# SUPPORTING MATERIAL

## Bovine β-Lactoglobulin Is Dimeric under Imitative Physiological Conditions: Dissociation Equilibrium and Rate Constants over the pH Range 2.5 to 7.5

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## MATERIALS AND METHODS

### Protein expression and purification

The βLg A and B variants were expressed and purified following the protocol described by Ariyaratne *et al.* (1) and Ponniah *et al.* (2). Briefly, βLg was over expressed, along with a chaperone disulfide bond isomerase DsbC on a pETDUET-1 plasmid in *Escherichia coli* Origami (DE3) cells, which offer a more oxidizing environment than standard cell lines, such as BL21, and lysed using a French press. Following a centrifugation step, the supernatant was subjected to ion-exchange chromatography at pH 6.5 (20 mM Bis-Tris, stepwise NaCl gradient from 0.05-1.0 M). the protein was then dialyzed against 50 mM phosphate buffer, pH 7.5, before dropping the pH to 2.6, to precipitate out unwanted *E. coli* proteins and misfolded βLg, followed by addition of NaCl to a final concentration of 30% (w/v) NaCl, which then precipitates βLg. This procedure follows that used to purify βLg from bovine milk (3). The βLg A and B variants were greater than 95% pure, as judged by SDS-PAGE. The protein was then stored at 4 °C. Mass-spectrometry confirmed that our recombinant proteins, being expressed in *E. coli*, retain the N-terminal methionine, which is cleaved in wild-type βLg. Mass-spectrometry also established that two disulfide bonds are present, as in native βLg (2). Immediately prior to the analytical ultracentrifugation experiments, the βLg A and B variants were further purified using size-exclusion chromatography. For each pH, a HiPrep 16/60 Sephacryl S-200 HR column (GE) was equilibrated with buffer prior to injection of the proteins. Protein fractions were collected and then quantified by measuring their absorbance at 280 nm. The buffers were chosen according to their buffering capacity at the desired pH value: 20 mM citrate buffer in 100 mM NaCl was used to study the proteins at pH 2.5, 3.5, 4.5, 5.5 and 20 mM MOPS buffer in 100 mM NaCl was used to perform the experiments at pH 6.5 and 7.5. MES buffer was used also at pH 5.5 and yielded similar results to the citrate buffer (see within). Given this and the chemical relatedness of MES and MOPS buffers, we do not consider the change of buffer from citrate to MOPS to cause any significant change in the dimer-monomer equilibrium under the constant 100 mM NaCl ionic-strength buffer employed in this study.

### Analytical Ultracentrifugation Experiments

Sedimentation velocity experiments were performed using a Beckman model XL-I analytical centrifuge (Brea, CA) and a run temperature of 25 °C. βLg A (and separately B) was pooled after gel filtration and diluted to three different concentrations for the analytical ultracentrifugation experiments [~5, ~15 and ~45 μM (~0.09, ~0.28 and ~0.83 mg/mL), see Table S2 for the precise experimental concentrations]. The density and the viscosity of the buffers used in the AUC runs are shown in the Supporting Material (Table S3) and were experimentally determined using a densitometer (Anton Parr) and a viscometer (Anton Parr) or, where possible, calculated using SEDNTERP (http://www.rasmb.bbri.org). V-bar was calculated using SEDNTERP and is based on the protein's amino-acid sequence. The protein concentration was determined prior to the experiments by measuring the absorbance at 280 nm and using the extinction coefficient

(determined also by SEDNTERP) of 0.96 L $g^{-1}$ $cm^{-1}$ (17,210 $M^{-1}$ $cm^{-1}$) for βLg A and for βLg B (4, 5).

For the sedimentation-velocity experiments, sample (380 μL) and buffer (400 μL) were loaded into the two sectors of a double-sector quartz cell and mounted in a Beckman eight-hole rotor. The samples were centrifuged at 50,000 rpm. Data were collected in continuous mode using a step size of 0.003 cm without averaging or a time lag between scans. To estimate the sedimentation parameters of the βLg A and B at different pH values, data were fitted to a $c(s)$ distribution or a $c(M)$ distribution using the software SEDFIT (6), available from http://www.analyticalultracentrifugation.com. The $c(s)$ analyses were performed with a regularization at a confidence level of 0.95 (maximum entropy), a floating frictional ratio and baseline, $S_{min}$ = 0.2 and $S_{max}$ = 6 and a resolution of 100. Weight-averaged $s$ values for the $c(s)$ distributions were determined using SEDFIT and converted to the standardized sedimentation coefficient ($s_{20,w}$) using SEDNTERP. Sedimentation coefficients and fit statistics for βLg A and B are provided in Supplementary Tables S4 and S5, respectively. A van Holde-Weischet analysis (7) was performed for all runs to examine the boundary shape; the results are shown in Figs. S18–S19..

Sedimentation-equilibrium experiments were also undertaken. Sample (100 μL) and buffer (120 μL) were loaded into a six-sector quartz cell. Sedimentation-equilibrium experiments were carried out at a run temperature of 25 °C and at four rotor speeds: 12,000, 15,000, 18,000 and 24,000 rpm. Once equilibrium was obtained at each speed, the data were collected in step mode using a step size of 0.001 or 0.003 cm. Sedimentation-equilibrium and sedimentation-velocity data were globally fitted to appropriate mathematical models implemented in the software SEDPHAT (8), available at http://www.analyticalultracentrifugation.com. Where only a single species was observed in solution, as at pH 4.5 and 5.5, a *single interacting species* model, which uses mass conservation, best fitted the data. Where two species were detected in solution, as at pH 2.5, 3.5, 6.5 and 7.5, a *monomer-dimer self-association* model was fitted to globally fit both the sedimentation velocity and sedimentation equilibrium data. The fit was refined by alternating simulated-annealing with the Marquardt-Levenberg and Simplex algorithms and including radial-invariant (RI) and time-invariant (TI) noise correction. The default weighting estimates employed by SEDPHAT for both the sedimentation velocity and sedimentation equilibrium data were used, and all parameters and experiments were checked to ensure they influenced the fit. Since the data were collected at various wavelengths, protein concentrations were determined using the absorbance at 280 nm prior to the experiment and fixed and the extinction coefficients were instead floated when fitted with SEDPHAT. The extinction coefficients were, however, constrained across experiments collected at the same wavelength. The s1 and s2 species, representing the monomer and dimer, were also floated (final values are given in Table S6). Error estimates were determined using conventional *F*-statistics, as implemented in SEDPHAT, and represent the 68% confidence interval. The sedimentation data and residuals are provided in Figs. S1–S7 and S11–S17 in the Supporting Material.

### Continuum electrostatic calculations of β-Lg dimer formation

Continuum electrostatic calculations have been performed on the βLg A dimer and on its dissociated monomers by solving a linear form of the Poisson-Boltzmann equation (PBE) in order

to characterize the relationship between dimer stability and ionic strength. At pH 6.5 and less, the βLg A dimer with the pH-gated EF loop associated with the Tanford transition at pH ~7.3 (9) is in the closed position with Glu89 buried (pdb code 3BLG) was used for calculations, while for calculations at pH 7.5 the βLg A dimer with the pH-gated EF loop in the open position with Glu89 exposed (pdb code 1BSY) was used. In the preliminary set-up, the radius and charge for each atom in the structure were calculated as defined by the PARSE force field using the PDB2PQR server (10). Hydrogen atoms were added assuming the protonation states of each residue at pH 2.5 as calculated by the PROPKA (11-13). Next, the structure was minimized by performing 1000 steps of steepest descent energy minimization in explicit solvent (SPC water model), as implemented in the GROMACS package. Continuum electrostatic calculations were then carried out using APBS software (14-16). The solvent probe radius was set at 1.4 Å, the dielectric constants (ε) were set at the values of 4.0 x $10^{-12}$ F $m^{-1}$ and 78.5 x $10^{-12}$ F $m^{-1}$ for the protein and the solvent respectively and the temperature was set to 25 °C. A cubic grid having dimensions 193 x 193 x 193 Å was adopted for all calculations in a multigrid approach. A first calculation was carried out with a 1.042 Å step per grid point, employing Debye-Hückel (full coulombic) boundary conditions. In these conditions βLg occupies approximately 50% of the grid space. The second calculation used a 0.523 Å step per grid point. In these new conditions, the βLg dimer occupies approximately the 90-95% of the grid. The electrostatic binding energy has been calculated by subtracting the energies of a pair of solvated dissociated βLg monomers from the energy of the solvated dimer. This work presents the results obtained by the second, more accurate calculation. To understand the ionic contribution to the binding energy, the same calculation was carried out over a range of ionic strengths spanning 0 to 500 mM by using NaCl as salt. The radii used to represent ionic species in solution were 0.95 Å and 1.81 Å for $Na^+$ and $Cl^-$ ions, respectively. The distribution of the ion charge around the βLg structure upon dimer formation at different ionic strengths has been calculated by subtracting the ion charge distribution of the monomers from the ion charge distribution of the dimer.

Electrostatic binding energy as a function of the ionic strength was analyzed assuming an extended Debye-Hückel model described by the following equation:

$$\ln K_{\mathrm{eq}} = \frac{-|z_+ z_-| A \sqrt{\text{Ionic strength}}}{1 + B \cdot \sqrt{\text{Ionic strength}}}$$

where $z_+$ and $z_-$ are the positive and negative charges on the ion in solution, and $A$ and $B$ are temperature-dependent constants for aqueous solutions, having values of 0.5085 and 1, respectively.

**Table S1:** Thermodynamic parameters characterizing the monomer-dimer equilibrium of bovine βLg at 20 °C (except where noted) and pH 1.6 to pH 8.8.

| βLg | pH | [NaCl] | $\Delta G_A$ (/kcal mol$^{-1}$) | $\Delta H_A$ (/kcal mol$^{-1}$) | $T\Delta S_A$ (/kcal mol$^{-1}$) | $K_A$ (20 °C) (/M$^{-1}$ x 10$^{-3}$) | $K_D$ (20 °C) (/μM) | Technique | Reference |
|---|---|---|---|---|---|---|---|---|---|
| B | 8.8 | 0.1 | | | | 4.2 | 238 | AUC | Georges et al (1962) (17) |
| **A**<br>**B** | **7.5** | **0.1** | | | | **71**<br>**116** | **11**<br>**8.6** | **AUC-SE/SV (25 °C)** | **This work** |
| A<br>B<br>C | 7.5 | 0.1 | -5.63 | ND | ND | 16<br>125<br>>200 | 63<br>8.0<br><5 | AUC-SE (20.5 °C) | McKenzie & Sawyer (1967) (18) |
| A<br>B | 7.0 | 0.1 | -6.20<br>-6.49 | -3.49<br>-5.77 | 2.71<br>0.72 | 42<br>69 | 24<br>14 | ITC<br>ITC | Bello *et al*. (2008) (19)<br>Bello *et al*. (2011) (20) |
| A | 7.0 | 0.1 | -6.27 | -15.52 | -9.25 | 47 | 21 | AUC-SE | Kelly & Reithel (1971) (21) |
| A<br>B | 6.9 | 0.13 | -6.29<br>-6.9 | ND | ND | 49<br>142 | 20<br>7.0 | AUC-SE/SV | Zimmerman *et al*. (1970) (22) |
| **A**<br>**B** | **6.5** | **0.1** | | | | **250**<br>**400** | **4.0**<br>**2.5** | **AUC-SE/SV (25 °C)** | **This work** |
| A* | 6.5 | 0.1 | -6.35 | ND | ND | 54 | 19 | AUC-SE | Sakai *et al*. (2000) (23) |
| A* | 6.5 | 0.02 | -7.11 | ND | ND | 203 | 4.9 | AUC-SE | Sakurai & Goto (2002) (24) |
| A | 5.2 | 0.1 | -8.08 | -6.91 | 1.17 | 1120 | 0.89 | AUC-SE | Kelly & Reithel (1971) (21) |
| C | 4.65 | 0.1 | -8.43 | ~0 | 85 | 1930 | 0.52 | AUC-SE | Sarquis & Adams (1976) (25) |
| **A**<br>**B** | **3.5** | **0.1** | | | | **250**<br>**714** | **4.0**<br>**1.4** | **AUC-SE/SV (25 °C)** | **This work** |
| B$^{Prep\ I}$ | 3.5 | 0.1 | -7.3 | | | 230 | 4.3 | Light scatt. (25 °C) | Townend *et al*. (1960) (26) |

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| βLg | pH | [NaCl] | $\Delta G_A$ (/kcal mol$^{-1}$) | $\Delta H_A$ (/kcal mol$^{-1}$) | $T\Delta S_A$ (/kcal mol$^{-1}$) | $K_A$ (20 °C) (/M$^{-1}$ x 10$^{-3}$) | $K_D$ (20 °C) (/μM) | Technique | Reference |
|---|---|---|---|---|---|---|---|---|---|
| A<br>A<br>A | 3.0 | 0.05 / 0.5<br>0.1<br>0.01 | -5.1 / -7.1 | -12 | -6.9 / -5.0 | 6.9 / 162<br>16<br>0.18 | 145 / 6.2<br>63<br>5,600 | AUC – ITC | Sakurai & Goto (2001) (4) |
| B$^{Prep\ II}$ | 3.0 | 0.1 | -5.8 | | | 18 | 56 | Light scatt. (25 °C) | Townend *et al*. (1960) (26) |
| B | 3.0 | 0.16 | | | | 41 | 24 | AUC-SE (25 °C) | Joss & Ralston (1996) (27) |
| A<br>B | 2.7 | 0.1 | -5.4 | -12.4<br>-12.8 | -7.0<br>-6.8 | 7.7<br>19.6 | 130<br>51 | Light scatt. (25 °C) | Townend *et al*. (1961) (28) |
| C | 2.7 | 0.1 | | | | ~17 | ~61 | AUC (25 °C) | Townend *et al*. (1964) (29) |
| B | 2.7 | 0.1 | | | | 6.3 | 160 | AUC-SE (25 °C) | Albright & Williams (1968) (30) |
| B$^{Prep\ II}$ | 2.7 | -5.7 | | | | 15 | 67 | Light scatt. (25 °C) | Townend *et al*. (1960) (26) |
| B | 2.64 | 0.16 | -5.84 | -10.4 | -4.47 | 19 | 53 | AUC-SE (25 °C) | Visser *et al*. (1972) (31) |
| B<br>B | 2.6 | 0.1<br>0.16 | -6.02 | -15.1 | -9.1 | 11<br>31 | 91<br>32 | AUC-SE | Joss & Ralston (1996) (27) |
| **A**<br>**B** | **2.5** | **0.1** | | | | **66**<br>**122** | **15**<br>**8.2** | **AUC-SE/SV (25 °C)** | **This work** |
| A | 2.46 | 0.1 | -4.71 | -17.1 | 12 | 3.22 | 310 | AUC-SE | Tang & Adams (1973) (32) |
| C | 2.46 | 0.1 | -5.39 | -16.5 | -11 | 10.5 | 95 | AUC-SE | Sarquis & Adams (1974) (33) |
| B$^{Prep\ II}$ | 2.2 | 0.1 | -5.5 | | | 11 | 91 | Light scatt. (25 °C) | Townend *et al*. (1960) (26) |

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| βLg | pH | [NaCl] | $\Delta G_A$ (/kcal mol$^{-1}$) | $\Delta H_A$ (/kcal mol$^{-1}$) | $T\Delta S_A$ (/kcal mol$^{-1}$) | $K_A$ (20 °C) (/M$^{-1}$ x 10$^{-3}$) | $K_D$ (20 °C) (/μM) | Technique | Reference |
|---|---|---|---|---|---|---|---|---|---|
| B<br>B | 2.3 | 0.07<br>0.107 | | | | 0.08/0.033<br>5/13 | 12,500/30,000<br>200/77 | Light/SAXS | Baldini *et al*. (1999) (34) |
| B$^{Prep\ II}$ | 2.0 | 0.1 | -4.9 | | | 3.9 | 260 | Light scatt. (25 °C) | Townend *et al.* (1960) (26) |
| B | 2.0 | 0.16 | | | | 21 | 48 | AUC-SE | Joss & Ralston (1996) (27) |
| B$^{Prep\ II}$ | 1.6 | 0.1 | -4.9 | | | 3.9 | 260 | Light scatt. (25 °C) | Townend *et al.* (1960) (26) |

**Pichia pastoris* expression, known to produce glycated protein

‡ Values of 4.9(12) x 10$^3$ and 6.4(8) x 10$^3$ M$^{-1}$ for pH 3, 50 mM NaCl and 20 °C are also found in this paper.

