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Proteostasis and the Regulation of Intra- and Extracellular Protein Aggregation by ATP-Independent Molecular Chaperones: Lens α -Crystallins and Milk Caseins

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Proteostasis and the Regulation of Intra- and Extracellular Protein Aggregation by ATP-Independent Molecular Chaperones: Lens α -Crystallins and Milk Caseins

Abstract

Conspectus: Molecular chaperone proteins perform a diversity of roles inside and outside the cell. One of the most important is the stabilization of misfolding proteins to prevent their aggregation, a process that is potentially detrimental to cell viability. Diseases such as Alzheimer's, Parkinson's, and cataract are characterized by the accumulation of protein aggregates. In vivo, many proteins are metastable and therefore under mild destabilizing conditions have an inherent tendency to misfold, aggregate, and hence lose functionality. As a result, protein levels are tightly regulated inside and outside the cell. Protein homeostasis, or proteostasis, describes the network of biological pathways that ensures the proteome remains folded and functional. Proteostasis is a major factor in maintaining cell, tissue, and organismal viability.

We have extensively investigated the structure and function of intra- and extracellular molecular chaperones that operate in an ATP-independent manner to stabilize proteins and prevent their misfolding and subsequent aggregation into amorphous particles or highly ordered amyloid fibrils. These types of chaperones are therefore crucial in maintaining proteostasis under normal and stress (e.g., elevated temperature) conditions. Despite their lack of sequence similarity, they exhibit many common features, i.e., extensive structural disorder, dynamism, malleability, heterogeneity, oligomerization, and similar mechanisms of chaperone action. In this Account, we concentrate on the chaperone roles of α -crystallins and caseins, the predominant proteins in the eye lens and milk, respectively. Intracellularly, the principal ATP-independent chaperones are the small heat-shock proteins (sHsps). In vivo, sHsps are the first line of defense in preventing intracellular protein aggregation. The lens proteins α A- and α B-crystallin are sHsps. They play a crucial role in maintaining solubility of the crystallins (including themselves) with age and hence in lens proteostasis and, ultimately, lens transparency. As there is little metabolic activity and no protein turnover in the lens, crystallins are very long lived proteins. Lens proteostasis is therefore very different to that in normal, metabolically active cells. Crystallins undergo extensive post-translational modification (PTM), including deamidation, racemization, phosphorylation, and truncation, which can alter their stability. Despite this, the lens remains transparent for tens of years, implying that lens proteostasis is intimately integrated with crystallin PTMs. Many PTMs do not significantly alter crystallin stability, solubility, and functionality, which thereby facilitates lens transparency. In the long term, however, extensive accumulation of crystallin PTMs leads to large-scale crystallin aggregation, lens opacification, and cataract formation. Extracellularly, various ATP-independent molecular chaperones exist that exhibit sHsp-like structural and functional features. For example, caseins, the major milk proteins, exhibit chaperone ability by inhibiting the amorphous and amyloid fibrillar aggregation of a diversity of destabilized proteins. Caseins maintain proteostasis within milk by preventing deleterious casein amyloid fibril formation via incorporation of thousands of individual caseins into an amorphous structure known as the casein micelle. Hundreds of nanoclusters of calcium phosphate are sequestered within each casein micelle through interactions with short, highly phosphorylated casein sequences. This results in a stable biofluid that contains a high concentration of potentially amyloidogenic caseins and concentrations of calcium and phosphate that can be far in excess of the solubility of calcium phosphate. Casein micelle formation therefore performs vital roles in neonatal nutrition and calcium homeostasis in the mammary gland.

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Proteostasis and the regulation of intra and extracellular protein aggregation by ATP-independent molecular chaperones: lens α -crystallins and milk caseins

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Conspectus

Molecular chaperone proteins perform a diversity of roles inside and outside the cell. One of the most important is the stabilization of misfolding proteins to prevent their aggregation, a process that is potentially detrimental to cell viability. Diseases such as Alzheimer's, Parkinson's and cataract are characterized by the accumulation of protein aggregates.

In vivo, many proteins are metastable and therefore under mild destabilizing conditions have an inherent tendency to misfold, aggregate and hence lose functionality. As a result, protein levels are tightly regulated inside and outside the cell. Protein homeostasis, or proteostasis, describes the network of biological pathways that ensures the proteome remains folded and functional. Proteostasis is a major factor in maintaining cell, tissue and organismal viability.

We have extensively investigated the structure and function of intra- and extracellular molecular chaperones that operate in an ATP-independent manner to stabilize proteins and prevent their misfolding and subsequent aggregation into amorphous particles or highly ordered amyloid fibrils. These types of chaperones are therefore crucial in maintaining proteostasis under normal and stress (e.g. elevated temperature) conditions. Despite their lack of sequence similarity, they exhibit many common features, i.e. extensive structural disorder, dynamism, malleability, heterogeneity, oligomerization and similar mechanisms of chaperone action. In this review, we concentrate on the chaperone roles of α -crystallins and caseins, the predominant proteins in the eye lens and milk respectively.

