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International Journal of Biological Macromolecules 37 (2005) 200–204

INTERNATIONAL JOURNAL OF
Biological
Macromolecules
STRUCTURE, FUNCTION AND INTERACTIONS

www.elsevier.com/locate/ijbiomac

Unfolding pathways of human serum albumin: Evidence for sequential unfolding and folding of its three domains

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Received 22 September 2005; received in revised form 26 October 2005; accepted 26 October 2005

Abstract

Human serum albumin (HSA) contains three α-helical domains (I–III). The unfolding process of these domains was monitored using covalently bound fluorescence probes; domain I was monitored by *N*-(1-pyrene)maleimide (PM) conjugated with cys-34, domain II was monitored by the lone tryptophan residue and domain III was followed by *p*-nitrophenyl anthranilate (NPA) conjugated with Tyrosine-411 (Tyr-411). Using domain-specific probes, we found that guanidium hydrochloride-induced unfolding of HSA occurred sequentially. The unfolding of domain II preceded that of domain I and the unfolding of domain III followed that of domain I. In addition, the domains I and III refolded within the dead time of the fluorescence recovery experiment while the refolding of domain II occurred slowly. The results suggest that individual domain of a multi-domain protein can fold and unfold sequentially.

Keywords: HSA; Pyrene maleimide; NPA; GdnHCl; Sequential unfolding

1. Introduction

Human serum albumin (HSA), a major protein component of blood plasma, has three structurally similar α-helical domains I–III [1,2]. The folding of a multi-domain protein could be a complex process because each domain can fold independently and inter-domain interactions can regulate the overall folding process [3–9]. The unfolding pathways of HSA have been examined in several studies [10–16]. Some studies detected an intermediate in the unfolding pathway whereas others concluded that unfolding occurs in a single concerted step [11,12,15,16]. The studies involving hydrophobic probes or fluorescence energy transfer between the two fluorophores that are located at two different domains suggested that unfolding occurred in two steps with the formation of an intermediate [15,16]. However, studies using tryptophan fluorescence and CD suggested one-step unfolding [10,16]. It is possible that each of three domains of HSA has different sensitivity towards chemical unfolding. Hydrophobic probes, such as nile red are highly environment sensitive [14,16]. Therefore, the apparent biphasic changes in the fluorescence intensity of nile red could arise from the average signal of sequential unfolding of different domains rather than unfolding of HSA in two steps [16]. Similar explanation is also plausible for the energy transfer studies involving two domains. It is interesting to examine whether unfolding of each domain of HSA can occur in a single concerted step or in multiple steps. The presence of a single cysteine residue in domain I, a single tryptophan residue in domain II and a highly reactive tyrosine residue in domain III provides a unique opportunity to follow the unfolding and folding pathways of each domain of HSA [1,14,15,17,18]. Previously, it has been shown that Tyrosine-411 (Tyr-411) located in domain III is the only tyrosine residue accessible for chemical modification by *p*-nitrophenyl anthranilate (NPA) [17,18].

In this study, we have monitored the unfolding process of each of the three domains of HSA by following the fluorescence signal of a probe that is covalently linked to a specific domain. The domain I was monitored by fluorescence of pyrene maleimide, which was covalently linked to cys-34, domain II was monitored by fluorescence of intrinsic tryptophan residue and domain III was examined by the fluorescence of NPA, which was covalently bound to Tyrosine-411. Using domain-specific

Abbreviations: GdnHCl, guanidine hydrochloride; HSAP, human serum albumin; NPA, *p*-nitrophenyl anthranilate; PM, pyrene maleimide

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doi:10.1016/j.ijbiomac.2005.10.009

probes, the unfolding of each domain of HSA was found to occur in a single concerted step. However, the unfolding of domain II preceded the unfolding of domain I and the unfolding of domain III followed the unfolding of domain I suggesting that three domains of HSA unfold sequentially. Further, the refolding experiments showed that the refolding of domains I and III was nearly complete with in the mixing time of the experiment while the refolding of domain II occurred slowly. The results show that multi-domain proteins can fold and unfold in a stepwise fashion and support the idea that each domain of a multi-domain protein can fold and unfold differently.

2. Materials and methods

2.1. Materials

HSA, fraction V, essentially fatty acid free was purchased from Calbiochem, USA. According to the manufacturer the purity level of HSA is ≥98%. We also confirmed the purity level of HSA by Coomassie blue staining of the SDS-PAGE (data not shown). Protein concentration was measured by Bradford method using bovine serum albumin as standard [19]. GdnHCl was obtained from Aldrich Chemical Co. BSA (fraction V), Coomassie blue and *p*-nitrophenyl anthranilate were obtained from Sigma Chemical Co. *N*-(1-Pyrene)maleimide (PM) was purchased from Molecular Probes.

