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

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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 occurs 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
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 formed in a JASCO J810 spectropolarimeter equipped with a water-circulating bath. All circular dichroism studies were performed using a procedure described recently [14]. Briefly, HSA 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 ($\Delta G_{m \rightarrow U}$) 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:

$$\Delta G_{m \rightarrow U} = \Delta G_{m \rightarrow U}^{(0)} - m[D]$$

where $m$ is the slope of the transition, $\Delta G_{m \rightarrow U}^{(0)}$ 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:

$$F = \frac{\exp[m(D) - (D)^{\alpha U} - m[D]]}{1 + \exp[m(D) - (D)^{\alpha U} - m[D]]}$$

where $\alpha_U$ and $\alpha_L$ are the intercepts and $\beta_L$ and $\beta_U$ are the slopes of the baselines of the equilibrium property ($F$) at low and high concentration of denaturant.

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 7. Then, five-fold molar excess of PM-mercapto ethanol to the reaction mixture. Reduced HSA was treated with 225 μM NPA for 7 h at 25 °C and the unfolded HSA (PM-HSA) 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].
high GdnHCl concentrations, respectively: $D_{m}(\{D\}_{50\%})$ is the GdnHCl concentration at which 50% of the protein is unfolded. $R$ is the universal gas constant and $T$ is the absolute temperature. The data were fitted to this equation using the nonlinear least-square method to obtain the best fitted $m$ and $D_{m}$. The curve fitting were performed using MICROSOFT ORIGIN (7.5) software.

2.7. Refolding kinetics of labeled and unlabeled HSA

Labeled (PM-HSA and NPA-HSA) or unlabeled HSA (100 $\mu$M) was incubated with 6 M GdnHCl for 30 min at 25 $^\circ$C in 25 mM phosphate buffer, pH 7. The unfolded sample was diluted 50 times in phosphate buffer to adjust the final concentration of HSA to 2 $\mu$M and measured the fluorescence at different time intervals. We measured the fluorescence intensity of tryptophan, PM and NPA for unlabeled HSA, PM-HSA and NPA-HSA, respectively, using the excitation and emission wavelength described previously. The fraction of refolding was calculated by using the following equation:

$$F_r = 1 - \frac{F}{F_N}$$

where $F_r$ is the fraction refolded, $F$ the observed fluorescence intensity at different time intervals, $F_N$ the fluorescence intensity of the labeled or unlabeled HSA in the native state and $F_D$ is the fluorescence intensity in the presence of 6 M GdnHCl.

3. Results

3.1. Unfolding of NPA-HSA by monitoring NPA fluorescence

The Tyr-411 positioned at domain III was covalently modified by NPA as described under Section 2. Under the conditions used $0.92 \pm 0.1$ mol of NPA was incorporated per mol of HSA. Fig. 1A shows the fluorescence spectra of NPA-HSA in the presence of different concentrations of GdnHCl. The changes in fluorescence intensity of NPA-HSA at 410 nm with increasing concentration of GdnHCl are shown in Fig. 1B. The fluorescence of NPA-HSA did not change significantly up to 2 M GdnHCl. Beyond 2 M GdnHCl, NPA fluorescence gradually decreased with increasing concentration of GdnHCl and the limiting fluorescence was attained at 6 M GdnHCl. The fluorescence intensity changes were fitted in a two-state transition model and the mid point of the unfolding transition ($D_{m}$) was calculated to be $2.9 \pm 0.2$ M and the standard free energy change was calculated to be $5.8 \pm 0.5$ kcal mol$^{-1}$.

3.2. Unfolding of HSA was monitored by PM-HSA fluorescence

The single sulfhydryl group, cys-34 located in domain I was chemically modified by PM. The changes in the fluorescence spectra of PM-HSA at different concentrations of GdnHCl are shown in Fig. 2A. There was no detectable change in fluorescence intensity up to 2 M GdnHCl (Fig. 2B). Further increase in the GdnHCl concentration reduced the fluorescence intensity of PM-HSA (Fig. 2B). The fluorescence intensity changes were fitted in a two-state transition model. The $D_{m}$ value for the unfolding transition of PM-HSA was calculated to be $4.4 \pm 0.2$ M and the standard free energy change was calculated to be $5.4 \pm 0.7$ kcal mol$^{-1}$.

