Discussion

Chapter – 2

Bilirubin (BR), the principal degradation product of heme metabolism in mammals is transported to the liver by albumin for conjugation and subsequent excretion from the body (Brodersen et al., 1990; Gray and Stroupe 1978). Endogenous substances such as bilirubin with low intrinsic (metabolic) clearances and low hepatic extraction ratios may also prove to be principle candidates for protein binding. The clinical importance of the analysis of the binding of bilirubin with human serum albumin (HSA), a crucial carrier of bilirubin in plasma, has attracted investigators over the last several decades (Athar et al., 1999). The interaction of bilirubin with bovine serum albumin (BSA) in aqueous solutions have been studied by various spectroscopic methods (Blauer 1970). The binding of BR to human serum albumin (HSA) has been a long term discussion with reference to the binding forces involved in BR-HSA interaction, nature of the binding site and conformation of the bound pigment (Jacobsen and Brodersen 1983; Lightner et al., 1984; Lightner et al., 1988). However to the best of our knowledge the interactions of bilirubin with thiol-proteinase inhibitors (cystatins) has not been investigated as yet. Therefore, an attempt was made to study the interactions of bilirubin with cystatin that was purified in our laboratory from buffalo liver as detailed in the materials and methods section.

Bilirubin (BR) remains a biologically important, chemically interesting and pedagogically useful molecule. BR is a bile pigment that is produced from the catabolism of haemoproteins at a rate of about 0.25–0.5 g per day and has toxic effects on many cellular functions (Karp, 1979; Wennberg et al., 1979).

Fluorescence spectroscopy is a useful technique which provides useful information on the interactions of macromolecules with proteins. Intrinsic fluorescence of proteins provides a considerable information about the protein structure and dynamics and has been used extensively to study protein folding and association reactions (Lakowicz 2006). Various measurable parameters of fluorescence viz. Quenching, enhancement of intensity, spectral shift etc. are used for interpretation of related structure-dynamics in proteins (Lakowicz 2006). When different concentrations of bilirubin (BR) solutions were incubated with a fixed concentration of cystatin, a remarkable decrease in fluorescence intensity of cystatin was observed, which indicated that BR could bind with cystatin (fig 3.9). Fluoresence quenching is the decrease of the quantum yield of fuorescence from a fluorophore induced by a variety of molecular interactions with a quencher molecule (Naik 2010). Taking advantage of our finding that bilirubin can

strongly quench the fluorescence intensity of cystatin, stern-volmer plot further confirmed that the binding between bilirubin and cystatin is purely static. Stern-volmer analysis of fluorescence quenching data showed the binding constant to be $2.1\pm 0.03\times10^5$ and the number of binding sites equal to one (fig 4.0). Binding constant determines the affinity of the ligand (bilirubin) with protein (cystatin). The value of the binding constant is in accordance with that reported for the binding of methotrexate with goat lung cystatin (Khan et al., 2010) and interaction of bilirubin with liver cystatin Shah and Bano (2010).

The absorption spectra of cystatin with BR was found to be in accordance with the fluorescence results for the binding of BR with the inhibitor. It was evidenced by increase in absorbance and red shift thus confirming the binding of cystatin to BR (fig 4.1). The results are in accordance with the binding of BR with albumin (Jacobson and Brodersen 1983; Tayyab 2001).

Upon complexation with bilirubin the thiol proteinase inhibitory activity was severely challenged suggesting that the binding of BR to cystatin was accompanied by functional inactivation of cystatin (Table 4.1). A similar decline in papain inhibitory activity of cystatin was seen upon binding of methotrexate with lung cystatin suggesting changes in the environment of the crucial amino-acid residues of the protein (khan et al., 2010) and interaction of bilirubin with liver cystatin Shah and Bano (2010).

FTIR spectroscopy was done to analyze the conformational changes during cystatin-Bilirubin interaction. The results confirm the damage caused to cystatin as shown in previous investigations. In the IR spectra of proteins, the secondary structure is determined by the amide I and II bands, particularly the former (Timasheff, S. N et al., 1967; Ruegg. M et al., 1975); the amide I band absorbs at 1657 cm^{-1} (mainly a C=O stretch), and the amide II band absorbs at 1542 cm^{-1} (C-N stretching coupled with N-H bending modes) (Bramanti.E et al., 1996; Krimm.S et al., 1986). Figure 4.2 shows the original spectra of native (untreated cystatin) along with cystatin co-incubated for 30 minutes with bilirubin (0.5- 5 μM). It was observed that upon bilirubin interaction, the α-helix structure was reduced as is evidenced by the decrease in intensity around amide I (1655 cm^{-1}) region in the liver cystatin (BLC). There is also a shift in peak in amide I region from 1655cm^{-1} towards 1637 cm^{-1} with increase

in the bilirubin concentration. The reduction of the α-helix in favor of the unordered structure towards the β-sheet conformation is indicative of the partial unfolding of the inhibitor in the presence of bilirubin. A similar conformational transition from α-helix to β-sheet structure was observed for the myelin protein upon heating the protein at 35 °C (Timasheff et al., 1967).

