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Intermediate formation at lower urea concentration in 'B' isomer of human serum albumin: a case study using domain specific ligands

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Abstract

The urea-induced unfolding of 'N' isomer (occurring at pH 7.0) and 'B' isomer (occurring at pH 9.0) of human serum albumin was studied by fluorescence and circular dichroism spectroscopic measurements. Urea-induced destabilization in different domains of both the isomers was monitored by using domain specific ligands, hemin (domain-I), chloroform, bilirubin (domain-II), and diazepam (domain-III). Urea-induced denaturation of N and B isomers of HSA showed a two-step, three-state transition with accumulation of intermediates around 4.8–5.2 M and 3.0–3.4 M urea concentrations, respectively. During first transition (0–4.8 M urea for N isomer and 0–3.0 M urea for B isomer) a continuous decrease in diazepam binding suggested major conformational changes in domain-III prior to intermediate formation. On the other hand, binding of hemin, a ligand for domain-IB and chloroform, whose binding site is located in domain-IIA remains unchanged up to 5.0 M urea for N isomer and 3.0 M urea for B isomer. Similarly, fluorescence intensity of Trp-214 that resides in domain-IIA remained unchanged up to the above-said urea concentrations and decreased thereafter. Absence of any decrease in hemin binding, chloroform binding, and Trp-214 fluorescence suggested the non-involvement of domain-IB and domain-IIA in intermediate formation. A significant increase in bilirubin binding prior to intermediate formation showed favorable conformational rearrangement in bilirubin binding cavity formed by loop 4 of domain-IB and loop 3 of domain-IIA. Further, a nearly complete abolishment of bilirubin binding to both isomers around 7.0 M and 6.0 M urea concentrations, respectively, indicated complete separation of domain-I from domain-II from each other. From these observations it can be concluded that N to B transition of human serum albumin shifted the intermediate formation towards lower urea concentration (3.0–3.4 M urea for B isomer as against 4.8–5.2 M urea for N isomer). Further both the intermediates were found to possess similar α-helical (~39%) content and ligand binding properties.

Keywords: Human serum albumin; Ligand binding; N ↔ B transition; Urea-induced unfolding; Equilibrium intermediate

Human serum albumin (HSA) is a single polypeptide chain, multi-domain protein that aids in the transport, metabolism, and distribution of exogenous and endogenous ligands [1]. HSA consists of three homologous domains encompassing the complete sequence. Its overall native three dimensional conformation is stabilized by various intra- and inter-domain forces such as salt bridges, hydrophobic interactions, and natural boundaries involving helical extensions h_{10} (domain-I)–h_1 (domain-II) and h_{10} (domain-II)–h_1 (domain-III) existing between three domains of albumin [2,3]. Under slightly alkaline conditions, between pH 7.0 and 9.0, HSA as well as its bovine counterpart, BSA undergo a conformational change known as N ↔ B transition. It is supposed to be a structural fluctuation, a loosening of the molecules with the loss of rigidity, particularly affecting the N-terminal region [4–8]. It has been suggested that N ↔ B transition involves breaking of salt bridges from domain-I to domain-III, causing increased flexibility of molecules [3,9,10]. Further in the alkaline pH range between pH 7.0 and 9.0, HSA showed a slight reduction in helical content and a small increase in β-sheet structure [3,10]. The recombinant domains of HSA did not show significant alteration in their

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secondary structure content in the alkaline pH region. It was therefore suggested that main losses in secondary structure were affecting the two inter-domain helices ($h_{10\ domain\text{-}I}$–$h_{1domain\text{-}II}$, $h_{10domain\text{-}II}$–$h_{1domain\text{-}III}$) of HSA and that the secondary structural integrity of the domains is not impaired in the N ↔ B transition [3].

Recent advances in physical techniques [11,12] have shown the presence of stable intermediate conformation in a number of proteins [13–15]. It has now become clear that folding of many proteins may proceed through these intermediate states [16]. The folding process is even more complex in multi-domain proteins [17–19] where each domain may be capable of unfolding/refolding independently and inter-domain interactions may affect the overall folding topology.

Albumin denaturation by urea at neutral pH has been studied by a number of workers. These studies have established that a stable intermediate exists during urea denaturation of serum albumin [20–23]. Tayyab et al. [22], by using domain specific ligands to BSA, have suggested major conformational changes in domain-III and partial but significant loss of native conformation in domain-I prior to intermediate formation.

In this study attempts have been made to characterize the urea-induced structural transition in N and B isomers of human serum albumin using domain specific ligands namely, hemin which binds to domain-IB [24,25], chloroform which binds in the close vicinity of single Trp-214 [26], bilirubin which binds to domain-II [27], and diazepam which binds to domain-III [24]. The study will help in understanding the structural and functional stability of B isomer of HSA as compared to N isomer. That the N ↔ B transition has physiological significance is suggested by the fact that under increased Ca^{2+}concentration in blood plasma, the B isomer predominates [28]. Moreover, it will also help in understanding the role of inter-domain interactions such as salt bridges and natural boundaries between different domains as B isomer of HSA is lacking these interactions [10].

