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ORIGINAL PAPER

Spectroscopic studies on the interaction of bilirubin with liver cystatin

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Abstract Studies on the role of endogenous metabolites such as bilirubin and their interactions with biomolecules have attracted considerable attention over the past several years. In this work, the interaction of bilirubin (BR) with purified goat liver cystatin (LC) was studied using fluorescence and ultraviolet (UV) spectroscopy. The fluorescence data proved that the fluorescence quenching of liver cystatin by BR was the result of BR–cystatin complex formation. Stern–Volmer analysis of fluorescence quenching data showed the binding constant to be 9.27×10^4 M^{-1} and the number of binding sites to be close to unity. The conformation of the BR–cystatin complex was found to change upon varying the pH of the complex. The BR–cystatin complex was found to have reduced papain inhibitory activity. Photo-illumination of BR–cystatin complex causes perturbation in the micro-environment of goat liver cystatin as indicated by red-shift. This report summarizes our research efforts to reveal the mechanism of interaction of bilirubin with liver cystatin.

Keywords Bilirubin (BR) · Cystatin · Fluorescence · Photo-illumination

Introduction

Bilirubin, the end product of haem degradation in humans, is a cytotoxic pigment which is responsible for jaundice in a variety of clinical disease states (Ostrow 1986). The pigment is a linear tetrapyrrole, consisting of two asymmetrical dipyrromethenones connected by a methylene bridge (Schmid 1978). Each dipyrrolic half-molecule carries a carboxyl ethyl side-chain that might be expected to render the pigment a highly polar, ionized and water–soluble molecule at physiological pH values. Hydrogen bonds stabilize the bilirubin molecule in a rigid conformation that resembles a ridge tile or partially folded book (Bonnett et al. 1976) in which planar dipyrromethenone constitutes one leaf of the book. In this conformation, which exists both in crystalline form (Bonnett et al. 1976) and in certain solutions (Kaplan and Navon 1981), these internal hydrogen bonds suppress ionization of the carboxyl groups and monopolize all polar groups in the bilirubin molecule.

Serum albumin (SA) serves as a vehicle for this haem metabolite, which is transported to the liver for conjugation by glucoronidation (Ostrow et al. 1994) and detoxification (McDonagh 1979; Schmid and McDonagh 1979; Lighterner and McDonagh 1984; Frydman and Frydman 1987; Peters 1975). Human serum albumin (HSA), the major plasma protein, binds bilirubin reversibly and acts as a buffer preventing its transfer from blood to tissues. Plasma contains two forms of bilirubin: the first is conjugated to glucuronic acid and is mainly unbound to proteins (direct bilirubin), and the second is an unconjugated form mainly bound to albumin (indirect bilirubin). A small portion of unconjugated form is unbound and is called unbound unconjugated "free" bilirubin; it increases in case of hyperbilirubinaemia and is responsible for bilirubin toxicity (Ostrow et al. 1994). Hyperbilirubinaemia may develop in neonates if either breakdown of haemoglobin is increased or BR binding capacity of albumin is decreased (Brodersen 1978; Hansen and Bratlid 1986). If untreated, deposition of excess BR in various tissues, especially the brain, leads to kernicterus, which may result in infant death (Karp 1979).

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The structure of bilirubin, possessing two propionic acid side-chains, which may contribute to stabilizing the pigment protein complex, has aroused great interest in studying the interaction of bilirubin with goat liver cystatins. Cystatins are proteins that tightly bind and inhibit the harmful effect of cysteine proteinases (Ekiel et al. 1997). These proteins are all related by structure and function to an inhibitor of cysteine proteinases first described in egg white called chicken egg-white cystatins. Cystatins have been evolutionarily related, forming the "cystatin superfamily". The members of the protein superfamily were grouped into three families on the basis of the location, size and complexity of their polypeptide chains (Barrett et al. 1986a, b). Members of family 1, the stefins, are found primarily intracellularly, contain about 100 amino acid residues (~11 kDa) and lack disulphide bonds. Members of family 2, the cystatins, are found primarily in body fluids and in tissues also. These contain about 120 amino acid residues (~14 kDa) and two intrachain disulphide bonds. Family 3 comprises the plasma kininogens and may therefore also be called the kininogen family, with molecular weight of ~70–120 kDa. They contain additional disulphide bonds and are also glycosylated (Barrett et al. 1986a). These inhibitors might protect cells from unwanted proteolysis, which may otherwise cause a number of pathologies (Shah and Bano 2009), such as rheumatoid arthritis (Trabant et al. 1991), osteoporosis (Delaisse et al. 1991), alzheimer's disease (Bernstein et al. 1996), metastasizing cancer (Koppel et al. 1984) and microbial invasion (North et al. 1990).

