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The interaction of clenbuterol hydrochloride with bovine hemoglobin using spectroscopic techniques and molecular modeling methods

Yun Wu [a], Hui Mao [b], Bo Zhao [b,*] and Jian Shen [b]

[a] *Department of Basic Science, China Pharmaceutical University, Nanjing, China*
[b] *Jiangsu Key Laboratory of Biofunctional Materials, College of Chemistry and Environmental Science, Nanjing Normal University, Nanjing, China*

Abstract. The interaction of clenbuterol hydrochloride (CL) to bovine hemoglobin (BHb) under physiological conditions was investigated by using UV-vis absorption, fluorescence, circular dichroism (CD) and molecular modeling. The fluorescence intensity of BHb decreased regularly with the gradual increasing concentration of CL. It is observed that there was a prominent interaction between CL and BHb. The fluorescence data revealed that the fluorescence quenching is a static process, and the thermodynamic parameters were calculated according to the Van't Hoff equation. The alternations of protein secondary structure in the presence of CL were determined by the evidence of CD. Molecular modeling study that corroborate our experimental results revealed that the binding mode of CL–BHb complex could be attributed to the hydrophobic interaction and hydrogen bonding, but electronic interaction cannot be excluded.

Keywords: Interaction, clenbuterol hydrochloride, bovine hemoglobin, spectroscopic techniques, molecular modeling methods

1. Introduction

Clenbuterol hydrochloride (CL) was once used to improve the percent conversion of feed and the percent content of meat. It is one of synthetic β-adrenoceptor agonist, an amine derivative of phenylethyl alcohol, and the chemical name is 1-(4-amino-3, 5-dichlorophenyl)-2-tertbutylaminoethanol hydrochloride. The chemical structural formula of CL showed in Fig. 1.

Hemoglobin (Hb) is one of the most significant proteins in the blood plasma of higher organisms. Hb is well known for its function in the blood circulatory system that working as a transporter of oxygen. It carries and releases oxygen to the low oxygen partial pressure tissues through the blood circulatory [11]. Bovine hemoglobin (BHb) has a molecular weight of 64,500 and contains four globin chains, two identical α-chains of 141 amino acids each and two identical β-chains of 146 amino acid each [8].

The harm to human health and the destruction to ecological food chain caused by clenbuterol hydrochloride residues in the domesticated animal products draws us a great attention in recent years. Since abuse of clenbuterol hydrochloride has led to potential toxicological risk to public health, it is necessary to investigate the interaction of clenbuterol hydrochloride with protein, as it can illustrate the

*Corresponding author: Bo Zhao, Jiangsu Key Laboratory of Biofunctional Materials, College of Chemistry and Environmental Science, Nanjing Normal University, Nanjing 210097, China. Tel.: +86 25 83598053; E-mail: zbchem@126.com.

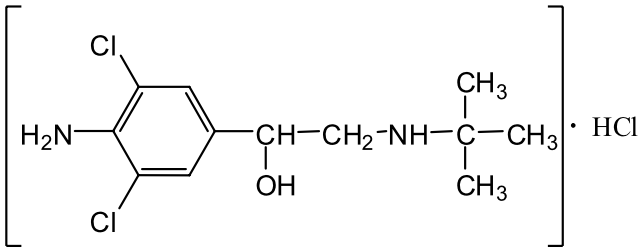

Fig. 1. The chemical structural formula of CL.

nature of CL-protein complex *in vitro* and provide important insight into the interaction of the physiologically important protein BHb with CL [2–4,9]. The above investigations may provide some important theoretic information for the improvement of the metabolism and distribution of CL in life science, biological technology, medicine, molecular functional design.

2. Materials and methods

2.1. Materials

Bovine hemoglobin (BHb) was purchased from Sigma Chemical Company and used without further purification. The BHb solution (5 μM) was prepared in Tris-HCl buffer solution (pH 7.4), and kept in the dark at 277 K. Tris-HCl buffer solution (pH 7.4) consists of Tris (0.2 M), HCl (0.1 M) and NaCl (0.01 M) maintaining the ionic strength. The pH measurements were carried out on a pHS-3C pH meter, which was calibrated with standard pH buffer solution. Clenbuterol hydrochloride was purchased from Sigma Chemical Company, and the stock solution (5×10^{-3} mol/l) was prepared in methanol. The fluorescence intensities were recorded after a series of assay solutions were placed in water bath for 12 h at 287, 297 and 307 K, respectively. All other reagents and solvents were of analytical reagent level. Newly double-distilled water was used during the whole experiments.