**Table S2:** Details of experimental conditions for analytical ultracentrifugation runs. The loading concentration was determined prior to the experiment using the absorbance at 280 nm and an extinction coefficient of 17210 $M^{-1}$ $cm^{-1}$. When the data were fitted using SEDPHAT, the extinction coefficient was floated, since the data were collected at various wavelengths.

| Buffer | pH | βLg A protein concentration from wavelength scans (μM) | βLg B protein concentration from wavelength scans (μM) |
|---|---|---|---|
| Citrate | 2.5 | 5.3<br>15.7<br>33.4 | 6.8<br>19.2<br>53.0 |
| Citrate | 3.5 | 4.8<br>15.8<br>26.7 | 5.1<br>15.0<br>44.1 |
| Citrate | 4.5 | 2.5<br>14.5<br>43.9 | 3.6<br>13.9<br>47.8 |
| Citrate | 5.5 | 6.5<br>16.8<br>47.3 | 6.5<br>17.6<br>50.0 |
| MES | 5.5 | 5.7<br>16.6<br>52.1 | 9.6<br>17.8<br>50.4 |
| MOPS | 6.5 | 3.5<br>11.0<br>33.0 | 4.3<br>11.9<br>36.5 |
| MOPS | 7.5 | 5.1<br>22.6<br>36.5 | 5.2<br>15.4<br>43.3 |

**Table S3:** Buffer density and viscosity values used for the AUC experiments at 25 $^{o}$C and an ionic strength of 100 mM NaCl. The values for citrate buffer were retrieved from SEDNTERP 1.09 database (http://www.Jphilo.mailway.com/download.htm). The density and viscosity for MOPS buffer at the different pH values were experimentally determined using a densitometer and a viscometer (Anton Parr).

| **pH** | **Buffer (20 mM in 100 mM NaCl)** | **Density ( $\rho$ , g cm$^{-3}$))** | **Viscosity ( $\eta$ , poise)** |
|---|---|---|---|
| 2.5-5.5 | Citrate | 1.0027 | 0.008904 |
| 5.5 | MES [2-(*N*-morpholino)ethanesulfonic acid] | 1.0025 | 0.00902 |
| 6.5-7.5 | MOPS [3-(*N*-morpholino)propanesulfonic acid] | 1.0022 | 0.00902 |

**Table S4:** Hydrodynamic properties and fit statistics for SV data of βLg A at three concentrations over the pH range 2.5-7.5.

| **Sample** | **Model** | $s_{20,w}$ peak (S) | Weight-averaged $s_{20,w}$ | Mass (Da) | $f/f_0$ | *Runs-Z test score* | *r.m.s.d.* |
|---|---|---|---|---|---|---|---|
| **Citrate pH 2.5** | | | | | | | |
| 5.3 μM | *c(s)* distribution | 2.1 | 2.2 | | 1.26 | 7.6 | 0.005 |
| 15.7 μM | *c(s)* distribution | 2.4 | 2.3 | | 1.15 | 13 | 0.006 |
| 33.4 μM | *c(s)* distribution | 2.6 | 2.5 | | 1.17 | 12 | 0.006 |
| | | | | | | | |
| **Citrate pH 3.5** | | | | | | | |
| 4.8 μM | *c(s)* distribution | 2.7 | 2.6 | | 1.23 | 1.3 | 0.008 |
| 15.8 μM | *c(s)* distribution | 2.7 | 2.7 | | 1.24 | 0.5 | 0.009 |
| 26.7 μM | *c(s)* distribution | 2.8 | 2.7 | | 1.25 | 1.7 | 0.009 |
| | | | | | | | |
| **Citrate pH 4.5** | | | | | | | |
| 2.5 μM | *c(s)* distribution | 2.7 | 2.6 | | 1.16 | 4.3 | 0.004 |
| 14.5 μM | *c(s)* distribution | 2.8 | 2.8 | | 1.20 | 5.8 | 0.004 |
| 43.9 μM | *c(s)* distribution | 2.8 | 2.8 | | 1.23 | 5.5 | 0.006 |
| | | | | | | | |
| **Citrate pH 5.5** | | | | | | | |
| 6.5 μM | *c(s)* distribution | 2.6 | 2.6 | | 1.20 | 2.5 | 0.004 |
| 16.8 μM | *c(s)* distribution | 2.7 | 2.7 | | 1.22 | 4.7 | 0.005 |
| 47.3 μM | *c(s)* distribution | 2.8 | 2.7 | - | 1.23 | 14 | 0.01 |
| 47.3 μM | *c(M)* distribution | - | - | ~33,000 | 1.24 | 12 | 0.01 |
| 47.3 μM | discrete species | 2.7 | - | 33,900±1,400 | - | 3.9 | 0.005 |

**Table S4 cont.:** Hydrodynamic properties and fit statistics for SV data of βLg A at three concentrations over the pH range 2.5-7.5 (follows from previous page).

| **Sample** | **Model** | $s_{20,w}$ peak (S) | Weight-averaged $s_{20,w}$ | Mass (Da) | $f/f_0$ | *Runs-Z test score* | *r.m.s.d.* |
|---|---|---|---|---|---|---|---|
| **MES pH 5.5** | | | | | | | |
| 5.7 μM | *c(s)* distribution | 2.6 | 2.5 | | 1.20 | 4.03 | 0.006 |
| 16.6 μM | *c(s)* distribution | 2.7 | 2.6 | | 1.22 | 5.73 | 0.006 |
| 52.1 μM | *c(s)* distribution | 2.7 | 2.6 | | 1.22 | 6.19 | 0.01 |
| 52.1 μM | *c(M)* distribution | | | ~33,000 | 1.24 | 8.7 | 0.01 |
| 52.1 μM | discrete species | 2.7 | - | 33,300±1,700 | - | 9.0 | 0.007 |
| | | | | | | | |
| **MOPS pH 6.5** | | | | | | | |
| 3.5 μM | *c(s)* distribution | 2.1 | 2.3 | | 1.30 | 4.3 | 0.007 |
| 11.0 μM | *c(s)* distribution | 2.5 | 2.6 | | 1.24 | 4.8 | 0.01 |
| 33.0 μM | *c(s)* distribution | 2.6 | 2.7 | | 1.37 | 10 | 0.007 |
| | | | | | | | |
| **MOPS pH 7.5** | | | | | | | |
| 5.13 μM | *c(s)* distribution | 2.1 | 2.0 | | 1.32 | 1.5 | 0.005 |
| 22.6 μM | *c(s)* distribution | 2.6 | 2.3 | | 1.30 | 6.8 | 0.006 |
| 36.5 μM | *c(s)* distribution | 2.9 | 2.4 | | 1.31 | 9.6 | 0.007 |
| | | | | | | | |

**Table S5:** Hydrodynamic properties and fit statistics for SV data of βLg B at three concentrations over the pH range 2.5-7.5.

| **Sample** | **Model** | $s_{20,w}$ peak (S) | Weight-averaged $s_{20,w}$ | Mass (Da) | $f/f_0$ | *Runs-Z test score* | *r.m.s.d.* |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| **Citrate pH 2.5** | | | | | | | |
| 6.8 μM | *c(s)* distribution | 2.2 | 2.3 | | 1.26 | 1.0 | 0.005 |
| 19.2 μM | *c(s)* distribution | 2.5 | 2.5 | | 1.17 | 0.11 | 0.006 |
| 53.0 μM | *c(s)* distribution | 2.7 | 2.6 | | 1.22 | 4.4 | 0.007 |
| | | | | | | | |
| **Citrate pH 3.5** | | | | | | | |
| 5.1 μM | *c(s)* distribution | 2.7 | 2.7 | | 1.27 | 11 | 0.008 |
| 15.0 μM | *c(s)* distribution | 2.8 | 2.8 | | 1.23 | 2.4 | 0.012 |
| 44.1 μM | *c(s)* distribution | 2.8 | 2.8 | | 1.26 | 3.5 | 0.008 |
| | | | | | | | |
| **Citrate pH 4.5** | | | | | | | |
| 3.6 μM | *c(s)* distribution | 2.7 | 2.7 | | 1.24 | 2.5 | 0.006 |
| 13.9 μM | *c(s)* distribution | 2.8 | 2.8 | | 1.23 | 2.3 | 0.004 |
| 47.8 μM | *c(s)* distribution | 2.8 | 2.8 | | 1.24 | 10.15 | 0.01 |
| | | | | | | | |
| **Citrate pH 5.5** | | | | | | | |
| 6.5 μM | *c(s)* distribution | 2.7 | 2.7 | | 1.21 | 4.6 | 0.004 |
| 17.6 μM | *c(s)* distribution | 2.8 | 2.8 | | 1.21 | 3.7 | 0.004 |
| 50.0 μM | *c(s)* distribution | 2.8 | 2.8 | - | 1.23 | 10.16 | 0.02 |
| 50.0 μM | *c(M)* distribution | - | - | ~33,000 | 1.25 | 14 | 0.02 |
| 50.0 μM | discrete species | 2.9 | - | 34,200±1,100 | - | 3.0 | 0.005 |

**Table S5 cont.:** Hydrodynamic properties and fit statistics for SV data of βLg B at three concentrations over the pH range 2.5-7.5 (follows from previous page).

| **Sample** | **Model** | $s_{20,w}$ peak (S) | Weight-averaged $s_{20,w}$ | Mass (Da) | $f/f_0$ | *Runs-Z test score* | *r.m.s.d.* |
|---|---|---|---|---|---|---|---|
| **MES pH 5.5** | | | | | | | |
| 9.6 μM | *c(s)* distribution | 2.7 | 2.6 | | 1.20 | 1.7 | 0.004 |
| 17.8 μM | *c(s)* distribution | 2.7 | 2.7 | | 1.20 | 5.0 | 0.005 |
| 50.4 μM | *c(s)* distribution | 2.7 | 2.7 | | 1.22 | 9.81 | 0.009 |
| 50.4 μM | *c(M)* distribution | - | - | ~33,000 | 1.24 | 10.7 | 0.01 |
| 50.4 μM | discrete species | 2.9 | - | 33,200±1,400 | - | 7.0 | 0.007 |
| | | | | | | | |
| **MOPS pH 6.5** | | | | | | | |
| 4.3 μM | *c(s)* distribution | 2.5 | 2.6 | | 1.33 | 7.8 | 0.007 |
| 11.9 μM | *c(s)* distribution | 2.8 | 2.7 | | 1.22 | 8.9 | 0.007 |
| 36.5 μM | *c(s)* distribution | 2.9 | 2.9 | | 1.23 | 7.8 | 0.009 |
| | | | | | | | |
| **MOPS pH 7.5** | | | | | | | |
| 5.2 μM | *c(s)* distribution | 2.3 | 2.2 | | 1.46 | 1.6 | 0.005 |
| 15.4 μM | *c(s)* distribution | 2.6 | 2.4 | | 1.29 | 0.69 | 0.006 |
| 43.3 μM | *c(s)* distribution | 2.7 | 2.7 | | 1.26 | 2.7 | 0.006 |
| | | | | | | | |

**Table S6:** The fitted values for monomer (s1) and dimer (s2).

| | **s1** | | **s2** | |
|---|---|---|---|---|
| | **βLg A** | **βLg B** | **βLg A** | **βLg B** |
| **pH 2.5 *citrate*** | 1.79 | 1.83 | 2.90 | 2.76 |
| **pH 3.5 *citrate*** | 2.27 | 2.28 | 2.79 | 2.76 |
| **pH 6.5 *MOPS*** | 1.98 | 1.91 | 2.91 | 2.90 |
| **pH 7.5 *MOPS*** | 1.40 | 1.66 | 2.93 | 2.92 |

**Table S7.** Equilibrium and rate constants at 25 $^{o}$C and at an ionic strength of 100 mM NaCl, calculated for βLg A and βLg B dimer dissociation at different pH[*] by global fitting of SE data for $K_D^{(2-1)}$ , of SV data for $K_D^{(2-1)}$ and $k_{off}$, and of SE and SV data for $K_D^{(2-1)}$ and $k_{off}$..

| **Protein** | **Global fit - equilibrium data only** | **Global fit - velocity data only** | | **Global fit – velocity and equilibrium data** | |
|---|---|---|---|---|---|
| | $K_D^{(2-1)}$ **(/μM)** | $K_D^{(2-1)}$ **(/μM)** | $k_{off}$ **(/s$^{-1}$)** | $K_D^{(2-1)}$ **(/μM)** | $k_{off}$ **(/s$^{-1}$)** |
| βLg A | | | | | |
| pH 2.5(citrate) | 19.3 (14.9-26.2) | 13.8 (10.1-17.7) | 0.008 (0.002-0.019) | 14.8 (11.2-18.4) | 0.009 (0.002-0.018) |
| pH 3.5(citrate) | 1.4 (0.2-4.4) | 4.2 (1.8-7.0) | 0.007 (ND)[†] | 4.0 (2.3-6.0) | 0.007 (ND)[†] |
| pH 6.5(MOPS) | 11.9 (5.5-23.7) | 3.9 (2.5-5.8) | Rapid equil. | 4.0 (2.7-5.6) | Rapid equil. |
| pH 7.5(MOPS) | 24 (13-43) | 10.4 (6.1-15.0) | Rapid equil. | 10.8 (7.6-15.8) | Rapid equil. |
| | | | | | |
| βLg B | | | | | |
| pH 2.5(citrate) | 15 (11-21) | 8.3 (5.5-11.1) | 0.009 (0.003-0.029) | 8.2 (5.8-11.1) | 0.013 (0.003-0.045) |
| pH 3.5(citrate) | 11.6 (6.0-20.6) | 1.1 (0.7-2.3) | 0.041 (ND)[†] | 1.4 (1.1-4.5) | 0.037 (ND)[†] |
| pH 6.5(MOPS) | 1.0 (0.3-4.8) | 2.2 (1.3-3.7) | Rapid equil. | 2.5 (1.0-7.1) | Rapid equil. |
| pH 7.5(MOPS) | 17 (10-27) | 8.4 (7.8-10.3) | Rapid equil. | 8.6 (6.3-10.9) | Rapid equil. |

[*] Calculated error ranges representing an estimated 69% confidence interval are reported in parentheses.

