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Anisotropic Interactions in Protein Mixtures: Self Assembly and Phase Behavior in Aqueous Solution

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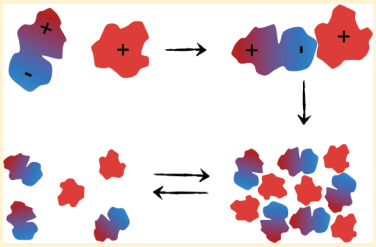
Supporting Information

ABSTRACT: Recent experimental studies show that oppositely charged proteins can self-assemble to form seemingly stable microspheres in aqueous salt solutions. We here use parallel tempering Monte Carlo simulations to study protein phase separation of lysozyme/α-lactalbumin mixtures and show that anisotropic electrostatic interactions are important for driving protein self-assembly. In both dilute and concentrated protein phases, the proteins strongly align according to their charge distribution. While this alignment can be greatly diminished by a *single* point mutation, phase separation is completely suppressed when neglecting electrostatic anisotropy. The results highlight the importance of subtle electrostatic interactions even in crowded biomolecular environments where other short-ranged forces are often thought to dominate.



SECTION: Biophysical Chemistry

In a set of experimental studies, it has been shown that 1:1 mixtures of lysozyme (lys) and α-lactalbumin (α-lac) self-assemble into well-defined micrometer-sized spheres.[1−3] This process is highly dependent on the salt concentration, and microspheres are formed only for the calcium depleted *apo* form of α-lac at salt concentrations below 100 mM, while for the calcium loaded *holo* form of α-lac, the self-assembly vanishes. Contrary to phase separation of similarly charged proteins,[4,5] lys and apo α-lac phase separate due to attractive electrostatic interactions between the two oppositely charged proteins. Interestingly, this electrostatic interaction is far from isotropic, and it has recently been shown that the anisotropic charge distribution of α-lac, combined with the high positive net charge of lys gives rise to strong directional protein−protein interactions.[6,7] The remaining question is how such long-ranged, noncentrosymmetric interactions from charged residues influence protein assembly and phase transition? We here present a mesoscopic simulation study of aqueous protein mixtures where we investigate the contribution of anisotropic protein−protein interactions on phase transition of proteins as well as on microstructuring in dense protein environments.

Previous studies on anisotropic protein−protein interactions[8−14] typically describe proteins as patchy spherical particles with attractive sites interacting via angularly dependent square well potentials. These models rely mainly on ambiguously placed attractive patches and not on anisotropy owing to nonuniform surface charge distributions of real proteins. One exception is the embedded discrete charge model[9,11,15] where charged residues are projected onto a hard sphere. Although approximately accounting for anisotropic electrostatics, this model treats the excluded volume as spherical symmetric. To remedy this, here we develop a protein model that captures anisotropic interactions due to both excluded volume and electrostatic interactions, while at the same time being fast enough for simulation studies of phase transitions involving *many* protein molecules.

Simulating phase behavior at an atomistic level is unfeasible due to the vast number of solvent and solute molecules in the system. We therefore gradually coarse-grained the proteins (see illustration in Figure 1) while maintaining (i) the salt-dependent potential of mean force between lys/α-lac and (ii) the distance-dependent average alignment of α-lac's dipole moment with respect to lys. The latter unitless property ranges between +1 and −1, where +1 corresponds to a fully aligned dipole. The final model, (c), maintains all charged residues as in the crystal structure, while the remaining part is represented by a central particle. As shown in the Supporting Information, this model closely reproduces the well-tested reference model (a).

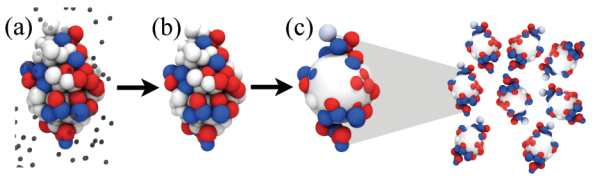


Figure 1. Coarse graining starting from an implicit solvent/explicit salt model where amino acids are represented by spheres (a).[6,16] From (a)→(b) Debye−Hückel electrostatics; (b)→(c) neutral residues are collected into a central sphere. See Supporting Information for details.

