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# Bromophenol Blue Binding as a Probe to Study Urea and Guanidine Hydrochloride Denaturation of Bovine Serum Albumin

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**Urea and guanidine hydrochloride (GdnHCl) denaturation of bovine serum albumin (BSA) were investigated using bromophenol blue (BPB) binding as a probe. Addition of BPB to BSA produced an absorption difference spectrum in the wavelength range, 525–675 nm with a minimum at 587 nm and a maximum at 619 nm. The magnitude of absorption difference (ΔAbs.) at 619 nm decreased on increasing urea/GdnHCl concentration and followed the denaturation curve. The denaturation was found to be a two-state, single-step transition. The transitions started at 1.75 and 0.875 M and completed at 6.5 and 3.25 M with the mid point occurring around 4.0 and 1.5 M urea and GdnHCl concentrations, respectively. The value of free energy of stabilization, $\Delta G_D^{H_2O}$ as determined from urea and GdnHCl denaturation curves was found to be 4041 and 4602 cal/mol, respectively. Taken together, these results suggest that BPB binding can be used as a probe to study urea and GdnHCl denaturation of BSA.**

**Key words: bovine serum albumin, bromophenol blue, denaturation, guanidine hydrochloride, urea.**

Abbreviations: ΔAbs., absorbance difference; ANS, 1-anilino-naphthalene-8-sulfonate; BSA, bovine serum albumin; BPB, bromophenol blue; GdnHCl, guanidine hydrochloride; HSA, human serum albumin; UV, ultraviolet.

Most of the proteins fold into their globular conformation which is required for the specific interactions needed for their biological functions. This makes the study of protein folding an important subject not only to get insight into the fundamental mechanism but also in producing large amount of refolded proteins from inclusion bodies (*1*, *2*). Such studies are also helpful in revealing whether the denaturation is a two-state or multi-state process and determining the conformational stability of proteins (*3–7*). Although many proteins undergo denaturation through the formation of an intermediate, the intermediate may or may not be detected depending upon the probe used to monitor conformational changes during denaturation process (*7–10*). Denaturation of serum albumin, a single chain multidomain protein has been studied by several workers using different probes like absorption difference at 287 nm (*3*), fluorescence at 340 nm upon excitation at 282 nm (*11*), tryptophan fluorescence upon excitation at 295 nm (*4*), ellipticity at 222 nm (*4*, *12*), 1-anilino-naphthalene-8-sulfonate (ANS) fluorescence (*4*), differential calorimetry (*13*), etc. Denaturation of serum albumin has been suggested to follow a single-step transition as well as two- or multiple-step transitions depending upon the reaction conditions and probes used (*4*, *11–14*). For example, urea denaturation of serum albumin has been shown to follow a two-step, three-state transition involving one intermediate when studied by fluorescence and ellipticity measurements (*12*) but became single-step, two-state transition when studied by UV difference spectroscopy (*3*). Similarly, guanidine hydrochloride (GdnHCl) denaturation of serum albumin has been reported to follow both single-step and two-step transitions (*4*, *10*). Different probes used in denaturation studies either require a sophisticated costly instrument or higher protein concentration to study protein denaturation. Although fluorescence and ellipticity probes are quite sensitive and require lesser protein concentration (~2 μM) (*4*), but suffer from the drawback of the unavailability of these instruments in all research laboratories. On the other hand, absorption difference at 287 nm offers the advantage of easy availability of spectrophotometer in all the laboratories but requires higher protein concentration (~23 μM) (*11*). There is a need of searching additional probes that require lesser protein concentration and the use of commonly available visible spectrophotometer. Bromophenol blue (BPB) has been reported to bind same hydrophobic sites on proteins where polarity sensitive fluorescence probes bind (*15*). Furthermore, absorbance changes of albumin-bound BPB has also been reported with change in pH (*16*). In view of the above, it would be of interest to use BPB binding as a probe to study denaturation of proteins. Here, we present our data on urea and GdnHCl denaturation of bovine serum albumin (BSA) using BPB binding as a probe.

## EXPERIMENTAL PROCEDURES

*Materials*—Bovine serum albumin (BSA), essentially fatty acid free (Product No A-6003), bromophenol blue (BPB) (Product No B-6896), urea (Product No U-0631) and guanidine hydrochloride (GdnHCl) (Product No G-3272) were obtained from Sigma-Aldrich Inc., USA.

