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Denaturation Mechanism of BSA by Urea Derivatives: Evidence for Hydrogen-Bonding Mode from Fluorescence Tools

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Abstract Urea and alkyl urea derivatives, which posses a free N-H moiety in the urea molecular framework is responsible for the fluorescence quenching of BSA. Fluorescence quenching accompanied with a blue initially and subsequently a red shift in the emission maximum of BSA is observed on the addition of urea derivatives containing N-H moieties. On the contrary, a fluorescence enhancement accompanied with a shift in the emission maximum towards the blue region is observed on the addition of tetramethylurea (TMU). Urea derivatives, which posses a free N-H moiety acts as a perfect denaturant by direct hydrogen-bonding interaction with BSA resulting in the unfolding process. The unfolding of the buried tryptophan moieties to the aqueous phase does not occur, when all the N-H moieties in the urea are methyl substituted (TMU). Fluorescence spectral techniques reveal that the direct hydrogen-bonding interaction of the N-H moiety of urea molecular framework with the carbonyl oxygen moieties of BSA results in the unfolding of the tryptophan moieties to the aqueous phase, while that of the carbonyl oxygen of urea with the N-H moieties of BSA is definitely not involved in the denaturation process. Steady state and time-resolved fluorescence studies illustrate that the extent of protein folding occurs at a relatively lower concentration of unsymmetrical alkyl urea derivatives (butyl urea (BU) and ethyl urea (EU)), compared to that of urea.

Keywords BSA · Urea derivatives · Tryptophan fluorescence · Denaturation · Protein unfolding · Hydrogen-bonding interaction

Introduction

Fluorescence spectroscopic techniques are used as a tool in elucidating the structure and the conformation of large globular protein, peptides and polypeptides both in their native and folded states [1]. Fluorescence spectral studies of biomolecules containing fluorescent amino acids, tryptophan and tyrosine provide useful information regarding the microenvironment of the medium in the presence of denaturing agents. Among the amino acids, tryptophan exhibits unique photophysical properties [2–6] compared to tyrosine and the fluorescence emanating from tyrptophan plays a key role in the denaturation process in many proteins.

Serum albumin, the most widely studied globular protein is present in blood plasma and circulatory system [7, 8]. Serum albumin involves in the transportation of fatty acids, amino acids, steroids and metal ions. It binds with ligands and contributes to 80% of the osmotic blood pressure [9], and is also responsible for the maintenance of blood pH [10].

BSA contains a single polypeptide chain folded into tertiary globular conformation forming three domains [7, 8, 11, 12]. The native form of BSA consists of a micro-heterogeneous population of amino acids [13, 14] and has a larger proportion of hydrophobic interior and less non-polar surface at the exterior. The protein-protein interactions in aqueous solutions are significantly important, which depends upon the number of charged groups and non-polar groups [15].

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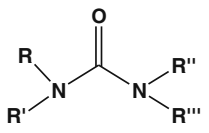
BSA contains two tryptophan moieties (Trp-214 and Trp-135), wherein Trp-214 is deeply buried in the hydrophobic interior. Trp-214 participates in the additional hydrophobic packing interaction at the IIA–IIIA interface. Trp-135 is situated in the second helix of the first domain and is buried in a hydrophobic pocket, which is in contact with the aqueous phase [16–18]. The binding affinity offered by site(I) is mainly through hydrophobic interactions, whereas site(II) involves a combination of hydrophobic, hydrogen-bonding and electrostatic interactions [16, 19]. Interestingly, the buried hydrogen bonds in proteins (intra and intermolecular hydrogen-bonding of peptide groups with water molecule) do not contribute significantly to the protein stability in aqueous solutions [20]. They are easily disrupted by the influence of heat, pH, mineral acids, surfactants and chemical denaturants.

Tryptophan is highly sensitive to the local environment, displaying a substantial spectral shift, which corresponds either to the native or denatured form. The degree of exposure of tryptophanyl side chain is depicted by the position of the fluorescence emission maximum [4, 21]. The aqueous solution of BSA exhibits emission at 340 nm (absence of buffer) and is assigned to the native tryptophan fluorescence, and the emission at 332 and 348 nm are assigned to the tryptophan moiety deeply buried in the hydrophobic pocket and exposed to the aqueous hydrophilic phase respectively. The denatured state is usually identified by the loss of biological function and the quenching of the native protein fluorescence. The process of protein denaturation results in the exposure of the buried tryptophan moieties from the hydrophobic core to the aqueous solvent phase [2, 20–25]. In a spectroscopic approach, the denatured state is accompanied with drastic changes in the shift in absorption and emission maxima. The variation in the photophysical properties of the protein molecule is attributed to the change in the solvent properties like hydrogen-bonding, increased viscosity and formation of aggregates. The extent of folding depends upon the concentration of the denaturant, which is a vital factor in denaturation process.

