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Unfolding mechanism of lysozyme in various urea solutions: Insights from fluorescence spectroscopy

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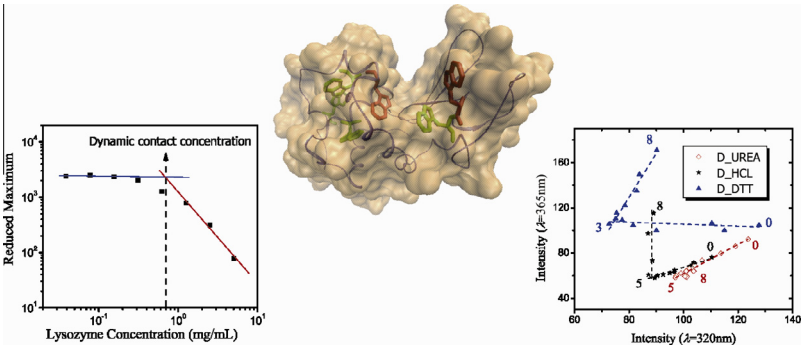
HIGHLIGHTS

- Description of fluorescence quenching effect with dynamic contact concentration.
- Except slight conformational variation, lysozyme cannot be unfolded by urea solely.
- Emergence of intermediate state for lysozyme by a combination of urea and HCl.

GRAPHICAL ABSTRACT



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ABSTRACT

Fluorescence spectroscopic technique is very popular in exploring the folding/unfolding process of proteins. In this paper, unfolding process of hen egg-white lysozyme was investigated in various denaturing solutions. Firstly, polymer solution theory was employed to comprehend the dependence of fluorescence quenching effect on protein concentration, and dynamic contact concentration was suggested as a critical value for related fluorescence experiment. Secondly, it was found that urea alone could not completely unfold lysozyme but did when together with DTT or HCl. Lysozyme was destabilized in concentrated urea solution, but still could maintain its spatial structure. Phase diagram of fluorescence intensities revealed that HCl could enhance the denaturing capacity of urea, resulting in the emergence of intermediate state in the thermodynamic unfolding process of lysozyme.

Introduction

Protein folding known as the delicate relationship between an extended, highly-disordered polypeptide chain and the compact, well-structured protein is a center problem in molecular structural biology [1–3]. Protein exhibits marginal stability and its folding depends not only on amino acid sequence but also on characteristics of surrounding environment, such as temperature, pressure, and solvent conditions [4,5]. As the other facet of this famous problem, protein's unfolding process by chemical denaturants can provide instrumental insights into the nature of protein stability and relative susceptibilities to denaturants. As one of the most used chemical denaturants, urea can exert its effect by binding to the protein and/or by changing the structure of the solvent. As a result, the extended forms of the unfolded protein is stabilized and the kinetic free-energy barrier is reduced [6]. Recently, Luan et al. found out that acid- and urea-induced denatured states of some proteins had very close radii of gyration but significantly different in the

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pattern of long-range contacts [7], indicating that there are still many unknowns for the unfolded proteins.

As an important model protein, hen egg-white lysozyme contains a single polypeptide chain (129 amino acids) folded into a globular conformation with two domains (Fig. 1), which has been investigated extensively for understanding the mechanism of protein stability, folding, denaturation and aggregation [5,8–18]. However, some nontrivial issues related to the possible existence of unfolded intermediates still cannot reach a consensus [10,11,19,20], especially in the acidic environment [8,10,13,14,21,22]. Haezebrouck et al. found that, at extreme low pH conditions, a populated equilibrium intermediate appeared during the denaturation of human and pigeon lysozyme [23,24]. However, similar intermediate for egg-white lysozyme had not been detected in the work of Ibarra-Molero and Sanchez-Ruiz [8]. Sasahara et al. [13] discovered that the intermediate state distinct from molten globule characteristics of hen egg-white lysozyme could not be observed at pH 2.2 but at pH 0.9.

In this paper, we investigated thermodynamic unfolding mechanism of hen egg-white lysozyme in acidic urea solutions using fluorescence spectroscopic technique. Fluorescence emission spectra is very sensitive to the polarity of the local surrounding of fluorophores [25], which intensities at two wavelengths (such as 320 nm and 365 nm) can be plotted in the form of a phase diagram. The corresponding dependence will be linear if it is a all-or-none unfolding process, otherwise the nonlinearity reflecting the existence of intermediates or partially-unfolded species [15,26,27]. In some special cases, polar solution can lead to the deduction of emission intensity but cannot alter the conformation of protein, resulting in a synchronous change of the emission spectrum. As an advantage, phase diagram can discriminate between this appearance and the all-or-none transition of a protein [27].