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*Analytical Procedures*—Protein concentration was determined spectrophotometrically on a Shimadzu double-beam spectrophotometer, model UV-2450, using a specific absorption coefficient of 6.61 at 280 nm (*17*).

*Absorption Difference Spectroscopy*—Absorption measurements were made on a UV-2450 spectrophotometer (Shimadzu, Japan) at 25°C using quartz cuvette of 1 cm path length. A stock solution containing equimolar (100 μM) mixture of BPB and BSA was prepared by dissolving 670 mg of BSA and 6.7 mg of BPB in 0.1 M Tris–HCl buffer, pH 8.0 in a total volume of 100 ml. The protein-free BPB solution was prepared by dissolving the same amount of BPB in 100 ml of 0.1 M Tris–HCl buffer, pH 8.0. Absorbance difference spectra of BPB-BSA (1:1) complex were recorded in the wavelength range 525–675 nm against free BPB. Similar concentrations of urea/GdnHCl were added in the blank solutions in experiments involving urea/GdnHCl.

*Fluorescence Spectroscopy*—Fluorescence measurements were carried out on a Hitachi Fluorescence Spectrophotometer, model F-2500. The fluorescence spectra were measured at a protein concentration of 0.2 μM with a 1 cm path-length cell. Excitation and emission slits were fixed at 10 nm each. Intrinsic fluorescence was measured by exciting the protein solution at 280 nm and the emission spectra were recorded in the range of 300–400 nm.

*Denaturation Experiments*—Solutions for the denaturation experiments were prepared in 0.1 M Tris–HCl buffer, pH 8.0. To a 0.5 ml stock solution of BPB-BSA (1:1) mixture (final concentration = 10 μM) or BSA (final concentration = 0.2 μM) taken in different tubes, different volumes of buffer were added first and different volumes of the stock denaturant solution (10 M urea/6.667 M GdnHCl) were added last so as to get the desired concentration of denaturant. The final solution mixture (5 ml) was incubated for 12 h at 25°C before difference spectral measurements/fluorescence measurements were made. Blank solutions containing similar concentrations of BPB and denaturant were also prepared. Absorption difference spectra of solutions containing BPB, BSA and denaturant were recorded against appropriate blanks. Though the reversibility of BSA denaturation has already been established (*3*), we further tested the reversibility of folding in view of any binding of BPB to partially folded state(s) of protein.

*Bromophenol Blue Binding*—Binding of BPB to BSA both in the absence and presence of different urea/GdnHCl concentrations was studied by fluorescence quench titration method as described earlier for other ligands (*18*). To 0.5 ml stock protein solution (1.8 μM) previously incubated with different denaturant (urea/GdnHCl) concentrations in a desired volume of 0.1 M Tris–HCl buffer, pH 8.0 for 12 h at 25°C, increasing volumes of stock BPB solution (17.9 or 35.8 μM) were added in order to get BPB/BSA molar ratio in the range of 0–1.0 and 0–2.0. The total volume of the mixture was 5.0 ml. After 20 min incubation at room temperature, fluorescence spectra were recorded in the wavelength range 300–400 nm upon excitation at 280 nm. The fluorescence intensity values at emission maxima were transformed into percentage by taking the fluorescence intensity of BSA in the absence of BPB as 100. Percentage fluorescence quenching at different denaturant concentrations was determined by taking the difference in fluorescence intensities of native BSA between BPB/BSA molar ratio of 0 and 1.0/2.0 as 100%. The data were plotted as fluorescence quenching (%) versus denaturant concentration.

*Data Analysis*—Denaturation data were analysed using two-state mechanism as described earlier (*4*). The denaturation curves were normalized to the apparent fraction of the denatured form, $F_D$, using the following Eq. 1

$$F_D = \frac{(Y - Y_N)}{(Y_D - Y_N)} \quad (1)$$

where $Y$ represents the observed variable parameter at a given denaturant concentration and $Y_N$ and $Y_D$ are the values of the variable characteristic of the native and denatured states, respectively. The values of $Y_N$ and $Y_D$ were obtained by linear extrapolation of pre- and post-transition regions. Values of $F_D$ ranging from 0.25 to 0.75 were used for $K_D$ calculation. The equilibrium constant, $K_D$ between the native and denatured states of a protein at a given denaturant concentration is given by Eq. 2

$$K_D = \frac{F_D}{(1 - F_D)} \quad (2)$$

These $K_D$ values were transformed into free energy change ($\Delta G_D$) using Eq. 3

$$\Delta G_D = -RT \ln K_D \quad (3)$$

where $R$ is the gas constant (1.987 cal/deg/mol) and $T$ is the absolute temperature. A least-squares analysis of $\Delta G_D$ values as a function of denaturant concentration, [D] was used to fit the data to the following equation for the determination of $\Delta G_D^{H_2O}$, the free energy of stabilization.