2.2. Apparatus and methods

A LS-55B spectrofluorophotomer (Perkin Elmer, American) was performed to measure the fluorescence emission spectra equipped with a 1.0 cm quartz cell. The emission spectra was recorded in the range of 300–500 nm, using 5 × 5 nm slit widths, and the excitation wavelength is 280 nm. Three milliliters BHb solution (5 μM) was titrated manually by 1×10^{-3} mol/l solution of CL (to give a final concentration of 0–40 μM). A series of experiments were operated at three different temperatures 288, 298 and 308 K. The temperature of sample was kept by recycled water throughout the experiment. UV-vis absorption spectra were recorded on a UV-2450 UV-vis recording spectrophotometer (Shimadzu, Japan). A 1.0 cm quartz cell was used throughout the experiment. The circular dichroism spectra were obtained on a JASCO −20 automatic recording spectropolarimeter (JASCO, Japan) with a 0.1 cm quartz cell at 298 K. The CD spectra were recorded in the range of 200–250 nm, and the speed of scanning was 10 nm/min. The concentration of BHb was 5 mM. The buffer solution was selected as the blank and automatically subtracted during scanning.

Protein-ligand docking study was performed using Discovery Studio 2.1 software where the pH is set to 7.4. The crystal structure of BHb (PDB entry code 1G09) was downloaded from the Protein Data Bank

and the potential of the 3D structure of HSA was assigned to the CHARMm force field. The structure of CL was built in Discovery Studio 2.1 and optimized by applying the CHARMm force field. During the docking process, a maximum of 10 conformers have been chosen for the final energy minimization. The conformation with the lowest energy was selected for final analysis.

3. Results and discussion

3.1. UV-vis absorption spectra

Figure 2 shows the UV absorption spectra of BHb in the absence and presence of CL. Hemoglobin has four heme groups, locating in the crevices near the surface of the molecule. As can be seen in Fig. 2, there were three peaks (216, 275 and 406 nm). The latter sharp peak (406 nm) was just the characteristic absorption of the porphyrin-Soret band. The absorption maximum of Soret band is decreased with the addition of CL, while the maximum absorption wavelength remains unchanged, which indicates that the heme is not exposed from the crevices at the exterior of the subunit and CL is easily integrated into the hydrophobic pocket of BHb. BHb has a strong absorbance with a peak at 216 nm and the absorbance of BHb decreased regularly with the addition of CL, and the maximum absorption wavelength is red shifted. The absorption peak (275 nm) of CL–BHb system showed a slight red-shift with the addition of CL. This means that the peptide strands of BHb extended and the hydrophobicity was decreases. The result of UV/vis absorption measurement results showed that there was a permanent interaction between BHb and CL, and the fluorescence quenching was due to the formation of BHb–CL complex [7].

3.2. Fluorescence spectra

Fluorescence quenching is a significant method to study the interaction of drug with protein because it is a sensitive and relatively easy method. The fluorescence of BHb originates from tryptophan (Trp),

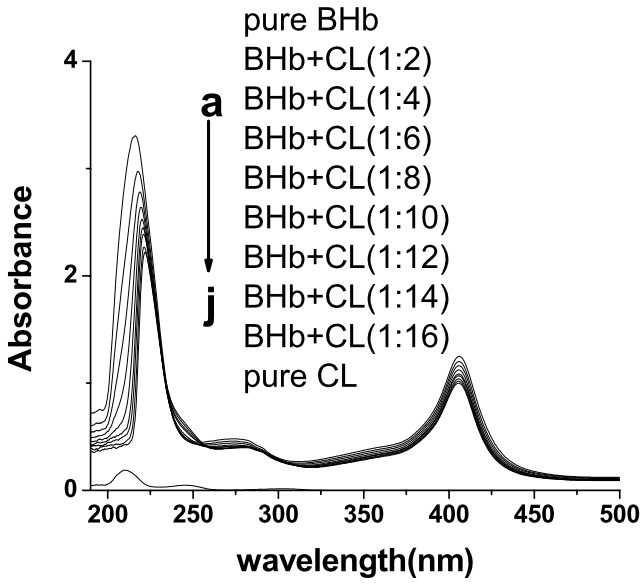

Fig. 2. UV-vis absorption spectra obtained in Tris-HCl buffer solution at pH 7.4. The C_{BHb} was 5 μM (a–i) and C_{CL} was 10, 20, 30, 40, 50, 60, 70, 80, 100 μM (b–j), respectively.

