Kinetics of charge-dependent reversible condensation of reflectin nanostructures

Electronic Supplementary Information

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Supplementary Materials and Methods

Materials

The reagents utilized were of the highest grade available. Ethylenediaminetetraacetic acid (EDTA), sodium phosphate dibasic heptahydrate, sodium phosphate monobasic monohydrate, Urea, Triton X-100, Lysozyme from chick egg, imidazole and trifluoroacetic acid (TFA) were obtained from Merck. DNase I from Roche. Sodium chloride (NaCl), sodium hydroxide (NaOH), methanol, ethanol and glycerol were obtained from PanReac. Guanidine hydrochloride (GndHCl), Dithiothreitol (DTT) and glacial acetic acid from Thermo Scientific, and hydrochloric acid (HCl) from VWR. For cloning, *Escherichia coli* Nzy5α and *Rosetta* (DE3) competent cells were purchased from Nzytech (Portugal) and Novagen. Tris-base, Luria Broth (LB) medium, ampicillin (Amp), chloramphenicol (Chlor), Agar, Yeast extract, Isopropyl β-D-1-thiogalactopyranoside (IPTG), and Tryptone were purchased from Nzytech (Portugal). For SDS-PAG Electrophoresis the sodium dodecyl sulfate (SDS) 10% solution and 30% Acrylamide/Bis Solution were purchased from Bio-Rad. Ammonium persulphate (PSA), N-N-N'-N'-tetramethylethylenediamine (TEMED), Blue bromophenol sodium salt and 2-Propanol were obtained from Carl Roth. Coomassie Blue R-250 dye was obtained from Sigma-Aldrich. The protein markers were the Low Molecular Weight protein marker (LMW) from Nzytech. For protein purification resin IMAC Q-Sepharose 6 Fast Flow (17-0921-07) and empty disposable PD-10 Columns (17-0435 01) using polyethylene frits with 15 mm of diameter were purchased from Cytiva. For protein quantification, the Bicinchoninic Acid assay kit, and the protein standard solution (9048-46-8) were purchased from Sigma-Aldrich.

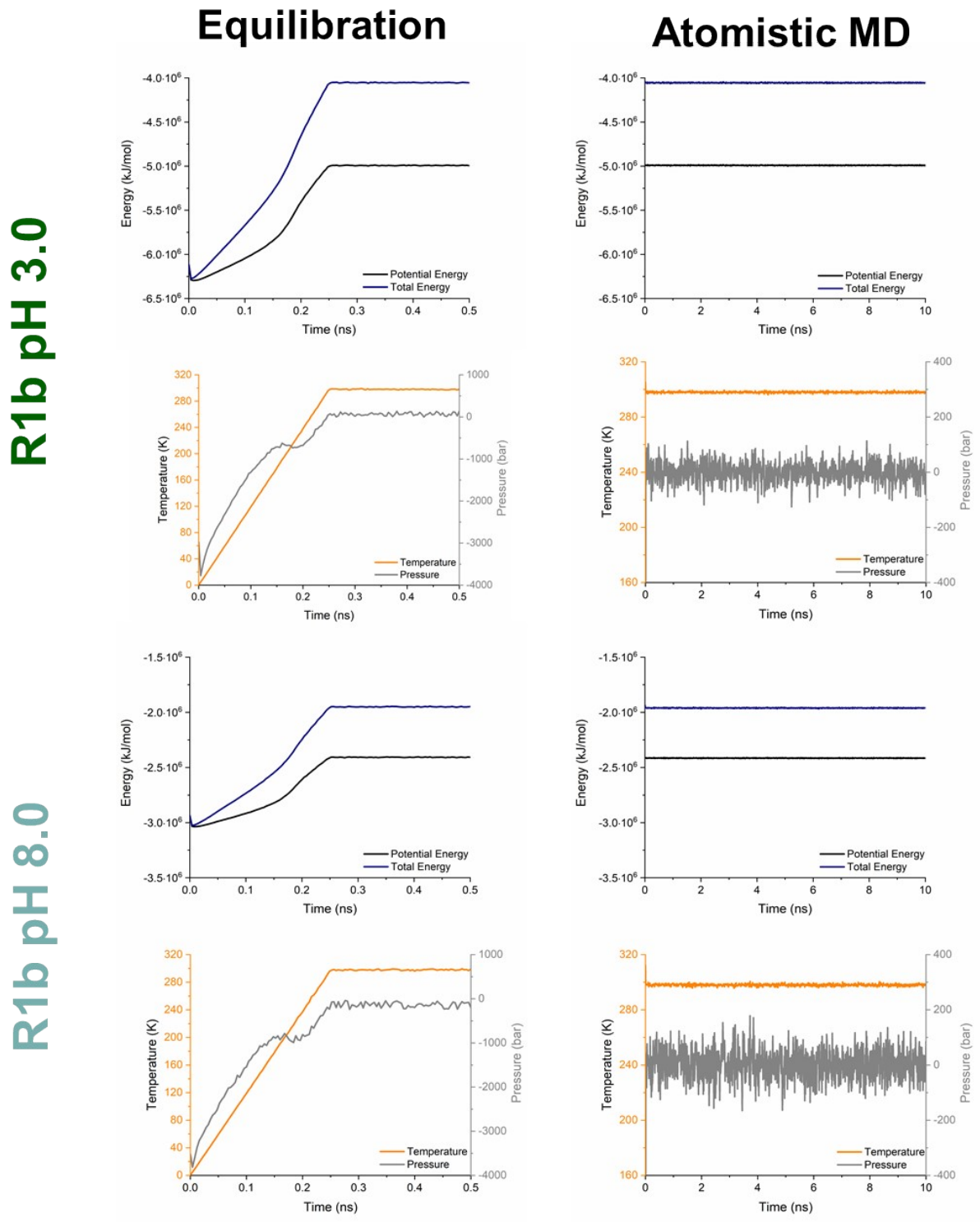

Fig. S1: R1b All-Atom MD Simulations at pH 3.0 and pH 8.0. Parameters evaluated during equilibration (1st column) and simulation runs (2nd column): i) Potential Energy (kJ/mol); ii) Total Energy (kJ/mol); iii) Temperature (K); iv) Pressure (bar).