$$\Delta G_D = \Delta G_D^{H_2O} - m\,[D] \quad (4)$$

where $m$ is a measure of the dependence of $\Delta G_D$ on denaturant concentration and its value was obtained from the slope of linear plot. Intercept on $Y$-axis yielded the $\Delta G_D^{H_2O}$ value.

## RESULTS AND DISCUSSION

Figure 1A shows absorption difference spectra of BPB–BSA complex both in the absence and presence of increasing urea concentrations. The difference spectrum was characterized by the presence of a negative peak with minima at 587 nm and a positive peak with a maxima around 619 nm. Addition of increasing concentrations of urea to BPB–BSA (1:1) mixture resulted in progressive decrease in absorbance difference (ΔAbs.) at 619 nm accompanied by a blue shift. The magnitude of decrease in ΔAbs. at 619 nm was found to be smaller at both lower (0.1–1.5 M) and higher (7.0–9.0 M) urea concentrations whereas the decrease became more pronounced in the middle range (1.75–6.5 M) of urea concentrations. This can be more clearly seen in Fig. 1B where the values of ΔAbs. at 619 nm are plotted against urea concentration. This figure is similar to urea denaturation curves of serum albumins reported earlier using other probes such

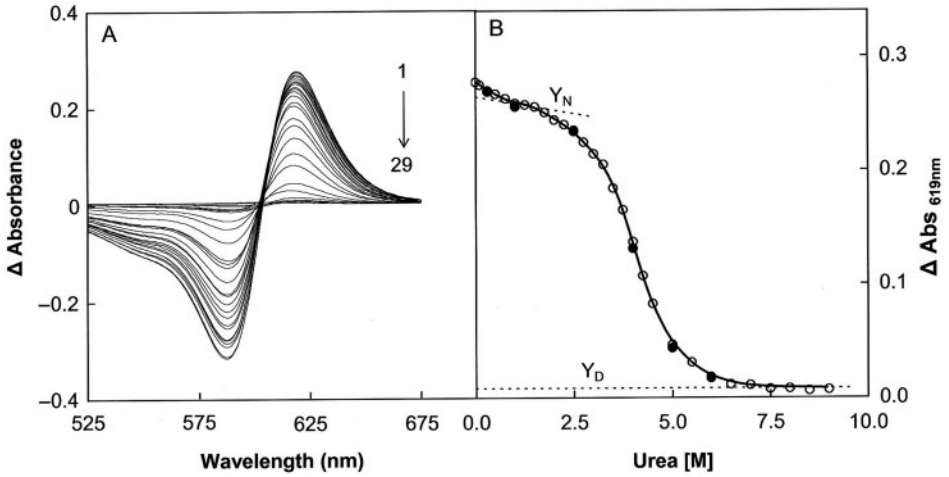


Fig. 1. **Urea-induced denaturation of BSA in 0.1 M Tris–HCl buffer, pH 8.0, 25°C as monitored by BPB binding.** (A) Difference absorption spectra of BPB–BSA (1:1) complex against BPB in the absence and presence of increasing urea concentrations. Spectra from top to bottom (*1–29*) correspond to urea concentration as 0, 0.1, 0.3, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25, 2.5, 2.75, 3.0, 3.25, 3.5, 3.75, 4.0, 4.25, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 M, respectively. (B) Urea denaturation curve of BSA as determined from ΔAbs. values at 619 nm against urea concentration. Open circles show denaturation points and solid circles represent renaturation data.

as ΔAbs. at 287–289 nm (*11*), fluorescence intensity at 340 nm on excitation at 280 nm (*9*), fluorescence intensity at 340 nm on excitation at 295 nm (*4*), mean residue ellipticity at 222 nm (*12*) and ANS fluorescence at 480 nm on excitation at 380 nm (*4*). The denaturation was found to be completely reversible for the transition studied by BPB binding as the experimental points for the unfolding and refolding experiments fall on the same curve.

