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Analysis of conformational change of human serum albumin using chiral capillary electrophoresis

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Abstract

The conformational change of human serum albumin (HSA) was studied through its binding with basic drug-mexiletine by chiral capillary electrophoresis. The effects of the conformational change of HSA resulted from pH, thermal, acute vibration, and alcohol on its chiral selectivity to mexiletine were investigated in detail. This study offers a simple and complementary method to investigate the binding of proteins with drugs and the characteristic of conformational change of protein. The method is easy to perform, high speed, low reagent consumption, and no modification is required to the commercially available CE instrument.

Keywords: Conformational change; Chiral separation; Capillary electrophoresis; Human serum albumin; Mexiletine

1. Introduction

The study of conformational change of protein is important for the understanding of protein stability and unfolding pathway, and it is a hard task because proteins are composed of huge number of atoms and the folding/unfolding process is complicated. The common techniques used to study the unfolding transitions of protein involve differential scanning calorimetry [1,2], spectroscopic methods (circular dichroism spectropolarimetry [2,3], UV spectroscopy [4] and fluorescence spectroscopy [2,5]) and nuclear magnetic resonance [6]. In the last decade, capillary electrophoresis (CE) has been successfully applied as an additional and complementary technique for characterizing protein unfolding [7–24]. Possessing the advantages of high separation efficiency, rapid analysis ability, low reagent consumption, simple performance in free solution and involving no mass transfer, CE is perfectly suitable for the monitoring of the protein unfolding. CE can provide a population profile of the species within folding/unfolding equilibria, and can provide some kinetic and thermodynamic information [8,10].

In 1991, Karger first reported the influence of column temperature on the electrophoretic behavior of myoglobin and α-lactalbumin by CE [7]. After that, CE was applied to study the thermal-dependent conformational changes of proteins [8,9,11–19], investigate the denaturant-induced unfolding of proteins [20,21], monitor the effect of ligand binding on conformation of proteins [22,23], and analyze the single-strand conformation polymorphism of *p53* mutation [24]. Ishihama [19] developed a new method to accurately estimate the temperature inside the capillary and to measure the transition temperature of proteins using the corrected electrophoretic mobility. In most of the cases, CE was used to estimate the thermal stability of protein, but the applications of CE were strongly limited due to the requirement of precise temperature control and the relative narrow operation temperature range of commercially available CE instrument (4–60 °C). Therefore, the analytes were limited in the proteins with low transition temperature [7,22]. Otherwise, the instrument must be modified for the requirement of wide operation temperature range [11–13,15].

Due to the chiral nature, proteins can interact differently with the enantiomers of chiral molecules, especially for chiral drugs. This is why proteins were widely used as chiral selectors in CE [25,26]. The geometric matching and specific

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interactions between proteins and chiral molecules, including hydrophobic interaction, electrostatic interaction and hydrogen bonding interaction, are the basis of the chiral recognition of protein to guest. As a kind of biomacromolecules, proteins are readily to vary their conformation (unfolding/folding) with the change of their physical or chemical environments, such as temperature, pH, acute vibration and adding some chemical denaturants (e.g., ethanol, propanol and urea, etc.) [27]. The conformational change of protein may affect the special interaction between protein and guest, therefore, the chiral recognition ability of protein to guest is affected correspondingly. But this kind of effect is always ignored in the experiment.

Human serum albumin (HSA) is the most abundant protein in blood plasma. It has a single peptide chain with 585 amino acids. The molecular mass of HSA is 67,000 Da. HSA is a heart-shaped molecule assembled by three homologous [28]. The conformational transitions of HSA at different environment have been studied previously [2,3,5,6,19,29]. Therefore, HSA was chosen as model protein in this work. The conformational change of HSA was monitored by its binding with the chiral drug-mexiletine in CE. To avoid the interference of UV detection with protein as buffer additive in CE, the partial filling technique [30–34] was applied.

