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Reconstitution of full-length human caveolin-1 into phospholipid bicelles: validation by analytical ultracentrifugation

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Abstract

A significant hurdle in obtaining biophysical information on membrane proteins is developing a successful strategy for their reconstitution into a suitable membrane mimic. In particular, utilization of the more 'native-like' membrane mimics such as bicelles is generally more challenging than simple micellar solubilization. Caveolin-1, an integral membrane protein involved in membrane curvature, endocytosis, mechano-protection, and signal transduction, has been shown to be particularly recalcitrant to standard reconstitution protocols due to its highly hydrophobic characteristics. Herein we describe a robust method to incorporate recombinantly produced full-length caveolin-1 into bicelles at levels needed for biophysical experimentation. The benchmark of successful reconstitution is the obtainment of protein in a homogeneous state; therefore, we developed a validation procedure to monitor the success of the reconstitution using analytical ultracentrifugation of density-matched bicelles. Our findings indicated that our protocol produces a very homogeneous preparation of caveolin-1 associated with bicelles, and that caveolin-1 is highly α -helical (by circular dichroism spectroscopy). We believe that this methodology will serve as a general strategy to facilitate biophysical studies on membrane proteins.

Graphical Abstract

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Keywords

caveolin-1; reconstitution; analytical ultracentrifugation; sedimentation equilibrium; bicelles; fluorescence spectroscopy

Introduction

Biophysical studies of homogeneously purified membrane proteins necessitate their reconstitution into membrane mimics that closely resemble the environment of the biological membrane. Most commonly, detergent micelles, liposomes, nanodiscs, or bicelles are employed with each system having particular strengths and weaknesses.^{1,2} The choice of a suitable membrane mimic is largely empirical; for some membrane proteins detergent micelles support full structure and function while others need more sophisticated mimics such as bicelles.^{3–6} Caveolin-1 is an integral membrane protein of great interest to the biological community as it is intimately involved in caveolae biogenesis, signal transduction, mechano-protection, and membrane curvature.^{7–9} The hallmark feature of caveolin is the intramembrane domain which is postulated to form an unusual hairpin structure within the hydrophobic core of the membrane.¹⁰ Thus, there is intense enthusiasm in characterizing caveolin structurally.¹¹ However to date, most structural studies on caveolin have been done in micellar environments which have produced a wealth of secondary structure data, but have fallen woefully short of producing compelling tertiary structure data.^{12–16} This paucity of data suggests indirectly that micelles may not adequately support the tertiary structure of caveolin. Therefore, it is imperative to assess caveolin behavior in an environment which better resembles the native bilayer. However, the employment of more native-like membrane mimics such as bicelles tends to be much more technically demanding than standard micellar reconstitution.^{17–19}

Phospholipid bicelles, are discoidal lipid aggregates comprised of mixtures short-chain phospholipids (DHPC) and long-chain phospholipids (DMPC), and have a number of features which make them particularly well-suited for biophysical studies on membrane proteins.²⁰ One benefit is the presence of an actual bilayer, the size of which can be controlled by the molar ratio of DMPC to DHPC, q . In addition, bicelles formed at q values 1.0 have thermotropic behavior (gel to fluid main-phase transition temperatures) which is indistinguishable from that of pure DMPC vesicles, indicating that a protein residing in the

center of the bicelle (i.e. not the rim) will experience a native-like environment in terms of membrane fluidity.^{21,22}

Here, we present a facile protocol for reconstituting recombinantly produced full-length human caveolin-1 into bicelles for biophysical studies. We then employ analytical ultracentrifugation using density-matched bicelles to prove that the procedure allows for the procurement of the protein in a homogenous state. Furthermore, circular dichroism spectroscopy revealed that the reconstituted caveolin-1 was highly helical. We believe that the methodology developed in this work will serve as a general strategy for incorporating membrane proteins in bicelles and evaluating their homogeneity.

Materials and Methods

DMPC and NBD-DMPE were purchased from Avanti Polar Lipids (Alabaster, AL). DPH was purchased from Sigma-Aldrich (St. Louis, MO). D₂O was purchased from Cambridge Isotope Laboratories (Tewksbury, MA). All other reagents were of standard laboratory grade.