Urea denaturation curve of BSA as shown in Fig. 1B was found to be a two-state, single-step transition. The transition started at 1.75 M urea concentration and completed around 6.5 M urea with a midpoint occurring around 4.0 M urea. This result was in agreement with previous reports on denaturation of BSA/HSA (*3*, *4*). Lack of detection of an intermediate in urea denaturation of BSA can be explained on the basis of the probe used for monitoring conformational changes as use of either tryptophan fluorescence or ANS fluorescence as probes also showed a single-step transition of urea denaturation of albumin (*4*). BPB has been shown to bind the same hydrophobic sites on proteins as polarity sensitive fluorescence probe, ANS (*15*). The lack of intermediate formation in urea denaturation of BSA as studied by BPB binding is understandable since ANS binding also showed the absence of an intermediate in urea denaturation of HSA (*4*). Involvement of domains I and III has been suggested in the formation of intermediate during urea denaturation of serum albumin (*4*). The absence of accumulation of the hydrophobic patches during urea denaturation of albumin can be justified by the more or less even distribution of non-polar residues (sites for ANS binding) in different domains of the protein (*19*).

To determine the free energy of stabilization, $\Delta G_D^{H_2O}$, data of Fig. 1B were normalized into $F_D$ values using Eq. 1 and plotted against urea concentration (Fig. 2A). Calculation of $\Delta G_D$ values from $F_D$ data using Eq. 3 and plotting these values against urea concentration yielded the linear plot shown in Fig. 2B. The least squares analysis was used to fit the data to Eq. 4 to determine $\Delta G_D^{H_2O}$ and a value of 4041 cal/mol was obtained for $\Delta G_D^{H_2O}$ of BSA. The '*m*' value as determined from the slope of the plot was found to be 1015 cal/mol/M of urea concentration.

In order to check the validity of BPB-binding probe to study other denaturation phenomena, we also studied GdnHCl-induced denaturation of BSA. Figure 3A shows absorption difference spectra of BPB–BSA (1:1) complex in the absence and presence of increasing GdnHCl concentrations. These spectra were qualitatively similar to those observed with increasing urea concentrations (Fig. 1A) in terms of the presence of minima and maxima. However, the complete denaturation was achieved at lower GdnHCl concentration compared to urea concentration as shown above. As can be seen from Fig. 3A, an initial decrease in ΔAbs. was noticed in the 0.125–0.25 M GdnHCl concentration range. The value of ΔAbs. remained more or less the same within the range, 0.375–0.75 M, showed a significant decrease during 0.875–3.0 M and finally became constant beyond 3.0 M GdnHCl concentrations. These changes can be clearly seen from Fig. 3B where values of ΔAbs. at 619 nm are plotted against GdnHCl concentration. In other words, transition started at 0.875 M and completed at 3.25 M with the mid point occurring around 1.5 M GdnHCl concentration, suggesting a single-step, two-state transition. GdnHCl-induced denaturation of BSA as monitored by BPB binding also showed complete reversibility (Fig. 3B).

The data of Fig. 3B were normalized into $F_D$ values as described above and plotted against GdnHCl concentration (Fig. 4A). A plot of $\Delta G_D$ versus GdnHCl concentration was obtained in the same way as reported above for urea denaturation and shown in Fig. 4B. The values of 4602 cal/mol and 3008 cal/mol/M were obtained for $\Delta G_D^{H_2O}$ and '*m*'