Materials and methods

Materials. Human serum albumin (HSA) essentially fatty acid free, lot no. 90K7604; ultra pure urea, lot no. 42K0133, and hemin were obtained from Sigma Chemical, USA. Bilirubin and chloroform were purchased from Sisco Research Laboratories, India.

Diazepam was a product of Ranbaxy Laboratories, India. All the other reagents were of analytical grade. HSA was freed from dimers and higher-mers by passing through Sephacryl-S100 (HR) (76 × 1.8 cm) gel filtration column.

Protein concentration was determined spectrophotometrically using $E^{1\%}_{1\,cm}$ of 5.30 at 280 nm [29] on a Hitachi spectrophotometer, model U-1500 or alternately by method of Lowry et al. [30]. Bilirubin concentration was also determined spectrophotometrically using a molar absorption coefficient of 47500 M^{-1} cm^2 at 440 nm [31].

CD measurements. CD measurements were carried out with a Jasco spectropolarimeter, model J-720 equipped with a microcomputer. The instrument was calibrated with D-10-camphorsulfonic acid. All the CD measurements were made at 25 °C with a thermostatically controlled cell holder attached to Neslab's RTE—110 water bath with an accuracy of ± 0.1 °C. Spectra were collected with a scan speed of 20 nm/min and a response time of 1 s. Each spectrum was the average of four scans. Far UV CD spectra were taken at protein concentrations of 1.8–2.0 μM with a 1-mm path length cell. The results were expressed as MRE (Mean Residue Ellipticity) in deg cm^2 mol^{-1} which is defined as $MRE = \theta_{obs}/(10 \times n \times l \times Cp)$ where θ_{obs} is the CD in milli-degree, n is the number of amino acid residues (585), l is the path length of the cell, and Cp is the mole fraction. Helical content was calculated from the MRE values at 222 nm using the following equation as described by Chen et al. [32]:

$$\%\ \alpha - \text{helix} = (MRE_{222\,nm} - 2340/30300) \times 100.$$

Fluorescence measurements. Fluorescence measurements were performed on Shimadzu spectrofluorimeter, model RF-540 equipped with a data recorder DR-3. The fluorescence spectra were measured at 25 ± 0.1 °C with a 1-cm path length cell. The excitation and emission slits were set at 5 and 10 nm, respectively. Intrinsic fluorescence was measured by exciting the protein solution at 280 or 295 nm and emission spectra were recorded in the range of 300–400 nm.

Denaturation experiments. Solutions for the denaturation experiments of N and B isomers of HSA were prepared in 60 mM sodium phosphate buffer of pH 7.0 and 20 mM glycine–NaOH (Gly–NaOH) buffer of pH 9.0, respectively. To a 0.5 ml stock protein solution, different volumes of the desired buffer were added first; followed by the addition of a stock denaturant solution (10 M urea) to get a desired concentration of the denaturant. The final solution mixture (3.0 ml) was incubated for 10–12 h at room temperature before optical measurements.

Chloroform binding. Binding of chloroform to N and B isomers of HSA at different urea concentrations was studied by fluorescence quenched titration method [26]. To a fixed volume (3.0 ml) of protein solution previously incubated with different urea concentrations for 10–12 h at room temperature, increasing volumes (1–15 μl) of chloroform were added. Fluorescence was measured after 1 h at 340 nm after exciting the protein samples at 295 nm. The data were plotted as relative fluorescence vs. chloroform concentration (mM).

Bilirubin binding. Bilirubin solution was prepared by dissolving 5 mg of solid bilirubin in 0.5 N NaOH containing 1 mM EDTA and immediately diluting it to the desired volume of 50 mM sodium phosphate buffer, pH 7.0, and/or 20 mM Gly–NaOH buffer, pH 9.0. The binding of bilirubin to albumin (at pH 7.0 as well as pH 9.0), at different urea concentrations was studied using fluorescence enhancement technique [33]. To a fixed volume of stock protein solution (3.5 μM), previously incubated with different urea concentrations for 10 h at room temperature, increasing volumes of stock bilirubin solution were added to achieve different bilirubin/albumin molar ratios. The fluorescence was measured at 530 nm after exciting the bilirubin–albumin complex at 466 nm. The spectra were recorded in dark after 15–20 min of the addition of bilirubin to protein solution. The data were plotted as relative fluorescence against bilirubin/albumin ratio.

Diazepam binding. To study the binding of diazepam to the urea—denatured HSA (at pH 7.0 and 9.0) stock protein solution (3.5 μM), previously incubated with different urea concentrations for 10–12 h at room temperature, was titrated with increasing diazepam concentrations to get different diazepam/protein molar ratios. The solution was excited at 280 nm after 30–40 min incubation at room temperature and the fluorescence emission was measured at 340 nm. The data were plotted as relative fluorescence vs. diazepam/protein molar ratio.

