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Protein nanoparticle electrostatic interaction: Size dependent counterions induced conformational change of hen egg white lysozyme

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ABSTRACT

In our earlier paper (Ghosh et al., 2013), we have shown that (i) the positively charged hen egg white lysozyme (HEWL), dispersed in water, binds electrostatically with the negatively functionalized iron oxide nanoparticles (IONPs), and (ii) the Na^+ counterions, associated with functionalized IONPs, diffuse into bound proteins and irreversibly unfold them. Having this information, we have extended our investigation and report here the effect of the size and the charge of alkaline metal counterions on the conformational modification of HEWL. In order to obtain a negative functional 'shell' on IONPs and the counterions of different size and charge we have functionalized IONPs with different derivatives of citrate, namely, tri-lithium citrate (TLC, $Li_3C_6H_5O_7$), tri-sodium citrate (TSC, $Na_3C_6H_5O_7$), tri-potassium citrate (TKC, $K_3C_6H_5O_7$) and tri-magnesium citrate (TMC, $Mg_3C_{12}H_{10}O_{14}$). The size of counterions varies as $Mg^{2+} < Li^+ < Na^+ < K^+$. After interaction with the functionalized IONPs, the unfolding of HEWL was the maximum in presence of Li^+, and was decreasing with increasing size of counterions. The UV–vis absorption measurements indicated that the unfolding of HEWL was due to modification in the hydrophobic environment around the tryptophan regions. The unfolding of HEWL was associated with the change of folding conformation from the α-helix to the β-sheet. In absence of counterions, ligand-IONPs have no effect on the native conformation of HEWL. An effective use of counterions in order to modify protein conformation (and, the functionality) via protein–nanoparticle electrostatic interaction is a new finding, and be useful for an alternative medical therapy.

1. Introduction

The study of protein–nanoparticle interaction is a fascinating area due to its particular application in medicine [1–3]. For their applications in physiological environment, nanoparticles need to be functionalized with appropriate ligands, e.g., polymers [4], or biomolecules [5–7]. As biological activities of proteins depend on their conformations, it is important to know the conformation of protein after interaction with ligand-nanoparticles. A number of publications have already addressed this issue [8–10]. A publication by Mahmoudi et al. [11] reported an irreversible change in the secondary conformation of iron saturated human transferrin after interaction with bare or PVA functionalized Fe_3O_4 nanoparticles.

The charged residues on the surface of a protein play an important role in binding ionic ligands from the surrounding solvent [12–15]. The capability of a protein to bind ions depends on the net charge on the protein and the distribution of polar residues. For example, in superoxide dismutase, the charged residues are organized to ensure efficient channeling of superoxide radical (O_2^-) to the active sites of the enzyme [16]. The negatively charged side chains around calcium-binding sites have been shown to enhance the affinity of calbindin D_{9k} for the metal ions [17]. The binding of ions to protein molecules also depends on the solution pH, the ionic size and the charge, and the ionic concentration [18,19]. Often metal ions coordinate to the binding sites located in loops or unstructured regions of the proteins. The mechanism of metal ion binding with proteins has been reported earlier [20,21]. The effective binding sites for the metal ions are rather flexible in unstructured proteins (α-synuclein) or in unstructured domains of proteins (e.g. prion protein) [22,23]. The small angle neutron scattering (SANS) [24] and the circular dichroism (CD) spectroscopy [25] measurements have

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revealed that the radius of gyration in transglutaminase increases on Ca^{2+} ion binding. Bovine serum albumin's (BSA) high affinity for various ligands has also been reported [26,27].

The circular dichroism (CD) spectroscopy is a sensitive tool for rapidly evaluating protein's secondary conformation and its change due to interaction with nanoparticles [28–30]. The fluorescence spectroscopy [11] and the Fourier-transformed infrared (FTIR) spectroscopy [31–33] have also been used to study the secondary conformation of proteins. The FTIR demonstrated that the proteins with predominantly α-helical conformation have the amide I and II absorptions in the spectral range of 1652–1657 and 1545–1551 cm^{-1} respectively, in aqueous solution, while those with predominantly β-sheet conformation exhibited similar absorptions in the range 1628–1635 and 1521–1525 cm^{-1}. The amide I absorption contains major contributions from the C═O stretching vibration of the amide group, while the amide II absorption appears from the N—H bending and the C—N stretching vibrations [34–37]. The exact frequency of the amide I and II vibrations would be influenced by the strength of hydrogen bonds involving the amide C═O and the N—H groups.

The changes in the ultraviolet (UV) absorption spectra of proteins associated with its denaturation have been reported [38,39]. The difference of the spectra of proteins in the native and the unfolded conformations in the region of 270 and 300 nm have been assigned to the alterations in the environment of tyrosine and tryptophan residues [40–42]. Glazer and Smith reported that alteration in the native conformation of protein is associated with the appearance of a peak between 230 and 235 nm in the difference spectrum [43], corresponding to helix-to-coil transformation. This is similar to the absorption changes near 280 nm, where the peptide bond does not contribute [44–46]. A recent report [47] uses the UV absorbance at 230 nm (A_{230}) as a probe to show the thermodynamic stability and the unfolding kinetic of the *E. coli* maltose binding protein (MBP) and the *E. coli* ribonuclease H (RNase H).

