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ORIGINAL PAPER

Relaxation of the folding of globulin around heme of hemoglobin of *Homo sapiens* by the food-grade additive molecule chlorophyllin

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Abstract Binding of chlorophyllin (Chln), a food-grade additive molecule with hemoglobin (Hb), has been studied by photophysical and photochemical methods with a view to unravel the biochemical transport pathway of it. The binding affinity constant and binding sites between Chln and Hb are determined and found to be 3.3×10^5 M^{-1} and 15 (on tryptophan basis), respectively. Fluorimetric quenching experiments entail that Chln is bound in the vicinity of the tryptophan residue of Hb. Circular dichroism studies suggest that Chln induces a change in the α-helical content of Hb. Chlorophyllin is bound in the vicinity of the tryptophan residue of hemoglobin, which has been confirmed from spectrofluorimetric studies, when a quenching in the tryptophan fluorescence occurs because of the chlorophyllin-induced exposure of the tryptophan residue to hydrophillic zone. The cyclic voltammetric studies indicate that the redox reaction of Fe^{II} of Hb is inhibited shielding of it by the Chln molecule.

Keywords Human hemoglobin · Chlorophyllin · Fluorescence quenching · α-Helical content · Binding sites

Introduction

Chlorophyllin (Chln) is a sodium-copper salt analogue of the ubiquitous green plant pigment chlorophyll. It is marketed as an over-the-counter drug [1–3]. Both chlorophyll and Chln are constituents of the human diet [4] and have been found to be effective anticarcinogens [5]. It possesses antioxidant properties and may decrease oxidative damage [1, 6]. It has been reported that Chln is radioprotective in *Drosophila* and laboratory mammals [7, 8]. It was recently used as a potent inhibitor of aflatoxin and B1 hepatocarcinogen in rainbow trout and rats [9, 10]. The antimutagenic property [11] of Chln has also been reported. The formation of strong complexes by Chln with mutagens and their intermediates, scavenging of free radicals and reactive oxygen species, and suppression of the metabolic activation of mutagens by specific cytochrome P_{450} and other metabolizing enzymes [12] have been suggested to be the mechanism of action.

Several earlier studies have reported the radioprotective [13], chemoprotective [14], antimutagenic, and anticarcinogenic properties of chlorophyllin. A lone communication indicated its in vitro interaction with DNA [2]. In studies, many drugs and agents have been implicated in the development of blood dyscrasias as methemoglobinemia and non-immune hemolytic anemia [15–19]; many simple organic hydrocarbons have been shown to convert oxy-Hb to methemoglobin [20, 21]. Very little information is available regarding the degree and specificity with which these drugs or agents may interact with human Hb [22]. Studies on the interaction of fish and human Hb with water have been reported previously [23]. However, till date, to our knowledge no investigation has been made on Hb-Chln interaction hinting at the transportation pathway of Chln through biological fluids. Keeping the above-mentioned facts in mind and as a part of our continued effort concerning the interaction of Hb with small molecules [24], our present work was designed to study the interaction of Chln with human Hb. It seems logical to conceive that Chln can also penetrate the membranes of the erythrocytes and in turn may affect their biological activities by

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interacting with Hb, which in turn may affect the well-being of the organisms.

Results and discussion

Spectrophotometric study

The isolated Hb in 1.5×10^{-1} M PBS buffer showed a characteristic absorption spectrum possessing three peaks at 415 nm (Soret band), 540, and 577 nm. The first peak, i.e., the Soret band, is obtained due to the heme group, whereas the latter two peaks are due to the vinyl group and porphyrin ring system, respectively. All these three peaks are due to π–π* transitions. The concentration of the stock solution was determined from its Soret absorbances using an extinction coefficient value of 125 mM^{-1} cm^{-1} (heme basis). The percent oxygenation for Hb was estimated [25] and found to be 99% oxygenated. The results of the spectrophotometric studies with increasing concentration of Chln are shown in Fig. 1. The binding affinity constant, K, for the interaction between Hb and Chln was determined [26] and found to be 1.2×10^5 M^{-1} (Fig. 1 inset).

