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Use of Domain Specific Ligands to Study Urea-Induced Unfolding of Bovine Serum Albumin

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Urea-induced structural transitions in different domains of bovine serum albumin (BSA) were studied fluorometrically using domain specific ligands; chloroform, bilirubin, and diazepam. Urea denaturation of BSA showed a two-step, three-state transition with the accumulation of an intermediate around 4.8–5.2 M urea. During first transition (0–5.0 M urea), a continuous decrease (starting from 1.0 M urea) in diazepam (a ligand for domain III) binding and a late (from 3.0 M urea onward) decrease in chloroform (a ligand primarily for domain I) binding suggested major conformational changes in domain III and partial but significant loss of native conformation in domain I prior to intermediate formation. Absence of any decrease in bilirubin (a ligand for domain II) binding up to 4.5 M urea indicated non-involvement of domain II in the unfolding of BSA in this region. However, decrease in bilirubin binding during second transition reflected the unfolding of domain II and its separation from domain I. **© 2000 Academic Press**

Key Words: **bovine serum albumin; urea denaturation; ligand binding; intermediate state.**

Bovine serum albumin (BSA) is a large single polypeptide chain, organized into three structurally homologous domains I, II, and III (1). These domains are in turn connected to each other through helical extensions involving helix10(I)-helix 1(II) and helix10(II)-helix1(III) which are supposed to be the natural borders existing between three domains of albumin (2, 3). The inter-domain clustering for the association of these domains involves both hydrophobic and salt bridge interactions (4, 5). BSA during urea denaturation has been shown to follow a two-step, three-state transition with the accumulation of an intermediate around 4.6–5.0 M urea (6, 7). The unfolding/refolding of BSA seems to be a complex process in view of the independent unfolding/refolding behaviour of all the three domains (2, 8). Further, it is not clear that which domain(s) bear(s) initial shock of denaturation by urea and is (are) involved in the intermediate formation. Recently, we have shown that domain III is primarily responsible for intermediate formation during urea denaturation of human serum albumin (HSA) (9). On the other hand, Khan *et al.* (6) earlier suggested the involvement of both domains II and III in the intermediate formation during urea denaturation of BSA. This seems to be a controversial point in terms of the relative stabilities of various domains during urea denaturation of albumin. Separation and/or unfolding of a particular domain at low urea concentration may destabilize the rest of the molecule due to disruption of inter-domain interactions which are responsible for maintaining the integrity of native conformation (4, 5). Therefore, in order to characterize the urea-induced structural transition in terms of unfolding of various domains of BSA, we studied the binding of domain specific ligands such as diazepam (domain III), chloroform (primarily for domain I), and bilirubin (domain II) at different urea concentrations. The data presented in this paper provide information regarding the preferential unfolding of albumin domains during urea denaturation and their separation from each other.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA), essentially fatty acids free (Lot 42F-9365), and urea (ultra pure) were obtained from Sigma Chemical Co., USA. Chloroform (99.9%) and bilirubin (extra pure) were the products of Sisco Research Laboratories, India. Diazepam (Lot 02399) was obtained from Ranbaxy Laboratories Ltd., India. All other reagents used were of high grade analytical reagents.

Protein concentration was determined spectrophotometrically using $E^{1\%}_{1cm}$ of 6.67 at 279 nm (10).

Fluorescence spectroscopy. Fluorescence measurements were made on a Shimadzu spectrofluorometer, 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. Excitation and emission slits were set at 5 and 10 nm, respectively. Intrinsic fluorescence was measured by exciting the protein solution either at 280 or 295 nm and the emission spectra were recorded in the range of 300–400 nm.