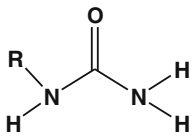
Many researchers have proposed different mechanisms [26–47] for protein denaturation by urea. However, two distinct mechanisms (Direct and Indirect) were proposed based on protein denaturation by urea. The direct mechanism, which is based on Shellmann, Kreschek-Schrega and Stokes (SKSS) model [48–50] and indirect mechanism (water mediated) is dependent on the concentration of the denaturant, presence of hydrophobic groups and the hydrogen-bonding properties of aqueous solution with the protein molecule. In general, urea and alkyl urea derivatives exhibit excellent hydrogen-bonding and hydrophobic properties. The presence of hydrophobic and hydrophilic groups in protein denaturants is of crucial importance in the context of protein folding. In order to unravel the exact mechanism involved in the denaturation of BSA by urea derivatives, photophysical properties of BSA in the presence of symmetrical and unsymmetrical alkyl urea derivatives are investigated in detail.

Symmetrical alkyl urea derivatives



Type	R	R'	R''	R'''
U	H	H	H	H
1,3-DMU	CH_3	H	CH_3	H
1,1-DMU	CH_3	CH_3	H	H
TMU	CH_3	CH_3	CH_3	CH_3

Unsymmetrical alkyl urea derivatives



Type	R
MU	CH_3
EU	C_2H_5
BU	C_4H_9

Experimental Methods

BSA fraction V powder pH-7, was purchased from SRL Chemicals India Ltd, and was refrigerated at 4°C throughout our studies. Urea (U) (Molecular biology grade) obtained from Merck Chemicals, India was used as such. The urea derivatives N-methylurea (MU), N-ethylurea (EU), N-butylurea (BU), 1,3-dimethylurea (DMU), 1,1-dimethylurea (1,1-DMU) and tetramethylurea (TMU) were purchased from Lancaster Chemicals, U.K, (above 97%

purity). Solutions of BSA and urea derivatives were prepared in triple distilled water. The concentration of urea in the range of 0 to 10.0 M was used, and for other urea derivatives to their maximum solubility in water was used.

Absorption and emission spectra were recorded using Agilent 8453 diode array spectrophotometer and Perkin-Elmer MPF-44B fluorescence spectrophotometer interfaced with PC through Rishcom-100 multimeter. Hitachi F4500 spectrophotometer was used for 3D spectral studies. Fluorescence decays were recorded using IBH TCSPC spectrometer [51]. The excitation wavelength was fixed at 295 nm for the present investigation to avoid the excitation of tyrosine and phenylalanine. Both the charge and energy transfer in BSA to the indole side chain of tryptophan moiety is avoided at 295 nm excitation. The emission spectrum of BSA exhibits a single peak centered at 340 nm in water, which is assigned to the tryptophan moieties in native BSA [1].

Results and Discussion

Absorption Spectral Studies

BSA exhibits an absorption maximum at 279 nm and the absorption at this wavelength involves a significant contribution from phenylalanine, tyrosine and tryptophan moieties in BSA. No significant change in the absorbance at 295 nm was observed on the addition of U (0 to 6.0 M) to BSA. The absorption spectrum of BSA as a function of urea in aqueous solution is shown in Fig. 1, and an increase in the absorbance in the range 200–250 nm is due to the

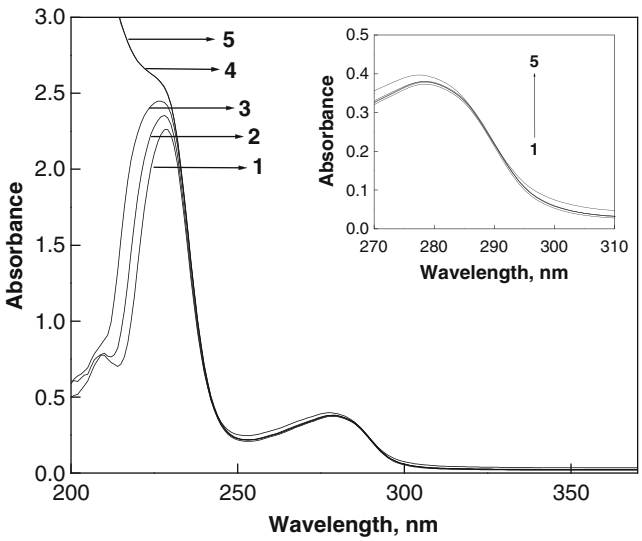


Fig. 1 Absorption spectra of BSA as a function of [urea] in M. (1) BSA alone, (2) BSA + U 1.0 M, (3) BSA + U 2.0 M, (4) BSA + U 4.0 M, (5) BSA + U 6.0 M. Inset: Absorption spectra of BSA-U in the spectral range of 270 to 310 nm

