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Salts enhance both protein stability and amyloid formation of an immunoglobulin light chain

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ABSTRACT

Amyloid fibrils are associated with sulfated glycosaminoglycans in the extracellular matrix. The presence of sulfated glycosaminoglycans is known to promote amyloid formation *in vitro* and *in vivo*, with the sulfate groups playing a role in this process. In order to understand the role that sulfate plays in amyloid formation, we have studied the effect of salts from the Hofmeister series on the protein structure, stability and amyloid formation of an amyloidogenic light chain protein, AL-12. We have been able to show for the first time a direct correlation between protein stability and amyloid formation enhancement by salts from the Hofmeister series, where SO_4^{2-} conferred the most protein stability and enhancement of amyloid formation. Our study emphasizes the importance of the effect of ions in the protein bound water properties and downplays the role of specific interactions between the protein and ions.

1. Introduction

Light Chain Amyloidosis (AL) is a protein misfolding disease caused by an abnormal proliferation of monoclonal plasma B cells that secrete free Light Chains (LC) into circulation. These free LC misfold and aggregate as amyloid fibrils in vital organs, causing organ failure and death. Somatic mutations are the most studied source of LC destabilization causing the protein to sample partially unfolded states that lead to amyloid formation. These amino acid substitutions have a global destabilizing effect, so that less energy is required to unfold the protein [1–3]. The propensity to form amyloid fibrils *in vitro* correlates with a decrease in folding stability for some AL proteins, suggesting that destabilizing interactions influence the kinetics of amyloid formation [4].

Amyloid fibril formation studies with AL proteins have been performed under non-physiological conditions, such as low pH for the variable domain (VL) protein 'SMA' and by addition of Na_2SO_4 in combination with incubation at the melting temperature (T_m) for the VL protein 'AL-09' [5–9]. Na_2SO_4 has been used to promote amyloid fibril formation with a number of amyloid-related and unrelated protein precursors, including AL-09, protein G B1 variant I6T53 and β2-microglobulin [9–11].

The determinants of amyloid formation and the role external factors play in this process are not well understood. In particular, little is known about the role and nature of the influence SO_4^{2-} plays in stabilizing folded states, folding intermediates and promoting amyloid formation. SO_4^{2-} is relevant to amyloid deposition *in vivo* because glycosaminoglycans (GAGs) are sulfated heterogeneous polysaccharides composed of repeating disaccharide units of glucuronic or iduronic acid and *N*-acetylglucosamine or *N*-acetylgalactosamine [12,13]. Several groups have shown that GAGs promote and stabilize amyloid fibril formation and deposition *in vitro* and *in vivo* [14–16]. It is thought that the multiple negative charges from the SO_4^{2-} ions present in these macromolecules are an important factor in promoting amyloid formation in the extracellular matrix [17].

In 1888, Lewith and Hofmeister published accounts of great differences between the minimum concentrations of various neutral salts required to precipitate a given protein from solution [18]. The results are compiled into what it is now known as the Hofmeister series, where various ions are ordered according to their effectiveness in promoting protein stability and solubility. The underlying concept is that interactions between biopolymers in aqueous media are mediated by water; cosolutes such as neutral salts can influence both the surfaces of the solid bodies and the structure of the water. The Hofmeister series includes kosmotropic ions that interact strongly with water and stabilize proteins by promoting stronger interactions among non-polar groups (salting-out) and chaotropic ions that destabilize proteins by breaking hydrogen bonds within the peptide groups (salting-in) [19]. Baldwin reports data from Jarvis and Scheiman [30] showing that surface tension of salt solutions follows the rank order of the Hofmeister series [19]. The surface tension increases with salt concentration. Work by Nandi and Robinson in 1972 [31], also reviewed by Baldwin showed that salting-out constants are proportional to the accessible surface area of the amino acid used. These salting-out constants are proportional to the surface tension

