Chapter – 2

Studies on the interaction of bilirubin with purified Buffalo liver cystatin

4.2 RESULTS

4.2 INTERACTION OF BUFFALO LIVER CYSTATIN WITH BILIRUBIN

Bilirubin (BR), a catabolic product of haemoglobin in mammals is transported to the liver by the albumin for further metabolism (Brodersen 1979; Gray and Stroupe 1978). Binding of BR with albumins from humans and pigeon have been reported (Khan et al., 2002). A comparative study of the binding of BR with serum albumin from different mammalian species viz, human (HSA), equine (ESA), dog (DSA) and guinea pig (GPSA) by various spectroscopic methods has been reported by (Tayyab et al., 2003). In the present study the binding mode of liver cystatin with bilirubin was studied using spectroscopic methods.

For macromolecules, the fluorescence measurements can give useful information about the binding of small molecules with proteins such as the binding constants, binding sites and binding mechanism. Fluoesence intensity of a compound may decrease or quench by a variety of molecular interactions, such as excited-state reactions, molecular rearrangements, energy transfer, ground state complex formation or collisional quenching. Binding of BR with BLC was studied using fluorescence and absorption spectroscopy. To a fixed concentration of protein (1μM) increasing concentrations of BR were added. The absorption spectra was recorded in the wavelength range of 200-300 nm, for fluorescence spectra the wavelength range was 300-400 nm after exciting the protein solution at 280nm and FTIR spectra was recorded in the range of 1600 and 1700 cm^{-1}. All spectral measurements were done in dim light to prevent undesired photo degradation of BR.

4.2.1 Structural changes and binding of Bilirubin with BLC

Intrinsic fluorescence is an excellent spectroscopic probe to investigate conformational changes in the tertiary structure of proteins. The modification of microenvironment of aromatic residues of BLC in the presence of bilirubn has been studied by monitoring the changes in intensity and wavelength of emission maximum as a function of bilirubin concentration.

The fluorescence intensity of BLC (1μM) in the presence of different concentrations (0.25-20 μM) of bilirubin were recorded in the range of 300-400nm upon excitation at 280nm. Bilirubin caused quenching of the intrinsic fluorescence of BLC (Fig 3.9). These results indicated that there was interaction between bilirubin and buffalo liver cystatin (BLC) and the binding reactions resulted in a non-fluorescent complex. The fluorescence quenching data was analysed by the stern-volmer plot equation:

$$F_0/F = 1+Ksv\ [Q]$$

Where F_0 and F are steady-state fluorescence intensities in the absence and presence of quencher, respectively; Ksv the stern-volmer plot for the binding of BLC-BR complex. The Ksv values obtained at three different concentrations are shown in the table 4.1.

Determination of binding constant (K) and the number of binding sites (n)

When small molecules bind independently to a set of equivalent sites on a macromolecule, the equilibrium between free and bound molecules is given by the equation (Feng et al., 1998; Gao et al., 2004):

$$\text{Log}\ [(F_0 - F)/F] = \log K + n \log [Q]$$

where K and n are the binding constant and the number of binding sites, respectively. Thus, a plot of Log $(F_0 - F)/ F$ versus [Q] can be used to determine k as well as n. The values of K and n obtained at three different concentrations are shown in table 4.1.

4.2.2 UV-vis absorption studies of BR-BLC complex.

The interaction between BR-BLC complex was studied from UV-vis absorption spectra data (fig 4.1). The UV absorption intensity of BLC increased with the increase in bilirubin concentration. The addition of bilirubin results in the distinct shift of BR-BLC spectrum toward longer wavelength (red shift). The results evince interaction and complex formation between bilirubin and BLC.

Figure 3.9: Intrinsic fluorescence study of BLC in the presence and absence of bilirubin (BR).

BLC (1μM) was incubated with various concentrations of bilirubin from 0.25 μM to 20μM for 30 mins in sodium phosphate buffer, pH 7.5. Fluorescence measurements were carried out on a Shimadzu Spectrofluorimeter model RF-540 equipped with a data recorder DR-3 at 25°C. The fluorescence was recorded in the wavelength region 300-400 nm after exciting the protein solution at 280 nm for total protein fluorescence. The slits were set at 5 nm for excitation and emission. The path length of the sample was 1 cm. Each spectrum is an average of three individual scans.