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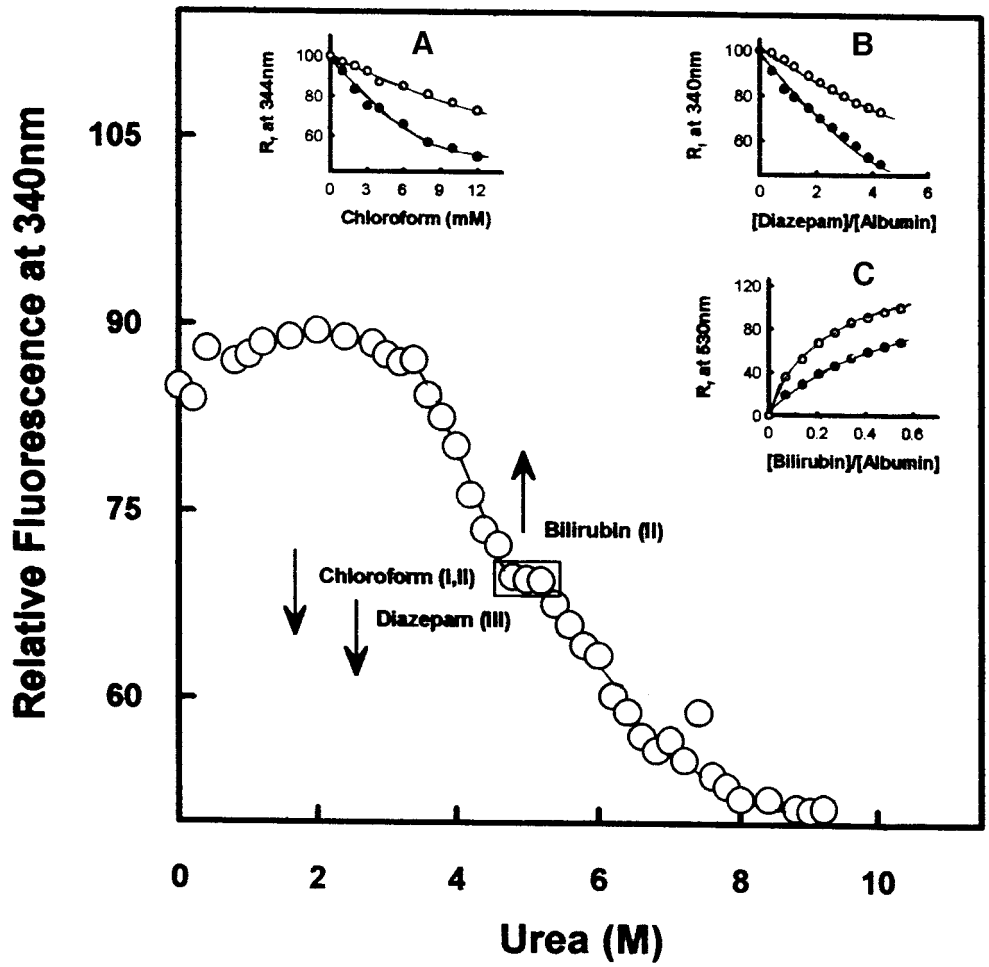

FIG. 1. Urea-induced denaturation of BSA in 0.06 M sodium phosphate buffer, pH 7.4 and at 25°C. The unfolding was monitored by measuring the intrinsic fluorescence at 340 nm after exciting the protein at 280 nm. Boxed region shows the urea concentration range (4.8–5.2 M) where a stable intermediate was detected. Insets A, B, and C show the binding isotherms of chloroform (A), diazepam (B), and bilirubin (C) to native BSA (●) and BSA previously denatured with 5 M urea (○). Each data point was the average of 2–3 independent observations.

Urea denaturation of BSA. Solutions for the denaturation experiment were prepared in 0.06 M sodium phosphate buffer, pH 7.4. To a 0.5 ml stock protein solution, different volumes of the same buffer were added first followed by the addition of a stock denaturant solution (10 M urea) to get a desired concentration of denaturant. The final solution mixture (5.0 ml) was incubated for 10–12 h at room temperature before fluorescence measurements were made. Reversibility of BSA denaturation was not studied because it has already been established (6, 9).