2. Experimental

2.1. Apparatus

The CE experiments were performed using an Agilent CE^{3D} CE system (Agilent Technology, Waldbronn, Germany) equipped with 50 cm (41.5 cm effective length) × 50 μm I.D. uncoated fused silica capillary (Yongnian Optical Fiber Factory, Hebei, China). Detection was carried out by diode-array detection (DAD) at 200 nm (at the cathode end).

2.2. Reagents

Analytical grade reagents were used in all our experiments and water was doubly distilled. Running buffer was 67 mM phosphate buffer in desired pH value, which was prepared by mixing disodium hydrogenphosphate and potassium dihydrogenphosphate with a certain proportion. Mexiletine and HSA (essential fatty acid free, Sigma) were dissolved in the running buffer to prepare 0.5 mM solutions. HSA solution was stored in the 4 °C. Unless noted otherwise, the pH of HSA was adjusted to match with the buffer.

2.3. Electrophoresis procedures

Prior to the first use, a new capillary was rinsed with 0.1 M NaOH, doubly distilled water and the running buffer for 30, 10, 10 min, respectively, and then pre-equilibrated with the running buffer under the separation voltage for 10 min. Between runs, the capillary was rinsed with 60 mM sodium dodecyl sulfate (SDS), 1 M NaOH, doubly distilled water and the running buffer each for 2 min. The capillary was partially filled with a protein zone in a certain plug length, i.e., from 0 to 13.5 cm, prior to the introduction of sample (5.0 kPa, 2 s). Then, electrophoresis was performed with the both end of capillary dipping in the running buffer (25 °C).

In the experiments of thermal denaturation of protein, the protein solution was heated in the water bath in the 30–100 °C temperature range for different time (1–48 min). After the protein solution was cool down to room temperature, electrophoresis experiments were carried out.

3. Results and discussion

3.1. Chiral separation

In the partial filling method, protein is partially filled into the capillary prior to the introduction of sample. Then electrophoresis is performed in the buffer without protein. At the experimental condition, basic drug is positively charged and migrates toward the cathode. The isoelectric point of HSA is 4.7, so it is negatively charged at pH higher than 4.7, and possessed electrophoretic mobility towards the anodic end. Therefore, drug, which moves in a faster apparent velocity than protein, can enter the protein zone and interacts with them. The interaction difference between protein and drug enantiomers leads to the enantioseparation of drug. Because of a faster apparent velocity than protein, drug reaches the detection window prior to protein in the partial filling mode. Therefore, the UV detection interference caused by protein can be effectively eliminated. The enantioseparation of mexiletine with HSA as selector was obtained by systematically optimizing the operation variables, and the electropherogram of mexiletine is shown in Fig. 1.

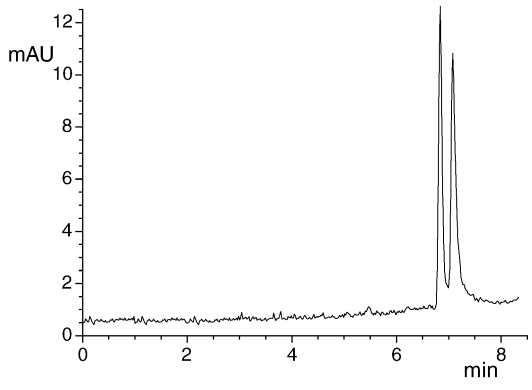

Fig. 1. The electropherogram of mexiletine. Running buffer, 67 mM phosphate buffer, pH 7.4; sample: 0.5 mM mexiletine; injection, 5.0 kPa, 2 s; selector: 0.4 mM HSA, the plug length of HSA zone, 9.0 cm; detection: 200 nm; voltage, 12 kV; 25 °C.

3.2. Effect of conformational change of protein on the chiral separation

The structure of protein is complicated; it can undergo many conformational transitions with the change of the environment. The multiple-sited specific interaction between protein and drug is hereby affected with the conformational change, which results in the corresponding variance of its chiral selectivity to drug. In this study, the resolution of mexiletine with HSA as chiral selector was determined to investigate the effects of the conformational change of HSA on its chiral selectivity.

