-N-G*-A*-V*-T*-T*-Y-N-T*-D-P-K*-T* (* denotes ^{15}N isotope labeling) corresponding to a the hypervariable region of loop 3 [Fig. 1(A)] and the ^{15}N , ^1H -HSQC spectrum of ^1H , ^{15}N labeled Opa₆₀ (full-length) recorded at 10°C.

Figure 5. Backbone assignment of loop resonances using a synthetic peptide. A: ^{15}N , ^1H -HSQC spectra were recorded for both peptide and Opa₆₀ (zoomed in regions of specific resonances are shown) and based on overlap (indicated by black dashed line) identical spins in the full length and peptide were identified. B: The homonuclear 2D ^{15}N -edited ^1H , ^1H -TOCSY strips (black strips) identified side chain spin systems and compared to the HNCA strips (gray strips) to obtain the sequential assignment. Connectivity between C_α , β and C_α , γ resonances are shown with a dashed black line.

Only twelve residues were ^{15}N labeled, but through both heteronuclear and homonuclear NMR experiments the assignment of the peptide resonances and, the full-length by comparison, could be assigned. The amino acid ^1H and ^{15}N spin systems were identified with ^1H , ^1H -COSY, ^1H , ^1H -TOCSY, and ^{15}N -edited versions of each. Using the spin

system identifications from the TOCSY spectrum of the peptide, the amino acid type for HNCA strips was identified and, thus, sequentially assigned [Fig. 5(B)]. The sequential assignment of the peptide could then be transferred to the full length 3D spectra (based on spectral overlap in the ^{15}N , ^1H -HSQC spectra) and verified with the HNCA of the full

length protein. Given the amount of spectral overlap, amino acid composition, and chemical shift degeneracy in this loop region, previous attempts to assign the resonances proved too difficult prior to the peptide comparison.

Conclusion

The results presented provide specific strategies for assigning resonances of β -barrel membrane proteins in an effort to accelerate solution NMR structure determination. The difficulty for β -barrel membrane proteins is exemplified with the Opa proteins in comparison to previously determined β -barrel membrane proteins solution NMR structures. The Opa proteins have two domains with very different dynamical properties (the unstructured extracellular loops and the membrane embedded β -barrel). An approach was used that would isolate these two domains such that they could be investigated independently. Protease cleavage was the most effective way to assign the β -barrel resonances and as observed in the ^{15}N , ^1H -HSQC spectra [Fig. 1(B–D)], protease cleavage for three different β -barrel proteins did not affect the global fold of the β -barrel. To assign the loops, temperature was used to modulate the dynamics of the loops and provided conditions in which loop resonances could be assigned. Temperature should be sought first before attempting the third strategy of synthetic peptides for two reasons (1) cost and (2) to provide direction in the peptide design that would be most helpful in obtaining the most assignment coverage. The peptide strategy is likely only helpful for assigning unstructured regions. Alternatively, proteolytic cleavage sites can be engineered in specific extracellular loops to reduce spectral crowding for assignment of other loop resonances or in cases where loop cleavage destabilizes the protein. In summary, the strategies described here are effective tools in a much needed toolbox for solution NMR structure determination of membrane proteins.

Materials and Methods

Expression and purification of Opa₆₀ and Opa₅₀

Protocols for expression and purification were previously published.²⁸ Briefly, the gene for Opa₆₀ and Opa₅₀ were sub-cloned into pET28b from pEX vector constructs (provided by Martine Bos, Utrecht University) and transformed into BL21 (DE3) cells. Opa₆₀ was sub-cloned such that both N- and C-terminal fusion tags were included (MGSSHHHH HHSSGLVPRGSHM and KLAAALEHHHHHHH, respectively) and Opa₅₀ was sub-cloned to include only the N-terminal His-tag. Cells cultures were expressed in D₂O (99.8%) minimal medium containing 4 g/l ^{13}C (99%)-glucose and 1 g/l ^{15}N (99%)-ammonium chloride (Cambridge Isotopes Lab) at 310°C to

an OD₆₀₀ of ≈ 0.8 then induced with 1 mM isopropyl- β -thio-D-galactoside for 8 h. The cells were resuspended and lysed in 50 mM Tris-HCl and 150 mM NaCl (lysis buffer). The lysate was then centrifuged at 12,000g for 30 min. The pellet was resuspended in extraction buffer (lysis buffer with 8M urea) and solubilized overnight. The resuspension was centrifuged at 12,000g for 30 min and the supernatant was loaded onto a Co²⁺ immobilized metal affinity chromatography (IMAC) column equilibrated with 10 column volumes (CV) of extraction buffer. The column was then washed with 15 CV of wash buffer (20 mM sodium phosphate, pH 7.8, 150 mM NaCl, 20 mM imidazole, 8M urea) followed by an elution with 5 CV of elution buffer (20 mM sodium phosphate, pH 7.0, 150 mM NaCl, 680 mM imidazole).