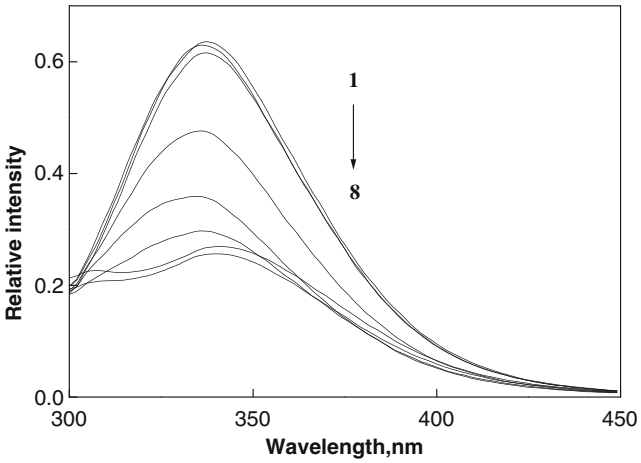


Fig. 2 Emission spectra of BSA in the absence and presence of [urea]. λ_{ex}295nm. (1) 0.0 M, (2) 1.0 M, (3) 2.0 M, (4) 4.0 M, (5) 5.0 M, (6) 6.0 M, (7) 8.0 M, (8) 10.0 M

absorption of urea [51, 52]. Addition of symmetrical and unsymmetrical urea derivatives to BSA results no significant change in the absorbance value at 295 nm. The absorption spectra of BSA with varying concentration of symmetrical (DMU and TMU) and unsymmetrical (EU and BU) alkyl urea derivatives are provided in the supporting information (S1 to S4).

Fluorescence Spectral Studies

Addition of urea upto 2.0 M to BSA results no significant change in the fluorescence intensity or shift in the emission maximum of BSA. An increase in the concentration of urea upto 6.0 M results in a fluorescence quenching, accompanied with a gradual shift in the emission maximum towards the blue region (340 to 334 nm). The emission observed at 334 nm is from the buried tryptophan moiety (Trp 214) in the hydrophobic interior. On increasing the concentration of urea, above 6.0 M and upto 10.0 M, results in a red shift of 334 emission maximum to 348 nm as shown in Fig. 2. The emission at 348 nm is attributed to the exposure of the buried tryptophan moiety (Trp 214) to a hydrophilic phase, which signifies that urea unfolds the buried tryptophan moieties resulting in the denaturation of protein. The pattern of shift in the emission maximum of BSA on the addition of urea in water is similar to that of urea interaction with BSA in tris buffer medium. It has been well documented that there exists an intermolecular hydrogen-bonding interaction between BSA and urea in tris buffer, which is influenced by the concentration of urea [38, 53–55]. In the present study, a marked variation in the emission of the tryptophan moieties of BSA observed by the addition of urea is a clear indication that urea promotes folding of BSA at low concentration (less than 6.0 M) and unfolds BSA at very high concentration (above 6.0 M).

Addition of unsymmetrical urea derivatives (BU and EU) to BSA results in a fluorescence quenching accompanied with a shift in the emission maximum towards the blue region at a relatively low concentration, when compared to urea. The emission spectrum of BSA as a function of EU and BU are shown in Figs. 3 and 4 respectively. Interestingly, a new emission peak in the spectral range of 400–410 nm is observed on the addition of EU and BU to BSA. The emission maximum around 400 nm is attributed to the complete exposure of both the tryptophan moieties to the aqueous phase resulting in a larger extent of unfolding. It has been well documented that interaction of protein denaturants like guanidine hydrochloride [2] and urea [25] with β-barrel protein [21] results in the formation of an emission peak around 400 nm, which is attributed to the complete exposure of the buried tryptophan moieties to the aqueous phase.

Addition of 1, 1-DMU and 1, 3-DMU to BSA results in a fluorescence quenching accompanied with a blue shift in the emission maximum similar to that of urea. But, no new emission is observed at higher concentration of DMU, as observed in the case of BU and EU. The emission spectrum of BSA as a function of 1, 3-DMU is shown in the supporting information (S5).