3.2.1. Effect of pH

The effect of running buffer pH on the chiral separation of mexiletine was studied (the pH of HSA solution was matched with that of the buffer); the result was shown in Fig. 2. It can be seen that the chiral separation cannot be realized at pH <6.0. When the pH is increased from 6.0 to 7.8, the resolution of mexiletine increases. While, further increasing the pH from 7.8 to 9.4, the resolution decreases gradually and finally reaches zero at pH 9.4. The fact indicates that HSA possesses the chiral recognition ability to mexiletine in the pH range of 6.0–9.4. The disappearance of the chiral separation of mexiletine at the slightly acidic and alkaline environment (pH <6.0 and >9.4) may be due to the conformational transitions of HSA. The unfolding conformation of HSA at acid environment has a large hydrated surface, which is not favorable to the hydrophobic interaction of the mexiletine enantiomers with HSA. As a result, the resolution of drug reduces. On the contrary, the unfolding transitions of HSA at basic environment form a closed subdomain structure with a small hydrated surface. But the drug molecule enters the hydrophobic cavity of HSA with more difficulty, so the resolution of mexiletine also decreases at higher pH.

Further study shows that when the pH of HSA solution is shifted from 7.4 to 2.0, then returned to 7.4, the resolution of mexiletine reduces from 1.89 to 0, and then increases to 1.39

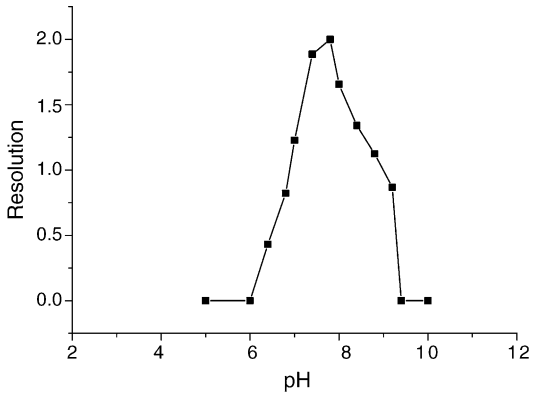

Fig. 2. The effect of pH on the resolution of mexiletine. Running buffer, 67 mM phosphate buffer at different pH; samples: 0.5 mM mexiletine; selector, 0.4 mM HSA at the same pH as buffer, the plug length of HSA zone, 9.0 cm. Other conditions as in Fig. 1.

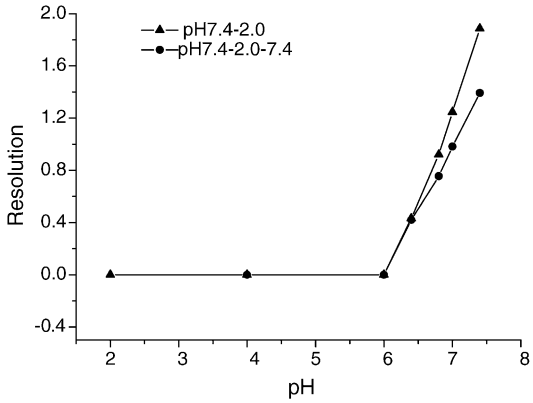

Fig. 3. The effect of pH-induced conformational change of HSA (7.4 → 2.0 → 7.4) on the resolution of mexiletine. Running buffer, 67 mM phosphate buffer at the same pH as HSA solution; selector, 0.4 mM HSA at different pH. The triangles represent the addition of HCl to the protein solution, while the circles reflect the reverse process upon addition of NaOH. Other conditions as in Fig. 2.

(Fig. 3). The trend was similar when the pH was adjusted from 7.4 to 11.0, then back to 7.4 (figure not shown). The results show that the conformational change of HSA in the range of 2.0–11.0 is reversible, and the unfolding and refolding are fast processes. The chiral recognition ability of HSA can recover about 70% when the pH of protein shifted either from neutral to acid, then back to neutral or from neutral to alkaline, then back to neutral. While it cannot recover completely, which may be due to the destruction of some salt bond at the extreme acid and alkaline environment, since this kind of destruction cannot be recovered completely.

