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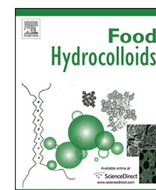

Complexation of lysozyme with sodium caseinate and micellar casein in aqueous buffered solutions

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We present an extended structural and morphological study of the complexation of lysozyme (Lys) with sodium caseinate (SC) and micellar casein (MC) by means of turbidity measurements, phase analysis, dynamic, static and electrophoretic light scattering, bright-field and confocal laser scanning (CLSM) microscopy, fluorescence anisotropy and circular dichroism measurements. The solution behavior, structure, effective charge and morphology of the formed complexes as well as the protein structure within the complexes are dependent on the state of the casein molecules (SC versus MC), pH, ionic strength, and the $[Cat^{+}]/[An^{-}]$ charge ratio (ChR). Absorption measurements indicate complexation of Lys with caseins at a pH as high as 11.29 ($I = 0.01$). At ChR>1, i.e. in excess of lysozyme, CLSM clearly showed formation of complex Lys/SC particles with a neutral core and an exterior part consisting exclusively of hydrophilic Lys macromolecules, whereas in the case of Lys/MC particles a uniform distribution of both proteins was observed. Binding of Lys with SC or MC leads to disruption of the secondary structure of Lys. Binding isotherms from fluorescence anisotropy are well described by an independent binding site model.

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1. Introduction

Protein-protein interactions drive many biophysical processes of proteins in solution, such as aggregation, solubilisation and desolubilisation, and phase transitions including crystallization, gelation, and amorphous precipitation. Many of these processes are of significant research interest because of their practical importance. In the biopharmaceutical industry, it is crucial to prevent therapeutic proteins from aggregation during the manufacturing process and storage in order to maintain safety and efficacy (Schmidt, Havekost, Kaiser, Kauling, &Henzler, 2005). In addition, protein crystallization and precipitation are used for industrialized recombinant protein purification processes (Gunton, Shiryayev, & Pagan, 2007). Furthermore, studying protein-protein interactions could shed light on the mechanisms of protein condensation (or phase transition) diseases, such as cataract and sickle cell disease (Uversky & Fink, 2006). Finally, protein-protein interactions may play an essential role in many human neurodegenerative diseases attributed to protein aggregation, such as Parkinson and Alzheimer diseases (Howel, 1992). Protein-protein interactions are also relevant for food and nutrition. They can affect the nutritional and organoleptic quality of food products during manufacture, storage and consumption. Many studies, to date, display interesting and technologically useful properties produced by protein interactions including enhanced gelation properties originating from synergistic interactions and new textural properties as a result of aggregation of oppositely charged proteins and phase separation (Bouhallab & Croguenneg, 2014; Howell, Sabila, Grootveld, & Williams, 1996). Clearly, knowledge of protein interactions can lead to a better understanding of biochemical changes in food products during processing and storage such as for example the aggregation of proteins in fish leading to toughening upon frozen storage (Howel, 1992). Furthermore, an understanding of the effect of protein structure on protein-protein interactions, for example, of smooth and skeletal muscle proteins permits the manipulation of protein side chains in order to enhance gelation properties (Howell et al., 1996; Bouhallab and Croguenneg, 2014). However, both experimental data and the corresponding understanding of how structural and conformational properties of the interacting proteins

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affect complex formation and the resulting morphology as well as the molecular and thermal aggregation properties of the proteins in complexes, is presently lacking for most proteins.

Proteins might exhibit long-lasting interactions when being part of a protein complex or may be carrying another protein merely for a limited amount of time, for example to modify it. Depending on the type and strength of the interactions, which are also largely affected by the physico-chemical conditions of the medium, different supramolecular structures such as fibrils, spherical particles or aggregates can be formed (Winkler, Roland, & Cherstvy, 2014). Hence, understanding the driving forces that trigger protein self-assembly and the successive steps leading to the development of supramolecular structures is of paramount importance for controlling the shape, size and properties of these structures. Proteins bind to each other through a combination of coulomb and hydrophobic interactions, van der Waals forces, and salt bridges between specific binding domains on each protein (Winkler et al., 2014). In general, the protein-protein interaction energy is only slightly higher than the thermal energy *kT*, enabling the proteins to rearrange locally with respect to each other allowing them to adopt their preferential orientations (Winkler et al., 2014). Comparisons of the relaxation phenomena of protein-protein complexes with that of polyelectrolyte complexes have shown (Winkler et al., 2014) that the main difference between protein molecules and polyelectrolytes is the distance between the opposite charges. The distance between opposite charges on different protein molecules is larger than for polyelectrolytes because optimal 3D packing is more complicated in proteins than in linear polyelectrolytes. It is therefore not surprising that when two proteins are different in size, it is difficult to obtain full charge neutralization (Desfougères, Croguennec, Lechevalier, Bouhallab, & Nau, 2010). A variety of macromolecular complexes of globular proteins has recently been characterized (Desfougères, et al., 2010; Van der Linden & Venema, 2007; Coers, Permyakov, Permyakov, Uversky, & Fink, 2002; Krebs et al., 2000; Sagis, Veerman, & van der Linden, 2004; Krebs, Delvin, & Donald, 2007). Linear and fibrillar assemblies, such as amyloid fibrils, are favored at pHs far from the isoelectric point (pI) and at low ionic strength when electrostatic repulsion is high. Contrarily, spherical objects are obtained by incubation of proteins at a pH close to their pI and at an ionic strength favoring electrostatic interaction (Desfougères et al., 2010; Krebs et al., 2007). Although plenty of studies on complexation of globular proteins have been performed to characterize macromolecular protein complexes, few studies are available (Anema & de Kruif, 2013; Pan, Yu, Yao, & Shao, 2007) on interactions in systems containing unordered protein(s).

