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INTERACTION OF CHLORPROMAZINE WITH MYOGLOBIN AND HEMOGLOBIN

A COMPARATIVE STUDY

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Abstract—The mode and nature of the binding of chlorpromazine (CPZ), a psychotropic drug, with myoglobin, a monomeric muscle protein, were studied spectrofluorometrically and the results compared with those from the binding of CPZ to hemoglobin, a tetrameric allosteric protein from red blood cells (RBC). CPZ interacted with myoglobin in a non-cooperative mode, with a binding constant of $8.4 \times 10^3\ M^{-1}$ in 0.145 M NaCl, pH 6.8, whereas in the case of hemoglobin this interaction was found to be positively cooperative with a binding constant of $4.2 \times 10^3\ M^{-1}$. The interaction of CPZ with myoglobin was not influenced by the NaCl molarity of the solution, whereas CPZ interaction with hemoglobin significantly decreased with increasing NaCl molarity, indicating that CPZ–hemoglobin binding is mostly electrostatic in nature, whereas that of the CPZ–myoglobin complex is of a non-electrostatic type. Thermodynamic analysis revealed that binding of CPZ to hemoglobin was exothermic ($\Delta H° = -2.65$ kcal/mol), whereas binding to myoglobin was endothermic ($\Delta H° = +1.39$ kcal/mol) with a high entropic contribution ($\Delta S° = +23$ cal/degree/mol), suggesting that CPZ binding to myoglobin is hydrophobic in nature. Such contrasting binding features of this drug have been discussed in the light of a typical subunit interaction property present and absent in hemoglobin and myoglobin, respectively.

Key words: chlorpromazine; myoglobin; hemoglobin

Chlorpromazine (CPZ†; molecular weight 315) is one of a non-planar phenothiazine group of drugs used widely in the treatment of psychological patients all over the world [1]. As is the case with most ligands, this drug manifests its functional activity at the molecular level by interacting with different receptor macromolecular components within the cell. CPZ is known to interact with DNA, RNA and protein [2, 3] and also with the cell membranes [4]. We have already established in our laboratory that CPZ binds in a positive cooperative mode with hemoglobin, a tetrameric allosteric protein from red blood cells. The binding is mostly electrostatic in nature, and oxygen was found to be released gradually from hemoglobin with the gradual increase in the extent of binding [5, 6].

Like hemoglobin, the muscle protein myoglobin (molecular weight 16,700) is another heme-containing protein involved in the storage and transfer of oxygen within the muscle cells [7]. The α and β polypeptide chains of hemoglobin and the myoglobin chain are structurally alike in that each has eight α-helical segments, the same percent α-helix content and a similar three-dimensional structural organization. However, unlike tetrameric hemoglobin with its four interacting subunits ($2_\alpha 2_\beta$), myoglobin is a single-chain monomeric protein and has no subunit interactions [8]. In the present study, we investigated the mode and nature of the binding of CPZ to myoglobin, which lacks the above mentioned subunit interaction property of tetrameric hemoglobin. Our results clearly indicate a distinct difference in the binding characteristics of CPZ to myoglobin as compared with CPZ binding to tetrameric hemoglobin.

MATERIALS AND METHODS

CPZ was obtained as a gift from Sun Pharmaceuticals, India. An aqueous stock solution of CPZ was made fresh before each set of experiments. Two absorption maxima were seen, one at 254 nm ($\varepsilon = 33{,}200\ M^{-1}\ cm^{-1}$) and the other at 305 nm ($\varepsilon = 4000\ M^{-1}\ cm^{-1}$). Solutions of CPZ up to a 1000 μM concentration obeyed Beer's law, and showed no significant absorbance in the visible wavelength region.

Preparation of tetrameric hemoglobin from human RBC and its characterization were performed according to a method described elsewhere [5]. Horse heart myoglobin was purchased from the Sigma Chemical Co., U.S.A. Stock solutions of myoglobin were prepared by dissolving myoglobin crystals in PBS buffer (0.145 M NaCl in 0.002 M

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† Abbreviations: CPZ, chlorpromazine; RBC, red blood cells; and ε, molar extinction coefficient.