Furthermore, the electrophoresis behavior of HSA at various pH was investigated. In order to eliminate the effect of the running buffer pH on the mobility of HSA, pH of the running buffer was fixed at pH 7.4. It was found that the mobility of HSA at pH 7.4 is faster than that at a higher or a lower pH (Fig. 4). The peak shape of HSA obviously broadened at

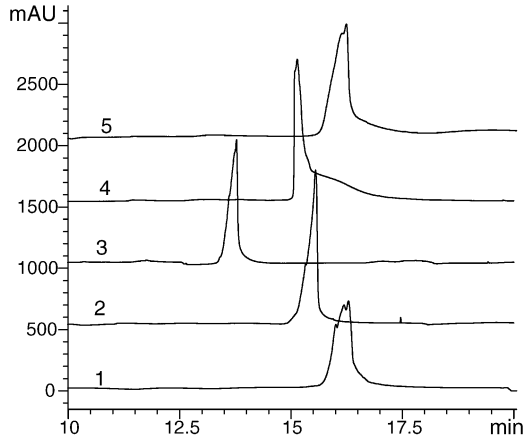

Fig. 4. The electropherograms of HSA at different pH. Running buffer, 67 mM phosphate buffer, pH 7.4; sample, 0.4 mM HSA at different pH, 1 = pH 2.0, 2 = pH 6.0, 3 = pH 7.4, 4 = pH 9.2, 5 = pH 11.0, injection, 5.0 kPa, 2 s; voltage, 12 kV.

pH >9.0 and <6.0. It is easy to understand that the mobility of HSA at pH 7.4 is larger than that at higher pH. HSA is negative charged when pH is above 4.7. The higher the pH is, the more the negative charge is, and the larger the mobility is. While, the migration velocity of HSA at pH lower than 7.4 is slower than that at pH 7.4, this is contrary with the above explanation. Therefore, there should be another force affecting the migration of the protein. It is known that the protein undergoes several pH-dependent conformational transitions [28,35]. The conformation is the most compact close to physiological pH (7.4). Moderate pH deviations from physiological values to either acidic or basic ones cause an increase in HSA globule size (unfolding) [36]. The increase in HSA size leads to a large resistance in its moving. Consequently, the mobility of HSA reduces. The peak broadening of HSA at pH >9.0 and <6.0 indicates that there maybe coexist several conformations in the pH range. It is very obvious to observe several splits in the electropherogram of HSA at pH 2.0 (Fig. 4(1)).

3.2.2. *Effect of temperature*

Unlike the common CE method of investigating the conformational change of proteins, the electrophoretic behavior of the proteins at different column temperatures was studied. In this work, the conformational change of thermal-induced HSA was investigated by determining the chiral selectivity of HSA, which was heated at different temperatures (30–100 °C) for 1 min and cooled to the room temperature. Therefore, no high column temperature is required and there is no need to modify the CE instrument. It shows in Fig. 5 that when protein is heated at the temperature below 60 °C for 1 min, no significant effect on the resolution of mexiletine is observed. When protein is heated over 60 °C, the resolution decreases sharply with the increase of temperature. At last, the chiral selectivity of HSA disappears above 90 °C. The sudden decrease of chiral selectivity over 60 °C indicates that the conformation of HSA changes suddenly over 60 °C. The result agrees well with Wu et al.'s report [37].

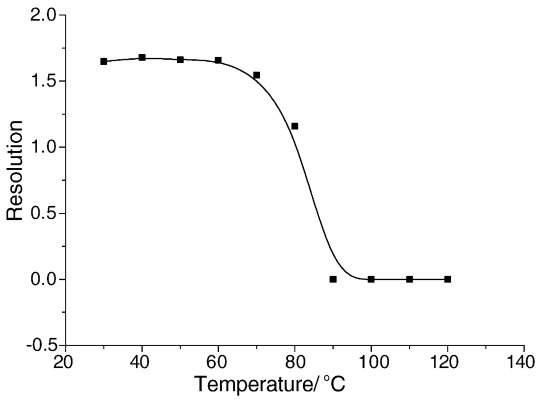

Fig. 5. The effect of thermal denaturation of protein on the resolution of mexiletine. Sample: 0.5 mM mexiletine; selector: 0.4 mM HSA (heated at different temperature for 1 min); other conditions as in Fig. 1.