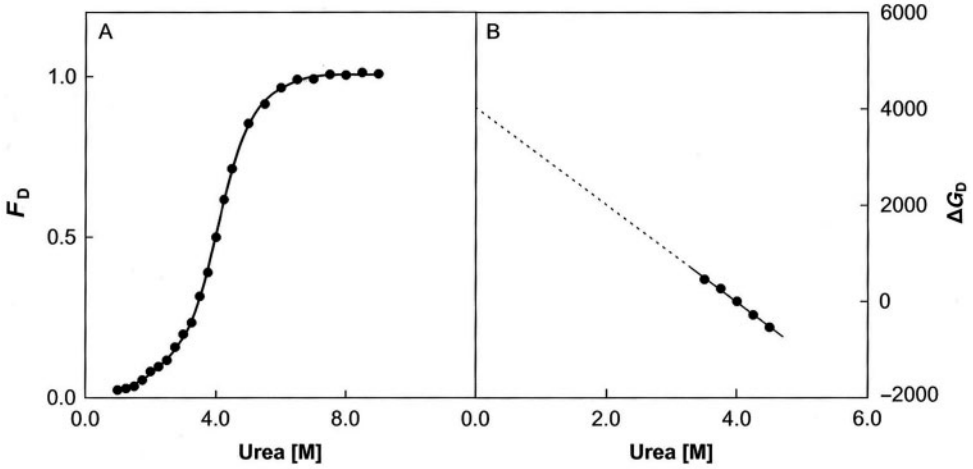


Fig. 2. **(A) Normalized denaturation curve for urea denaturation of BSA shown in Fig. 1B.** (B) Plot of $\Delta G_D$ against urea concentration for the transition shown in Fig. 2A.

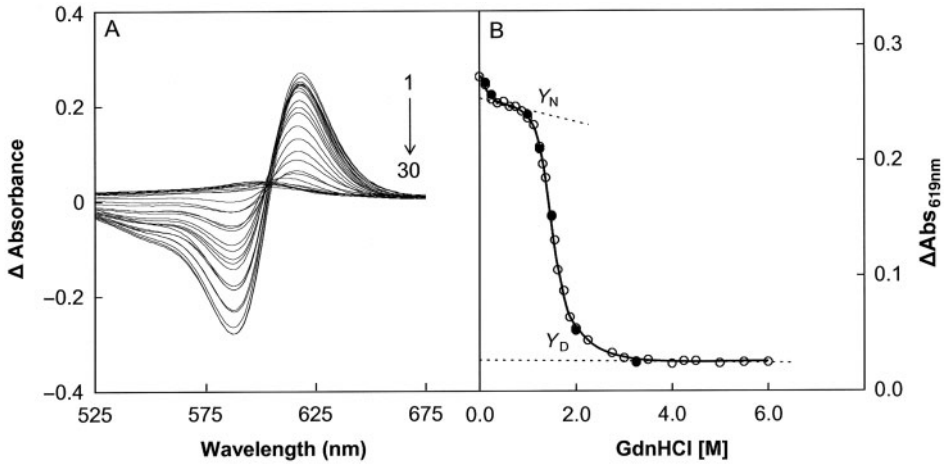


Fig. 3. **GdnHCl-induced denaturation of BSA in 0.1 M Tris–HCl buffer, pH 8.0, 25°C as monitored by BPB binding.** (A) Difference absorption spectra of BPB–BSA (1:1) complex against BPB in the absence and presence of increasing GdnHCl concentrations. Spectra from top to bottom (*1–30*) correspond to GdnHCl concentration as 0, 0.125, 0.25, 0.375, 0.5, 0.625, 0.75, 0.875, 1.0, 1.125, 1.25, 1.3, 1.375, 1.5, 1.56, 1.625, 1.75, 1.875, 2.0, 2.25, 2.5, 2.75, 3.0, 3.25, 3.5, 4.0, 4.25, 4.5, 5.0, 5.5 and 6.0 M, respectively. (B) GdnHCl denaturation curve of BSA as determined from ΔAbs. values at 619 nm against GdnHCl concentration. Open circles show denaturation points and solid circles represent renaturation data.

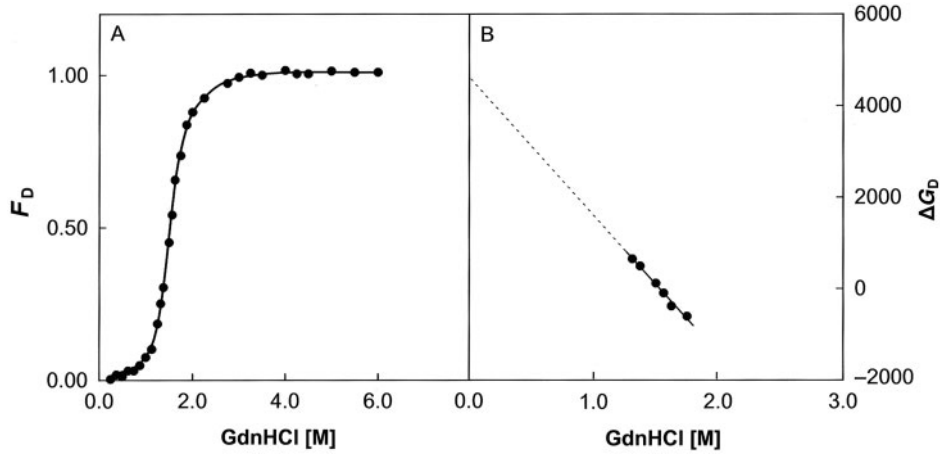


Fig. 4. **(A) Normalized denaturation curve for GdnHCl denaturation of BSA shown in Fig. 3B.** (B) Plot of $\Delta G_D$ against GdnHCl concentration for the transition shown in Fig. 4A.