Intracellularly, the principal ATP-independent chaperones are the small heat-shock proteins (sHsps). *In vivo*, sHsps are the first line of defense in preventing intracellular protein aggregation. The lens proteins α A- and α B-crystallin are sHsps. They play a crucial role in maintaining solubility of the crystallins (including themselves) with age, and hence in lens proteostasis and, ultimately, lens transparency. As there is little metabolic activity and no protein turnover in the lens, crystallins are very long-lived proteins. Lens proteostasis is therefore very different to that in normal, metabolically active cells. Crystallins undergo extensive post-translational modification (PTM), including deamidation, racemization, phosphorylation and truncation, which can alter their stability. Despite this, the lens remains transparent for tens of years, implying that lens proteostasis is intimately integrated with crystallin PTMs. Many PTMs do not significantly alter crystallin stability, solubility and functionality, which thereby facilitates lens transparency. In the long term, however, extensive accumulation of crystallin PTMs leads to large-scale crystallin aggregation, lens opacification and cataract formation.

Extracellularly, various ATP-independent molecular chaperones exist that exhibit sHsp-like structural and functional features. For example caseins, the major milk proteins, exhibit chaperone ability by inhibiting the amorphous and amyloid fibrillar aggregation of a diversity of destabilized proteins. Caseins maintain proteostasis within milk by preventing deleterious casein amyloid fibril formation via incorporation of thousands of individual caseins into an amorphous structure known as the casein micelle. Hundreds of nanoclusters of calcium phosphate are sequestered within each casein micelle through interaction with short, highly phosphorylated casein sequences. A stable biofluid results containing a high concentration of potentially amyloidogenic caseins and concentrations of calcium and phosphate that can be far in excess of the solubility of calcium phosphate. Casein micelle formation therefore performs vital roles in neonatal nutrition and calcium homeostasis in the mammary gland.

Proteostasis and molecular chaperones

Proteostasis, a portmanteau word of protein and homeostasis, has attracted much interest because it succinctly describes the tight regulation of protein levels, conformations and localizations both inside and outside the cell. Proteostasis also encompasses the synthesis, trafficking, folding and degradation of the proteome and is therefore intimately related to cell and organismal well-being and viability.¹ If proteostasis is dysregulated, diseases that are associated with protein unfolding, misfolding and aggregation, for example Alzheimer's, Parkinson's and cataract, may develop due to the accumulation of partially folded and aggregated proteins.

Molecular chaperones are important components in proteostasis because they interact with target proteins to prevent their inappropriate association.² Many intra and extracellular chaperones have been described. There are two broad types: (i) ATP-dependent chaperones which have a variety of energy-requiring regulatory roles, for example to facilitate protein folding, and (ii) ATP-independent chaperones which prevent protein aggregation, for example under conditions of cellular stress such as elevated temperature.²

Recent reviews^{2,3} have appeared on proteostasis, including discussion of the role of molecular chaperones which have mainly addressed how correct protein folding is regulated via the action of intracellular, ATP-dependent chaperones. We, and others, have recently reviewed the functional role of small heat-shock proteins (sHsps), the major intracellular ATP-independent chaperones, in proteostasis including in relation to disease.^{4,5} ATP levels are low extracellularly, so extracellular chaperones also function ATP-independently. Little comparison has been made of the role of sHsps and extracellular chaperones in proteostasis. In this article, we address this aspect. A comprehensive analysis of intra and extracellular ATP-independent chaperones is outside the scope of this review. We concentrate on the sHsps α A- and α B-crystallin, the predominant eye lens proteins. The functional roles of extracellular chaperones, including in proteostasis and disease, is described in a recent review.⁶ With respect to extracellular chaperones, we discuss milk casein proteins. Despite their lack of sequence similarity, sHsps and caseins share many structural and functional features, including the ability to prevent deleterious protein aggregation.

Lens crystallin proteins and lens proteostasis

The lens is a unique organ; it lacks a blood supply, there is no protein turnover in its center (the nucleus), it grows throughout life and it has little metabolic activity. Crystallins in the nucleus are as old as the individual. Figure 1 gives a schematic representation of the structural features of the lens and its constituent lens fiber cells, which are unique cell types. The lens is a mixture of α , β and γ -crystallin proteins at very high concentration (up to 300-400 mg/mL) which are tightly packed together in a supra-molecular array to enable unimpeded passage of light and hence proper refraction and focussing of light onto the retina.⁸