Caveolin-1 expression and purification

The DNA for full-length human caveolin-1 (1–178) with a C-terminal myc-tag was synthesized by Genscript Corporation (Piscataway, NJ). Caveolin-1 has three sites of cysteine palmitoylation (C133, C143, C156) that have been shown to be nonessential for proper caveolin-1 trafficking.²³ Therefore, each cysteine was mutated to serine. The construct was cloned into the pET-24a vector which appends a C-terminal hexahistidine tag (EMD Millipore, Billerica, MA) yielding a final construct of caveolin-1-myc-H6. Next, the plasmid was transformed into BL21(DE3) *E. coli* cells, and over-expressed using the auto-induction method at 25°C for 24 hours.²⁴ Please note, that the caveolin-1-myc-H6 construct has previously been shown to retain key functional properties which strongly suggests that the fold of the protein is not altered by the tags.²⁵ Cells from a 1 L growth were harvested by centrifugation and washed with 0.9% (w/v) NaCl. The pellet was resuspended in 40 mL of lysis buffer (50 mM phosphate, 300 mM NaCl, pH 8, 2% (v/v) Empigen BB® detergent (Sigma, St. Louis, MO)). Cells were lysed at 4°C by sonication with magnetic stirring for 20 minutes (Bronson 450 watt sonifer, power level 4, duty cycle 30%). The lysate was cleared by centrifugation at 50,000 x *g* for 1.5 hours, and the supernatant was filtered through a 0.2 µm syringe filter and passed over a 25 mL Ni-Sepharose column (GE Healthcare, Piscataway, NJ) equilibrated with lysis buffer. The column was washed with 125 mL of wash buffer (50 mM phosphate, pH 8, 300 mM NaCl, 35 mM imidazole, 0.5% (v/v) Empigen BB®), and eluted using elution buffer (50 mM phosphate, pH 8, 300 mM NaCl, 250 mM imidazole, 0.5% (v/v) Empigen BB®). The protein-containing fractions were pooled and concentrated to 5 mL using ultrafiltration (MWCO 10,000). The concentrated sample was re-filtered through a 0.2 µm syringe filter and injected onto a Sephacryl 300 HR 16/60 column (GE Healthcare, Piscataway, NJ) equilibrated with gel filtration buffer (50 mM phosphate, pH 7.4, 150 mM NaCl, 0.5% (v/v) Empigen BB®). The purified protein was stored at 4°C and quantitated using the micro-BCA assay (ThermoFisher Scientific, Waltham, MA). Protein concentrations were estimated by A₂₈₀

measurements using a calculated extinction coefficient of $30,940 \text{ M}^{-1}\text{cm}^{-1}$ (Protparam, <https://web.expasy.org/protparam/>)

Bicelle Preparation Calculations

The DHPC component of a bicellar mixture is in rapid equilibrium between a free monomer in solution and the rim of the bicelle, and regardless of the concentration of the bicellar solution, the concentration of free monomeric DHPC is constant at $\sim 7 \text{ mM}$. Therefore, the parameter q used to prepare a bicellar solution can deviate significantly from the the actual molar ratio of DMPC and DHPC within the bicelle (q_{eff}). To prepare bicelles at a particular q_{eff} , the following equation was used:

$$q_{\text{eff}} = [\text{DMPC}] / ([\text{DHPC}]_{\text{total}} - [\text{DHPC}]_{\text{free}})$$

For this study, $q_{\text{eff}} = 1.0$ bicelles were prepared which corresponds to a value of $q = 0.7$.

Incorporation of caveolin-1 into bicelles

To incorporate caveolin-1 (1–178) into bicelles, roughly $350 \mu\text{g}$ of purified caveolin-1 was applied to a Ni-NTA spin column (Qiagen, Valencia, CA) equilibrated with gel filtration buffer. The nickel spin column was washed twice with $600 \mu\text{L}$ of a $q_{\text{eff}} = 1.0$ bicellar solution (2.3% (w/w) total lipid, 10 mM HEPES pH 7.4, and 100 mM NaCl). The protein was eluted with $2 \times 200 \mu\text{L}$ of the above buffer containing 250 mM imidazole. The elution was then processed using a Zeba™ desalting column (Pierce, Rockford, IL.) to remove the imidazole. The desalting column was equilibrated with 10 mM HEPES pH 7.4, 100 mM NaCl, 7 mM DHPC. From this stock three samples at various concentrations ($30 \pm 2 \mu\text{M}$, $15 \pm 2 \mu\text{M}$, and $7.5 \pm 2 \mu\text{M}$) were prepared for analytical ultracentrifugation by diluting with the desalting column equilibration buffer.

