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The effect of fatty acid binding in the acid isomerizations of albumin investigated with a continuous acidification method

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ABSTRACT

The protein Human Serum Albumin (HSA) is known to undergo conformational transitions towards partially unfolded forms triggered by acidification below pH 4.5. The extent of Fatty Acids (FA) binding has been thought to have an impact on the conformational equilibrium between the native and acid forms and to be a possible explanation for the observation of more than one band in early electrophoretic migration experiments at pH 4. We compared the acid-induced unfolding processes of commercial FA-free HSA, commercial "fatted" HSA and FA-HSA complexes, prepared at FA:HSA molar ratios between 1 and 6 by simple mixing and equilibration. We used a method for continuous acidification based on the hydrolysis of glucono-δ-lactone from pH 7 to pH 2.5, and followed the average protein changes by the blue shift of the intrinsic fluorescence emission and by performing a small angle X-ray scattering analysis on selected samples. The method also allowed for continuous monitoring of the increase of turbidity and laser light scattering of the protein samples related to the release of the insoluble ligands with acidification. Our results showed that the presence of FA interacting with albumin, an aspect often neglected in biophysical studies, affects the conformational response of the protein to acidification, and slightly shifts the loss of the native shape from pH 4.2 to pH 3.6. This effect increased with the FA:HSA molar ratio so that with three molar equivalents a saturation was reached, in agreement with the number of high-affinity binding sites reported for the FA. These findings confirm that a non-uniform level of ligand binding in an albumin sample can be an explanation for the early-observed conformational heterogeneity at pH 4.

1. Introduction

The protein Human Serum Albumin (HSA) is one of the main macromolecular constituents of extracellular fluids. In the circulation it serves as a carrier and depot for many molecules that can interact with the multiple binding sites located in the three-domain albumin structure [1–3].

This unique ligand-binding ability of the protein is due to a favorable combination of hydrophobic pockets and side-chain charges and to a pronounced flexibility, which, to a large degree, is caused by inter-subdomain linkers and flexible loops [4].

Unesterified fatty acids (FA) are the main physiological ligands for albumin and this specific binding to a highly abundant and soluble protein allows for a FA concentration in plasma and interstitial compartments far beyond the low water solubility of these nutrients, which is important for energy metabolism and synthesis of membrane and signaling lipids. FA-albumin interaction ensures FA transport and delivery to the utilization sites with mechanisms of loading and release, which are not yet fully clarified at a molecular level, but probably rely on the dynamic nature of the FA-albumin complexes, which are rapidly equilibrating [5,6]. The FA binding is also heterogeneously distributed in terms of binding degree and involved binding sites. Indeed, crystallographic studies were able to identify a total of seven common binding sites for FA across the protein structure, in presence of a large excess of the ligands. On the other hand, when performing crystallization experiments at lower molar ratios, the solved structures always showed an average electron density in several pockets, thus supporting the idea of heterogeneity [7]. Previous binding affinity studies had predicted two or three highest affinity sites [8] and it was shown that two of them are located in domain III, whereas a third high affinity pocket should be located at the interface between domains I and II (Supplementary data, Fig. S1) [9].

The relatively easy availability of albumin, especially from bovine serum (BSA) made it one of the most used model proteins

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in physical-chemical and biophysical studies. It should be noted, however, that many biophysical studies did neglect to specify the "fatted" or "defatted" nature of the albumin product used.

Albumins have many applications in colloidal science and they are increasingly used in biomedical and engineering applications. They are prone to conformational changes during adsorption (and desorption) when applied as medical device coating to prevent adhesion from bacteria, platelets and other proteins [10–12].

In many formulations as drug nanocarriers, albumin is prepared in the form of nanoparticles [13,14]. It can also be used to stabilize colloidal particle dispersions against sedimentation, for example with multi-layer polyelectrolyte nanoparticles. Generally, the adsorption of the protein onto the particles' surfaces yields an albumin-mediated steric stabilization. Additionally, albumin coating diminishes phagocytosis of nanocarriers by macrophages and increases their colloidal stability and circulation lifetime in albumin-containing media [15,16]. In the context of tuning the monodispersity as well as the maximization of the coverage and of an irreversible adsorption, knowledge of the protein behavior under various pHs and ionic strengths is essential for robust protocol development.

Specific and well-defined pathways of structural rearrangements have been reported for albumin in responses to different triggers such as denaturant addition [17–20], acidification [21–24] and oxidation [25]. In particular, the acid isomerizations of the protein occurring below pH 4.5 have been object of several studies. According to a well-established view, the native form of the protein in solution (N), which resembles the crystal structure, is replaced by intermediately unfolded conformers referred to as the F form below pH 4.5, whereas an expansion of the molecule towards extended isomers, the E-form, occurs below pH 3.5 [1]. A substantial amount of biophysical data in a variety of buffer conditions has been collected on serum albumin in acid environment, where the N-F and F-E transitions take place [21,22,26–28]. In the early literature, heterogeneity in the acid-induced unfolding or protein precipitation processes at low pH was noticed and the uncontrolled presence of FA was suggested as the main reason behind these inconsistencies [29]. One of the early observations was the presence of more than one band in the electrophoretic migration of the protein at pH 4 [30,31], with relative intensity of the bands linked to titration with oleic acid [32].

