Brazilian Journal of Medical and Biological Research (2004) 37: 963-968
ISSN 0100-879X Short Communication

Quenching of the intrinsic fluorescence of bovine serum albumin by chlorpromazine and hemin

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Abstract

The binding of chlorpromazine (CPZ) and hemin to bovine serum albumin was studied by the fluorescence quenching technique. CPZ is a widely used anti-psychotic drug that interacts with blood components, influences bioavailability, and affects function of several biomolecules. Hemin is an important ferric residue of hemoglobin that binds within the hydrophobic region of albumin with high specificity. Quenching of the intrinsic fluorescence of bovine serum albumin (BSA) was observed by selectively exciting tryptophan residues at 290 nm. Emission spectra were recorded in the range from 300 to 450 nm for each quencher addition. Stern-Volmer graphs were plotted, and the quenching constant estimated for BSA solution titrated with hemin at 25ºC was 1.44 (± 0.05) x 10^5 M^{-1}. Results showed that bovine albumin tryptophans are not equally accessible to CPZ, in agreement with the idea that polar or charged quenchers have more affinity for amino acid residues on the outer wall of the protein. Hemin added to albumin solution at a molar ratio of 1:1 quenched about 25% of their fluorescence. The quenching effect of CPZ on albumin-hemin solution was stronger than on pure BSA. This increase can be the result of combined conformational changes in the structure of albumin caused firstly by hemin and then by CPZ. Our results suggest that the primary binding site for hemin on bovine albumin may be located asymmetrically between the two tryptophans along the sequence formed by subdomains IB and IIA, closer to tryptophan residue 212.

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Research supported by CAPES.

Received April 10, 2003
Accepted April 22, 2004

Key words
- Chlorpromazine
- Hemin
- Albumin
- Fluorescence quenching

Chlorpromazine (CPZ) is a phenothiazine drug widely used in psychiatric treatment, and possesses a tricyclic ring bend at the N-S axis. Similar to other anti-psychotic drugs, its chronic use may cause severe side effects (1-3), such as parkinsonism, cornea opacity and respiratory troubles, and is suspected to be related to iron sediments in blood vessels and nervous cells (4). It is also known that high acute doses of CPZ induce hyperglycemia in people with latent diabetes mellitus. The cause of many of these side effects is still unclear.

CPZ binds to three important blood com-

ponents: red blood cell membranes, albumin and lipoproteins. CPZ also interacts with lipid, the bilayer and proteins of biomembranes, influencing their permeability (5,6), although the mechanisms responsible are still in discussion. Recently, Rukhadze et al. (7) investigated the association of CPZ with bovine serum albumin (BSA), concluding that concentration of both the drug and protein influenced the absolute and relative values of the free fraction of CPZ.

Binding to plasma proteins is an important pharmacological parameter, since it frequently affects the distribution and elimination of a drug, as well as the duration and intensity of its physiological effects (8). Difficulties in predicting the clinical effects of the plasma level of a drug may reflect the biological variability in its absorption and elimination. Indeed, it is well known that the interaction of CPZ with blood components influences bioavailability and affects the function of several biomolecules (9).

On the basis of these considerations, we studied the interaction between CPZ and albumin, and determined if CPZ could influence the hemin-albumin interaction. Hemin is an important porphyrin residue of hemoglobin that binds to the hydrophobic region of albumin with high specificity (10). It is known that highly hydrophobic porphyrins can penetrate lipidic regions of membranes, while moderately hydrophobic ones preferentially distribute into polar regions of the cell (11). Porphyrins and related compounds have been used widely as therapeutic drugs. Most clinical applications have focused on fluorescence detection and photodynamic therapy of cancer (12).

Albumin represents 52-60% of the total plasma protein and plays an important role in the transport and storage of hormones, ions, fatty acids and drugs, acting by regulating their plasma concentrations (9,13). It is the major binding protein for most anionic drugs, and binds cationic drugs with moderate affinity, but more avidly than other plasma proteins such as alpha-acid glycoproteins and lipoproteins (14). Phenothiazines are cationic (basic) compounds (8).

Most drugs bind reversibly to a number of binding sites on albumin, and there is evidence of conformational changes in protein induced by its interaction with low molecular weight drugs. These changes appear to affect the secondary and tertiary structure of albumin. Hushcha et al. (15) reported that albumin globules have the most compact configuration at physiological pH, and either increasing the pH of the medium to 8.0 or decreasing it to 5.4 resulted in the increase of globule size. The interaction with CPZ causes conformational changes of albumin similar to basic transitions. Verbeeck et al. (8), using the equilibrium dialysis technique, reported that the binding of CPZ by albumin is a high capacity and low affinity interaction.

