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The effects of allantoin, arginine and NaCl on thermal melting and aggregation of ribonuclease, bovine serum albumin and lysozyme

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ABSTRACT

Allantoin is widely used as a skin care agent and readily forms crystals, which were recently shown to bind endotoxins and high molecular weight aggregates in cell culture harvests. Here, we have investigated the effects of allantoin on thermal stability and aggregation of protein using ribonuclease, bovine serum albumin and lysozyme using temperature-regulated circular dichroism (CD) and differential scanning microcalorimetry (DSC).

Ribonuclease showed no change in thermal stability and aggregation by the addition of allantoin. While allantoin showed no effects on the thermal stability of bovine serum albumin, it enhanced aggregation. Similarly, allantoin showed no stabilizing effects on lysozyme, but it strongly suppressed aggregation. Such suppressed aggregation resulted in reversibility of thermal unfolding of lysozyme. These effects of allantoin were then compared with those of NaCl and arginine hydrochloride. Arginine was similar to allantoin at low concentrations, where both solvent additives can be compared due to limited solubility of allantoin.

1. Introduction

Allantoin, an inexpensive natural product, is a cell metabolite present in a variety of plants, including wheat germs, tobacco seeds and comfrey roots [1,2]. Allantoin is also found in animals and can be chemically synthesized from urea [3]. Allantoin has anti-inflammatory, anti-ulcer and cell-growth promoting activities and is thus widely used in a variety of commercial non-pharmaceutical (e.g. cosmetic), and pharmaceutical (e.g. as a wound healing skin care agent) [4–9]. It is slightly soluble in aqueous solution and readily forms crystals, and is chemically unstable in alkaline solution. Recently, a novel application of crystalline allantoin has been reported by Gagnon et al. [10–15]. Allantoin crystals were found to bind endotoxins and high molecular weight aggregates, which contain variable amounts of chromatin, in cell culture harvests with high specificity and affinity. To our knowledge, no studies have been reported on the effects of soluble allantoin on protein solution.

Allantoin is chiral as shown in Fig. 1 (circle) and contains amide groups and as such, its structure is similar to amine-containing low molecular weight compounds that have been shown to suppress protein aggregation during thermal melting [16–18]. Arginine has also been found to be a highly effective aggregation suppressor at high concentration. Compared with the synthetic amine compounds, arginine is a natural amino acid and thus safe to be used as an aggregation suppressing agent [19–23]. In this paper, we have investigated the effects of soluble allantoin on heat-induced structure changes and resultant aggregation of globular proteins and compared the results with the effects of NaCl and arginine.

2. Materials and methods

Three commercial proteins, BSA (bovine serum albumin), Lyso (lysozyme) and RNase (ribonuclease), were used as received. These proteins were dissolved in phosphate-buffered saline (PBS, 10 mM phosphate, 0.15 M NaCl, pH 7.0) at 20 mg/ml and used as a stock solution. A stock solution of allantoin was prepared at 200 mM in PBS by heating at 95 °C. Once dissolved, the stock solution was kept at 75 °C without precipitation. The stock solution was prepared daily. Stock solution containing 1 M arginine or 2 M NaCl were also prepared in PBS.

Circular dichroism (CD) analysis was carried out on a Jasco J-1500 spectropolarimeter equipped with a Peltier temperature controller. Since arginine and allantoin have strong absorbance in the far UV region, the near UV region was used for CD measurements. Near UV CD spectra and melting curves were determined using 1 cm path-length quartz cell with 1 cm width. A 2 mL aliquot

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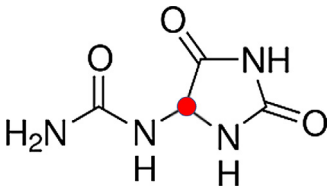

Fig. 1. Structure of allantoin. Asymmetric carbon is circled.

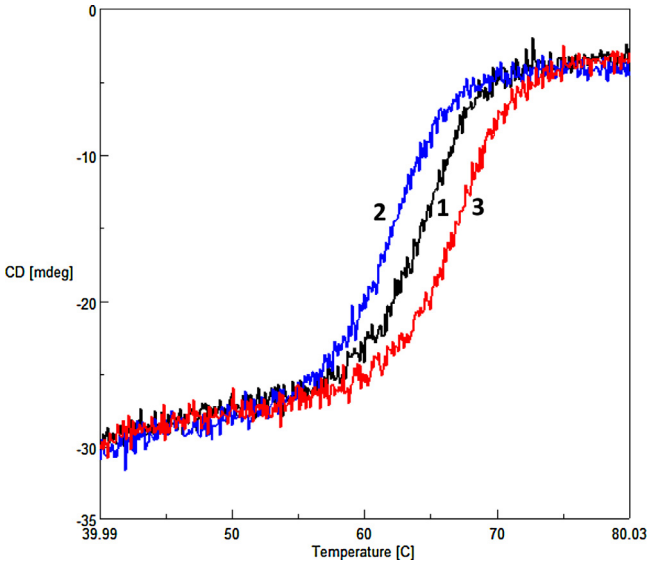

Fig. 2. Thermal melting of RNase in the absence (curve 1) and presence of 0.9 M arginine (curve 2) and 1.8 M NaCl (curve 3).
Melting of 2 mg/ml RNase was followed at 276 nm.

of the samples was loaded in the cell. Melting was followed at the indicated wavelength with a temperature increment of 1 °C/min.