Recently a method based on the hydrolysis kinetics of glucono-δ-lactone (GdL) for continuous acidification of the protein solution, was applied to characterize with fluorescence spectroscopy and small angle X-ray scattering (SAXS) the rearrangements involved in the acid isomerization of HSA, without the variability of multiple sample preparation [24]. In the same work preliminary results showed that the presence of 1:3 molar equivalents of palmitic acid induced a difference in the pH unfolding efficiency of albumin, compared to the defatted protein alone. This effect was tentatively explained by a suppression of the partially denatured conformer F in the presence of the FA ligands, analogous to the suppression of an intermediate state in the path of urea-dependent unfolding [18]. However this hypothesis could only partially explain the delay of the fluorescence blue-shift at lower pH values. In addition, further experiments involving the binding of other FA than palmitic acid to albumin and at different molar equivalents, could extend this preliminary observation to a more general effect.

In the present work we aimed at using this continuous acidification method to further investigate the effect of FA binding on the conformational changes of the protein in acid environment. For this purpose, we compared the acid unfolding of samples of HSA with different degrees of FA binding. In particular solutions prepared from commercial FA-free protein ("defatted" HSA), from commercial globulin-free but not delipidated albumin ("fatted" HSA, HSAFatted) and from defatted HSA with known additions of saturated FA (lauric acid C12:0 – HSALau, myristic acid C14:0 – HSAMyr, palmitic acid C16:0 – HSAPalm, stearic acid C18:0 – HSASte) at HSA:FA molar ratios 1:1, 1:3 and 1:6 were studied. The fluorescence analysis of the protein's tryptophan emission during acidification revealed that the characteristic blue shift observed concurrently to the loss of the native conformation occurred at slightly lower pH values in the presence of the FA ligands. Correction for UV–vis absorption suggested no mis-interpretation due to the increase in turbidity. The analysis of SAXS data sets of the structural rearrangements of HSA and HSAFatted as a function of decreasing pH suggested that the effect of delay of the fluorescence blue-shift in the presence of FA could be driven by a shift of the appearance of the partially unfolded intermediate F-form and of the extended form to slightly lower pH values. These findings demonstrated a dependence of the acid response of HSA on the presence of FA and provided a possible approach to correlate the stability of complexes formed by HSA and specific classes of ligands with the unfolding propensity in acid conditions.

2. Materials and methods

2.1. Sample preparation

For the FA-free HSA and the FA-HSA complexes, HSA lyophilized powder, fatty acid- and globulin-free (≥ 99%, type A3782, Sigma) was used, whereas for the "fatted" HSA the Sigma product A8763 was employed. Stock solutions at concentrations between 10 and 20 g/l were prepared by dissolving the powders in 10 mM sodium phosphate buffer at pH 7.4. Hence, the solutions were centrifuged at 12×10^3 g for 5 min and the protein concentration was estimated by measuring the absorbance at 280 nm and using the extinction coefficient [33] 35,700 M^{-1} cm^{-1}. Finally, diluted working solutions were prepared to reach a protein concentration of 3 g/l (around 45 μM), suitable for the SAXS experiments or 1 g/l (around 15 μM) for the spectroscopic and light scattering measurements. For the preparation of FA-HSA samples, comprehensive experimental reports are given in the Supplementary data, where details are reported which account for nontrivial aspects highlighted in the literature for a successful FA-HSA complex formation [34]. The acidification was started in all cases by adding a known volume of the protein working solution to a weighted amount of glucono-δ-lactone (GdL, ≥99% type G4750, from Sigma) and mixing for 30 s to induce complete solubilization of the lactone. For the blanks, the same procedure was applied using the buffer rather than the protein solution. The GdL amount added in the samples was 2% and 5% in the SAXS experiments, and 2% in the spectroscopy and light scattering experiments, expressed as weight of GdL (g)/volume of protein solution (ml).

2.2. pH-time calibration

A calibration of the pH variation over time in the same conditions applied during the experiments (Supplementary data, Fig. S2) allowed for reporting the variation of the experimental parameters as a function of pH rather than as a function of the time elapsed from the GdL addition. For samples identical to those analyzed and at the same temperature of the experiments, pH-vs.-time profiles were measured with a Hamilton minitrode electrode connected to a Crison 2002 pH-meter and calibrated with standard buffer solutions at pH 7.00 and 4.01.

2.3. Fluorescence measurements

The intrinsic fluorescence was measured by exciting the protein solution at λ_{ex} = 295 nm with a band-pass of both excitation and emission monochromators of 5 nm. Emission spectra were

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