[†] Indicative value, as no error range could be determined.

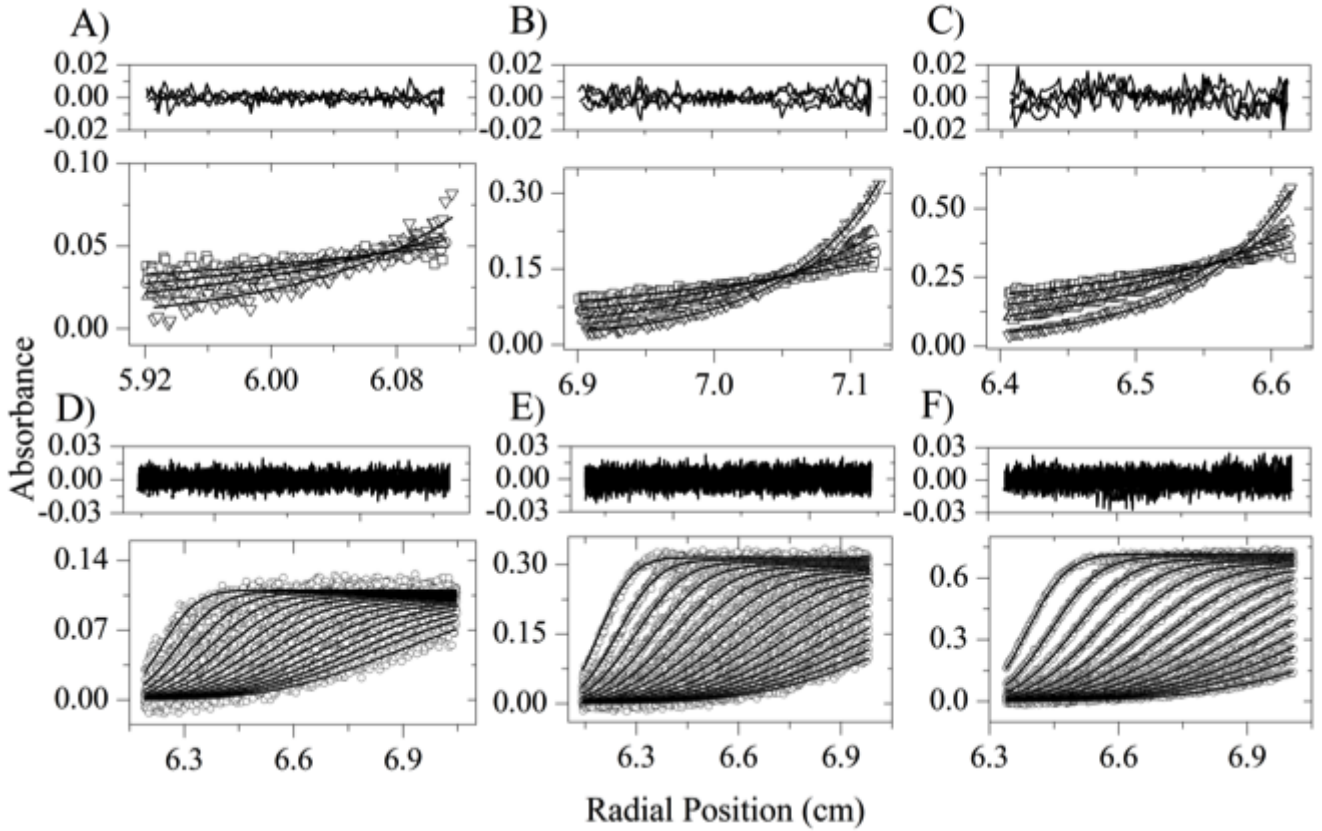


**Fig. S1:** Global fit of sedimentation equilibrium (*A-C*) and velocity (*D-F*) data for β-lactoglobulin A at pH 2.5 (*20 mM citrate, 100 mM NaCl, 25 $^{o}$C*), fitted to a *monomer-dimer equilibrium* model, as implemented in SEDPHAT. The protein concentrations were 5.3 μM (*A and D*), 15.7 μM (*B and E*) and 33.4 μM (*C and F*). The equilibrium data (*A-C*) were collected at 4 speeds (*12,000 – triangle down, 15,000 – triangle up, 18,000 – circle, and 24,000 – square, rpm*) at a wavelength of 250 nm. The velocity data were collected at 50,000 rpm at 280 nm wavelength. Residual plots of the fit are shown above. Statistics for the non-linear least-squares fits were: *(A)* r.m.s.d. = 0.003, Z-score = 2, *(B)* r.m.s.d. = 0.004, Z-score = 9, *(C)* r.m.s.d. = 0.006, Z-score = 9, *(D)* r.m.s.d. = 0.005, Z-score = 11, *(E)* r.m.s.d. = 0.006, Z-score = 3, *(F)* r.m.s.d. = 0.005, Z-score = 7. The global reduced chi-squared was 0.344.

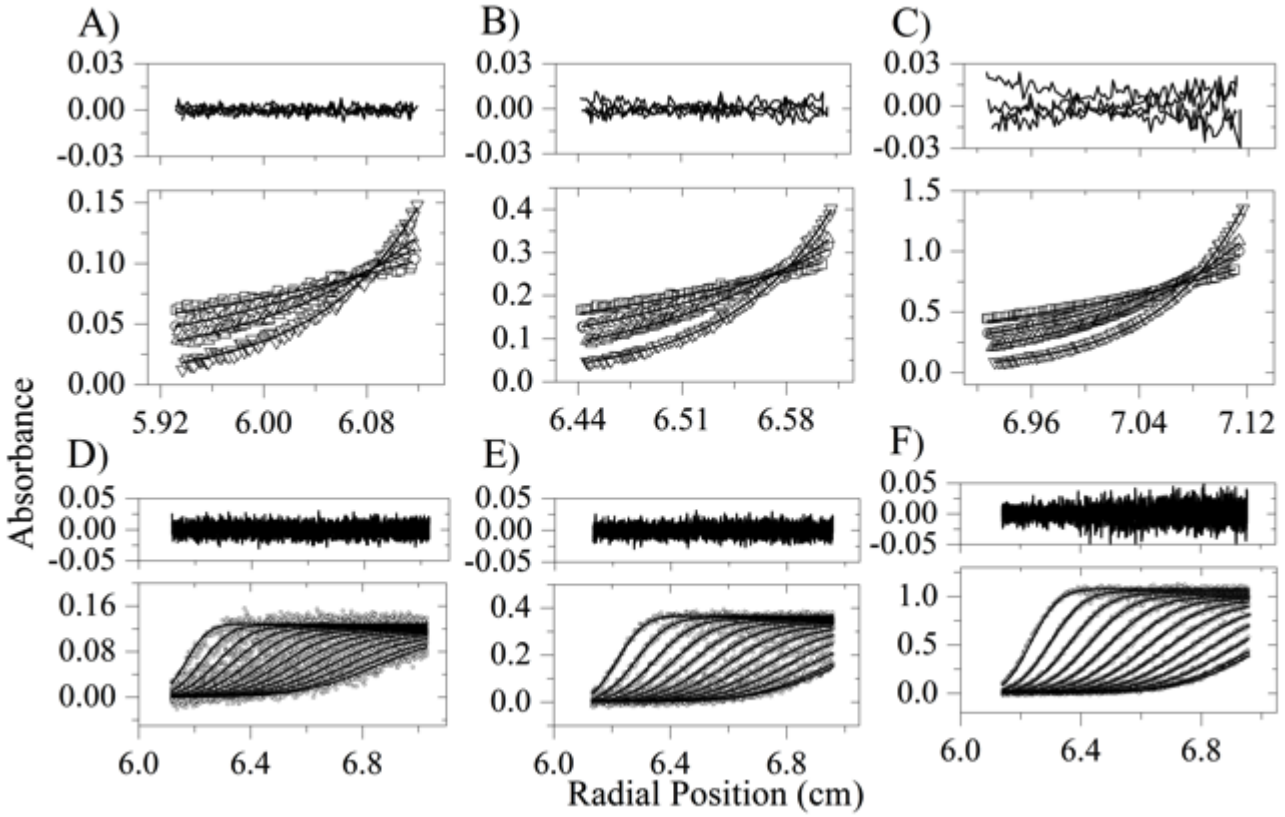


**Fig. S2:** Global fit of sedimentation equilibrium (*A-C*) and velocity (*D-F*) data for β-lactoglobulin A at pH 3.5 (*20 mM citrate, 100 mM NaCl, 25 $^{o}C$*), fitted to a *monomer-dimer equilibrium* model, as implemented in SEDPHAT. The protein concentrations were 4.8 μM (*A and D*), 15.8 μM (*B and E*) and 26.7 μM (*C and F*). The equilibrium data (*A-C*) were collected at 4 speeds (*12,000 – triangle down, 15,000 – triangle up, 18,000 – circle, and 24,000 – square, rpm*) at a wavelength of 260 nm. The velocity data were collected at 50,000 rpm at 237 nm wavelength. Residual plots of the fit are shown above. Statistics for the non-linear least-squares fits were: *(A)* r.m.s.d. = 0.002, Z-score = 2, *(B)* r.m.s.d. = 0.003, Z-score = 4, *(C)* r.m.s.d. = 0.005, Z-score = 10, *(D)* r.m.s.d. = 0.008, Z-score = 3, *(E)* r.m.s.d. = 0.009, Z-score = 0.1, *(F)* r.m.s.d. = 0.009, Z-score = 1.6. The global reduced chi-squared was 0.700.

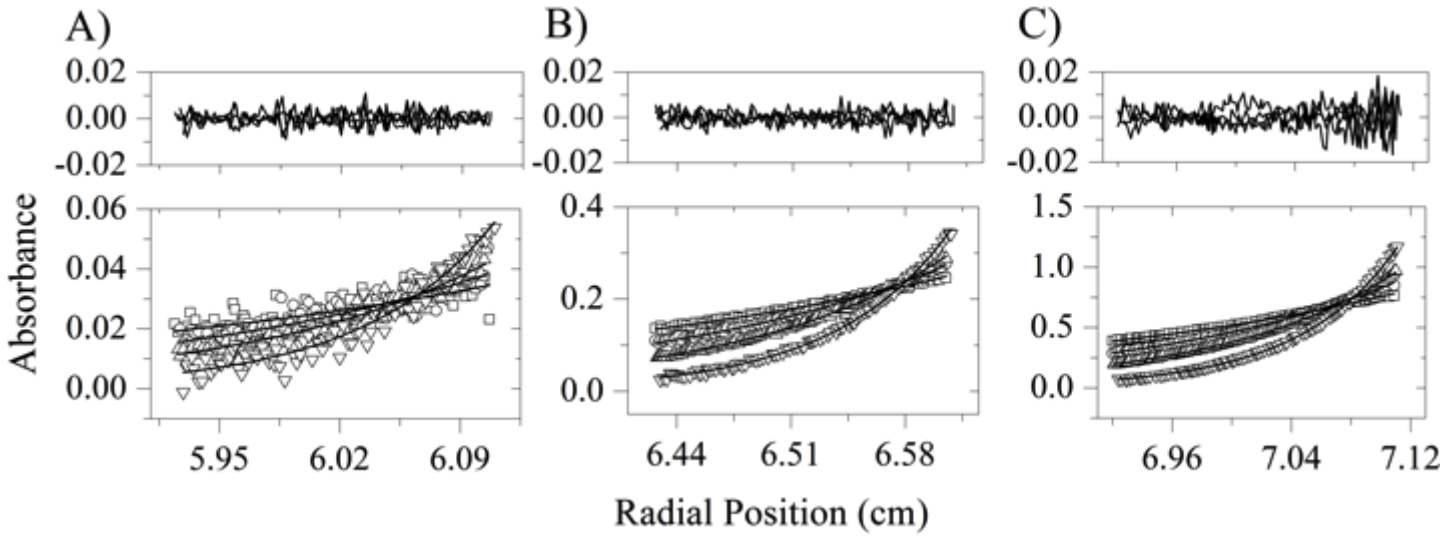


**Fig. S3:** Single species analysis of sedimentation equilibrium (*A*-*C*) data for β-lactoglobulin A at pH 4.5 (*20 mM citrate, 100 mM NaCl, 25 $^{o}$C*), fitted to a *single species of interacting system* model, as implemented in SEDPHAT. The protein concentrations were 2.5 μM (*A*), 14.5 μM (*B*) and 43.9 μM (*C*). The equilibrium data (*A*-*C*) were collected at 4 speeds (*12,000 – triangle down, 15,000 – triangle up, 18,000 – circle, and 24,000 – square, rpm*) at a wavelength of 255 nm. Residual plots of the fit are shown above. The derived molecular weight (and range) is 33,916 (32,130 – 34,860). Statistics for the non-linear least-squares fits were: *(A)* r.m.s.d. = 0.003, Z-score = 0.62, *(B)* r.m.s.d. = 0.003, Z-score = 2.31, *(C)* r.m.s.d. = 0.005, Z-score = 3.4.

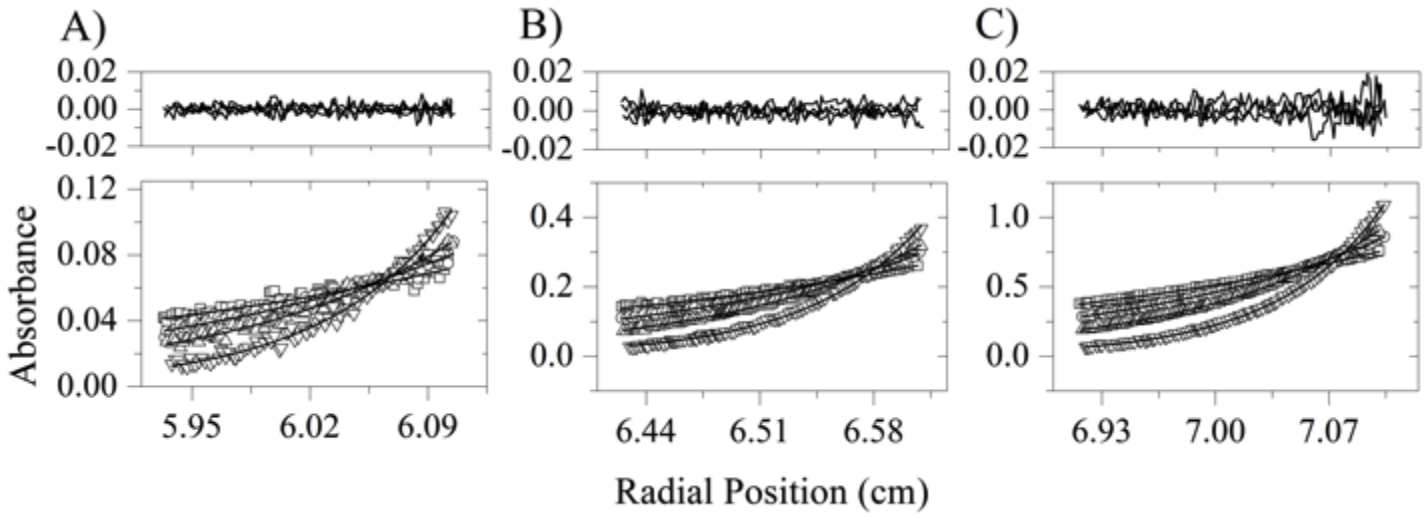


**Fig. S4:** Single species analysis of sedimentation equilibrium (*A*-*C*) data for β-lactoglobulin A at pH 5.5 (*20 mM citrate, 100 mM NaCl, 25 $^{o}$C*), fitted to a *single species of interacting system* model, as implemented in SEDPHAT. The protein concentrations were 6.5 μM (*A*), 16.8 μM (*B*) and 47.3 μM (*C*). The equilibrium data (*A*-*C*) were collected at 4 speeds (*12,000 – triangle down, 15,000 – triangle up, 18,000 – circle, and 24,000 – square, rpm*) at a wavelength of 255 nm. Residual plots of the fit are shown above. The derived molecular weight (and range) is 33,880 (32,521 – 35121). Statistics for the non-linear least-squares fits were: *(A)* r.m.s.d. = 0.003, Z-score = 1.5, *(B)* r.m.s.d. = 0.003, Z-score = 2.6, *(C)* r.m.s.d. = 0.005, Z-score = 3.9.