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increments of the salts, suggesting that these physical properties of the solutions are not protein specific. James Hallewell St. Johnston reported in 1927 that the surface tension of Na_2SO_4 albumin and caseinogen solutions changed following the same pattern [20]. $NaNO_3$ and NaCl caseinogen solutions increased surface tension as a function of salt concentration, but to a lesser extent compared to Na_2SO_4, in agreement with Jarvis and Scheiman [30]—reviewed in [19]. Baldwin concludes that Hofmeister interactions are unusual because they show a similar pattern, not only with different globular proteins but with DNA and collagen. The Hofmeister series is dominated by anions, with Cl^- having little effect even at high concentrations and being considered a null (middle) point where the opposite interactions (salting-in and salting-out) cancel out. SO_4^{2-} is among the most stabilizing of the anions described in the Hofmeister series. Experimentally, SO_4^{2-} confers thermodynamic stability in multiple systems, including AL-09 and prion proteins [9–22]. Other anions besides SO_4^{2-} have also been shown to affect the formation of fibrils for other amyloid precursors. Anions induce partial folding of the natively unfolded α-synuclein, forming a critical amyloidogenic intermediate that leads to the acceleration of the rate of fibrillation. The magnitude of acceleration correlated with the position of anions in the Hofmeister series [23]. Salts have an effect on the aggregation of Aβ(1-40) amyloid fibrils by promoting different degrees of nucleation and growth [24]. Klement et al. reported little effect of salts on the freshly dissolved unfolded Aβ(1-40) but they demonstrated strong effect on the fibrillar peptide. It is possible that salts affect amyloid precursor proteins that are natively folded in different ways that they affect small unfolded polypeptides, such as Aβ(1-40). The effect of SO_4^{2-} and other anions in amyloid precursor proteins with a folded conformation may possibly shift from salting-out stabilizing effects to salting-in destabilizing effects during the aggregation process. We are interested in understanding this role and correlating these effects to the sulfated glycosaminoglycans promoting amyloid formation.

We conducted a proof of principle study in which we tested the effect of physiologically relevant anions and cations on AL-12 protein structure, thermodynamic stability and amyloid formation. The salts tested did not affect protein structure. Thermodynamic stability and amyloid formation were enhanced according to the rankings of the Hofmeister series. The largest enhancement was seen with SO_4^{2-} anions and Mg^{2+} cations.

2. Materials and methods

2.1. Cloning/expression/extraction/purification

Patient AL-12 presented with cardiac AL. AL-12 VL sequence was previously deposited in GenBank (the first amino acid begins at nucleotide 51 and ends with nucleotide 374) with the accession number AF490912 [25]. Upon resequencing, we confirmed that codon 88 corresponded to cysteine. cDNA was created according to the methods in Abraham et al. [25]. Briefly, RNA was extracted from bone marrow and cDNA was produced by RT-PCR and cloned into the pCR®II-TOPO® cloning vector (Invitrogen, Carlsbad, CA). The DNA was then subcloned into the pET12a vector (Novagen, Madison, WI). The plasmid was transformed into BL21 (DE3) Gold competent cells (Stratagene, La Jolla, CA) and protein expression was induced with 0.8 mM IPTG (isopropyl-beta-D-thiogalactopyranoside). After 17–20 h of post induction growth, the bacteria were collected, pelleted and frozen. Protein was extracted from the periplasmic space of the bacteria with a freeze–thaw step followed by osmotic shock using 20% sucrose in 10 mM Tris HCl pH 9.0 and eluted with 10 mM Tris HCl pH 9.0. The periplasmic fraction was then dialyzed into 10 mM Tris HCl pH 7.4. The protein was purified using size exclusion chromatography (HiLoad 16/60 Superdex 75 column) on an AKTA FPLC (GE Healthcare, Piscataway, NJ). Pure fractions were checked by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Blue. A Western Blot was used to confirm the purified protein identity by using a sheep anti human kappa light chain as the primary antibody (The Binding Site Inc., San Diego, CA). The secondary antibody was a rabbit anti sheep polyclonal IgG H & L (HRP) antibody (Abcam Inc., Cambridge, MA). Protein concentration was determined by its absorbance at 280 nm, the calculated extinction coefficient and the cuvette pathlength. The extinction coefficient of 13,610 $(M\ cm)^{-1}$ was calculated using the peptide property calculator (www.basic.northwestern.edu/biotools/proteincalc.html) and the protein sequence. Pure fractions were combined, concentrated, flash frozen and stored at −20 °C.