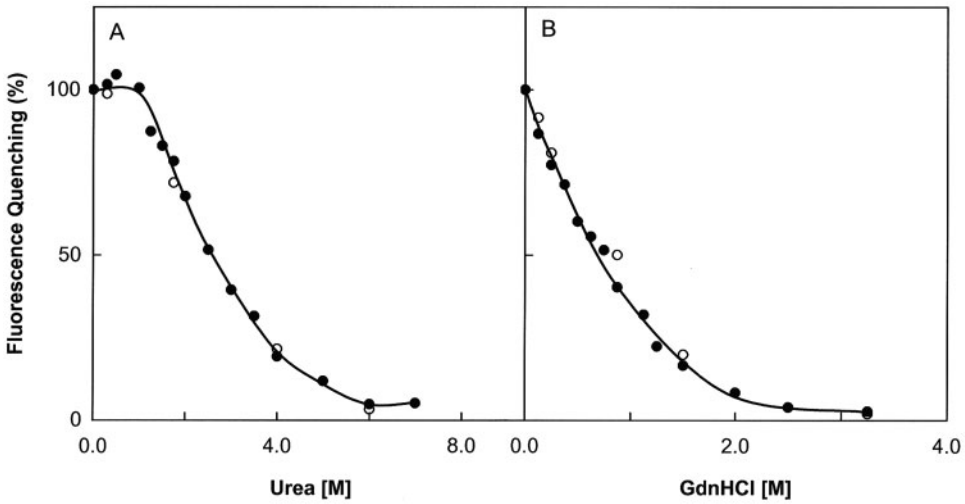


Fig. 5. **Effect of increasing urea (A) and GdnHCl (B) concentrations on the binding of BPB to BSA.** BPB/BSA molar ratio was kept as 1:1 (solid circle) and 2:1 (open circle). Fluorescence data were transformed into percentage fluorescence quenching as described in Experimental section.

respectively using least squares analysis. The value of $\Delta G_D^{H_2O}$ was similar to the one obtained with urea denaturation curve and agreed well with the $\Delta G_D^{H_2O}$ values reported for a number of proteins (*20*). Studies on GdnHCl denaturation of BSA using 2:1 BPB–BSA complex (data not shown) yielded similar type of transition as obtained with 1:1 BPB–BSA complex in terms of start, mid and end-point values of transition. Furthermore, $\Delta G_D^{H_2O}$ value (4623 cal/mol) was also found similar to the one (4602 cal/mol) obtained with 1:1 BPB–BSA complex. These results ruled out the possibility of any effect of BPB binding to BSA on the determination of $\Delta G_D^{H_2O}$.

Figures 5A and B show the effect of urea and GdnHCl respectively on the binding of BPB to BSA as monitored by fluorescence quenching. As shown in Fig. 5A, BPB binding remained unaltered at low (<1.0 M) urea concentration and decreased sharply beyond 1.0 M. However, there was a sharp decrease in binding from the start in the presence of GdnHCl (Fig. 5B). Binding was found negligible at 6.0 M urea and 3.25 M GdnHCl concentration. These results were in agreement with the denaturation results where end points of transitions were found as 6.5 M and 3.25 M for urea and GdnHCl denaturation, respectively (Figs 1B and 3B). Since BPB binding was studied using protein fluorescence quenching at 340 nm, most of which caused by tryptophan residues, 134 and 212 of BSA, located in domains I and II, respectively (*19*), it is possible that urea and GdnHCl change their environment differently compared to the absorbance signal. This may account for the differences in the start and mid points of transitions observed in the equilibrium unfolding and BPB-binding experiments. In view of a single high-affinity BPB-binding site on BSA as reported earlier (*21*), we studied BPB binding at 1:1 BPB/BSA molar ratio. When the stoichiometry of BPB binding to BSA was determined using Job's plot, a molar ratio of two BPB molecules bound to one BSA molecule was obtained (unpublished observation) which agreed well with the published value (*15*). We also performed the BPB-binding experiments at different denaturant concentrations using fluorescence quenching at 2:1 BPB/BSA molar ratio. The results (open circles in Fig. 5A and B) were found to be similar to the one obtained with 1:1 BPB/BSA molar ratio.