In the present study, quenching of the intrinsic fluorescence of BSA was observed by selectively exciting tryptophan residues. BSA consists of a chain of 580 amino acid residues forming a single polypeptide of known sequence, which contains three homologous α-helix domains (I-III) (13,16) and two tryptophan residues. These are located at positions 134 and 212 of the chain. Stern-Volmer graphs were plotted, and the primary binding sites of CPZ and hemin to BSA were analyzed.

We have performed *in vitro* studies on the interaction of CPZ and hemin with BSA. CPZ and hemin were purchased from Sigma. To prevent photodegradation, CPZ stock solutions were kept in the dark at -10ºC. Hemin stock solutions (5 mM in 20 mM NaOH) were also stored at -10ºC. Fluorescence measurements were performed with a PTI-QM1 Fluorescence System (Pontifícia Universidade Católica, Rio de Janeiro, RJ, Brazil). The intrinsic fluorescence of albumin was observed by exciting tryptophan residues at 290 nm. Emission spectra were recorded in the range from 300 to 450 nm for each quencher addition. The final concentra-

tions of CPZ employed, 60 to 260 nM, were within the plasma levels measured after the administration of this drug to psychiatric patients (1). Emission and excitation bandwidth were set at 3 nm. The quenching of the fluorescence of BSA by CPZ was observed under two different conditions: with a BSA solution and with 1:1 BSA/hemin solution (BSA-hemin mixture).

Quenching measurements were made in 3 ml of 2 µM BSA in sodium phosphate buffer, pH 7.4. CPZ and hemin were added from concentrated stock solutions so that the increase in volume was negligible. Experiments were performed at 25ºC.

To study the interaction of CPZ with the BSA-hemin mixture, titration with CPZ was started after adding hemin to BSA solution at 1:1 molar ratio. Using data from all experiments, graphs were plotted according to the Stern-Volmer equation (17):

$$F_0 = F\ (1 + K_S\ [Q])$$

where F_0 and F are the relative fluorescence intensities of the albumins in the absence and presence of CPZ, respectively (F_0 is taken to be always 100%), K_S is the quenching constant, which is related to the bimolecular collision process, and [Q] is the quencher concentration.

Measurement of quenching of albumin's natural fluorescence is an efficient method to study its interaction with several substances. It can reveal the accessibility of quenchers to albumin's fluorophore groups, help understand albumin's binding mechanisms to drugs, and provide clues to the nature of the binding phenomenon (1,17). A solution of BSA excited at 290 nm emits fluorescence attributable mainly to its tryptophan residues. One residue is located at position 134 in subdomain IB, and another at position 212, in subdomain IIA. The adherence of two subdomains with their grooves towards each other forms a domain, and three of such domains make up an albumin molecule (9,16). An important feature of intrinsic protein fluorescence is the high sensitivity of tryptophan to its local environment (17).

The BSA solution was titrated with hemin at 25ºC and quenching data are presented as a Stern-Volmer plot in Figure 1. The quenching constant was 1.44 (± 0.05) x 10^5 M^{-1} (see inset in Figure 1). Only regions of low concentrations were considered to calculate this constant. At 1:1 ligand/protein molar ratio, hemin quenches about 25% of original BSA fluorescence, and 50% is quenched at the 7:2 molar ratio. This indicates that 3.5 mol hemin quench one-half the fluorescence emitted by 1 mol BSA. This fast fall in fluorescence intensity shows that hemin readily binds to BSA, indicating that hemin reaches subdomains where tryptophan residues of this molecule are located. This also suggests that the primary binding site of hemin on BSA is very close to these residues, since the occurrence of quenching requires molecular contact between fluorophore and quencher (17).