Verification of detergent removal

To determine that all of the Empigen BB® was removed under these conditions, a trial experiment was run. For this experiment, DPH was added to the $350 \mu\text{g}$ sample of caveolin-1 to yield a final concentration of $\sim 1 \mu\text{M}$. Next, the fluorescence was measured in a $1 \times 0.1 \text{ cm}$ cuvette held at 25°C using an excitation wavelength of 355 nm and an emission wavelength of 430 nm . The excitation and emission slits were each set to 5 nm respectively. Next, the sample was reconstituted into bicelles using the method discussed above. The DPH fluorescence was then re-measured at the end of the reconstitution (post desalting step) to assess the completeness of detergent removal.

Density matching experiments

All sedimentation equilibrium experiments were performed at 25°C using a Beckman XL-A analytical ultracentrifuge and a 4-hole AnTi-60 rotor. Density matching samples were prepared on a $300 \mu\text{L}$ scale. First, 3.58 mg of DMPC and $0.43 \mu\text{g}$ of NBD-DMPE (1:10,000 mole ratio) were co-dissolved in chloroform followed by evaporation to dryness. To each sample, $7.5 \mu\text{L}$ of a $40\times$ concentrated stock buffer solution was added which yielded final concentrations of 10 mM HEPES pH 7.4 and 100 mM NaCl. Next, the appropriate quantity

of H₂O and D₂O were added to achieve the desired composition (0, 10, 25, 40, 50, 60, 70, 80, 90% D₂O (v/v)). Following this, the samples were vortexed until a homogeneous milk-like suspension was obtained. Finally, 13.03 µL of a 25% (w/w) DHPC solution was added. After adding the DHPC, the solution became clear. The samples were loaded into a 6-channel charcoal-filled epon centerpiece using a reference solution containing only buffer. A volume of 120 µL of sample was loaded per channel. Equilibrium absorbance measurements (468 nm) were taken from 10,000 rpm to 35,000 rpm stepping up the speed in 1,000 rpm increments. Data was collected at each rotor speed. Achievement of equilibrium was determined using the computer program WinMatch. For each percentage of D₂O, a plot of the natural log of the absorbance versus the square of the radius was generated, fitted to a linear function and the buoyant molecular weight (M_{eff}) was extracted from the slope. Next, M_{eff} was averaged over all speeds. The density of the bicellar solutions at each percentage was measured using a Kyoto Electronics Density/Specific Gravity Meter (model #DA-210). Finally, M_{eff} was plotted versus the density of the solution at each amount of additive, fitted to a linear function, and interpolated to a buoyant molecular weight of zero to determine the density matched concentration of D₂O.

Sedimentation equilibrium of caveolin-1 in bicelles

In a fashion similar to that stated for the density matching experiments above, a bicellar solution of caveolin-1 was analyzed at three different speeds (25,000, 30,000 and 35,000 rpm) and three different concentrations. Final scans at each speed (step-size 0.001 cm and 99 replicates) were acquired once equilibrium had been established using the match feature in the software Heteroanalysis (version 1.1.0.58, University of Connecticut, Storrs, CT). The reference was a sample which did not contain protein. All experiments were carried out at 25 °C. The data were fitted globally to a single-species model using a non-linear least squares approach using Heteroanalysis. During fitting, the molecular weight, baseline, and reference concentration were all allowed to float.

