Colloids and Surfaces B: Biointerfaces xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Secondary structure and folding stability of proteins adsorbed on silica particles – Pressure versus temperature denaturation

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ARTICLE INFO

Article history:
Received 23 January 2015
Received in revised form 17 March 2015
Accepted 18 March 2015
Available online xxx

Keywords:
Protein adsorption
Protein unfolding
FTIR spectroscopy
Temperature effects
Pressure effects

ABSTRACT

We present a systematic study of the pressure and temperature dependent unfolding behavior of proteins that are adsorbed on silica particles. Hen egg white lysozyme and bovine ribonuclease A (RNase) were used as model proteins, and their secondary structures were resolved by Fourier transform infrared (FTIR) spectroscopy in the temperature range of 10–90 °C and the pressure range of 1–16,000 bar. Apparently, the secondary structures of both proteins do not change significantly when they are adsorbing on the silica particles. Remarkably, the changes of the secondary structure elements upon protein unfolding are very similar in the adsorbed and the free states. This similarity could be observed for both lysozyme and RNase using both high pressures and high temperatures as denaturing conditions. However, the pressures and temperatures of unfolding of lysozyme and RNase are drastically lowered upon adsorption indicating lower folding stabilities of the proteins on the silica particles. Moreover, the temperature ranges, where changes in secondary structure occur, are broadened due to adsorption, which is related to smaller enthalpy changes of unfolding. For both proteins, free or adsorbed, pressure-induced unfolding always leads to less pronounced changes in secondary structure than temperature-induced unfolding. In the case of lysozyme, high pressure also favors a different unfolded conformation than high temperature. Overall, the results of this study reveal that adsorption of proteins on silica particles decreases the folding stability against high pressures and temperatures, whereas the unfolding pathways are mainly preserved in the adsorbed state.

1. Introduction

When proteins are used in biotechnological applications, they are often immobilized on carrier particles [1,2]. Using carrier particles allows for an easier way to remove proteins from the reaction solution by centrifugation, sedimentation, filtration or even magnetic forces [3]. For example, expensive enzymes can then be reused in additional reaction cycles. However, proteins are biologically active in their native conformation only, which is marginally stable against unfolding. Any deviation from the natural environment, which is mostly a neutral aqueous solution at ambient pressure and slightly elevated temperatures, can cause denaturation due to partial or complete unfolding. For example, proteins can be unfolded by increasing and lowering the temperature, increasing the pressure, or changing the chemical environment, such as addition of cosolvents or exposure to interfaces [4–7]. Thus, immobilization of proteins on carrier particles can also lead to a loss of biological activity.

From a thermodynamic point of view, the stability of the native, folded conformation is given by the standard Gibbs energy of unfolding, ΔG°, which is $G^\circ_{\text{unfolded}} - G^\circ_{\text{folded}}$. It characterizes a two-state model of a folded conformation that is in equilibrium with unfolded conformations via [6]

$$\Delta G^\circ = -RT \ln K \tag{1}$$

where $K = c_{\text{unfolded}}/c_{\text{folded}}$ is the equilibrium constant. The standard state is given by $c^\circ = 1\,\text{mol}\,\text{L}^{-1}$. By increasing the temperature, T, the protein unfolds, when ΔG° becomes negative. This temperature dependence can be expressed by [4]

$$\Delta G^\circ(T) = \Delta G^\circ(T_{\text{unf}}) + \left(\frac{\partial \Delta G^\circ}{\partial T}\right)_p \times (T - T_{\text{unf}})$$
$$= -\Delta S(T_{\text{unf}}) \times (T - T_{\text{unf}}) \tag{2}$$

Here, we neglect the second order derivative, which is related to the heat capacity change of unfolding, and we use $\Delta G^\circ(T_{\text{unf}}) = 0$, where T_{unf} is the temperature of unfolding. ΔS is the entropy change of