Hemin binding. Immediately prior to use, hemin was dissolved in 10.0 mM NaOH to obtain a stock concentration of 4.0 mM. To a fixed volume of stock protein solution (3.5 μM), previously incubated with different urea concentrations for 10 h at room temperature, increasing volumes of stock hemin solution were added to achieve different hemin/albumin molar ratios and the fluorescence was measured at

340 nm after exciting the solution at 280 nm. The spectra were recorded after 15–20 min of the addition of hemin to protein solution. The data were plotted as relative fluorescence against hemin/albumin ratio.

Results and discussions

Urea-induced denaturation of N isomer of HSA

Figs. 1A and B show the urea-induced denaturation of N isomer of HSA as monitored by the measurements of intrinsic fluorescence at 340 nm (after exciting the protein at 280 nm) and MRE at 222 nm, respectively. As can be seen from Figs. 1A and B urea-induced denaturation of N isomer of HSA, which started from 2.5 M urea and completed around 8.4 M urea, followed a two-step, three-state transition with accumulation of an intermediate (I) state around 4.8–5.2 M urea concentration. The 'I' state was characterized by abundant secondary structure, i.e., ~40% α-helix as compared to ~58% α-helix found in the native protein as calculated from MRE values at 222 nm. These results are in agreement with the earlier observations made for a number of proteins including HSA and BSA [20–23,34–38].

Urea-induced denaturation of B isomer of HSA

Urea-induced denaturation of basic (B) isomer of HSA (Figs. 2A and B) was also found to be a two-step process with accumulation of an intermediate (I′) state at lower urea concentration (~3.0–3.4 M). The first transition which corresponds to transformation of B state to I′ state started at ~2.0 M urea and completed around 3.0 M urea concentration. The second transition which corresponded to the unfolding of I′ state started at around 3.5 M urea and finally sloped off to the unfolded (U) state at 8.5 M urea concentration. The 'I''

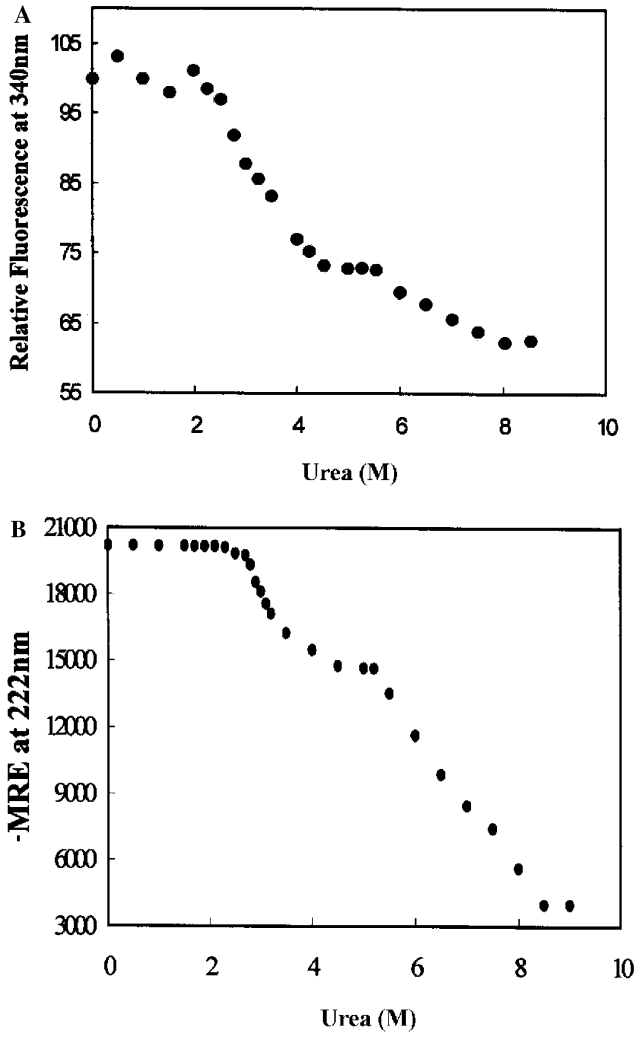

Fig. 1. (A) Urea denaturation profile of 'N' isomer of HSA as monitored by intrinsic fluorescence measurement at 340 nm and (B) by MRE measurement at 222 nm.