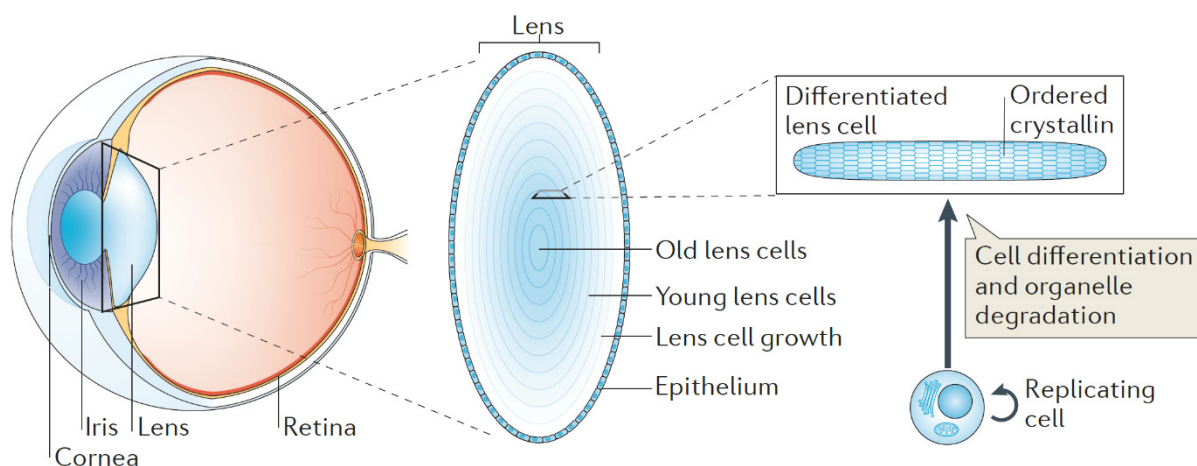


Figure 1. Schematic cross-section of the eye and its lens.⁷ Cells in the lens epithelium are metabolically active and synthesize crystallins. Subsequently, cell differentiation leads to loss of intracellular organelles and the production of lens fiber cells containing crystallins at high concentration, arranged in a well-ordered array. Reproduced with permission from ref. [7]. Copyright 2013 Springer-Nature.

Human α -crystallin is comprised of two closely related small proteins, α A- and α B-crystallin, of 173 and 175 amino acids in length respectively. They are the major lens crystallins. Both are sHsps that act as molecular chaperones to prevent lens crystallins from aggregating and precipitating. α A- and α B-crystallin co-associate to form a large heterogeneous and dynamic complex of ~650 kDa in mass. They contain large regions of structural disorder localized to the N- and C-terminal regions which flank the well-structured, β -sheet-rich, central α -crystallin domain (ACD).⁹ The β - and γ -crystallins are structurally related to each other (but not to α -crystallin) with each protein having a mass in the 21-28 kDa range. Structurally, they form well-ordered, β -sheets arranged in four Greek keys and two domains (Figure 2). The β -crystallins are oligomers (dimers, tetramers and octamers) whereas the γ -crystallins are monomers.⁹

