Chapter -3

Unfolding studies in the presence of chemical denaturants-
GdnHCl and Urea
4.3 RESULTS

4.3.1 Effect of denaturants on HMGBC and LMGBC

Inactivation of HMGBC and LMGBC by urea

Results of the loss in the inhibitory activity of HMGBC and LMGBC after incubation for 2 hours in urea solution of different concentration are shown in Fig. 6.0A. HMGBC was found to be stable (89% activity is retained) up to 2.0 M urea while 85% activity was retained at same concentration of urea for LMGBC. The activity was lost with increasing concentration of urea reaching 50% loss at around 3.0 M for both the cystatins. Only 25% of inhibitory activity of HMGBC was left at 4.0 M urea however 46% was obtained for LMGBC at the same concentration. Both the cystatins were left with very negligible activity (less than 5%) at 7.0M urea concentration.

89% inhibitory activity of HMGBC and 94% inhibitory activity for LMGBC were retained upon renaturation with 50 fold dilution of the preincubation mixtures at urea concentration of 8M (Fig. 6.1A).

Effect of guanidium hydrochloride (GdnHCl) on HMGBC and LMGBC inactivation

Both HMGBC and LMGBC completely lost their activities at 6.0 M GdnHCl concentration as shown in Fig.6.0B. Both cystatins retained around 89% activity up to 1.5 M GdnHCl concentrations. At 3.0 M concentration only 33.3% of inhibitory activity was retained by HMGBC while only 25% of LMGBC activity was left.

In case of 6M GdnHCl 92% inhibitory activity of HMGBC and 96% inhibitory activity of LMGBC was recovered back upon renaturation at 40 fold dilution of the preincubation mixtures (Fig. 6.1B).
Fig 6.0 A Urea inactivation of HMGBC (●) and LMGBC (▲)

Native cystatins (1μM) were incubated with increasing concentration of urea (0-8M) for 2 h at room temperature. The cystatin activity was assayed for loss of antiproteinase activity by caseinolytic assay of Kunitz (1947). Values are mean of four independent determinations.

Fig 6.0B Guanidine hydrochloride inactivation of HMGBC (●) and LMGBC (▲)

Native cystatins (1μM) were incubated with increasing concentration of GdnHCl (0-6M) for 2 h at room temperature. The cystatin activity was assayed for loss of antiproteinase activity by caseinolytic assay of Kunitz (1947). Values are mean of four independent determinations.
Fig. 6.1A Renaturation of Urea induced inactivated HMGBC (●) and LMGBC (○) upon dilution

Native cystatins (1μM) were incubated with 8M urea (0-8M) for 12 h at room temperature in the presence of different fold dilutions of the same buffer as used under native conditions. The cystatin activity was assayed for regain of antiproteinase activity by caseinolytic assay of Kunitz (1947). Values are mean of four independent determinations.

Results are expressed as % regain of inhibitory activity upon dilution as compared to native. Inhibitory activity at 8M urea concentration is considered as 0% while in the absence of denaturant the inhibitory activity was considered as 100%.

Fig. 6.1B Renaturation of GdnHCl induced inactivated HMGBC (●) and LMGBC (○) upon dilution

Native cystatins (1μM) were incubated with 6M GdnHCl (0-6M) for 12 h at room temperature in the presence of different fold dilutions of the same buffer as used under native conditions. The cystatin activity was assayed for regain of antiproteinase activity by caseinolytic assay of Kunitz (1947). Values are mean of four independent determinations.

Results are expressed as % regain of inhibitory activity upon dilution as compared to native. Inhibitory activity at 6M GdnHCl concentration is considered as 0% while in the absence of denaturant the inhibitory activity was considered as 100%.
4.3.2 Intrinsic fluorescence studies of HMGBC and LMGBC in the presence of denaturants

Effect of urea on intrinsic fluorescence of HMGBC and LMGBC

Intrinsic fluorescence is an excellent spectroscopic probe to investigate conformational changes in the structure of any protein. Results in Fig. 6.2A and 6.2B indicate the maxima for HMGBC native state to be at 335 nm. Upon denaturation by 8M urea, the maxima was shifted to 337 nm showing a red shift of 2 nm (Fig. 6.2A). From 0.5M to 1.0M change in urea concentration an increase in the intrinsic fluorescence intensity was observed. At urea concentration higher than 1M there is a decrease in emission intensity with a red shift of 2 nm, leading to the lowest at 2.0 M. Further increasing the denaturant concentration from 3.0 M, there is gradual increase in the fluorescence intensity upto 8M.

