Micro-scale NMR Experiments for Monitoring the Optimization of Membrane Protein Solutions for Structural Biology

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[Abstract] Reconstitution of integral membrane proteins (IMP) in aqueous solutions of detergent micelles has been extensively used in structural biology, using either X-ray crystallography or NMR in solution. Further progress could be achieved by establishing a rational basis for the selection of detergent and buffer conditions, since the stringent bottleneck that slows down the structural biology of IMPs is the preparation of diffracting crystals or concentrated solutions of stable isotope labeled IMPs. Here, we describe procedures to monitor the quality of aqueous solutions of [^{2}H, ^{15}N]-labeled IMPs reconstituted in detergent micelles. This approach has been developed for studies of β-barrel IMPs, where it was successfully applied for numerous NMR structure determinations, and it has also been adapted for use with α-helical IMPs, in particular GPCRs, in guiding crystallization trials and optimizing samples for NMR studies (Horst *et al.*, 2013). 2D [^{15}N, ^{1}H]-correlation maps are used as “fingerprints” to assess the foldedness of the IMP in solution. For promising samples, these “inexpensive” data are then supplemented with measurements of the translational and rotational diffusion coefficients, which give information on the shape and size of the IMP/detergent mixed micelles. Using microcoil equipment for these NMR experiments enables data collection with only micrograms of protein and detergent. This makes serial screens of variable solution conditions viable, enabling the optimization of parameters such as the detergent concentration, sample temperature, pH and the composition of the buffer.

Materials and Reagents

Studies of IMPs

1. Phosphocholine-detergents (Avanti Polar Lipids)
2. Tris Base (Thermo Fisher Scientific, catalog number: BP1521)
3. HCl (Thermo Fisher Scientific, catalog number: A144212)
4. Urea (Thermo Fisher Scientific, catalog number: BP169212)
5. Ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher Scientific, catalog number: BP1201)
6. L-Arginine (L-Arg) (Sigma-Aldrich, catalog number: A5131100G)

7. Phosphate buffer (Sigma-Aldrich, catalog number: P7994)
8. Sodium Azide (NaN_3) (Sigma-Aldrich, catalog number: S803225G)
9. NaCl (Thermo Fisher Scientific, catalog number: BP358212)
10. Stock solutions of unfolded protein (see Recipes)
11. Refolding buffer (see Recipes)
12. NMR buffer (see Recipes)

Equipment

NMR data collection

1. NMR experiment set-ups used in this protocol are either part of the Bruker standard pulse sequence library or are described in the Appendix. These experiments were implemented on a Bruker DRX spectrometer equipped with microprobes (Bruker Corporation，model：1 mm TXI, 1.7 mm TXI)
2. The following experiments were used to monitor the quality of aqueous solutions of [^{2}H, ^{15}N]-labeled IMPs: 2D [^{15}N, ^{1}H]-TROSY experiments (Pervushin *et al.*, 1997), ^{1}H-TRO-STE (Horst *et al.*, 2011) and TRACT (Lee *et al.*, 2006).

Procedure

1. NMR sample preparation
   a. Add 100 µl of freshly prepared stock solution of unfolded protein to 600 µl refolding buffer and stir overnight at 4 °C.
   b. Exchange with NMR buffer by repeated dilution/concentration cycles. ‘‘Upconcentration’’ of the detergent during these dilution/concentration cycles was taken into account when adjusting the detergent concentration in the NMR sample. For details on how to adjust these cycles, see Stanczak *et al.* (2009). For example, 10 mM 30-Fos in the NMR buffer results in approximately 160 mM 30-Fos in the NMR sample (Stanczak *et al.*, 2012).
   c. Added 5 µl of D_2O and 1 µl of a 100 mM solution of 4, 4-dimethyl-4-silapentane-1-sulfonic acid (DSS) as an internal reference for the ^{1}H chemical shifts to 45 µl of the protein solution.
2. Evaluation of different sample conditions based on 2D [^{15}N, ^{1}H]-TROSY experiments. The following criteria were used (see Zhang *et al.*, 2008; Stanczak *et al.*, 2012 for examples):
   a. Completeness of NMR observation of the IMP by comparison of the number of observed backbone ^{15}N–^{1}H correlation peaks with the number expected from the amino acid sequence.
   b. High average peak intensity and uniform distribution of peak intensities.

c. Analysis of the peak line shapes to support the interpretation of the peak intensity measurements.