The sample and reference cells (made of tantalum) were loaded with ~0.5 mL of degassed sample and buffer, respectively. A MicroCal VP-DSC was programmed to scan from 10 to 95 °C at a rate of 60 °C/hr with an 8 s filtering period and no feedback. Several buffer vs. buffer scans were recorded before running the sample vs. buffer experiment to bring the thermal environment of the instrument to a steady state and to obtain a pre-experiment buffer-buffer baseline. After each sample-buffer scan, the cell was cleaned in cycle with 10% Contrad, followed by an exhaustive water wash. The raw data were processed and analyzed using Origin 7.0. A buffer-buffer scan was subtracted from each sample-buffer scan, and the baseline was calculated and processed using the Origin software according to the manufacture's instructions. The baseline-corrected C_p profiles were normalized to protein concentration (expressed as kcal/mol/°C).

3. Results and discussion

3.1. Ribonuclease

The near UV CD spectra of 1.5 mg/ml RNase in PBS were compared at 20 and 90 °C (data not shown), from which the optimal protein concentration and wavelength to monitor thermal melting were determined to be 2 mg/ml and 276 nm. Fig. 2 shows the change in CD intensity at 276 nm of 2 mg/ml RNase in PBS with increasing temperature from 40 to 80 °C (curve 1). The negative CD signal at this wavelength started increasing at 57 °C with a transition mid-temperature (Tm) of ~64 °C. This melting was accompanied by no increase in HT[V] signal at 276 nm (data not shown), which closely follows the absorbance property and hence the light scattering of the sample. It thus does not appear that thermal unfolding of RNase accompanies aggregation. Fig. 2 also shows the melting curves of RNase in 0.9 M arginine (curve 2) and 1.8 NaCl (curve 3). The melting curve was shifted to a higher temperature in NaCl and to a lower temperature in arginine, indicating stabilization by NaCl and destabilization by arginine of RNase structure. Neither case involved increase in HT[V] intensity, indicating that neither NaCl nor arginine caused visible aggregation of RNase, as in the buffer solution. Fig. 3 plots the melting temperature of RNase as a function of additive concentration. It is evident that both arginine and NaCl had no effects on melting temperature at low concentrations and that at higher concentration NaCl increased the melting temperature while arginine decreased it. Allantion showed no effects on melting temperature and HT[V] signals of RNase, although higher concentration of allantoin could not be used due to its limited solubility. It may be summarized here that RNase does not appear to aggregate upon melting and that NaCl and arginine have an opposite effect on its thermal stability. Allantoin is inert to thermal stability and aggregation of RNase.

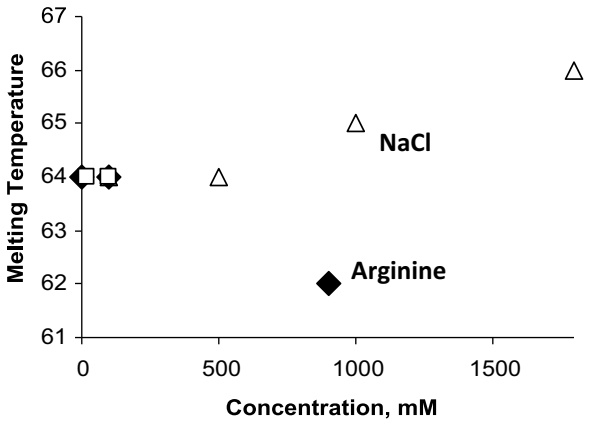

Fig. 3. Melting temperature of RNase as a function of allantoin (square), arginine (diamond) and NaCl (triangle) concentration.
Melting of 4 mg/ml BSA was followed at 270 nm.

3.2. BSA

Similarly, the near UV CD spectra of 2 mg/ml BSA in PBS were compared at 30 and 90 °C (data not shown), from which the optimal wavelength to monitor BSA melting was determined to be 270 nm. Fig. 4A shows the melting of 4 mg/ml BSA in PBS (curve 1). The CD intensity at 270 nm started increasing at ~55 °C with a Tm of 65 °C. In this case, the HT[V] intensity started increasing around 55 °C as shown in Fig. 4B (curve 1), reflecting thermal unfolding of BSA. Fig. 4A also shows the melting curves of 4 mg/ml BSA in 0.8 M arginine (curve 2) and 0.8 M NaCl (curve 3). Both additives shifted the melting curves to higher temperature, stabilizing the BSA. Fig. 5 plots the Tm against arginine (diamonds) and NaCl (triangles) concentration. While NaCl linearly increased the Tm, arginine appeared to level-off at 500 mM. Fig. 5 also shows the effects of allantoin (squares), which resulted in no changes in Tm up to 100 mM.

BSA at 4 mg/ml showed aggregation above 55 °C as described above (Fig. 4B). Fig. 4B also shows the HT[V] signal at 270 nm as a function of temperature in 10 mM (curve 2) and 40 mM (curve 3) allantoin. It is evident that the rate of HT[V] increase was greater in the presence of allantoin, more so at 40 mM and thus that allantoin enhanced aggregation of BSA. The difference in HT[V] (ΔHT[V]) between 40 and 80 °C is plotted in Fig. 6 as a function of arginine (diamonds), NaCl (triangles) and allantoin (squares) concentration. Allantoin resulted in a steady increase in ΔHT[V], indicating its enhancing effects on BSA aggregation. Arginine (diamond) initially followed the same trend, sharply increasing ΔHT[V]. However, the