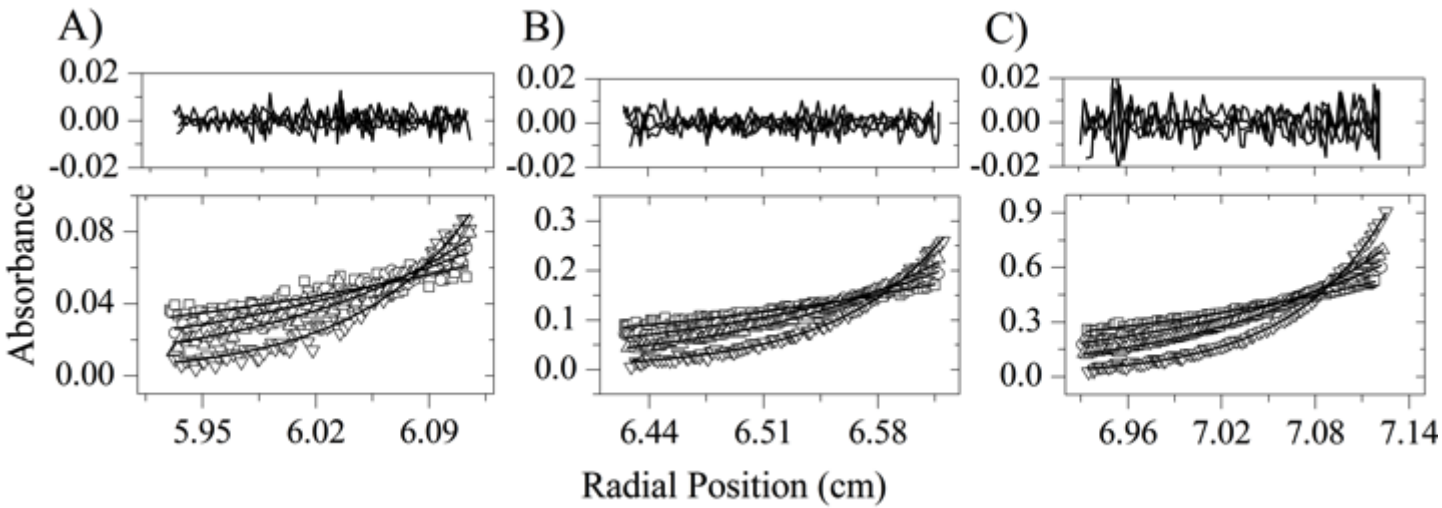


**Fig. S5:** Single species analysis of sedimentation equilibrium (*A*-*C*) data for β-lactoglobulin A at pH 5.5 (*20 mM MES, 100 mM NaCl, 25 $^{o}$C*), fitted to a *single species of interacting system* model, as implemented in SEDPHAT. The protein concentrations were 5.7 μM (*A*), 16.6 μM (*B*) and 52.1 μM (*C*). The equilibrium data (*A*-*C*) were collected at 4 speeds (*12,000 – triangle down, 15,000 – triangle up, 18,000 – circle, and 24,000 – square, rpm*) at a wavelength of 250 nm. Residual plots of the fit are shown above. The derived molecular weight (and range) is 35808 (33,808 – 38,058). Statistics for the non-linear least-squares fits were: *(A)* r.m.s.d. = 0.003, Z-score = 0.33, *(B)* r.m.s.d. = 0.004, Z-score = 5.7, *(C)* r.m.s.d. = 0.006, Z-score = 4.8.

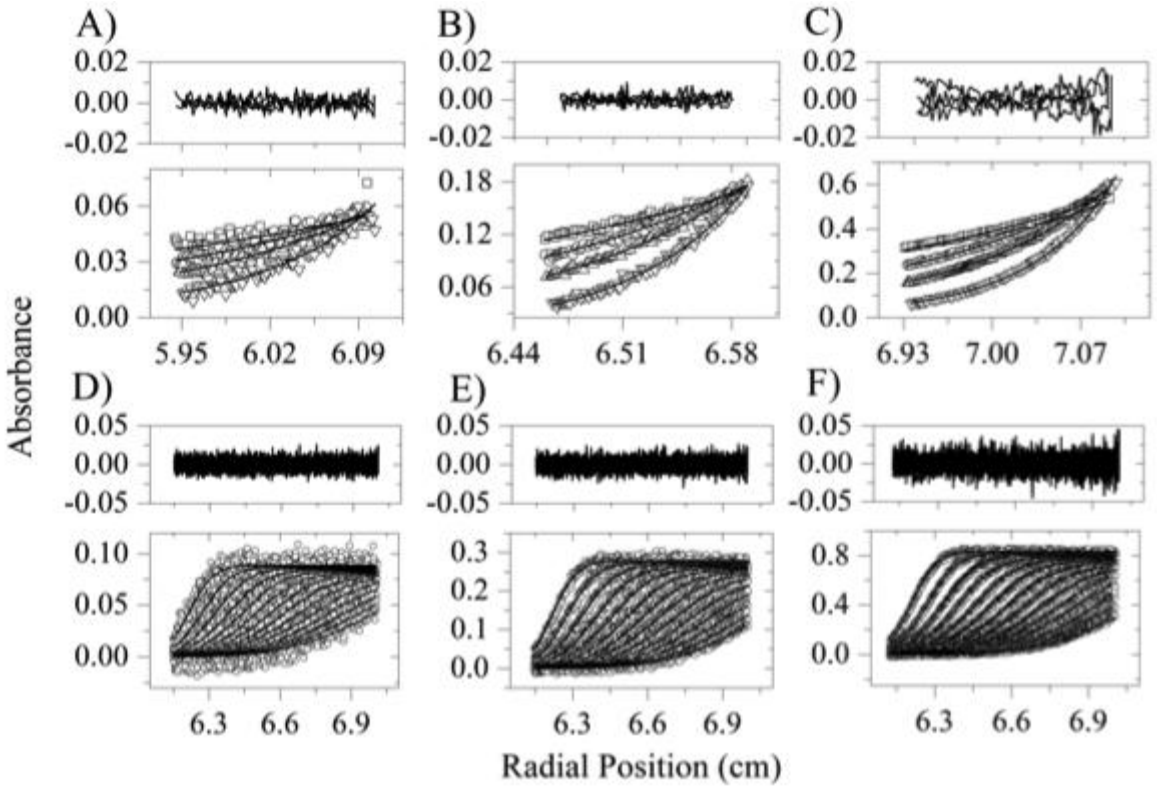


**Fig. S6:** Global fit of sedimentation equilibrium (*A-C*) and velocity (*D-F*) data for β-lactoglobulin A at pH 6.5 (*20 mM MOPS, 100 mM NaCl, 25 $^{o}C$*), fitted to a *monomer-dimer equilibrium* model, as implemented in SEDPHAT. The protein concentrations were 3.5 μM (*A and D*), 11.0 μM (*B and E*) and 33.0 μM (*C and F*). The equilibrium data (*A-C*) were collected at 4 speeds (*12,000 – triangle down, 15,000 – triangle up, 18,000 – circle, and 24,000 – square, rpm*) at a wavelength of 260 nm. The velocity data were collected at 50,000 rpm at 237 nm wavelength. Residual plots of the fit are shown above. Statistics for the non-linear least-squares fits were: *(A)* r.m.s.d. = 0.003, Z-score = 0.4, *(B)* r.m.s.d. = 0.003, Z-score = 0.4, *(C)* r.m.s.d. = 0.006, Z-score = 7.7, *(D)* r.m.s.d. = 0.007, Z-score = 2.8, *(E)* r.m.s.d. = 0.007, Z-score = 13, *(F)* r.m.s.d. = 0.01, Z-score = 38. The global reduced chi-squared was 0.7.

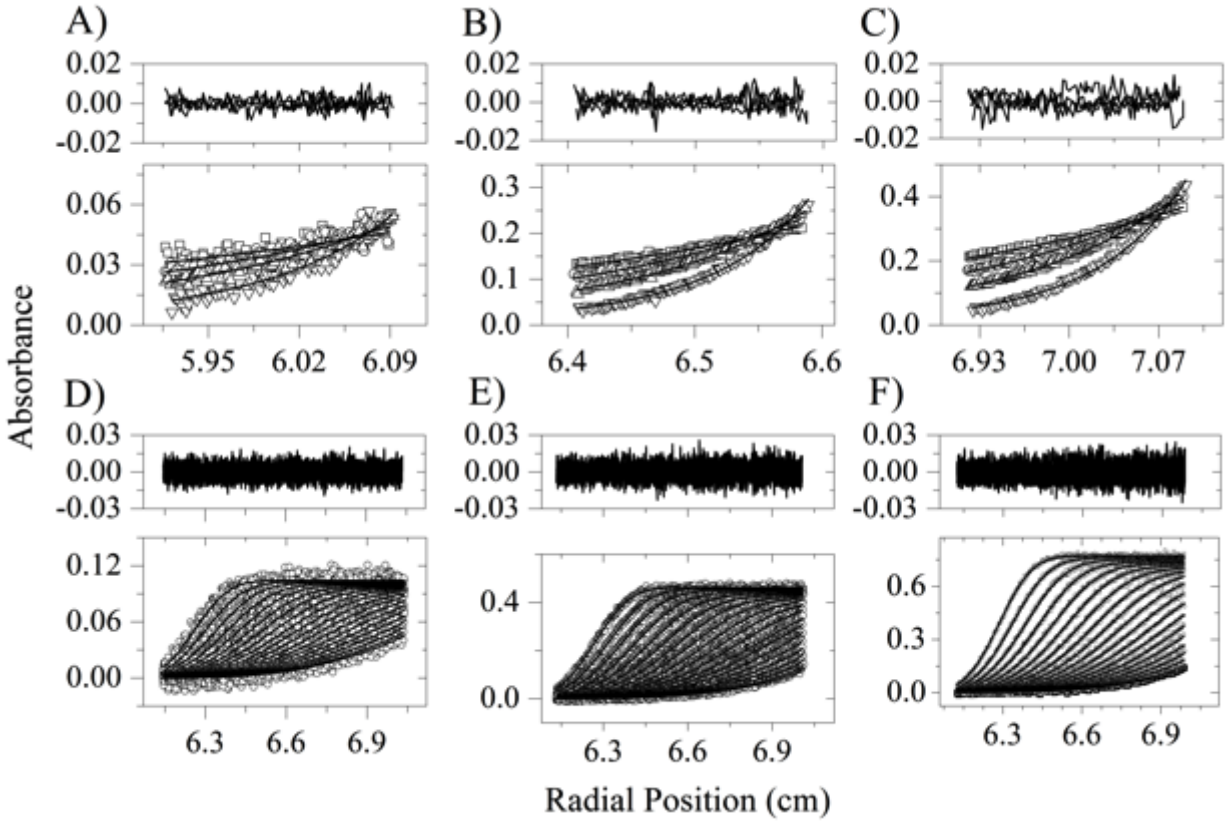


**Fig. S7:** Global fit of sedimentation equilibrium (*A-C*) and velocity (*D-F*) data for β-lactoglobulin A at pH 7.5 (*20 mM MOPS, 100 mM NaCl, 25 $^{o}C$*), fitted to a *monomer-dimer equilibrium* model, as implemented in SEDPHAT. The protein concentrations were 5.1 μM (*A and D*), 22.6 μM (*B and E*) and 36.5 μM (*C and F*). The equilibrium data (*A-C*) were collected at 4 speeds (*12,000 – triangle down, 15,000 – triangle up, 18,000 – circle, and 24,000 – square, rpm*) at a wavelength of 250 nm. The velocity data were collected at 50,000 rpm at 280 nm wavelength. Residual plots of the fit are shown above. Statistics for the non-linear least-squares fits were: *(A)* r.m.s.d. = 0.005, Z-score = 10.2, *(B)* r.m.s.d. = 0.004, Z-score = 6, *(C)* r.m.s.d. = 0.002, Z-score = 2, *(D)* r.m.s.d. = 0.005, Z-score = 1.6, *(E)* r.m.s.d. = 0.005, Z-score = 0.7, *(F)* r.m.s.d. = 0.006, Z-score = 2.7. The global reduced chi-squared was 0.4.

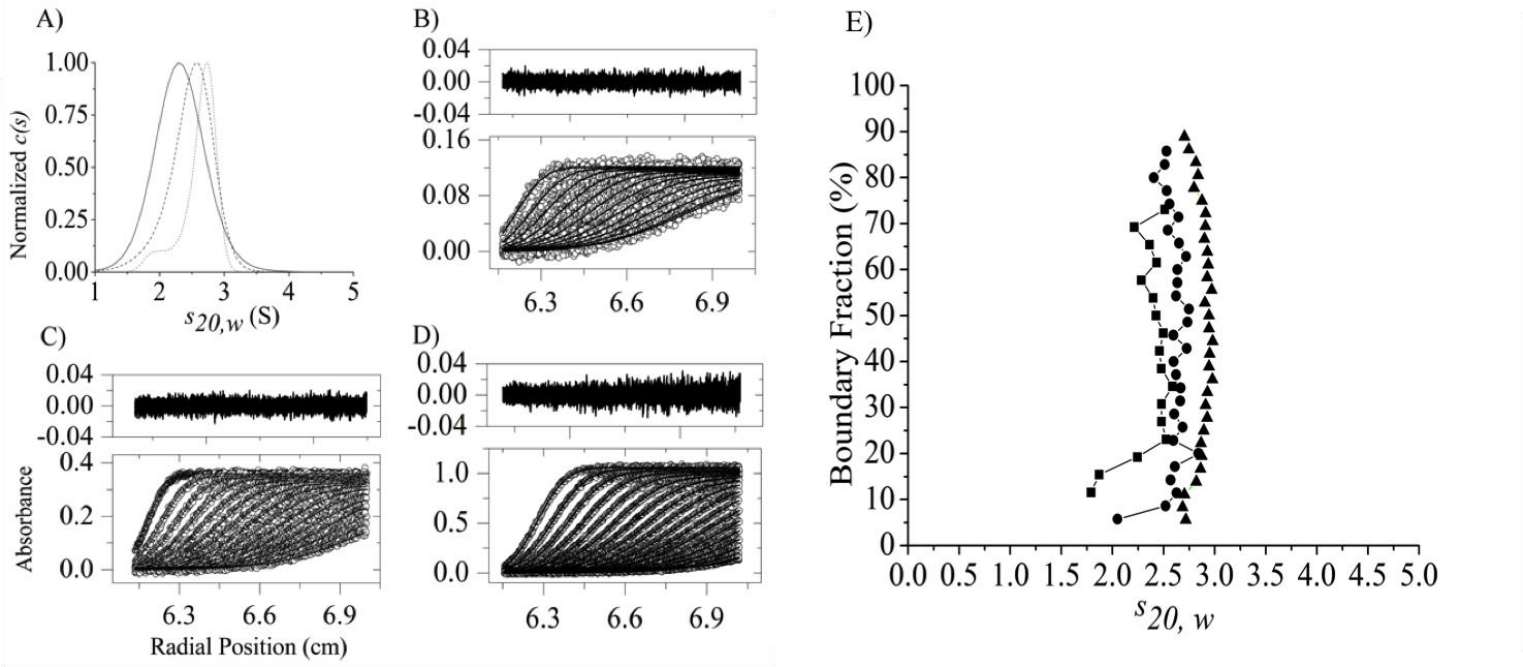


**Fig. S8.** Sedimentation velocity data for β-lactoglobulin B at pH 2.5 (*20 mM citrate, 100 mM NaCl, 25 $^{o}$C*). ***(****A)* Continuous *c*(*s*) analysis derived from SV data for β-lactoglobulin B at loading concentrations of 6.8 µM (*solid line; SV data and least-squares fits and residuals shown in panel B*), 19.2 µM (*dashed line; panel C*) and 53.0 µM (*dotted line; panel D*). Statistics for the non-linear least-squares fits: *(B)* r.m.s.d. = 0.005, test-*Z*-score = 0.95; *(C)* r.m.s.d. = 0.006, *Z*-score = 0.11; *(D)* r.m.s.d. = 0.007, *Z*-score = 4.4. In *(E)* the van Holde-Weischet full integration plot is shown at the three different loading concentrations of 6.8, 19.2 and 53.0 µM represented respectively using squares, circles and up-triangles.