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http://dx.doi.org/10.1016/j.colsurfb.2015.03.043
0927-7765/

unfolding, which has a positive value. The enthalpy change of unfolding can be derived from $\Delta H = T_{unf}\,\Delta S$. In a similar way, the effect of pressure, p, on the standard Gibbs energy of unfolding can be written as [4]

$$\Delta G^{\circ}(p) = \Delta G^{\circ}(p_{unf}) + \left(\frac{\partial \Delta G^{\circ}}{\partial p}\right)_{T} \times (p - p_{unf}) = \Delta V(p_{unf}) \times (p - p_{unf}) \quad (3)$$

where p_{unf} is the pressure of unfolding and $\Delta G^{\circ}(p_{unf}) = 0$. ΔV is the volume change of unfolding, which is usually negative. There are different contributions to ΔV. Most importantly, void volumes (cavities) in the folded conformation of a protein are filled with water molecules upon unfolding, which reduces the total volume of the system, but other contributions are also discussed in literatures [8–10].

Upon adsorption at an aqueous-solid interface, proteins can undergo some changes in conformation and partial unfolding. There are many studies in the literature that characterize these changes in terms of the secondary or the tertiary protein structure [11–20]. Of course, the extent of an adsorption-induced partial unfolding is related to the strength of protein–interface interactions and the folding stability of the protein. However, so far, there seems to be no clear picture or way to predict what kind of secondary structure is formed at aqueous-solid interfaces. On the other hand, there are a few studies in the literature indicating that the temperature range of protein unfolding is broadened and shifted to lower temperatures, when the protein is adsorbed at aqueous-solid interfaces [13,21,22]. Moreover, we have also found recently that the pressure range of protein unfolding is broadened and shifted to lower pressures at interfaces [23]. In this way, adsorption-induced conformational changes of a protein might simply be regarded as the onset of protein unfolding that already occurs at ambient conditions.

Therefore, the broad aim of this study is to investigate, if the conformation of an adsorbed protein can be related to any conformation of a free protein under denaturing conditions. In a systematic way, we study two proteins, free in solution and adsorbed on silica particles, and determine their secondary structures in the course of unfolding under conditions of high pressures and high temperatures. Hen egg white lysozyme and ribonuclease A (RNase) are used as model proteins. To unfold a protein by pressure, pressures in the kbar region are needed. Therefore, we have applied the diamond anvil technique. Using Fourier transform infrared (FTIR) spectroscopy, the secondary structures of the proteins under all conditions has been derived from the amide I′ band, which is composed of subbands arising from the various secondary structure elements of a protein [24]. As we will show here, proteins undergo the same changes in secondary structure in the adsorbed and the free state, when they are unfolded under high pressures or temperatures. The main effect of the interface is only a lowering of the pressure and temperature of unfolding of the proteins. This finding supports the idea that conformations of adsorbed proteins are mostly reflecting the normal unfolding equilibrium that is shifted to the unfolded side.

2. Materials and methods

2.1. Sample preparation

Hen egg white lysozyme (product No. 10837059001) was purchased from Roche Diagnostics (Mannheim, Germany) and ribonuclease A from bovine pancreas (RNase; product No. R5500) from Sigma–Aldrich (Steinheim, Germany). All measurements were performed in D_2O buffer solutions, because the amide I′ band, which is sensitive to the secondary structure of a protein, is overlapping with the bending mode of liquid H_2O. 10 mM phosphate–D_2O buffer with pD = 7.8 (pD = pH-meter reading + 0.4 [25]) and 10 mM Tris–D_2O buffer with pD = 7.8 were used for temperature-dependent and pressure-dependent experiments, respectively. They are characterized by only small pD-shifts when they are heated (phosphate) or pressurized (Tris) [26]. Protein solutions were prepared by dissolving 2 mg of lysozyme or RNase in 200 μL of D_2O followed by heating to 68 °C (lysozyme) or 63 °C (RNase) for 15 min. During this heating, labile H atoms of the protein are exchanged for D atoms. After lyophilization, 100 μL of D_2O buffer solution (phosphate or Tris) were added to generate a 2 wt% protein solution, which was used to study the free protein in the absence of silica particles. In a similar way, a 10 wt% protein solution was also prepared that was used to adsorb protein molecules on silica particles.