In case of LMGBC, the maxima was obtained at 336 nm for native (Fig. 6.2B). With the increasing concentration of urea from 0.5M to 8M, there is prominent increase in the fluorescence intensity reaching its maximum at 8M with a red shift of 1 nm.

Effect of GdnHCl on intrinsic fluorescence of HMGBC and LMGBC

Both the cystatins showed different behaviour in presence of guanidine hydrochloride. HMGBC showed a gradual increase in the intrinsic fluorescence with increasing concentration of GdnHCl indicating an unfolding of the cystatin molecule. The inhibitor showed maximum emission intensity at 6M GdnHCl with 1 nm red shift only (Fig. 6.3A).

LMGBC showed an intermediate state while unfolding in the presence of GdnHCl (Fig. 6.3B). At 0.5 M urea concentration the intensity was increased. However increasing the concentration from 0.5 to 1.5 M the intensity decreased showing the presence of some intermediate state. While increasing the GdnHCl concentration from 2.0M to 6.0 M there is steady increase in
Fig 6.2A **Intrinsic fluorescence analysis of HMGBC on interaction with various concentrations of urea.**

The concentration of HMGBC was 5µM. It was pre incubated for 2hrs at room temperature in 50mM sodium phosphate buffer, (pH 7.5) containing the indicated concentrations of urea (0.5-8M). Fluorescence was measured at an excitation wavelength of 280nm in the emission range of 300-400nm with a slit width of 5nm. Fluorescence spectra of HMGBC was measured in different concentrations of urea (1-8M). Spectra of native, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7 and 8M are shown in the Fig.6.2A.

Fig 6.2B **Intrinsic fluorescence analysis of LMGBC on interaction with various concentrations of urea.**

The concentration of LMGBC was 5µM. It was pre incubated for 2hrs at room temperature in 50mM sodium phosphate buffer, (pH 7.5) containing the indicated concentrations of urea (0.5-8M). Fluorescence was measured at an excitation wavelength of 280nm in the emission range of 300-400nm with a slit width of 5nm. Fluorescence spectra of LMGBC was measured in different concentrations of urea (1-8M). Spectra of native, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7 and 8M are shown in the Fig.6.2B.
A

Intrinsic fluorescence intensity

Wavelength (nm)

8M
7M
6M
5M
4M
3M
2M
1M
0.5M
Native

B

Intrinsic fluorescence intensity

Wavelength (nm)

8M
7M
6M
5M
4M
3M
2M
1.5M
1M
0.5M
Native
Fig 6.3A  Intrinsic fluorescence analysis of HMGBC on interaction with various concentrations of GdnHCl

The concentration of HMGBC was 5μM. It was pre incubated for 2hrs at room temperature in 50mM sodium phosphate buffer, (pH 7.5) containing the indicated concentrations of GdnHCl (0.5-6M). Fluorescence was measured at an excitation wavelength of 280nm in the emission range of 300-400nm with a slit width of 5nm. Fluorescence spectra of HMGBC was measured in different concentrations of GdnHCl (1-6M). spectra of native, 0.5, 1, 1.5, 2, 3, 4, 5, and 6M are shown in the Fig.6.3A.

Fig 6.3B  Intrinsic fluorescence analysis of LMGBC on interaction with various concentrations of GdnHCl

The concentration of LMGBC was 3μM. It was pre incubated for 2hrs at room temperature in 50mM sodium phosphate buffer, (pH 7.5) containing the indicated concentrations of GdnHCl (0.5-6M). Fluorescence was measured at an excitation wavelength of 280nm in the emission range of 300-400nm with a slit width of 5nm. Fluorescence spectra of LMGBC was measured in different concentration GdnHCl (1-6M). spectra of native, 0.5, 1, 1.5, 2, 3, 4, 5, and 6M are shown in the Fig. 6.3B.
fluorescence intensity of the cystatin showing complete unfolding at 6.0M GdnHCl. No marked shift of wavelength was observed.