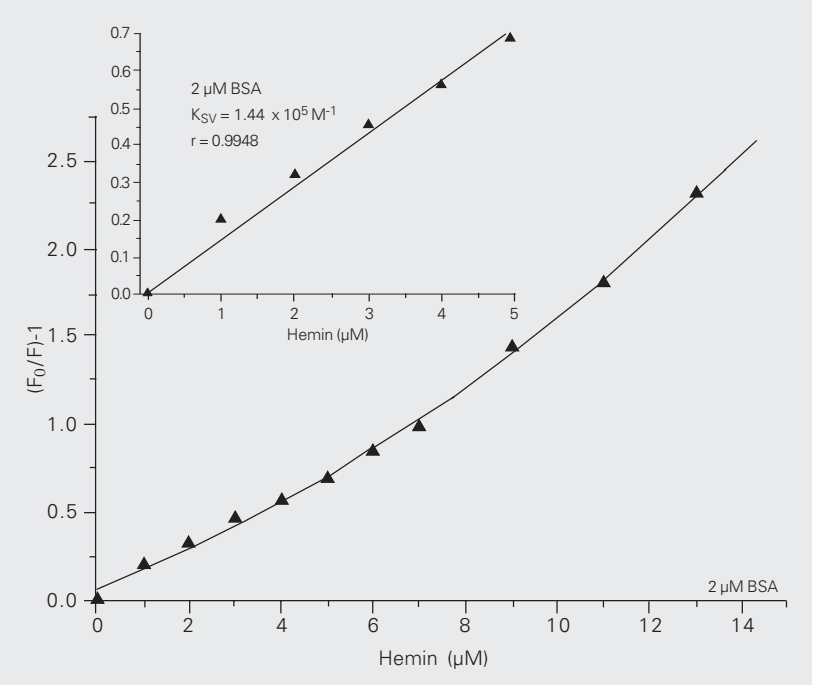

Figure 1. Stern-Volmer plots of the quenching of bovine serum albumin (BSA) by hemin at 25ºC. Excitation wavelength = 290 nm; BSA = 2 µM in 10 mM sodium phosphate buffer, pH 7.4.

It has been shown that hemin binds to human serum albumin (HSA), which has a structure similar to BSA, with a high association constant, 5.0 x 10^7 M^{-1} at 23ºC, pH 7.5 (6,9). Based on the magnitude of the binding constants and results of kinetic studies, Hrkal et al. (18) suggested that this primary binding site is located in the 124-298 amino acid sequence of HSA. They proposed that the presence of the C-terminal part of albumin was essential for the spatial configuration of the site. In both HSA and BSA, fragment 124-298 consists of a part of subdomain IB plus part of subdomain IIA, including the hinge region of this domain. It is important to emphasize that both tryptophan residues of BSA are located within that fragment.

Stern-Volmer plots for BSA, the BSA-hemin mixture and pure tryptophan solutions titrated with CPZ are shown in Figure 2. At 1:1 CPZ/BSA molar ratio, CPZ quenched about 7% of pure albumin fluorescence. Quenching reached 50% when CPZ concentration was 13 times that of BSA.

Addition of hemin (1:1) immediately quenched about 25% of BSA fluorescence. In the Stern-Volmer plot of the BSA-hemin mixture (Figure 2), the initial Fo value was the fluorescence intensity measured after hemin addition. Titration of this BSA-hemin mixture with CPZ at 25ºC showed differences in quenching curves when compared to the titration of pure albumin. At a 1:1 CPZ/BSA molar ratio, CPZ quenched about 12% of the original mixture fluorescence. In this case, the quenching effect of CPZ on the BSA-hemin mixture was 1.7-fold greater than on pure BSA. Quenching reached 50% at 4:1 CPZ/BSA. This ratio was about three times smaller than for pure BSA (13:1), and the quenching effect on the BSA-hemin mixture was three times greater than on BSA.