On the contrary, the addition of TMU to BSA results in a fluorescence enhancement accompanied with a gradual shift in the emission maximum towards the blue region. The emission spectrum of BSA as a function of TMU is shown in Fig. 5.

From the emission spectral studies it is clearly evident that the fluorescence enhancement of BSA is observed only when there is no free N-H moiety in urea, whereas a fluorescence quenching of BSA is observed for urea derivatives containing free N-H moieties. It is also evident that the hydrogen-bonding interaction between the N-H moieties of urea derivatives is definitely involved in the unfolding of the tryptophan moieties of BSA and the carbonyl oxygen of urea derivatives is not involved in the denaturation.

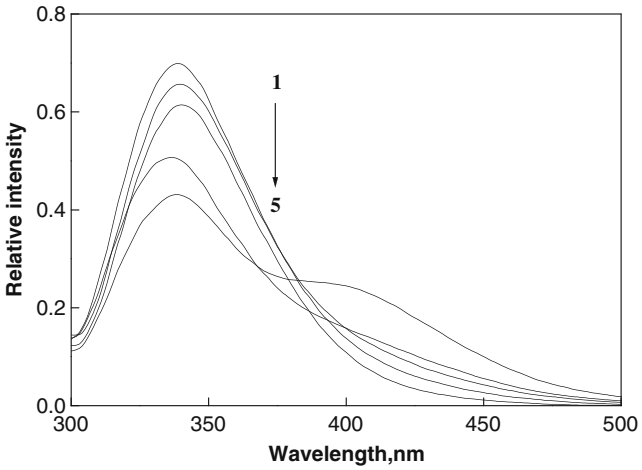


Fig. 3 Emission spectra of BSA in the absence and presence of [EU]. λ_{ex} 295 nm. (1) 0.0 M, (2) 0.6 M, (3) 1.2 M, (4) 2.4 M, (5) 4.8 M

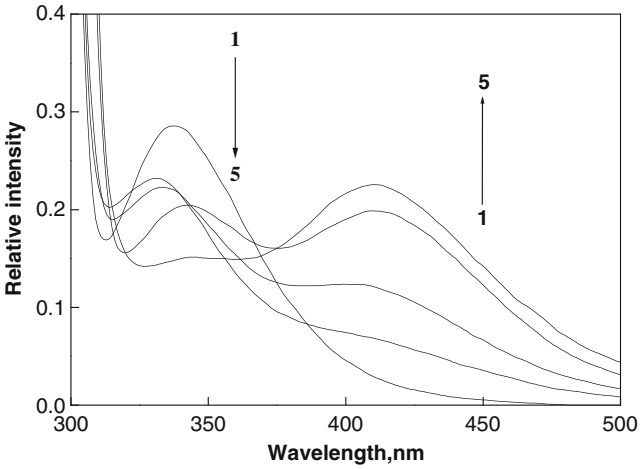


Fig. 4 Emission spectra of BSA in the absence and presence of [BU]. λ_{ex} 295 nm. (1) 0.0 M, (2) 0.6 M, (3) 1.2 M, (4) 2.4 M, (5) 4.8 M

The extent of fluorescence quenching of BSA by BU is increased by 8 when compared to urea, which signifies that a very low concentration of BU (0.5 M) is required when compared to that of urea (4.0 M) for the denaturation process. The above observation reveals that the presence of hydrophobic moiety and a free N-H group in the urea molecular framework, results in a larger fold of exposure of the buried tryptophan moieties of BSA to the aqueous phase, and act as an efficient denaturant compared to that of urea. BU, which is more hydrophobic than all other urea derivatives used in our studies, is an excellent protein

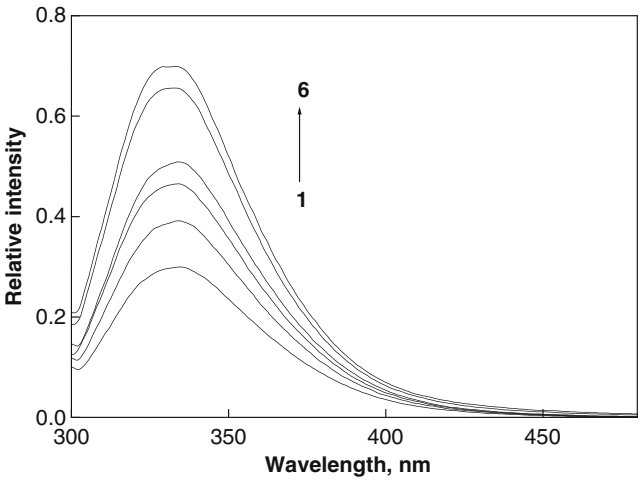


Fig. 5 Emission spectra of BSA in the absence and presence of [TMU]. λ_{ex} 295 nm. (1) 0.0 M, (2) 0.5 M, (3) 1.0 M, (4) 2.0 M, (5) 4.0 M, (6) 6.0 M