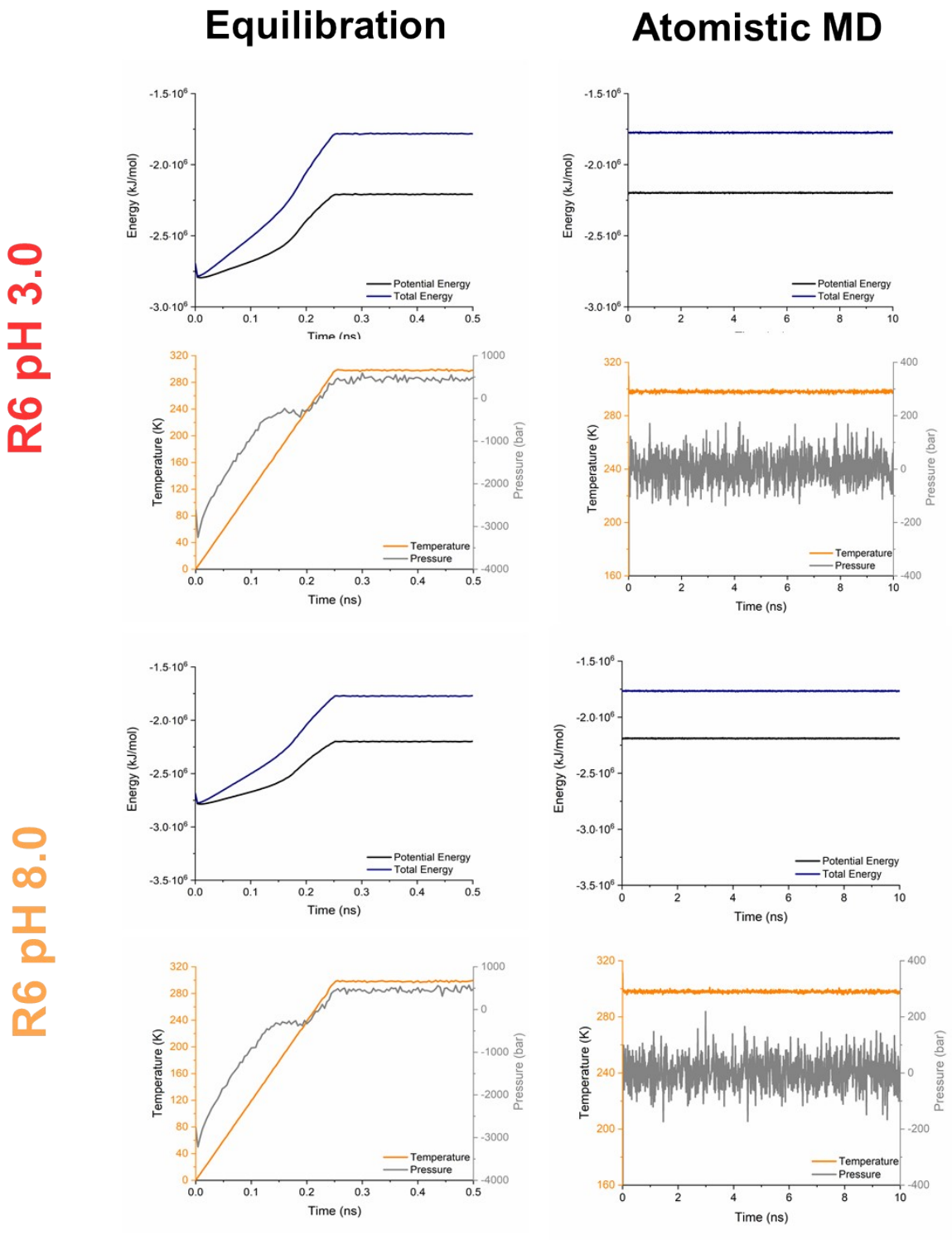

Fig. S2: R6 Atom MD Simulations at pH 3.0 and pH 8.0. Several parameters were evaluated during equilibration (1st column) and during the simulation run (2nd column): i) Potential Energy (kJ/mol); ii) Total Energy (kJ/mol); iii) Temperature (K); iv) Pressure (bar).