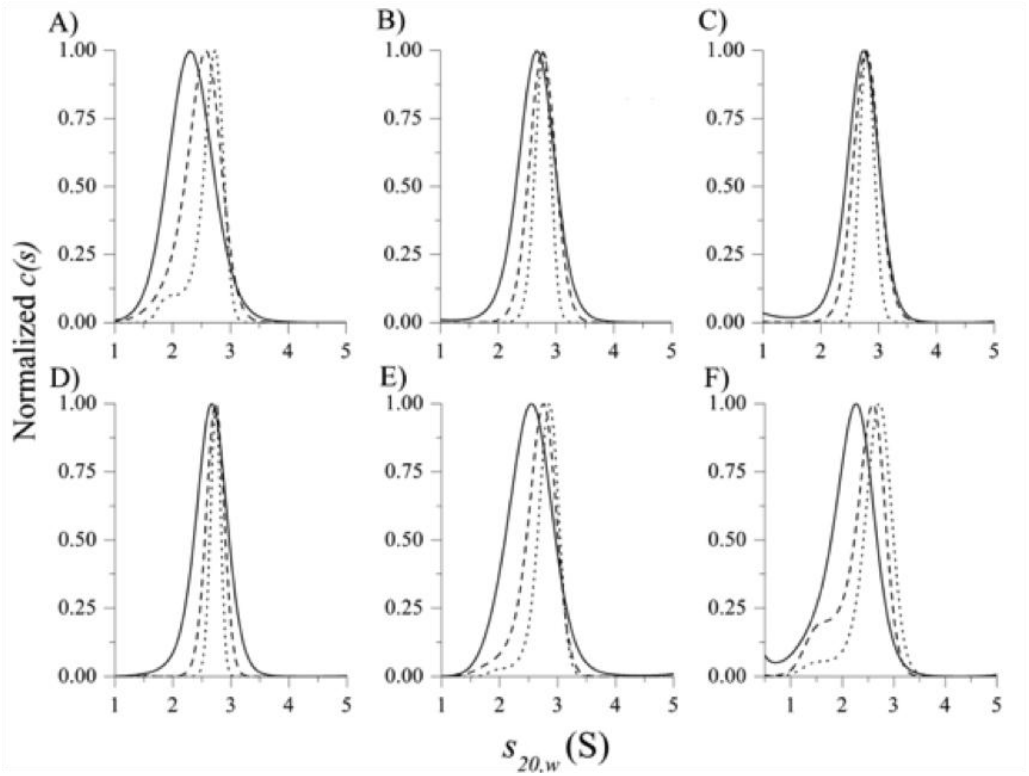


**Fig. S9.** Normalized $c(s_{20,w})$ distribution plots for β-lactoglobulin B over the pH range of 2.5 to 7.5. Figures show the distribution plots for β-lactoglobulin B obtained from the fitting of sedimentation velocity isotherms using a continuous $c(s)$ distribution model. The three protein concentrations explored are approximately 5 μM (*solid line*), 15 μM (*dashed line*) and 45 μM (*dotted line*); actual concentrations are given in parentheses. In 20 mM citrate and 100 mM NaCl: *(A)* pH 2.5 (6.76, 19.2, 53.0 μM), *(B)* pH 3.5 (5.1, 15.0, 44.1 μM), *(C)* pH 4.5 (3.6, 13.9, 47.8 μM) and *(D)* pH 5.5 (6.5, 17.6, 50.0 μM). In 0.0200 M MOPS and 100 mM NaCl: *(E)* pH 6.5 (4.3, 11.9, 36.5 μM) and *(F)* pH 7.5 (5.22, 15.4, 43.3 μM). Statistics for the fits can be found in the supporting material (*Figs. S11-S17*).

A) B)

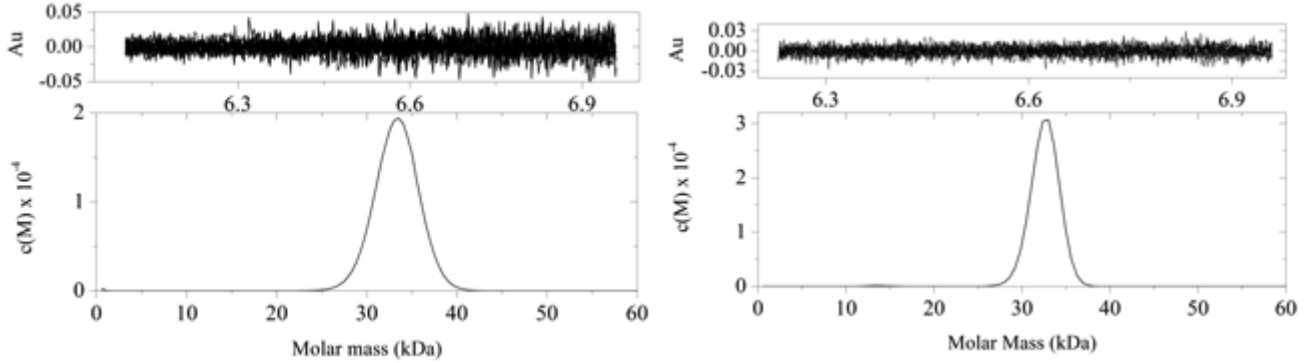


**Fig. S10.** Continuous mass distribution *c*(*M*) obtained from the fitting of SV data at pH 5.5 (*20 mM citrate*, *100 mM NaCl*) for *(A)* βLg A at 47.3 μM and *(B)* βLg B at 50.4 μM. The apparent molar mass of the species represented by the peak has been found to be 33,900 ± 1,300 Da for βLg A and 34,200 ± 900 for βLg B.

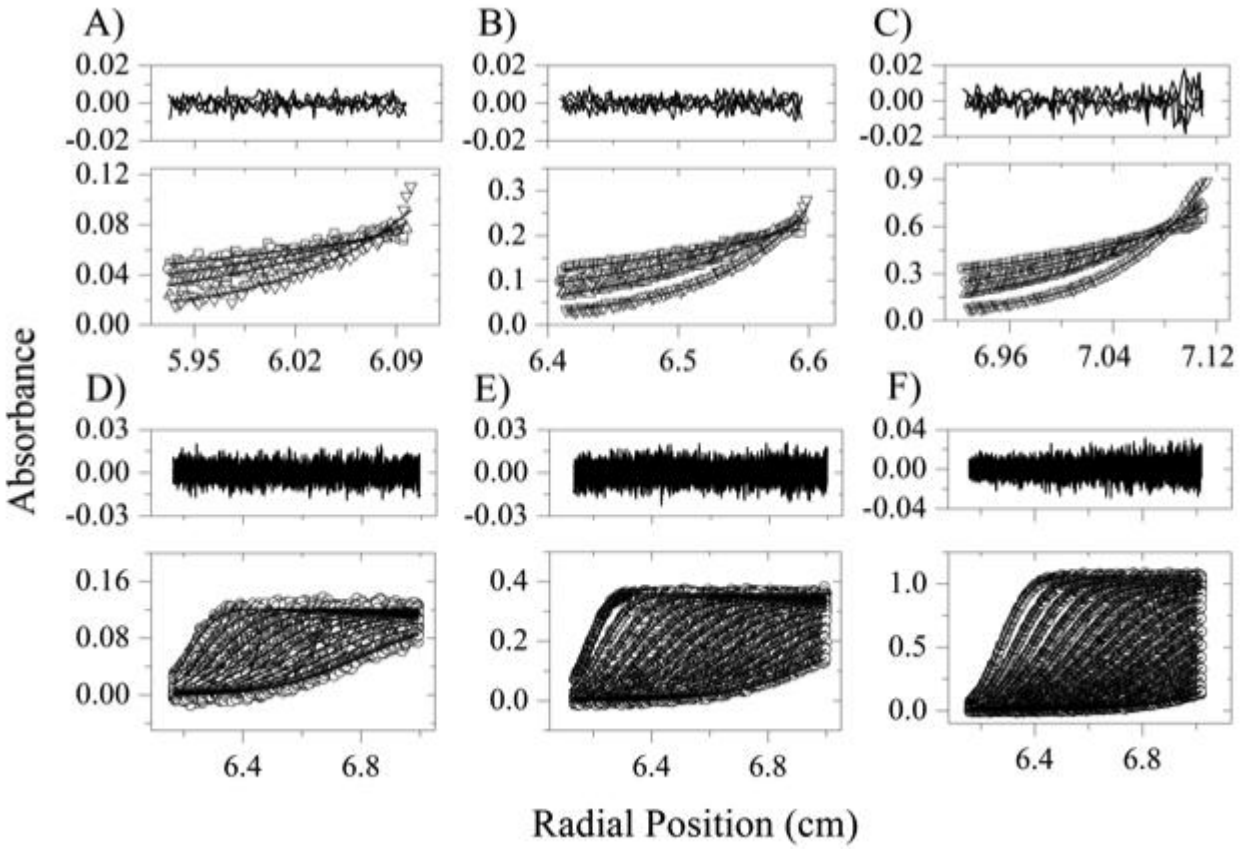


**Fig. S11:** Global fit of sedimentation equilibrium (*A-C*) and velocity (*D-F*) data for β-lactoglobulin B at pH 2.5 (*20 mM citrate, 100 mM NaCl, 25 $^{o}$C*), fitted to a *monomer-dimer equilibrium* model, as implemented in SEDPHAT. The protein concentrations were 6.76 μM (*A and D*), 19.2 μM (*B and E*) and 53.0 μM (*C and F*). The equilibrium data (*A-C*) were collected at 4 speeds (*12,000 – triangle down, 15,000 – triangle up, 18,000 – circle, and 24,000 – square, rpm*) at a wavelength of 250 nm. The velocity data were collected at 50,000 rpm at 280 nm wavelength. Residual plots of the fit are shown above. Statistics for the non-linear least-squares fits were: *(A)* r.m.s.d. = 0.003, Z-score = 0.7, *(B)* r.m.s.d. = 0.003, Z-score = 4.6, *(C)* r.m.s.d. = 0.005, Z-score = 4.1, *(D)* r.m.s.d. = 0.005, Z-score = 0.9, *(E)* r.m.s.d. = 0.006, Z-score = 0.2, *(F)* r.m.s.d. = 0.007, Z-score = 5. The global reduced chi-squared was 0.394.

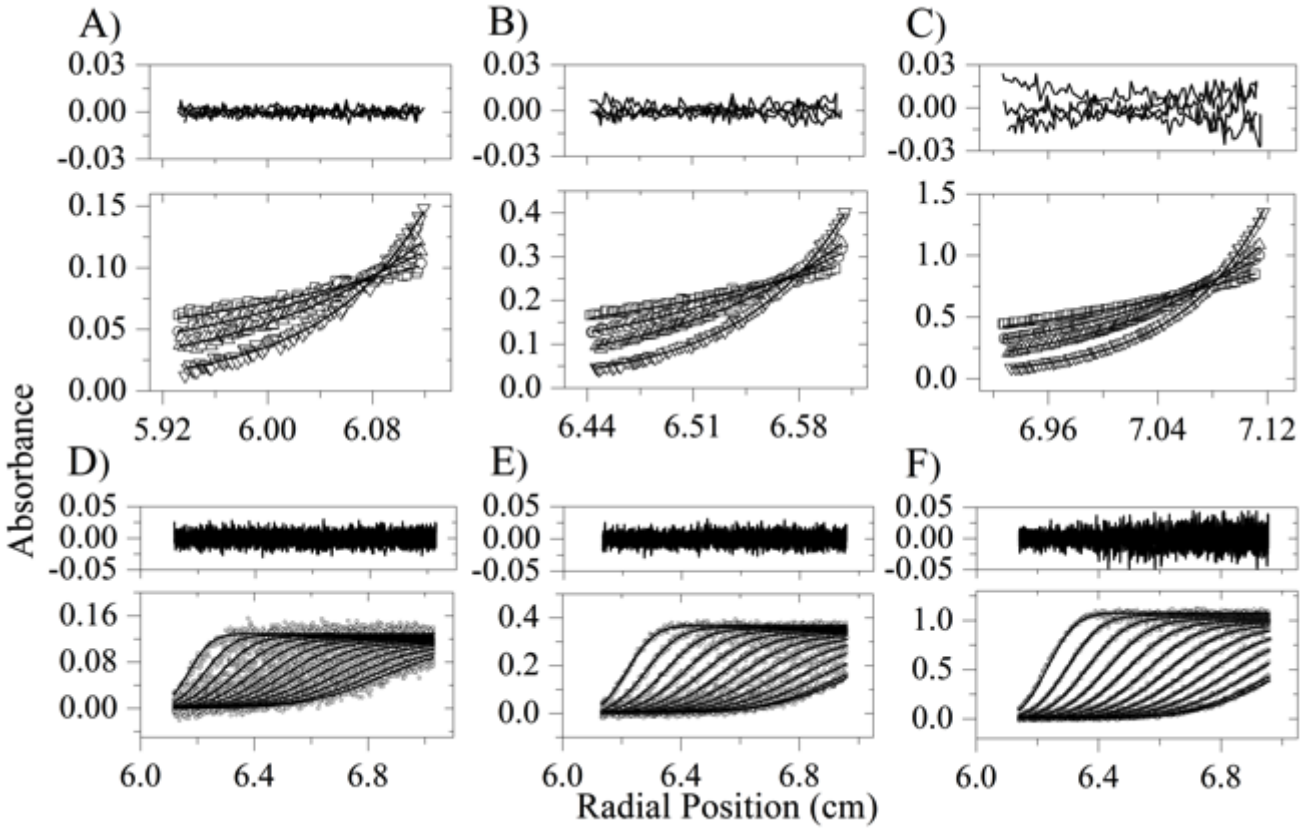


**Fig. S12:** Global fit of sedimentation equilibrium (*A-C*) and velocity (*D-F*) data for β-lactoglobulin B at pH 3.5 (*20 mM citrate, 100 mM NaCl, 25 $^{o}$C*), fitted to a *monomer-dimer equilibrium* model, as implemented in SEDPHAT. The protein concentrations were 5.1 μM (*A and D*), 15.0 μM (*B and E*) and 44.1 μM (*C and F*). The equilibrium data (*A-C*) were collected at 4 speeds (*12,000 – triangle down, 15,000 – triangle up, 18,000 – circle, and 24,000 – square, rpm*) at a wavelength of 260 nm. The velocity data were collected at 50,000 rpm at 237 nm wavelength. Residual plots of the fit are shown above. Statistics for the non-linear least-squares fits were: *(A)* r.m.s.d. = 0.002, Z-score = 0.15, *(B)* r.m.s.d. = 0.004, Z-score = 5.7, *(C)* r.m.s.d. = 0.009, Z-score = 13, *(D)* r.m.s.d. = 0.008, Z-score = 11, *(E)* r.m.s.d. = 0.008, Z-score = 3.6, *(F)* r.m.s.d. = 0.011, Z-score = 3. The global reduced chi-squared was 0.9.

