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Interactions between sodium oleate and α-lactalbumin: The effect of temperature and concentration on complex formation

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ABSTRACT

Complexes of α-lactalbumin and oleic acid have previously been shown to be cytotoxic to cancer cells. In this study oleic acid is replaced by the more soluble sodium oleate and complexes of α-lactalbumin and sodium oleate are formed. Dynamic light scattering results showed that there was a small linear increase in the particle size of α-lactalbumin when it was titrated with sodium oleate. The fluorescence spectra of α-lactalbumin showed a linear increase in the emission maximum when sodium oleate was added up to a molar ratio of 8–11 oleate molecules per α-lactalbumin. Differential scanning calorimetry results show that the thermal unfolding of α-lactalbumin is altered by the presence of the sodium oleate. There is a decrease in size of the endothermic peak of apo α-lactalbumin when sodium oleate is added. The temperature at which unfolding occurred decreased for both apo and holo α-lactalbumin. FTIR measurements showed no significant effect of sodium oleate in the amide I region of the α-lactalbumin spectrum indicating the presence of oleate has little or no effect on the secondary structure of α-lactalbumin. The interactions between α-lactalbumin and sodium oleate/oleic acid are pH dependent, turbidity and dynamic light scattering measurements showed that the association between the two was optimal between pH 6.0 and 8.0.

The results obtained here suggest that α-lactalbumin can bind at least a 20 fold molar excess of oleate, most likely in a non-specific manner.

1. Introduction

The formation of a bioactive complex between α-lactalbumin (α-la) and oleic acid known as BAMLET/HAMLET (Bovine/Human α-la made lethal to tumours) has been studied for over a decade now (Svensson, Hakansson, Mossberg, Linse, & Svanborg, 2000). HAMLET/BAMLET has been shown to be cytotoxic to a range of cancer cell lines at levels where healthy cells are unaffected, the properties and action of the complex have been extensively reviewed (Mok, Pettersson, Orrenius, & Svanborg, 2007; Mossberg, Hun Mok, Morozova-Roche, & Svanborg, 2010).

Several chromatographic methods have been developed to form the complex (Brinkmann et al., 2011; Svensson et al., 2000). The chromatographic methods used for the preparation of HAMLET/BAMLET involve preconditioning an ion-exchange column with oleic acid and subsequently passing apo α-la through the column. The active complex can be eluted using a salt gradient. Oleic acid is insoluble in water so it is dissolved in ethanol to facilitate application to the column. It is worth noting however that the buffers used for the chromatography are all above the reported pKa of oleic acid, 7.6 (Hamilton & Cistola, 1986). This means that the majority of the oleic acid may be in the charged oleate form on the column.

The formation of HAMLET/BAMLET has been attributed to a misfolding of α-la and has been purported to be an example the beneficial misfolding of proteins (Svensson et al., 1999). However, α-la which has been extensively heat denatured still forms tumoricidal complexes (Liskova, Kelly, O'Brien, & Brodkorb, 2010). Furthermore, two other proteins have been used to form cytotoxic complexes; equine lysozyme (Wilhelm et al., 2009), which is structurally similar to α-la, and bovine β-lactoglobulin (Liskova et al., 2011), which while being a globular whey protein has very little structural similarity to α-la. Peptides of α-la also form cytotoxic complexes with oleic acid (Tolin et al., 2010). Recently it has been proposed that oleic acid is the cytotoxic component of these complexes (Permyakov et al., 2012).

The mechanism of BAMLET action on cancer cells has been widely studied as has the protein component of the complex. In light of the findings outlined above, interest in the oleic acid portion of the complex has increased recently. It had been shown that simply

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mixing α-la and oleic acid would not form complexes with cytotoxicity comparable to HAMLET/BAMLET (Svensson et al., 2000, 2002). More recently, several papers have demonstrated that it is possible to form cytotoxic complexes by mixing the components under the correct conditions of pH and temperature (Kamijima et al., 2008; Knyazeva et al., 2008). Kamijima et al. (2008) mixed α-la with a 120 fold molar excess of oleic acid (14 mM). To form the molten globule of the protein the solution was heated at 50 or 60 °C, excess oleic acid was removed by centrifugation. Knyazeva et al. (2008) formed complexes by titrating α-la in solution at pH 8.3 with oleic acid. The concentrations of oleic acid used in the study were below the critical micelle concentration (cmc); the hypothesis behind using the lower concentrations of oleic acid was that structures formed by the oleic acid at higher concentrations would prevent its interaction with the α-la to form a complex. A greater number of oleic acid molecules bound to α-la at higher temperatures, 2.9 at 17 °C vs. 9 at 45 °C. Agger and Bro (2009) formed cytotoxic complexes by mixing oleic acid and α-la under high shear conditions. Permyakov et al. (2011) titrated α-la at 45 °C with oleic acid. The titrations were made in a buffer at pH 12.0 under these conditions the oleic acid is in its deprotonated form, oleate. The study found that up to 34 oleic acid molecules could be bound to the α-la. This is much greater than any values previously reported for BAMLET/HAMLET.