In this study, the interaction of bilirubin with cystatin was studied using fluorescence spectroscopy. Cystatin used in the present study was purified in our laboratory using goat liver. Fluorescence spectroscopy is a powerful tool to study the interaction of ligands with proteins. The intrinsic fluorescence of the protein and the fluorescence intensity of bilirubin–cystatin complex were evaluated, and taking advantage of our finding that bilirubin can strongly quench cystatin fluorescence, the association constant was accurately calculated. These observations were further confirmed by supporting results from UV–vis spectrophotometry. We have also extended these studies to cover the effect of pH and photo-irradiation on the cystatin-bilirubin complex.

Materials

Papain, Sephacryl-S100HR, casein, acrylamide, ethylene diamine tetra acetic acid (EDTA), acetone, Coomassie Brilliant Blue R-250 and cysteine were from Sigma Chemical Company, St. Louis, USA. Medium-range molecular weight markers were from Genei. BR was purchased from Sisco Laboratories, India. All other chemicals used were of analytical grade.

Methods

Purification of liver cystatin

Cystatin was isolated and purified from goat liver with yield of 370 by using a three-step procedure. Fresh liver tissue (100 g) was homogenized in 200 ml extraction buffer (0.05 M sodium phosphate buffer, pH 7.5, 1.0% NaCl, 3 mM EDTA, 2.0% *n*-butanol). The homogenized tissue was subjected to alkaline treatment. The supernatant of pH treatment was then subjected to 40% ammonium sulphate saturation. The supernatant obtained was thereafter subjected to 1:1 acetone fractionation and later to 60% ammonium sulphate saturation. The precipitated protein after extensive dialysis was applied to a Sephacryl S-100 column, pre-equilibrated with 0.05 M sodium phosphate buffer (pH 7.5). Eluted fractions were analysed for protein concentration and inhibitory activity. Purified liver cystatin (LC) gave a single band on polyacrylamide gel electrophoresis (PAGE). The molecular weight of the inhibitor was found to be 38.8 kDa as calculated by its subunit structure on sodium dodecyl sulphate (SDS) PAGE.

Protein estimation

Protein content was quantitated by Folin's phenol reagent by the method of Lowry et al. (1951).

Assay of thiol proteinase inhibitory activity

The inhibitory activity of cystatins was assessed by its ability to inhibit caseinolytic activity of papain by the method of Kunitz (1947).

Preparation of solutions

Stock solutions of bilirubin were prepared by dissolving 2 mg bilirubin in 2 ml 0.05 M sodium phosphate buffer, pH 7.5, containing 1 M sodium carbonate and 1 mM EDTA and immediately diluting it to the desired volume with the above buffer. The solution was filtered and stored in the dark. The concentration of bilirubin was determined spectrophotometrically by taking the absorbance of the bilirubin solution at 440 nm using a molar extinction coefficient of 47,500 M^{-1} cm^{-1} (Jacobsen and Wennberg 1974). The solution was prepared fresh and used within 2 h. All experiments involving bilirubin were done in dark.

Absorption spectroscopy

Absorption spectra of cystatin as well as cystatin bound to BR were taken on UV–vis spectrophotometer in the wavelength range 220–400 nm using a cell holder with 1 cm path length.