The results summarized in Figure 4.2 further confirms the damage caused to cystatin as evidenced by a significant shift in the peak intensity, i.e., from 1655 to 1637 and significant changes in the structure of cystatin from that of α-helix towards β structure after co-incubating cystatin (BLC) for 30' with bilirubin (0.5-5μM).

We extended the studies to cover the effect of pH, time and photo irradiation on the cystatin-bilirubin complex. The cystatin-BR complex used for further study was in the ratio of (1:1). The cystatin-bilirubin complex was incubated in the buffers of different pH values (6.0, 7.0, 8.0, 9.0 and 10.0) with same ionic strength. In the pH range of 6.0 to 7.0 fluorescence intensity was found to increase accompanied with a blue shift. However, in the pH range of 7.0 and 9.0, a red shift was observed accompanied by a significant increase in fluorescence intensity (fig 4.4). Further there was an increase in fluorescence intensity in the pH range of 9.0 to 11.0 with a slight blue shift. Changes in the state of ionization in the pH range between 6.0 to 9.0 of various ionisable groups of bilirubin (pyrrole nitrogen, propionic acid and carboxyl groups) may change the mode of interaction between the complex components. In case of pH 10 and pH 11 the slight blue shift indicates the refolding or transition in the structure towards a more compact form. It can be due to extreme change in the pH towards the alkaline side but there was no regaining of inhibitory activity at higher pH (pH 10-pH 11). These observations were further confirmed with supporting results from UV-vis spectrophotometer. The absorption spectra of cystatin with BR shows a peak at 225 nm suggesting the formation of cystatin bilirubin complex with no indication of dissociation of bilirubin (fig 4.3). The gradual increase in absorption in the pH range of 6.0 to 10.0 is most likely due to 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. Similar conformational transistion have been reported upon varying the pH of the BSA-bilirubin complex (Blauer and King 1968).

To investigate the effect of varying time of incubation of cystatin with bilirubin on the activity of cystatin, the complex was incubated for varying time intervals 15-120 mins. Results indicated that increasing the time of incubation led to a sharp decrease in fluorescence intensity, which tended towards a plateau when the fluorescence intensity had reached about three-fourth of its initial value (figure 4.6). The decrease in the fluorescence intensity upon incubating the BLC with bilirubin in the ratio of 1:1 over a different length of time shows the structural changes leading to the futher damage to BLC with respect to time. The fact that the cystatin-BR complex reached saturation was further confirmed by the sharp peak at 250 nm by the UV difference spectra which remained constant thereafter (figure 4.5). Wavelength shifts have also been reported upon increasing the time of incubation of the bilirubin-BSA complex (Blauer and king 1968). These spectral changes were also accompanied by the loss of the inhibitory activity of complex as compared to the BLC-BR (1:1) complex used for the study.

Irradiation of BR-cystatin complexes under white light for varying time periods (0-30 mins) was also studied by fluorescence and UV spectroscopy. The decrease in the fluorescence intensity along with a red shift of 5 nm was seen upon exposing the BR-cystatin complex to white light (fig 4.8). These photo-induced changes could be due to structural-isomerazation resulting in the conformational twisting of the bound BR. The photochemical properties of different bilirubin-albumin complexes of human (HSA), equine (ESA), dog (DSA) and guinea pig (GPSA) have been reported by Tayyab et al. (2002). The photo-induced changes were also confirmed by UV spectroscopy upon exposing the BR-cystatin complex towards white light (fig 4.7). These results are in accordance with that of lamola (1983). Photo-illuminated BR-cystatin complex lost its papain inhibitory activity as compared to the unexpected BLC-BR complex (1:1) used for the study suggesting that the functional inactivation of complex occurs upon exposing the complex to white light.

There is a plausible correlation between bilirubin and cystatin which may play a significant role in various hepatic diseases such as jaundice characterized by high plasma bilirubin levels (Buyukberber 2010). The accumulated bilirubin may bind to liver cystatin resulting in its functional inactivation, which may further lead to the increase in cathepsins level, the hallmark of liver cirrhosis. Such correlations may have significant advantages in clinical interpretations and warrant further study to

elucidate the underlying mechanisms. Further there is a need to determine the effective and safe ways to clear the body of the excess bilirubin in case of some pathophysiological conditions like presence of *Fasciola hepatica* (liver fluke) in case of mammals and humans, which act as host to this parasite leading to liver cirrhosis and possibly jaundice or normal blockade of bile duct leading to increased level of bilirubin in blood.