These approaches demonstrate different means to overcome the two central issues in forming complexes between α-la and oleic acid: firstly the α-la protein needs to be partially unfolded and secondly the insoluble oleic acid had to be dispersed and brought into contact with the α-la. The solubility of oleic acid in water is low. In the chromatographic methods it is solubilised in ethanol before being applied to the column where it is dispersed over the resin to make it accessible to α-la. By mixing the oleic acid with the protein at high pHs the oleic acid is deprotonated and more soluble. A method has been developed which uses more soluble sodium oleate (NaOle) to for the formation of complexes (Brodkorb & Liskova, 2010; Liskova et al., 2011).

To unfold the protein two approaches have been used; in the methods using a column, Ca is removed from α-la using a chelating agent, such as EDTA, causing the protein to partially unfold. An alternative method is to unfold the protein by heating; this approach has been used in methods where the two components of the complex are mixed together. The quantity of oleic acid bound to the protein has also been widely debated; initially it was thought that it was a one to one ratio (Svensson, Mossberg, Pettersson, Linse, & Svanborg, 2003). However, more recent studies have shown that the ratio is in fact much higher (Brinkmann et al., 2011; Pettersson-Kastberg et al., 2009). Tolin et al. (2010) showed that oleic acid could bind to several different peptides from α-la, meaning that there could be several different binding sites within α-la for oleic acid.

To date there is limited information on the interaction between NaOle and α-la and the stability of complexes formed between the two. This paper will investigate the interaction between NaOle and α-la as a function of temperature and NaOle concentration.

2. Material and methods

α-La (95% purity, approximately 3% β-lactoglobulin) was sourced from Davisco Foods International (Eden Prairie, Mn, USA). NaOle (≥95 purity) and phosphate buffered saline (PBS) was from Sigma Aldrich (Arklow, Ireland).

2.1. Dynamic light scattering: titration of protein with Na oleate

An α-la solution 424 μM (6 g L^{-1}) was prepared in a 2× concentration of PBS (pH 7.4). A stock solution of 10 mM NaOle was prepared in milliQ® water. Various quantities of the NaOle solution was added to 0.5 mL aliquots of the protein solution and the final volume was adjusted to 1.0 mL, giving solutions containing 212 μM (3 g L^{-1}) α-la containing a molar excess of NaOle ranging from 0 to 20 in a PBS solution. The hydrodynamic diameter of the aggregates was determined using a Zetasizer Nano system (Malvern Instruments Inc., Worcester, UK). The measurements were carried out at 25 °C measuring the backscatter at 173°. The cumulative method was used to find the mean size of a particle that corresponded to the mean of the volume distribution. The viscosity of the solvent was assumed to be the same as water, given the low concentration of protein. The size of the aggregates present in NaOle solutions in the same concentration range was also measured.

2.2. Change in particle size during heating and protein refolding

A solution containing 3 g L^{-1} α-la and 1.06 mM NaOle (5 fold molar excess) was prepared as outlined above. The particle size of solution was measured at 5 °C temperature increments from 20 to 60 °C. The cuvette containing 1 mL of the mixture was equilibrated for 2 min at each temperature prior to measuring the particle size as outlined above. A control solution containing 3 g L^{-1} was subjected to the same measurement regime. The solutions were cooled from 60 °C back to 20 °C and measured again. Solutions that had been heated in a waterbath for 60 °C for 1 h were also measured at 20 °C for comparison.

Samples were also prepared as outlined above containing 0–20 fold molar excess NaOle, the particle size of the solutions was measured and they were heated at 60 °C for 1 h. After cooling the particle size was measured again at 20 °C.

2.3. Fluorescence: titration of α-lactalbumin with oleate

Fluorescence measurements were carried out using a Cary eclipse fluorometer (Varian, Inc., USA) with temperature controlled cuvette holder. The measurements were made at an α-la concentration of 25 μM in PBS with various excess of NaOle added. The solutions were equilibrated to the desired temperature while stirring constantly. Emission scans between 300 and 400 nm were taken using an excitation wavelength of 280 nm. The slits were both set to 5 nm. Samples were measured at 10, 25, 35, 45, 55 and 60 °C. The spectra were converted to the wavenumber scale and the curves fitted to a Gaussian distribution to calculate the emission maximum. The emission maximum was plotted against excess NaOle for each temperature.

2.4. Fluorescence: the formation of a complex during protein refolding

Solutions containing α-la with various molar excesses of NaOle were prepared as outlined in the dynamic light scattering section. The solutions were heated at 60 °C for 1 h and cooled on ice. For fluorescence measurements an aliquot of the solution taken before and after heating was diluted to 25 μM with PBS and the fluorescence spectrum measured at 20 °C as outlined above. The emission maximum was determined as described above.

2.5. Differential scanning calorimetry (DSC)

The DSC measurements were made using a Steraram DSC III micro calorimeter. PBS was used in the reference cell. A 100 g L^{-1} (7.05 mM) α-la solution in PBS with a 0, 1, 2 or 5 fold molar excess of NaOle added was placed in the reference cell. The cells contained ~900 mg of solution balanced to ±0.5 mg. The solutions were heated from 20 °C to 80 °C at 0.5 °C min^{-1}.