3. Evaluation of the hydrodynamic radius of IMP/detergent mixed micelles, using ^{1}H-TRO-STE (Horst *et al.*, 2011) and TRACT (Lee *et al.*, 2006) experiments.
   a. Determine the translational diffusion coefficient, D_t, using the ^{1}H-TRO-STE experiment.
   b. Determine the rotational diffusion coefficient, D_r, using the TRACT experiment.
   c. Calculate the hydrodynamic radius, R_h, using the formula $R_h = (3D_t/4D_r)^{1/2}$.
   d. Optimize sample conditions towards small R_h values, which favors the recording of high-quality NMR data.

Data analysis

Processing and analysis of NMR datasets:

Process all NMR experiments with the Bruker standard software Topspin. For all experiments, the data matrices are multiplied with an exponential window function in the ^{1}H-dimension, and for 2D [^{15}N, ^{1}H]-TROSY a 75°-shifted sine bell window (De Marco and Wüthrich, 1976) is applied in the ^{15}N-dimension The 2D [^{15}N, ^{1}H]-TROSY data sets were analyzed using the XEASY module (Bartels e*t al.*, 1995) of the CARA release 1.5.5 (www.cara.nmr.ch). The ^{1}H-TRO-STE datasets are analyzed using the Bruker T1/T2-software package, as described in the Topspin DOSY application manual, chapter 3.2. The TROSY and anti-TROSY components of the TRACT data set are first separated using the Bruker standard AU program *split*, and then individually integrated using the Bruker integration module. The integrals were fitted to a mono-exponential decay, using the program XMGRACE (http://plasma-gate.weizmann.ac.il).

Notes

Optimization of NMR acquisition parameters for the 2D [^{15}N, ^{1}H]-TROSY, ^{1}H-TRO-STE and TRACT experiments:

1. For all experiments
   a. Determine the lengths of high power radio-frequency pulses as described in the user manual for the spectrometer. Typical pulse lengths are 8 µs and 35 µs for ^{1}H and ^{15}N, respectively.
   b. Set the carrier frequency to the water line by minimizing the residual water signal in the Bruker standard pulse sequence *zggppr*.
   c. Optimize the water flip-back pulses *sp2* and *sp3*, using the experiment shown in Listing 1 of the Appendix to interactively minimize the residual water signal in the topspin *gs* acquisition mode.
2. 2D [^{15}N, ^{1}H]-TROSY and TRACT experiments (listings 2 and 4 of the Appendix)

a. Adjust the WATERGATE soft pulse *sp1*, using the Bruker standard pulse sequence *zggpwg* by minimizing the residual water signal interactively in the topspin *gs* acquisition mode.
b. Adjust the [^{15}N, ^{1}H]-INEPT transfer delay, *d2*, to maximize the signal from the protein amide moieties in the first increment of the TRACT experiment. Typical values for *d2* are between 2.0 and 2.5 ms.
c. Adjust the number of complex points and the maximum evolution time, t_{1max}, for the ^{15}N-dimension in the 2D [^{15}N, ^{1}H]-TROSY experiment. Typical values for IMP's reconstituted in detergent micelles are 100 points and 35 ms, respectively. Use the same t_{1max} value for the TRACT experiment.

3. ^{1}H-TRO-STE experiment (listing 3 in the Appendix)
   a. Calibrate the pulsed-field gradient strengths with the residual ^{1}H signal of 99.9% D_2O, using the Bruker standard pulse sequence *ledgp2s1d* (D_t = (1.902 ± 0.09) × 10^{-9} m^2s^{-1} for HDO at 25 °C).
   b. Optimize the [^{15}N,^{1}H]-CRINEPT transfer delay, *d2*, by maximizing the signal from the protein amide moieties in the first increment of the spectrum.
   c. Optimize the diffusion delay and the gradient duration, *d20* and *p30*, respectively, using the standard procedures described in the Topspin DOSY application manual, Chapter 2.1.

Recipes

1. Stock solutions of unfolded protein
   [^{2}H, ^{15}N]-labeled protein (12 mg/ml) in 20 mM Tris–HCl at pH 8.0 and 6 M urea
2. Refolding buffer
   20 mM Tris–HCl at pH 7.5
   5 mM EDTA
   600 mM L-Arg
   45 mM detergent
3. NMR buffer
   5 mM phosphate buffer at pH 6.8
   10 mM NaCl, 0.3 % (v/w) NaN_3
   10 mM detergent

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This work was adapted from previously published studies on the *E. coli* outer membrane protein X (OmpX) (Stanczak *et al.*, 2009), and was used as a platform for the structure determination of *E. coli* OmpW (Horst *et al.*, 2014). The procedures described in this protocol were also used to characterize *E. coli* OmpA in lipid bilayer nanodiscs and

detergent micelles (Susac *et al.*, 2014). This work was supported by the Roadmap initiative grant P50 GM073197 for technology development. Kurt Wüthrich is the Cecil H. and Ida M. Green Professor of Structural Biology at the Scripps Research Institute.

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