Materials and methods

Materials

Hen egg-white lysozyme (81,989 units/mg), sodium hydroxide (NaOH, purity ~98%), potassium dihydrogen phosphate (KH_2PO_4, 98%), disodium hydrogen phosphate (Na_2HPO_4, 99%), sodium chloride (NaCl, 99.5%) and dithiothreitol (DTT, 99%) were obtained from Sigma Chemical Co. (St. Louis, USA). Urea and ethylene diamine tetraacetic acid (EDTA) were purchased from Sangon Biotech. Co. (Shanghai, China). All chemical reagents were of analytical grade without further purified. All buffer solutions were prepared with Milli-Q super purified water.

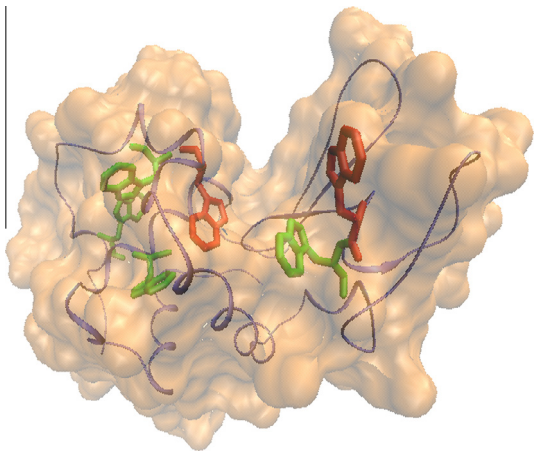

Fig. 1. Three dimensional structure of lysozyme (PDB ID: 2LYZ) in carton mode (blue) and surface mode (transparent orange). Trp62 and Trp108 are indicated in red, and other Trp residues in green. The picture was drawn by VMD software [28]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Unfolding of native lysozyme

Native lysozyme (6 mg/ml) was dissolved in phosphate buffer (50 mM, pH = 7), and corresponding concentration of proteins was determined by Bradford method. The denaturing solution was made up based on the phosphate buffer containing 1 M EDTA and certain concentrations of urea, denoted as D_UREA in this work. HCl solution was added into D_UREA until pH reached 2.0 (D_HCl). To reduce the disulfide bond in lysozyme, D_DTT was made up based on D_UREA containing DTT (30 M). Protein solution was mixed with denaturing solutions and then incubated overnight at 4 °C prior to spectral measurements.

Fluorescence spectroscopic measurements

Lysozyme containing six tryptophan (Trp) residues and three tyrosine (Tyr) residues is a good object for fluorescence analysis. As shown in Fig. 1, Trp62 and Trp108 are partially exposed to the solvent and not close to cystine or methionine sulfurs, thus responsible for most of lysozyme intrinsic fluorescence in native state [9].

Intrinsic fluorescence measurements were performed on fluorescence spectrophotometer (Hitachi F-7000, Tokyo, Japan) with a cuvette of 1 cm light path. The excitation wavelength (λ_{ex}) of fluorescence spectrum was set at 295 nm, and the emission spectra were recorded in the wavelength range of 300–500 nm. The bandwidth of excitation and emission slits was set as 10 nm. The emission maximum (λ_{max}) and fluorescence intensity were determined according to the peak of fluorescence emission spectra.

Results and discussion

A critical concentration of lysozyme in fluorescence experiment

It is well known that fluorescence intensity is obviously affected by fluorophore concentration [25]. There might be self-quenching effect if protein molecular is too close to each other or form a dimer [29]. Fluorescence emission spectra were obtained for lysozyme in denaturing D_DTT solution with 8 M urea, as shown in Fig. 2A. There is a complex relationship between fluorescence intensity and lysozyme concentration. In order to investigate the dependence of lysozyme fluorescence intensity at λ_{max} on the protein concentration, an order parameter "reduced intensity" was suggested here. Generally, emission intensity is contributed by all fluorophores, while reduced intensity only counts emission intensity per mg lysozyme, and its value can be simply determined through the division of emission maximum (λ_{max}) by lysozyme concentration. Therefore, reduced intensity can be looked as an intensive parameter while emission intensity as an extensive one. As shown in Fig. 2B, the reduced intensity decreased slightly with lysozyme concentration until about 0.5 mg/ml, beyond which the intensity decayed exponentially, suggesting a self-quenching effect of lysozyme.

Since DTT can disrupt the disulfide bonds of protein, lysozyme became a linear chain when denatured in D_DTT solution. Basically, quenching effect will disappear if one protein molecule cannot feel the existence of another one in Brownian movement. In view of polymer solution theory [30], the dynamic contact concentration (C_s) is a critical concentration corresponding to this case, which is about 0.5 mg/ml for some polymers with similar molecular weight [31]. In Fig. 2, two fitting lines were drawn based on the first three or the last three data points, and their cross-point is