Refolding of Opa₆₀ and Opa₅₀

The elution was concentrated to 200 μM and subsequently diluted 20-fold with 20 mM Tris-HCl, pH 8.0, 500 mM NaCl with 4.5 mM *n*-dodecyl-phosphocholine (FC-12, Anatrace). The sample was incubated at room temperature for five days. Protein folding was monitored based on the shift of apparent molecular weight on SDS-PAGE, until the final sample lacked the higher apparent molecular weight band of the unfolded species. The solution was concentrated and dialyzed against 4 L of 20 mM sodium phosphate, pH 6.2, and 150 mM NaCl; three times for an hour each. Final NMR samples were concentrated to 400–800 μM and contained 110–150 mM FC-12 as measured by comparing NMR peak intensities with standard FC-12 concentrations.

Expression, purification, and refolding of OprH

OprH expression plasmid was provided by Lukas Tamm (University of Virginia). Previously published protocols for expression, purification, and folding were followed.³ OprH was extracted in 50 mM sodium phosphate, pH 8, 300 mM NaCl, 20 mM imidazole, and purified over a Ni²⁺ IMAC column with 15 CV of wash buffer (50 mM sodium phosphate, pH 8, 300 mM NaCl, 20 mM imidazole, and 8M urea) followed by 5 CV of elution buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 250 mM imidazole, and 8M urea). After purification, OprH was concentrated to 400 μM and diluted 10-fold into OprH refolding buffer (20 mM Tris-HCl, pH 8.5, 5 mM EDTA, 600 mM L-arginine with 3% 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC, Anatrace)) and incubated at 310°C for 72 h. The diluted sample was concentrated and dialyzed against 2.5 L of 20 mM Tris-HCl, pH 8.5, 5 mM EDTA, and 50 mM KCl. The solution was then exchanged over a concentrator into sodium phosphate, pH 6.0, 50 mM KCl, 0.05% sodium azide for a final concentration of 600 μM OprH in 90 mM DHPC.

Trypsin proteolysis

Trypsin from porcine pancreas (Sigma-Aldrich) was added to the proteins in their respective final buffers at a trypsin:protein sample molar ratio between 1:50 and 1:100. After incubating overnight at room temperature, trypsin-treated samples were assessed with SDS-PAGE. SDS-PAGE samples were either boiled for 5 min or remained unboiled in SDS loading buffer and run alongside untreated samples both boiled and unboiled on 10% Tris-HCl gels. Gels were stained with Coomassie brilliant blue. With confirmation of complete proteolysis from the SDS-PAGE gel, trypsin was removed by flowing the solution over 0.5 mL of *p*-aminobenzamidinium-agarose resin (Sigma-Aldrich). The flow-through was then dialyzed against 4 L of 20 mM sodium phosphate, pH 6.2, and 150 mM NaCl; three times for an hour each. Final NMR samples were concentrated to 400–800 μ M and contained 110–150 mM FC-12 as measured by comparing NMR peak intensities with standard FC-12 concentrations.

Preparation of peptide

A 20 amino acid peptide corresponding to a region in the third extracellular loop of Opa₆₀ (Ac-TVPSNAPNGAVTTYNTDPKT-NH₂) was synthesized by Anaspec with ¹⁵N amide nitrogen incorporation for all threonine, valine, alanine, and lysine residues. The lyophilized peptide was resuspended in 20 mM sodium phosphate, pH 6.2, and 150 mM NaCl at a concentration of 1.0 mM.

NMR spectroscopy

NMR spectra were collected on Bruker AVANCE spectrometers operating at proton frequencies of 600 MHz and 800 MHz equipped with Bruker 5 mm TXI cryoprobes and recorded at 10, 20, 30, and 40°C for Opa₆₀ and the synthetic peptide, 40°C for Opa₅₀, and 45°C for OprH. Spectra were processed with Topspin and assigned using CARA.²⁹ In order to assign the backbone, TROSY versions of HNCA, HN(CO)CA, HNCO, HN(CA)CO, HN(CA)CB, and HN(COCA)CB pulse sequences were recorded for both the full-length and trypsin-treated samples. ¹⁵N, ¹H TROSY-HSQC spectra were recorded over a series of temperatures from 40°C to 10°C to observe chemical shifts changes of assigned resonances for both Opa₆₀ and the synthetic peptide. 2D ¹H, ¹H TOCSY, and COSY spectra and ¹⁵N-edited versions of each were recorded for the peptide to assign the side chains.

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