Metropolis Monte Carlo simulations in the canonical ensemble, *NVT*, were used for two-protein interactions when coarse-graining model (a), while simulations in the isobaric−isothermal ensemble, *NpT*, were used to study many-protein

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interactions and phase behavior with model (c). The system energy for a given configuration was $U = \sum_{i<j} 4\varepsilon_{LJ}[(\sigma_{ij}/r_{ij})^{12} - (\sigma_{ij}/r_{ij})^{6}] + (e^2 z_i z_j/4\pi\varepsilon_0\varepsilon_r r_{ij})\zeta$, where ε_{LJ} is the depth of the Lennard-Jones potential, σ_i is the diameter, and $\sigma_{ij} = (\sigma_i + \sigma_j)/2$. r_{ij} is the separation between particle i and j, z_i is the charge valency, e is the electron unit charge, and $\varepsilon_0\varepsilon_r$ is the dielectric permittivity of water. For explicit salt, $\zeta = 1$; for implicit salt, $\zeta = e^{-\kappa r_{ij}}$, where κ is the inverse Debye screening length. For NpT simulations, the histogram method was used to sample volume probability distributions, $P(V)$, related to the constrained Gibbs free energy, $G = -kT \ln P(V)$ where k is Boltzmann's constant.[17] Minima in G correspond to (meta-)stable phases, and to obtain coexistence concentrations, G was reweighted, $G' = G \pm \Delta pV$, such that two minima had equal free energies.[18] The Helmholtz free energy is $A = G' - pV$. We used parallel tempering in both p and κ to efficiently sample configurational space at low salt concentrations where strongly attached clusters form. For simulation details, see Supporting Information.

Using model (c), we performed NpT simulations with 40 proteins to investigate the Helmholtz free energy of bulk 1:1 protein mixtures containing lys and four different forms of α-lac: apo, holo, smeared, and mutated apo (see Table 1 and Figure 2). Phase separation, indicated by a common tangent of two points in the Helmholtz free energy curve, was observed only for mixtures containing the apo forms and at low salt concentrations. This is in perfect agreement with experimental observations.[2] Increasing the salt concentration leads to reduced electrostatic interactions between the oppositely charged lys and α-lac, and eventually suppresses phase separation. The same is the case for holo α-lac, where the bound calcium ion reduces the net charge of α-lac.

Table 1. Model Details for Lys and α-Lac[a]

	α-lac				
protein	apo	holo	mutated[b]	smeared[c]	lys
PDB entry	1F6R	1F6S	1F6R	1F6R	4LZT
$N_{residues}$	124	125	124	124	130
net charge, pH 7.5	−5.9	−4.5	−5.9	−5.9	7.0
dipole moment[d] (D)	78	68	34	3	20

[a]For all particles, ε_{LJ} =0.05 kT in models (a) and (b), ε_{LJ} = 0.075 kT in model (c). σ_i = 30 Å for the central sphere (see ref 6 for σ of other particles). [b]Residues D79 and K99 are swapped. [c]Net charge is uniformly smeared over all titratable sites. [d]Calculated with respect to the protein mass center.

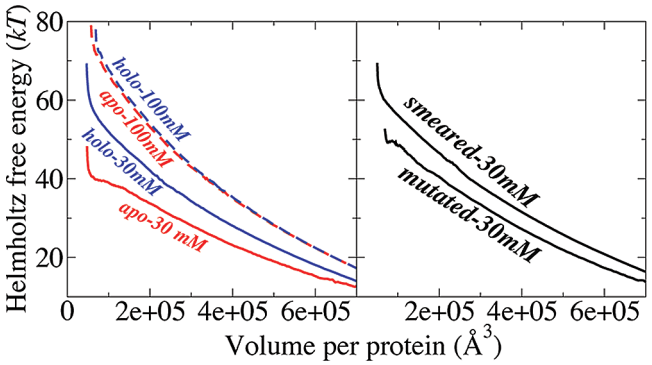

Figure 2. Helmholtz free energy for 1:1 mixtures of lys with apo, holo, mutated, and smeared α-lac at 30 mM (solid lines) and 100 mM (dashed lines) salt concentrations.

To identify the contribution of anisotropic electrostatics to phase separation, we artificially reduced the α-lac dipole moment from 78 to 34 D by swapping merely two charged amino acids (Table 1). As seen in Figure 3, this mutation, which does not alter the net charge, drastically decreases the critical salt concentration at coexistence and widens the immiscibility gap of lys/apo α-lac. We also approached isotropic electrostatics by smearing out the total charge of apo α-lac on *all* titratable residues and, as seen in Figure 2, lack of directional interactions completely obstructs phase separation in the investigated salt concentration range.