2.2. Spectroscopic studies

All fluorescence studies were performed using JASCO FP-6500 spectrofluorometer equipped with a constant temperature water-circulating bath. All circular dichroism studies were performed in a JASCO J810 spectropolarimeter equipped with a Peltier temperature controller. The secondary structure was monitored over the wavelength range of 200–260 nm using a 0.1 cm path length cuvette and ellipticity was determined at 220 nm. We used 5 μM of the labeled and unlabeled HSA for the measurement of secondary structures. Each spectrum was recorded using an average of five scans.

2.3. Chemical modification of cys-34

Chemical modification of cys-34 of domain I was performed using a procedure described recently [14]. Briefly, HSA (200 μM) in 25 mM phosphate buffer (pH 7) was incubated with 6 M GdnHCl at 25 °C for 4 h. Then, five-fold molar excess of PM was added to the unfolded reaction mixture and incubated for an additional 5 h at 25 °C. The labeling reaction was quenched by adding excess β-mercapto ethanol to the reaction mixture. The unbound dye was removed by extensive dialysis against 25 mM phosphate buffer of pH 7.We measured the concentration of HSA in the PM-labeled protein by Bradford method. PM concentration was determined by measuring absorbance at 337 nm using the molar extinction coefficient of 40,000 M^{-1} cm^{-1}. The incorporation ratio of PM per mol of HSA was calculated by dividing the bound PM concentration by the HSA concentration. The incorporation ratio was found to be 0.95 ± 0.05. GdnHCl-induced unfolding of PM-labeled HSA (PM-HSA) was studied using 337 nm as an excitation wavelength and measuring the fluorescence intensity at 380 nm.

2.4. Chemical modification of Tyr-411

Tyrosine-411, located in domain III, has an unusually low pK_a of 8.3 and it is almost 20 times more reactive than the rest of the 19 tyrosine residues in HSA [17,18]. It was established that only Tyrosine-411 could be covalently modified by *p*-nitrophenyl anthranilate, a tyrosine-specific probe [20]. Therefore, NPA-labeled HSA (NPA-HSA) can be used as a probe to monitor the unfolding process of domain III. HSA (200 μM) was incubated with 225 μM NPA for 7 h at 25 °C in 25 mM phosphate buffer, pH 8. The reaction mixture was exhaustively dialyzed against 25 mM phosphate buffer, pH 7 at 4 °C to remove the free NPA and *p*-nitrophenol. Unfolding of NPA-HSA (5 μM) was studied in the presence of different concentrations of GdnHCl by exciting the labeled HSA at 360 nm and measured the fluorescence at 410 nm. The distance between tryptophan located in domain II and tyrosine located in domain III was found to be 24.3 Å using fluorescence resonance energy transfer, which is consistent with the previously reported distance [20].

2.5. Equilibrium unfolding of labeled and unlabeled HSA

For equilibrium unfolding studies, 5 μM HSA (labeled or unlabeled) in 25 mM phosphate buffer (pH 7) was incubated in the presence of different concentrations (0–7 M) of GdnHCl for 30 min at 25 °C and the unfolding of HSA was measured either by monitoring fluorescence (tryptophan, NPA or PM) or circular dichroism. All the excitation and emission parameters were the same as described in the previous section. We used 0.3 cm path length fluorescence cuvette for fluorescence measurements.

2.6. Calculation of D_m values for steady-state unfolding of HSA

We have calculated the mid point of unfolding transition as described previously [16]. Briefly, the free energy (ΔG_{U-F}) of unfolded proteins in the presence of different concentrations of GdnHCl [*D*] has a linear relationship with denaturant concentrations and it can be written as:

$$\Delta G^{D}_{U-F} = \Delta G^{H_2O}_{U-F} - m[D] \quad (1)$$

where *m* is the slope of the transition, $\Delta G^{H_2O}_{U-F}$ is the free energy of unfolding in the absence of denaturant. Change of any physical property (e.g. secondary structure or fluorescence) of a protein in the presence of different concentrations of GdnHCl can be expressed as:

$$F = \frac{(\alpha_F + \beta_F[D]) + (\alpha_U + \beta_U[D]) \times \exp\{m([D] - [D]_{50\%})/RT\}}{1 + \exp\{m([D] - [D]_{50\%})/RT\}} \quad (2)$$

where α_F and α_U are the intercepts and β_F and β_U are the slopes of the baselines of the equilibrium property (*F*) at low and