4.3.3 Changes in extrinsic fluorescence of HMGBC and LMGBC in the presence of denaturants

Effect of urea

The fluorescence emission of 1-anilinonapthalene-8-sulphonic acid (ANS) is known to increase when the dye binds to the hydrophobic regions of the protein (Stryer, 1965). Fig. 6.4 and 6.5 shows the ANS fluorescence emission spectra of cystatins.

For HMGBC, the maxima was observed at 480nm (Fig. 6.4A). At 0.5 M to 1.0 M, there is an increase in the ANS fluorescence while from 1.0 to 4.0 M urea concentration there is a decrease in the extrinsic fluorescence with least at 2M urea indicating the formation of intermediates, may be the protein gets aggregated. The ANS fluorescence was found to be maximum at 5M urea concentration, showing the exposure of hydrophobic patches. Increasing the concentration of urea from 5M – 8M there is significant decrease in extrinsic fluorescence owing to the disruption of hydrophobic patches.

In the presence of urea, extrinsic fluorescence of LMGBG was first gradually increased because of the exposure of hydrophobic patches till 4.0M urea concentration. At higher concentration, the ANS fluorescence was decreased as the ANS bound patches were disrupted (Fig. 6.4B).

Effect of guanidium hydrochloride

GdnHCl induced HMGBC denaturation leads to continuous increase of ANS binding to the cystatin molecules till 3M GdnHCl. At 3M concentration, the fluorescence intensity was found to be maximum as highest numbers of hydrophobic patches are exposed. At 4.0 M and higher GdnHCl concentration fluorescence intensity was found to be decreased (Fig. 6.5A).
Fig 6.4A Extrinsic fluorescence analysis of HMGBC in the presence of urea

The concentration of HMGBC was 5µM. It was pre incubated for 2hrs at room temperature in 50mM sodium phosphate buffer, (pH 7.5) containing the indicated concentrations of urea (0.5-8M). Fluorescence was measured at an excitation wavelength of 380nm in the emission range of 400-600nm with a slit width of 5nm. The protein: ANS concentration was taken as 1:60. Fluorescence spectra of HMGBC was measured in different concentrations of urea (1-8M). Spectra of native, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7 and 8M are shown in the Fig.6.5A.

Fig 6.4B Extrinsic fluorescence analysis of LMGBC in the presence of urea

The concentration of LMGBC was 3µM. It was pre incubated for 2hrs at room temperature in 50mM sodium phosphate buffer, (pH 7.5) containing the indicated concentrations of urea (0.5-8M). Fluorescence was measured at an excitation wavelength of 380nm in the emission range of 400-600nm with a slit width of 5nm. The protein: ANS concentration was taken as 1:60. Fluorescence spectra of LMGBC was measured in different concentrations of urea (1-8M). Spectra of native, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7 and 8M are shown in the Fig.6.5B.
Fig 6.5 A Extrinsic fluorescence analysis of HMGBC in the presence of GdnHCl

The concentration of HMGBC was 5μM. It was pre incubated for 2hrs at room temperature in 50mM sodium phosphate buffer, (pH 7.5) containing the indicated concentrations of GdnHCl (0.5-6M). Fluorescence was measured at an excitation wavelength of 380nm in the emission range of 400-600nm with a slit width of 5nm. The protein: ANS concentration was taken as 1:60. Fluorescence spectra of HMGBC was measured in different concentrations of GdnHCl (1-6M). Spectra of native, 0.5, 1, 1.5, 2, 3, 4, 5 and 6M are shown in the Fig.6.6A