Chloroform binding. Binding of chloroform to BSA at different urea concentrations was studied by fluorescence quench titration method (11). To a fixed volume (5.0 ml) of protein solution, previously incubated with different urea concentrations for 10–12 h at room temperature, increasing volumes (1–20 μl) of chloroform were added and the fluorescence was measured after 1 h at 344 nm after exciting the protein samples at 295 nm. Fluorescence was also recorded at 340 nm after exciting the protein at 280 nm which was comparable to that obtained at 295 nm (data not shown). The data were plotted as relative fluorescence versus 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 with 0.06 M sodium phosphate buffer, pH 7.4. The concentration of bilirubin was determined spectrophotometrically using a molar absorption coefficient as 47,500 M^{-1} cm^2 at 440 nm (12). The binding of bilirubin to albumin was studied using fluorescence enhancement technique as described earlier (13, 14). To a fixed volume of stock protein solution (3.5 μM), previously incubated with different urea concentrations for 10–12 h at room temperature, increasing volumes (1–20 μl) of stock bilirubin solution (1.0×10^{-3} M) were added to achieve different bilirubin/albumin molar ratios and the fluorescence was measured at 530 nm after exciting the bilirubin–albumin complex at 466 nm. The total volume of incubation mixture was 3 ml. The spectra were recorded in dark after 15–20 min of the addition of bilirubin to the protein solution. The data were plotted as relative fluorescence against bilirubin/albumin molar ratio.

Diazepam binding. To study the binding of drug to native as well as urea-denatured BSA, stock protein solution (3.5 μM), previously incubated with different urea concentrations for 10–12 h at room temperature, was titrated with increasing drug concentrations to get different (0–4.0) drug/protein molar ratios. The solutions were excited at 280 nm after 30–40 min incubation at room temperature and the fluorescence was measured at 340 nm. The data were plotted as relative fluorescence versus drug/protein molar ratio.