Table-1.8: THERMODYNAMIC PARAMETERS OF BLC-BR INTERACTION AT DIFFERENT CONCENTRATIONS

Parameters	0.25 µM	0.5 µM	0.75 µM
Ksv (M^{-1})	$1.9\pm0.05 \times10^5$	$2.1\pm 0.03\times10^5$	$3.15\pm0.02 \times10^5$
K (M^{-1})	$4.17\times10^5\pm0.07$	$4.22\times10^5 \pm0.03$	$4.31\times10^5 \pm0.06$
Binding constant(n)	1.045	1.082	1.034

Figure 4.0: Stern-Volmer plot for the binding of BLC with BR at different concentrations.

The fluorescence data was analysed by Stern-volmer equation. BLC (1μM) was incubated with various concentrations of (BR) from 0.25 μM to 20 μM for 30 mins at temperatures 298k and their fluorescence spectra were recorded between 300-400nm after exciting BLC at 280nm. The fluorescence quenching data was further analyzed by stern-volmer equation as described in methods. The plot of F0/F vs concentration (log [Q]) of bilirubin gives binding constant (K) and the number of binding sites (n) between BR-BLC complex. Regression R^2 = 0.914.

4.2.3 Inhibitory activity of BLC in presence of (BR)

Changes in the inhibitory activity of buffalo liver cystatin (BLC) after incubation at different concentrations of BR is shown in Table 4.1. The result showed that BLC lost significant amount of inhibitory activity at 0.5 μM concentration after 30 mins incubation. It was further shown that the inhibitory activity of BR showed decrease in its inhibitory activity at constatnt concentration of BR (0.5μM) but with increase in time interval upto 1 and a half hour. The obtained data also indicates that inactivation of buffalo liver cystatin by BR is concentration dependent.

4.2.4 FT-IR measurements of BLC in the presence of bilirubin (BR).

Further evidence regarding the BLC-BR interaction comes from FT-IR spectroscopy, showing BR-BLC complexes. Infrared spectra of proteins exhibit a number of the so called amide bonds, which represent different vibrations of the peptide moiety. This vibration mode originates from the C = O stretching vibration of the amide group (coupled to the in phase bending of the N – H bond and the stretching of the C – N bond and gives rise to infrared bands in the region between approximately 1600 and 1700 cm^{-1} (Wiltod et al., 1993). The protein amide bands have a relationship with the secondary structure.

Figure 4.2 shows the FT-IR spectra of BR and BR bound with the BLC. The spectra in the figure 4.2 was obtained by subtracting the absorption of phosphate buffer from the spectrum of inhibitor solution. The evident peak shift of amide I band from 1655.1 to 1640.3 cm^{-1} indicates that the secondary structure of the BLC is changed when BR binds with it.

INHIBITORY ACTIVITY OF LIVER CYSTATIN IN THE PRESENCE OF BILIRUBIN (BR)

Effect of bilirubin on liver cystatin function was assessed by monitoring th changes in its anti-proteolytic activity which was measured by the caseinolytic assay of papain (Kunitz 1947). Buffalo liver cystatin (1 μM) was incubated with increasing concentrations of bilirubin (0.25-20 μM) for 30 minutes. The results obtained are

Figure 4.1: UV-vis spectroscopy of BLC in the presence of bilirubin (BR)

Absorption difference spectra of bilirubin-cystatin complex in the presence of different concentrations of bilirubin obtained in sodium phosphate buffer, pH 7.5. BLC concentration was fixed at 1 μM while the BR concentration was varied from 0.25 μM- 20 μM. Absorption spectra of the native BLC and in presence of BR were recorded in the range of 200-300 nm on a double beam Shimadzu UV-vis spectrophotometer UV-1700 using a cuvette of 1 cm path length.

Figure 4.2: FT-IR spectra of BLC in the presence of BR

BLC was incubated with different concentrations of BR (0.25-1μM) for 30 mins. The absorbance spectra were taken in the region 1800-1400 cm^{-1} as described in methods.The spectrum is the average of three individual scans.

summarized in Table 1.9. Data indicates that exposure of liver cystatin to the presence of bilirubin (1 μM) resulted in rapid decline of anti-proteolytic activity towards papain with half of the inactivation (55%) taking place at 0.75 μM bilirubin concentration. More than 80% loss in activity took place upon incubation of the cystatin with 10 μM BR.