Fig 6.5 B Extrinsic fluorescence analysis of LMGBC in the presence of GdnHCl

The concentration of LMGBC was 3μM. It was pre incubated for 2hrs at room temperature in 50mM sodium phosphate buffer, (pH 7.5) containing the indicated concentrations of GdnHCl (0.5-6M). Fluorescence was measured at an excitation wavelength of 380nm in the emission range of 400-600nm with a slit width of 5nm. The protein: ANS concentration was taken as 1:60. Fluorescence spectra of LMGBC was measured in different concentrations of GdnHCl (1-6M). Spectra of native, 0.5, 1, 1.5, 2, 3, 4, 5 and 6M are shown in the Fig.6.6B.
For LMGBC, the extrinsic fluorescence was found to increase at 0.5 M GdnHCl, it then decreased till 2M concentration with a maximum at 1.5M. From 2 M onwards it increased till 4M and then again it decreased at 5M and 6M concentration because of disruption of hydrophobic patches (Fig. 6.5B).

4.3.4 CD measurements

CD studies of urea induced denaturation of HMGBC and LMGBC

Far UV-CD spectroscopy was used to monitor the changes in the secondary structure content of cystatins upon urea and guanidium hydrochloride induced denaturation.

The native spectra of HMGBC and LMGBC showed the presence of significant secondary structure content (Fig. 6.6A, 6.6B). Both HMGBC and LMGBC showed the loss of secondary structure as the concentration of urea was increased. At 8M urea concentration the secondary structure of both the cystatins was completely lost.

CD studies of guanidium hydrochloride induced denaturation of HMGBC and LMGBC

In presence of GdnHCl, the secondary structure of HMGBC was lost as the concentration of the denaturant was increased. At 6M GdnHCl concentration the secondary structure was completely lost (Fig. 6.7A).

The GdnHCl induced LMGBC denaturation occurred in two steps. Fig.6.7B shows that at 0.5 M concentration the secondary structure component was less than that of native. But as the concentration is further increased from 0.5 to 2.0 the secondary structure content was increased showing that at this concentration the LMGBC was more compact as compared to the native (Fig.6.7B). As the concentration was further increased there is loss of the secondary structure content with complete loss at 6M concentration.
5.3 DISCUSSION

The unfolding of proteins in denaturing compounds has been widely investigated. GdnHCl and urea are the most common denaturants used for the conformational, stability and unfolding studies of proteins. In the present study, denaturation and renaturation of two very important mammalian (goat) brain proteins namely HMGBC and LMGBC in presence of urea and GdnHCl have been reported. For several proteins difference in the behaviour has been reported from classical behaviour towards denaturants. Both the cystatins used in the study showed different behaviour while denaturing in the presence of urea and GdnHCl. High molecular mass goat brain cystatin (HMGBC) showed completely different way of unfolding with urea and GdnHCl. The denaturation of HMGBC in presence of urea takes place via intermediate stage. Firstly at lower concentrations the activity was retained till 2.0M concentration, however a decrease in extrinsic fluorescence and intrinsic fluorescence intensity was observed maximally at 2.0 M urea and further increase in urea concentrations showed the formation of some intermediate state between native and completely denatured forms. At 2M urea concentration, the non native state or intermediate state is of the aggregated species formed during the urea induced unfolding. These aggregated species were found to have lesser activity, decreased intrinsic and extrinsic fluorescence as compared to the native state because of the burial of aromatic amino acids and hydrophobic patches of HMGBC at that urea concentration. As the concentration of urea was further increased till 8M there is complete unfolding of HMGBC. The secondary structure was found to be maximum for native HMGBC and its continuous decrease was found at higher concentrations leading to the complete loss at 7M and 8M urea concentration. Aggregation of protein molecules at lower urea concentration have been reported earlier also (Jaenicke, 1987).

Although there is retention of 89% of activity during this aggregation, there is marked and very prominent conformational change in HMGBC as
shown by the results obtained from intrinsic fluorescence, extrinsic fluorescence and far UV CD measurements.

The mode of unfolding of LMGBC in the presence of urea was found to be highly cooperative process, as no significant intermediates are present during the transition of LMGBC from native to denatured states. Such type of two state model behaviour was generally found in small globular proteins and is previously reported for other proteins (Aune and Tanford, 1969).