Circular Dichroism Spectroscopy Measurements

Circular dichroism measurements were performed at 25°C using a JASCO CD Spectrophotometer (Easton, MD). The bicellar caveolin-1 sample (q_{eff} = 1.0, 2.3% (w/w) total lipid, 10 mM HEPES pH 7.4, and 100 mM NaCl) was at a concentration of 30 µM. A 0.1 mm path length quartz cuvette was utilized for measurements. Data were collected by scanning every wavelength from 260–190 nm in step mode at a speed of 200 nm/minute taking 16 accumulations for signal averaging. A background spectrum of DMPC-DHPC bicelles lacking protein was used as a blank. The data were converted from machine units (θ) to units of mean residue ellipticity (Θ) using the following equation:

$$[\theta] = \theta \times \frac{(0.1 \times MRW)}{(P \times C)}$$

Here, MRW is the mean residue weight (protein MW/number of residues in the protein), P is the pathlength in cm, and C is the protein concentration in mg/mL yielding units of M⁻¹ cm⁻¹.

SDS-PAGE and Immunoblotting

Protein samples taken at various stages of the purification procedure were resolved on 15% polyacrylamide gels and transferred to a PVDF membrane. The blots were blocked with 1% (w/v) casein, and probed using an anti-myc antibody. Signals were detected using chemiluminescence. The immuno-blots were imaged using a BioRad ChemiDoc XRS+ system and Image Lab software (BioRad, Hercules, CA).

Results and Discussion

Reconstitution of caveolin-1 into phospholipid bicelles

To date, no tertiary structural data has been collected for full-length caveolin-1. This is likely due to the exceptionally insoluble (hydrophobic) character of caveolin-1 which has resulted in most structural studies being carried out in strong detergent micelles.¹¹ While micelles are advantageous because reconstitution is not technically demanding, it is becoming apparent that they may not adequately support the tertiary structure of caveolin-1.¹³ Bicelles on the other hand appear to be well suited to caveolin-1; they contain an actual lipid bilayer and they are composed of saturated phosphatidylcholine lipids which predominate in caveolae where caveolin-1 is natively found.²⁶

We sought to develop a non-denaturing protocol for reconstituting recombinantly-produced homogeneously purified caveolin-1 efficiently into bicelles. As an expression source, *E. coli* is appropriate as over-expression of full-length caveolin-1 in the host has been shown to result in caveolae formation, a property that clearly indicates that the protein is properly folded and functional.^{27,28} A number of factors were considered when choosing a detergent for the initial lysis and purification steps. Ideally, the detergent would be native-like and non-denaturing. The zwitterionic detergent, Empigen BB® was identified as being an optimal choice for the purification as it has a zwitterionic headgroup that mimics phosphocholine, is not cost prohibitive, and most importantly it readily solubilized caveolin-1. Initial purification was done via nickel affinity chromatography followed by gel filtration chromatography (SI Figure 1). The gel filtration chromatography step was absolutely necessary to get a pure caveolin-1 sample as significant aggregates and/or impurities remained after the nickel affinity chromatography step (SI Figure 1B). The material sharply eluted from a sephacryl S-300 HR gel filtration column and at nearly the same volume in empigen micelles as was observed for prior studies utilizing dodecylphosphocholine micelles (DPC) where caveolin-1 was shown to be in a singular oligomeric state.²⁹ In addition, this step removes the imidazole used to elute the protein in the first nickel affinity chromatography step. This allows for the reassociation of the protein with the Ni-NTA spin column in the subsequent step. After binding to the Ni-NTA spin column, the protein was eluted into $q_{eff} = 1.0$ DMPC-DHPC bicelles, a choice that was based on studies which showed that bicelles with q_{eff} values less than 1.0 showed a significant loss of lipid segregation and we did not desire to risk exposing caveolin-1 to the detergent-like DHPC which could disrupt its tertiary fold.^{19,22} A final step employing a mini-desalting column pre-equilibrated with DHPC at the critical bicelle concentration (7 mM) was used to remove the imidazole, while maintaining a minimal concentration of the DHPC to maintain the desired q_{eff} of the caveolin loaded bicelles.³⁰ Using A_{280}

measurements, the amount of protein in the final bicellar preparation was estimated. It was found that nearly all of the protein initially loaded onto the column was present in the bicelle elution fraction, indicating that the reconstitution process was effective. Western blot analysis of the purification procedure confirmed that full length caveolin-1 was obtained in the bicelle elution, migrating to the approximate distance of a 23 kD molecular weight standard (SI Figure 2).

