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A zinc complex of heparan sulfate destabilises lysozyme and alters its conformation

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ABSTRACT

The naturally occurring anionic cell surface polysaccharide heparan sulfate is involved in key biological activities and is implicated in amyloid formation. Following addition of Zn–heparan sulfate, hen lysozyme, a model amyloid forming protein, resembled β-rich amyloid by far UV circular dichroism (increased β-sheet: +25%), with a significantly reduced melting temperature (from 68 to 58 °C) by fluorescence shift assay. Secondary structure stability of the Zn–heparan sulfate complex with lysozyme was also distinct from that with heparan sulfate, under stronger denaturation conditions using synchrotron radiation circular dichroism. Changing the cation associated with heparan sulfate is sufficient to alter the conformation and stability of complexes formed between heparan sulfate and lysozyme, substantially reducing the stability of the protein. Complexes of heparan sulfate and cations, such as Zn, which are abundant in the brain, may provide alternative folding routes for proteins.

1. Introduction

The formation of amyloid fibrils is an intrinsic property of many proteins [1], some of which are implicated in amyloid conditions including Alzheimer's disease (AD), congestive heart failure and Parkinson's disease (PD). Whether fibril formation is a harmful event *per se* remains unclear; a benign role has been proposed [2] but, increasingly, cytotoxicity is being attributed to soluble oligomers rather than fibrils [3,4]. Protein folding in general may occur *via* several routes which, for correctly folded proteins, converge to an active conformation [5], while amyloid formation has been described as a multiple-phase process [6]. Lysozyme has been proposed as a paradigm for protein folding and misfolding [7] with core structures involving amino acid regions 26–123 and 32–108 implicated in amyloid fibril formation [8]. It is also proposed that the rate of fibril formation is driven by the stability of the protein in its native state [1] but, "seeds" can promote fibril formation considerably [9], and seeding is not confined to proteins of the same sequence [9,10].

The linear, anionic, polysaccharide, heparan sulfate (HS) (Scheme 1) is the carbohydrate component of heparan sulfate proteoglycans (HSPGs), which are involved in many disease processes and interact with several amyloid-forming proteins. These include, α-synuclein, a component of Lewy bodies in Parkinson's disease (PD) and, in non-Aβ components of amyloid, the Aβ peptide fragment of APP in Alzheimer's disease (AD) as well as lysozyme, which is also implicated in amyloid disease. Heparan sulfate proteoglycans are involved in the early formation of plaques, including in AD and PD [11,12] and, indeed, are already *in situ* on cell surfaces before the onset of disease. There is a strong electrostatic component to the interaction between glycosaminoglycans (GAGs) and amyloid forming proteins [13,14]. In particular, HS, widely expressed on mammalian cells and a ligand for many proteins [15] has been proposed as a potential initiator of amyloid formation [16]. Glycosaminoglycans also promote suspected amyloid structures, rich in β-sheet, for example, in gelsolin; an ability that is related to the level of O-sulfation [14], as well as in apomyoglobin [2].

The formation of fibrils, in association with GAGs, has been under scrutiny for many years [12,17] and interactions with GAGs might provide routes to influence the formation, or stability of fibril forming proteins. Work on systematically modified heparin derivatives, which serve as models of the sulfated S-domains of HS, has established that GAG conformation is sensitive to both substitution pattern and cation binding [18], which can be highly selective for particular structural features of HS [19]. It is increasingly thought that the cytotoxic components in amyloid diseases are soluble oligomers but, it is not established whether the role of GAGs is as a participant in an undesirable process, or whether it assists in the clearance of toxic soluble oligomers. In either case, however, it will be important to understand the relationships between HS structure, its initial interactions with amyloid forming

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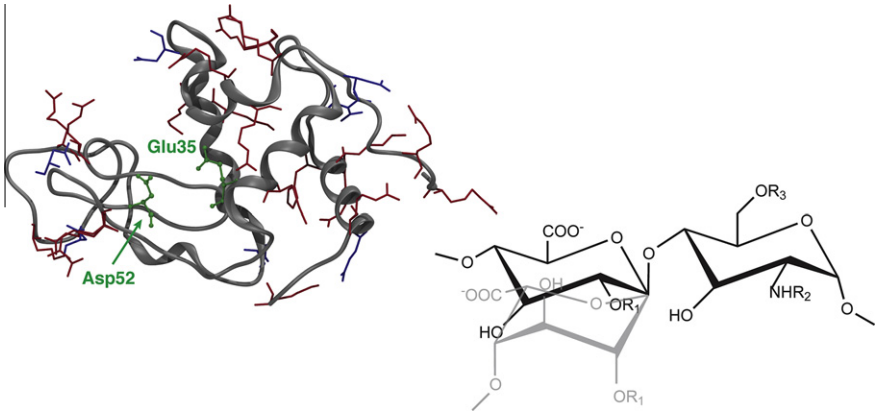