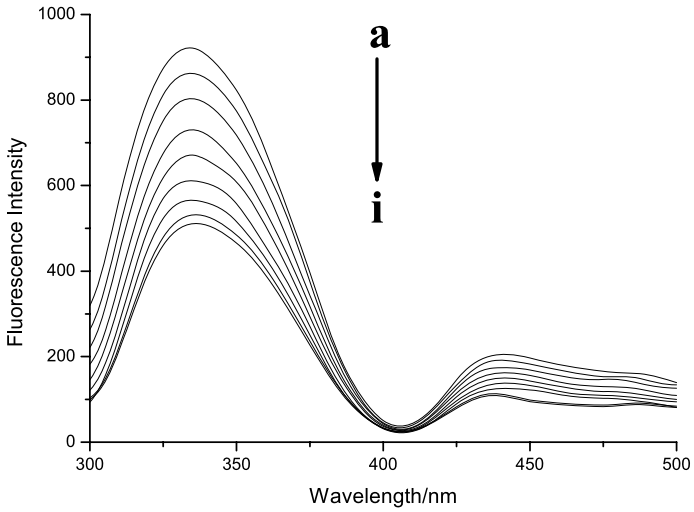

Fig. 3. The fluorescence emission spectra of 5 μM BHb in the presence of CL at 298 K, the concentration of CL corresponds to 0; 5.0; 10.0; 15.0; 20.0; 25.0; 30.0; 35.0; 40.0 μM from a to h.

tyrosine (Tyr) and phenylalanine (Phe) residues [13]. The fluorescence intensity ratio of Trp, Tyr and Phe is 100:9:0.5 because of the difference among their structures. Therefore, the fluorescence of BHb is mainly due to the contribution of Trp residue. The fluorescence emission spectra of BHb in the presence and absence of CL were shown in Fig. 3. There is a maximum emission peak at 336 nm when the excitation wavelength is 280 nm in the fluorescence spectra. The fluorescence intensity of BHb decreased regularly with the increasing concentration of CL. Slight blue shift of the maximum emission wavelength was observed which reflected that the microenvironment around fluorophore in BHb solution in the presence of CL is different from that of pure BHb solution. It is obvious that the fluorescence quenching of BHb is attributed to the affection of CL. The changes in the fluorometric behavior of the fluorophore with the addition of CL in buffer solution can be rationalized in terms of binding of the drug with the protein leading to a more polar microenvironment around the fluorophore. A blue shift in the fluorescence maximum also suggests an enhancement in the non-polarity of the microenvironment.

The three possible quenching mechanisms for fluorescence quenching are dynamic quenching, static quenching and the influence of non-radiative energy transfer. Generally speaking, dynamic quenching and static quenching could be determined by the experimental data at different temperatures. For dynamic quenching, the quenching constant will increase with the rising temperature, because the increase of effective ionic collision and the aggravation of electron transfer; while for static quenching, the quenching constant will decrease with the rising temperature, which may be owing to the decrease of BHb–CL complex stability.

In order to determine the quenching mechanism of CL–BHb, the Stern–Volmer equation curves for the binding of CL with BHb at three temperatures (288, 298 and 308 K) was made. Figure 4 shows the regression curve which was plotted according to the well-known Stern–Volmer equation [6]:

$$F_0/F = 1 + K_{\mathrm{q}}\tau_0[Q] = 1 + K_{\mathrm{SV}}[Q], \tag{1}$$

where F_0 and F represent the steady-state fluorescence intensities in the absence and presence of quencher, respectively, and $[Q]$ is the concentration of quencher. K_{q} is the quenching rate constant

