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Molecular evidence of stereo-specific lactoferrin dimers in solution

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ABSTRACT

Gathering experimental evidence suggests that bovine as well as human lactoferrin self-associate in aqueous solution. Still, a molecular level explanation is unavailable. Using force field based molecular modeling of the protein–protein interaction free energy we demonstrate (1) that lactoferrin forms highly stereo-specific dimers at neutral pH and (2) that the self-association is driven by a high charge complementarity across the contact surface of the proteins. Our theoretical predictions of dimer formation are verified by electrophoretic mobility and N-terminal sequence analysis on bovine lactoferrin.

1. Main

This combined theoretical and experimental study of the pair interaction of bovine lactoferrin (LF) shows that the protein self-assembles into dimers. Free energy calculations reveal a well defined, stereo-specific complex that is stabilized by a region of complementary amino acid residues. Historically, LF was first isolated from bovine milk but the glycoprotein is found in all mammals. It is secreted in various exocrine fluids such as bile, saliva, pancreatic fluid and tears [1]. Inflammatory reactions and viral infections are often followed by increased plasma concentrations of LF and the protein is considered part of the acute-phase immune response. More specifically, LF has antibacterial, antiviral and anti-inflammatory activity, underlining that the protein is an important part of the immune system [2]. LF is also interesting from a technical perspective, for instance, cationic protein-stabilized emulsions have been prepared of the protein and LF is known to be a fouling component of contact lenses [3,4]. LF has also received attention as a nutritional additive in milk formulas [5]. The protein consists of two major lobes and approximately 700 amino acids. The macromolecule has the shape of a dumbbell and is well described by a bi-axial ellipsoid with half-axis of 47 Å and 26 Å. Several experimental studies imply that the protein self-assembles: dimers of bovine LF have been misstaken for IgG2, that has a molecular weight twice of the LF monomer and sedimentation equilibrium has revealed a concentration-dependent average molecular weight [6,7]. Further, LF is found to elute with molecular weights corresponding to monomers, dimers and trimers, according to chromatographic analysis of bovine milk [8,9]. Scattering studies have also revealed large aggregates of human LF and that these dissolve at high ionic strength [10]. These observations all suggest that LF is able to self-assemble into larger structures, although no molecular level explanation has been presented. Using Metropolis Monte Carlo simulations we investigate the angularly averaged free energy of interaction between two LF molecules in an aqueous salt solution using the Faunus open source software package [11,12]. This software package has been applied with success for computation of virial coefficients of lysozyme and binding constants of lysozyme and *apo*-α-lactalbumin [13,14]. The simulation procedure is described in detail in the Appendix as well as elsewhere and we shall give here only a brief overview [13,15]: each protein is treated as a rigid body consisting of spherical amino acid beads that may be either charged or neutral, depending on the type and protonation state; PDB entry 1BLF was used to generate the coarse grained *holo*-Lactoferrin structure. A rigid model is reasonable since the structure of the *holo*-form is considered stiff and is resistant to proteolytic degradation[1,16]. The solvent is treated as a dielectric continuum and particles interact via a combined Lennard–Jones (LJ) and salt screened Debye–Hückel potential, where the total energy for each configuration, U, is given by the sum of the amino acid pair interactions.

$$U = \sum_{i<j}^{N} \left[\frac{q_i q_j}{4\pi\epsilon_0\epsilon_r r_{ij}} e^{-\kappa r_{ij}} + 4\epsilon \left(\left(\frac{\sigma_{ij}}{r_{ij}} \right)^{12} - \left(\frac{\sigma_{ij}}{r_{ij}} \right)^{6} \right) \right] \quad (1)$$

r_{ij} is the separation between amino acids, q is the charge of an amino acid and κ is the inverse Debye length, related to the ionic strength. ϵ_0 is the permittivity of vaccum and ϵ_r the dielectric constant of water,