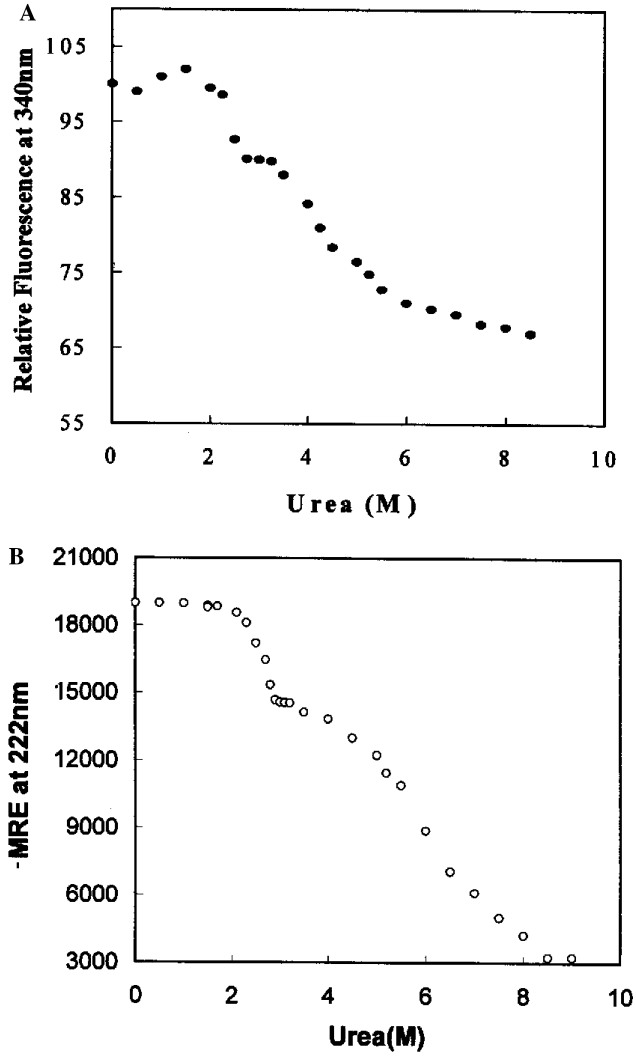

Fig. 2. (A) Urea denaturation profile of 'B' isomer of HSA as monitored by intrinsic fluorescence measurement at 340 nm and (B) by MRE measurement at 222 nm.

state possesses ~39% α-helical structure as compared to 45% in basic HSA. Therefore, urea-induced denaturation of both N and B isomers of HSA may be approximated to a two-step, three-state transition and mechanism for unfolding for the two forms may, respectively, be represented as:

$$N \leftrightarrow I \leftrightarrow U$$

$$B \leftrightarrow I' \leftrightarrow U$$

In order to monitor the loss of native conformation in different domains of HSA (both N and B isomers) during intermediate formation, we studied the binding of different ligands hemin (for domain-I), chloroform and bilirubin (for domain-II), and diazepam (for domain-III) to native as well as urea denatured HSA. Further, conformational changes in domain-II were also investigated by monitoring the fluorescence of single Trp-214, which resides in domain-II of the HSA.

Conformational transition in domain-I

Hemin binding

X-ray crystal structure of HSA-hemin complex and binding studies indicated a single site for hemin in domain-IB [24,25]. To get insight into structural alterations in domain-IB, the binding of hemin to HSA (both N and B isomers) was studied at different urea concentrations. The fluorescence quench titration data at increasing hemin/albumin molar ratio are shown in Fig. 3A (data at 3.0 and 5.0 M urea for N and B isomers of HSA omitted for brevity). Absence of any significant decrease in hemin binding to N isomer of HSA denatured with urea up to 5.0 M concentrations (Fig. 3B) suggested that domain-IB did not undergo any significant structural changes up to 5.0 M urea. Above 5.0 M urea concentration, binding of hemin decreased continuously up to 7.0 M urea concentration. Decrease in hemin binding to HSA denatured with urea at a concentration higher than 5.0 M urea indicated no structural perturbation in domain-IB during intermediate formation. As can be seen from Fig. 3B, there was a significant decrease in the extent of hemin-induced fluorescence quenching of B isomer of HSA as compared to N isomer indicating significant alteration of domain-I during N ↔ B transition. Further, we observed no significant decrease in hemin binding to B isomer of HSA up to 3.0 M urea concentrations. Above 3.0 M urea concentration, hemin binding decreased gradually up to 7.0 M urea. These results showed non-involvement of domain-IB during intermediate formation in either case.

Conformational transition in domain-II

Tryptophanyl fluorescence

Figs. 4A and B show the urea-induced denaturation of N and B isomer of HSA, respectively, by measuring