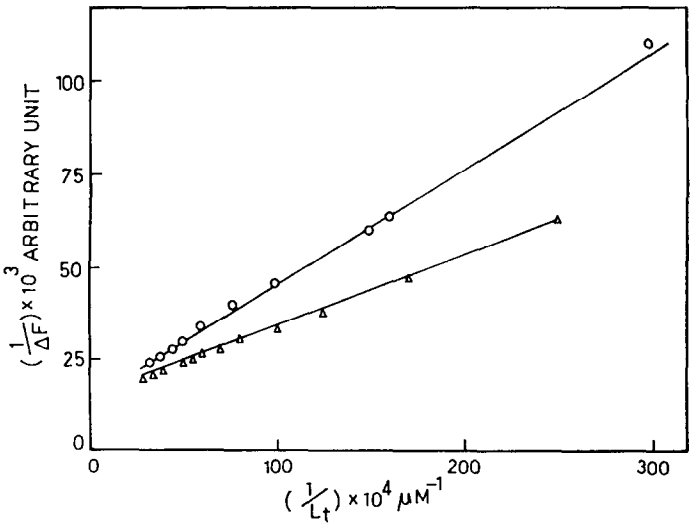

Fig. 1. Plot of $1/\Delta F$ versus $1/L_t$, where L_t is the concentration of CPZ added to tetrameric hemoglobin (○) and myoglobin (△).

sodium phosphate buffer, pH 6.8). The molar concentration of the myoglobin solution was determined from its absorbance at 418 nm (ε = 128 $mM^{-1}\,cm^{-1}$) [9].

The binding parameters were estimated from the quenching of the fluorescence intensity of a fixed concentration of hemoglobin or myoglobin solution in the presence of an increasing added CPZ concentration within the 5–400 μM range. All fluorescence measurements were performed in a Perkin Elmer MPF-44B Spectrofluorometer using a 1-cm path length quartz cuvette. CPZ from a concentrated stock solution was added to 3 mL of myoglobin or hemoglobin solution by a Tarson micropipette so that the volume increment in the cuvette was negligible. The emission maxima of hemoglobin and myoglobin were at 324 nm when excited at 285 nm. Quenching of the tryptophan fluorescence intensity of myoglobin or hemoglobin in the presence of the added CPZ was measured from the change of the respective emission intensity at 324 nm. The drug CPZ has its absorption minimum at 285 nm, and when excited at 285 nm, it does not contribute significantly to the fluorescence at the above emission wavelength. The fluorescence intensity of even 400 μM CPZ at 324 nm is 30-fold less than that of 8 μM myoglobin or hemoglobin excited similarly at 285 nm. The quenching of protein fluorescence by CPZ is mainly due to the formation of a ground state complex between the protein and CPZ. This has already been shown for the hemoglobin–CPZ complex [5, 6].

The temperature dependence of the binding constant was measured spectrofluorometrically from the emission spectra of the hemoglobin or myoglobin solution in the presence of the drug in the temperature range of 5° to 35° using a circulating water bath, the temperature of which can be controlled within ±0.1°.

Table 1. Dependence of the binding affinity constant (K) on the sodium chloride (NaCl) molarity of the solution

NaCl (M)	Affinity constant K ($\times 10^3\ M^{-1}$) for CPZ binding to: Hemoglobin	Myoglobin
0.002		10.0 ± 1.0
0.075	5.4 ± 0.28	
0.145	4.2 ± 0.61	8.4 ± 0.71
0.30	1.7 ± 0.35	8.0 ± 0.78

Values are means ± SEM of measurements, in each case, from five individual experiments.

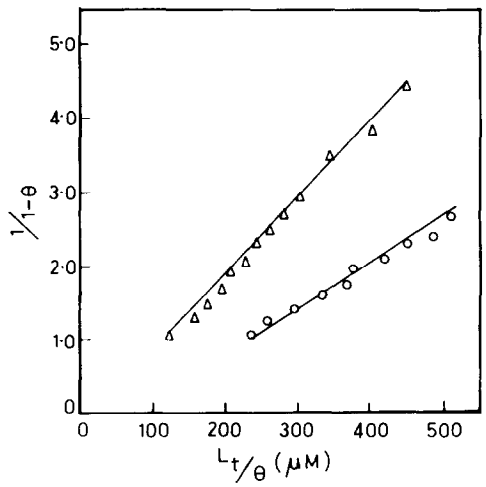

Fig. 2. Plot of $1/1 - \theta$ versus L_t/θ where θ is the extent of binding and L_t is the concentration of added CPZ. The concentrations (A_t) of hemoglobin (○) and myoglobin (△) were 16 and 8 μM, respectively.