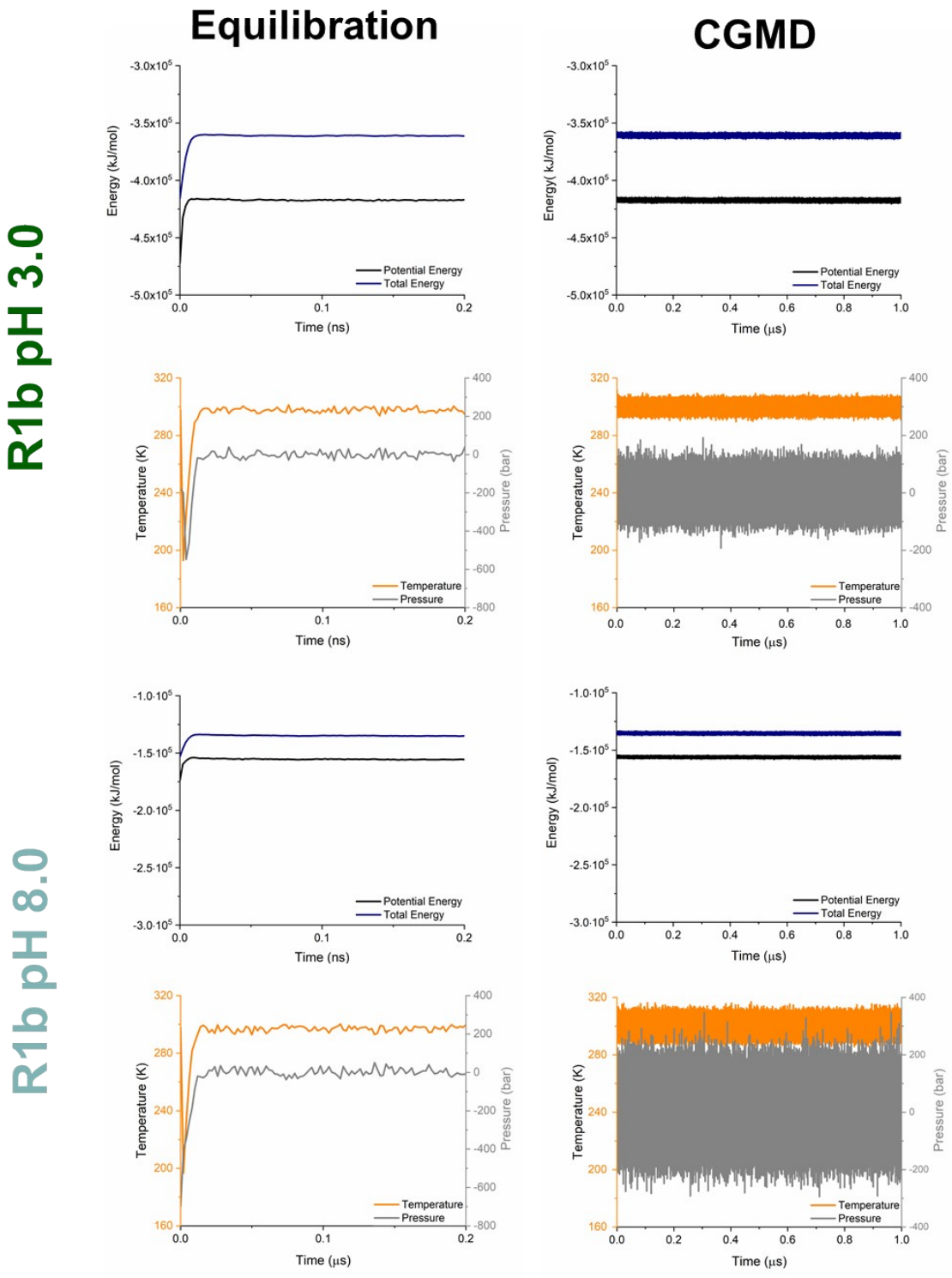

Fig. S3: R1b Coarse-grained MD Simulations at pH 3.0 and pH 8.0. Several parameters were evaluated during equilibration (1st column) and during the simulation run (2nd column): i) Potential Energy (kJ/mol); ii) Total Energy (kJ/mol); iii) Temperature (K); iv) Pressure (bar).

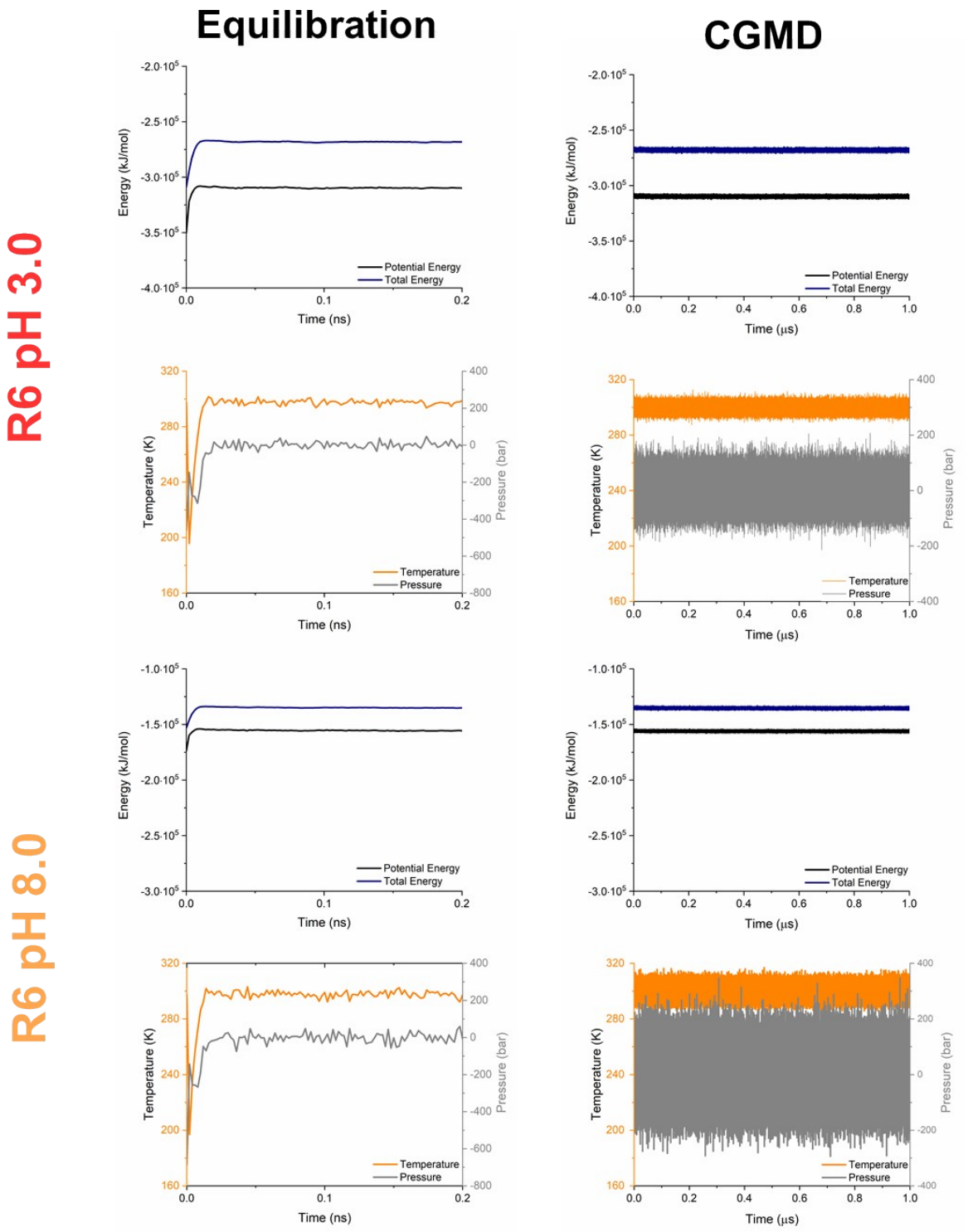

Fig. S4: R6 Coarse-grained MD Simulations at pH 3.0 and pH 8.0. Several parameters were evaluated during equilibration (1st column) and during the run (2nd column): i) Potential Energy (kJ/mol); ii) Total Energy (kJ/mol); iii) Temperature (K); iv) Pressure (bar).

