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Minireview

Equine lysozyme: The molecular basis of folding, self-assembly and innate amyloid toxicity

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Abstract **Calcium-binding equine lysozyme (EL) combines the structural and folding properties of c-type lysozymes and α-lactalbumins, connecting these two most studied subfamilies. The structural insight into its native and partially folded states is particularly illuminating in revealing the general principles of protein folding, amyloid formation and its inhibition. Among lysozymes EL forms one of the most stable molten globules and shows the most uncooperative refolding kinetics. Its partially-folded states serve as precursors for calcium-dependent self-assembly into ring-shaped and linear amyloids. The innate amyloid cytotoxicity of the ubiquitous lysozyme highlights the universality of this phenomenon and necessitates stringent measures for its prevention.**

Keywords: Lysozyme; Molten globule; Calcium-binding; Amyloid; Oligomers; Cytotoxicity

1. Introduction

EL occupies a special position within the extended family of structurally homologous proteins – lysozymes and α-lactalbumins. These are among the most studied objects providing a wealth of information on the mechanisms of structural organization, folding and amyloidogenicity of polypeptides. EL is viewed as an evolutionary link between them, as it possesses the structural and folding features of both subfamilies [1,2]. Specifically, it contains the active-site residues Glu 35 and Asp 52, involved in lysozyme enzymatic (EL) activity [3,4], and the conserved, high-affinity calcium-binding site of α-lactalbumins [5] (Fig. 1). Consequently, EL acts as a bacteriolytic enzyme similar to lysozymes, ubiquitous components in the many body fluids and tissues of all mammalians, while α-lactalbumins take part in lactose biosynthesis in the mammary glands [5]. Due to EL's extremely low stability and cooperativity compared to non-calcium-binding c-type lysozymes, it forms equilibrium partially folded states similar to α-lactalbumins [6–8]. However, like c-type lysozymes it populates well-defined transient kinetic intermediates [9], which enables us to compare the equilibrium and kinetic species of the same protein. EL can also self-assemble into amyloids with a very distinctive ring-shaped and linear morphology [10,11]. They differ from the fibrils of both lysozymes involved in systemic amyloidoses [12,13] and α-lactalbumins, forming amyloids in vitro [14]. Here we review the EL folding, misfolding and amyloid properties in view of their general implications for understanding the underlying mechanisms and causes of these processes.

2. Calcium-binding property

Lysozymes/α-lactalbumins consist of two sub-domains (Fig. 1): the α-domain is rich in α-helical structure, while the β-domain contains a triple-stranded β-sheet and several loops. They possess also a conserved pattern of four disulfides. The EL calcium-binding property was initially predicted [15], since in the domain interface it has the same calcium-binding ligands as α-lactalbumins (Fig. 1). Then the binding constant of $2 \times 10^6\ M^{-1}$ was determined experimentally [1] and the structure of the binding-site confirmed by crystallographic and NMR structural analysis [2–4]. The presence of the calcium-binding site most likely contributes to EL's significantly lower stability and cooperativity compared to c-type lysozymes [6–9]. If hen and human lysozymes undergo highly cooperative two-state thermal denaturation above 70 °C, and even the amyloidogenic variants of human lysozyme are destabilized by only ca. 12 °C [16,17], then, by contrast, apo-EL displays a three-state transition in a wide range between room temperature and 80 °C (Fig. 2a) [18,19]. The first denaturation transition is calcium-dependent and leads to molten globule formation; upon calcium-binding it is shifted to significantly higher temperatures. EL's unfolding behaviour, however, cannot be described by two-state model even at very high calcium content. Similarly, the three-state unfolding of EL was observed upon chemical denaturation [7]. The thermodynamic behaviour of EL within the first thermal unfolding transition was described by a four-state scheme [18], where N and I correspond to apo-native and apo-intermediate states, and NM and IM – to holo-forms, respectively.

$$\begin{array}{ccc} & \alpha(T) & \\ N & \leftrightarrow & I \\ + & & + \\ M & & M \\ K_n(T) \updownarrow & & \updownarrow K_u(T) \\ NM & \leftrightarrow & IM \\ & \beta(T) & \end{array} \qquad (1)$$

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Abbreviations: EL, equine lysozyme

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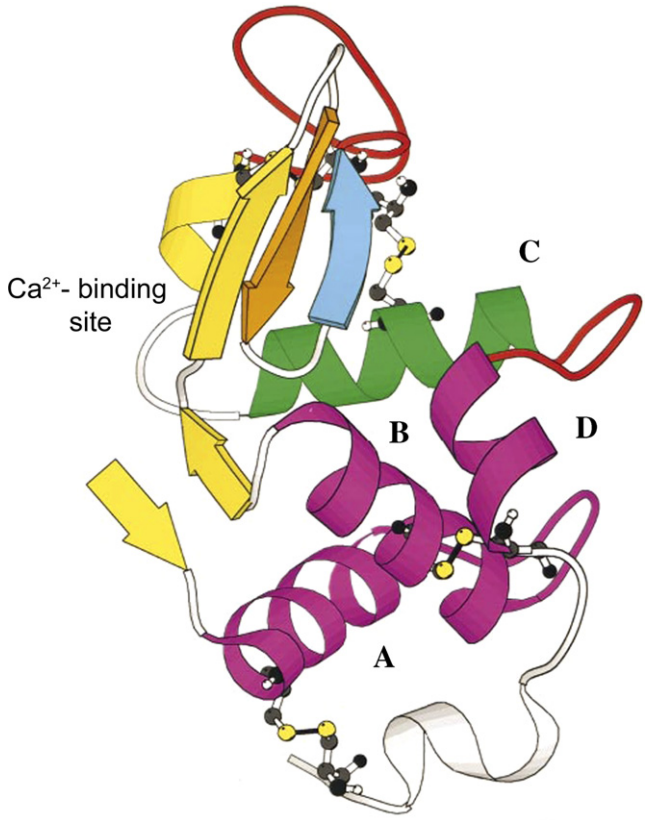