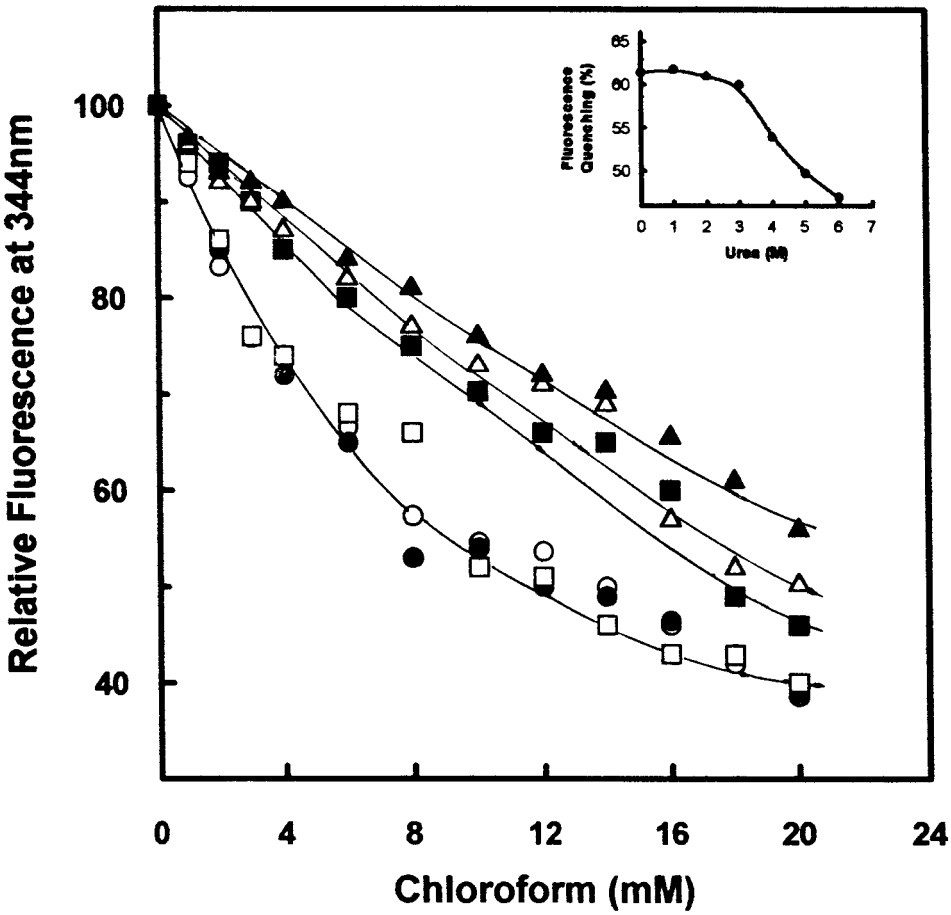

FIG. 2. Effect of chloroform on the tryptophan fluorescence of native BSA (●) and BSA denatured with different urea concentrations viz. 2 M (□), 3 M (○), 4 M (■), 5 M (△), and 6 M (▲) at pH 7.4. The tryptophan fluorescence was recorded at 344 nm after exciting the protein at 295 nm at a protein concentration of 3.5 μM. The inset shows the effect of increasing urea concentration on the chloroform-induced tryptophan fluorescence quenching of BSA at a fixed chloroform concentration (20 mM). Each data point was the average of 2 independent observations.

RESULTS AND DISCUSSION

Figure 1 shows urea-induced denaturation of BSA as monitored by measuring the intrinsic fluorescence at 340 nm after exciting the protein at 280 nm. Urea denaturation curve of BSA was characterized by a two-step, three-state transition with the accumulation of a stable intermediate around 4.8–5.2 M urea. These results were in agreement with earlier observations (6, 7). Previous studies suggested major structural alterations in domain III and minor changes, if any, in domain II during intermediate formation (6, 7). Involvement of domain I in the first transition has been excluded. In view of the involvement of inter-domain interactions in stabilizing the native conformation of albumin (4, 5), it seems that disruption of these interactions due to structural alteration in one or more domains of BSA may destabilize the rest of the molecule. In order to monitor the loss of native conformation in different domains of BSA during intermediate formation, we studied the binding of three different ligands, namely, chloroform (11), bilirubin (13, 14), and diazepam (3) to native as well as urea-denatured (5 M) BSA. Binding isotherms for these ligands are shown in the insets (A, B, and C) of Fig. 1. Binding of both chloroform and diazepam (ligands for domains I and III, respectively) decreased significantly indicating the loss of native conformation both in domains I and III (see Fig. 1, insets A and B). On the other hand, binding of bilirubin when studied by fluorescence enhancement technique, did not show any decrease rather it increased in 5 M urea compared to native BSA (Fig. 1, inset C). At a bilirubin/albumin molar ratio of 0.6:1.0, nearly 40% increase in fluorescence enhancement was observed in 5 M urea as compared to that observed with native BSA solution. These observations led us to study in detail the binding of these ligands to BSA denatured with different urea con-