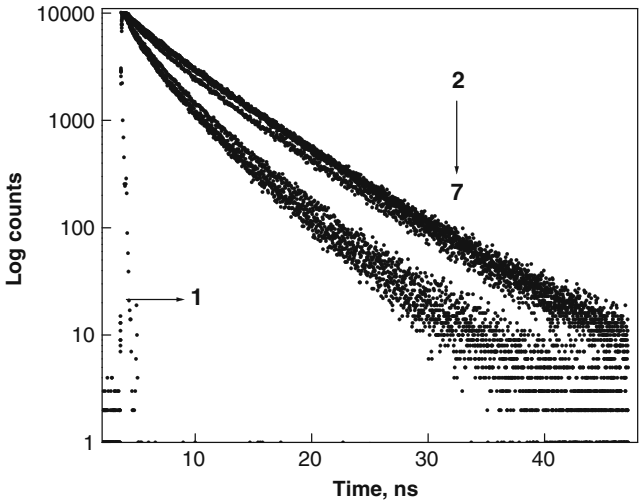


Fig. 6 Fluorescence decay of BSA in the absence and presence of [urea]. (1) Laser Profile, (2) 0.0 M, (3) 0.6 M, (4) 1.2 M, (5) 2.4 M, (6) 4.8 M, (7) 6.0 M, (8) 8.0 M

denaturant compared to other urea derivatives and this is in agreement with the hydrophobicity order of urea derivatives (BU > EU > MU > urea).

3D Emission Contour Spectral Studies

The 3D spectrum of BSA in the absence and presence of U, DMU or TMU is provided in the supporting information (S6), which shows no significant change in the pattern of contour spectra around 400 nm. Interestingly, a variation in the contour pattern is clearly observed at 400 nm on the addition of EU or BU to BSA as provided in the supporting information (S7). The contour pattern of BSA in the presence of BU and EU shows an emission at 340 and 410 nm, which correspond to the emission of both the tryptophan (Trp-214 and Trp-135) and tryptophan (Trp-214) exposed to the hydrophilic exterior respectively. The increase in the steady state fluorescence intensity of BSA (410 nm) on the addition of EU or BU is attributed to increase in the proportion of the buried tryptophan (Trp-214) moieties exposed to the aqueous phase (Figs. 3 and 4).

Time-Resolved Fluorescence Studies

The advantage of time-resolved fluorescence decay measurements over circular dichroism measurements to understand the denaturation of BSA with urea derivatives is clearly illustrated in the present study. The possibility of estimating the extent of the buried tryptophan moieties in BSA exposed to the aqueous phase at very high concentration of urea derivatives by TCSPC is distinctly established. The extent of folding is of utmost importance in protein chemistry, which could not be ascertained from CD studies due to the strong absorbance of alkyl urea derivatives.

The fluorescence lifetime of BSA exhibits a bi-exponential decay pattern with fluorescence lifetime of 6.4 and 3.3 ns as reported [1, 17, 18, 53]. The 6.4 ns lifetime component is assigned to trp-214 and the 3.3 ns component is assigned to that of trp-135 moiety. Denaturation is indicated by the decrease in the fluorescence lifetime of both the components [1, 2, 21–25], albeit there are reports which signify that the fluorescence lifetime may increase or decrease upon denaturation [56–59]. The extent of denaturation is estimated from the amplitude of the components very clearly.

Influence of Symmetrical Urea Derivatives

Addition of urea results in a gradual decrease in the fluorescence lifetime of both the tryptophan moieties. The fluorescence decay of BSA as a function of urea is shown in Fig. 6. The fluorescence lifetime and the relative amplitude of the two tryptophan moieties on the addition of urea (0 to 8.0 M) is provided in Table 1, wherein τ_1 and τ_2 are the fluorescence lifetime of trp-214 and trp-135 moieties, B_1 and B_2 are the relative amplitudes of the two tryptophan moieties respectively. The relative amplitude of the trp-135 moiety increases and that of trp-214 moiety decreases on the addition of urea. A decrease in the fluorescence lifetime of BSA on addition of urea is attributed to the denaturation, which results in the gradual exposure of the trp-214 moiety to the aqueous phase. Addition of DMU to BSA also shows a similar behavior.