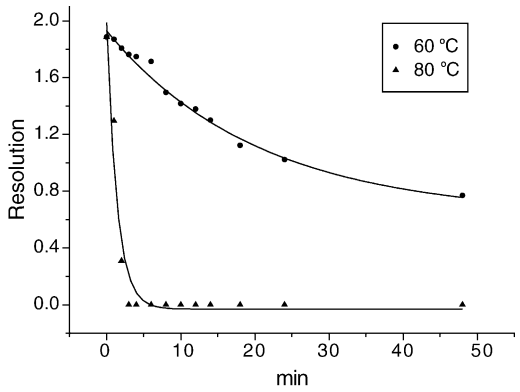

Fig. 6. The effect of heating time of HSA on the resolution of mexiletine. Sample, 0.5 mM mexiletine; selector: 0.4 mM HSA heated at 60 and 80 °C for different time; other conditions as in Fig. 1.

Their study by two-dimensional near-infrared spectroscopy indicated that the secondary structure of HSA changes suddenly near 60 °C, and hydration changes markedly near 60 °C [37]. After heated (70–90 °C heating 1 min) HSA was stored at 4 °C for 1–48 h, its chiral selectivity to mexiletine was studied by CE. The result shows that the chiral recognition ability of HSA cannot recover even 48 h later. This suggests that the thermal-induced conformational change of HSA is irreversible over 70 °C.

The influence of the heating time on the recognition ability of protein was studied at both 60 and 80 °C. Fig. 6 shows that when HSA is heated at 60 °C, the resolution of mexiletine decreases generally with the increase of heating time, i.e., the chiral recognition ability of HSA decreases generally. The recognition ability of HSA remains 41% when it is heated at 60 °C for 48 min. However, when HSA is heated at 80 °C, its chiral selectivity reduces sharply and disappears in 3 min. This indicates that the thermal-induced conformational change of HSA is a rapid kinetic process, the time of unfolding process is shorter than the sum of the time of thermal treatment and the electrophoretic running (12 min). Furthermore, the conformational change is dependent much on the temperature than on heating time. Consequently, combined with the fact that the chiral selectivity of HSA has no obvious change at temperature below 60 °C, it can be speculated that the conformational change of HSA in this temperature range is reversible. The speculation is in good accordance with the results of Lumry and Eyring [29], and Picó [5]. In addition, the electrophoresis behavior of heated HSA was studied in the pH 7.4 phosphate buffer (Fig. 7). All of the mobility of heated HSA is slower than that of native protein (pH 7.4), and the shape of peak broadens as well as the height of peak decreases at the higher temperature. The decrease of the mobility of thermal denatured protein is due to the thermal-induced conformational change of HSA. The broadening in shape and the decrease in height is a consequence of a mixture of several conformation states with different migration times.

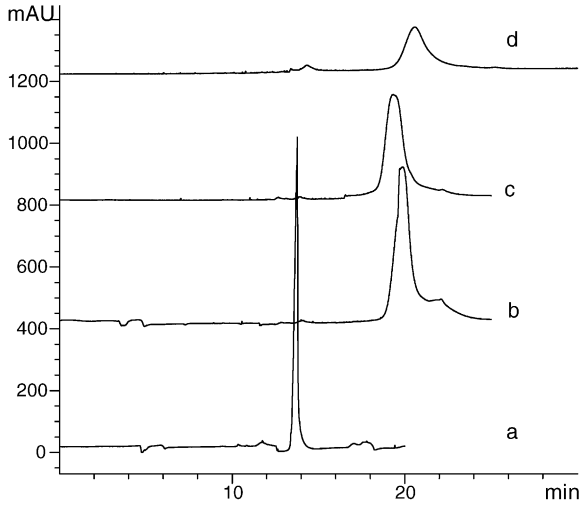

Fig. 7. The electropherograms of thermal denatured HAS. Running buffer, 67 mM phosphate buffer, pH 7.4; sample, 0.4 mM HSA heated at different temperatures; a = native, b = 60 °C, 48 min, c = 80 °C, 2 min, d = 80 °C, 3 min. Other conditions as in Fig. 4.

