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Preferential interactions between protein and arginine: Effects of arginine on tertiary conformational and colloidal stability of protein solution

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ABSTRACT

The purpose of this study was to better understand the preferential binding behavior of arginine to protein as well as the impact of arginine on the conformational and colloidal stability of protein solution. Physical stabilities of model proteins, bovine serum albumin (BSA) and ovalbumin (OVA), were investigated by fluorescence-based and dynamic light scattering techniques in the absence and presence of arginine. We investigated the interactions between arginine and tryptophan or tyrosine residues by conducting solubility and fluorescence studies of two amino acid derivatives, *N*-acetyl-L-tryptophanamide (NATA) and *N*-acetyl-L-tyrosinamide (NAYA), in arginine solutions. The result showed that arginine preferentially bond to the aromatic amino acids of proteins mainly through hydrogen bonds and Van der Waals' forces, while the binding constant K of arginine with BSA and OVA at 298 K was 41.92 and 5.77 L/mol, respectively. The fluorescence quenching, the decreased fluorescence lifetime and the red-shifted ANS peak position revealed that arginine perturbed the local environment of tryptophan and tyrosine residues. We also found the attenuated electrostatic repulsion among BSA and OVA molecules after adding arginine. These findings provided strong evidence that arginine possessed negative effects on tertiary conformational and colloidal stability of BSA and OVA during the preferential binding process.

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1. Introduction

A great deal of attention has been paid to the stability of protein solution due to the tremendous momentum and widespread recognition of protein therapy (Global, 2012). Protein formulations are susceptible to aggregation during processes of refolding, purification, sterilization, shipping and storage (Chi et al., 2003). Two types of stability, including conformational and colloidal, are largely related to the aggregate formation in protein solutions (Chaudhuri et al., 2014; Chou et al., 2012; Goldberg et al., 2011). Excipients, like sugars, polyols and amino acids, are widely incorporated in industrial protein formulations to improve stability of protein solutions (Kamerzell et al., 2011). Furthermore, the extensive application of arginine is attributed to increasing protein solubility (Arakawa et al., 2007), improving protein refolding yield (Tsumoto et al., 2004), relieving the light induced aggregation (Maity et al., 2009) and reducing viscosity of high protein concentration formulation (Ohtake et al., 2011). However, it has been reported that similar to urea and guanidine hydrochloride, arginine is a protein disturbing solute, which can result in the instability and inactivation of protein as well as perturb both the functional and structural properties of proteins (Ishibashi et al., 2005; Manikwar et al., 2013; Thakkar et al., 2012a,b; Xie et al., 2004; Yancey et al., 1982). At present, arginine selection for a particular protein formulation is generally based on trial-and-error.

The interactions between proteins and cosolvents have been classified into three typical systems as follows. One is preferential exclusion, one is preferential binding, and one is neutral binding. Sugars, polyols and amino acids possess stabilizing effects due to their preferential exclusion from the protein. However, interaction with peptide groups and disturbing the conformational stability of proteins are favorable for some cosolvents (such as urea and guanidine hydrochloride), which belongs to the preferential binding effect (Timasheff, 1998). Some popular techniques have been used to assess the interaction between arginine and protein, such as steady-state fluorescence spectroscopy (Kheddo et al., 2014), circular dichroism spectroscopy (Thakkar et al., 2012a), differential scanning calorimetry (Vagenende et al., 2013),

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high-resolution X-ray analysis (Ito et al., 2011), size exclusion chromatography (Xie et al., 2004) and molecular dynamics simulation (Mason et al., 2009). To the best of our knowledge, it remains unclear how arginine interacts with protein and how physicochemical properties of protein determine the interaction behavior of arginine with protein. In particular, little is known about the effects of arginine on the conformational and colloidal stability of protein formulations.

Synchronous fluorescence spectroscopy can separately investigate the local environment of tryptophan and tyrosine residues by selecting suitable wavelength intervals (Bozoğlan et al., 2014). Time-resolved fluorescence spectroscopy and fluorescent probe technique are reliable and frequently used methods for tertiary conformation analysis of proteins. The interaction parameter (k_D) of proteins can be obtained from dynamic light scattering (DLS) rapidly and noninvasively, which is a rational representation of colloidal stability (Roberts et al., 2014). Positive and negative k_D values manifest the presence of repulsive and attractive interactions, respectively. A protein formulation with repulsive interactions is preferable as attractive interactions lead to protein aggregation.

In this study, we investigated the interactions between arginine and tryptophan or tyrosine residues by conducting solubility and fluorescence studies of two amino acid derivatives, *N*-acetyl-L-tryptophanamide (NATA) and *N*-acetyl-L-tyrosinamide (NAYA), in arginine solutions. Effects of arginine on tertiary conformational stability of bovine serum albumin (BSA) and ovalbumin (OVA) were assessed through synchronous fluorescence spectroscopy, fluorescent probe technique and time-resolved fluorescence. DLS was used to evaluate the colloidal stability of BSA and OVA in the presence of arginine. In addition, we also investigated the binding parameters, thermodynamic parameters and binding forces between arginine and BSA or OVA by fluorescence quenching titration.