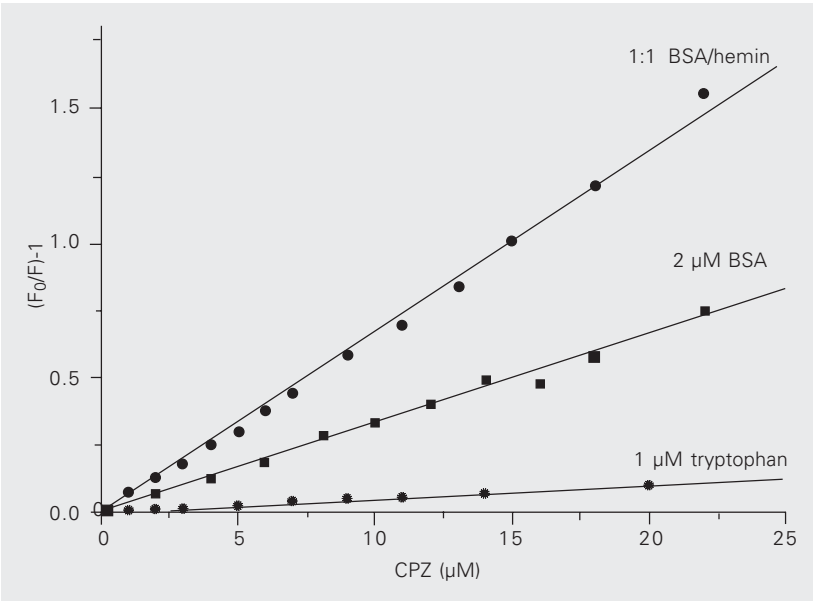

Figure 2. Stern-Volmer plot of the quenching of bovine serum albumin (BSA) solution, BSA-hemin 1:1 molar solution (BSA-hemin) and tryptophan by chlorpromazine (CPZ) at 25ºC. Excitation wavelength = 290 nm; BSA = 2 µM in 10 mM sodium phosphate buffer, pH 7.4; tryptophan = 1 µM in 10 mM sodium phosphate buffer.

Figure 2 shows a linear plot for tryptophan ($r = 0.9885$, $P < 0.0001$). According to Lakowicz (17), a linear plot is generally indicative of a single class of fluorophore molecules, all equally accessible to the quencher. Figure 2 also shows that BSA and BSA-hemin titrated with CPZ yielded linear plots, with correlation coefficients of $r = 0.9945$ ($P < 0.0001$) and $r = 0.9982$ ($P < 0.0001$), respectively. This suggests that at pH 7.4 the tryptophan residues of BSA were equally accessible to CPZ for CPZ/BSA molar ratios lower than 13:1.

Albumin domains have a hydrophobic interior and polar exterior, and almost all hydrophobic residues are located between the helices and inside the trough, whereas the great majority of polar residues are on the outer wall of the structure (9). Steinhardt et al. (19) suggested that the environments of the tryptophan residue(s) in albumin solutions are relatively polar, and the environment of the tryptophan residue located at position 134 of BSA presents higher polarity than that at position 212.

Hrkal et al. (18) suggested that the primary binding site for hemin is located in the amino acid sequence 124-298 of HSA, in agreement with the observation that hemin binds close to the middle of the albumin

molecule (10), where tryptophan 212 is located. According to these data and on the basis of the non-linearity of the BSA Stern-Volmer plot (Figure 1), we suggest that the primary binding site for hemin in BSA is located asymmetrically between the two tryptophans along the sequence formed by subdomains IB and IIA, closer to the tryptophan residue 212.

Experiments with HSA fragments revealed that different peptides in amino acid sequence 124-298 were able to bind hemin with relatively high affinity (18). The gradual loss of linearity of the Stern-Volmer plots with continuous addition of hemin (Figure 1) suggests that the secondary sites for hemin are located near tryptophan residue(s).

Adams and Berman (20) proposed that the binding of hemin to HSA to form a complex follows a two-stage process. The first step being a chemically controlled process involving an interaction of hemin with a group on the protein surface, which would be followed by an entropy-controlled internalization of the hemin molecule, suggesting a process of "albumin adaptability".

Comparison of BSA and BSA-hemin plots reveals that the addition of hemin can increase the quenching effect of CPZ on BSA three times, showing that hemin makes tryptophan residues more accessible to the drug. One reason for this increase is the conformational change caused by binding of hemin to BSA, which can facilitate the CPZ insertion into the structure of the protein molecule or result in exposure of the previously shield tryptophan residue. Indeed, according to Steinhardt et al. (19), interaction between albumin and ligands is often accompanied by conformational changes of the protein and/or simple coverage of the residue(s) by certain parts of the bound ligand molecules. Binding of anionic ligands results in a more compact structure, and binding of cationic ligands apparently results in an increase in the overall volume of albumin (9). On the other hand, Hushcha et al. (15) reported that binding of CPZ to albumin is able *per se* to cause conformational changes in protein similar to basic transitions.

In summary, the quenching constant estimated for BSA solution titrated with hemin at 25ºC was 1.44 (± 0.05) x 10^5 M^{-1}. At 1:1 ligand/protein molar ratio, hemin quenches about 25% of original BSA fluorescence, while CPZ quenched about 7%. Comparison of BSA and BSA-hemin plots showed that the presence of hemin in solution makes tryptophan residues more accessible to the drug, since it was able to increase three times the quenching effect of CPZ on BSA.

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