Table 1 Fluorescence lifetime decay analysis of BSA with urea

[U] M	τ_1(ns)	τ_2 (ns)	τ_3(ns)	B_1	B_2	B_3	χ^2
0	3.32±0.09	6.42±0.03	–	17	83	–	1.18
0.6	3.0±0.1	6.22±0.02	–	15	85	–	1.06
1.2	2.73±0.06	6.22±0.02	–	21	79	–	1.04
2.4	2.31±0.06	6.05±0.02	–	27	73	–	1.10
4.8	1.84±0.03	7.72±0.02	–	44	56	–	1.23
6.0	1.44±0.04	4.35±0.03	–	40	60	–	1.33
8.0	1.49±0.03	4.30±0.03	–	37	63	–	1.29

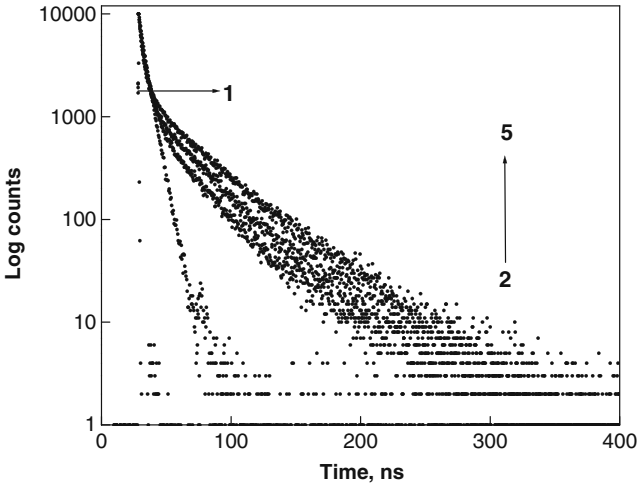


Fig. 7 Fluorescence decay of BSA in the absence and presence of [TMU]. Laser Profile, (2) 0.0 M, (3) 1.0 M, (4) 2.0 M, (5) 4.0 M

Addition of TMU to BSA results in a triexponential decay behavior. The fluorescence decay of BSA as a function of TMU is shown in Fig. 7. The fluorescence lifetime and relative amplitude distribution of the tryptophan moieties as a function of TMU is provided in Table 2. A decrease in the fluorescence lifetime and the relative amplitude distribution of trp-214 and trp-135 moieties accompanied with the formation of a new long lifetime component of 40 ns as the major component results on the addition of 4.0 M of TMU. The observation of longest lifetime component (40 ns) with 70% amplitude is assigned to the location of the tryptophan moiety in the newly formed hydrophobic microenvironment. The displacement of water molecules in the vicinity of the protein molecule results in the creation of a new and very high hydrophobic microenvironment around BSA. Fluorescence spectral studies clearly reveal that the carbonyl oxygen of TMU does not form a hydrogen-bonding interaction with the N-H moieties in the peptide linkage of BSA and the (C=O) of TMU forms hydrogen-bonding only with water molecules in its close vicinity.

Influence of Unsymmetrical Urea Derivative

A gradual decrease in the fluorescence lifetime of trp-135 and trp-214 moieties was observed on the addition of MU, EU and BU as shown in the supporting information (S8 and S9). Addition of MU, EU and BU to BSA is accompanied with an increase in the relative amplitude of the tryptophan moiety exposed to the aqueous phase, and gradual decrease in the relative amplitude of the tryptophan moiety situated in the hydrophobic interior as shown in Table 3, which similar to the urea observation.

A decrease in the fluorescence lifetime and the relative amplitude of the tryptophan moieties situated in the hydrophobic interior signifies that alkyl urea derivatives containing free N-H moieties in the urea molecular framework act as a protein denaturant, which clearly authenticates further that N-H moiety in urea derivatives alone unfolds the protein.

The denaturing efficiency of urea derivatives is further revealed from time-resolved fluorescence studies. 1.2 M of BU alone is required in the exposure of 50% of buried tryptophan moieties to the aqueous phase, whereas 6.0 M of urea is required for the same. This clearly illustrates that BU is a more efficient protein denaturant than urea.