2.2. Analytical size exclusion chromatography

The oligomeric state of the purified protein was determined on a Superdex 75 10/30 size exclusion column on an AKTA FPLC (GE Healthcare, Piscataway, NJ). Albumin, carbonic anhydrase, cytochrome *C* and aprotinin (Sigma, St. Louis, MO) were used as molecular weight standards. Each standard was prepared in 10 mM Tris HCl pH 7.4, 0.15 M NaCl. The column was calibrated with each standard at a flow rate of 0.3 ml/min. The elution volume for each standard peak was determined. Blue dextran was injected onto the column to determine its void volume, which was used to calculate the ratio of elution to void volume (Ve/Vo) for each molecular weight standard. A calibration curve was produced by plotting the logarithm of molecular weight standards as a function of their Ve/Vo and determining the line of best fit. The purified AL-12 was injected and eluted through the column at a flow rate of 0.3 ml/ min in the same buffer as the standards. After determining the Ve/Vo of AL-12, the molecular weight was calculated by using the equation for the line of best fit.

2.3. Circular dichroism spectroscopy (CD)

Secondary structure of AL-12 was measured by following the Far UV-CD spectrum (Jasco Spectropolarimeter 810, JASCO Inc, Easton, MD) from 260–200 nm, taking measurements every 1 nm with a scanning speed of 10 nm/ min at 4 °C in a 0.2 cm cuvette. Protein concentration was 20 μM. Samples were done triplicate with the average of the 3 scans reported. Individual samples for ionic strength of 1 were analyzed. Secondary structure of AL-12 ± 0.5 M Na_2SO_4 at various temperatures were followed by Far UV-CD in a 0.1 cm cuvette following the same parameters listed above. Samples were incubated at each temperature for 15 min prior to the Far UV-CD scan.

Thermal denaturation experiments were done by following ellipticity at 216 nm, from 4–90 °C every 2 °C with a temperature slope of 30 °C/h and response time of 32 s. Refolding data were acquired immediately after the unfolding curve from 90–4 °C using the same parameters listed above. Thermal denaturation data was processed following a two-state transition model. Folded and unfolded baselines were linearly extrapolated from the data with a minimum of 10 points. The fraction folded (FF) at each temperature was calculated by using the following equation: FF = (ellipticity observed − ellipticity of the unfolded)/(ellipticity of folded − ellipticity of unfolded). The T_m was calculated at the temperature in the midpoint transition where 50% of the protein was folded. Solutions of AL-12 (20 μM) in the presence of various salts (Na_2SO_4, NaCl, $MgSO_4$, $MgCl_2$, NH_4Cl, Na_2HPO_4) at a concentration of 0.5 M were prepared and allowed to equilibrate overnight at 4 °C before acquisition of CD data. Samples were done in triplicate. ANOVA single factor analysis was done using the T_m of the various salts to determine the *p*-values.

A chemical denaturation experiment was done by equilibrating individual samples of AL-12 (20 μM) with various urea concentrations (0–6 M) for 42 h at 4 °C. Parameters previously described were used to acquire the Far UV-CD spectra from 260–200 nm. The ellipticity at 212 nm was used as a function of urea concentration because it provided the greatest difference between 0–6 M urea. Each urea concentration was verified using refractometry and calculated using the following equation: $[\text{urea}] = 117.66 * \Delta N + 29.753 * B(\Delta N)^2 + 185.56 * (\Delta N)^3$, where ΔN is the difference in refraction between each sample and buffer