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Effects of macromolecular crowding on alkaline phosphatase unfolding, conformation and stability

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ABSTRACT

The interior of the cell is tightly packed with various biological macromolecules, which affects physiological processes, especially protein folding process. To explore how macromolecular crowding may influence protein folding process, alkaline phosphatase (ALP) was chosen as a model protein, and the unfolding process of ALP induced by GdnHCl was studied in the presence of crowding agents such as PEG 4000, Dextran 70 and Ficoll 70. The effect of macromolecular crowding on the denatured state of ALP was directly probed by measuring enzyme activities, fluorescence spectroscopy and circular dichroism. From the results of circular dichroism, GdnHCl induced a biphasic change, suggesting that a three-state unfolding mechanism was involved in the denaturation process irrespective of the absence or presence of crowding agents. It was also found that crowding agents had a little impact on the unfolding process of ALP. The results of phase diagrams also demonstrated that the unfolding process of ALP induced by GdnHCl was three-state mechanism. Moreover, the results of fluorescence spectra demonstrated that with the increase of GdnHCl concentration, the structure of protein had changed, but existence of crowding agents can make protein structure more stable. Our results can provide valuable information for understanding the protein folding *in vivo*.

1. Introduction

In cell, protein folding is an important and complex process that is conducted by a stochastic search of the lowest energy structure corresponding to the natively folded state [1]. Most studies of protein folding and conformation are often conducted in dilute solution rather than crowding milieu. However, cells are tightly packed with various biological macromolecules (proteins, nucleic acids, ribonucleoproteins, polysaccharides, etc.). These macromolecules occupy a significant fraction (~40%) of the total cellular volume, which make the concentration of cells reach the range of 80–400 mg/mL [2–4]. It makes the cells very crowded, with considerably limited amounts of free water [5–7] and very restricted amounts of available space. This phenomenon is called as macromolecular crowding effect. To better understand and mimic the crowding environment, high concentrations of polymers such as polyethylene glycol, dextran or ficoll are often used as macromolecular crowding reagents in vitro experiments. A number of theoretical and experimental studies have revealed that macromolecular crowding affects protein folding, aggregation and stability [8–23], as well as protein function and activity [24–29].

Generally, the excluded volume hypothesis is used to explain the influences of macromolecular crowding on proteins. Excluded volume effects decrease the effective volume available for the proteins in the cell and promote more compact protein conformations. However, other studies show the excluded volume effects is not the only factor of macromolecular crowding affecting the behaviors of proteins. These inconsistencies are usually explained by the so-called "soft interaction", such as hydrophobic, electrostatic, and van der Waals interactions between the crowding reagents and proteins, in addition to the hard nonspecific steric interactions [30–34]. Regard to protein folding, it is commonly believed that macromolecular crowding directly affects the conformation of unfolded state, and makes the structure more compact and increases the free energy

Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; ALP, alkaline phosphatase; GdnHCl, guanidine hydrochloride; Tris, tris(hydroxymethyl) aminomethane; CD, circular dichroism; p-NPP, *p*-nitrophenyl phosphate; UV–vis, ultraviolet–visible spectroscopy.

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of unfolded state. This makes the folded state more stable, and also promotes protein folding.

Recently, there are many studies about the influences of macromolecular crowding on protein folding and stability. Simanta Sarani Paul et al. found that crowding media such as Ficoll 70 and Dextran 70 affected the native state equilibrium of yeast cytochrome c between compact and expanded states, shifting its population toward the compact conformation [35]. Ashima Malik et al. showed that macromolecular crowding agents were not always stable in nature. In regard to myoglobin, Ficoll 70 was observed to be typically unstable in its influence on myoglobin unfolding [36]. Ruchira Engel et al. showed that crowding agents destabilized the unfolded state of apoflavodoxin, and made apoflavodoxin become more compact and acquire secondary structure. Due to the addition of crowding media, the midpoint of thermal unfolding of native apoflavodoxin increased by 2.9 °C [37]. Manish Kumar Suthar et al. demonstrated that the refolding rate of plasmodium falciparum purine nucleoside phosphorylase denatured by urea was not affected by the presence of crowding agents [38]. Yong-Qiang Fan et al. studied the refolding of recombinant human brain-type creatine kinase. They found that there was more aggregation rather than correct refolding in the presence of crowding agents than in the absence of crowding agents [39]. Macromolecular crowding has complex influences on protein folding, which changes with the type, size and shape of proteins and crowding agents and so on. Therefore, in order to understand the protein folding in vivo deeply, the effects of macromolecular crowding on protein folding may require a case-by-case analysis.