4.2.5 EFFECT OF pH ON CYSTATIN-BR COMPLEX

Intrinsic fluorescenece study of BLC in the presence of bilirubin (BR) at different pH values

The effect of pH on the fluorescence of cystatin bilirubin complex is shown in figure 4.3. The cystatin-BR complex shows a two step transistion in the pH range of 6.0 to 11.0. In the ph range of 6.0 to 7.0 there is a blue shift of 5 nm from 340 to 335 nm along with a decrease in the fluorescence intensity. However, in the pH range of 8.0 to 9.0 there is a red shift of 5 nm from 340 nm to 345 nm accompanied by a significant increase in fluorescence intensity.

UV-vis spectroscopy of BLC in the presence and absence of bilirubin (BR) at different pH values

The difference absorption spectrum (the system containing bilirubin and cystatin measured against cystatin alone) in the ultraviolet region in the pH range of 6.0 to 10.0 has been shown in figure 4.4. The difference spectra shows peak at 255 nm suggesting the formation of the cystatin-bilirubin complex with no indication of dissociation of bilirubin. The gradual increase in absorption in the pH range of 6.0 to 10.0 is most likely due to the conformational changes of the bilirubin molecule bound at specific sites. There was a loss in inhibitory activity of cystatin-BR complex upon decreasing the pH to 6.0 or increasing the pH to slight alkaline conditions, thereby suggesting that conformational changes are accompanied by functional inactivation of cystatin.

Table 1.9: INHIBITORY ACTIVITY OF BLC IN THE PRESENCE OF BILIRUBIN AT DIFFERENT CONCENTRATIONS

Concentration of bilirubin (BR) (μM)	% Remaining activity
0.25	85 ± 3.2
0.5	62 ± 1.3
0.75	55 ± 2.5
1.0	42 ± 1.1
5.0	29 ± 2.2
10	14 ± 3.1
15	11 ± 3.3
20	6 ± 4.2

Figure 4.3: Intrinsic fluorescence study of BLC in the presence of BR at different pH values.

The figure shows the fluorescence emission spectra of the cystatin bilirubin complex at different pH values (pH 6.0-pH 10.0). Various buffers of different pH and same ionic strength were used.Tris glycine buffer (pH 6.0, 50mM), sodium phosphate buffer (pH 7.0 and 8.0, 50 mM) and tris-NaOH buffer (pH 9.0, 50 mM). The spectrum is the average of three individual scans. BLC (1 μM) was incubated with various pH buffers (6-10) in presence of BR 0.5 μM for 30 mins. Fluorescence measurements were carried out on a Shimadzu Spectrofluorimeter model RF-540 equipped with a data recorder DR-3 at 25°C. The fluorescence was recorded in wavelength region 300-400 nm after exciting the protein solution at 280 nm for total protein fluorescence. The slits were set at 5 m for excitation and emission. The path length of the sample was 1 cm.

Figure 4.4: UV-vis spectroscopy of BLC in the presence of BR at different pH values.

With BLC concentration fixed at 1 μM while the BR concentration was taken as 0.5 μM in solution varying in pH values (pH 7-pH 10) at room temperature, UV difference spectra of the bilirubin cystatin complex was obtained at different pH values, (pH 6.0-pH 10.0). Various buffers of different pH and same ionic strength were used. Tris glycine buffer (pH 6.0, 50 mM), sodium phosphate buffer (pH 7.0 and 8.0, 50mM) and tris-NaOH buffer (pH 9.0 and pH 10.0, 50 mM). Absorption spectra of the native BLC and in presence and absence of bilirubin were recorded in the range of 200-300 nm on a double beam Shimadzu UV-vis spectrophotometer UV-1700 using a cuvette of 1 cm path length.

4.2.6 EFFECT OF VARYING TIME OF INCUBATION ON BR-CYSTATIN COMPLEX

Intrinsic fluorescence of BLC in the presence of bilirubin incubated for varying time intervals

The bilirubin-cystatin complex was incubated for varying time intervals, 10', 20', 30', 60', 90' and 120' (Figure 4.5). It was found that till 30' incubation there was a decrease in fluorescence intensity, however there was no change in the emission maxima of the bilirubin-cystatin complex. Upon increasing the time of incubation to 60', 90' and 120' there was a continuous decrease in the fluorescence intensity and a blue shift of 5 nm from 340 nm to 355 nm which remained constant afterwards indicating that bilirubin cystatin complex has reached saturation.