Fluorescence spectroscopy

Fluorescence measurements were made on a Shimadzu spectrofluorometer (25°C) using a quartz cell with 1 cm path length. The excitation and emission slits were set at 5 and 10 nm, respectively. The fluorescence of BR bound to cystatin was recorded in the wavelength range 250–400 nm after exciting the complex at 280 nm.

Photochemical experiments

Photochemical experiments were carried out to measure photo-induced changes in the optical properties of the BR–cystatin complex. Samples were exposed to 40-W white fluorescent light (FTL; Anchor Electronics and Electricals Ltd., India) for various time periods. The distance of the sample from the light source was 2 cm. Photo-induced changes in the BR–cystatin complex were measured by fluorescence and absorption measurements.

Statistical analysis

All experiments were repeated three times to document reproducibility. Wherever applicable, data are expressed as mean ± standard error of the mean (SEM). Significance of difference in mean values was evaluated using one-way analysis of variance (ANOVA). A probability level of $P < 0.05$ was selected as indicating statistical significance.

Results and discussion

BR binding studies

Binding of BR to liver cystatin (LC) was studied using fluorescence and absorption spectroscopy. To a fixed concentration of protein (1 μM) taken in various test tubes, increasing concentrations of BR were added. Absorption and fluorescence spectra were recorded after incubating the solution for 30 min. Absorption spectra were recorded in the wavelength range 220–400 nm. Fluorescence was recorded in the wavelength range 250–400 nm after exciting the protein solution at 280 nm for total protein fluorescence. All spectral measurements were done in dim light to prevent undesired photo-degradation of BR.

Binding of BR to cystatin resulted in a blue-shift from 340 to 335 nm. Increasing the concentration of bilirubin resulted in increased quenching of the cystatin–BR complex. Figure 1 shows the fluorescence emission spectra of the complex of BR with cystatin in the presence of increasing concentration of BR. It was found that maximum quenching occurred at 10 μM bilirubin concentration.

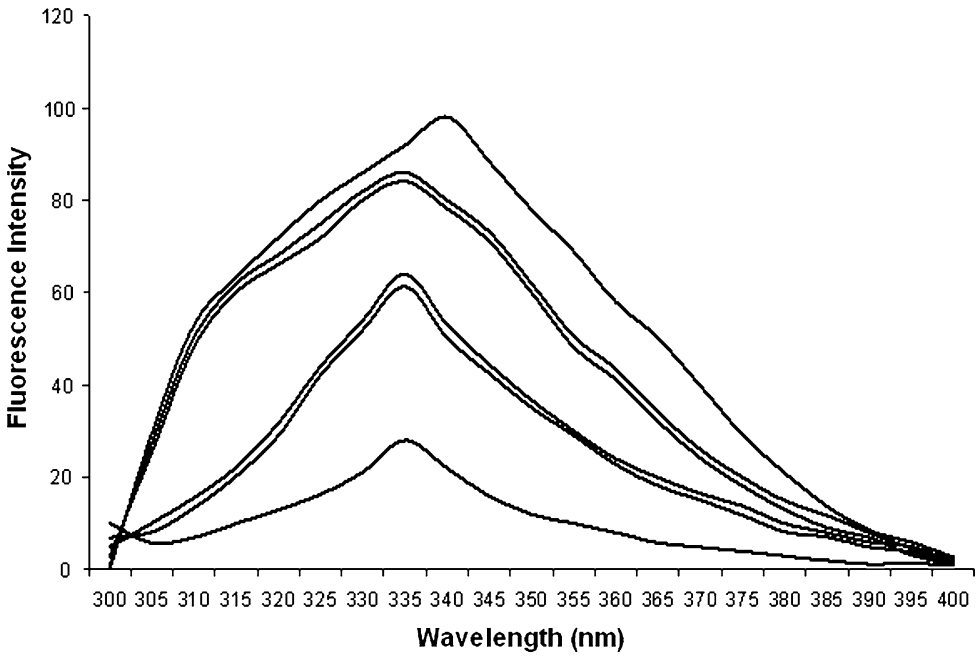

Fig. 1 Fluorescence emission spectra of bilirubin–cystatin complex in the presence of different concentrations of bilirubin obtained in sodium phosphate buffer, pH 7.5 viz. (from *top* to *bottom*) liver cystatin 1 (μM), bilirubin 1, 3, 5, 7, and 10 (μM). Each spectrum is the average of three individual scans