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set to 80 in this work. σ_{ij} is the mean diameter of the amino acids and ϵ is the LJ interaction parameter (0.05 kT). k is Boltzmann's constant and T is the temperature. During the MC simulation, proteins are translated as well as rotated to explore configurations in the canonical ensemble at 298 K. The free energy of interaction (or potential of mean force, PMF) is obtained by sampling the protein–protein radial distribution function as a function of the mass center separation, R. In contrast to typical docking methods, the present approach correctly takes into account thermal motion and hence the entropy of the protein–protein association process. Fig. 1A shows the calculated lactoferrin–lactoferrin free energy of interaction at pH 5, 7 and 9 and at an ionic strength of 5 mM ($\kappa = 0.023$ Å^{-1}). At these pH values the LF net charge is $+23e$, $+14e$ and $+4e$. Titration simulations estimate pI to 9.4, slightly above the experimental range, 8–9 [17–19]. At pH 5 the interaction is purely repulsive due to a large net charge of the proteins. However, as pH is increased, a deep narrow minimum appears at approximately 47 Å. The attraction broadens at pH 9, close to isoelectric conditions, but the minimum is, interestingly, very distinct. We now demonstrate that the narrow free energy minimum is due to a few highly specific residue–residue interactions that lock the entire complex into a unique constellation. Firstly, by inspecting the MC generated configurations (see animation in Appendix) it is clear that when the proteins are in contact, only few orientations are observed. Secondly, we identify a close to orthogonal set of vectors, spanned from the proteins mass center to the mass centers of E385 and E66 and correlate the E385 angles with the E66 dihedrals (inset of Fig. 2). This allows us to estimate the orientational free energy and, as evident from Fig. 2, the two proteins are aligned in a strongly stereo-specific manner, dominated by a narrow set of protein–protein orientations. The choice of E385 was motivated by the fact that it is parallel to the line connecting the protein mass centers at the free energy minimum. The total interaction between the two proteins is influenced by both electrostatic and van der Waals interactions. Due to the non-zero molecular net charge, any attractive electrostatic contribution must be due to charge patches on the protein surface. To qualitatively investigate the effect of this patchiness, we identify a few amino acids with high inter-protein charge-complementarity. These are selected by inspecting the protein–protein contact surface of different configurations at the free energy minimum. Two such candidates are glutamate E535 and lysine K498. In Fig. 1B we present the PMF between mutated forms of LF as well as for the wildtype at pH 8, where the charge of the wildtype is $+11e$. Reverting the negative charge of E535 has dramatic effects on the potential of mean force: this single point mutation leads to a complete loss of the attractive free energy minimum. The mutation increases the net charge of the protein by two units and an increased repulsion can be expected. Conversely, the protein net charge is maintained by swapping charges

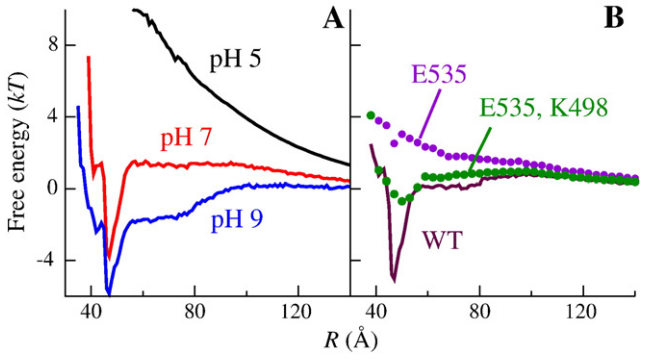

Fig. 1. Free energy of interaction (PMF) between lactoferrin molecules as a function of the mass center separation, R, at (A) different pH values for the wildtype, (B) different mutations and wildtype at pH 8. The ionic strength is 5 mM in all cases. Note, one kT equals 2.48 kJ/mol at 298 K. The maximum error is about 0.2 kT.

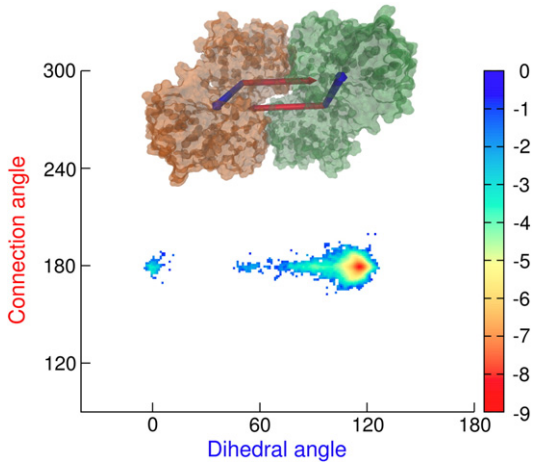

Fig. 2. Angular free energy (kT) of the connection angle (red vectors) and dihedral (blue vectors) at pH 8 and at 5 mM ionic strength. Sampling is performed within a 60 mass center separation. The inset shows the two proteins and the reference frames. The scale is relative to a free orientation.