Ludox AM colloidal silica (density 1.21 g mL^{-1}, specific surface area > 198 m^2 g^{-1}) from Sigma–Aldrich was used as the source for silica particles. The H_2O solvent of this colloidal dispersion has been replaced by D_2O using Amicon Ultra centrifugal filters (500 μL, 10 kDa). This type of filter retains the silica particles, and only the solvent can pass. A filter was loaded with a 1:1 mixture of the original colloidal dispersion and D_2O buffer solution. After intense mixing and centrifugation at 10,000 × g for 10 min, the volume was reduced from 500 to 300 μL, because 200 μL of the solvent had passed through the filter. The loss of solvent was compensated by addition of 200 μL of fresh D_2O buffer solution. This procedure was repeated eight times. Finally, no residual H_2O could be detected by FTIR spectroscopy.

For protein adsorption on silica particles, the 10 wt% protein solution was mixed with the silica-D_2O dispersion in a volume ratio of 1:4 to generate a 2 wt% protein solution with silica particles that was used in the FTIR experiments. Silica particles have a negative surface charge, whereas the proteins, lysozyme and RNase, have a positive net charge at neutral pD-values (the isoelectric points of lysozyme and RNase are found at pH 11.0 and 9.4, respectively [27,28]). This electrostatic attraction between the protein molecules and the silica particles leads to complete adsorption, as has been confirmed experimentally. After 15 min of incubation, protein-silica sample solutions were filled into Amicon Ultra centrifugal filters (volume 500 μL, pore size 50 kDa) and were centrifuged at 10,000 × g for 15 min. In this way, all non-adsorbed protein is passing through the filter, whereas all proteins that are adsorbed on the silica particles are retained by the filter. The filtrate was analyzed by UV-spectroscopy at 270–300 nm, however, no UV-absorption was detected proving that essentially all lysozyme or RNase molecules are adsorbed on the silica particles. The surface coverage of the silica particles can be calculated as follows. Ludox AM colloidal silica has a surface area of at least 239 m^2 mL^{-1}. During sample preparation, the colloidal dispersion has been diluted in two steps by factors of 2 and 1.25 resulting in a surface area of 95 m^2 mL^{-1}. Lysozyme and RNase have molar masses of 14,300 g mol^{-1} and 13,700 g mol^{-1}, respectively, and a specific volume of 0.703 mL g^{-1} [6]. Assuming spherical shapes, we obtain a radius of $r = 1.6 \times 10^{-9}$ m and a molecular footprint area of $\pi r^2 = 7.8 \times 10^{-18}$ m^2 for both proteins. Samples contain 2 wt% of protein corresponding to 8.6×10^{17} molecules mL^{-1}. Thus, the surface coverage is $8.6 \times 10^{17} \times 7.8 \times 10^{-18}$ m^2/95 m^2 = 0.07 = 7% by lysozyme or RNase.

2.2. FTIR spectroscopy

Infrared absorption spectra of free and adsorbed lysozyme and RNase were recorded using the Nicolet 6700 Fourier transform infrared spectrometer from Thermo Fisher Scientific operating with a liquid nitrogen-cooled MCT detector. The whole spectrometer was continuously purged with dry air. Temperature-dependent

Please cite this article in press as: S. Cinar, C. Czeslik, Secondary structure and folding stability of proteins adsorbed on silica particles – Pressure versus temperature denaturation, Colloids Surf. B: Biointerfaces (2015), http://dx.doi.org/10.1016/j.colsurfb.2015.03.043