The fluorescence data were analysed by fitting to the Stern–Volmer equation:

$$F_0/F = 1 + K_{SV}[Q],$$

where F_0 and F are the steady-state fluorescence in the absence and presence of quencher (bilirubin), respectively, K_{SV} is the Stern–Volmer quenching constant, and [Q] is the concentration of the quencher (BR).

The plot of $\mathrm{Log}(F_0 - F)/F$ versus Log[Q] can be used to determine the binding constant and the number of binding sites, using the following equation given by Feng et al. (1998) and Gao et al. (2004):

$$\mathrm{Log}\,[(F_0 - F)/F] \;=\; \mathrm{Log}\,K^{+} n\,\mathrm{Log}[Q],$$

where K and n are the binding constant and the number of binding sites, respectively (Fig. 2).

The binding constant was found to be $9.27 \times 10^4\ M^{-1}$, and the number of binding sites was found to be 1. The absorption spectra of cystatin with BR were also computed, and in accordance with the fluorescence results, binding of BR to inhibitor was evidenced by increase in absorbance and red-shift. The results are in accordance with the binding of BR with albumin (Jacobson and Brodersen 1983; Harmatz and Blauer 1975; Tayyab et al. 1995; Khan and Tayyab 2001).

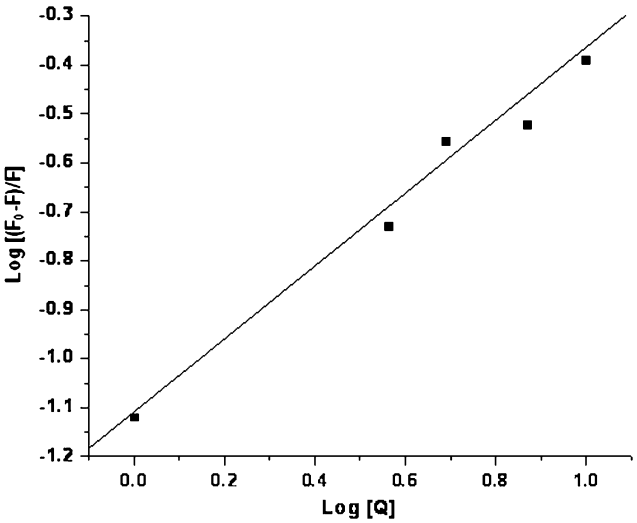

Fig. 2 Determination of number of binding sites and binding constant by Stern–Volmer. Each *data point* represents an average of three separate experiments. Regression $R^2 = 0.9695$

Functional inactivation of liver cystatin

Effects of bilirubin on liver cystatin function were assessed by monitoring the changes in its anti-proteolytic activity using caseinolytic assay of papain (Kunitz 1947). Liver cystatin (1 μM) was incubated with increasing concentrations of bilirubin (1–10 μM) for 30 min. The results obtained are summarized in Table 1. As shown in the table, exposure of liver cystatin to bilirubin (1 μM) resulted in rapid decline of anti-proteolytic (39%) loss towards papain, with half of the inactivation (56%) taking place at 3 μM bilirubin concentration. More than 80% loss in activity took place upon incubating the cystatin with 10 μM BR.

Effect of pH on cystatin-BR complex

The effect of pH on fluorescence of cystatin–bilirubin complex is shown in Fig. 3. The cystatin–BR complex shows a two-step transition in the pH range 6–9. In the pH range 6–7 there is blue-shift of 5 nm from 340 to 335 nm along with a decrease in fluorescence intensity. However, in the pH range 8–9 there is a red-shift of 5 nm from 340 to 345 nm, accompanied by a significant increase in fluorescence intensity. Changes in the state of ionization in the pH range 6–9 of various ionizable groups of bilirubin (pyrrole nitrogens, propionic acid, carboxyl groups etc.) may change the mode of interaction between the complex components.