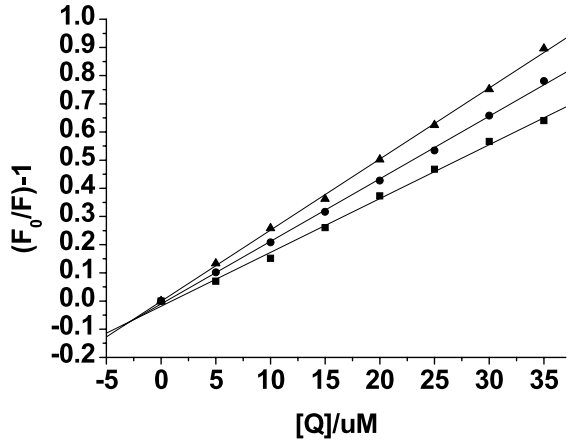

Fig. 4. The Stern–Volmer curves for the binding of CL with BHb at different three temperatures. (▲) 290 K; (●) 300 K; (■) 310 K; BHb = 5 μM, pH 7.4.

Table 1
The Stern–Volmer quenching constants of CL–BHb complex at different temperatures

Temperature (K)	K_{SV} ($\times 10^4$ M^{-1})	K_q ($\times 10^{12}$ M^{-1}/s)	Linear regression equation	R
288	2.524	2.524	$F_0/F = 0.999 + 2.524 \times 10^4[Q]$	0.9995
298	2.223	2.223	$F_0/F = 0.996 + 2.223 \times 10^4[Q]$	0.9992
308	1.915	1.915	$F_0/F = 0.998 + 1.915 \times 10^4[Q]$	0.9982

of BHb, while τ_0 is the average life-time of BHb without the quencher and its value is 10^{-8} s. K_{SV} is the Stern–Volmer quenching constant, which was determined by linear regression of Stern–Volmer equation. The Stern–Volmer quenching constants at different temperatures were listed in Table 1.

It can be seen that the Stern–Volmer quenching constants (K_{SV}) is inversely correlated with temperature, which indicates that the mechanism of the fluorescence quenching process may be a static quenching process rather than a dynamic quenching process. Furthermore, the quenching rate constants (K_q), deduced from K_{SV} and τ_0, are much greater than the maximum diffusion collision quenching rate constants (2.0×10^{10} l/mol s) of the most quenchers with biomacromolecule [5]. All of these above revealed that the fluorescence quenching of BHb by CL could be mainly owing to the static quenching, and the dynamic collision could be negligible.

For the static quenching process, the fluorescence quenching data were analyzed to calculate the binding parameters using the following equation [1]:

$$\log[F_0 - F/F] = \log K_a + n \log[Q], \tag{2}$$

where K_a the binding constant and n is the number of the binding sites per BHb.

The binding constants K_a and binding sites n at different temperatures were summarized in Table 2. The binding constants decreased with the rising temperature, which indicate that the interaction between CL and BHb is weakening when the temperature rises, and the formation of an unstable complex. The complex may partly decomposed when the temperature raise. The values of n at the experimental temperatures approximately equal to 1 indicated that CL bind to BHb with high affinity and there is about one binding site on BHb.

Table 2
Binding constants K_a and binding sites n at different temperatures

Temperature (K)	10^{-4} K_a (M^{-1})	n	R
288	10.5	1.16	0.9987
298	3.39	1.04	0.9999
308	1.93	0.98	0.9927

3.3. Thermodynamic parameters for CL–BHb complex

As we know, there are mainly four types of mode on the binding of drug to biomacromolecule, including hydrogen bond, van der Waals, hydrophobic and electrostatic interactions. According to the temperature dependence of the thermodynamic parameters, enthalpy (ΔH) and entropy (ΔS), the binding mode of CL–BHb could be determined.

The experiments were operated at 288, 298 and 308 K. The enthalpy (ΔH) could be seen as a constant during the calculation, when the temperature does not vary significantly. The values of ΔH and ΔS could be obtained from Van't Hoff equation in Table 3.

It can be seen in Table 3 that the negative free energy ΔG reveals that the binding of CL–BHb is spontaneous. The values of ΔH and ΔS are negative and positive, respectively. Ross et al. [10] has characterized the sign and magnitude of the thermodynamic parameters associated with various individual kinds of interaction that may take place in protein binding process. From the point of view, the positive ΔS is frequently regarded as an evidence for hydrophobic interaction. But for hydrophobic interaction, ΔH and ΔS are both positive. Negative ΔH cannot be attributed to electrostatic interaction, because the value of ΔH should be very small, almost zero for electrostatic interaction. Therefore, the binding mode of CL–BHb complex could be attributed to the hydrophobic interaction, but electronic interaction cannot be excluded.