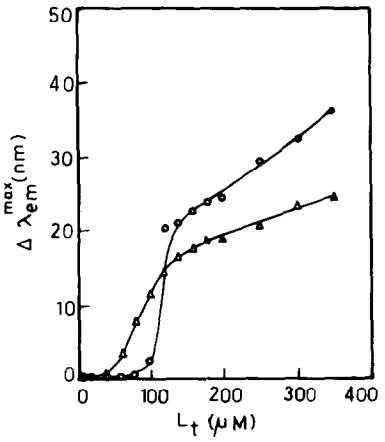

Fig. 3. Plot of the change in wavelength of the emission maximum ($\Delta\lambda_{em}^{max}$) of hemoglobin (○) and myoglobin (△) as a function of added CPZ concentration, L_t.

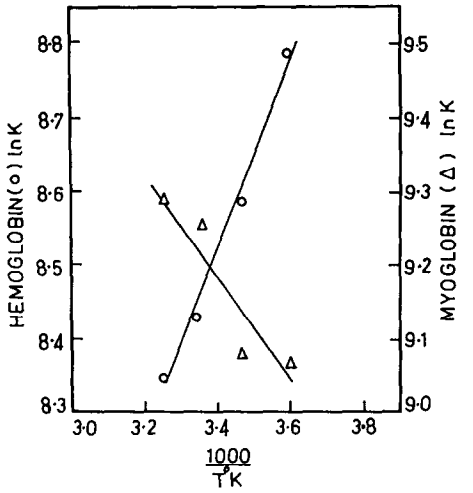

Fig. 5. Plot of ln K versus 1/T where K is the association constant for CPZ binding to hemoglobin (○) and myoglobin (△). T is the temperature in absolute scale.

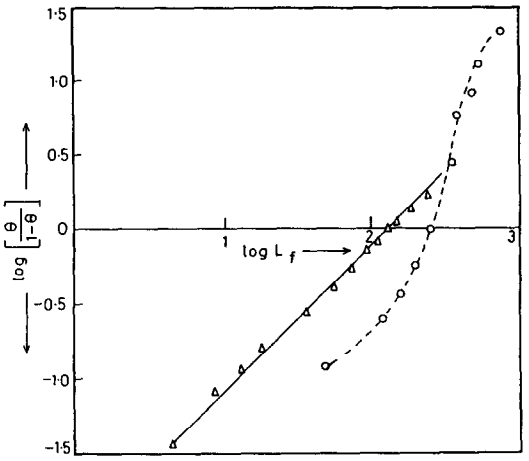

Fig. 4. Hill plot analysis of CPZ binding to hemoglobin (○) and myoglobin (△). The concentration of free drug, L_f, is expressed in μM.

RESULTS AND DISCUSSION

Figure 1 is the linear plot of $1/\Delta F$ versus $1/L_t$ following the equation [10]:

$$\frac{1}{\Delta F} = \frac{1}{\Delta F_{max}} + \frac{1}{KL_t} \cdot \frac{1}{\Delta F_{max}}$$

where $\Delta F = F_0 - F$; F_0 and F represent the fluorescence intensity of myoglobin or hemoglobin at 324 nm in the absence and presence of the added total CPZ concentration (L_t), respectively. ΔF_{max} is the maximum change in fluorescence intensity. The intercept of the above plot on the $1/\Delta F$ axis corresponding to $1/L_t = 0$ measures $1/\Delta F_{max}$, and the slope gives the estimate for the affinity constant, K. The binding affinity constant for CPZ to myoglobin in 0.145 M NaCl was found to be 8.4 ± $0.71 \times 10^3\,M^{-1}$, which was markedly higher than that of the CPZ-hemoglobin complex ($K = 4.2 \pm 0.61 \times 10^3\,M^{-1}$) (Table 1).

Figure 2 is the linear plot of $1/1 - \theta$ versus L_t/θ, where θ ($= \Delta F/\Delta F_{max}$) is the extent of binding. The intercept of this plot on the L_t/θ axis gives the estimate of the possible number of binding sites (p) according to the equation

$$\frac{1}{1-\theta} = \frac{KL_t}{\theta} - KpA_t,$$

where A_t is the fixed concentration of hemoglobin or myoglobin. The number of binding sites (p) for myoglobin was estimated to be 2 ± 0.2, while that for tetrameric hemoglobin was measured to be 5 ± 0.3. From the equilibrium dialysis experiment, the number of binding sites for tetrameric hemoglobin was independently estimated to be 6 [5]. Note that the number of tryptophans in tetrameric hemoglobin and monomeric myoglobin is 6 and 2, respectively [8]. This result might tempt one to speculate that perhaps tryptophan residues are in or near the possible binding site. However, the exact nature of the binding site is still to be elucidated clearly, and we leave this issue to be resolved by further experiments.