The difference absorption spectrum (the system containing bilirubin and cystatin measured against cystatin alone) in the ultraviolet region in the pH range 6–9 is shown in Fig. 3 (inset). The difference spectra shows a peak at 250 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 6–9 is most likely due to conformational changes of the bilirubin molecule bound at specific sites. There was a loss in inhibitory activity of cystatin upon decreasing the pH to 6 or increasing the pH to slightly alkaline conditions,

Table 1 Effect of bilirubin on papain inhibitory activity of liver cystatin

Bilirubin (μM)	0	1	3	5	7	10
Liver cystatin activity (%)[a]	100 ± 4.1	61.08 ± 2.2* (−39)	44.37 ± 1.8* (−56)	33.10 ± 1.6* (−66)	28.11 ± 1.2* (−72)	20.23 ± 1.3* (−83)

Liver cystatin (LC) (1 μM) was incubated with increasing concentration of bilirubin for 30 min

[a] LC was assayed for loss in anti-proteolytic activity by caseinolytic method of Kunitz (1947). Activity of native LC is taken to be 100

Results are mean ± SEM of three or more separate experiments

* Significantly different from native LC (control) at $P < 0.05$ by one-way ANOVA

Values in parenthesis represent percentage change from control

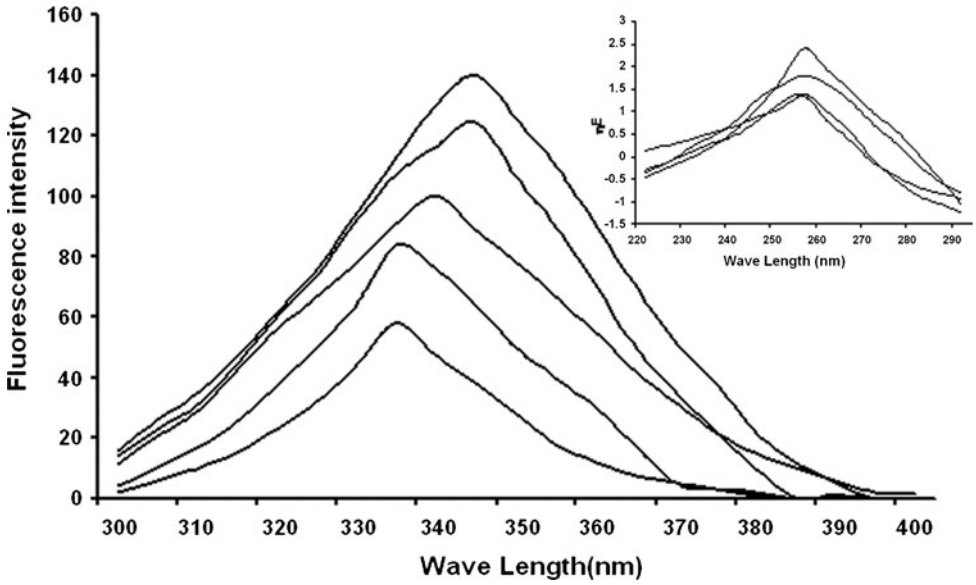

Fig. 3 Fluorescence emission spectra of the bilirubin cystatin complex obtained at different pH values, viz. (from *top* to *bottom*) 9, 8, LC (native) 7, 6. Various buffers of different pH and same ionic strength were used. The spectrum is the average of three individual scans. *Inset*: UV difference spectra of the bilirubin cystatin complex obtained at different pH values, viz. (from *top* to *bottom*) 9, 8, LC (native) 7, 6. Various buffers of different pH and same ionic strength were used. ηE on *y*-axis represents absorbance complex subtracted from absorbance of cystatin alone. Each spectrum is the average of three individual scans

thereby suggesting that conformational changes are accompanied by functional inactivation of cystatin.