Scheme 1. Schematic of hen lysozyme (left) [41]. Amino acids Asp52 and Glu35 (green) are the primary constituents of the enzymatic site, which are involved in the hydrolysis of the β-(1 → 4) glycosidic linkage between N-Ac glucosamine and N-Ac muramic acid of bacterial cell wall polysaccharides, the natural substrate. Highlighted in red are the basic amino acids, which may interact with the acidic heparan sulfate and are clustered in three regions; left hand side of the catalytic site cleft, right hand side of the molecule near the C- and N-termini and in a cluster above amino acid Glu35. Highlighted in blue are the acidic amino acids, possible sites for the interaction with cations. Major repeating disaccharide of HS (right). Heparan sulfate is composed of a repeating disaccharide of uronic acid (glucuronic acid (black) or its epimer iduronic acid (grey)) 1 → 4 linked to N-glucosamine. The sugar can have varying substitution patterns: uronic acid position 2 (R_1)/N-glucosamine position 6 (R_3) – hydroxyl or O-sulfate, N-glucosamine position 2 (R_2) – N-acetyl, N-sulfate or free amine. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

proteins, and to evaluate the role of their associated cations. Amyloid formation in lysozyme has been associated with helix-C (residues 88–99; ITASVNCAKKIV), which contains two adjacent Lys residues, providing a potential binding site for HS [20]. Lysozyme also contains several tryptophan residues, many of which reside on the surface including either side of the substrate-binding cleft.

There is increasing evidence that cations, in particular Zn, play a role in amyloid diseases. Zinc is the most abundant trace metal in brain tissue, where concentrations are between 0.15 and 0.20 mM, which is only about six times less abundant than calcium [21] and it has also been reported that Zn ions bind to Aβ peptides [22,23].

Here, we investigate the consequences of the binding of HS and HS associated with Zn ions, to the stability and structure of hen lysozyme (Scheme 1), a proxy for mammalian lysozyme. The influence of this GAG structure and its associated cations on the surface accessibility of lysozyme was measured using a fluorescent shift assay employing the dye Sypro Orange™, which binds to the exposed hydrophobic amino-acids of proteins as they unfold, causing a change in fluorescence characteristics. A melting temperature (T_m) was derived in the absence or presence of the binding GAG [24]. Secondary structure of lysozyme and lysozyme complexed with the different forms of HS was then examined by far UV circular dichroism (CD) (185–260 nm), together with the surface binding of the HS forms to lysozyme by near UV CD (250–330 nm) spectroscopy. Circular dichroism was also used to follow the thermal- and UV-induced denaturation of lysozyme and HS complexes with lysozyme.

2. Materials and methods

2.1. Materials

Hen lysozyme was obtained from Fluka, (Sigma–Aldrich, Gillingham, Dorset, UK). Zinc–HS was prepared from HS (10 mg/ml, in deionized water), shaken with Zn-cation exchange resin (Dowex W-50, Zn form, 1 h), spun and the supernatant removed.

2.2. Circular dichroism spectroscopy

Far UV synchrotron radiation circular dichroism (SRCD) experiments (185–260 nm) were conducted at beam line B23, Diamond Light Source (0.2 mm CaF_2 (Hellma UK Ltd., Southend on Sea, Essex, UK), 20 °C). Lysozyme was used at 0.5 mg/ml (12.5 mM DPBS). HS forms were added to a final concentration of 0.08 mg/ml. Denaturation experiments (UV) were conducted using 60 scans, 0.5 mm slits, ~12 h after preparation. Near UV CD (250–330 nm) employed a Chirascan Plus spectrometer (Applied Photophysics Ltd., Leatherhead, Surrey, UK), 4 mm quartz cell (Hellma UK Ltd., Southend on Sea, Essex, UK) with lysozyme at 0.25 mg/ml (DPBS 12.5 mM) (abs. < 0.8, HT < 600 V, 1 s dwell time, 0.5 nm resolution, 2.0 bandwidth). HS samples were added to a final concentration of 0.04 mg/ml. Protein secondary structure content was determined using CDNN version 2.1 [25].

