Conformational analysis of membrane proteins in phospholipid bilayer nanodiscs by hydrogen exchange mass spectrometry

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SUPPORTING INFORMATION: Detailed Materials and Methods

Materials

All reagents were purchased from Sigma-Adrich (St. Louis, MO) unless otherwise noted. Ni-NTA Agarose resin was purchased from QIAGEN. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was obtained from Avanti Polar Lipids (Alabaster, Al). Solutions of DOPC were prepared in chloroform and the concentration determined by phosphate analysis.[1, 2] Deuterium Oxide, >99%, was purchased from Cambridge Isotope Laboratories (Andover, MA). Anti-HPC4 monoclonal antibody coupled Sepharose resin was obtained from Dr. Charles T. Esmon (Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK).[3]

Purification of Recombinant Wild-type Carboxylase

Wild-type human gamma-glutamyl carboxylase (GGCX) with a carboxyl terminus 12-amino acid epitope tag (EDQVDPRLIDGK) was isolated from Sf9 cells as previously described.[4] Solubilized GGCX microsomes were purified using a calcium dependent HPC4 antibody resin and eluted in 25 mM MOPS pH 7.5, 100 mM NaCl, 0.5% CHAPS, 0.2% DOPC, 10 mM EDTA,

and 15% glycerol stabilized by a protease inhibitor cocktail. Protein concentration and purity was determined by enzymatic activity assay measurements and SDS-PAGE.

Purification of Membrane Scaffold Protein (MSP1D1)

MSP1D1 is a construct of apolipoprotein A1 with the first 54 residues replaced by a poly histidine tag and linker with a TEV protease cleavage site. MSP1D1 expression and purification was carried out as previously described.[5] In short, the MSP1D1 plasmid with a polyhistidine tag was obtained from Add Gene (Cambridge, MA) and expressed in *E. coli* BL21 Codon Plus (DE3). For purification, the protein was isolated by nickel affinity chromatography and purity was confirmed by polyacrylamide gel electrophoresis. Fractions containing MSP1D1 were pooled and dialyzed against standard buffer (20 mM Tris/HCl pH 7.4, 100 mM NaCl, 0.5 mM EDTA, and 0.01% NaN_3).

Self-assembly of Nanodiscs

GGCX embedded nanodiscs were prepared in a similar manner to other protein embedded nanodiscs.[6-8] Briefly, a DOPC/deoxycholate solubilized mixture (2:1) was added to purified GGCX with modified amounts of phospholipid to optimize self-assembly. The mixture was incubated for one hour at 4 °C. Purified MSP1D1 stock in standard buffer was then added to the reconstitution mixture in a 20:1 MSP1D1: GGCX active target protein ratio and incubated for an additional hour at 4 °C. After incubation, detergent was removed during a 2 hour gentle rotation with damp Biobeads SM-2 (BioRad) at 4 °C. The disk preparations were purified by size exclusion chromatography by monitoring the absorbance at 280 nm on a Tosoh Biosciences TSKGEl BioAssist $G3SW_{XL}$ 7.8 × 300 mm (5 μm, 250 Å) column run with 50 mM Tris/HCl pH

7.0, 150 mM NaCl, 0.02% NaN_3 (TBS) at 0.5 mL/minute (see Figure S1A). SDS-PAGE was used to confirm the presence and purity of GGCX containing nanodiscs (see Figure S1B). Fractions containing purified nanodiscs were isolated and concentrated by Millipore Microcon YM-30 centrifugal filters. The same protocol was used to prepare empty nanodiscs, except GGCX was not added.

Hydrogen exchange reactions and mass spectrometry

Continuous labeling exchange experiments[9] were initiated by 10-fold dilution of 50 pmol GGCX (100 pmol MSP1D1) incorporated nanodiscs (~2 μM GGCX) in 99% deuterium oxide buffer (50 mM Tris/HCl, 150 mM NaCl, 0.02% NaN_3, ^2H_2O, pD 7.0) at 21 °C. At selected times ranging from 10 seconds to 4 hours after the introduction of ^2H_2O, the sample was quenched to pH 2.5 by addition of formic acid and placed on ice. To limit back-exchange, the pH was held at 2.5 and 0 °C - 4 °C for all sample handling prior to analysis by mass spectrometry.[10] Upon quenching, nanodiscs were immediately disassembled with addition of ice-cold sodium cholate to a 25:1 ratio of sodium cholate:DOPC while maintaining quench conditions at pH 2.5. Porcine pepsin immobilized on Poros 20AL resin (Applied Biosystems)[11] was added to digest the sample during incubation on ice for five minutes. In the last minute of digestion, three milligrams of zirconia coated silica particles (Supelco HybridSPE) were added to the digestion mixture to facilitate phospholipid precipitation. The sample was spun filtered (0.45 μm cellulose acetate filter, pre-chilled) at 4 °C for 60 seconds to capture both the pepsin and ZrO_2 beads. Flowthrough from the spin filtration was immediately injected to the UPLC system. Empty nanodisc samples were prepared in a similar manner.