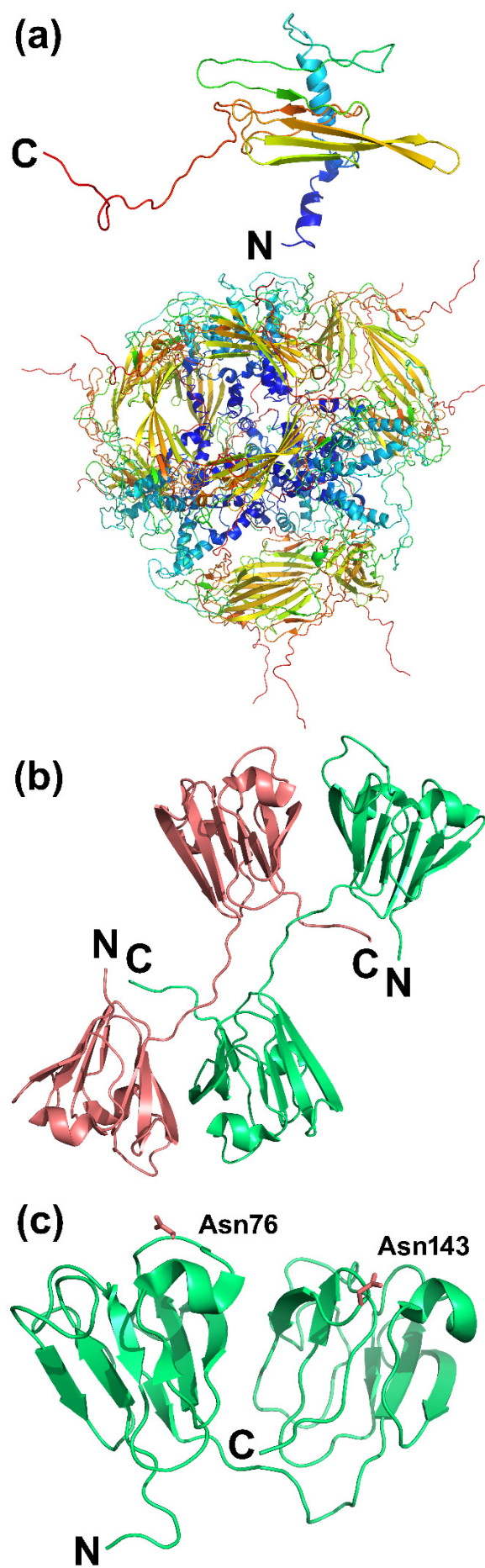


Figure 2. Representative structures of the three families of human crystallins. (a) Monomeric subunit (top) of 24-mer model of α B-crystallin (below), based on SAXS, solid-state NMR and electron microscopic data (PDB: 3J07)¹⁰. The monomers are colored in rainbow: N-terminal region (blue-green), ACD (green-orange) and C-terminal region (orange-red). Other models have been proposed for the α B-crystallin oligomer which vary in structural detail, most likely due to the protein's structural heterogeneity. (b) Crystal structure of β B2-crystallin dimer (PDB: 1YTQ)¹¹. The individual subunits are colored green and salmon. (c) Crystal structure of monomeric γ S-crystallin (PDB: 2M3T)¹². The major sites of deamidation in γ S-crystallin (Asn76 and Asn143) are displayed and colored salmon. N- and C-termini are denoted 'N' and 'C', respectively.

Standard mechanisms to maintain proteostasis, as occur for metabolically active cells, cannot operate in lens fiber cells because they contain neither cellular organelles nor the typical regulatory mechanisms for protein synthesis, maintenance, transport and degradation. Proteostasis in the lens is therefore very different to that in normal cells. So, how does the eye lens maintain functionality, i.e. transparency, over the lifespan of the individual by keeping its constituent crystallin proteins soluble?

With age and due to the lack of protein turnover, many different PTMs occur to lens crystallins including deamidation of Gln and Asn, racemization, phosphorylation of Ser, crosslinking and truncation from both termini.¹³ With regard to Asn deamidation, which involves four possible products including the conversion from L-Asn to L-Asp, the sites of modification of the various lens crystallins have been defined.¹³ Potentially, these PTMs may lead to crystallin destabilization, misfolding and aggregation and hence lens opacification. However, despite this myriad of PTMs, the lens remains transparent for decades. In fact as described below, many crystallin PTMs are relatively benign with respect to crystallin stability and hence solubility. In other words, there is major structural redundancy built into the crystallins.

Deamidation to form the Asp- or Glu-altered crystallins (i.e. the introduction of additional negative charge(s)) has minimal effect on the overall structure and function of the particular crystallin *in vitro*, for example deamidation in γ S-crystallin at Asn76¹⁴ and Asn143¹⁵ (which are both surface-exposed, Figure 2c) and in Gln147 α A-crystallin.¹⁶ However, the additional negative charge in the deamidated crystallins leads to greater stability of the crystallins to temperature, but does promote association.

Phosphorylation of the two α -crystallin subunits is extensive in the lens, and occurs to a significant extent even *in utero*.¹⁷ In the main, mimicking of phosphorylation by incorporating Asp for phosphoserine is associated with enhanced chaperone action of α B-crystallin, particularly against amorphously aggregating target proteins.¹⁸ It also leads to greater polydispersity and enhanced stability of the protein at neutral pH. Thus, phosphorylation and/or deamidation of crystallins increase the negative charge on the protein which facilitates interactions with the surrounding water to enhance thermal stability. In a similar manner, proteins from thermophilic organisms have much greater charge on their surface compared to their temperate counterparts which promotes stability because the charged sidechains can hydrogen bond with water, in addition to forming salt bridge electrostatic interactions with nearby oppositely charged sidechains.

α -, β - and γ -Crystallins all contain flexible and unstructured extensions at their N- and/or C-termini that protrude from their domain cores (Figure 2).^{19,20} In α A- and α B-crystallin, these regions encompass the last 10 and 12 amino acids respectively and in β B2-crystallin, they refer to the first 15 and last 10 residues. In γ -crystallin, the first four residues are flexible. Loss of parts of the C-terminal extension of α A-crystallin (as occurs with age) does not significantly affect its structure or chaperone ability,¹⁹⁻²¹ although truncation past the C-terminal extension into the C-terminal region of α A-crystallin does have a deleterious effect.²¹ A similar lack of effect on the β -sheet structure of β -crystallins is also observed for truncation within their flexible extensions.^{19,20}

In humans, no unmodified α -crystallin is present in the lens nucleus after around 40 years of age.²² Despite the associated structural changes, aged α -crystallin retains functionality. For example, the highly aggregated form of α -crystallin has reasonable chaperone ability,²³ and the central ACD of α B-crystallin itself is an effective chaperone,²⁴ as are peptide fragments of the ACD.²⁵

What other factors may alter lens proteostasis and lead to crystallin aggregation and hence lens opacification?