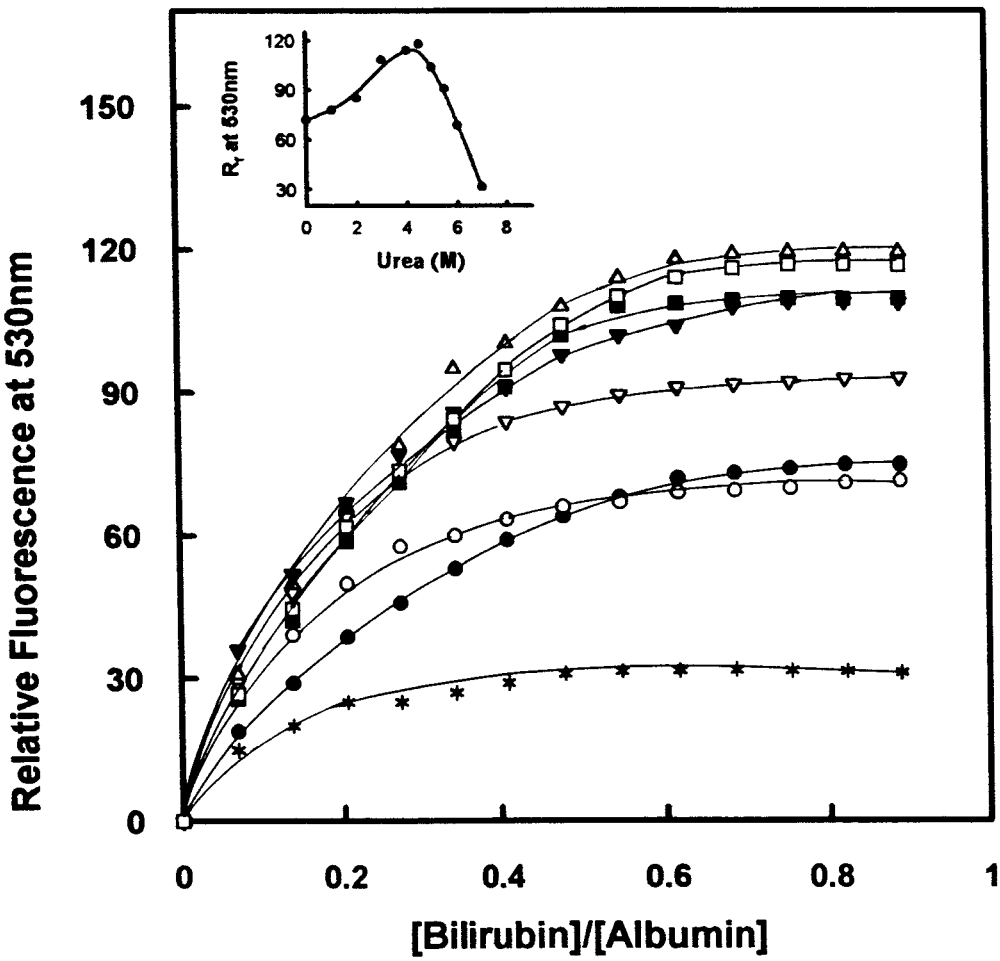

FIG. 3. Relative fluorescence of bilirubin bound to native BSA (●) and BSA denatured with different urea concentrations viz. 3 M (■), 4 M (□), 4.5 M (△), 5 M (▼), 5.5 M (▽), 6 M (○), and 7 M (∗) urea at increasing bilirubin/albumin molar ratios. The fluorescence was recorded at 530 nm after exciting the bilirubin-albumin complex at 466 nm at pH 7.4. The inset shows the effect of urea concentration on the binding of bilirubin to albumin at a fixed bilirubin/albumin molar ratio of 0.6:1.0. Each experiment was repeated twice at different urea concentration and each point was the average of two data sets.

centrations so as to monitor fine loss of native conformation in different domains of BSA prior to intermediate formation.

Accumulating evidences suggest that in BSA, chloroform binds to subdomains I B and II A in close vicinity to the two tryptophan residues, Trp-134 and Trp-212, respectively. The more water exposed Trp-134 site (in subdomain IB) has considerably higher affinity than the site in subdomain IIA (11). To get more insight about structural alterations in subdomain IB of domain I, the binding of chloroform to BSA was studied at different urea concentrations and the data are shown in Fig. 2. Absence of any significant decrease in chloroform binding to BSA denatured with urea up to 3 M concentration (Fig. 2, inset), suggested that domain I did not undergo any significant structural change up to 3 M urea concentration. Above 3 M urea concentration, binding of chloroform decreased continuously up to 6 M urea. Decrease in chloroform binding to BSA denatured with urea at a concentration higher than 3 M indicated the structural perturbation in domain I during intermediate formation.