2.3. NMR spectroscopy

NMR spectra were recorded with a 600 MHz Bruker Avance III spectrometer (20 mg/ml, D_2O (0.002% TSP), 25 °C). The following pulse sequences were used: ^1H, zgcppr (32 scans, 32 k FID); HSQC, hsqcetgpsisp2.2 (16 scans, 1024 – *direct* by 320 – *indirect* points) and NOESY, noesyphpr (24 scans, 2084 – *direct* by 320 – *indirect* points).

3. Results

3.1. Interaction of Zn–HS with lysozyme induces distinct lysozyme secondary structures and altered melting temperatures

The interaction of Zn-HS with lysozyme gave rise to different melting temperatures (T_m), 57.6 (1.6) °C, to either that of HS with lysozyme, 65.3 (3.6) °C, or lysozyme alone, 67.7 (0.7) °C, as measured by the fluorescent shift assay [24] (method can be found in Supplementary Data), as well as subtly different secondary structures (Fig. 1A). The Zn-HS lysozyme (pH ~6.0) interaction resulted in changes from the original structure of lysozyme to a structure featuring an increased proportion of β-sheet (+25%) strongly resembling non-cytotoxic amyloid formed between heparin and apomyoglobin at pH 5.5 [2]. The lysozyme–HS complex exhibited a similar increase in β-sheet (+20%) (complete secondary structures can be found in Supplementary Data). The changes induced in lysozyme by the Zn-HS complex were not reproduced by Zn ions alone (Supplementary Data S1), while the addition of $ZnCl_2$ to lysozyme raised its Tm to 72.1 (0.3) °C.

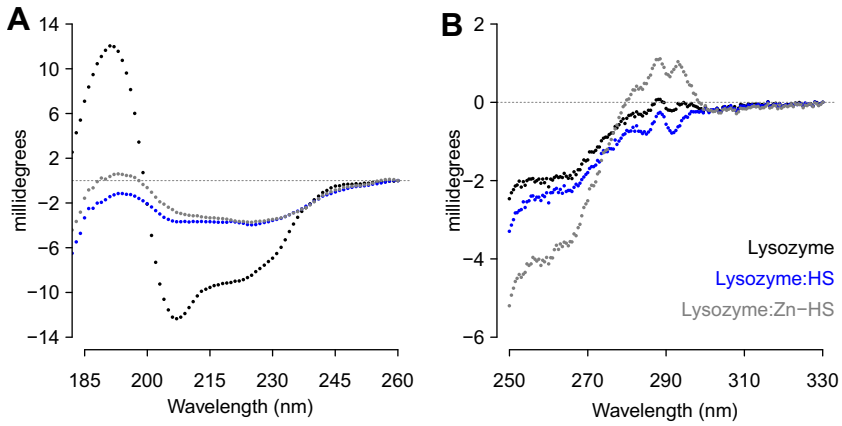

Fig. 1. (A) The Zn form of HS induces subtle secondary structural changes in lysozyme. Far UV SRCD spectra (185–260 nm) of lysozyme (0.50 mg/ml) [black], lysozyme with HS (0.08 mg/ml) [blue] and Zn–HS (0.08 mg/ml) [grey]. Major structural changes, producing similar and predominantly ordered β-sheet and unstructured regions were induced by HS and Zn–HS. (B) Zn–HS induced a distinct near UV spectrum indicating binding in and around the substrate cleft of lysozyme. Near UV CD spectra (250–330 nm) of lysozyme (0.25 mg/ml) [black], lysozyme with HS (0.04 mg/ml) [blue] and lysozyme with Zn-HS (0.04 mg/ml) [grey]. The tryptophan residues of the active site gave rise to the peak at 291 nm and the trough at 293 nm [26]. The Zn-HS form induced considerable changes in these signals compared to lysozyme alone or lysozyme with HS. Changes either side of these signals originating from tryptophan residues elsewhere on the protein surface were also evident with Zn–HS. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Near UV CD confirmed interactions between Zn–HS and lysozyme and demonstrated interactions with residues both inside and outside the active catalytic site of lysozyme