Spectrofluorimetric study

A fluorimetric emission study was used to investigate the interaction of Chln with Hb. Due to the presence of tryptophan (Trp) in the Hb molecule, it shows a fluorescence emission peak at 331 nm when excited at 280 nm. It contains three intrinsic fluorophores: tryptophan, tyrosine, and phenylalanine (a weak fluorophore). In our study, 10 μM hemoglobin solutions were excited in the presence of increasing concentrations of Chln at an excitation wavelength of 280 nm, and emission spectra were taken in the wavelength region of 290–500 nm. There is no characteristic fluorescence of Chln under the experimental conditions in the above region. Fluorescence emission spectra showed (Fig. 2) considerable quenching in the fluorescence intensity of Hb in the presence of different concentrations of Chln ranging from 10 to 300 μM. Binding constant data indicate the nature of Chln with Hb. The binding constant (Fig. 3) was obtained using a method described in the literature [26] and was found to be 3.3×10^5 M^{-1}.

The possible number of binding sites (p) of Chln per Hb molecule was determined (Fig. 4) from the relation [27]. The p value was calculated to be 15 (on tryptophan basis). Thus, the binding of Chln induces a certain conformational change of Hb.

The quenching study was analyzed further by the Stern-Volmer equation [28]. The Stern-Volmer quenching constant (K_{SV}) was found to be 1.4×10^5 M^{-1}. The Stern-Volmer plots can be used to characterize the type of quenching (i.e., either dynamic or static). Spectrofluorimetric observation indicates a strong interaction between Chln and Hb. Fluorescence of Hb tetramer is mainly due to the six tryptophan residues, since tryptophan has the highest quantum yield among all the amino acid residues. At the excitation wavelength of 280 nm, the fluorescence emission spectrum of Hb at 331 nm is attributed to the tryptophan residue of Hb [29], which is embedded in the hydrophobic zone of the protein. With increasing concentration of Chln, the fluorescence intensity of Hb gradually

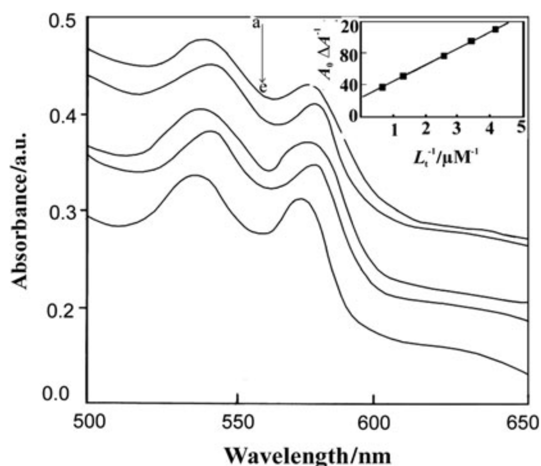

Fig. 1 Absorption spectra of 10 μM Hb titrated with varying concentrations of Chln. The Chln concentrations were used during titration of the protein: (*a*) 0 μM, (*b*) 50 μM, (*c*) 100 μM, (*d*) 150 μM, (*e*) 200 μM. *Inset* plot of $A_0/\Delta A$ versus $1/L_t$ for the estimation of the binding affinity constant in the interaction of Hb with Chln. $\Delta A = A_0 - A$; A_0 and A are the protein absorbances in the absence and presence of Chln (L_t), respectively

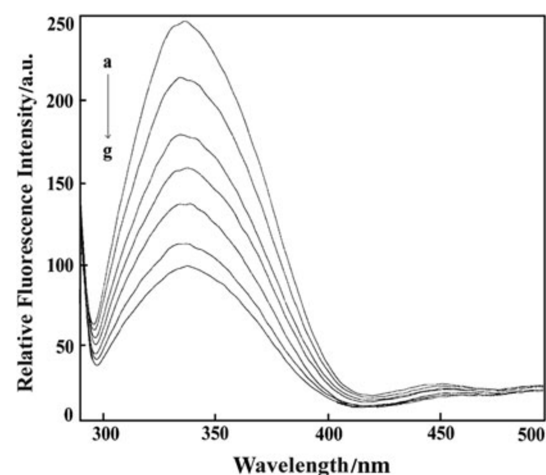

Fig. 2 Fluorescence emission spectra of 10 μM Hb in the absence and presence of Chln. The Chln concentrations were used during titration of the protein: (*a*) 0 μM, (*b*) 50 μM, (*c*) 100 μM, (*d*) 150 μM, (*e*) 200 μM, (*f*) 250 μM, (*g*) 300 μM