Glutathione levels are high in the young human lens but decrease with age, particularly in the nucleus, i.e. from around 2.5 to below 1.0 $\mu\text{mol/g}$ of lens wet weight.²⁶ A barrier to glutathione transport also develops with age in the periphery of the lens nucleus.²⁷ Combined, these factors potentially contribute to oxidative modification of the crystallins. Indeed, cataract lens crystallins, particularly specific β - and γ -crystallins, contain significantly more disulfide bonds than age-matched, normal lens crystallins.²⁸

In aged human lenses, there is a significant reduction in free water, perhaps due to greater crystallin aggregation.²⁹ Furthermore, cataract lenses have less free water than normal lenses, which increases crystallin concentration.²⁹ Minton³⁰ has hypothesized that dehydration due to macromolecular crowding and the associated increase in protein concentration exacerbate the onset of age-related, protein misfolding diseases. Similar behavior could occur in the aged lens (where protein concentration is very high). As a result, crystallin aggregation is promoted because of the increased crystallin concentration due to dehydration and the presence of more crystallins since the lens synthesizes crystallins throughout an individual's lifespan.

Frederikse³¹ observed an amyloid-like structural arrangement of the crystallin proteins in the lens. He concluded that lens crystallins are arranged in a β -sheet supramolecular order and are therefore potentially 'primed' to form amyloid fibrils. Crystallin fibril formation³² may be advantageous as it leads to a well-defined crystallin β -sheet array that ensures proper transparency. In the lens, it is unlikely that fibrillar crystallins would be cytotoxic because of the inert nature (i.e. lack of metabolic activity) of lens fiber cells. Consistent with this, amyloid fibrillar forms of α -crystallin and α B-crystallin alone function well as molecular chaperones,²³ as do the amyloidogenic α -crystallin mini-chaperone peptides.²⁵

Thus, in the lens environment where significant temporal changes occur, i.e. in glutathione levels, water arrangement and possibly the formation of amyloid fibrils, crystallin PTMs may provide a means to adapt to this changing environment and therefore to retain solubility. So, why does cataract eventually occur, and often with rapid onset? We hypothesize that

eventually, the many crystallin PTMs accumulate to such an extent that the lens proteostasis network cannot cope and large-scale crystallin destabilization occurs leading to their aggregation, insolubilization, lens opacification and ultimately cataract.

The lens is potentially a very good model for age-related molecular changes in other tissues where long-lived proteins occur that are involved in the development of protein misfolding diseases.¹³ The absence of metabolic activity within lens fiber cells means that all crystallin changes, for example PTMs, are caused by ageing. Thus, changes in crystallins in the lens can be regarded as a baseline measure of age-related changes in other tissues. It is well recognized that many of the age-related protein misfolding diseases are promoted by specific mutations that upset the delicate balance in proteostasis inside and outside the cell, which are superimposed on age-related PTMs.

Extracellular molecular chaperones: caseins

Although caseins are best known for very high levels of expression in mammary epithelial cells, their widespread occurrence has been reported in other normal tissues and biofluids with individual casein transcripts being present at a high level.^{33,34} In the milk of various species, whole casein comprises three to five gene products at around 26 mg/mL in the cow but as high as 150 mg/mL in other species. Although highly variable in sequence and post-translational modifications, typically caseins are small proteins of around 200 amino acids in length.

We have worked mainly with bovine α_{S1} -, α_{S2} -, β - and κ -caseins. In bovine milk, there is approximately four times as much β - or α_{S1} -casein as κ - or α_{S2} -casein but the ratios vary among species, individuals, through stages of lactation and the effect of proteolysis. Nevertheless, in spite of considerable variation in casein composition, all milks contain polydisperse, more-or-less spherical, colloidal particles called casein micelles (Figure 3) which scatter light strongly to give milk its white appearance. The term ‘micelle’ identifies a biological structure formed by association but does not imply any other similarity to detergent micelles. Bovine milk is rich in calcium and inorganic phosphate compared to other biofluids with nearly two-thirds of the calcium and half the inorganic phosphate being present in the form of nanoclusters of an amorphous calcium phosphate (ACP) salt sequestered within the casein micelles.³⁵

Caseins were among the first proteins to be recognized as functional intrinsically disordered proteins (IDPs).³⁶ The functional role of the unfolded casein conformation was first thought to be ease of digestion by proteases. An additional role was later proposed in which the unfolded conformation was required for effective binding to the nanoclusters of calcium phosphate.^{37,38} Many other proteins associated with mineralization are now known or predicted to be IDPs.^{39,40}

We have identified four different linear functional motifs in casein sequences:⁴¹⁻⁴³ (i) an N-terminal hydrophobic signal sequence, (ii) between zero and six phosphate centers comprising a cluster of (predicted) phosphorylated residues, (iii) a number of hydrophilic sequences flanking the phosphate centers to form the calcium phosphate binding motif and (iv) one or two longer, polar tract^{44,45} sequences, rich in Pro and Gln (PRO-GLN-rich), which are involved in casein-casein non-covalent binding.