UV-vis absorption of BR-BLC complex incubated for different time intervals

The difference absorption spectrum (the system containing bilirubin and cystatin measured aganist cystatin alone) in the ultraviolet region for varying time intervals is shown in figure 4.6. The absorption spectra shows a peak at 250 nm for 20' of incubation. Upon increasing the time of incubation to 30', 60', 90' and 120' there is a marked increase in absorbance with a red shift of 5 nm which remained constant thereafter. These spectral changes were also accompanied by loss of inhibitory activity of cystatin-BR complex.

Figure 4.5: Intrinsic fluorescence study of BLC in the presence of BR at different time intervals.

Fluorescence emission spectra of bilirubin cystatin complex obtained for different time intervals (1'-90'). BLC (1μM) was incubated in presence of BR 1 μM for different time intervals. Fluorescence measurements were carried out on a Shimadzu spectrofluorimeter model RF-540 equipped with a data recorder DR-3 at 25°C. The fluorescence was recorded in wavelength region 300-400 nm after exciting the protein solution at 280 nm for total protein fluorescence. The slits were set at 5 m for excitation and emission. The path length of the sample was 1 cm. The spectrum is the average of three individual scans.

Figure 4.6: UV-vis spectroscopy of BLC in the presence of BR at different time intervals.

UV difference spectra of the bilirubin cystatin complex obtained for different times of incubation. With BLC concentration taken as 1 μM while the BR concentration was taken as 1 μM in solution and incubated for different time intervals (1' to 120') at room temperature. Absorption spectra of the native BLC and in presence of BR were recorded in the range of 200-300 nm on a double beam Shimadzu UV-vis spectrophotometer UV-1700 using a cuvette of 1 cm path length.

Figure 4.7: Intrinsic fluorescence study of BLC in the presence and absence of bilirubin (BR) upon photo-illumination.

Fluorescence spectra of bilirubin cystatin complex obtained for different time of exposure to white light.Native protein (BLC) was present in 1μM concentration. Cystatin and unexposed BR-cystatin complex, was exposed for 5, 10, 20 and 30 mins. Fluorescence measurements were carried out on a Shimadzu Spectrofluorimeter model RF-540 equipped with a data recorder DR-3 at 25°C. The fluorescence was recorded in wavelength region 300-400 nm after exciting the protein solution at 280 nm for total protein fluorescence. The slits were set at 5 m for excitation and emission. The path length of the sample was 1 cm.

4.2.7 EFFECT OF PHOTO-ILLUMINATION ON BR-CYSTATIN COMPLEX

Intrinsic fluorescence of BLC in the presence and absence of bilirubin upon photo-illumination

BR-Cystatin complex was exposed to white light for varying time intervals and the effect of this photoillumination on BR-cystatin complex was studied by fluorescence spectroscopy. As can be seen in Figure 4.7 there was a red shift of 5 nm accompanied by the decrease in the fluorescence intensity. The conformational twisting of bound BR leading to conformational or structural isomerisation upon photo-illumination might be responsible for photo induced fluorescence changes.

UV-vis spectroscopy of BLC in the presence and absence of bilirubin upon photo-illumination

The difference absorption spectra for BR cystatin complex under white light for varying time periods (0-30 mins) is shown in Figure 4.8. A blue shift of 10 nm was observed upon 10' photo-illumination. A significant blue shift of 10 nm was observed upon increasing the time of exposure to 30'. The significant blue shift depicts the sensitivity of the BR-cystatin complex towards white light. Photo-illuminated BR-cystatin complex significantly lost its papain inhibitory activity suggesting that the functional inactivation of protein occurs upon exposing the complex to white light.

Figure 4.8: UV-vis spectroscopy of BLC-BR complex in presence and absence of Fluorescent light.

UV difference spectra of the bilirubin cystatin complex is shown in this figure. Native protein (BLC) concentration was fixed at 1 μM. Cystatin bilirubin complex was exposed to white light and scans obtained for different time of exposure to white light. The unexposed LC-BR complex was exposed for 10, 20, and 30 min. Absorption spectra of the native BLC and in presence of BR were recorded in the range of 200-300 nm on a double beam Shimadzu UV-vis spectrophotometer UV-1700 using a cuvette of 1 cm path length.