3.2.3. *Effect of vibration*

Acute vibration (such as ultrasound) can also bring about the conformational change of proteins [38,39]. In this study, the HSA solution, which was ultrasonically vibrated for 2 min, lost its chiral recognition ability to the enantiomers. Its chiral recognition ability cannot recover after placement at 4 °C for 1–48 h. This fact suggests that the ultrasonic-induced conformational change of HSA is irreversible.

3.2.4. *Effect of alcohol*

Alcohol has been proven to cause a conformational transition of protein [40,41]. The effect of alcohol on the chiral separation efficiency of HSA was studied. It can be seen in Fig. 8 that with the increase of ethanol, the resolution of mexiletine decreases gradually, and reaches zero when the percent of ethanol in the protein solution reaches to 5% (v/v). Similarly, the protein solution containing 1.1% (v/v) 1-propanol has no chiral selectivity ability. It has been reported that high content alcohol (>50%, v/v) could cause the conformational transition of protein. Whereas at low content of alcohol (<20%, v/v), the conformation of protein does not change, it maintains the compact native-like structure, but decreased stability [40]. In our experiment, it is found that the percent of alcohol leading to the disappearance of the chiral separation of drug is very low (≤5%, v/v). This result suggests that the protein conformation does not change and the addition of alcohol affects the interaction between drug and protein. The stronger the hydrophobicity of alcohol, the low the concentration of alcohol leading to the disappearance of chiral separation. Therefore, it can be concluded that the hydrophobic interaction may play an important role in the interaction between HSA and mexiletine.

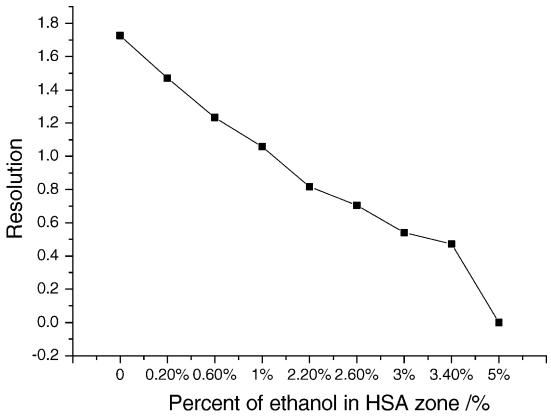

Fig. 8. The effect of ethanol on the resolution of mexiletine. Sample: 0.5 mM mexiletine; selector, 0.4 mM HSA containing different percent of ethanol. Other conditions as in Fig. 1.

4. Conclusion

Passing through the study of the effects of conformational change of HSA on its chiral recognition ability to mexiletine by capillary electrophoresis, the results can provide a series of useful information to understand the interaction between HSA and drugs and the characteristics of conformational change of HSA. Capillary electrophoresis technique can provide a simple and assistant method to reveal the conformation characteristics of protein by studying the influence of conformational change of protein on its chiral selectivity. The method possesses the advantages of easy performance, high speed, and low reagent consumption. The following conclusions can be drawn from the present work.

(1) The HSA interacts differently with enantiomers of the chiral drugs by reversible binding, the binding interaction mainly involves hydrophobic interaction.
(2) Ultrasonic-induced conformational change of HSA is irreversible, while pH-induced conformational change is reversible in the pH range of 2.0–11.0. The conformation of HSA is the most compact close to physiological pH (pH 7.4).
(3) The thermal-induced conformational change of HSA strongly depends on the temperature, and it is a rapid dynamic process. The conformational change is reversible below 60 °C, but it is irreversible above 70 °C. HSA undergoes a sudden conformational change near 60 °C.

Acknowledgements

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