Effect of photo-illumination on BR–cystatin complex

Effect of photo-illumination of BR–cystatin complex was studied by fluorescence spectroscopy. As can be seen in Fig. 4, there was a red-shift of 5 nm accompanied by the decrease in the fluorescence intensity, the maximum decrease being for 30 min exposure. The conformational twisting of bound BR leading to configurational or structural isomerisation upon photo-illumination was responsible for photo-induced fluorescence changes.

The difference absorption spectra for BR–cystatin complex under white light for varying time periods (0–30 min) are shown in Fig. 4 (inset). A blue-shift of

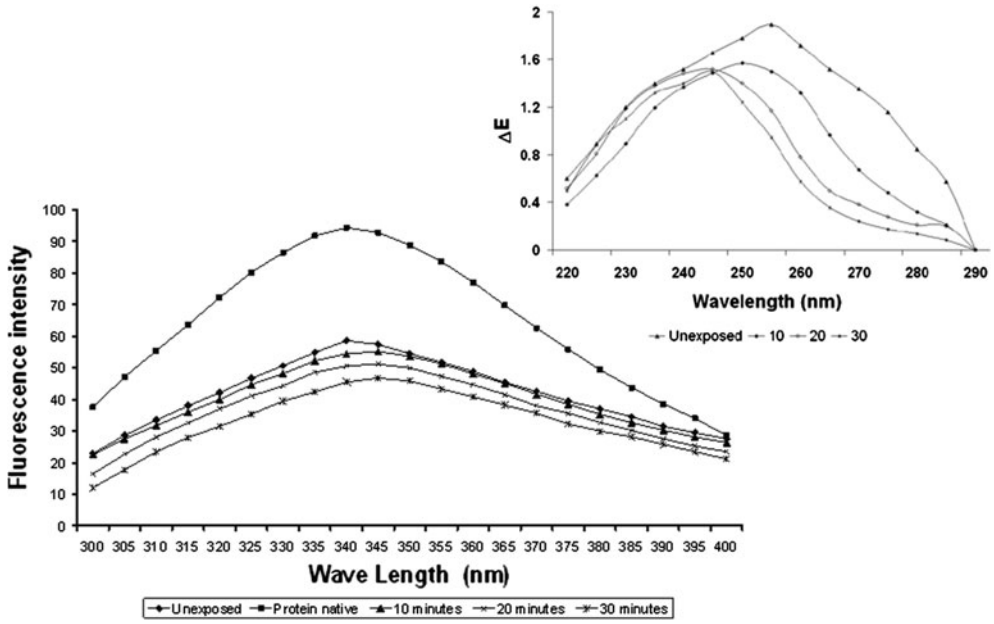

Fig. 4 Fluorescence spectra of bilirubin cystatin complex obtained for different time of exposure to white light. viz. (*top* to *bottom*) Protein (LC) native, unexposed BR-LC complex, exposed for 10, 20, and 30 min. Each spectrum is the average of three individual scans. *Inset*: UV difference spectra of the bilirubin cystatin complex obtained for different time of exposure to white light. viz. (*top* to *bottom*) unexposed LC-BR complex, exposed for 10, 20, and 30 min. ηE on the *y* axis represents absorbance complex subtracted from absorbance of cystatin alone. The spectrum is the average of three individual scans

5 nm was observed upon 10 min photo-illumination. A significant blue-shift of 10 nm was observed upon increasing the time of exposure to 30 min. The significant blue-shift indicates the sensitivity of the BR–cystatin complex to white light. Photo-illuminated BR–cystatin complex lost its papain inhibitory activity, suggesting that functional inactivation of protein occurs upon exposing the complex to white light.

Conclusions

Our study reveals correlations between bilirubin and cystatin which may play a significant role in various hepatic diseases such as jaundice, which is accompanied by increase of bilirubin levels. The accumulated bilirubin upon binding with liver cystatin results in its functional inactivation, which further leads to increase in cathepsin levels, the hallmark of liver cirrhosis. Such correlations may have significant advantages in clinical interpretation and warrant further study to elucidate the underlying mechanisms.

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