of K498 with E535 — both of which are in the contact region. This also significantly diminishes the free energy minimum, underlining that the contact surface is highly tailored to facilitate a dimeric complex, assembled via complementary electrostatic interactions. Coordinates of a typical dimer configuration is supplied in the online material. Lastly, we provide experimental data to back our theoretical predictions. LF was separated using 12% Tris–Glycine SDS-PAGE under non-reducing conditions followed by staining with Coomassie blue R-250. Results indicate a primary band at 80 kDa, consistent with the molecular weight of the protein in its monomeric form. Two distinct bands, consistent with the molecular weight of dimeric and trimeric LF are also clearly visible at 160 kDa and 240 kDa (Fig. 3). To

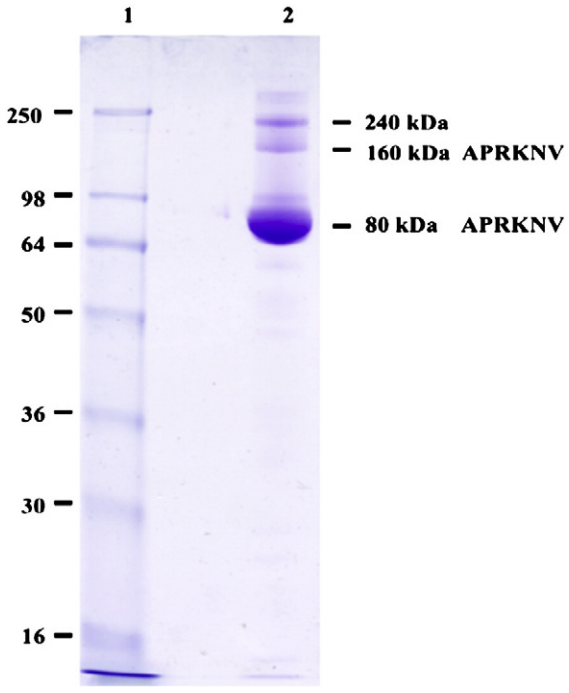

Fig. 3. SDS PAGE analysis of LF. Proteins were separated using 12% Tris–Glycine SDS PAGE under non-reducing conditions and pH 8.3 following by staining with Coomassie blue R-250. Lane 1: Molecular weight marker; Lane 2: LF. N-terminal sequence analysis of the electroblotted bands is indicated.

Table 1
Properties of coarse grained amino acids.

Particle	Radius (Å)	pKa
Ala	3.1	–
Arg	4.0	12.0
Asp	3.6	4.0
Gly	2.9	–
Glu	3.8	4.4
His	3.9	6.3
Lys	3.7	10.4

PVDF membranes (Invitrogen). The 80 kDa and 160 kDa electro-blotted bands were excised and amino acid sequence analyses were performed on an Applied Biosystems PROCISE HT protein sequencer with on-line identification of phenylthiohydantoin-derivatives using standard programmes. Lactoferrin was identified in both bands.

While experimental data and the present simulations suggest the formation of LF dimer, it is desirable to further investigate special orientation of the aggregates by experimental scattering techniques. It would also be of importance to evaluate if the model is able to predict trimers. We intend to continue with such studies and to further scrutinize the driving force in the simulated system. Initial examination of more detailed models shows consistent results with this work.

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Appendix A

To coarse grain the protein we assume that each amino acid has a density of 0.9 g/ml and use the molecular weight to compute the radius. The sphere is centered at the mass center in the atomistic amino acid structure. It has been shown that this provides a good topological description of proteins and reproduces atomistic results [13]. To evaluate the average charge of each amino acid we perform Monte Carlo simulations with single proteins. In such calculations, each amino acid is allowed to titrate in a Markov chain, according to a Boltzmann weight that is modified by the pKa-value and pH of the solution. This provides us with an estimate of the charge distribution of the proteins that are being used as input when we evaluate the pair-interaction. A complete description of simulation details and parameters can be found in the reference [13] and [15]. The radii of some of the different amino acids, along with pKa-values used to generate average charges, are provided in Table 1.

Lactoferrin is here modeled in its *holo*-form. This is achieved by placing a unit charge at the site of each ferric ion in the original coordinates, approximating a ferric ion synergistically bound with a carbonate. The magnitude of the Lennard–Jones interaction parameter is obtained from a Hamaker argument. Since the densities of proteins are fairly constant one may estimate the contribution of the interactions across a water medium on an amino acid level. The magnitude in this work is chosen so it reproduces that of a previously published model [13]. Since the range of interaction depends on the size of the amino acid, large residues will effectively attract each other more than smaller which is reasonable. This does not capture any sort of specific hydrophobic interactions. What is being modeled is rather an averaged dispersion–hydrophobic interaction and the geometrical influences of the experimental structure. In the simulations we restrict the sampling to distances shorter than 200 Å and the radial distribution function is sampled with a bin width of 1 Å. Each PMF is generated with approximately 10^7 configurations, which was found sufficient to produce static results with low noise. The animation provided displays a representative trajectory of the Monte Carlo simulation at pH 8 and clearly shows how the two proteins bind in a specific orientation. Red colored particles represent negatively charged amino acids while blue colored are positively charged.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bpc.2010.06.005.