Alkaline phosphatase (ALP) is a homologous dimers metalloenzyme, widely existing in nature and animal organs. It plays a very important role in the process of biological phosphorus metabolism. It not only can non-specifically catalyze the decomposition of phosphate esters, but also can catalyze the phosphorylation of alcohols [40]. It is a set of isoenzyme, and it can be divided into the tissue specificity (intestinal/placenta/germ cell type) and the tissue non-specificity (liver/bone/kidney type) [41]. For the tissue non-specific ALP, some studies have reported its phosphate monoester enzyme, phosphordiesterase and focal phosphatase activities [42]. ALP is associated with human diseases such as high phosphatase leukemia, seminoma, and lung injury. In medicine, only the change of serum ALP in the form of dimers can have good activities [43]. Therefore, it has great significance and important guiding role in understanding the ALP folding process in simulated physiological environment. There have been some reports about ALP folding and resilience in the presence of magnesium ions and artificial molecular partner [44,45]. But most of all studies were performed in dilute solution system, and did not consider the influence of the crowded environment in the cell, so the results cannot accurately reflect the real behavior of ALP within the cell. Therefore, it is quite meaningful to understand the characteristic of ALP unfolding or folding in an intracellular environment.

In this study, we have used the macromolecular crowding agents (PEG 4000, Dextran 70 and Ficoll 70) to probe the impact of crowding on the unfolding process of ALP. PEG, Ficoll 70 and Dextran 70 are common macromolecular crowding agents for *in vitro* studies. PEG, a non-charged highly water-soluble polymer with well-known effects on water dynamics, has been widely employed as a solubilizing agent and stabilized in chemoenzymatic synthesis. Ficoll 70 is a highly cross-linked sucrose or epichlorohydrin copolymer that behaves like a semirigid sphere [46,47]. Dextran 70 is a flexible and linear polymer of D-glucopyranose, which is a ribbon-like, quasirandom coil molecule with few and short branches [48,49]. They are chemically inert and not found to interact with proteins. In this study, the enzyme activity and intrinsic fluorescence spectra of unfolding ALP were measured, and the equilibrium denaturation of ALP were quantitatively studied in both dilute solutions and crowding milieu. In addition, the phase diagrams, ANS fluorescence spectra, three-dimensional fluorescence spectra, and synchronous fluorescence spectra were also examined. The goal of this investigation was to develop a deep understanding of the effects of macromolecular crowding on unfolding process of ALP induced by GdnHCl.

2. Materials and methods

2.1. Materials

Calf intestine alkaline phosphatase, PEG 4000, Dextran 70, Ficoll 70 and 8-anilino-1-naphthalenesulfonic acid fluorescence (ANS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The protein was used without further purification. Guanidine hydrochloride (GdnHCl), tris(hydroxymethyl) aminomethane (Tris), *p*-nitrophenyl phosphate (p-NPP) and other chemicals and solvents were commercially purchased.

2.2. Enzyme assay

The ALP-catalyzed hydrolysis of *p*-nitrophenyl phosphate was assayed as described [50]. The absorbance at 405 nm was continuously monitored at 25 °C with a UV-spectrophotometer (TU-1900, Puxi General, Inc., China). The increase of absorbance at 405 nm was always colinear with time. ALP activity was determined by following the increasing absorbance at 405 nm accompanying the hydrolysis of the substrate p-NPP and was recorded as the change in absorbance per min at 405 nm. The relative enzyme activity was the percentage of the actual enzyme activity compared to the enzyme activity without GdnHCl.

2.3. Intrinsic fluorescence spectra

The fluorescence spectra of ALP were recorded on a fluorescence spectrophotometer (Cary Eclipse, Agilent Technologies, Inc., USA), equipped with a quartz cell of 1 cm path length. 3 mL of the mixed solution containing 0.12 mg/mL ALP and different concentrations of GdnHCl and 100 g/L crowding agents were pipetted in a 1.0 cm quartz cuvette. Excitation light at 280 nm and emission data over a range of 290–500 nm were recorded with a bandwidth of 5 nm.

2.4. Phase diagram method

The phase diagram method of fluorescence is a sensitive tool for the detection of unfolding intermediates of proteins [51], which can be described by building up a diagram based on Eq. (1):

$$I\left(\lambda_1\right) = a + bI\left(\lambda_2\right) \tag{1}$$

where $I(\lambda_1)$ and $I(\lambda_2)$ are the spectral intensity values measured on wavelengths λ_1 and λ_2 under different experimental conditions to for a protein undergoing structural transformations; a and b are the intercept and slope of the $I(\lambda_1)$ *vs.* $I(\lambda_2)$ plot. In principle, λ_1 and λ_2 are arbitrary wavelengths of the spectrum, but in practice such diagrams will be more informative if λ_1 and λ_2 will be on different slopes such as 320 nm and 365 nm for fluorescence spectrum. The fluorescence spectra of ALP were recorded on a fluorescence spectrophotometer (Cary Eclipse, Agilent Technologies, Inc., USA), equipped with a quartz cell of 1 cm path length. The excitation wavelength was set to 280 nm.

2.5. Circular dichroism (CD)

CD experiments were performed on a JASCO J-810 CD spectropolarimeter (Jasco Inc., Japan) over a wavelength range of 200–260 nm