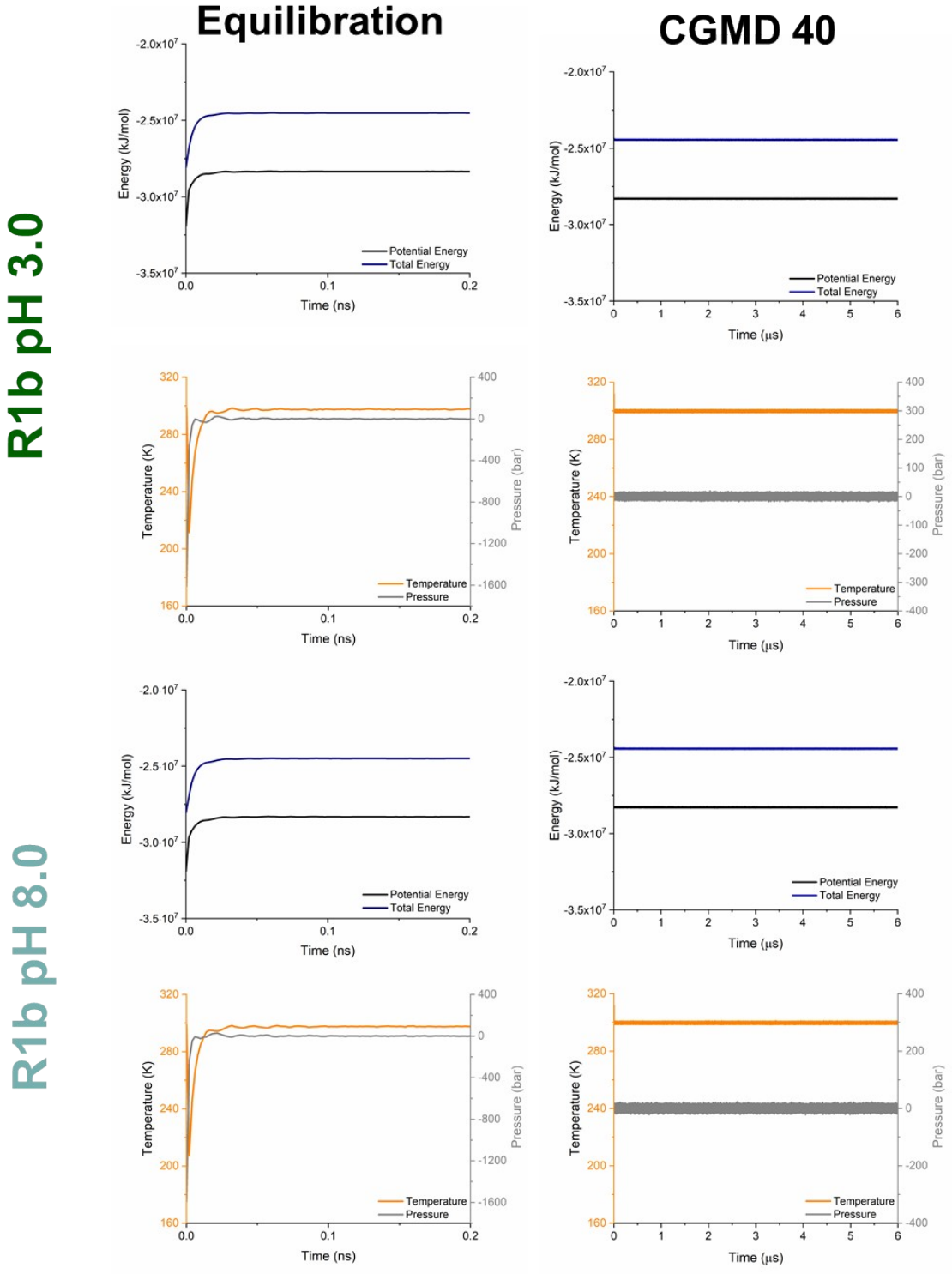

Fig. S5: R1b Coarse-grained MD Simulations with 40 proteins at pH 3.0 and pH 8.0. Several parameters were evaluated during equilibration (1st column) and during the simulation run (2nd column): i) Potential Energy (kJ/mol); ii) Total Energy (kJ/mol); iii) Temperature (K); iv) Pressure (bar).

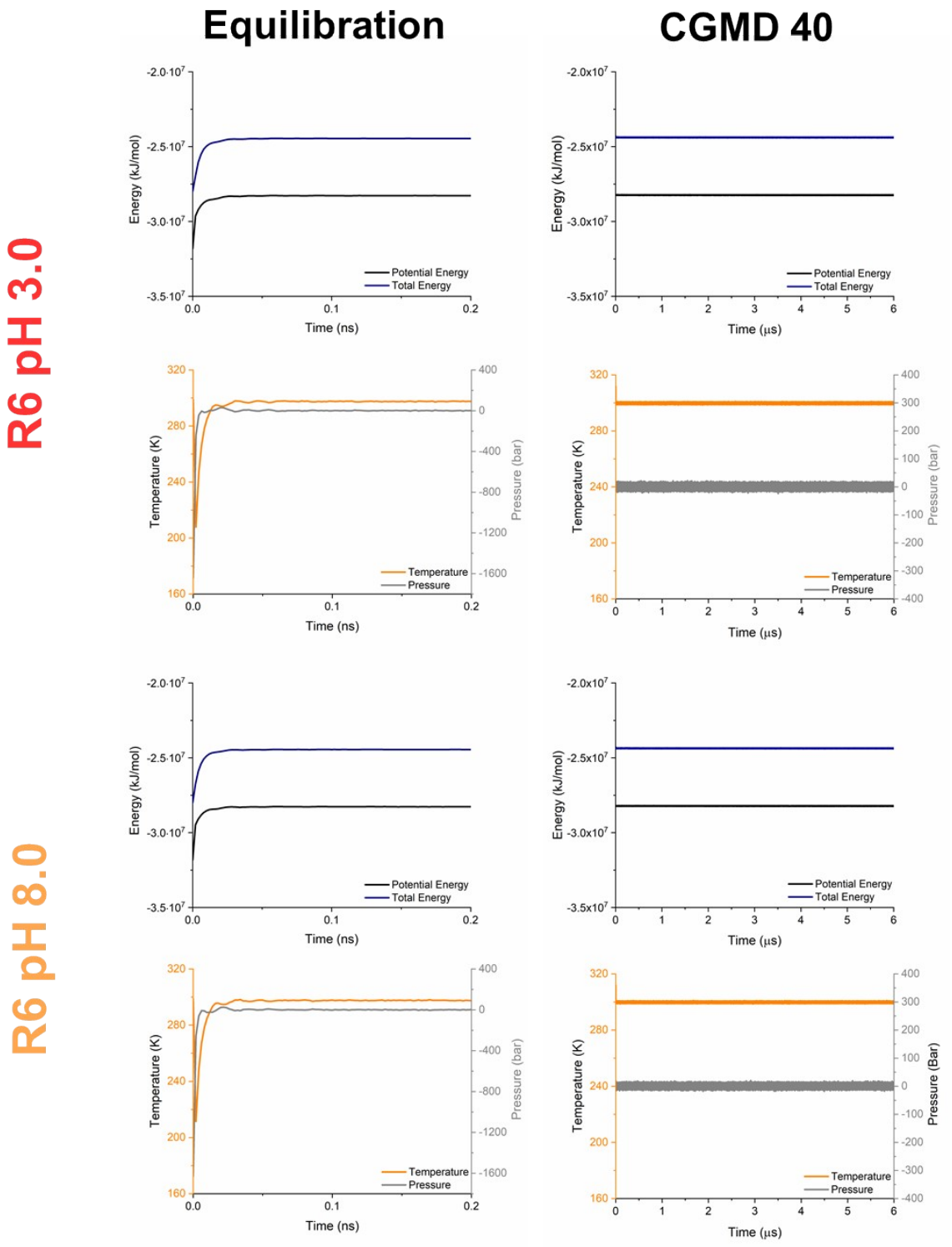

Fig. S6: R6 Coarse-grained MD Simulations with 40 proteins at pH 3.0 and pH 8.0. Several parameters were evaluated during equilibration (1st column) and during the run (2nd column): i) Potential Energy (kJ/mol); ii) Total Energy (kJ/mol); iii) Temperature (K); iv) Pressure (bar).