3.4. CD spectra studies

CD is a quantitative method to analysis the secondary structure change of proteins. The far UV CD spectrum is very sensitive upon the change of BHb conformation, so the secondary structure could be calculated from the data of the far UV CD spectrum.

To get a full comprehension of how the structure of BHb is affected by CL, CD measurement was performed on BHb and the CL–BHb complex. Figure 5 shows the CD spectra of BHb with and without CL. The CD spectra of BHb displays two distinct negative minima in the ultraviolet region, one at 209 nm and the other at 220 nm, which is the characteristic of α-helical structure of a protein. The reasonable explanation is that the negative peak 209 nm is contributed by $\pi \rightarrow \pi*$ transfer for the peptide bond of α-helical, while the negative peak 220 nm is contributed by $n \rightarrow \pi*$ transfer for the peptide bond of α-helical [14]. As can be seen from Fig. 5, the addition of CL–BHb leads to a decrease in the ellipticity without significant shift of the peaks, indicating that the binding of CL–BHb induces a decrease in the α-helical content of BHb. However, the CD spectra of BHb in presence and absence of CL are similar in shape, indicating that the structure of the BHb is also predominated by α-helix after the binding of CL [12]. From the above results, it is apparent that the binding of CL–BHb causes a conformational change of the protein, with the loss of α-helix stability. The CD result was expressed as MRE (Mean Residue Ellipticity) in deg cm^2/dmol, which is defined as:

$$MRE = \theta_{obs}/(10nlC_p), \tag{3}$$

Table 3
Thermodynamic parameters for CL–BHb complex

Temperature (K)	K ($\times 10^4$ M^{-1})	ΔG^0 (kJ/mol)	ΔS^0 (J/mol·K)	ΔH^0 (kJ/mol)	R
288	2.543	−3.958	48.99	−10.15	0.9927
298	2.205	−4.448			0.9999
308	1.930	−4.938			0.9987

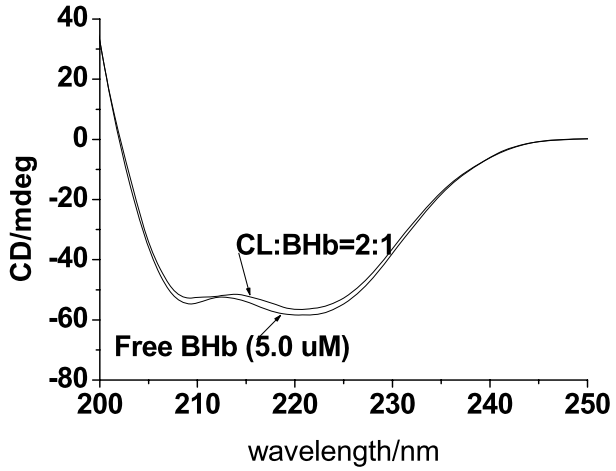

Fig. 5. The CD spectra of free BHb and BHb–CL complex at pH 7.4.

where θ_{obs} is the CD in millidegree, n is the number of amino acid residues (574), l is the path length of the cell (0.1 cm), and C_{p} is the mole fraction of BHb. The α-helical contents could be calculated from the MRE values at 209 nm using the following equation [12]:

$$\alpha\text{-helical} = \{(-MRE_{209} - 4000)/(33000 - 4000)\} \times 100. \tag{4}$$

The results revealed that the content of α-helix was decreased from 51.97% in free BHb to 49.61% at molar ratio CL/BHb of 2:1.

3.5. Molecule modeling

The application of molecular modeling has been employed to study the interaction between CL and BHb. The stereoview of the docking pose of CL with BHb is shown in Fig. 6. As shown in Fig. 6, CL is best located on the centre of the binding pocket of site 2. The CL is in close proximity to the heme ring and this imply the interference in the oxygen binding property of the protein. The binding free energy (ΔG) was found to be -24.9 l/mol. Negative value of ΔG shows that the binding reaction is thermodynamically favorable.