Mechanism of Protein Denaturation

Urea and alkylurea derivatives containing N-H moieties in the urea framework acts as an excellent hydrogen-bonding donor as well as an acceptor. There exists a urea-urea, urea-water and water-water hydrogen-bonding interaction which is unevenly distributed in the solvent phase and this hydrogen-bonding arrangement largely depends upon the concentration of urea derivatives in solution. We have well established that the addition of urea results in the perturbation of the structure of water-water hydrogen-bonding network and this leads to a decrease in the number of water molecules in the shell phase and the position of water molecules are altered [51]. At higher concentration of urea results in a larger displacement of water molecules from the shell phase (predominantly hydrophobic) to the hydrophilic bulk phase and this is well supported by molecular modelling studies based on the SKSS model [48–50]. Experimental evidences and molecular dynamics studies justify that water molecules are largely displaced on the addition of urea [48–50, 60–69], and the water molecules situated in the buried protein molecule are not involved in the protein stability and its conformational changes.

Table 2 Fluorescence lifetime decay analysis of BSA with TMU

[TMU] M	τ_1(ns)	τ_2 (ns)	τ_3(ns)	B_1	B_2	B_3	χ^2
0	3.32±0.09	6.42±0.03	–	17	83	–	1.18
1.0	1.56±0.06	5.86±0.02	33.4±0.1	13	48	39	1.21
2.0	1.42±0.06	4.92±0.02	34.43±0.08	12	31	57	1.28
4.0	2.26±0.03	6.72±0.03	40.0±0.1	20	19	62	1.07

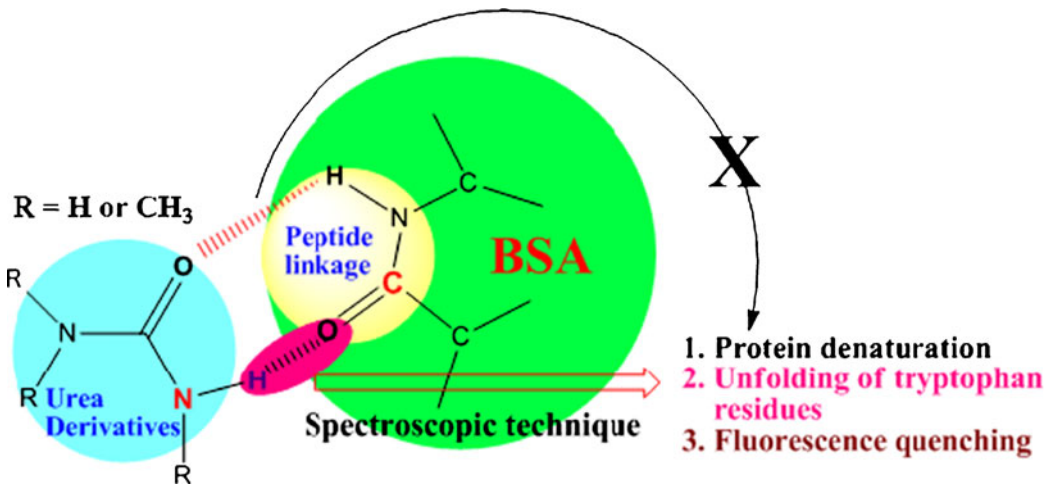
Table 3 Fluorescence lifetime decay analysis of BSA with unsymmetrical alkylurea derivatives

Urea Type	Conc. [M]	τ_1(ns)	τ_2(ns)	τ_3 (ns)	B_1	B_2	B_3	χ^2
MU	0.6	3.05±0.07	6.41±0.02	–	24	76	–	1.10
	1.2	2.50±0.09	6.29±0.03	–	15	85	–	1.00
	2.4	2.46±0.08	6.21±0.03	–	19	81	–	1.23
	4.8	1.95±0.03	5.39±0.03	–	37	63	–	1.23
	7.2	1.85±0.03	5.40±0.03	–	36	64	–	1.08
EU	0.6	2.83±0.08	6.62±0.02	–	20	80	–	1.10
	1.2	2.65±0.05	6.62±0.02	–	23	77	–	1.18
	2.4	2.32±0.06	6.50±0.02	–	27	73	–	1.22
	4.8	2.09±0.04	5.97±0.02	–	41	59	–	1.22
BU	0.6	1.89±0.03	5.27±0.02	–	37	63	–	1.16
	1.2	1.76±0.05	4.93±0.02	–	48	52	–	1.20
	2.4	1.62±0.03	5.50±0.04	–	40	60	–	1.30
	4.8	2.83±0.05	6.4±0.1	0.68±0.05	38	50	12	1.30

In aqueous solution of BSA, water and urea molecules compete with each other to form a hydrogen-bonding network with BSA thereby triggering in an uneven pattern of hydrogen-bonding arrangement throughout the phase. Further, this hydrogen-bonding interaction is largely influenced by the concentration, the hydrophobicity of the urea derivatives and the availability of the hydrogen-bonding donor and acceptor groups in the urea molecular framework. In the present study, we probe whether the mechanism of protein denaturation is governed by either direct or indirect mechanism or by both.