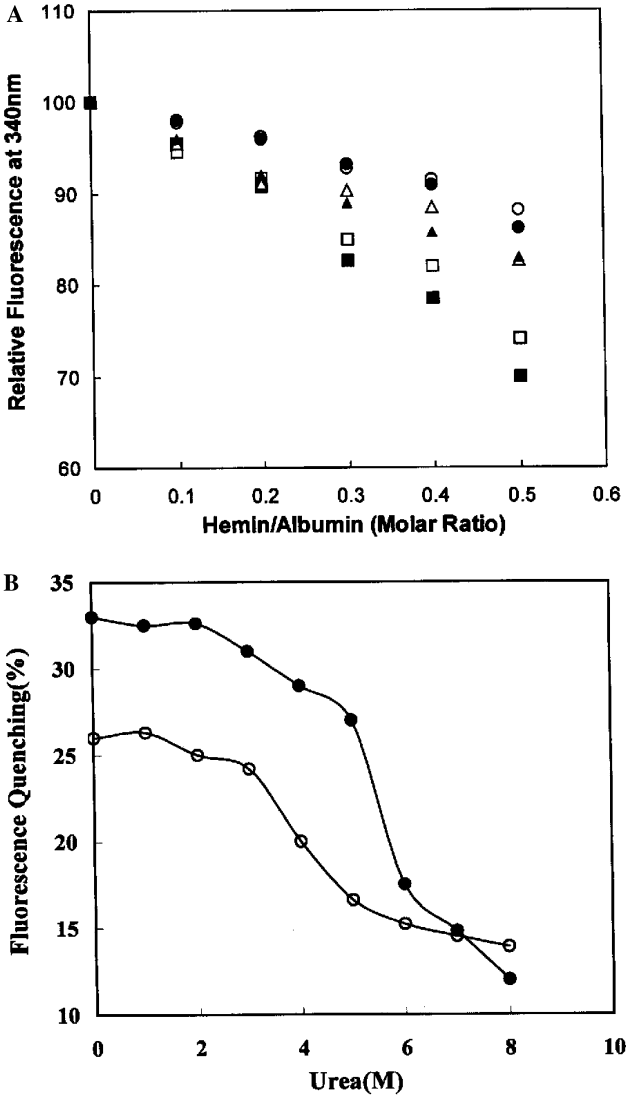

Fig. 3. (A) Fluorescence quench titration results of hemin binding to 'N' isomer of HSA (■) and 'N' isomer denatured with 7.0 M (●) and 8.0 M (○) urea concentrations and 'B' isomer of HSA in the absence (□) and presence of 7.0 M (▲) and 8.0 M (△) urea. (B) shows the binding isotherm of hemin to 'N' (●) and 'B' (○) isomers of HSA as function of urea concentration at a fixed hemin/albumin molar ratio of 0.5:1.0.

the tryptophan emission fluorescence at 344 nm. As can be seen from the figure, transitions are single step with no apparent intermediate state. The transitions started at around 5.2 M urea for N isomer and 3.3 M urea for B isomer of HSA and completed at 8.0 and 6.0 M urea concentrations, respectively. Since HSA contains only one tryptophan residue (Trp-214), which resides in domain-II, changes in fluorescence intensity observed after exciting the protein at 295 nm may be ascribed to the conformational changes in domain-II. Thus, it can be inferred that no structural change occurred in domain-II in the urea concentration range 0–5.0 M for N isomer 0–3.2 M for B isomer of HSA. Further, the change in

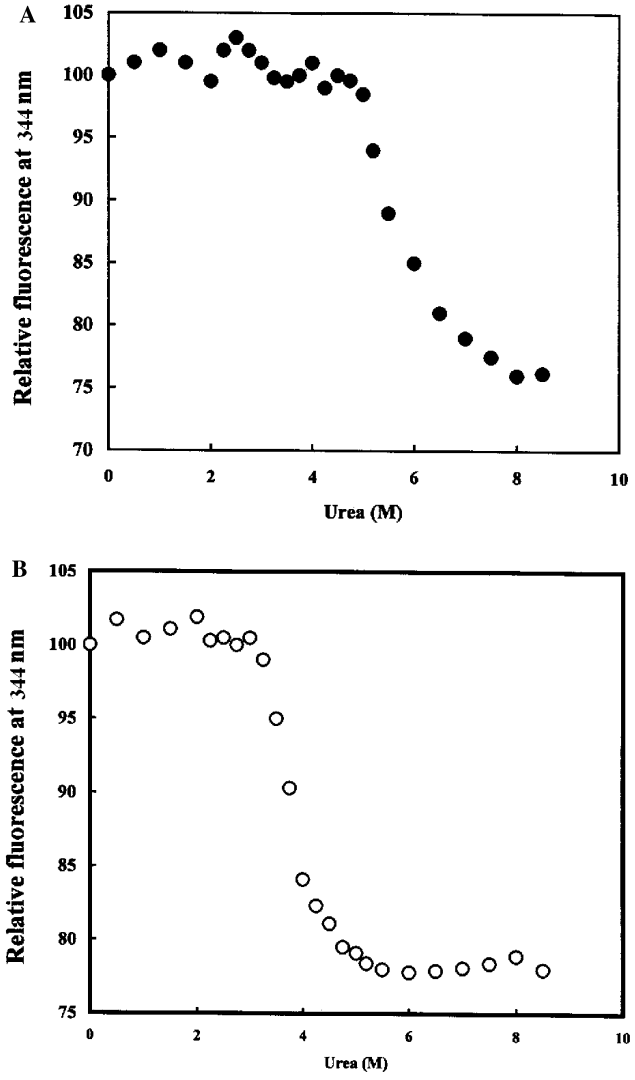

Fig. 4. (A) Urea denaturation profile of 'N' isomer of HSA and (B) of 'B' isomer of HSA as monitored by Trp-214 fluorescence at 344 nm after exciting the protein at 296 nm.