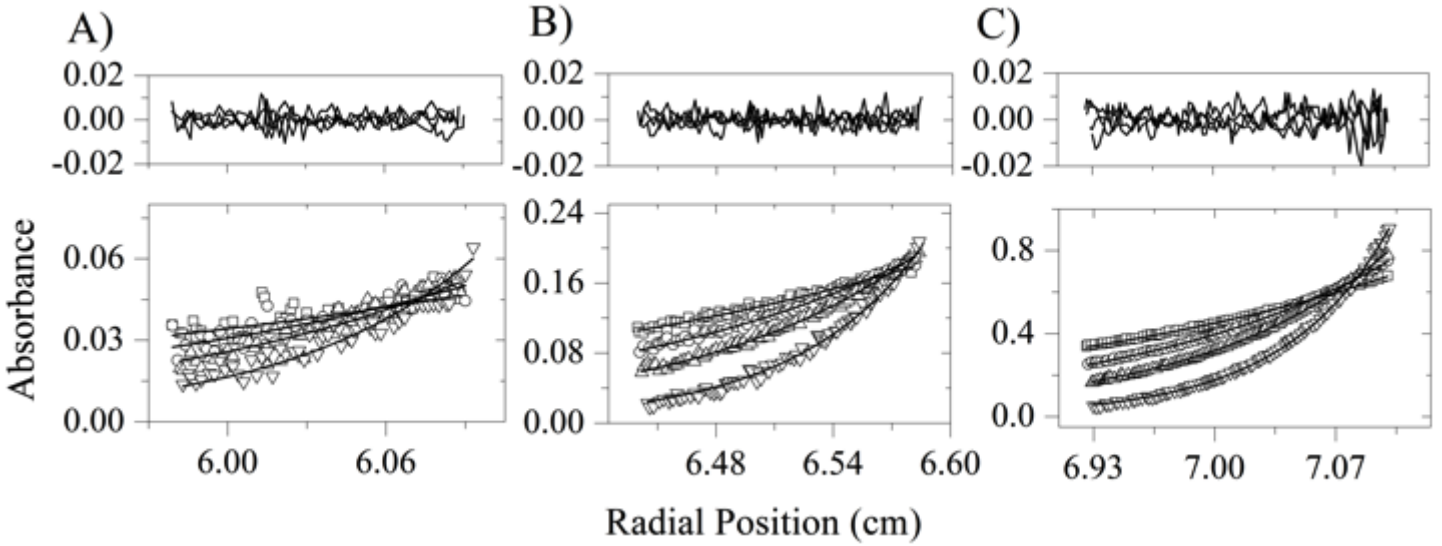


**Fig. S13:** Single species analysis of sedimentation equilibrium (*A*-*C*) data for β-lactoglobulin B at pH 4.5 (*20 mM citrate, 100 mM NaCl, 25 $^{o}$C*), fitted to a *single species of interacting system* model as implemented in SEDPHAT. The protein concentrations were 3.6 μM (*A*), 13.9 μM (*B*) and 47.8 μM (*C*). The equilibrium data (*A*-*C*) were collected at 4 speeds (*12,000 – triangle down, 15,000 – triangle up, 18,000 – circle, and 24,000 – square, rpm*) at a wavelength of 255 nm. Residual plots of the fit are shown above. The derived molecular weight (and range) is 33,270 (32,070 – 34,570). Statistics for the non-linear least-squares fits were: *(A)* r.m.s.d. = 0.003, Z-score = 1.24, *(B)* r.m.s.d. = 0.003, Z-score = 1.19, *(C)* r.m.s.d. = 0.005, Z-score = 7.38.

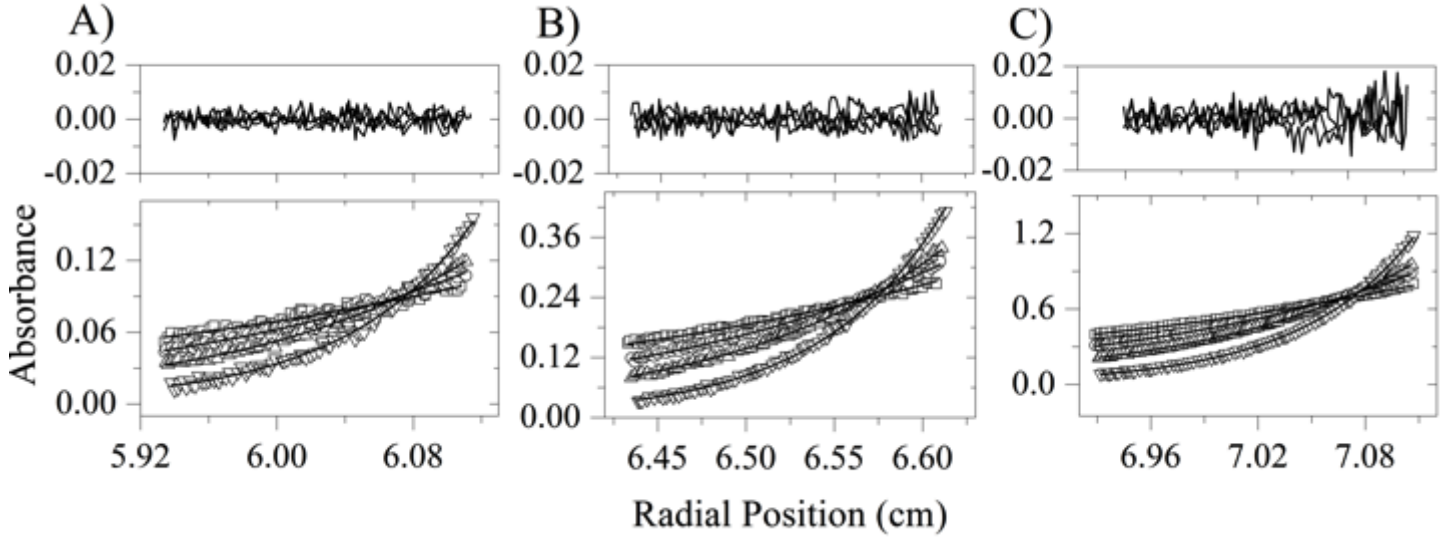


**Fig. S14:** Single species analysis of sedimentation equilibrium (*A*-*C*) data for β-lactoglobulin B at pH 5.5 (*20 mM citrate, 100 mM NaCl, 25 $^{o}$C*), fitted to a *single species of interacting system* model as implemented in SEDPHAT. The protein concentrations were 6.5 μM (*A*), 17.6 μM (*B*) and 50.0 μM (*C*). The equilibrium data (*A*-*C*) was collected at 4 speeds (*12,000 – triangle down, 15,000 – triangle up, 18,000 – circle, and 24,000 – square, rpm*) at a wavelength of 255 nm. Residual plots of the fit are shown above. The derived molecular weight (and range) is 34,162 (33,262 – 35,261). Statistics for the non-linear least-squares fits were: *(A)* r.m.s.d. = 0.002, Z-score = 0.85, *(B)* r.m.s.d. = 0.003, Z-score = 4.35, *(C)* r.m.s.d. = 0.005, Z-score = 2.98.

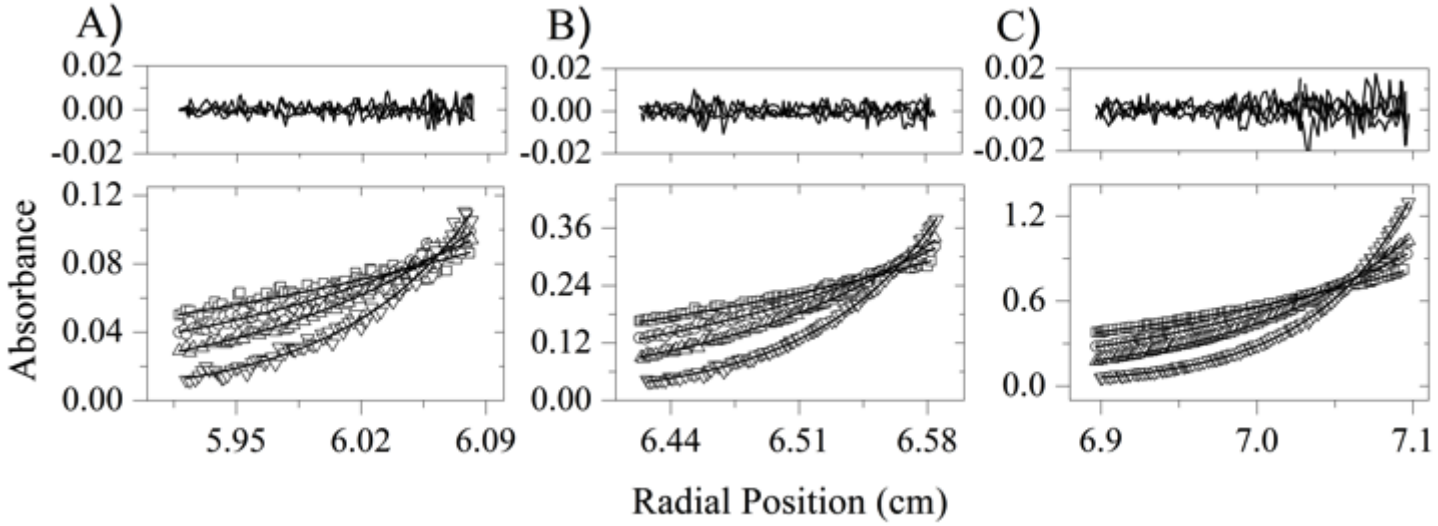


**Fig. S15:** Single species analysis of sedimentation equilibrium (*A*-*C*) data for β-lactoglobulin B at pH 5.5 (*20 mM MES, 100 mM NaCl, 25 $^{o}$C*), fitted to a *single species of interacting system* model as implemented in SEDPHAT. The protein concentrations were 9.6 μM (*A*), 17.8 μM (*B*) and 50.4 μM (*C*). The equilibrium data (*A*-*C*) were collected at 4 speeds (*12,000 – triangle down, 15,000 – triangle up, 18,000 – circle, and 24,000 – square, rpm*) at a wavelength of 255 nm. Residual plots of the fit are shown above. The derived molecular weight (and range) is 34939 (33,808 – 36,039). Statistics for the non-linear least-squares fits were: *(A)* r.m.s.d. = 0.003, Z-score = 0.27, *(B)* r.m.s.d. = 0.003, Z-score = 4.6, *(C)* r.m.s.d. = 0.005, Z-score = 3.8.

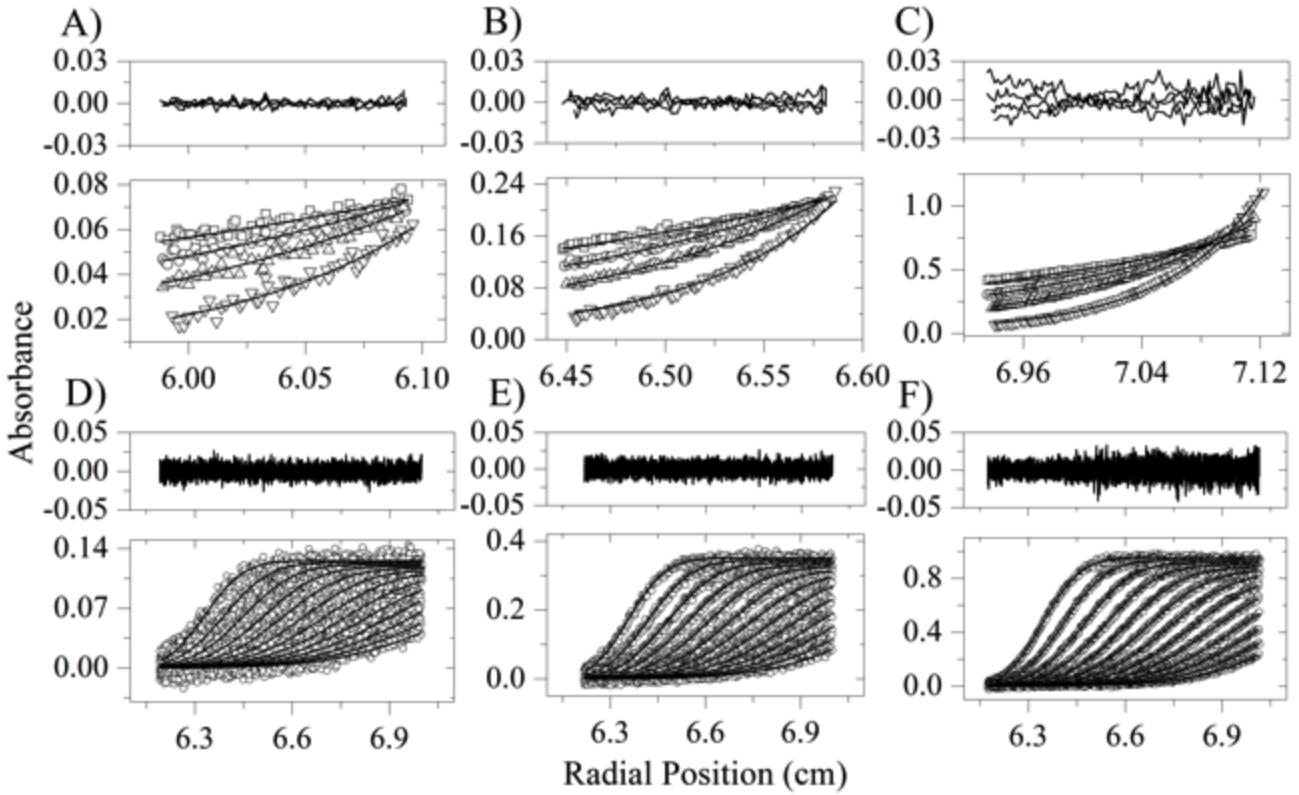


**Fig. S16:** Global fit of sedimentation equilibrium (*A-C*) and velocity (*D-F*) data for β-lactoglobulin B at pH 6.5 (*20 mM MOPS, 100 mM NaCl, 25 $^{o}$C*), fitted to a *monomer-dimer equilibrium* model as implemented in SEDPHAT. The protein concentrations were 4.3 μM (*A*) and 5.5 μM (D), 11.9 μM (*B*) and 15.4 μM (*E*), and 36.5 μM (*C*) and 42.0 μM (*F*). The equilibrium data (*A-C*) were collected at 4 speeds (*12,000 – triangle down, 15,000 – triangle up, 18,000 – circle, and 24,000 – square, rpm*) at a wavelength of 260 nm. The velocity data were collected at 50,000 rpm at 237 nm wavelength. Residual plots of the fit are shown above. Statistics for the non-linear least-squares fits were: *(A)* r.m.s.d. = 0.002, Z-score = 0.08, *(B)* r.m.s.d. = 0.004, Z-score = 5.6, *(C)* r.m.s.d. = 0.008, Z-score = 10.7, *(D)* r.m.s.d. = 0.007, Z-score = 7.8, *(E)* r.m.s.d. = 0.007, Z-score = 12, *(F)* r.m.s.d. = 0.009, Z-score = 6.6. The global reduced chi-squared was 0.733.