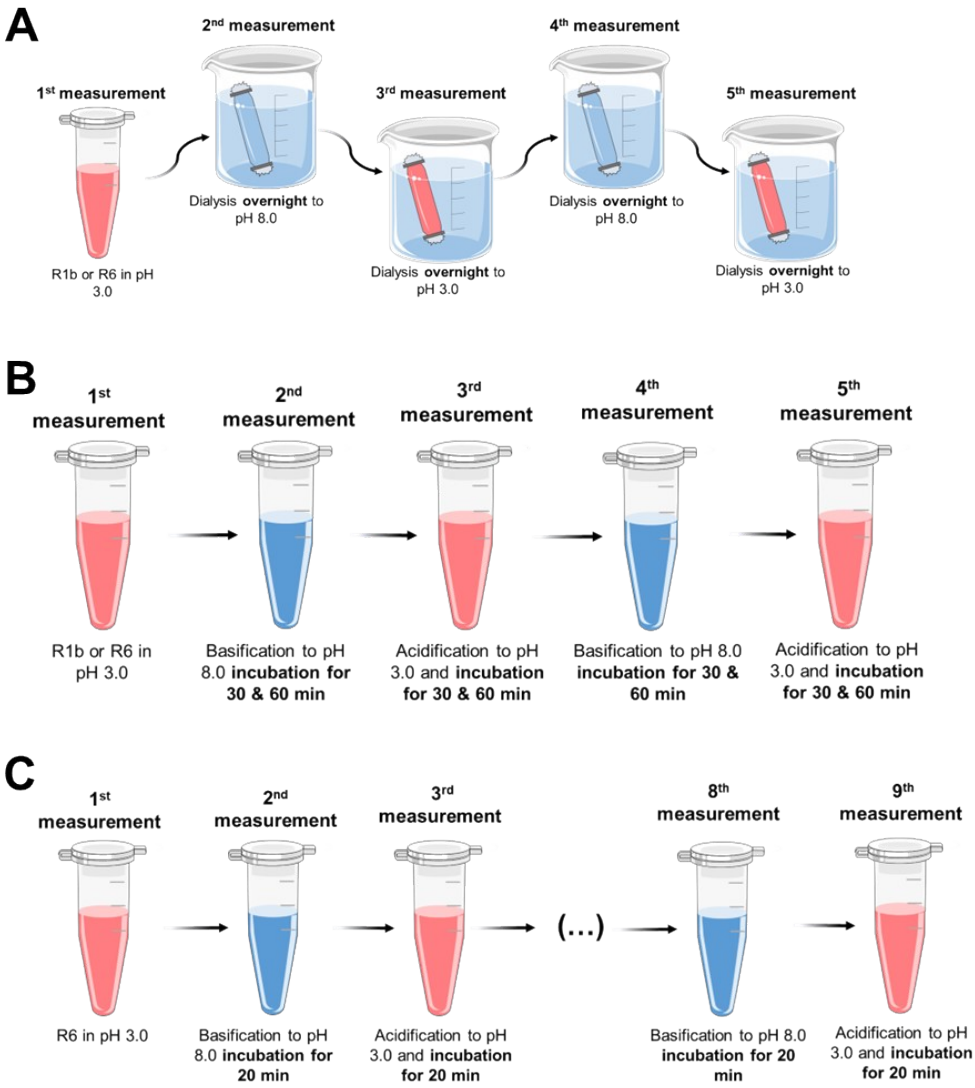

Fig. S7: Schematic representation of three experimental protocols for the study of R1b and R6 self-assembly reversibility with cyclical variation of the pH. (A) Protein samples (R1b or R6) were initially measured at pH 3.0, and then subjected to alternating pH conditions (8.0 and 3.0) using overnight dialysis. (B) R1b or R6 were initially at pH 3.0, then alternately basified to pH 8.0 and acidified to pH 3.0. Incubations of 30 and 60 minutes were used for each pH transition, with measurements taken at each stage. (C) R6 samples underwent multiple cycles of basification to pH 8.0 and acidification to pH 3.0, with 20-minute incubations for each transition. Measurements were taken after each pH change, with the process extending to at least 9 measurements.