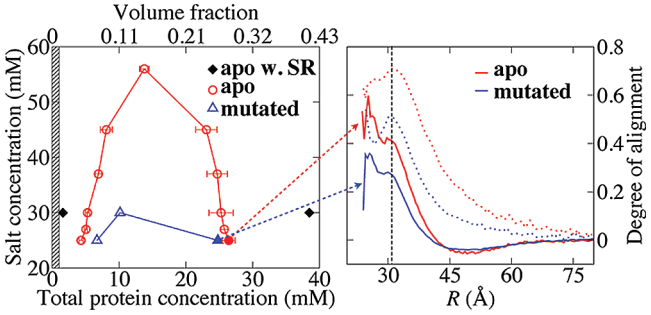

Figure 3. Left: Fluid−fluid coexistence curve of lys and α-lac in different forms and with extra short-ranged attraction (SR). The shaded area shows the experimentally observed concentration range of the dilute phase; error bars reflect the standard deviation of five independent Monte Carlo runs. Right: α-lac dipole moment alignment with respect to lys (25 mM salt) as a function of protein separation in the dense phases (solid) and at infinite dilution (dotted); the vertical line indicates the position of the $w(r)$ minima.

It is instructive to follow the alignment of α-lac's dipole moment with respect to the mass center of lys (see Figure 3, right): at infinite protein dilution, the apo form of α-lac is aligned by more than 70% when in contact with lys and then levels off to zero when the proteins separate. As expected, by diminishing the dipole moment with a point mutation, this alignment is reduced. A similar behavior is seen in the dense protein phases but to a lesser degree due to the surrounding proteins. Still, the apo form of α-lac is aligned by ∼40%, underlining that anisotropic electrostatics are involved in the dense phase structuring.

Experimentally, microsphere formation occurs at low salt and at total protein concentrations between 0.075 and 1.05 mM,[2] implying that the equilibrium concentration of the dilute phase is in the sub-millimolar range. Our model predicts a dilute phase concentration 5−70 times higher, and thus underestimates the width of the coexistence curve. Similar issues have been reported for lys.[11,19] One reason could be the lack of charge regulation in our model, although this is probably of minor importance.[6,20] It has also been proposed that apo α-lac undergoes conformational changes into a semifolded molten globule state upon binding to lys, leading to exposure of hydrophobic residues.[3] Such a mechanism, which will be temperature dependent,[8,19] likely leads to a short-range attraction unaccounted for by our rigid protein model. Although quantitative agreement is not the chief goal of this work, we attempted to remedy the above discrepancy by pragmatically incorporating a square well potential (depth 1 kT,

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width 3.1 Å, corresponding to a water layer) between the neutral spheres in model (c). This spherically symmetric potential is justified by lack of angular correlations in the hydrophobic interactions for the present system (see Supporting Information). Figure 3 shows that this simple addition significantly improves the agreement with the experimental dilute phase density. Further, the simulated two-body potential of mean force, $w(R)$, is related to the thermodynamic dissociation constant, $K_d^{-1} \approx 4\pi\int_{\text{contact}}^{\infty}(e^{-w(R)/kT} - 1)R^2\,dR$.[21] Without short ranged attraction, K_d = 737 μM at 39 mM salt and pH 7.5, merely 32% off the experimental value of 490 ± 70 μM.[7] Including the above square well potential, we obtained K_d = 446 μM. Thus, the short-ranged attraction improves agreement with the experimental phase behavior as well as consistency at the two-protein level. Note that our reference model (a) was originally used to reproduce lys−lys osmotic second virial coefficients[16] and that we have made no efforts to fit it to experimental data for the current lys/α-lac system.

We have shown that anisotropic electrostatic interactions resulting from a nonuniform distribution of amino acid charges play an important role in protein self-assembly. Due to the high charge anisotropy of α-lac, this protein aligns in the electric field of lys, both in dilute and concentrated protein environments, showing that directional electrostatics are influential even at crowded conditions. Phase separation is sensitive to subtle charge changes, and a single amino acid point mutation that minimizes the protein dipole moment of α-lac, while retaining the net charge, is enough to significantly shift the immiscibility gap to lower salt concentrations. This dipole moment reduction changes the close contact alignment and, consequently, the structure of the dense phase. Artificially smearing out the charge over the protein surface, and thus approaching isotropic electrostatics, completely inhibits phase separation. These results show that phase behavior of aqueous protein mixtures is sensitive to anisotropic electrostatics, both qualitatively and quantitatively, and that inclusion of directionality in theoretical models is important for accurate prediction of coexistence curves.

■ ASSOCIATED CONTENT

Supporting Information

Simulation details and model development. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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