Concomitant with the binding of CPZ to hemoglobin or myoglobin, a change in the conformation of these two proteins always occurred in such a way that the tryptophan moieties of the protein molecules were more exposed to the polar region. This was reflected by an increase in the wavelength of the emission maximum (λ_{em}^{max}) of the proteins as a function of the increase in the added CPZ concentration (Fig. 3). It is also evident from Fig. 3 that upon binding of CPZ, hemoglobin exhibited greater cooperativity in the conformational change than did myoglobin.

To ascertain whether such binding was cooperative

Table 2. Estimated values of thermodynamic parameters for binding of CPZ to hemoglobin and myoglobin

CPZ binding to:	Estimated values of thermodynamic parameters		
	ΔG° (kcal/mol)	ΔH° (kcal/mol)	ΔS° (cal/degree/mol)
Hemoglobin	−4.99	−2.65	+7.87
Myoglobin	−5.46	+1.39	+23.0

or not, binding data were analysed by the Hill equation [5]:

$$\log\frac{\theta}{1-\theta} = n_H \log K + n_H \log L_f,$$

where n_H is the Hill coefficient measuring the degree of cooperativity and L_f is the free CPZ concentration given by the relation L_f = total drug − bound drug = $L_t - \theta pA_t$. Figure 4 shows the Hill plot analysis of the CPZ binding to hemoglobin or myoglobin. From the slope of the above plot in the region $\theta = 0.5$, n_H was measured to be 1 for myoglobin, indicating that the mode of CPZ binding to myoglobin is non-cooperative. This was in sharp contrast to the positive cooperative mode in case of the CPZ – hemoglobin complex for which n_H was about 3.

Another contrasting feature of the binding of CPZ to myoglobin was that the affinity constant K did not change significantly with the increasing NaCl molarity of the solution in the range 0.002 to 0.3 M (Table 1), whereas in the case of the CPZ–hemoglobin complex it decreased appreciably with the increase in NaCl concentration. Our results thus indicate that CPZ binding to tetrameric hemoglobin is positively cooperative and mostly electrostatic in nature while in the case of myoglobin it is of a non-cooperative and non-electrostatic type. Subunit interactions amongst the four polypeptide chains ($2_\alpha 2_\beta$) of tetrameric hemoglobin enable it to exhibit a cooperative binding feature, not only with oxygen but also with this drug molecule, CPZ. Myoglobin, lacking the above subunit interaction, binds with CPZ in a completely different mode.

Thermodynamic analysis of the temperature dependence of the binding constant (K) also corroborated the above view. Figure 5 shows the plot of ln K versus 1/T where T is the temperature (absolute scale) at which the CPZ binding to hemoglobin or myoglobin was studied. The slope of this plot measures the standard enthalpy change (ΔH°) following the equation

$$\ln K = -\frac{\Delta G^\circ}{RT} = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R},$$

where ΔG° is the standard free energy change upon binding of this drug to the respective protein, and ΔS° is the corresponding standard entropy change. Assuming no significant temperature dependence of ΔH° in the above temperature range (5° to 35°), the values of ΔG°, ΔH° and ΔS° were estimated and tabulated in Table 2. CPZ binding to tetrameric hemoglobin was exothermic ($\Delta H^\circ = -2.65$ kcal/mol) and ΔS° was positive (+7.87 cal/degree/mol) so that ΔG° was always negative in the above temperature range. Electrostatic binding of CPZ to tetrameric hemoglobin is of a salt-linkage type. With a rise in temperature, these linkages become weaker and hence the association constant K decreases with the increase of temperature (ΔH° is negative). On the other hand, in case of the CPZ–myoglobin complex, although ΔH° was positive (+1.39 kcal/mol; endothermic process), ΔS° assumed such a high positive value (+23 cal/degree mol) that the entropic contribution ($T\Delta S^\circ$) in the equation $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$ ultimately enables ΔG° to become negative, thus making the binding process a favourable one. It thus leads to the notion that binding of CPZ to myoglobin is entropically driven and most likely hydrophobic in nature. Such hydrophobic interaction possibly involves an intermediate collisional process that becomes enhanced with the rise in temperature and hence the overall association constant increases with the increase of temperature (ΔH° is positive).

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