Based on their recently discovered biology and the explosive growth in understanding of other IDPs, we have proposed responses to some of the most perplexing basic questions about caseins which hitherto were unanswered.^{38,41-43} For example, why is casein not rich in essential amino acids? Why in milk is the micelle always formed by a mixture of caseins? Why are casein micelles necessary? Why is milk remarkably stable compared to other biofluids? How did milk evolve from a dilute precursor biofluid into a concentrated nutritional resource? In providing answers to each of these questions, we were informed by our findings of close functional similarities between caseins and sHsps.

Intra- and inter-molecular interactions in caseins

Caseins do not form condensed structures like globular proteins but they readily associate with themselves or other caseins to form amorphous aggregates or interact promiscuously with many other partly unfolded proteins. In their associated states, there is no evidence of the caseins adopting a more compact conformation. Indeed, the highly expanded structure of the monomeric caseins appears to be retained. A detailed molecular explanation of casein association is not to hand but significant factors include^{38,41-43} (i) relatively uncommon but invariably intermolecular disulfide bonds, (ii) polar tract interactions through the Pro,Gln-rich sequences in which hydrogen bonding by the backbone is more important than the hydrophobic interactions required for a condensed fold, (iii) prolines in the polar tracts inhibit condensation because they are difficult to de-solvate and they restrict the backbone chain dihedral angles; Pro-rich sequences, in consequence, tend to form highly hydrated expanded structures such as gels, mucus and slime.^{46,47} The strength and extent of intermolecular interactions can be fine-tuned by PTMs involving, for example, proteolytic processing, covalent modifications such as phosphorylation and glycosylation and modulation of electrostatic interactions through pH, ionic strength and the binding of metal ions such as Ca^{2+} .

Caseins stabilize milk against calcium phosphate precipitation

In most species, milk contains high calcium and phosphate concentrations that would lead to precipitation of calcium phosphate if it were not for the action of caseins. Instead of forming a precipitate, nanocluster complexes of an amorphous form of calcium phosphate are formed with the caseins creating a thermodynamically stable biofluid.⁴⁸ The equilibrium size of the nanoclusters can be expressed in terms of the free energy of forming the core of calcium phosphate and the free energy of sequestration by the outer shell of caseins.⁴⁹ In a typical casein micelle of 100 nm radius, there are about 800 nanoclusters that give rise to the characteristic substructure of casein micelles on a scale of 18 nm.⁵⁰⁻⁵²

Caseins act as molecular chaperones and can form amyloid fibrils

Caseins are promiscuous chaperones that stabilize a wide range of target proteins,^{53,54} including proteins destabilized by various types of stress (reviewed in^{41,55}). Moreover, caseins prevent amyloid fibril formation by proteins, including $\alpha_{\text{S}2}$ - and κ -caseins,⁵⁶⁻⁵⁸ ovalbumin⁵⁹ and the amyloid- β peptide.^{60,61} Like sHsps, they do not require ATP and cannot refold proteins into their native conformation.

Caseins from all species examined contain sequences (steric zippers⁶²) compatible with the cross- β structures found in amyloid fibrils.⁶³ After taking PTMs into account, the zipper

sequences are found in the Pro-Gln-rich polar tract sequences.³⁸ Amyloid fibril formation has been studied only with bovine caseins but the presence of casein amyloid structures (mammary corpora amylacea) in the mammary glands of other species suggests that the phenomenon is general. Among the bovine caseins, only purified κ - and α_{S2} -caseins form amyloid fibrils under physiological conditions⁵⁶⁻⁵⁸ but amyloid formation by either is slowed or halted by the chaperone action of any of the other caseins.^{57,58,64}

Proteostasis of caseins in bovine milk

Females of most species go through repeated cycles of pregnancy, lactation, involution and tissue remodeling during their reproductive life. Proteostasis is important because the proteins and milk salts are secreted into the cisterns and ducts of the mammary gland at high concentrations where they may be stored for hours, days, weeks, or even months before parturition, depending on the reproductive strategy of the species.⁶⁵ The avoidance of pathological processes is essential at all stages of the cycle because they would endanger not just the neonate(s) of a single cycle but the future reproductive success of the mother.³⁸ The challenges of pathological calcification and amyloidosis have been overcome in large part by the organization of caseins into the casein micelle.^{38,41} The micelle is an amorphous aggregate that is formed by the promiscuous interactions of the polar tract sequences from three or more caseins. It provides an effective means of controlling casein fibril formation and, through its calcium phosphate binding motifs, an effective means of controlling pathological calcification.