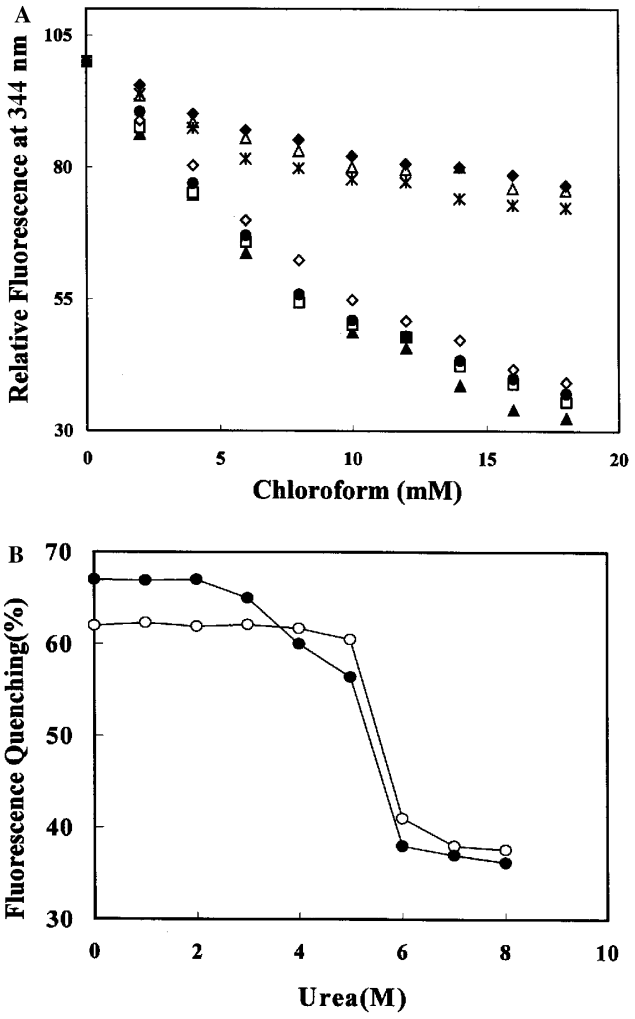

Fig. 5. (A) Chloroform-induced tryptophan fluorescence quenching of 'N' isomer of HSA in the absence (▲) and presence of 5.0 M (□), 6.0 M (∗), and 7.0 M (○) urea concentration and 'B' isomer of HSA in the absence (●) and presence of 3.0 M (◇) and 6.0 M (◆) urea concentration. (B) shows the effect of increasing urea concentrations on the chloroform-induced tryptophan fluorescence quenching of 'N' (○) and 'B' (●) isomers of HSA at a fixed chloroform concentration (18 mM).

fluorescence intensity observed by exciting the protein at 280 nm as shown in Figs. 1A and 2A, in the urea concentration range 2.5–4.6 M for N form and 2.0–3.25 M for B isomer may be attributed to conformational changes in domain-III and/or domain-I.

Chloroform binding

Recent studies have demonstrated that chloroform binds to the albumin in the close vicinity of tryptophan residue (Trp-214) [26]. Since the lone Trp-214 resides in domain-IIA, to get more insight into structural changes in sub domain-IIA, binding of chloroform to N and B isomers of HSA was studied at different urea concentrations using tryptophan fluorescence (excitation wavelength, 295 nm). Fig. 5A shows the chloroform-binding isotherm of HSA (both N and B isomers) in the absence and presence of different urea concentrations. The equilibration of HSA with chloroform caused concentration dependent quenching of tryptophan fluorescence with no apparent shift in the emission maximum (344 nm), which is suggestive of the chloroform binding to HSA. Absence of any significant decrease in chloroform binding to HSA (N and B isomers) up to 5.0 and 3.0 M urea, respectively (Fig. 5B), suggested that domain-II did not undergo any significant structural change during intermediate formation. Binding of chloroform decreased above 5.0 M urea in the case of N isomer and above 3.0 M urea in the case of B isomer of HSA continuously up to 7.0 M urea concentration. These observations indicated that the structural perturbations in domain-II occurred only in second transition. These results were in agreement with earlier reports suggesting non-involvement of domain-II in

conformational transition of HSA during intermediate formation [21,39]. The above chloroform binding studies together with tryptophan fluorescence studies indicated that unfolding of domain-II of B isomer started at a lower urea concentration (3.0 M) as compared to N isomer (5.0 M urea) of HSA. These studies confirmed the strong effect of N ↔ B transition in domain-II and non-involvement of the domain-II in intermediate formation.