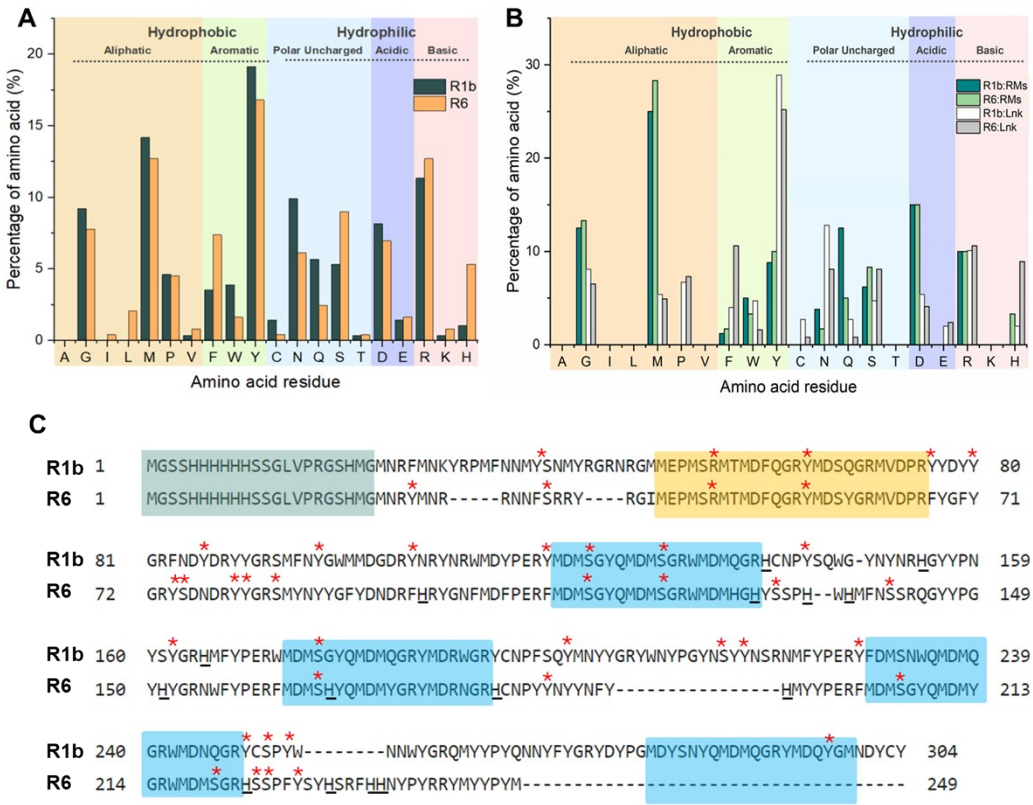

Fig. S8: (A) Comparison of the amino acid composition between R1b and R6 wild-type sequences. (B) Comparison of the amino acid composition between R1b and R6 repeating motifs (RM) and linkers (Lnk). Note: only RMs (highlighted in blue) and the respective linkers between them were included in this analysis. (C) Sequences of the recombinantly expressed R1b and R6 were used in this study. The conserved N-terminal motifs and reflectin repeating motifs are highlighted in yellow and blue respectively. The histidine tag together with the sequence used as a spacer between tag and reflectin sequence is highlighted in green. Linkers are shown in black. Histidine residues of the wild-type sequence are underlined. Predicted phosphorylation sites are identified with red '*'.

Table S1: Sequence properties of wild-type (WT) and recombinantly produced (RP) R1b and R6. The number of amino acids (Nº AA), molecular weight (MW) and isoelectric point (pI) were predicted using the expasy: get pI/Mw tool[8]. The number of phosphorylation sites was predicted using NetPhos - 3.1 tool[9] allows the prediction of generic phosphorylation sites in eukaryotic proteins using ensembles of neural networks.

Protein ID	R1b	R6
Nº RMs	4	3
N-terminal	Yes	Yes
Nº His (WT / RP)	3 / 9	13 / 19
Nº AA (WT / RP)	282 / 304	227 / 249
MW (kDa, WT / RP)	36.3 / 38.5	29.1 / 31.5
pI (WT / RP)	8.8 / 8.9	9.2 / 9.3
Nº Phosphorylation sited (WT / RP)	22 / 24	21 / 23

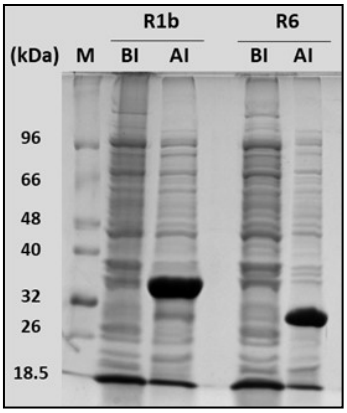

Fig. S9: Recombinant expression of reflectins. SDS-PAGE 12.5% gel of crude extracts obtained before induction (BI) and after induction of expression with 0.4 mM IPTG at 30 °C overnight (ON). Gels were stained with Coomassie blue. The marker lane (M) contained Nzytech Low Molecular Weight marker.

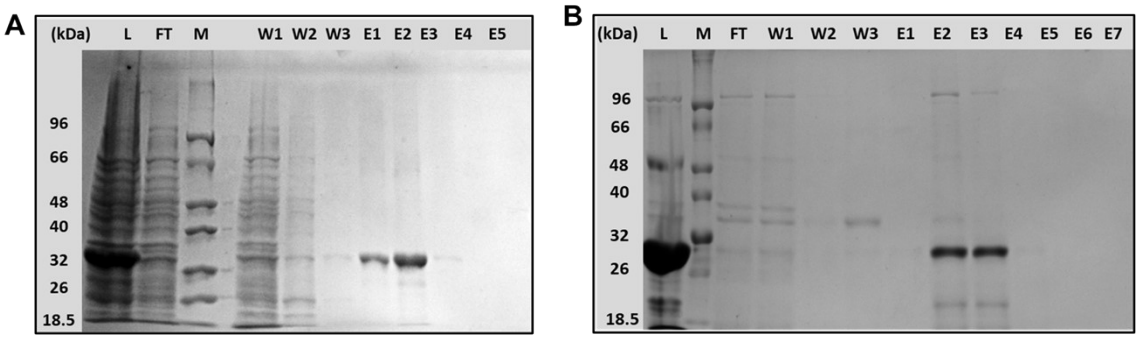

Fig. S10: Chromatographic purification of reflectins. SDS-PAGE 12.5% polyacrylamide gel after purification using Immobilized metal affinity chromatography under denaturant conditions. (A) R1b and (B) R6. L – Loading, FT – Flowthrough, W – Wash and E – Elution. R1b was eluted by increasing to 300mM imidazole. In the case of R6 step gradient was used where E1-E2 contained 100mM imidazole, E3-E4 contained 300 mM imidazole and E5-E8 contained 500 mM of imidazole. As marker (M) was used Low Molecular Weight (Nzytech) and the gel was stained with Coomassie blue.

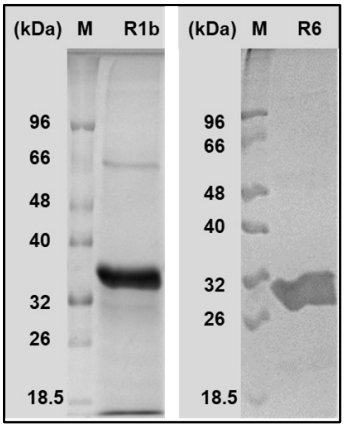

Fig. S11: Purified fractions of R1b and R6. SDS-PAGE 12.5% gel of pure R1b and R6, after purification through IMAC. The marker lane (M) contained the Nzytech Low Molecular Weight marker and both gels were stained with Coomassie blue