Farrell et al. noted⁶⁶ that for the casein micelle to achieve its functions, a number of necessary types of interaction must be facilitated along the physiological pathway, whereas interactions favoring dysfunctional pathways; for example, those leading to the formation of amyloid fibrils or different amorphous structures, or proteolysis, must be avoided. Figure 3 shows a central chemical equation in which the four caseins of bovine milk associate together and bind to and sequester nanoclusters of ACP to form a thermodynamically stable solution of casein micelles. The off-axis reactions are deleterious but all are normally inhibited. For example, precipitation of calcium phosphate is prevented by having an excess of competent, sequestering, α_{S1} -, α_{S2} - or β -caseins. Amyloid formation by α_{S2} - and κ -caseins is prevented by the chaperone action of the other caseins. Formation of an amorphous precipitate of casein is prevented by the chaperone action of a mobile sub-fraction of caseins not bound to the calcium phosphate; in normal bovine milk the mobile fraction is largely κ -casein. The casein micelle, however, provides no defense against premature proteolytic degradation. Premature proteolysis is limited because the main indigenous milk proteinase, plasmin, is overwhelmingly present as plasminogen.⁶⁷ It becomes physiologically activated during involution or pathologically activated through mastitis.

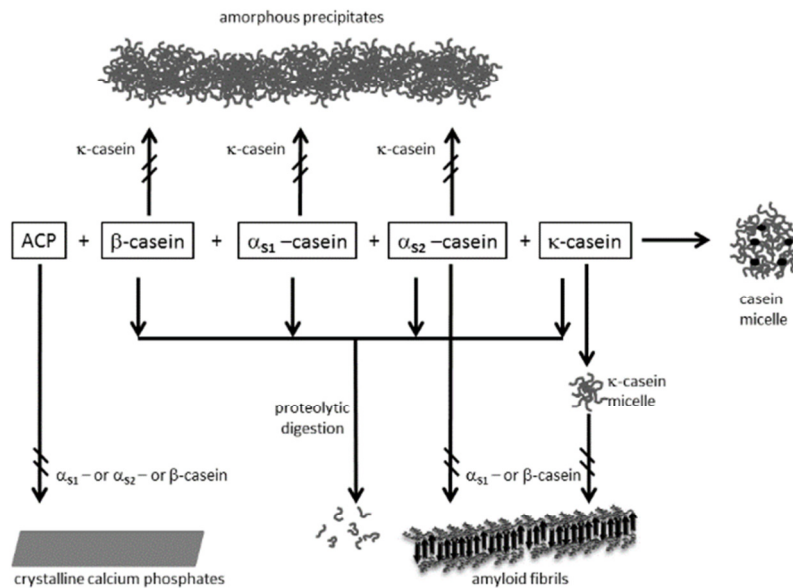


Figure 3. The central reaction is the functional formation of casein micelles whereas the off-axis reactions lead to dysfunctional outcomes such as pathological calcification, premature proteolysis, uncontrolled amorphous aggregation or amyloid fibril formation. Side reactions are inhibited by interaction with the relevant casein as indicated by a double strike through the arrow.

Comparison of caseins, α -crystallins and other sHsps

We have analyzed the amino acid composition of caseins, α -crystallins and other sHsps using the IDP disorder propensity scale^{68,69} in a plot against the fractions of positively and negatively charged residues, called the protein diagram of states^{36,70} (Figure 4). All of the caseins lie close to the nominal boundary between folded and unfolded states in a narrow region around the plane of electrical neutrality. No allowance has been made for the effect of phosphorylation which would tend to move the caseins into, or further into, the disordered domain and away from the plane of electrical neutrality. More sophisticated prediction methods based on sequences rather than just composition, confirm experimental findings that bovine caseins are IDPs.^{39,71,72} The α -crystallins from six species and the eight other human sHsps occupy a very similar region of the diagram of states to the caseins even though these groups of proteins show little or no sequence similarity, have apparently very different biological functions and no evident evolutionary relationship.