Structural alteration between domain-IB and domain -IIA

Bilirubin binding

To study conformational changes in the bilirubin binding cavity of N and B isomers of HSA, binding of bilirubin to the two isomers of HSA was studied at different urea concentrations. Since the bilirubin binding cavity is formed by loop 4 of domain-IIA and loop 3 of domain-IB, separation and unfolding of domain-II from domain-I can be selectively investigated by bilirubin binding studies. Fig. 6A shows the binding of bilirubin to N and B forms of HSA. As can be seen from Fig. 6B, binding of bilirubin to B isomer of HSA increases significantly up to 3.0 M urea and then decreases thereafter. At 5.0 M urea, extent of bilirubin binding is similar to that of B isomer in the absence of urea, suggestive of the retention of native like conformation of domain-II at this urea concentration. Above 5.0 M urea, a marked decrease in bilirubin binding was observed which was abolished completely at 6.0 M urea. Similar effect was also observed with N isomer of HSA. Binding of bilirubin to N isomer of HSA increased significantly up to 5.0 M urea. At 6.0 M urea it became similar to N isomer in the absence of urea and binding abolished completely at 7.0 M urea again suggesting native like conformation of domain-II; this is consistent with previous reports in the case of BSA [22]. Increase in bilirubin binding to HSA at increasing urea concentration cannot be due to non-specific interaction of urea with bilirubin as no fluorescence was observed with free bilirubin at different urea concentrations. Since the occurrence of bilirubin fluorescence is due to acquisition of helicity in the pigment upon binding to albumin [27], a significant increase in bilirubin fluorescence upon binding to urea denatured HSA as compared to N and B isomers in the absence of urea suggests that domain-II undergoes rearrangement as to allow the bound pigment to acquire a tight helical twisting. Marked decrease in bilirubin binding beyond 5.0 M urea and complete abolishment around 6.0 M urea in B isomer of HSA are suggestive of disruption of bilirubin binding cavity, which is formed by loops 4 of domain-IIA and loop 3 of domain-IB. Thus, it can be concluded that around 6.0 M urea, unfolding and complete separation of domain-II from domain-I occurred. Similarly a marked decrease in bilirubin binding to N isomer of HSA at 7.0 M urea is suggestive of unfolding/

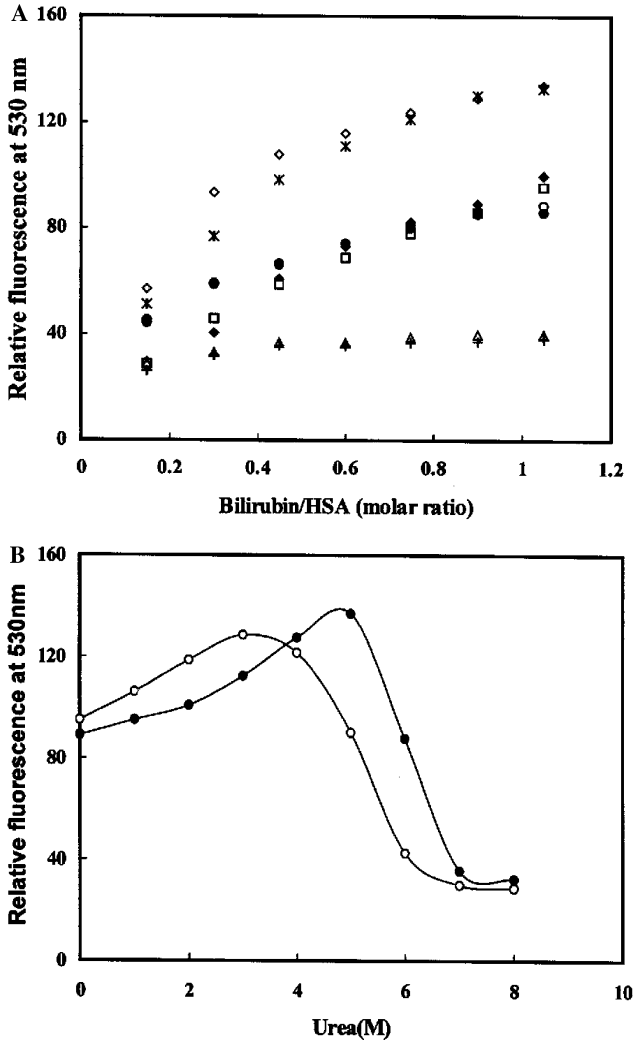

Fig. 6. (A) Relative fluorescence of bilirubin bound to 'N' isomer of HSA in the presence of 0.0 M (●), 5.0 M (∗), 6.0 M (○), and 7.0 M (+) urea and 'B' isomer of HSA in the presence of 0.0 M (□), 3.0 M (◇), 5.0 M (◆), and 6.0 M (△) urea concentrations. The fluorescence was recorded at 530 nm after exciting the bilurubin–albumin complex at 466 nm. (B) Effect of urea concentrations on the binding of bilirubin to 'N' (●) and 'B' (○) isomers of albumin at a fixed bilirubin/albumin molar ratio of 1.0:1.0.

separation of domains-I and II from each other at higher urea concentrations. Loss in bilirubin binding cavity at lower urea concentrations in the case of B isomer of HSA as compared to N isomer of HSA was again suggestive of significant effect of N ↔ B transition on domains-I and II.