The amino acid residues involved in the binding of CL–BHb were predicted in Fig. 7 where only residues around 8 Å of CL were displayed.

The benzene ring of drag is making hydrophobic interactions with Leu105, Ala130, Phe98, Pro95, Trp37 and Val96. The interaction between CL and BHb is not exclusively hydrophobic as there are

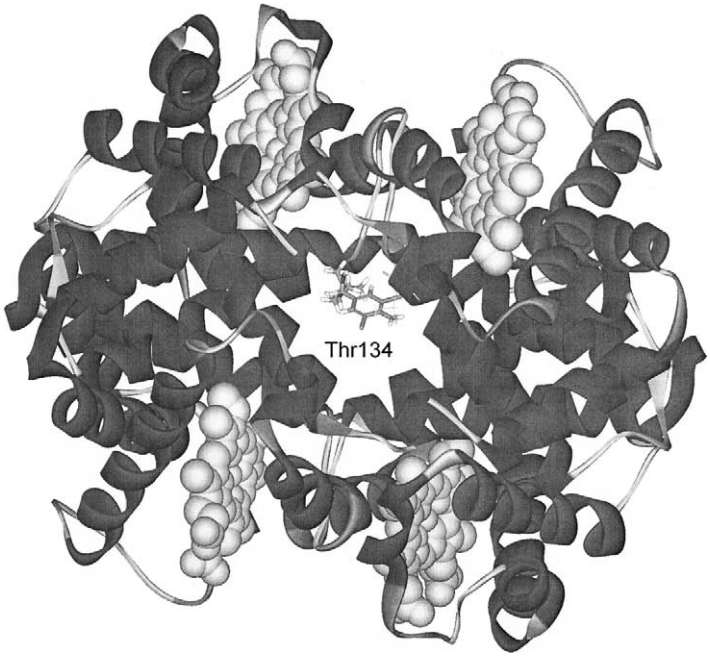

Fig. 6. The stereoview of the docking pose of CL with BHb.

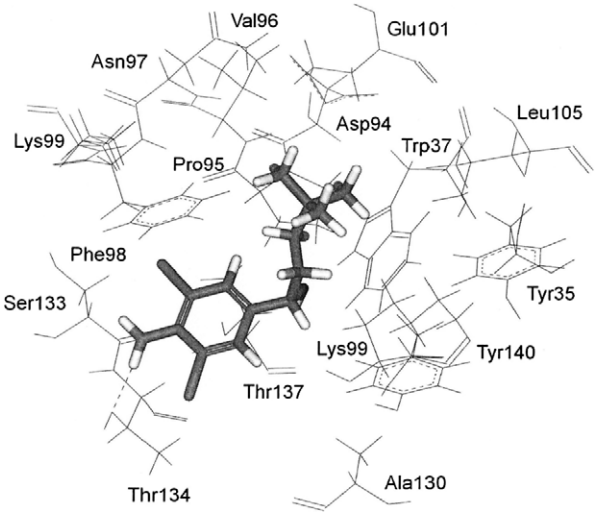

Fig. 7. The amino acid residues involved in the binding of CL–BHb.

several ionic and polar residues (Ser133, Asn97, Thr134, Thr137, Tyr140, Tyr35, Lys99, Asp94 and Glu101) in the proximity of bound ligand (within 8 Å). These polar residues play an important role in stabilizing drag via H-bonds and electrostatic interactions. Thr134 is in a suitable position involved in making H-bonds with the carboxylic groups of the side chain. The hydrogen-bonding or electrostatic interaction acts as an anchor and this helps to attain the 3D space position of CL in its biding pocket. This facilitates the hydrophobic interaction of the benzene rings with the side chain of BHb residues.

4. Conclusions

BHb was used as a model protein to explore the protein binding properties of CL. The binding of CL–BHb was investigated by UV/vis absorption, fluorescence, circular dichroism (CD) and molecular modeling under physiological conditions. The results revealed that the interaction between CL and BHb is a static process. The hydrophobic interaction and hydrogen bonding play a major role in the reaction, but the electronic interaction cannot be excluded. The conformational alternations were investigated by CD. And the molecular modeling was also utilized to explore the binding of CL–BHb.

Acknowledgements

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