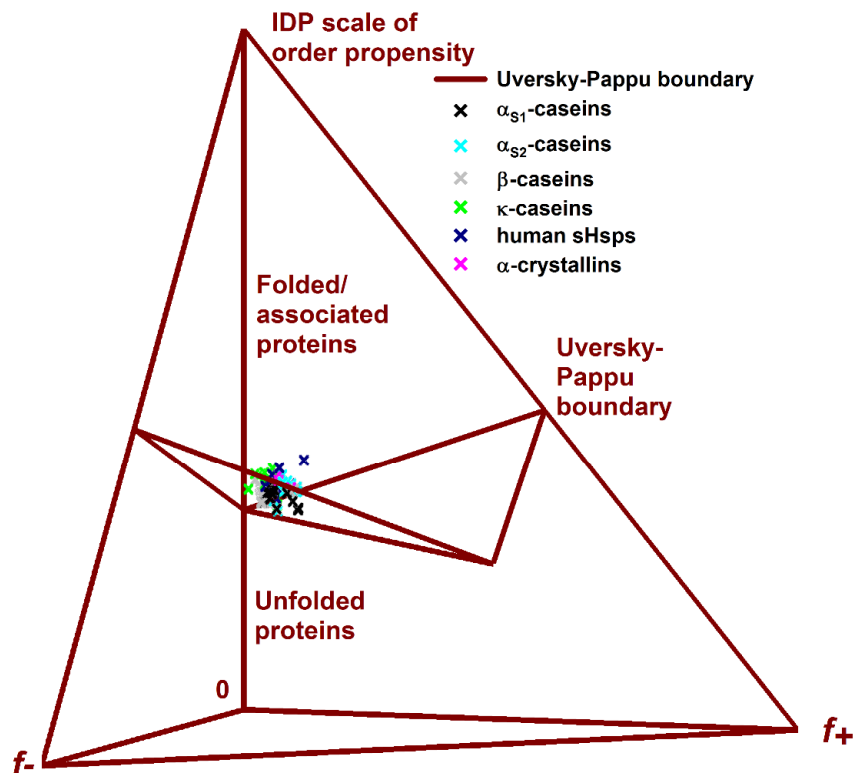


Figure 4. Protein diagram of states showing the fractions of positive (f_+) and negative (f_-) residues versus order propensity in unphosphorylated caseins, α -crystallins and human sHsps. The Uversky-Pappu boundary that separates unfolded (IDPs) from folded (globular) proteins is shown. The boundary intersects the faces of the pyramid to form a butterfly-like outline. In the right-angled pyramid, all the axes are of unit length with their origin at zero. Casein sequences are the mature forms from 20 species, but without other PTMs.⁴³ The α A- and α B-crystallin sequences are from the ExPASy Bioinformatics Resource Portal with accession codes: human α A P02489 (HSPB4), human α B P02511 (HSPB5), rat α A P24623, rat α B P23928, cow α A P02470, cow α B P02510, pig α B Q7M2W6, chicken α B Q05713 and orangutan α B Q5R9K0. The eight remaining human sHsp sequences are HSPB1 P04792, HSPB2 Q16082, HSPB3 Q12988, HSPB6 O14558, HSPB7 Q9UBY9, HSPB8 Q9UJY1, HSPB9 Q9BQS6 and HSPB10 Q14990.

Conclusions: caseins and sHsps have compositional and functional similarities.

1. Each group has extensive conformational disorder. For example, the disordered N- and C-terminal regions of sHsps are proposed to interact with partially unfolded target proteins during chaperone action, which provides a local crowded environment for the target proteins to facilitate their return to the native state.⁹ Casein polar tracts, likewise, bring the individual caseins together and may create conditions of local crowding of calcium phosphate binding motifs allowing them to form complexes with the nanoclusters.

2. Both groups exist as large, heterogeneous oligomers with inherent dynamism. Subunit exchange is a feature of sHsp chaperone mechanism and caseins in the micelle not bound to the nanoclusters are in dynamic exchange with the surrounding serum and interact with their target and/or partner casein proteins. The chaperone action of caseins and sHsps inhibits both amorphous and amyloid fibrillar aggregation.
3. Caseins and sHsps undergo extensive PTM, for example phosphorylation and truncation, to modify their function and enhance their contribution to proteostasis within their particular environment.
4. Caseins and sHsps contain steric zipper sequences. In sHsps, they are largely confined to the ACD.⁹ In caseins, they are mostly in the polar tracts.
5. In crowded conditions, caseins and crystallins both form gels but of very different appearance. Caseins form gels that are invariably white in appearance, whereas those formed by crystallins are transparent although, with crystallin aggregation, they can become opaque. The structural inhomogeneity in casein gels that causes the white appearance may arise from the contrast between the highly hydrophilic regions responsible for calcium phosphate sequestration and the less hydrophilic (but not hydrophobic) polar tracts.

In summary, caseins and sHsps exhibit markedly similar structural and functional characteristics despite their lack of evolutionary relationship. As a result, via a similar mode of action, they efficiently accomplish their proteostatic roles within their different cellular locales.

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Heath Ecroyd completed his B.Sc.(Honours) and Ph.D. at the University of Newcastle (Australia). He undertook a post-doctoral fellowship at the Institut National de la Recherche Agronomique, France and a Peter Doherty Fellowship from the National Health and Medical Research Council, working with John Carver at UoA on sHsps. In 2009, Heath took up a Lectureship at UoW, where he investigates protein aggregation and molecular chaperones.

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