Near UV CD (330–250 nm) of lysozyme reports CD signals from a series of tryptophan residues on the surface of the protein and are either in the catalytic active site, comprising a peak at 291 nm and a trough at 293 nm, or elsewhere on the surface, and these appear either side of these spectral features [26]. Analysis of the spectral features provides details about the interaction of lysozyme with polysaccharides. The Zn-HS polysaccharide caused significant changes in the tryptophan signals arising from the active site, and from the other surface tryptophan residues, compared to lysozyme alone or lysozyme with native HS (Fig. 1B). The near UV SRCD spectra of lysozyme alone and lysozyme bound to HS are relatively similar, despite their resulting secondary structures being clearly distinct (Fig. 1A). The spectrum of HS and lysozyme exhibited a more pronounced peak at 291 nm and trough at 293 nm, as well as slight differences elsewhere, indicating that HS binds both inside and outside the substrate cleft of lysozyme. The near UV CD spectrum of the lysozyme:Zn-HS complex is distinct to those of lysozyme alone and lysozyme bound to HS, despite the similar secondary structures exhibited by lysozyme:HS and lysozyme:Zn-HS.

3.3. The susceptibility of the complex formed between Zn–HS and lysozyme to denaturation by UV radiation was distinct to those of HS bound to lysozyme and lysozyme alone

The susceptibility of the HS–lysozyme complexes to denaturation by far UV radiation was followed over 60 scans using far UV SRCD (Fig. 2) and showed subtle differences, confirming that the complexes were distinct. The Zn–HS interaction with lysozyme destabilised the protein (Tm decreases by ~10 °C) when measured using the fluorescent shift assay. Employing the more extreme UV denaturation confirmed that Zn–HS had a different effect on the stability of lysozyme to that of HS. These results indicated that the form of lysozyme induced by Zn–HS, had a less stable surface structure while, its core secondary structure is comparable in stability to that induced by HS but, is structurally distinct (Fig. 2). The combined results confirm that Zn–HS binds to several locations on the surface of lysozyme, and this causes some major changes in secondary structure, in addition to the melting temperature being significantly lowered. Principal component analysis (PCA) of the UV denaturation spectra suggests that denaturation is directly proportional to the number of times the sample is scanned (Fig. 2D).

Lysozyme alone exhibited the most resistant structure to thermal denaturation, as determined by measuring the far UV SRCD spectra over a range of temperatures (10–85 °C in 5 °C steps, with 5 s increments) (Fig. 3). It retained its secondary structure until 60 °C (green line) before rapidly losing its integrity to a more disordered structure as the temperature increased. This was illustrated by principal component analysis (PCA) (Fig. 3D) that showed clearly two structurally distinct groups diverging between 65 and 70 °C. Lysozyme:HS appears to be less stable, because after 30 °C, secondary structure is gradually lost with each increase in temperature. The lysozyme:Zn-HS complex does not appear to offer a defined barrier to thermal denaturation and the transition from starting structure to disordered proceeds smoothly (Fig. 3D). This result is consistent with the significantly reduced stability observed in the fluorescence shift assays.

3.4. The interaction of Zn with HS

The interaction of Zn with HS was primarily through iduronic acid (IdoA), with differences observed at position-5 and -4 of IdoA, position-2 of 2-O-sulfated (2S-) IdoA and position-1 of de-2-O-sulfated (de-2-S) IdoA. This suggests that the ion prefers to interact with the acid group of IdoA, as signals associated with 2S- and de-2-S-IdoA were affected. Heparan sulfate heterogeneity, due to the high percentage of N-acetylation (~50%) and unmodified (NH_2, ~22%) position-2 of glucosamine (GlcN), made measuring NOEs difficult due to overlapping signals, so no conformational information could be extracted. To circumvent this problem HS was replaced by the more homogeneous bovine lung heparin (BLH). Zinc interacted with BLH in a similar manner to HS, with signals from IdoA position-5, 2-S-IdoA position-1 and -2 and de-2-S-IdoA position-1 being modified.

NOESY spectra were recorded for BLH and a 20:1 (Zn–BLH) molar equivalent; Zn–BLH exhibited no difference in NOE values across the glycosidic linkages, hinting that Zn binds to heparin or HS in a similar fashion to Na and unlike other transition metals such as Cu which, when coordinated with GAGs, causes a conformational change [19].