Fig. 1. Schematic diagram of EL demonstrating the folding areas with local cooperatively [9]. They are colour-coded as follows: violet, corresponding to the rapidly folded A, B and D α-helical core, blue – the 59–61 residue β-strand stabilized with 50–100 ms time constants, green-the C-helix, 120–150 ms time constants, yellow – the elements of secondary structures attaining persistent structure with 200–250 ms time constants, orange – the middle 51–55 residue β-strand, ca. 400 ms time constants, and red, denoting the most slowly stabilized loops. The disulphide bridges are shown in ball-and-stick.

Determining all thermodynamic parameters of the scheme, the "phase-diagram" was build, visualizing the population of EL conformations in the coordinates of free-calcium concentration and temperature. The "phase diagrams" of EL and α-lactalbumin both share a similarity (Fig. 2b) [18,20], indicating that stabilization of the structure around the calcium-binding site and domain interface is critical for both proteins.

3. EL structural characterization

The EL crystal structure has been determined at a 2.5 Å resolution, demonstrating a structural homology with lysozymes and a calcium-binding loop similarity to α-lactalbumins [4]. The ^{1}H NMR spectrum of holo-EL shows the high degree of resonance dispersion characteristic of lysozymes [3,21]. Backbone chemical shifts as well as sequential and long-range NOEs closely resemble those of human and hen lysozymes, providing evidence of the similarity between their solution structures. As in crystallographic studies the major chemical shift deviations were observed for the residues located in the calcium-binding loop [3,21]. The calcium-binding site was analyzed by ^{113}Cd and ^{43}Ca NMR, confirming its match with those of bovine and human α-lactalbumins [22,23].

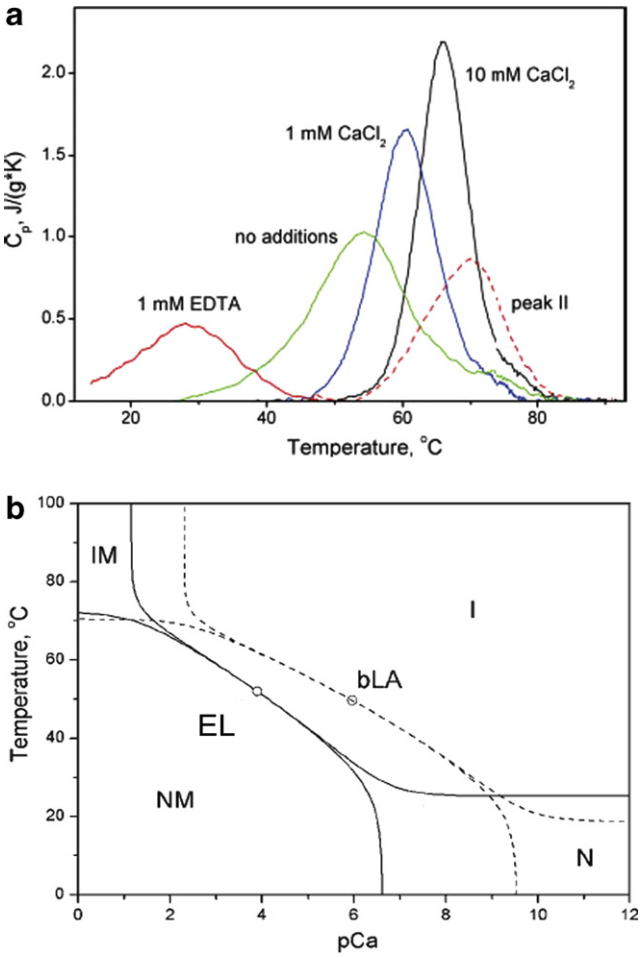

Fig. 2. (a) Specific heat capacity of EL at various calcium concentrations [18]. Concentration of calcium was controlled by adding 1 mM EDTA (red), 1 mM $CaCl_2$ (blue), 10 mM $CaCl_2$ (black) or no additives in control measurements (green). (b) Phase-diagrams of EL (solid lines) and bovine α-lactalbumin (dashed curve); EL states are denoted as in Eq. (1).

The amide hydrogen exchange protection measurements of holo-EL, probing both the structure and dynamic of molecule, show a pattern similar to those of human and hen lysozymes, i.e. the protection of amides in all major secondary structure elements [21]. However, the protection factors are on average 600-fold lower than in human and hen lysozymes. This correlates well with the EL's lower stability [6–8,18] and with the thermodynamic calculations of the protein equilibrium folding pathways [24].

4. The origin of molten globule stability

The molten globules of α-lactalbumins were the earliest discovered and most studied partially folded states [25,26]. Classic molten globules are compact states with native-like secondary structures, but with a few tertiary contacts compared to native proteins. Understanding the differences between the molten globule and native states is critical for clarifying the mechanisms of protein folding and stability. Most lysozymes do not readily form equilibrium molten globules, but EL is a notable exception, as it enters into various molten globule