In the presence of GdnHCl, HMGBC was observed to follow simple two state models for unfolding where no intermediates were found between native and denatured state. Although the inactivation of HMGBC was found to be stronger at lower concentration of GdnHCl i.e., at 3M concentration as compared to 3M urea where 56% activity was retained while with GdnHCl only 33% activity was found. The changes obtained with the guanidine hydrochloride induced denaturation of HMGBC indicated the equilibrium unfolding where only native and denatured states are significantly populated. This type of denaturation has been reported in literature (Tanford, 1968; Ternstom et al., 2005).

In contrast to HMGBC, LMGBC showed the existence of intermediate state while unfolding in the presence of GdnHCl. During the transition of LMGBC from native to denatured state there is an intermediate or non native state which was found to have decreased inhibitory activity, intrinsic, extrinsic fluorescence and more pronounced secondary structure as compared to the native form. This formation of intermediates can have several explanations. These can be due to micro environmental changes in the aromatic region of the protein, small local rearrangements of the native state (Ferreon and Bolen, 2004) or the stabilizing effects of GdnHCl at low concentrations (Mayr and Schmid, 1993; Smith and Scholtz, 1996). There are several reports of GdnHCl and urea showing different denaturation behaviour for different proteins (Pace, 1975; Yao and Bolen, 1995; Park et al., 2003; Deshpande et al., 2003; Wang et al., 2000; Inui et al., 2003; Inouye et al., 2000).
Refolding was also observed both in HMGBC and LMGBC with urea and GdnHCl upon dilution. Almost 92% of HMGBC and 96% of LMGBC inhibitory activity was retained with 6M GdnHCl upon 40fold dilution with the buffer. In case of urea 50 fold dilutions with the buffer results in the retention of 89% of HMGBC and 94% of LMGBC inhibitory activity (Fig. 6.1A and 6.1B). Although urea and GdnHCl have similar modes of action, the different behaviour of proteins towards these denaturants may be because urea has only chaotropic effects whereas GdnHCl is a monovalent salt having both ionic and chaotropic effect.

The unfolding behaviour of HMGBC and LMGBC with urea and GdnHCl indicate that these proteins follow different pathways and mechanisms for unfolding. The results obtained for these two cystatins in the presence of the denaturants are presented in the following proposed scheme A and B.

\[\text{0.5M-2.0M urea}\]

\[
\begin{align*}
\text{HMGBC} & \rightarrow \text{Non-native intermediate state} \\
& \quad /\text{Aggregates} (I) \\
& \downarrow \\
& \text{0.5M-6M GdnHCl} \\
& \downarrow \\
& \text{Denatured /unfolded state} (D)
\end{align*}
\]

\textbf{Scheme A:} Schematic representation of the changes involved during HMGBC denaturation denaturation in presence of denaturants.

\[\text{1.0M-2.0M GdnHCl}\]

\[
\begin{align*}
\text{LMGBC} & \rightarrow \text{Non-native intermediate state} (I) \\
& \downarrow \\
& \text{0.5M-8M Urea} \\
& \downarrow \\
& \text{2M-6M GdnHCl} \\
& \downarrow \\
& \text{Denatured /unfolded state} (D)
\end{align*}
\]

\textbf{Scheme B:} Schematic representation of the changes involved during LMGBC denaturation in presence of denaturants.
As the process of protein unfolding is very complex understanding the conformational changes that result in a protein by various treatments would provide a powerful tool for drug and comprehension of cellular organization at the molecular level. Although tremendous work has been carried out in this field, our study is of great significance as it deals with the denaturation mechanism of two different proteins (cystatins) of the same superfamily, from the same tissue which is of utmost importance, brain. As cystatins play important roles in mammalian body specifically in brain owing to their cysteine proteinase inhibitory activity it is of extreme consequence that their conformation should be stable for maximum functional activity. The above observations have shed some light on the structural alterations and loss of function of cystatins which result due to exposure of these proteins towards denaturants leading to effect the normal functioning of the protein.