Structural transition in domain-III

Diazepam binding

Earlier studies have shown that domain-III is primarily responsible for intermediate formation in the urea-induced unfolding transition of HSA and BSA [21–23]. Dockal et al. [3], on the basis of their studies on

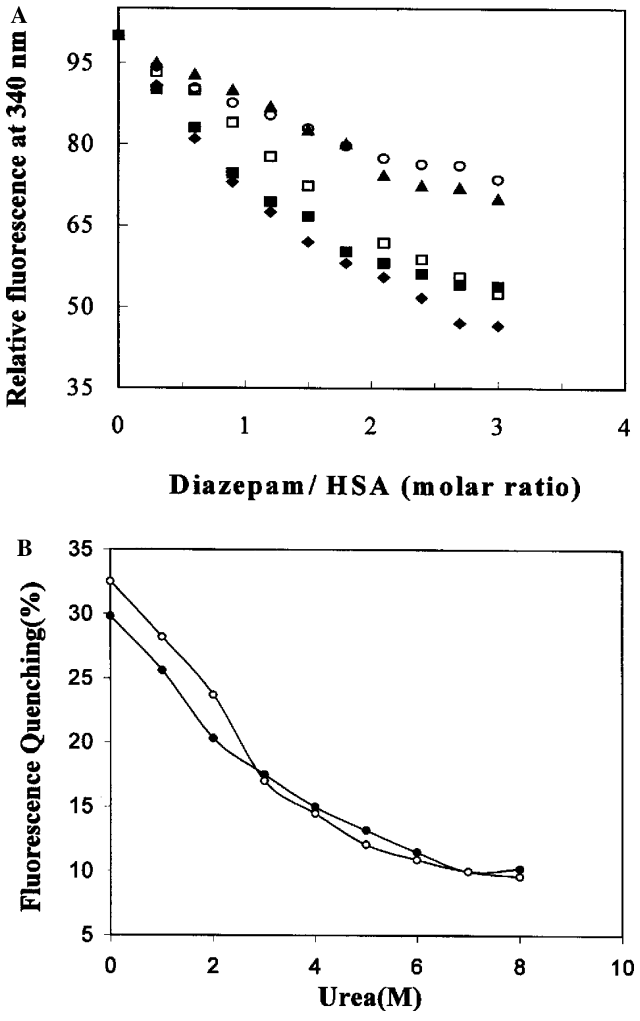

Fig. 7. (A) Fluorescence quench titration results of diazepam binding to 'N' isomer of HSA (■) and 'N' isomer denatured with 3.0 M (▲) urea concentrations and 'B' isomer of HSA in the absence (◆) and presence of 1.0 M (□) and 3.0 M (○) urea. (B) shows the binding isotherm of diazepam to N (●) and B (○) isomers of HSA as a function of urea concentration at a fixed diazepam/albumin molar ratio of 1.2:1.0.

conformational transition of recombinant HSA domains and intact HSA, concluded that domain-III of HSA is not involved in the N ↔ B transition of intact molecule. In view of the above information, we checked the urea-induced structural alterations in both isomers of the HSA by measuring the binding affinity of diazepam at different urea concentrations. Fig. 7A shows the binding isotherm of diazepam with N and B isomers of HSA, both in the absence and presence of increasing urea concentrations. A continuous decrease in diazepam binding to both isomers of HSA was noticed at increasing urea concentrations (Fig. 7B). A significant decrease in diazepam binding to both isomers of HSA denatured up to 3.0 M urea (Fig. 7A) indicated gradual structural alterations in domain-III against domain-I which retained its conformation up to this urea concentration. Diazepam binding to N and B isomers in the absence and presence of increasing concentrations of urea followed an almost similar pattern (Fig. 7B). Hence, these results were also indicative of no effect of N ↔ B transition on domain-III. These results are in agreement to earlier reports, suggesting that domain-III is much more labile to urea denaturation [20,22,39].

Conclusion

Taken together, these results, i.e., urea-induced unfolding curves and binding of domain specific ligands to HSA indicate that domain-III is much more labile to denaturation by urea and mainly involved in intermediate formation. Moreover, domain-II does not unfold initially, instead it undergoes structural rearrangement that is favorable for binding of its specific ligand, bilirubin. Interestingly, above 5.0 M urea concentration for N isomer and 3.0 M urea for B isomer, hemin and chloroform binding significantly decreased indicating loss of native conformation of domains-IB and IIA only after intermediate formation. Non-involvement of domain-IB during intermediate formation was further confirmed by increased binding of bilirubin to HSA in this concentration range of urea. Since high affinity bilirubin binding site is located between loops 4 and 3 of domains-IIA and IB, respectively, therefore, it appears that the formation of intermediate in the unfolding transition of human serum albumin mainly involves unfolding of domain-III. Since the 'B' isomerization particularly affects N-terminal region [6–10] and observed intermediate is at lower urea concentrations, therefore, it appears that formation of intermediates in the unfolding transition of human serum albumin also involves unfolding of domain-IA.

Acknowledgments

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