References

[1] L. Adlerova, A. Bartoskova, M. Faldyna, Lactoferrin: a review, Vetetinarni Midicinia 53 (2008) 457–468.
[2] J.H. Brock, The physiology of lactoferrin, Biochem. Cell Biol. 80 (2002) 1–6.
[3] A. Sarkar, K.K.T. Goh, H. Singh, Colloidal stability and interactions of milk-protein-stabilized emulsions in an artificial saliva, Food Hydrocolloids 23 (2009) 1270–1278.
[4] L. Meagher, H.J. Griesser, Interaction between adsorbed lactoferrin layers measured directly with the atomic force microscope, Colloids and Surfaces B 23 (2002).
[5] M. Tomita, H. Wakabayashi, Kouichirou, K. Yamauchi, T. Yaeshima, K. Iwatsuki, Twenty-five years of research on bovine lactoferrin applications, Biochimie 91 (2009) 52–57.
[6] F.L. Schanbacher, K.L. Smith, L.C. Ferguson, The similarity of bovine lactoferrin dimer to igg2, Fed. Proc. 30 (1971) 532.
[7] F.J. Castellino, W.W. Fish, K.G. Mann, Structural studies on bovine lactoferrin, J. Biol. Chem. 245 (1970) 4269–4275.
[8] R.J. Harmon, F.L. Schanbacher, L.C. Fergusson, K. Larry, Changes in lactoferrin, immunoglobulin g, bovine serum albumin and alpha-lactalbumin during acute experimental and natural coliform mastitis in cows, Infect. Immun. 13 (1976) 533–542.
[9] H. Wang, W.L. Hurley, Identification of lactoferrin complexes in bovine mammary secretions during mammary gland involution, J. Dairy Sci. 81 (1998) 1896–1903.
[10] S.E. Babina, F.V. Tuzikov, N.A. Tuzikova, V.N. Buneva, G.A. Nevinskii, Effect of nucleotides on the oligomeric state of human lactoferrin, Mol. Biol. 40 (2006) 121–131.
[11] N.A. Metropolis, A.W. Rosenbluth, M.N. Rosenbluth, A. Teller, E. Teller, Equation of state calculations by fast computing machines, J. Chem. Phys. 21 (1953) 1087–1097.
[12] M. Lund, M. Trulsson, B. Persson, Faunus: An object oriented framework for molecular simulation, Source Code Biol. Med. 3 (2008) 3:1.
[13] M. Lund, B. Jönsson, A mesoscopic model for protein–protein interactions in solution, Biophys. J. 85 (2003) 2940–2947.
[14] B.A. Persson, M. Lund, Association and electrostatic steering of alpha-lactalbumin–lysozyme heterodimers, Phys. Chem. Chem. Phys. 11 (2009) 8879–8885.
[15] M. Lund, B. Jönsson, On the charge regulation of proteins, Biochemistry 44 (2005) 5722–5727.
[16] E.N. Baker, H.M. Baker, A structural framework for understanding the multifunctional character of lactoferrin, Biochimie 91 (2009) 3–10.
[17] S. Belegrinou, I. Mannelli, P. Lisboa, F. Bertagnol, A. Valsesia, G. Ceccone, P. Colpo, H. Rauscher, F. Rossi, ph-dependent immobilization of proteins on surfaces functionalized by plasma-enhanced chemical vapor depostion of poly(acrylic acid)- and poly(ethylene oxide)-like films, Langmuir 24 (2008) 7251–7261.
[18] M.C. Wahlgren, T. Arnebrant, M.A. Paulsson, The adsorption from solutions of beta-lactoglobulin mixed with lactoferrin or lysozyme onot silica and methylated silica surfaces, J. Coll. Interface Sci. 158 (1993) 46–53.
[19] F. Superti, R. Siciliano, B. Rega, F. Giansanti, P. Valenti, G. Antonini, Involvement of bovine lactoferrin metal saturation, sialic acid and protein fragments in the inhibition of rotavirus infection, Biochim. Biophys. Acta 1528 (2001) 107–115.