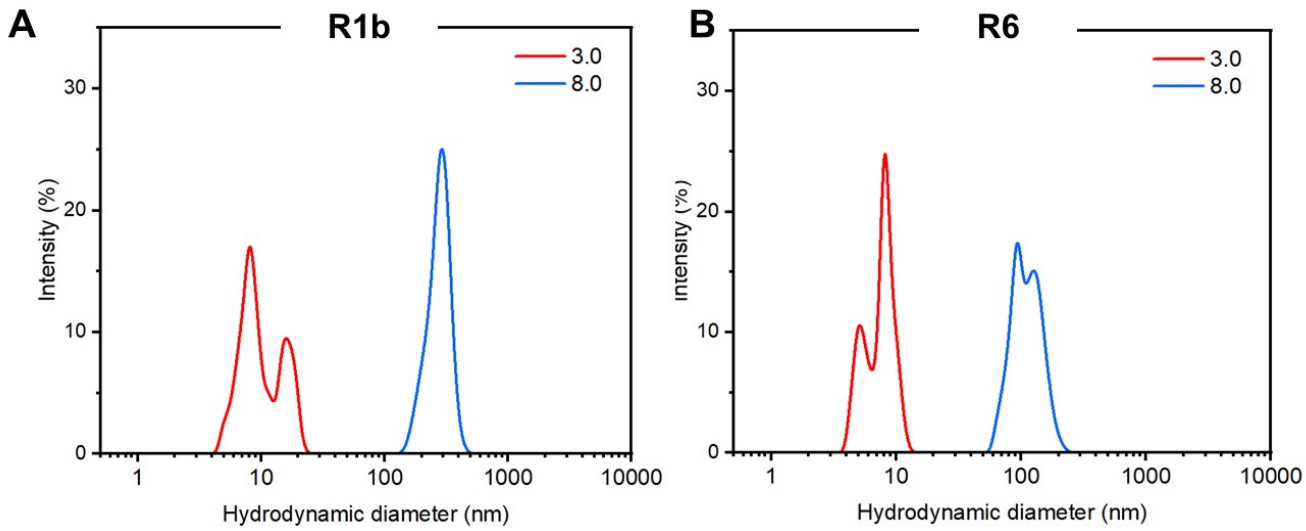

Fig. S12: DLS intensity distribution of R1b (A) and R6 (B) at pH 3.0 (red) and pH 8.0 (blue),

Table S2: Physicochemical properties of R1b and R6 protein samples in different solvent conditions obtained during DLS measurements. The table presents hydrodynamic diameter (nm), zeta potential (mV), and polydispersity index (PDI) measurements for both proteins in acidic (5 mM Na-Acetate/Acetic acid, pH 3.0) and basic (5 mM Tris-HCl, pH 8.0) environments.

	R1b			R6		
Solvent	**Hydrodynamic diameter (nm)**	**Zeta potential (mV)**	**PDI**	**Hydrodynamic diameter (nm)**	**Zeta potential (mV)**	**PDI**
5 mM Na-Acetate / Acetic acid (pH 3.0)	11.4 ± 5.1	+27 ± 6	0.48 ± 0.29	9.2 ± 5.6	+57 ± 25	0.20 ± 0.22
5 mM Tris-HCl (pH 8.0)	272.9 ± 75.6	+10 ± 15	0.26 ± 0.13	125.7 ± 60.4	-8 ± 6	0.19 ± 0.08

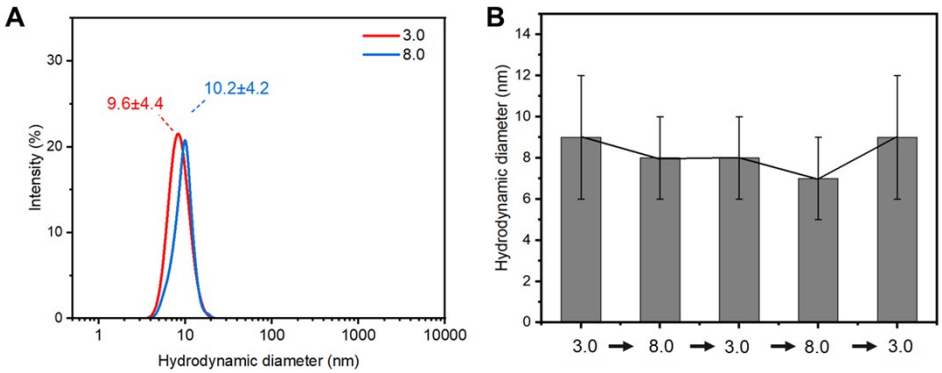

Fig. S13: pH-dependent behavior of Bovine Serum Albumin (BSA) obtained using DLS. (A) DLS intensity distribution of BSA monomers at pH 3.0 (red) and pH 8.0 (blue), showed no significant change in hydrodynamic diameter. (B) Variation of the average hydrodynamic diameter of the BSA particles measured by DLS during sequential pH changes between 3.0 and 8.0, demonstrating negligible size variation.

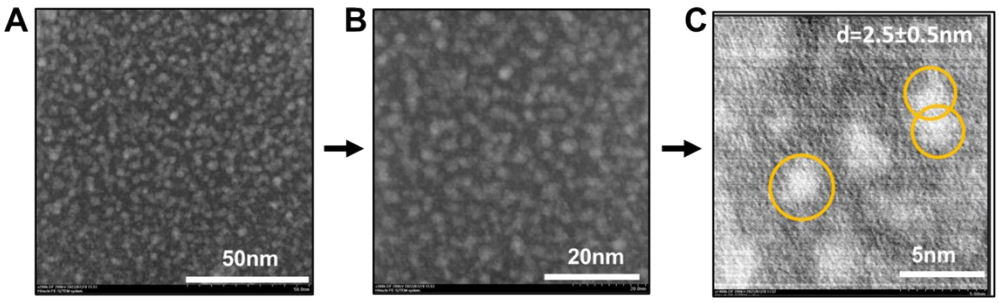

Fig.S14: Scanning Transmission Electron Microscope (STEM) images of R6 assemblies at pH 8.0 in Dark Field mode. Zoom in one cluster with magnifications of (A) x200k, (B) x300k, and (C) x1400k. The individual particles of the R6 monomer are shown in a yellow circle. The average diameter of these particles was determined using the ImageJ [1] particle analysis tool and corresponds to 2.5±0.5 nm (n=50)