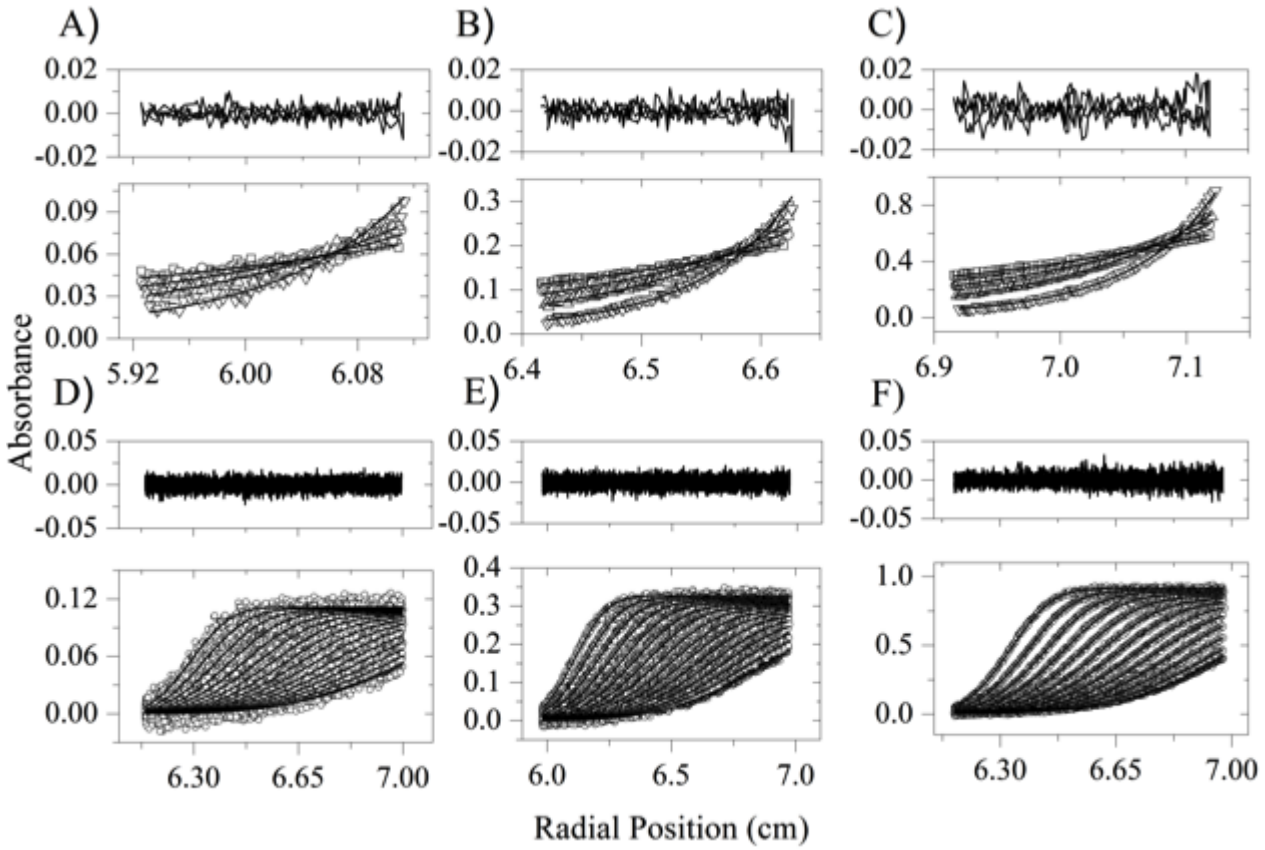


**Fig. S17:** Global fit of sedimentation equilibrium (*A-C*) and velocity (*D-F*) data for β-lactoglobulin B at pH 7.5 (*20 mM MOPS, 100 mM NaCl, 25 $^{o}$C*), fitted to a *monomer-dimer equilibrium* model as implemented in SEDPHAT. The protein concentrations were 5.22 μM (*A and D*), 15.4 μM (*B and E*) and 43.3 μM (*C and F*). The equilibrium data (*A-C*) were collected at 4 speeds (*12,000 – triangle down, 15,000 – triangle up, 18,000 – circle, and 24,000 – square, rpm*) at a wavelength of 250 nm. The velocity data were collected at 50,000 rpm at a wavelength of 280 nm. Residual plots of the fit are shown above. Statistics for the non-linear least-squares fits were: *(A)* r.m.s.d. = 0.003, Z-score = 0.6, *(B)* r.m.s.d. = 0.004, Z-score = 3, *(C)* r.m.s.d. = 0.005, Z-score = 3.4, *(D)* r.m.s.d. = 0.006, Z-score = 10, *(E)* r.m.s.d. = 0.006, Z-score = 5.6, *(F)* r.m.s.d. = 0.007, Z-score = 9.7. The global reduced chi-squared was 0.375.

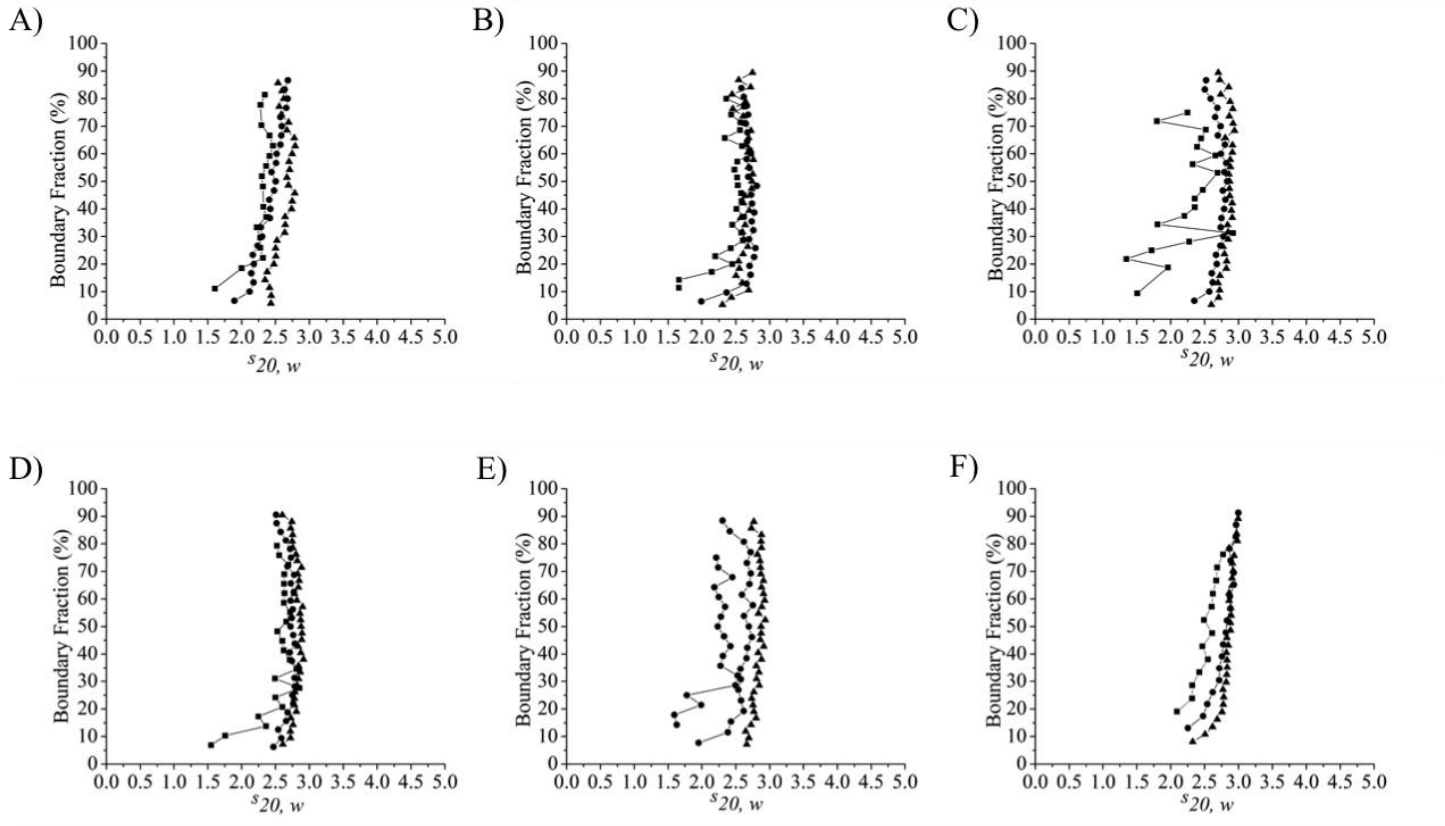


**Fig. S18:** van Holde-Weischet integral distribution plots for βLg A over the pH range of 2.5 to 7.5: *(A)* pH 2.5 in 20 mM citrate, 100 mM NaCl, *(B)* pH 3.5 in 20 mM citrate, 100 mM NaCl, *(C)* pH 4.5 in 20 mM citrate, 100 mM NaCl, *(D)* pH 5.5 in 20 mM citrate, 100 mM NaCl, *(E)*, pH 6.5 in 20 mM MOPS, 100 mM NaCl, *(F)* pH 7.5 in 20 mM MOPS, 100 mM NaCl. The van Holde-Weischet analysis is shown in each graph at three different loading concentrations of approximately 5, 15 and 45 μM represented respectively as squares, circles and triangles. Precise concentrations are given in the captions to Figures S1–S4 and S6–S7, respectively.

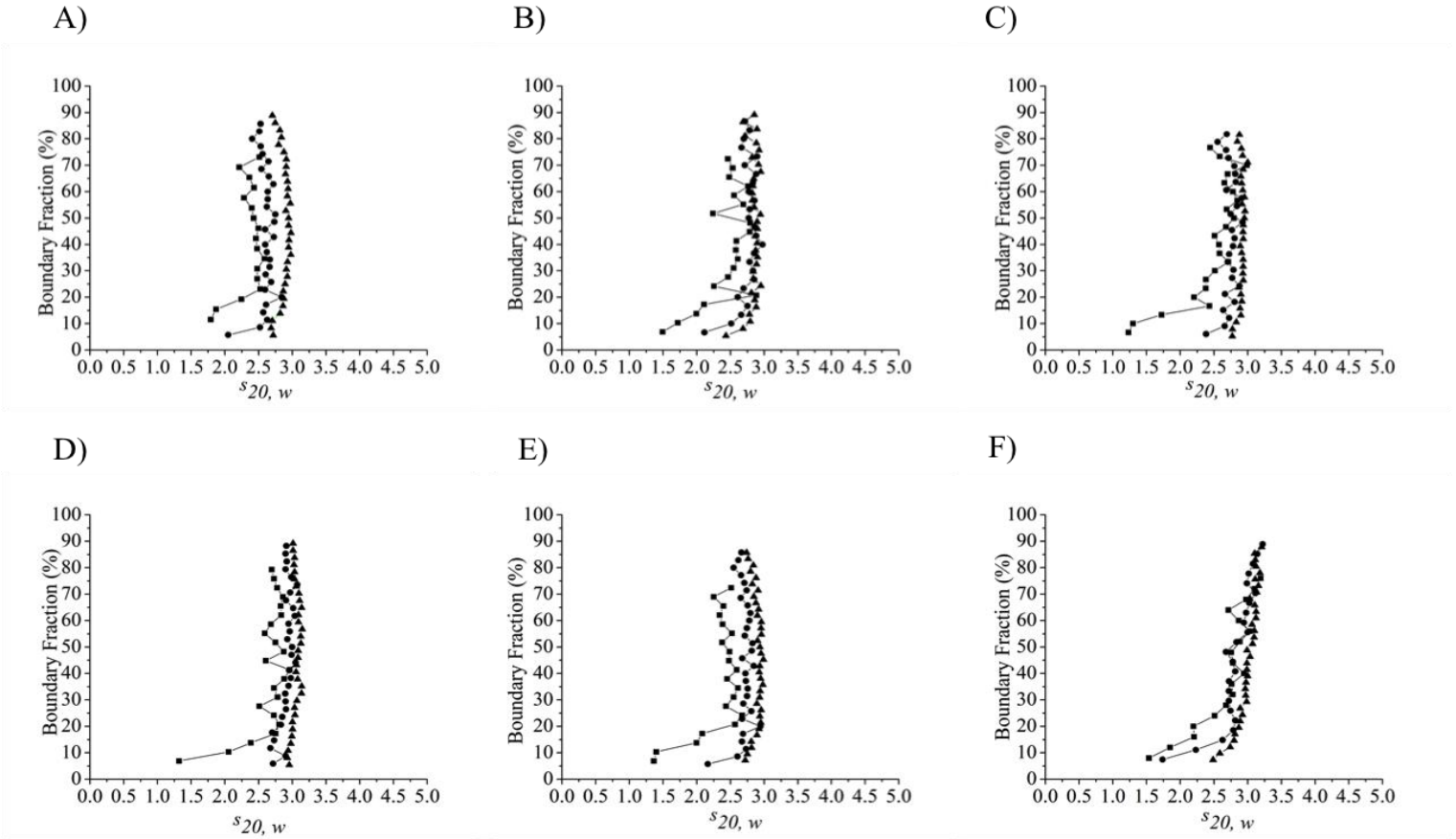


**Fig. S19:** van Holde-Weischet integral distribution plots for βLg B over the pH range of 2.5 to 7.5: *(A)* pH 2.5 in 20 mM citrate, 100 mM NaCl, *(B)* pH 3.5 in 20 mM citrate, 100 mM NaCl, *(C)* pH 4.5 in 20 mM citrate, 100 mM NaCl, *(D)* pH 5.5 in 20 mM citrate, 100 mM NaCl, *(E)*, pH 6.5 in 20 mM MOPS, 100 mM NaCl, *(F)* pH 7.5 in 20 mM MOPS, 100 mM NaCl. The van Holde-Weischet analysis is shown in each graph at three different loading concentrations of approximately 5, 15 and 45 μM represented respectively using squares, circles and triangles. Precise concentrations are given in the captions to Figures S11–S14 and S16–S17, respectively.