Coupled-HSQC spectra were recorded to investigate whether Zn caused conformational changes in the IdoA residues of BLH. Proton–carbon couplings were measured for position 1 2-S-IdoA, posi-

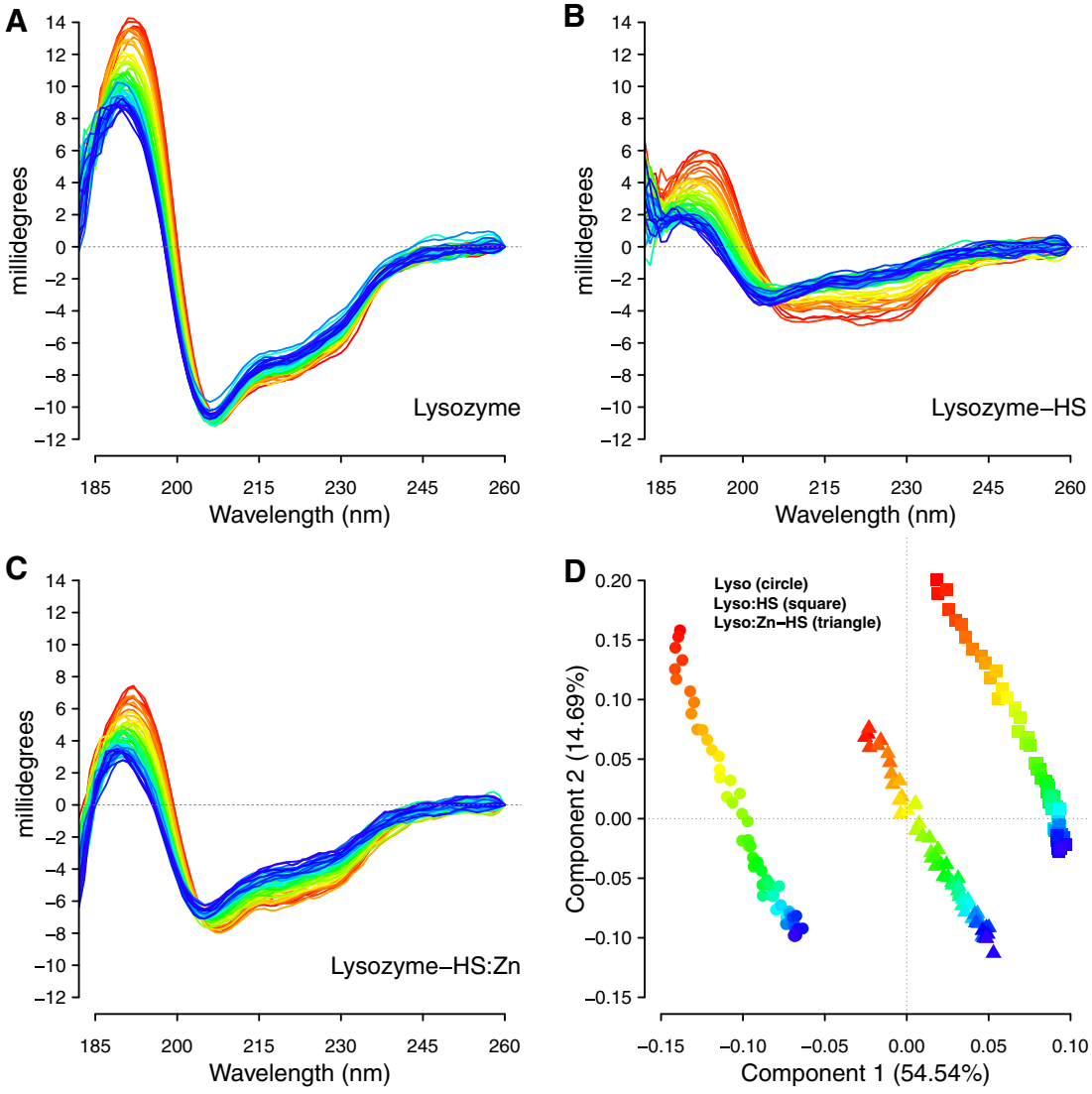

Fig. 2. Exposure of the lysozyme and lysozyme complexes to prolonged UV radiation resulted in denaturation confirming that protein secondary structures in lysozyme and complexes of lysozyme with native HS or Zn–HS are distinct. The stability of complexes of lysozyme alone (A) with native HS (B) and Zn–HS (C) to degradation by UV radiation was followed over 60 scans between 180 and 260 nm, which can detect the changes in secondary structure produced by UV induced denaturation. The red line indicates the starting spectrum, proceeding progressively to blue. PCA of the SRCD spectra of lysozyme and lysozyme:HS complexes undergoing UV denaturation (D). Lysozyme, free and bound, undergoes a gradual denaturation when exposed to UV radiation, the process is linear with two components describing the event.