In our earlier paper [48], we have shown that (i) the positively charged hen egg white lysozyme (HEWL) in water binds electrostatically with the negatively functionalized iron oxide nanoparticles (IONPs), and (ii) the Na^+ counterions, associated with the functionalized IONPs, diffuse into the bound proteins and irreversibly unfold them. In this paper, we report the effect of counterions having different size and charge on the HEWL conformation. In order to obtain the negative functional 'shell' and the counterions of different size and charge on IONPs we have used different derivatives of citrate, namely, tri-lithium citrate (TLC, $Li_3C_6H_5O_7$), tri-sodium citrate (TSC, $Na_3C_6H_5O_7$), tri-potassium citrate (TKC, $K_3C_6H_5O_7$) and tri-magnesium citrate (TMC, $Mg_3C_{12}H_{10}O_{14}$) where the size of respective counterions varies as $Mg^{2+} < Li^+ < Na^+ < K^+$. We have also reported the effect of aged ligand-IONPs on the HEWL conformation. Several techniques, like, dynamic light scattering (DLS), Fourier transform infrared (FTIR) spectroscopy, surface ζ-potential measurement, UV–vis absorption spectroscopy, circular dichroism (CD) spectroscopy and differential scanning calorimetry (DSC) have been used for the HEWL–ligand-IONPs interaction studies.

2. Experimental

2.1. Materials

$FeCl_3$ (98%) was purchased from Burgoyne Burbidges & Co (India) and $FeCl_2$ (99.5%) was purchased from Alfa Aesar (USA), NH_4OH (30% conc.) was purchased from Merck (India). Tri-lithium citrate (TLC, $Li_3C_6H_5O_7$, 98.5%), tri-sodium citrate (TSC, $Na_3C_6H_5O_7$, 98%), tri-potassium citrate (TKC, $K_3C_6H_5O_7$, 99%) and tri-magnesium citrate (TMC, $Mg_3C_{12}H_{10}O_{14}$, 97%) were purchased from S.D. Fine Chem. Ltd., Mumbai, India. HEWL ($\langle M_W \rangle$ = 14,300 g mol^{-1}; L-6876, Grade-1) was purchased from Sigma, USA. All chemicals were used without any further purification. The milli-Q water, obtained from a three-stage purification system (Millipore, USA), was further filtered through a 0.22 μm filter and then autoclaved. The pH and the electrical resistivity of this water were found to be 6.5 and 18.2 MΩ cm^{-1}, respectively. This water was used for sample preparations and measurements.

2.2. Preparation of functionalized IONPs dispersions

Iron oxide nanoparticles (IONPs) were synthesized by the chemical co-precipitation reaction between Fe^{3+} and Fe^{2+} salts (Fe^{3+}:Fe^{2+} = 3:2) in basic medium [49]. Briefly, 2.06 g of $FeCl_3 \cdot 6H_2O$ and 1.28 g of $FeCl_2$ (anhydrous) were dissolved in 10 ml of water in a 50 ml round bottom flask and magnetically stirred for 30 min at 60 °C to remove oxygen dissolved in the solution. Afterward the magnetic bar was removed, and 3.3 ml of NH_4OH (30% conc.) was added quickly to the solution with vigorous shaking, and a black precipitation was obtained. The black precipitate was separated out using a strong magnet (~1 Tesla) underneath and the supernatant was discarded. The precipitate was washed several times using water until the supernatant became neutral (pH 6.5). Finally, the precipitate was washed with acetone and was left at room temperature for drying. The phase of the nanoparticles formed by this process is well reported to be Fe_3O_4 [48,49]. The nanoparticles surface was functionalized with TLC, TSC, TKC and TMC following the procedure as reported earlier [48]; and named as, TLC-IONPs, TSC-IONPs, TKC-IONPs and TMC-IONPs, respectively. They were dispersed in water at a concentration of 2 wt% and pH of 6.5.

2.3. Preparation of HEWL solution and incubation

The stock solution of 0.1 wt% HEWL was prepared in water. The pH of this solution was found to be 6.5, i.e., there was no change in pH of the solution after dispersing HEWL. The 0.05 wt% dispersions of TLC-IONPs, TSC-IONPs, TKC-IONPs and TMC-IONPs in water were prepared from 2 wt% dispersions. For aging, the freshly prepared 0.05 wt% ligand-IONPs dispersions were left undisturbed at 25 °C for about four months and termed as 'aged' dispersions. The stock solution of HEWL was further diluted (see below) to match the scale in CD measurement. HEWL and ligand-IONPs were mixed at a weight ratio of 1:1 in each final mixture as described below, and left on a shaker at 4 °C for 48 h.

(i) 100 μl 0.1 wt% HEWL stock solution + 200 μl milli-Q water;
(ii) 100 μl 0.1 wt% HEWL stock solution + 200 μl 0.05 wt% ligand-IONPs.

The incubation process can be described using following relation

$$\text{HEWL(native)} + \text{ligand-IONPs} \rightarrow \text{HEWL(interacted)} + \text{HEWL} - \text{ligand-IONPs} \quad (1)$$

where 'HEWL (native)' and 'HEWL (interacted)' correspond to the HEWL before and after interaction with ligand-IONPs, respectively, and 'HEWL–ligand-IONPs' corresponds to the conjugates of HEWL and ligand-IONPs. Eq. (1) indicates that due to interaction of ligand-IONPs with proteins (HEWL), the ligand-IONPs develop a dynamic 'corona' of protein whose number varies over time due to continuous association and dissociation in equilibrium. After 48 h of incubation, the dispersions were kept on the magnet to separate out the 'interacted' HEWL to carry out the conformational analysis of the protein. The remaining solutions of HEWL–ligand-IONPs