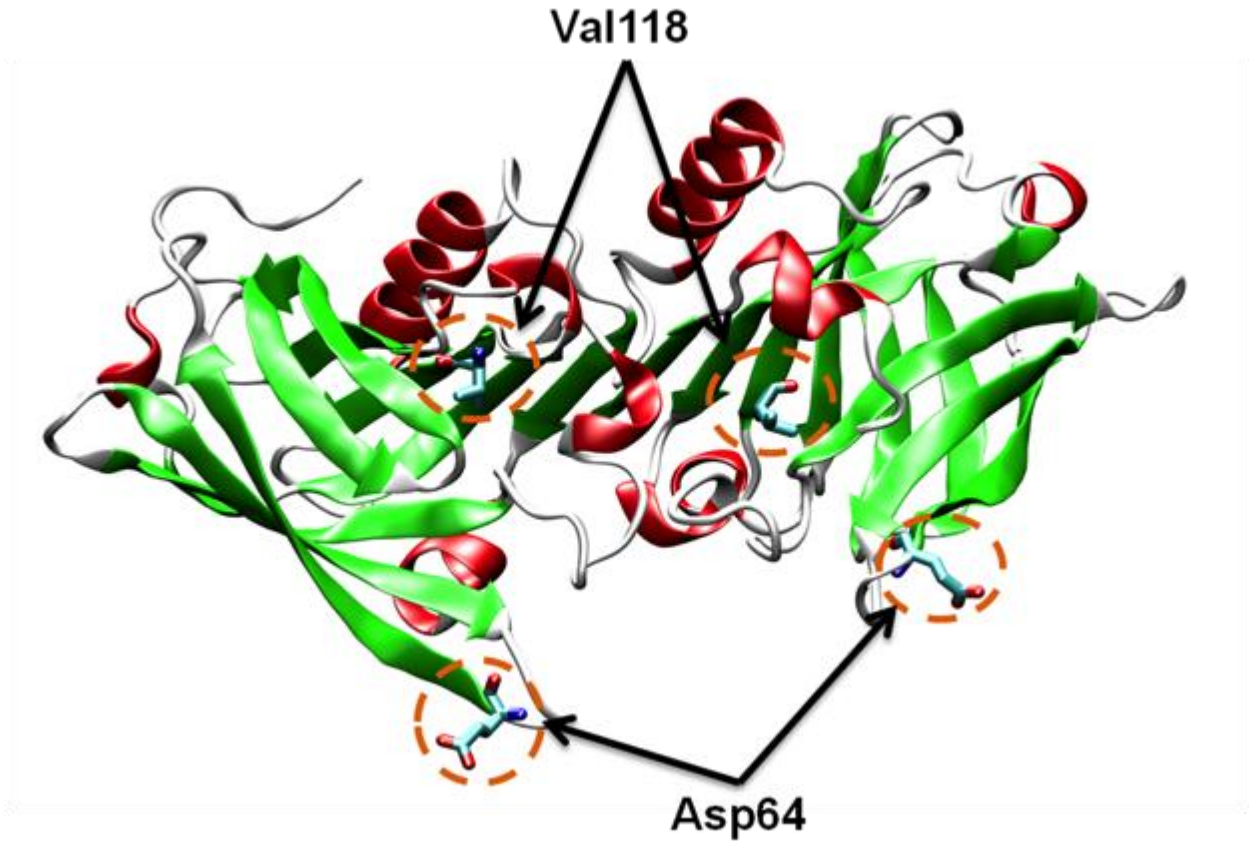


**Fig. S20.** Cartoon representation of the βLg A dimer. Color code is the same as that used for Fig. 1. The residues Asp64 and Val118 are represented within orange circles on both monomers in licorice and colored by atom type. These residues are substituted by Gly64 and Ala118 in βLg B.

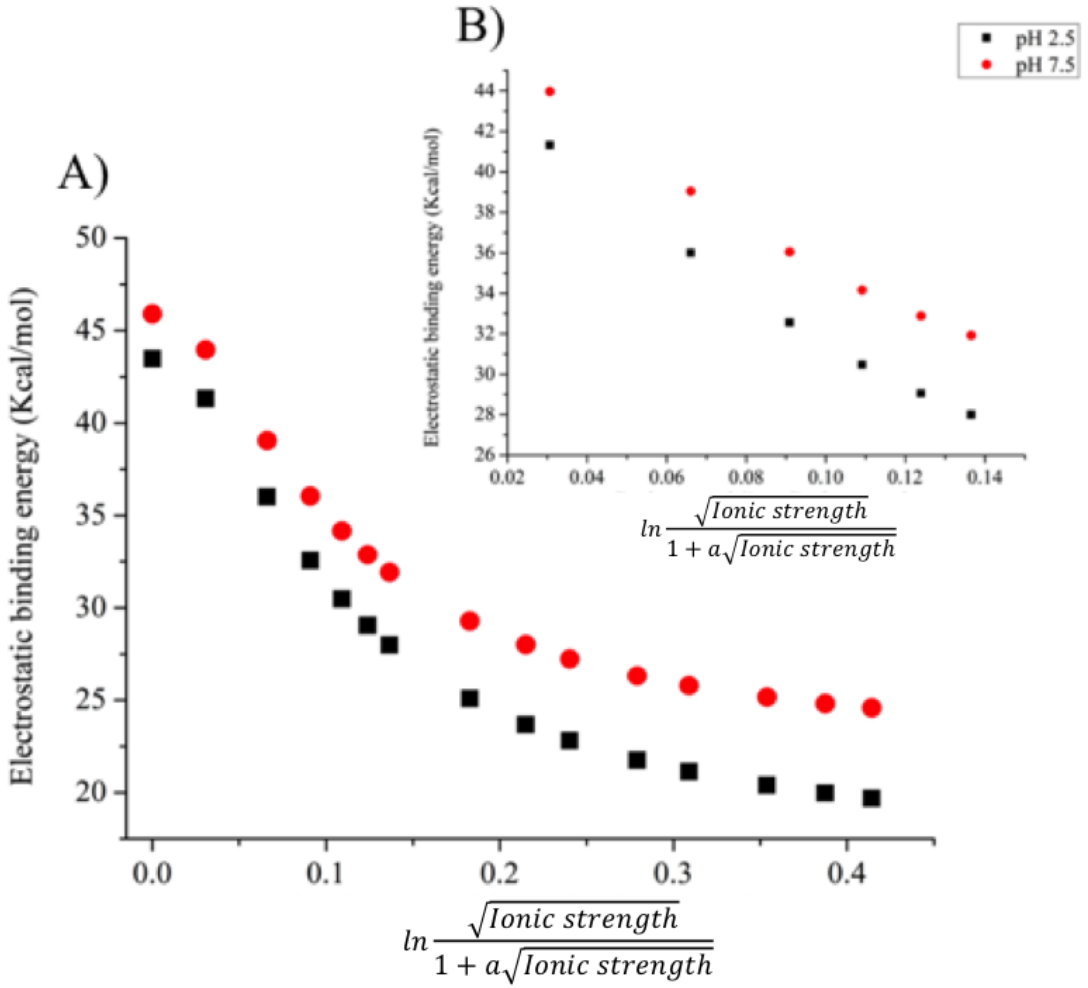


**Fig. S21.** *(A)* Calculated electrostatic binding energy for the βLgA dimer as a function of the ionic strength in NaCl solutions at pH 2.5 (*squares*) and 7.5 (*circles*). *(B)* Close up of *(A)* in the ionic strength range 1 to 25 mM NaCl showing the linear fit to the extended Debye-Hückel law.

**SUPPORTING REFERENCES**


1. Ariyaratne, K. A. N. S., Brown, R., Dasgupta, A., de Jonge, J., Jameson, G.B., Loo, T.S., Weinberg, C., Norris, G.E. 2002. Expression of bovine β-lactoglobulin as a fusion protein in Escherichia coli: a tool for investigating how structure affects function Int. Dairy J. 12:311-318.
2. Ponniah, K., T. S. Loo, P. J. Edwards, S. M. Pascal, G. B. Jameson, and G. E. Norris. 2010. The production of soluble and correctly folded recombinant bovine beta-lactoglobulin variants A and B in Escherichia coli for NMR studies. Protein Expr Purif 70:283-289.
3. Mailliart, P., and B. Ribadeau-Dumas. 1988. Preparation of beta-lactoglobulin and beta-lactoglobulin-free proteins from whey retentate by NaCl salting out at low pH. J. Food Sci. 53:743-752.
4. Sakurai, K., M. Oobatake, and Y. Goto. 2001. Salt-dependent monomer-dimer equilibrium of bovine beta-lactoglobulin at pH 3. Protein Sci. 10:2325-2335.
5. Townend, R., R. J. Winterbottom, and S. N. Timasheff. 1960. Molecular interactions in β-lactoglobulin. II. Ultracentrifugal and electrophoretic studies of the association of β-lactoglobulin below its isoelectric point. J. Am. Chem. Soc. 82:3161-3168.
6. Schuck, P. 2000. Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and lamm equation modeling. Biophys. J. 78:1606-1619.
7. Van Holde, K. E., and W. O. Weischet. 1978. Boundary analysis of sedimentation-velocity experiments with monodisperse and paucidisperse solutes. Biopolymers 17:1387-1403.
8. Houtman, J. C., P. H. Brown, B. Bowden, H. Yamaguchi, E. Appella, L. E. Samelson, and P. Schuck. 2007. Studying multisite binary and ternary protein interactions by global analysis of isothermal titration calorimetry data in SEDPHAT: application to adaptor protein complexes in cell signaling. Protein Sci. 16:30-42.
9. Qin, B. Y., M. C. Bewley, L. K. Creamer, H. M. Baker, E. N. Baker, and G. B. Jameson. 1998. Structural basis of the Tanford transition of bovine beta-lactoglobulin. Biochemistry. 37:14014-14023.
10. Dolinsky, T. J., J. E. Nielsen, J. A. McCammon, and N. A. Baker. 2004. PDB2PQR: an automated pipeline for the setup of Poisson-Boltzmann electrostatics calculations. Nucleic Acids Res. 32:W665-667.
11. Bas, D. C., D. M. Rogers, and J. H. Jensen. 2008. Very fast prediction and rationalization of pKa values for protein-ligand complexes. Proteins 73:765-783.
12. Li, H., A. D. Robertson, and J. H. Jensen. 2005. Very fast empirical prediction and rationalization of protein pKa values. Proteins 61:704-721.
13. Olsson, M. H. M., C. R. Søndergard, M. Rostkowski, and J. H. Jensen. [in press] 2011. PROPKA3: Consistent treatment of internal and surface residues in empirical pKa predictions. J. Chem. Theor. Comp. .
14. Baker, N. A., D. Sept, S. Joseph, M. J. Holst, and J. A. McCammon. 2001. Electrostatics of nanosystems: application to microtubules and the ribosome. Proc. Natl. Acad. Sci. USA 98:10037-10041.
15. Holst, M., R. E. Kozack, F. Saied, and S. Subramaniam. 1994. Protein electrostatics: rapid multigrid-based Newton algorithm for solution of the full nonlinear Poisson-Boltzmann equation. J. Biomol. Struct. Dyn. 11:1437-1445.

16. Holst, M., R. E. Kozack, F. Saied, and S. Subramaniam. 1994. Treatment of electrostatic effects in proteins: multigrid-based Newton iterative method for solution of the full nonlinear Poisson-Boltzmann equation. Proteins 18:231-245.
17. Georges, C., S. Guinand, and J. Tonnelat. 1962. Thermodynamic study of the reversible dissociation of β-lactoglobulin B at pH values over 5.5. Biochim. Biophys. Acta. 59:737-739.
18. McKenzie, H. A., and W. H. Sawyer. 1967. Effect of pH on β-lactoglobulins. Nature. 214:1101-1104.
19. Bello, M., G. Perez-Hernandez, D. A. Fernandez-Velasco, R. Arreguin-Espinosa, and E. Garcia-Hernandez. 2008. Energetics of protein homodimerization: effects of water sequestering on the formation of β-lactoglobulin dimer. Proteins: Struct., Funct., Bioinf. 70:1475-1487.
20. Bello, M., M. d. C. Portillo-Tellez, and E. Garcia-Hernandez. 2011. Energetics of ligand recognition and self-association of bovine β-lactoglobulin: differences between variants A and B. Biochemistry 50:151-161.
21. Kelly, M. J., and F. J. Reithel. 1971. Thermodynamic analysis of the monomer-dimer association of β-lactoglobulin A at the isoelectric point. Biochemistry. 10:2639-2644.
22. Zimmerman, J. K., G. H. Barlow, and I. M. Klotz. 1970. Dissociation of β-lactoglobulin near neutral pH. Arch. Biochem. Biophys. 138:101-109.
23. Sakai, K., K. Sakurai, M. Sakai, M. Hoshino, and Y. Goto. 2000. Conformation and stability of thiol-modified bovine β-lactoglobulin. Protein Sci. 9:1719-1729.
24. Sakurai, K., and Y. Goto. 2002. Manipulating monomer-dimer equilibrium of bovine β-lactoglobulin by amino acid substitution. J. Biol. Chem. 277:25735-25740.
25. Sarquis, J. L., and E. T. Adams, Jr. 1976. Sedimentation equilibrium in researching systems. X. Self-association of β-lactoglobulin C in acetate buffers. Biophys. Chem. 4:181-190.
26. Townend, R., L. Weinberger, and S. N. Timasheff. 1960. Molecular interactions in β-lactoglobulin. IV. The dissociation of β-lactoglobulin below pH 3.5. J. Am. Chem. Soc. 82:3175-3179.
27. Joss, L. A., and G. B. Ralston. 1996. β-Lactoglobulin B: a proposed standard for the study of reversible self-association reactions in the analytical ultracentrifuge? Anal. Biochem. 236:20-26.
28. Townend, R., C. A. Kiddy, and S. N. Timasheff. 1961. Molecular interactions in β-lactoglobulin. VII. The hybridization of β-lactoglobulins A and B. J. Am. Chem. Soc. 83:1419-1423.
29. Townend, R., T. T. Herskovits, H. E. Swaisgood, and S. N. Timasheff. 1964. The solution properties of β-lactoglobulin C. J. Biol. Chem. 239:4196-4201.
30. Albright, D. A., and J. W. Williams. 1968. Combined sedimentation and chemical equilibrium of β-lactoglobulin B in acid solution. Biochemistry. 7:67-78.
31. Visser, J., R. C. Deonier, E. T. Adams, Jr., and J. W. Williams. 1972. Self-association of β-lactoglobulin B in acid solution and its variation with temperature. Biochemistry. 11:2634-2643.
32. Tang, L.-H., and E. T. Adams, Jr. 1973. Sedimentation equilibrium in reacting systems. VII. Temperature-dependent self-association of β-lactoglobulin A at pH 2.46. Arch. Biochem. Biophys. 157:520-530.
33. Sarquis, J. L., and E. T. Adams, Jr. 1974. Sedimentation equilibrium in reacting systems. IX. Temperature-dependent self-association of β-lactoglobulin C in glycine buffers. Arch. Biochem. Biophys. 163:442-452.

34. Baldini, G., S. Beretta, G. Chirico, H. Franz, E. Maccioni, P. Mariani, and F. Spinozzi. 1999. Salt-induced association of β-lactoglobulin by light and X-ray scattering. Macromolecules 32:6128-6138.