In this study we examine the association behavior in aqueous buffered solutions of two acid proteins (sodium caseinate and micellar casein), both with an intrinsically disordered structure, but having a different dispersion state (molecularly versus colloidally dispersed state) with a basic globular protein (lysozyme).The scope of the study is to elucidate the interaction and complexation of casein and lysozyme, including the structure and composition of the complex particles, the allocation of the proteins within the complex, as well as the solution properties and peculiarities of the morphology of the complex system. Therefore dynamic and static light scattering, confocal laser scanning microscopy (CLSM), optical microscopy, phase analysis, electrophoretic mobility, and absorption measurements are utilized.

Casein is a member of the group of secreted calcium (phosphate)-binding phosphoproteins. It is a major milk protein, which naturally occurs as micellar casein (MC), with each micelle containing around 20,000–150,000 casein molecules with α_{s1}, α_{s2}, β, and κ caseins in the proportion 3:1:3:1 and 8% in mass of phosphate and calcium ions (Pitkowski, Durand, & Nicolai, 2008; Holt, Carver, Ecroyd, & Thorn, 2013). Casein micelles are roughly spherical core-shell particles with outer diameters ranging from 50 to 500 nm and an average size of 120–150 nm (Dalgleish, Spagnuolo, & Goff, 2004). The core is now generally described as a homogeneous web of caseins in which calcium phosphate nanoclusters are uniformly distributed (Horne, 2002; Marchin, Putaux, Pignon, & Léonil, 2007). The shell is essentially made of κ-caseins that extend into the aqueous phase as a polyelectrolyte brush and in this way produce short-range repulsions between the micelles (Tuinier & De Kruif, 2002). Sodium caseinate (SC) is derived from native micellar casein and forms small star-like associates in aqueous solution (Pitkowski et al., 2008). Lysozyme (Lys) is a well-studied 14.3 kDa globular protein with enzymatic activity that has a net positive charge in the pH range up to its pI (10.5).

At present, there is only limited knowledge about the effects of the dispersion state of casein (SC versus MC) on the segregative phase behavior of casein with other proteins (Polyakov, Grinberg, Antonov, & Tolstoguzov, 1979) or polysaccharides (Antonov, Lefebvre, & Doublier, 2007). However, the associative phase behavior of casein with other proteins as a function of its dispersion state is unexplored. This limited knowledge combined with the potential applications of lysozyme-casein systems provides a strong motivation to study and compare their complexation and solution behavior, the morphology of the complexes formed in Lys/SC and Lys/MC systems as a function of concentration, and pH, as well as the structure of the proteins in the complexes, aiming at gaining additional insight in protein/protein interactions.

2. Experimental section

2.1. Materials

2.1.1. Proteins and reagents

Lys from chicken egg white (dialyzed, lyophilized powder) was purchased from Sigma-Aldrich and used without further purification. The SC sample (14.1% protein nitrogen, 90% protein, 5.5% water content, 3.8% ash, 0.02% calcium) was procured from Sigma-Aldrich. Its isoelectric point is around pH = 4.7–5.2 (Swaisgood, 1992). The weight average molecular mass of the SC sample in 0.15 M NaCl solutions is 320 kDa ± 20 kDa (Antonov & Moldenaers, 2009). β–casein was purchased from Sigma-Aldrich and used without further purification. The MC sample, supplied by Laboratoire de Recherche et de Technologie Laitière (P. Schuck, LRTL, INRA Rennes, France), was a native calcium phosphocaseinate sample purified by ultrafiltration and diafiltration and then freeze-dried (Bourriot, Garnier, & Doublier, 1999; Schuck et al., 1994). It had the following characteristics: total protein content 90.7%, protein nitrogen 14.21%, non-casein protein 5.0%, lactose 0.5%, salts 8.3%, calcium 2.7%. All other reagents were of analytical reagent grade. Milli-Q ultrapure water was used in all experiments. Most experiments were performed in a dilute mono/bisphosphate (KH_2PO_4+K_2HPO_4) buffer with $I = 0.01$, where I is made dimensionless with $m^0 = 1$ mol/kg.

2.1.2. Preparation of the protein solutions and protein/protein mixtures

To prepare solutions of SC and β–casein with the required concentrations, the weighed amount of biopolymer sample was gradually added to phosphate buffer (pH 7.0, $I = 0.01$) at 23°C and stirred, first for 1 h at this temperature and then for 1 h at 45°C. Lys solutions were prepared by dispersing the protein in the buffer under stirring for 1 h at 23°C. The resulting solutions of SC, β–casein, and lys were centrifuged at 50,000 g and 23 °C for 1 h to remove insoluble particles. Colloidal solutions of MC were prepared by dispersing the protein in the buffer under stirring for 14 h at 23°C with subsequent centrifugation at 2000 *g* and 23 °C for 1 h.

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