As Empigen BB® has no convenient handle for detection, we evaluated the ability of our method to remove diphenylhexatriene (DPH), a hydrophobic fluorescent probe which produces a strong signal when incorporated into membranes. This was spiked into the sample before applying it to the nickel-NTA spin column. Analysis of the DPH fluorescence before and after the bicelle reconstitution step showed that a negligible amount of probe was present in the final sample (Figure 1). This indicated that the wash steps utilized were sufficient to remove all of the Empigen BB® detergent. Finally, CD spectroscopy was used to probe the protein's secondary structure in bicelles. The CD spectrum of caveolin-1 in bicelles had a maximum around 190 nm and two minima at 208 and 222 nm which indicate strong α -helical character for caveolin-1 in agreement with other biophysical studies (Figure 2).^{15,16,31} Analysis of the CD spectrum using the CDSSTR algorithm (Dichroweb) indicated that caveolin-1 is approximately 67% α -helical which compares favorably with determinations based on secondary structure evaluation of a functional caveolin-1 construct comprised of residues 62–178 reconstituted into lysomyristoylphosphatidylglycerol micelles by NMR (72%).¹⁵ This finding is also in agreement with studies performed in the presence of many other micelle types, including DPC which is structurally similar to Empigen BB®.¹¹

Density matching of $q_{eff} = 1.0$ bicelles

Determining the dispersity of a membrane protein sample can be challenging due to the presence of the membrane mimic. For example, gel filtration is of little utility because the large size of the membrane mimic dominates the size determination and prevents one from assessing the aggregation state of the protein itself. On the other hand, analytical ultracentrifugation is particularly attractive because one can eliminate the contribution of the membrane mimic straightforwardly through density matching.^{32–34} Density matching represents a condition where the density of the solution is matched to the inverse of the partial specific volume of the membrane mimic. When this condition occurs, the buoyant molecular weight of the membrane mimic becomes zero; therefore it will have no influence on the determination of the aggregation state of the protein.

Density matching of the bicelles was carried out by increasing the density of solution using heavy water (i.e. D₂O). Figure 3 shows the plot of the buoyant molecular weight (M_{eff}) versus solution density (i.e. various concentrations of D₂O). The density matched condition for bicelles was determined by interpolation of the line equation in Figure 3 to a buoyant molecular weight of 0. The matched condition was determined to be a density of 1.084 g/cm³ (71.7% (v/v) D₂O, SI Figure 3). Using this value, the partial specific volume of the bicelle was determined and yielded a value of 0.923 cm³/g. This data represents,

to our knowledge, the first instance where $q = 1.0$ bicelles have been density-matched, and the partial specific volume of a bicelle determined. The amount of D₂O required to density match the bicelles was close to the maximum amount of D₂O that can practically be added. Therefore, if significant quantities of denser phospholipids (e.g. phosphatidylserine or glycopospholipids) need to be added, one may need to use D₂¹⁸O instead which will allow for higher solution densities.

Although glycerol or sucrose were also convenient options for density matching experiments, for the study discussed here, we chose to use D₂O for two reasons: 1) it is quite similar to H₂O and 2) it simplifies data interpretation as glycerol and sucrose could distort the data by altering the bicelle partial specific volume in a concentration dependent manner due to density gradient formation and hydrostatic pressure.³²

Sedimentation equilibrium analysis of caveolin-1 in bicelles

The sedimentation profile of caveolin-1 (Figure 4) was obtained at three different speeds (25,000, 30,000, and 35,000 rpm) and three different concentrations ($30 \pm 2 \mu\text{M}$, $15 \pm 2 \mu\text{M}$, and $7.5 \pm 2 \mu\text{M}$). The three concentrations employed correspond to lipid to protein ratios of 1400:1, 2800:1, and 5600:1 which yields a theoretical density of caveolin-1 molecules per μm^2 bicelle of 0.6×10^3 for the lowest concentration and 2.5×10^3 for the highest concentration when considering just the surface area of the planar region of bicelles.³⁵ This compares favorably to the theoretical density of caveolin-1 in caveolae, which is estimated to be on the order of 10^3 caveolin molecules per μm^2 .^{26,36}

Using the computer program Heteroanalysis (University of Connecticut, Mansfield, CT), the data were fit simultaneously to an ideal single species model (This model provides an average molecular weight of all populations present in solution). After fitting the data, a molecular weight of $23.0 \pm 0.1 \text{ kD}$ was obtained. This agreed very well with the calculated molecular weight of caveolin-1 which was 22.7 kD. Employment of other models (i.e. monomer-dimer, etc) resulted in poor fits (data not shown) and confirmed the single species models was the best. Based on these results it was clear that caveolin-1 is present in a single state in the bicellar environment (monomer), and is not displaying any significant degree of sample heterogeneity.