The direct mechanism which is based on SKSS model illustrates that the urea participates in the solvation of hydrocarbon chains and the polar head groups of the amphifiles by replacing water molecules in the solvation layer. Indirect mechanism (water mediated) depicts that urea results in the breaking of the water structure, thereby triggering a collapse in the protein quaternary structure resulting in protein unfolding. Recent studies on the denaturation of proteins by urea suggest that urea denatures proteins via both direct and indirect mechanism. Apart from the urea-solvent hydrogen-bonding influences and the hydrophobic interactions, the origin of electrostatic interactions play a role in the unfolding process as reported by Thirumalai et al. [70, 71]. Urea and alkylurea derivatives exhibit hydrophobic nature and this favours the protein molecule to orient towards the hydrophobic environment, thereby resulting in the displacement of water molecules from the vicinity of BSA.

Our experimental findings clearly unravel the mechanism as direct hydrogen-bonding interaction of the N-H moiety of urea with the hydrogen-bonding functional groups of BSA that results in the unfolding of tryptophan moieties. If the carbonyl oxygen of urea derivatives had been involved in the fluorescence quenching of BSA, a similar pattern should have been observed for all urea derivatives, irrespective of the nature of the alkyl group substitution (N-H or N-CH_3). On the contrary, TMU



Scheme 1 Mechanistics approach of BSA denauration by urea derivatives containing N-H moiety

exhibits a gradual shift towards the shorter wavelength accompanied with a fluorescence enhancement, when compared to other urea derivatives containing N-H moieties, which results in a blue shift at lower concentration and a red shift at higher concentration of urea accompanied with fluorescence quenching. Further from the interaction of TMU with BSA, we envisage that removal of water molecules from the vicinity of BSA or breaking up of the polymeric water structure does not result in the denaturation process.

TMU is a well known water structure breaker compared to other urea derivatives. TMU-water complexes are formed in aqueous solutions and the only mode of interaction of TMU with water molecule is hydrogen-bonding interaction through the carbonyl oxygen. TMU involves in the effective removal of water molecules from the close vicinity of BSA molecule, thereby a larger hydrophobic microenvironment around BSA is created. If the denaturation had occurred by the water structure breaker mechanism, a similar spectral behavior should have been observed for all urea derivatives, which is in contrast with our observation.

Further, TMU does not form any hydrogen-bonding interaction with BSA as it does not possess any N-H moieties in molecular framework. The presence of four bulky (methyl) hydrophobic groups imparts a hydrophobic layer around the protein moiety such that it renders the tryptophan moieties to more hydrophobic microenvironment. The increase in the fluorescence intensity of BSA around 340 nm in the presence of TMU, reveals that it does not expose the tryptophan residue of BSA to the aqueous phase.

From the interaction of urea derivatives with BSA, it clearly justifies that that urea and alkylurea derivatives act as protein denaturants only when they possess a free N-H moiety in the urea molecular framework and the presence of hydrophobic group in urea framework promotes unfolding, whereas in the absence of N-H moiety BSA does not undergo denaturation as shown in Scheme 1. It is further emphasized that unsymmetrical alkylurea derivatives results in a larger extent of exposure of the buried tryptophan moieties to the aqueous phase and are found to be efficient protein denaturants among the urea derivatives used in this study.

Conclusion

The interaction of urea derivatives with BSA in aqueous solution reveals that the N-H moiety of urea is involved in a hydrogen-bonding interaction with the carbonyl oxygen of the peptide back bone of BSA, resulting in the unfolding process. The carbonyl oxygen in the urea molecular framework is not responsible for the protein denaturation. Urea derivatives does not act as a denaturant by breaking the structure of water, rather it forms a direct hydrogen-bonding interaction with BSA through its N-H functional group resulting in unfolding process. Further, the presence of a free N-H moiety along with alkyl groups in the urea molecular framework promotes unfolding of the tryptophan moieties at lower concentration than that of urea and the extent of denaturation increases with an increase in the hydrophobic nature and the concentration of the denaturant.

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