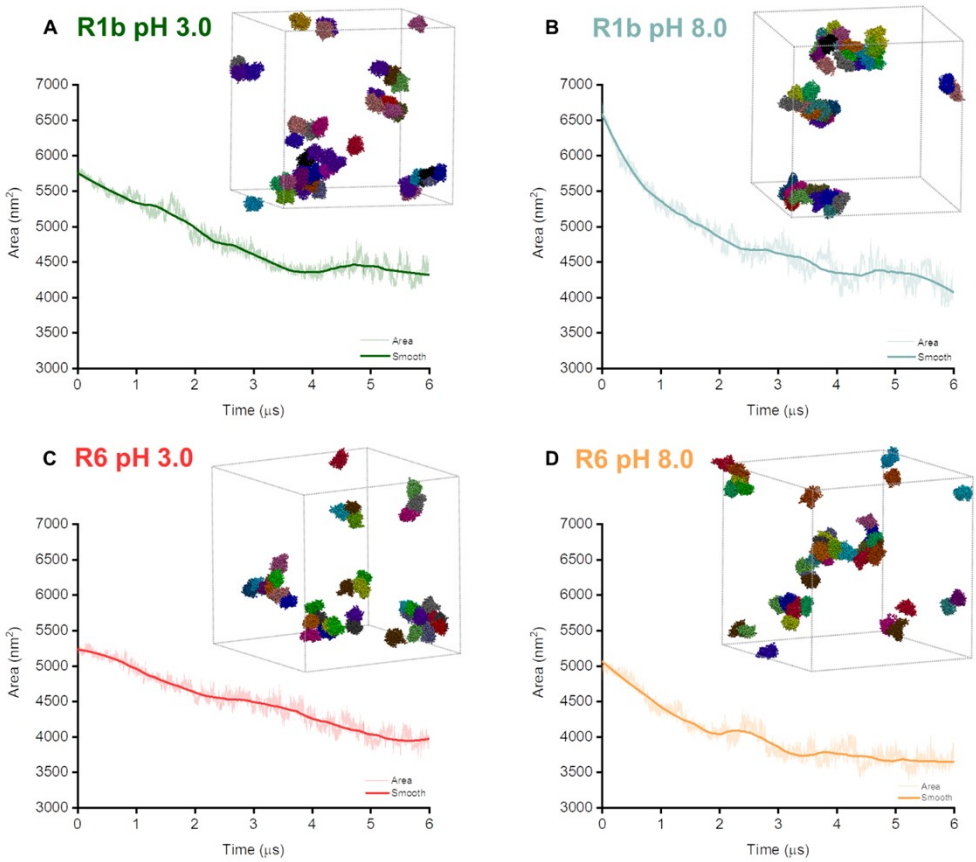

Fig. S15: Reflectins Coarse-grained MD simulation with 40 proteins at different pH conditions: pH 3.0 and pH 8.0. (A) R1b pH 3.0 Solvent accessible surface area (nm^2) over simulation time. (B) Reflectin 1b pH 8.0 Solvent accessible surface area (nm^2) over simulation time. (C) R6 pH 3.0 Solvent accessible surface area (nm^2) over simulation time. (D) Reflectin 6 pH 8.0 Solvent accessible surface area (nm^2) over simulation time.

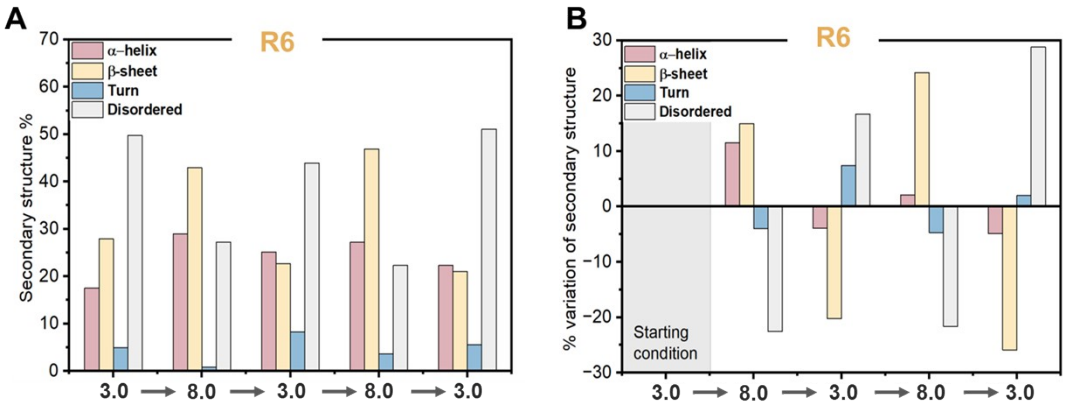

Fig. S16: Monitorization of alterations in R6's folding dynamics using Circular Dichroism spectroscopy under controlled cyclical pH variations. (A) Percentage of the secondary structure of R6 during reversibility study estimated using BeStSel web tool[10]. (B) Variation of the percentage of each secondary structure of R6 when compared to the previous condition. The starting condition corresponds to protein dissolved at pH 3.0. The pH was cyclically changed through overnight dialysis. Positive variation indicates the increase of the secondary structure, negative variation indicates the decrease of the determinant secondary structure in comparison to the previous condition.

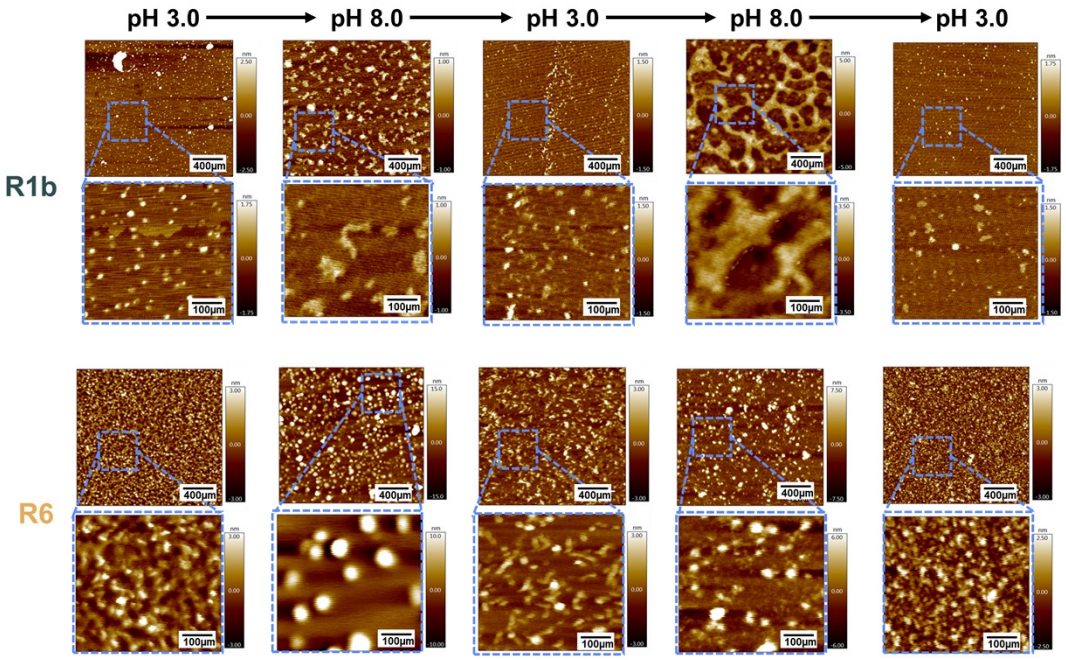

Fig. S17: Morphological changes in reflectin assemblies during pH-induced reversibility study. AFM height images of R1b (top) and R6 (bottom) assemblies through four cycles between pH 3.0 and 8.0. Overnight dialysis was used for all pH transitions. R1b shows limited reversibility with increasing heterogeneity over cycles. R6 exhibits consistent reversibility, forming larger assemblies at pH 8.0 and returning to small, uniform particles at pH 3.0. Each set: 400 nm overview (top) with 100 nm magnified inset (bottom, blue box). Scale bars: 400 nm (top), 100 nm (bottom). Color scale indicates height.

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