3.3. Unfolding of HSA was monitored by intrinsic tryptophan fluorescence

GdnHCl-induced unfolding of unlabeled HSA was monitored by the fluorescence intensity changes of the single tryptophan residue located in domain II. The fractional unfolding of HSA with increasing concentrations of GdnHCl is shown in Fig. 3
Fig. 2. Unfolding of PM-labeled HSA by GdnHCl. Fluorescence spectra of PM-HSA in the absence (○) and presence of 2 M (□), 4 M (▽) and 6 M GdnHCl (△) are shown in panel (A). Panel (B) shows the change of PM fluorescence intensity of PM-HSA at 380 nm against different concentrations of GdnHCl.

Using a two-state transition model, the $D_m$ of the unfolding transition was calculated to be $2.0 \pm 0.05$ M and the standard free energy change was calculated to be $10.1 \pm 1.6$ kcal mol$^{-1}$.

3.4. Circular dichroism study of unlabeled, NPA-labeled and PM-labeled HSA

The covalent modification of HSA by NPA and PM did not detectably perturb the far-UV CD spectra of HSA suggesting that the secondary structures of HSA did not change because of the covalent modification of the protein by PM or NPA (data not shown). In addition, HSA, NPA-HSA and PM-HSA displayed similar changes in the far-UV CD signals with increasing concentration of GdnHCl (Fig. 4). For example, the $D_m$ values were found to be $2.2 \pm 0.2$, $2.7 \pm 0.1$ and $2.2 \pm 0.3$ M GdnHCl for HSA, NPA-HSA and PM-HSA, respectively. The results showed that unfolding pattern of HSA was not perturbed by chemical modification and indicated that the chemical modification did not alter the stability of the proteins against GdnHCl.

3.5. Refolding kinetics of labeled and unlabeled HSA

We studied the refolding of HSA by monitoring the refolding of individual domain. Like unfolding studied, refolding of domains I–III were examined by monitoring the fluorescence of PM-HSA, unlabeled HSA and NPA-HAS, respectively. The refolding kinetics of unlabeled HSA, NPA-HSA and PM-HSA were found to be different (Fig. 5). For example, the refolding of HSA was found to be nearly completed in the dead time of the experiment when monitored by NPA or PM fluorescence.
However, the recovery of tryptophan fluorescence occurred slowly. The complete recovery of the fluorescence for all the constructs demonstrated that HSA unfolds reversibly.

4. Discussion

The unfolding patterns and \( D_{\text{m}} \) values of GdnHCl-induced unfolding of the three HSA constructs (PM-HSA, NPA-HSA and HSA) were found to be similar by far-UV CD spectroscopy. However, the \( D_{\text{m}} \) values for the three constructs differed significantly when examined by fluorescence spectroscopy; the \( D_{\text{m}} \) values were found to be 4.4 ± 0.2, 2.9 ± 0.2 and 2 ± 0.05 M GdnHCl for PM-HSA, NPA-HSA and HSA, respectively. The differences in the \( D_{\text{m}} \) values obtained using different probes can be explained by considering that each fluorescence probe reports the unfolding of a specific domain of HSA to which it is covalently linked, rather than the global unfolding of HSA. Thus, the changes in PM, tryptophan and NPA fluorescence may be more representative of the unfolding of domains I–III, respectively, rather than the global unfolding of the protein. The equilibrium unfolding of each of the three domains of HSA, monitored by tryptophan, NPA and PM fluorescence, was found to occur in a single step from the N → U suggesting that the unfolding process of a particular domain of HSA is cooperative in nature. Interestingly, studies involving energy transfer between two residues that are located in different domains of HSA [15] and environment-sensitive fluorescence probes reported that the unfolding of HSA occurred in multiple steps with the formation of at least one intermediate [14–16]. The unfolding process monitored by fluorescence energy transfer may appear to occur in multiple steps if each domain has different sensitivity to GdnHCl and they unfold sequentially. Similarly, the unfolding of HSA measured by hydrophobic probes, such as nile red could be biphasic because the binding of nile red to HSA might depend on the integrity of multiple domains [16].

The fluorescence recovery of PM, tryptophan and NPA demonstrated that the folding kinetics of the three domains of HSA were different (Fig. 5). The refolding rate of domain II was slower than the refolding rates of domains I and III. Probably the refolding of domain II was dependent on the refolding of domains I and III. Overall, the folding and unfolding patterns of the three domains of HSA were different and their stabilities were also different. This indicates the possibility of sequential folding mechanism. The different stabilities of the domains indicate that domains may unfold–refold independently at least at the initial phase of unfolding–refolding. Subsequently, the folding follows a sequential pattern guided by some inter-domain interactions. The results show that the folding pattern of a multi-domain protein like HSA is complex and that the use of multiple probes is necessary for understanding its folding mechanism.

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