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Molecular mechanism of polyethylene glycol mediated stabilization of protein

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ABSTRACT

The effect of different molar ratios of polyethylene glycol (PEG) on the conformational stability of protein, bovine serum albumin (BSA), was studied. The binding of PEG with BSA was observed by fluorescence spectroscopy by measuring the fluorescence intensity after displacement of PEG with chromophore ANS and had further been confirmed by measuring the intrinsic fluorescence of tryptophan residues of BSA. Co-lyophilization of BSA with PEG at optimum BSA:PEG molar ratio led to the formation of the stable protein particles. Circular dichroism (CD) spectroscopy study suggested that a conformational change had occurred in the protein after PEG interaction and demonstrated the highest stability of protein at the optimum BSA:PEG molar ratio of 1:0.75. Additional differential scanning calorimetry (DSC) study suggested strong binding of PEG to protein leading to thermal stability at optimum molar ratio. Molecular mechanism operating behind the polyethylene glycol (PEG) mediated stabilization of the protein suggested that strong physical adsorption of PEG on the hydrophobic core of the protein (BSA) along with surface adsorption led to the stability of protein.

Introduction

Proteins, in general, are known to unfold/refold through different denaturing states. It is crucial to know the differences in residual structure between different denatured states along the pathway of unfolding/folding [1]. Such denatured states will provide useful information in understanding the mechanism of protein stability. Stabilization of proteins in various conditions is one of the major challenges associated with the encapsulation of protein biopharmaceuticals in nano- and microspheres. There are different strategies for the stabilization of proteins; chemical modification, protein engineering, immobilization, and the use of polyhydroxy compounds [2]. Different sizes of PEG are frequently used as polyhydroxy stabilizers to improve the biocompatibility of biomolecules for both in vivo and ex vivo applications [3]. Interaction of PEG with biological macromolecules is an important study because of the many applications of polymers in various fields, such as biology, medicine and biomedical engineering [4]. This interaction often induces structural changes that may affect the entire molecule by affecting its functional or conformational activity. However, the effect of PEG at a molecular level is not fully understood. It is presumed that the conformational stability of protein is probably maintained by altering the hydration shell of the protein. Maintaining the protein integrity and its stability during interaction is a major challenge of utmost importance to overcome this problem. In some cases, structural changes may be induced in the protein by the physicochemical nature of the polymer surface while in others this may be due to intrinsic properties of the protein [5]. The conformational behavior of proteins on interacting with polymer particles is therefore of great importance in various biomedical applications, particularly drug delivery or receptor targeting. However, the molecular mechanism of the changes in the structure of protein after interaction with polymer are not very clear and need some basic understanding before such complexes are used for biomedical applications.

The structure and properties of BSA, the most abundant protein in the plasma, are well investigated and therefore used as a model for the studies on conformational change. BSA is composed of a single polypeptide chain made up of 583 amino acid residues and 17 disulfide bonds. These disulfide bridges are the basis for making the native BSA molecule quite compact as well as for providing stability to the helical structure. The primary structure of BSA at physiological pH is predominantly α-helical which undergoes reversible conformational isomerization with changes in pH (between 55–48% at pH 7.4) and the remaining polypeptide occurring in turns with extended or flexible regions between sub-domain with no β-sheet [6]. In this paper, we report the molecular mechanism operating behind the polyethylene glycol (PEG) mediated stabilization of the protein (BSA). The interaction of BSA with PEG and the conformational changes of the protein have been thoroughly characterized and monitored by using fluorescence, circular dichroism (CD) and differential scanning calorimetry (DSC).

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Materials and methods

Materials

BSA, 8-anilino-1-naphthalenesulfonic acid (ANS), polyethylene glycol (PEG) MW 8000 and urea were obtained from Sigma–Aldrich (St. Louis, USA), and all other chemicals used were of analytical grade or highest grade available.

Methods

Preparation of PEG coated BSA. PEG coated protein samples of different BSA to PEG ratio were prepared by mixing different concentration of PEG with BSA (1 mg/mL) in 50 mM PBS (phosphate buffer saline, pH 7.4). The samples were lyophilized in a freeze dryer (Martin Christ Alpha 1-2 LD, Osterode, Germany) at −54 °C for 12 h. Following sonication of the lyophilizates for 5 min (in 30 s on and 30 s off mode), the sonicated samples were centrifuged for 5 min at 10,000 rpm and the precipitates were collected after discarding supernatants. This process was repeated three times to remove the unbound PEG from the protein and then, precipitates were again lyophilized for 12 h under above said conditions.

Release of the BSA from PEG coated BSA. The protein (BSA) was released from PEG coated particles by overnight stirring of these particles in PBS (50 mM, pH 7.4) at 150 rpm in an orbital shaker. The concentrations of released BSA were measured by a UV–visible spectrophotometer (CARY 100 Bio, Victoria, Australia) at 280 nm and used for characterization studies.

Computational studies. The structure of BSA was retrieved from the Protein Data Bank (PDB) structural database (http://www.rcsb.org/) with the PDB ID 2BXA and was analyzed using the PyMOL program (http://pymol.sourceforge.net/). The docking of PEG was done by using freely available autodock (ADT4.2) (http://autodock.scripps.edu/).