In initial method validation experiments deuterium uptake was monitored on a HPLC-MS system and did not include disassembly of the nanodiscs or lipid removal. Pepsin digested samples were injected onto a Poros 20R2 (2 mm × 20 mm) trap placed in the sample injection loop and desalted with a three times (v/v) wash with ice-cold 0.05% TFA in water. A Waters (Milford, MA) VanGuard pre-column 3.5 μm C18 (2.1 mm × 5 mm) was used to facilitate lipid trapping prior to analytical separation. The HPLC separation was carried out on a Waters Xbridge 3.5 μm C18 column (1.0 × 100 mm). To minimize deuterium loss, the injector, trap, column and tubing were immersed in an ice bath. The peptides were eluted by 10-50% ACN over 15 minutes followed by a five minute ramp to 85% ACN in mobile phases containing 0.05% TFA and a flow rate of 50 μL/minute. Mass analysis was carried out with a Waters LCT Premier™ equipped with a standard ESI source.

The final optimized experiments (Figure 1) were run on a Waters nanoAcquity UPLC™ with HDX Technology.[12] After digestion, ZrO_2 removal and spin filtering (described above), the eluate was injected to a trap (Waters VanGuard pre-column 1.7 μm C18, 2.1 mm × 5 mm) and desalted with 0.05% formic acid in water for 5 minutes. The trap was placed in line with a second pre-column (Waters VanGuard pre-column 1.7 μm C18, 2.1 mm × 5 mm) directly connected to the analytical column (Waters Xbridge 1.7 μm C18, 1.0 mm × 100 mm). The second pre-column between the trap and the analytical column was used as a precaution to catch any residual lipid that was not removed by the ZrO_2 resin. The peptides were eluted with an 8-40% gradient of acetonitrile in 0.05% formic acid (pH 2.5) over six minutes at a flow rate of 40 μL/minute. Mass spectral analyses were carried out on a Waters QTof Premier™ equipped with a standard ESI source and Glu-Fibrinopeptide lockmass calibration. Peptic peptides were identified in undeuterated control samples for both GGCX containing and empty nanodiscs with

Waters MS^E analysis on the same UPLC/Qtof system used for HX MS experiments. Waters ProteinLynx Global Server 2.4 (PLGS) was used to search the spectra and identify peptic peptides. In this experimental setup, the error of determining the deuterium level at each time point did not exceed ±0.3 Da based on replicate measurements with loaded nanodiscs, more than three replicates of empty nanodiscs and the analysis of standard proteins and peptides.

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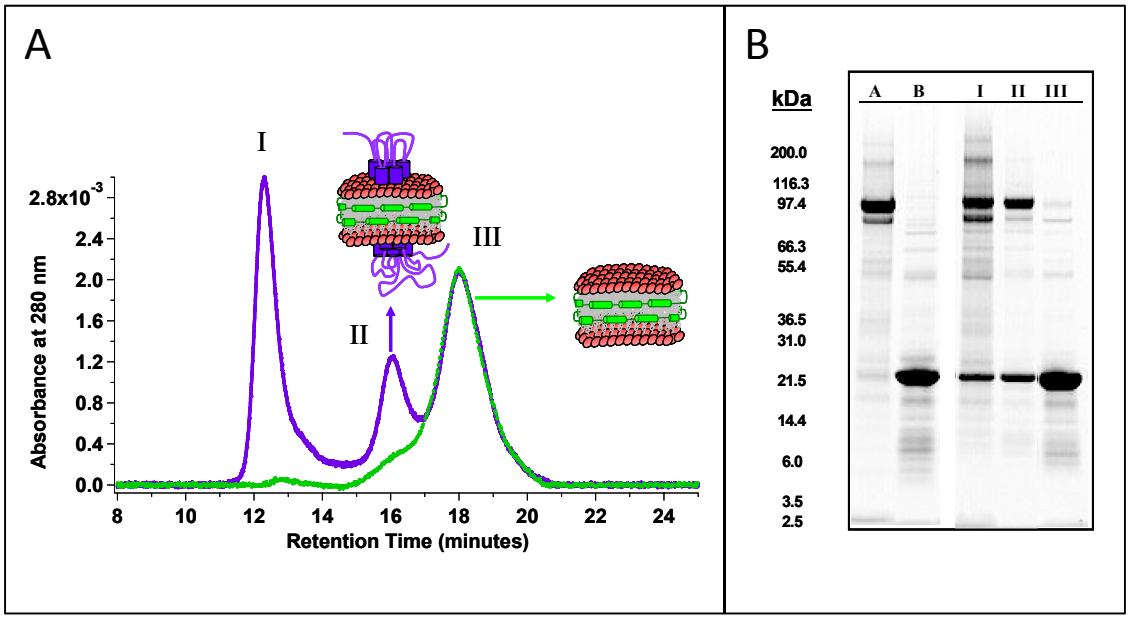

FIGURE S1.

(A). Purification of Gamma-Glutamyl Carboxylase (GGCX) containing nanodiscs by size exclusion chromatography. Assembly occurred in a reconstitution mixture with a 1:20:1200 mole ratio of GGCX target protein:MSP1D1:DOPC. Following self-assembly, samples were separated on a TSKGel BioAssist G3SW$_{XL}$ column (blue trace): Void volume (V_o) Peak I, GGCX containing nanodisc Peak II, Empty nanodisc Peak III. The green trace shows empty nanodiscs. **(B)**. Reducing SDS-PAGE gel confirming GGCX incorporation into nanodiscs. Lane A: purified GGCX; lane B: purified MSP1D1; lane I: aggregate eluting at V_o; lane II: GGCX nanodisc SEC fractions (panel A, peak II); lane III: empty nanodisc SEC fractions (panel A, peak III).