Conclusions

To date, to our knowledge, there is no reported bicellar reconstitution strategy that has been presented for full-length caveolin-1. Our method is advantageous because it is both gentle and easily carried out. Importantly, we have circumvented strategies which would destabilize the protein's tertiary fold by instead performing the reconstitution step "on-column" where the protein is immobilized. This method likely prevents excessive or biologically irrelevant self-association of the protein as it gently integrates into the bicelle. Additionally, we have found that our strategy is far more efficient and is likely to be both more general and reliable than other "mild" bicelle reconstitution strategies that first incorporate the protein into liposomes followed by the addition of DHPC at the desired molar ratio to generate bicelles, as vesicle reconstitution strategies often need to be systematically determined for a given protein.³⁷ Our method is also useful as a diagnostic for protein aggregation because bicelles

are very compatible with analytical ultracentrifugation and can be easily density-matched using the ultra-mild density modifier, D₂O. It is conceivable that this reconstitution strategy will also be of use for those who wish to investigate protein-protein interactions.

Overall the studies presented here reveal that full-length caveolin-1 can be readily reconstituted into $q = 1.0$ bicelles, and that the protein exists in a singular aggregation state (monomer, >98%). To our knowledge this is the first report of the oligomeric state of caveolin-1 in a bilayered membrane-like environment, and our approach further highlights the importance of using biophysical methods, like analytical ultracentrifugation, to understand membrane protein behavior in cells. One caveat of this study is that the influence of reconstituted protein on the morphology of the bicelle is unknown and future efforts should be devoted to probing this issue. CD studies of reconstituted caveolin-1 show that the full-length protein is highly α -helical (Figure 2), which is consistent with previous studies of caveolin-1, and also show that caveolin-1 is likely properly folded in bicelles.^{12,15,16,31} With the successful reconstitution of caveolin-1 in hand, we can now focus our attention on the numerous important structural questions that can only be addressed in a bilayered environment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations and Symbols

CD	circular dichroism spectroscopy
DMPC	1,2-dimyristoyl- <i>sn</i> -glycero-3-phosphocholine
DHPC	1,2-dihexanoyl- <i>sn</i> -glycero-3-phosphocholine
DPH	1,6-Diphenyl-1,3,5-hexatriene
NBD-DMPE	1,2-dimyristoyl- <i>sn</i> -glycero-3-phosphoethanolamine-N-(7-nitro-2- 1,3-benzoxadiazol-4-yl)
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

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Highlights

- Reconstitution of full-length caveolin-1 into bicelles.
- Caveolin-1 is in a highly homogeneous unaggregated state.
- Caveolin-1 displays helical content.
- Detergent removal can be monitored by fluorescence spectroscopy.

Figure 1. Fluorescence emission spectra of DPH before (*black*) and after exchange of the protein into bicelles (*red*). The signal is significantly reduced after the reconstitution into bicelles indicating that this method is effective for detergent exchange.

Figure 2.
Circular dichroism spectra of caveolin-1 reconstituted into $q = 1.0$ bicelles.

Figure 3. Linear plot of the buoyant molecular weight, M_{eff} , versus solution density for bicellar ($q = 1.0$) solutions prepared with various concentrations of D_2O . The dashed line represents a M_{eff} of zero which is the amount of D_2O required to density match $q = 1.0$ bicelles.

Figure 4. Sedimentation equilibrium profiles of caveolin-1 in density-matched (with D₂O) bicellar solutions ($q = 1.0$). Panels A-C represent a concentration of 30 μ M, D-F a concentration of 15 μ M, and G-I a concentration of 7.5 μ M. Residuals are displayed underneath each equilibrium profile.