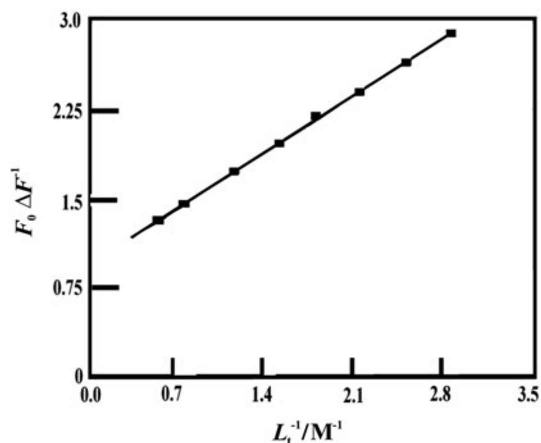

Fig. 3 Plot of $F_0/\Delta F$ versus $1/L_t$ for the determination of binding affinity constant (*K*) of Hb-Chln interaction, where F_0 is the fluorescence emission of the protein (10 μM) at 331 nm, and ΔF is the quenched fluorescence after successive addition of Chln (L_t); 10 μM Hb titrated with 10–300 μM Chln

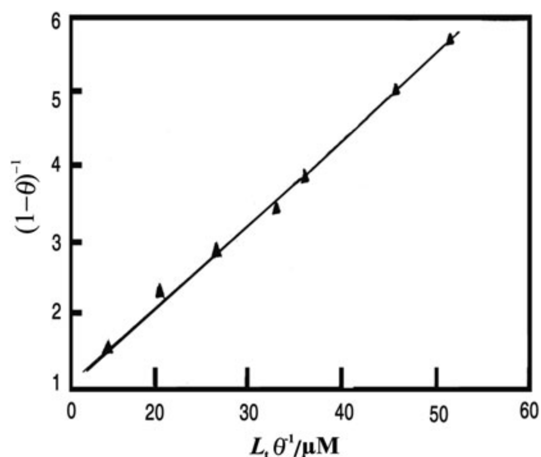

Fig. 4 Plot of $1/(1-\theta)$ versus L_t/θ for the spectrofluorometric estimation of the possible number of binding sites (*p*) involved in Hb-Chln interaction. θ is the extent of binding ($\Delta F/\Delta F_{max}$), and L_t is the Chln concentration; 10 μM Hb titrated with 10–300 μM Chln

decreases, indicating the binding of Chln to Hb. Thus, Hb, tryptophan and Chln gradually come into close proximity. The binding constant of Chln with Hb (3.3×10^5 M^{-1}) is very high, which indicates that Chln is strongly associated around the aromatic residue, the tryptophan of Hb. Hence, by this close association Chln exposes the tryptophan of Hb to the hydrated interfacial zone, thereby inducing a quenching in fluorescence intensity [30].

Circular dichroism study

Molar ellipticity [θ] values and the α-helical contents of the protein in the presence and absence of Chln were estimated by the methods described elsewhere [31, 32]. To understand the conformational status of Hb upon binding with Chln, CD spectra were recorded. Figure 5 shows the CD spectra of Hb in the range 210–250 nm in the absence and presence of various concentrations of Chln. In this wavelength region, CD measurement of a protein gives information about its conformation in relation to the secondary structure. Unreacted Hb shows its characteristic CD spectrum with negative ellipticity at 222 nm (due to $n \rightarrow \pi^*$), asserting its 75% α-helix content. The negative ellipticity of the protein at 222 nm gradually reduced with an increased addition of Chln, which indicates unambiguously that the α-helicity of hemoglobin subunits is gradually decreased [31], and was calculated to be ~40% when treated with the maximum of 300 μM of Chln. This experiment suggests that the interaction of Chln with Hb causes an alteration in the secondary structure of the protein that is reflected by the decrease in their α-helical content (Fig. 5; Table 1), thereby inducing a conformational change in the globular part of the protein molecule. Thus, the α-helix content of the Chln-treated hemoglobin gradually decreases (Fig. 6) relative to the untreated protein, indicating a change in the folding pattern of the macromolecule.