2. Material and methods

2.1. Materials

NATA (purity of >99%) and NAYA (purity of ≥99%) were obtained from Sigma (USA) and Sichuan Tongsheng Amino Acid Co., Ltd. (China), respectively. BSA (purity of ≥99%) and OVA (purity of >99%) were purchased from Shanghai Bio. Science & Technology Co., Ltd. (China) and Nanjing Duly Biotech Co., Ltd. (China), respectively. Arginine (purity of 99.5%) was supplied by Shanghai Huishi Bio. Reagent Co., Ltd. (China), and 8-Anilino-1-naphthalenesulfonic acid (1,8-ANS) was provided from Chengdu HX-R Reagent Co., Ltd. (China). All other chemicals were of analytical reagent grade, and double-distilled water was used throughout all the experiments. The working solutions used for fluorescence-based techniques and DLS were prepared in phosphate buffer solution (ion strength of 100 mM, pH 7.40), and the pH of arginine stock solution was adjusted to 7.40.

2.2. Preferential binding of arginine to NATA and NAYA

2.2.1. Solubility measurements of NATA and NAYA

Solubility of aromatic amino acids was determined using model compounds (NATA and NAYA). Briefly, an excess amount of NATA (10 mg) was added to a 2-mL centrifuge tube containing 1 mL of arginine solution with different concentrations (0 M, 0.25 M, 0.5 M, 0.75 M and 1 M). In parallel, 15 mg NAYA was operated in the same way. The samples were maintained at 25 °C for 24 h on a thermostatic water bath oscillator (HZS-H, Harbin Donglian Electronic Technology Development Co., Ltd.) at 180 rpm. Subsequently, samples of NATA or NAYA were centrifuged at 18,000 × *g* for 10 min at 25 °C. The supernatants were filtered through 0.22-μm membrane filters, and then filtrates of NATA or NAYA were diluted in 1:200 and 1:80, respectively. The absorbance of NATA and NAYA was determined at a wavelength of 280 nm and 275 nm on Cary100 UV–vis spectrophotometer (Varian Company, USA), respectively. The absorbance of the corresponding buffer was subtracted in order to correct the absorption background. A standard curve was prepared for NATA and NAYA in water using the above-mentioned procedures. All the experiments were performed in triplicate. The transfer free energy of the aromatic amino acids from water to arginine solution was assessed by the Eq. (1) (Arakawa et al., 2007).

$$\Delta G_{\text{transfer}} = -RT\ln\left(\frac{S}{S_{\text{water}}}\right) \quad (1)$$

where $\Delta G_{\text{transfer}}$ is the transfer free energy of NATA or NAYA from water to arginine solution; R is the universal gas constant; T is the temperature in Kelvin; S and S_{water} are the solubility of NATA or NAYA in arginine solution and water, respectively.

2.2.2. Fluorescence measurements of NATA and NAYA

The interaction of arginine with NATA or NAYA was investigated using the fluorescence emission spectra. NATA and NAYA were incubated in arginine solution with different concentrations at 25 °C for 12 h. The intrinsic fluorescence spectra of NATA and NAYA were then determined using a RF-5301PC spectrophotometer (Shimadzu, Japan) with an excitation wavelength of 280 nm and 275 nm, respectively. The baseline corresponding to the arginine solution was subtracted for correcting the fluorescence background.

2.3. Binding parameters between arginine and BSA or OVA

Briefly, 3.0 μmol/L BSA, 3.0 μmol/L OVA and 0.2 M arginine solutions were prepared for the fluorescence quenching titration experiment. At the fixed temperatures of 25 °C and 30 °C, the fluorescence quenching was achieved by adding 1 μL arginine solution to 2 mL BSA or OVA solution. All the solutions were mixed and rested for 2 min prior to analysis. The excitation wavelength was set at 280 nm, and the intrinsic fluorescence emission spectra of BSA or OVA were monitored at 25 °C and 30 °C, respectively. Subsequently, the binding parameters and binding sites were obtained according to the Stern–Volmer equation (Lakowicz, 2006) and Scatchard equation (Zhang et al., 2012). Furthermore, the main intermolecular forces between arginine and BSA or OVA were determined according to the Van't Hoff equation (Wang et al., 2006) as well as the view of Ross (Shao and Qiu, 2009).

2.4. Effects of arginine on tertiary conformational stabilities of BSA and OVA

2.4.1. Synchronous fluorescence measurements of BSA and OVA

For the synchronous fluorescence measurement, concentrations of BSA and OVA were maintained at 3.0 μmol/L, while the arginine concentration ranged from 0 to 0.8 mol/L. BSA and OVA were incubated in arginine solutions with different concentrations at 25 °C for 12 h. The synchronous fluorescence spectra of tyrosine and tryptophan residues were recorded with the $\Delta\lambda$ between excitation wavelength and emission wavelength fixed at 15 or 60 nm, respectively (Bozoğlan et al., 2014). The excitation and emission slits were set at 5 nm, while the scanning rate was slow. The baseline corresponding to the arginine solution was subtracted for correcting the fluorescence background.

2.4.2. Time-resolved fluorescence measurements of BSA and OVA

BSA and OVA were incubated in arginine solutions at different concentrations in the same way as described in synchronous fluorescence measurement. Fluorescence lifetime measurement was carried out on an FLS920 spectrometer (Edinburgh Instruments,

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