For studying the structural alterations in domain II, binding of bilirubin to BSA was studied at different urea concentrations and the results are shown in Fig. 3. Binding of bilirubin to BSA increased significantly on increasing urea concentration reaching a maximum value at 4.5 M urea (Fig. 3, inset) and then decreased becoming similar to the one obtained with native albumin, at 6 M urea. Beyond 6 M urea, a marked decrease in bilirubin binding was noticed which abolished completely around 7 M urea. Increase in bilirubin binding to BSA denatured with different urea concentrations cannot be ascribed to the non-specific interaction of urea with the pigment as no fluorescence was observed with free bilirubin in different urea concentrations. Since the occurrence of bilirubin fluorescence is due to acquisition of helicity in the pigment upon binding to

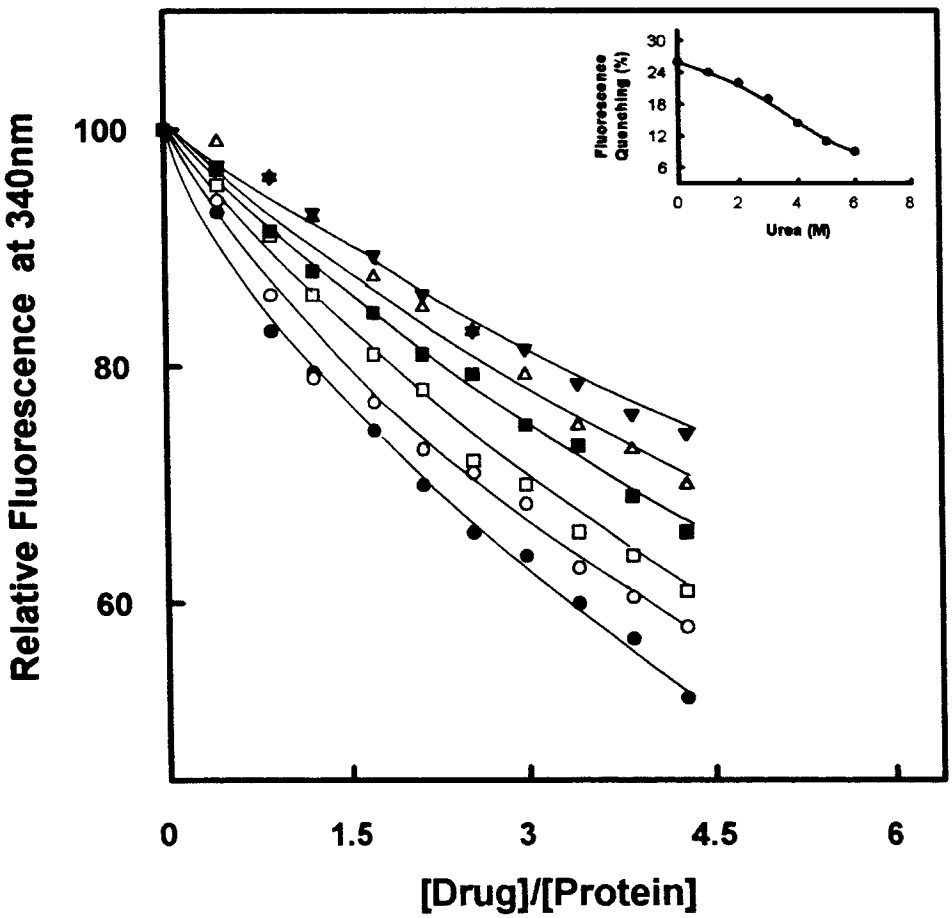

FIG. 4. Fluorescence quench titration results of diazepam binding to native BSA (●) and BSA denatured with different urea concentrations viz. 2 M (○), 3 M (□), 4 M (■), 5 M (△), and 6 M (▼) urea at pH 7.4. The molar ratio of drug/albumin was varied as 0–4.0 and the fluorescence was recorded at 340 nm after exciting the protein at 280 nm. The inset shows the binding isotherm of diazepam to BSA as a function of urea concentration at a fixed drug/albumin molar ratio of 1.7:1.0. Data points were the average of 2–3 independent observations.