Cyclic voltammetric study

The cyclic voltammograms of Hb in the absence and presence of Chln are presented in Fig. 7. The oxidative peak of Hb appears at +0.64 V, whereas the reductive peak appears at −0.27 V. Chln does not exhibit any oxidative peak under the experimental setup, but a reductive peak of Chln appears at −0.03 V. When Chln was added into the Hb solution, the current of the oxidative peaks of Hb

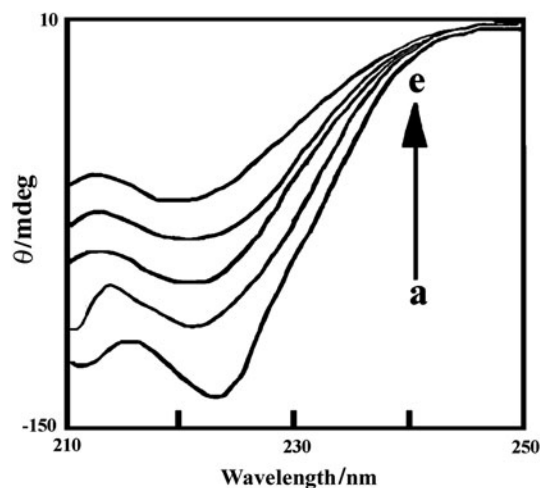

Fig. 5 Circular dichroism spectra of 10 μM Hb in the absence and presence of Chln. Chln concentrations were used: (*a*) 0 μM, (*b*) 50 μM, (*c*) 100 μM, (*d*) 200 μM, and (*e*) 300 μM. In the *Y* axis, θ measures the ellipticity of Hb

Table 1 Effect of Chln on α-helix contents of 10 μM Hb

Concentration of Chln (μM)	α-Helicity of Hb (%)
0	75.00
50	65.58
100	58.10
200	47.38
300	33.99

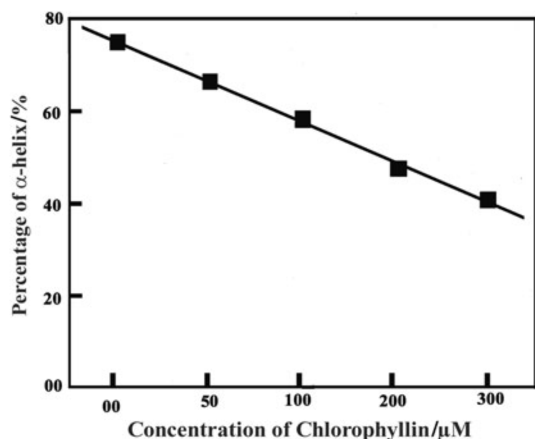

Fig. 6 Plot of percent α-helicity of tetrameric hemoglobin as a function of Chln concentration

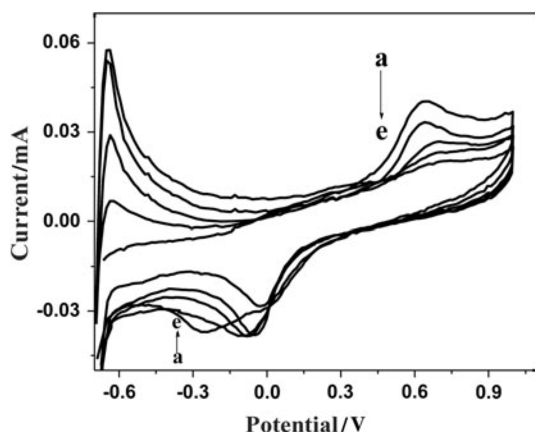

Fig. 7 Cyclic voltammograms of (*a*) 0.1 mM Chln, (*b*) 0.1 mM hemoglobin, (*c*) 0.1 mM hemoglobin and 0.02 mM Chln, (*d*) 0.1 mM hemoglobin and 0.06 mM Chln, and (*e*) 0.1 mM hemoglobin and 0.1 mM Chln. Rest time 5 s and scan rate 75 mV s^{-1}

decreased greatly and completely disappeared at the concentration ratio of Hb:Chln = 1:1, with the shift of peak potentials of about 0.1 V, and the reductive peak disappeared completely at any concentration ratio of Hb:Chln. It is proposed that it occurs because Chln binds the Hb in such a fashion that it shields the Fe^{II} from further oxidation and reduction, while the decrease in the reduction peak of Chln indicates that while shielding Fe^{II} of Hb, the Cu^{II} of Chln suffers reduction.

Conclusion

This investigation indicates that the food-grade additive molecule Chln binds strongly to the Hb molecule. The fluorimetric studies show that Chln binds strongly in the vicinity of the tryptophan of the Hb, thereby exposing it to the hydrated interfacial environment. Moreover, this binding induces a gain in coil structure of the Hb conformation, which is evident from a change in the α-helical pattern upon binding by Chln. The cyclic voltammetric studies also suggest that Chln binds Hb in such a way that a redox reaction of iron in Hb is inhibited. The strong binding of this molecule to Hb is an indication that Chln is probably bound tightly to the vehicle Hb first and then transported to its metabolic site.