tion 5 IdoA (2 couplings), position 2 2-S-IdoA (2 couplings), position 2 N-sulfated (NS) N-glucosamine, position 6 6-O-sulfated GlcN (2 couplings) and position 6 6-de-O-sulfated GlcN. All couplings changed on the addition of Zn to BLH apart from the proton–carbon coupling for position 2 NS-GlcN but, these changes were due to the addition of Zn and not as a consequence of a conformational change in the iduronic acid, because the ratios of the NOEs between IdoA position 5 and 2, 4 remained constant, indicating that the population of the $^{1}C_{4}$ and $^{2}S_{0}$ chair forms remained constant on the addition of Zn. NMR spectra can be found in Supplementary Data.

4. Discussion

Heparan sulfate is widely expressed on cell surfaces and in the extracellular matrices of higher organisms, including in all the organs relevant to amyloid forming diseases. HS has been identified *in vitro* as promoting Aβ formation [27,28] and the addition of the divalent cations, magnesium and calcium, were reported to inhibit interactions with heparin [29]. The binding of cations to heparin derivatives, as a proxy for HS, in copper [18], can have dramatic effects on biological activity [30].

Zinc binding to heparin (a close structural relative of HS, which resembles the more highly sulfated S-domains of HS) indicates two types of binding, one high and one low affinity [31] and appears to prefer heparin to other GAGs (although HS was not specifically tested) [32]. Heparin complexed with zinc ions promotes heparin cofactor II binding [33] and zinc binding to the beta A4 amyloid precursor protein (APP) has also been reported [34].

We show that the binding of the Zn-HS complex has clear effects on lysozyme, as a model amyloid forming protein distinct from HS or zinc ions alone. There is strong similarity between the SRCD spectrum of the Zn–HS complex with lysozyme and that observed between apomyoglobin and heparin at pH 5.5 [2]. This supports the contention that common amyloid structures can be induced in distinct proteins and under a variety of circumstances, in the present case by particular cations binding to HS at physiologically relevant pH values. It is also shown, through the addition of zinc ions alone to lysozyme, that the ion alone is not responsible for the destabilisation. In fact the opposite was