Circular dichroism (CD) measurements. The CD spectra were recorded in the far-UV range from 190 to 250 nm in optical cell with a path length of 0.1 cm (JASCO J-180) at 25 °C. The concentration of released BSA solution was adjusted to 100 μg/mL in 5 mM PBS (pH 7.4). The results were expressed as molar ellipticities [θ] using the equation,

$$\theta = 3300\ \Delta\mathrm{Abs}/c \cdot l$$

where, ΔAbs is the observed difference in absorbance for the left and right circular components of the incident light, l the path length (in cm) and c the concentration in mol l^{-1}.

Measurement of the PEG binding to BSA using ANS. The saturation concentration of ANS (40 μM) was determined from a standard curve of ANS prepared using constant concentration of BSA. This concentration of ANS was used in experiments involving released BSA from PEG coated BSA particles (BSA:PEG molar ratios of 1:0.25, 1:0.5, 1:0.75, 1:1, 1:2, 1:3, and 1:4). All experiments were performed at room temperature with incubation time of 15 min. The ANS binding to BSA was followed by measuring florescent intensity using a spectrofluorometer (Cary Elipse, Victoria, Australia) with an excitation wavelength of 375 nm and emission wavelength of 475 nm.

Fluorescence quenching measurements. The released BSA from PEG coated BSA particles (BSA:PEG molar ratios of 1:0.25, 1:0.5, 1:0.75, 1:1, 1:2, 1:3, and 1:4), were appropriately diluted and analyzed for intrinsic fluorescence emission by a spectrofluorometer with an excitation and emission wavelength at 280 and 340 nm, respectively.

Stability and denaturation of BSA in the presence of urea. To determine the minimum urea concentration causing denaturation of BSA (at constant BSA concentration of 1 mg/mL), the emission wavelength of maximum fluorescence intensity of BSA solutions at different urea concentrations (0.25–7.0 M) was measured. The selected urea concentration (6.25 M) was used for further experiment involving different BSA:PEG ratios (1:0.25, 1:0.5, 1:0.75, and 1:1) along with controls (BSA, BSA + Urea). The fluorescence emission spectra of the BSA after 15 min of incubation at room temperature were measured at an excitation wavelength of 290 nm and an emission wavelength of 340 nm.

Thermal stability and thermal unfolding analysis by DSC. Thermal denaturation of BSA was monitored with a high sensitivity differential scanning calorimeter (Model NANO-DSC, TA-instrument, USA). Thermograms of different BSA:PEG molar ratios (1:0.25, 1:0.5, 1:0.75, and 1:1) were obtained between 60 and 90 °C, at a scan rate of 30 °C/h. All results were averages of, at least, three independent measurements. The calorimetric data was analyzed by using the software NANOANALYZER (TA-instrument, USA) to obtain the temperature (T_m) at which maximum heat exchange occurs.

Results and discussion

Circular dichroism (CD) spectroscopy for conformational change

The conformational changes of BSA after its binding with PEG molecules were investigated using circular dichroism (CD) spectroscopy. BSA has a high percentage (48%, depending upon the pH 7.4 in present case) of α-helical structure, which shows a characteristic CD signal in the far-UV region. Changes in the ellipticity at 208 and 222 nm are useful probes for visualizing varying α-helical content which is expressed as MRE in deg cm^2 $dmol^{-1}$. The value of MRE was obtained using the equation $\mathrm{MRE} = [\theta]_{208}/10(n)(l)(C)$ where $[\theta]_{208}$ was the CD in millidegrees, n was the number of amino acid residues in protein (total of 583 for BSA), l was the path length of the cell (0.1 cm), and C is the concentration of protein. The helical content of BSA was thus calculated from the $[\theta]_{208}$ values according to the equation [7],

$$\%\mathrm{helix} = [(-[\theta]_{208} - 4000)/(33000 - 4000)] \times 100.$$

Since, PEG did not show any CD signal in the spectral range between 200 and 300 nm, the CD spectrum of BSA–PEG mixture was only due to the protein (BSA) [8]. The effect of bioprocessing such as lyophilization and sonication on BSA was investigated by taking a mixture of BSA and PEG at different molar ratio (1:0.25, 1:0.5, 1:0.75, 1:1, 1:2, 1:3, and 1:4), then removing the unbound PEG from the protein PEG aqueous mixture and releasing the protein in the same 50 mM PBS buffer after overnight incubation. The CD spectra of released proteins from PEG coated BSA particles showed that BSA:PEG molar ratio of 1:0.75 exhibited maximum conformational stability (retaining 38% of α-character as compared to 45% in case of native BSA molecule and MRE-15103.1) in comparison to other molar ratios of BSA:PEG. Lyophilization or sonication did not make any changes in the conformational stability of the protein (Fig 1).

Measurement of the PEG binding to BSA using ANS

The fluorescence dye 8-anilino-1-naphthalene sulfonate (ANS) is a valuable probe for the detection and analysis of conformational changes in proteins and for the evaluation of folding unfolding processes in proteins [9]. ANS, itself, does not show any fluorescence but on binding non-covalently with hydrophobic residues (such as tryptophan) of a protein like BSA, the fluorescence intensity is greatly enhanced [10]. As the affinity of BSA for PEG is very low, an indirect method was employed to evaluate the binding of PEG to BSA. There are two tryptophan residues in one BSA molecule: Trp-134 in the 8th helix of D129-R144 in domain I, and Trp-213