To confirm that the two-state model as studied above represents the unfolding transition of BSA, we studied the GdnHCl unfolding transition curve of BSA by measuring protein intrinsic fluorescence at 340 nm upon excitation at 280 nm (Fig. 6A). As can be seen from the figure, transition started after 1.0 M GdnHCl concentration and ended around 3.25 M GdnHCl concentration with a midpoint occurring at 2.125 M GdnHCl. These values of start and end points of transitions were similar to those obtained using BPB binding as a probe (Fig. 3B), suggesting requirement of similar concentrations of GdnHCl effective for protein unfolding. Transformation of data of Fig. 6A into $F_D$ values and calculation of $\Delta G_D$ values from $F_D$ data were performed in the same way as described above. Figures 6B and C show plots of $F_D$ and $\Delta G_D$, respectively, against GdnHCl concentration. A least squares analysis of the data of Fig. 6C yielded values of 4628 cal/mol and 2173 cal/mol/M for $\Delta G_D^{H_2O}$ and '*m*', respectively. These values were similar to the values obtained using BPB binding (Fig. 4B) as a probe.

Taken together, these results suggest that BPB binding can be used as a probe to monitor urea and GdnHCl denaturation of BSA. This probe offers the advantage of studying denaturation of BSA in the laboratory with the help of one visible spectrophotometer and the requirement of lower protein concentration over to UV difference spectroscopy and other techniques.

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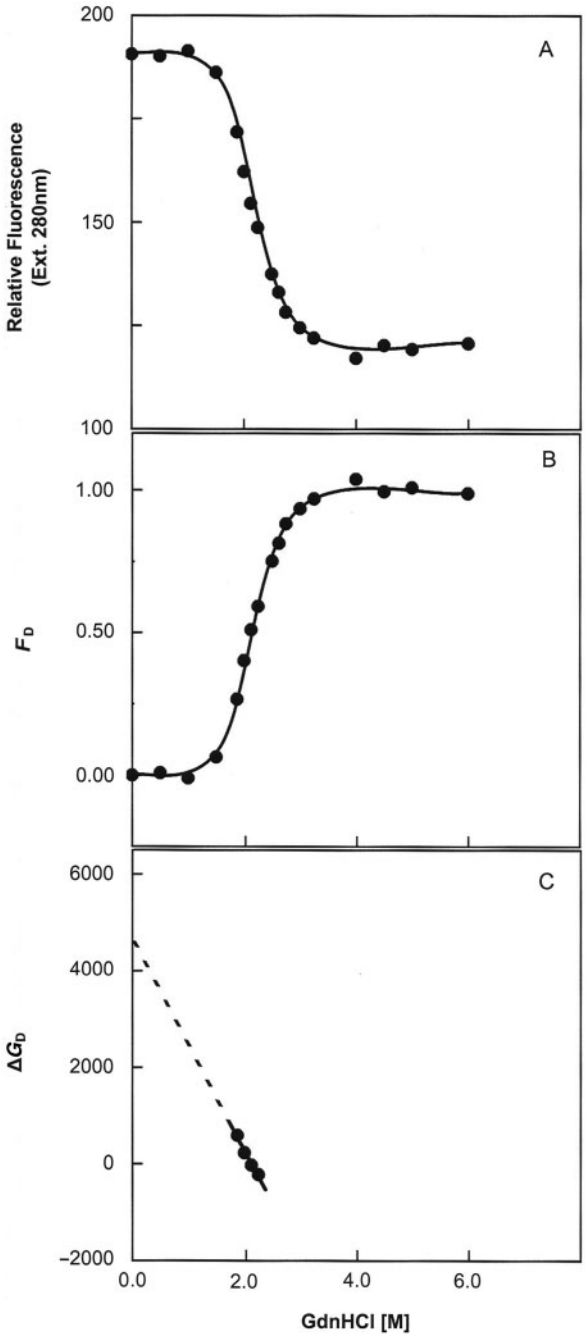


Fig. 6. **(A) GdnHCl-induced denaturation of BSA in 0.1 M Tris–HCl buffer, pH 8.0, 25°C as followed by intrinsic fluorescence measurement at 340 nm on excitation at 280 nm.** (B) Normalized denaturation curve for GdnHCl denaturation of BSA shown in Fig. 6A. (C) Plot of $\Delta G_D$ against GdnHCl concentration for the transition shown in Fig. 6B.