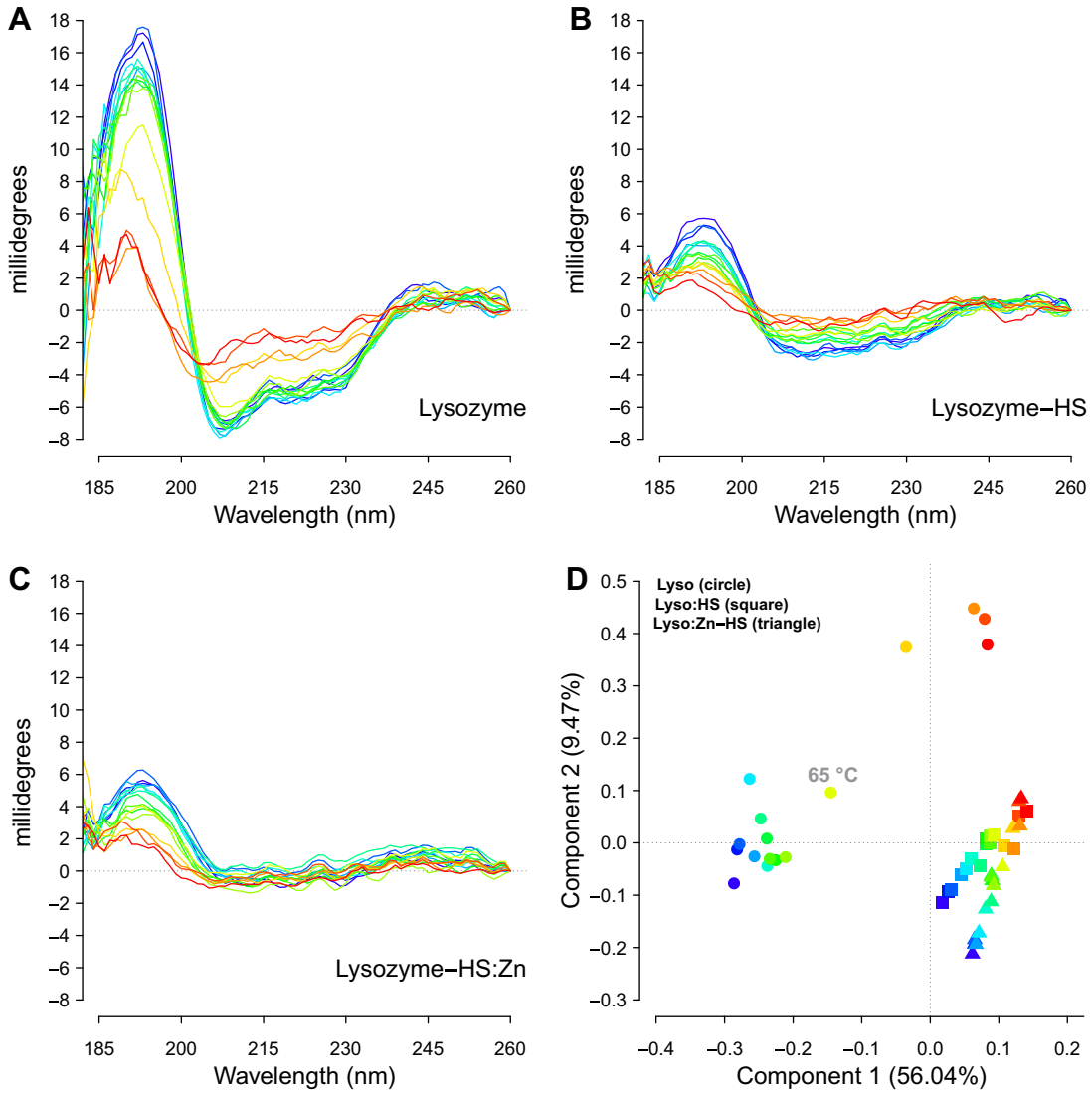

Fig. 3. Exposure of the lysozyme and lysozyme:HS complexes to a temperature gradient (A–C). The temperature ranged from 10 to 85 °C in 5 °C steps, with 5 s increments (progressively blue to red) resulting in distinct thermal denaturation profiles. PCA of the SRCD spectra of lysozyme and lysozyme:HS complexes undergoing thermal denaturation (D). Two distinct phases were revealed, for lysozyme alone, with the transition occuring between ~65 and 70 °C. This unfolding route is distinct from those evident when lysozyme was bound to HS or Zn-HS, which do not exhibit distinct phases, but a linear change of state. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

observed; the fluorescence shift assay reported a 5.5 °C stabilisation of lysozyme in the presence of zinc ions without HS.

Complexes of native HS and lysozyme or, Zn-HS and lysozyme were more fully converted to ordered β sheet, resembling non-cytotoxic amyloids [2]. Furthermore, although both HS forms seem to bind lysozyme in similar, if not identical ways according to their near UV SRCD spectra, their effects on melting temperature (measured by the accessibility to the fluorescent dye) and the stability of the secondary structures formed (far UV CD experiments) were distinct. Zinc is the most abundant of the rarer metal ions in the brain and has been associated with plaques [35,36]. Its role in amyloid diseases has been controversial although there is now considerable evidence of its involvement particularly in increased aggregation of Aβ [36,37] but, also other amyloid forming proteins [38,39]. Zinc ions are known to inhibit the binding of heparin, which shares some structural features with HS, to lysozyme [40]. It is also interesting that complexes of lysozyme and Zn-HS, although exhibiting similar secondary structure to those of lysozyme with HS, as well as similar stability to UV denaturation, had substantially reduced melting temperatures. This implies that the Zn-HS complex with lysozyme has higher surface accessibility but, similar stability for its core secondary structural elements, to the complex of HS and lysozyme. This suggests a partially relaxed structure that is susceptible to further interactions, particularly with more hydrophobic agents. The order of addition of the components appears to be vital. Here, we show that pre-formed complexes of HS with zinc interact with lysozyme, to induce structures which differ in stability and structure suggesting that GAG-cation interactions with proteins could provide an alternative route by which lysozyme can overcome the free energy barriers required to refold into other forms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.154.

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