albumin (13), a significant increase in bilirubin fluorescence upon binding to BSA denatured by 4.5–5 M urea as compared to native albumin suggests that rearrangement in domain II takes place in such a way as to allow the bound pigment acquiring a tight helical twisting. In other words, lack of any decrease in bilirubin binding to BSA in the presence of 4.5–5 M urea was indicative of the retention of native-like conformation of domain II at this urea concentration. Marked decrease in bilirubin binding beyond 6 M urea and complete abolishment around 7 M urea were suggestive of unfolding and separation of domains I and II from each other. This seems to be plausible since earlier studies have suggested the location of high affinity bilirubin binding site in the form of a cavity involving mainly loop 4 and partly loop 3 of subdomains IIA and IB, respectively (15). Recently, it has been suggested that native albumin conformation is stabilized by intra- and inter-domain forces and that individual domains cannot be expected to fully reflect their behaviour as when they are associated with each other in the whole protein (2). Our bilirubin binding results at different urea concentrations are fully in accord with this contention.

Earlier studies have shown the involvement of domain III of BSA in the intermediate formation during urea denaturation (6). We checked the unfolding of domain III of BSA by measuring its binding affinity to diazepam (3) at different urea concentrations. A continuous decrease in diazepam binding to BSA was noticed at increasing urea concentrations (see Fig. 4). A significant decrease in diazepam binding to BSA denatured up to 3 M urea (Fig. 4, inset) indicated gradual structural alterations in domain III against domain I which retained its conformation up to this urea concentration. These results were in agreement to earlier reports suggesting that domain III is much more labile to urea denaturation (2, 6, 7, 16).

What does the intermediate state indicate? Whether the transition is localized to a particular domain of the protein leaving the rest of the molecule (other domains) undisturbed or the whole protein is under strain due to the transition in one particular domain, are some of the

questions that remain to be answered in multidomain proteins. Denaturation of a multidomain protein is a complex process but the latter possibility seems to be plausible in view of the involvement of both inter- and intra-domain interactions in the stabilization of native protein conformation (4, 5). Further, relative stabilities of various domains (which may be different) in multidomain proteins decide the fate of their domains towards unfolding in a particular denaturing medium. Therefore, it seems inconceivable to think of the independent unfolding of any domain without affecting the rest of the molecule. This is reflected from our present study which indicates that in BSA domain III is much more labile to denaturation by urea followed by domain I whereas domain II does not unfold initially, instead undergoes a rearrangement for the favourable binding of specific ligand, bilirubin. Interestingly, above 3 M urea i.e., 4 and 5 M urea concentrations, chloroform binding significantly decreased indicating loss of native conformation of domain I prior to intermediate formation. We assign the decrease in chloroform binding to the unfolding of domain I for two reasons: First, the affinity of chloroform for first site in domain I (Trp-134) is significantly higher (11) compared with that of domain II (Trp-212) and second that domain II undergoes a rearrangement without significantly losing its integrity as indicated from increased bilirubin binding, thus decrease in chloroform binding to 4 M and 5 M urea denatured BSA, should mainly be attributed to the loss of native conformation in domain I and not in domain II. A gradual decrease in the diazepam binding with increasing urea concentrations starting from 1 M urea indicates that domain III is much more labile to urea denaturation as compared to domain I which retains its chloroform binding ability up to 3 M urea that is lost thereafter as shown in the inset of Fig. 2.

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