Experimental

Materials

Chlorophyllin was purchased from Sigma Chemical Company, USA. Other chemicals were either of AR or GR grades. Triple distilled (all glass) water was used throughout.

Preparation of Alsever's solution

Both the Alsever's solution used for the transportation of cells and the PBS buffer used for the studies were prepared according to a method described in the literature [24].

Preparation of PBS buffer

Phosphate buffer saline (1.5×10^{-1} M, PBS) was prepared, and the pH of the solution was 7.2.

Isolation and purification of human hemoglobin

Fresh Hb was purified from human blood donated by our colleague Arpita whenever required. For this purpose, 20 cm^3 of blood was collected in a reagent bottle containing ~50 cm^3 Alsever's solution, and Hb was extracted according to the method described in the literature [24, 33]. Processing the cells involved: (1) centrifuging the blood mixture at 2,000 rpm for 10 min in a Beckman J2-21 refrigerated centrifuge at 4 °C; (2) removal of the supernatant; (3) gently stirring the packed cells with four volumes of 1% NaCl solution, followed by centrifugation. Step 3 was performed three times. Among them, the last centrifugation was carried out at 3,000 rpm for 10 min; this would give the highest yield of Hb and least formation of methemoglobin. All centrifugations were performed at

4 °C. That the isolation and purification of Hb by the above method ensures sufficient purity for spectroscopic work has been documented in the literature [34]. The purified Hb was suspended in 0.15 M PBS buffer, and solutions were stored in small aliquots at 0 °C until use. Experiments were performed preferably on the day of isolation, but in other cases for each experiment, one aliquot was thawed and used immediately.

Preparation of sodium copper chlorophyllin solution

About 2.4×10^{-3} g Chln was taken in a 10-cm^3 volumetric flask and dissolved in 1.5×10^{-1} M PBS buffer. This solution showed the characteristic absorption spectrum possessing two peaks at 406 and 627 nm. The concentration of the stock solution was determined from its absorbance at 627 nm using an extinction coefficient value of 4×10^4 M^{-1} cm^{-1} [35].

Absorption spectroscopy

The interaction of Chln with Hb was studied spectrophotometrically and by other spectroscopic tools. The spectrophotometric experiments were done on a Hitachi U-3410 spectrophotometer using a 3-cm^3 quartz cell of path length 1 cm. For this study a fixed concentration of Hb (10 μM) was taken, and a varying amount of Chln solution was added to the test solution whereby the total volume of the test solution remained the same (5 cm^3) in all cases. Reference solutions (baseline) were made by the addition of a corresponding amount of Chln solution in PBS buffer. The absorbances of the mixture (Hb and Chln) were measured after 2 h of incubation at 37 °C. Absorption spectra from 500 to 600 nm were recorded. The Soret band of Hb at 415 nm was not used for monitoring the interaction because of the closeness of this band with the band of Chln at 406 nm.

Fluorescence spectroscopy

A Perkin-Elmer LS-55 spectrofluorimeter was used to study the interaction. The binding parameters for the interaction between Hb and Chln were determined from the quenching of protein fluorescence maxima with subsequent additions of Chln. A 10-μM Hb solution was incubated with different concentrations of Chln for 2 h.

Circular dichroism experiments

Circular dichroism (CD) measurements of 10 μM Hb and Hb treated with different concentrations of Chln were done after 2 h of incubation at 37 °C, and spectra were recorded on a Jasco-600 spectropolarimeter using a quartz cuvette of 1-cm path length in the wavelength range 210–250 nm at a scan speed of 10 nm min^{-1}, band width 2.0 nm, and data pitch 0.1 nm. The results were an average of 15 scans.

Cyclic voltammetric experiments

Cyclic voltammetric experiments (CV) were performed with a Ministate Systems (Sycopel Scientific Ltd., UK) Model AEW2-10 electrochemical analyzer. Voltammetric measurements were carried out in a one-compartment cell using a Pt disk working electrode, a Pt flag counter electrode, and a saturated calomel reference electrode (SCE). The electrolyte solutions were prepared with 0.15 M PBS buffer. The voltammetric experiments were carried out in the potential range from −0.7 to 1.0 V (vs. SCE). Under the same conditions, the solutions of Chln and blank were also recorded. The voltammetric peak current (I_p) was obtained, and the difference of the peak currents ($\Delta I_p = I_{p0} - I_p$) was used to show the changes of electrochemical responses of the reaction system.

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