## REFERENCES


1. Shakhnovich, E. and Fersht, A.R. (1998) Folding and binding. *Curr. Opin. Struct. Biol.* **8**, 65–67
2. Fink, A.L. (1998) Protein aggregation: folding aggregates, inclusion bodies and amyloid. *Fold Des.* **3**, 9–23
3. Khan, M.Y., Agarwal, S.K., and Hangloo, S. (1987) Urea-induced structural transformations in bovine serum albumin. *J. Biochem.* **102**, 313–317
4. Muzammil, S., Kumar, Y., and Tayyab, S. (2000) Anion-induced stabilization of human serum albumin prevents the formation of intermediate during urea denaturation. *Proteins: Struct. Funct. Genet.* **40**, 29–38
5. Kamatari, Y.O., Konno, T., Kataoka, M., and Akasaka, K. (1998) The methanol-induced transition and the expanded helical conformation in hen lysozyme. *Protein Sci.* **7**, 681–688
6. Jansens, A., van Duijn, E., and Braakman, I. (2002) Coordinated nonvectorial folding in a newly synthesized multidomain protein. *Science* **298**, 2401–2402
7. Konno, T. (1998) Conformational diversity of acid-denatured cytochrome c studied by a matrix analysis of far-UV CD spectra. *Protein Sci.* **7**, 975–982
8. Muzammil, S., Kumar, Y., and Tayyab, S. (2000) Anion-induced refolding of human serum albumin under low pH conditions. *Biochim. Biophys. Acta* **1476**, 139–148
9. Tayyab, S., Ahmad, B., Kumar, Y., and Khan, M.M. (2002) Salt-induced refolding in different domains of partially folded bovine serum albumin. *Int. J. Biol. Macromol.* **30**, 17–22
10. Santra, M.K., Banerjee, A., Krishnakumar, S.S., Rahman, O., and Panda, D. (2004) Multiple-probe analysis of folding and unfolding pathways of human serum albumin. *Eur. J. Biochem.* **271**, 1789–1797
11. Ahmad, N. and Qasim, M.A. (1995) Fatty acid binding to bovine serum albumin prevents formation of intermediate during denaturation. *Eur. J. Biochem.* **227**, 563–565
12. Kumar, Y., Muzammil, S., and Tayyab, S. (2005) Influence of fluoro, chloro and alkyl alcohols on the folding of human serum albumin. *J. Biochem.* **138**, 335–341
13. Tavirani1, M.R., Moghaddamnia, S.H., Ranjbar, B., Amani, M., and Marashi, S.A. (2006) Conformational study of human serum albumin in pre-denaturation temperatures by differential scanning calorimetry, circular dichroism and UV spectroscopy. *J. Biochem. Mol. Biol.* **39**, 530–536
14. Flora, K., Brennan, J.D., Baker, G.A., Doddy, M.A., and Bright, F.V. (1998) Unfolding of acrylodan-labeled human serum albumin probed by steady state and time-resolved fluorescence method. *Biophys. J.* **75**, 1084–1096
15. Bertsch, M., Mayburd, A.L., and Kassner, R.J. (2003) The identification of hydrophobic sites on the surface of protein using absorption difference spectroscopy of bromophenol blue. *Anal. Biochem.* **313**, 187–195
16. Nishikimi, M. and Yoshino, M. (1972) Fluorometric and spectrophotometric studies on the binding of sulfonphthalein dyes to proteins. *J. Biochem.* **72**, 1237–1244
17. Pace, C.N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.* **4**, 2411–2423
18. Tayyab, S., Sharma, N., and Khan, M.M. (2000) Use of domain specific ligands to study urea-induced unfolding of bovine serum albumin. *Biochem. Biophys. Res. Commun.* **277**, 83–88
19. Peters, T., Jr. (1996) *All About Albumin. Biochemistry, Genetics and Medical Applications.* Academic Press Inc., New York
20. Pace, C.N. (1990) Measuring and increasing protein stability. *Trends Biotech.* **8**, 93–98
21. Murakami, K., Sano, T., and Yasunaga, T. (1981) Kinetic studies of the interaction of bromophenol blue with bovine serum albumin by pressure-jump method. *Bull. Chem. Soc. Jpn.* **54**, 862–868